Original Article

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Standardisation of Antimicrobial Compounds from *Aspergillus flavus* against the *Escherichia coli* and *Staphylococcus aureus*: An In-vitro Analysis

Microbiology Section

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ABSTRACT

Introduction: The increasing prevalence of antibiotic resistance among pathogenic microorganisms poses a significant challenge to global public health. Previously effective treatments against common pathogens such as *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S.aureus*) are becoming progressively less effective. These organisms are responsible for a wide spectrum of diseases, ranging from minor infections to severe systemic illnesses. As resistance to conventional antibiotics continues to rise, the need for novel antimicrobial agents has become increasingly urgent. Natural products have long served as a rich source of antimicrobial substances, and among them, fungi are well recognised for their ability to produce a diverse array of bioactive metabolites with potential therapeutic applications.

Aim: The aim of the current study was to isolate and identify bioactive metabolite-producing *Aspergillus flavus* (*A.flavus*) strains from soil samples and to evaluate their antimicrobial activity against clinical isolates of *E.coli* and *S.aureus*.

Materials and Methods: The present in-vitro experimental study was conducted in the Department of Microbiology, SRM Medical College Hospital and Research Centre, Kattankulathur, Tamil Nadu, India, from January to December 2023. The current study involved the isolation of *A.flavus* from soil samples collected from dump yards and the isolation of *E.coli* and *S.aureus* from clinical specimens. The total sample size was 50, comprising 25 *E.coli* and 25 *S.aureus* isolates obtained from various clinical samples, including urine, pus, sputum, blood, ear swabs, wound swabs, tracheal aspirates, and tissue samples. Soil samples were collected from four different dump yard sites surrounding the SRM Medical College Hospital and Research Centre. Only *A.flavus* was isolated from soil samples using the spot method, while *E.coli* and *S.aureus* isolated from clinical samples were included in the study. Other fungal and bacterial isolates were excluded. Antimicrobial activity was assessed using the perpendicular cross-streaking method and the agar well diffusion method. Bioactive compounds were extracted through fermentation, followed by separation using Thin-Layer Chromatography (TLC). Compound identification was performed using Gas Chromatography-Mass Spectrometry (GC-MS). Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) software, and all data were expressed as mean±standard deviation.

Results: The isolated *A.flavus* strains demonstrated antimicrobial activity against both *E.coli* and *S.aureus*. TLC analysis revealed four distinct bioactive compounds with Rf values of 0.70, 0.80, 0.87, and 0.89. GC-MS analysis identified a total of 23 volatile compounds. The antimicrobial activity produced Zones Of Inhibition (ZOI) ranging from 23 to 18 mm against *E.coli* and from 16 to 24 mm against *S.aureus*.

Conclusion: The bioactive compounds extracted from *A.flavus* exhibited significant antibacterial activity, particularly against the Gram-positive organism *S.aureus*. These findings highlight the potential of *A.flavus* as a promising source of novel antimicrobial agents, especially in the context of increasing antibiotic resistance. Further studies are warranted to optimise the production and purification of these compounds and to explore their potential clinical applications.

Keywords: Antibacterial agents, Chromatography, Secondary metabolites

INTRODUCTION

The in-vitro experimental study involved controlled laboratory procedures to isolate, extract, and evaluate antimicrobial compounds from *A.flavus* against two bacterial strains. The term "unstable to stable" refers to the process of standardising and stabilising the bioactive antimicrobial compounds extracted from *A.flavus*. Initially, these compounds exist in a crude or unpurified (unstable) form with uncertain composition, potency, and reproducibility. Through a series of extraction, separation using TLC, and characterisation by GC-MS, the compounds were identified, isolated, and evaluated, thereby transforming them into a more stable and standardised form suitable for antimicrobial testing and potential therapeutic applications [1].

The increasing threat of pathogenic microorganisms becoming resistant to nearly all currently available antibiotics is now recognised as a major global public health concern. These escalating issues have prompted researchers to explore innovative pharmacological scaffolds with broad-spectrum antibacterial activity [2]. Such compounds may be derived from natural sources by exploring under-investigated environments, which could enhance the likelihood of discovering bioactive substances such as phytochemicals, antibiotics, fungicides with antibacterial activity, or other bioactive metabolites [3].

Microorganisms are classified into several groups, including viruses, bacteria, archaea, fungi, and protists. Among these, bacteria and fungi represent important reservoirs of novel antibacterial substances. Examples of antimicrobial compounds derived from bacteria include cyclic peptides such as mathiapeptide A, destotamide B, and marfomycins A, B, and E; spirotetronate polyketides such as abyssomycin C and lobophorins F and H; as well as alkaloids and sesquiterpene derivatives such as caboxamycin and mafuraquinocins A and D. These compounds

have demonstrated antimicrobial activity against clinically resistant bacteria, including *S.aureus*, methicillin-resistant *S.aureus*, *Bacillus subtilis*, and *Enterococcus faecalis* [4].

Antibiotic metabolites are produced by a wide range of fungal species worldwide, and the majority of these compounds have been identified as antibiotics. Recently, natural products have gained considerable attention due to their potential role in developing novel antibacterial drugs to combat bacterial infections. According to the Antimicrobial Peptide Database, antibacterial peptides have been synthesised and isolated from various sources, including soil fungi [5]. A wide spectrum of microorganisms, such as fungi, actinomycetes, and eubacteria isolated from soil and other environments, have yielded a substantial number of secondary metabolites with potent antimicrobial properties. Several fungal species have been shown to produce antibiotic metabolites, including cephalosporins and fusidic acid, which have been approved for medicinal use [6].

Fungi possess a remarkable ability to produce a diverse array of bioactive compounds, making them highly valuable to the pharmaceutical industry [7]. They have played a crucial role in drug development by contributing to the discovery of novel bioactive compounds and the production of antibiotics such as penicillin, as well as anticancer agents. Researchers continue to evaluate the antimicrobial efficacy of fungal extracts derived from solid-state waste and to identify their active constituents [8]. Antibiotics constitute one of the primary groups of secondary metabolites produced by microorganisms and are extensively used in the pharmaceutical sector to treat a wide range of infections [9].

However, bacterial infections have evolved into more virulent forms that pose significant challenges in clinical management due to increased pathogenicity and heightened resistance to antibiotic therapy. Hence, the current study was done to identify alternative sources of bioactive compounds to address the growing problem of multidrug resistance. Under varying environmental conditions, different *Aspergillus* strains can produce a wide range of secondary metabolites [10,11]. The development of fungi is influenced by complex interactions between internal and external factors. Various combinations of temperature, time, moisture, gas composition, and antimicrobial agents are evaluated for their fungistatic or fungicidal effects. Although synthetic antimicrobials are commonly used to prevent mould formation, natural antimicrobials have also demonstrated significant antifungal properties.

Many fungal species produce antibacterial substances that are effective in eliminating pathogenic microorganisms. For instance, penicillin-the first antibiotic drug-was derived from the fungus *Penicillium notatum* [12]. Typically, fungal metabolites are separated from the culture medium through fermentation followed by extraction using various solvents. Fungi possess multiple spore-dispersal mechanisms that exhibit varying resistance to airborne conditions as a result of the evolutionary adaptation of plant pathogens [13].

Therefore, the current study was undertaken to explore under-investigated environments, such as soil samples from dump yards, in the search for natural products capable of combating multidrug-resistant pathogens. The novelty of the current study lies in its methodological integration, targeted isolation from neglected soil environments, and comprehensive chemical and biological analysis of fungal secondary metabolites for antibacterial activity. This approach represents a practical, region-specific strategy to combat antibiotic resistance using natural resources.

The present study aimed to isolate and identify bioactive metabolite-producing *A.flavus* from soil samples and to evaluate their antimicrobial activity against *E.coli* and *S.aureus*. The primary objective was to isolate *A.flavus* from dump yard soil samples, while the secondary objectives included extraction of bioactive metabolites from the isolated fungus and evaluation of the antimicrobial activity of the extracted compounds using antibacterial susceptibility test.

MATERIALS AND METHODS

The current in-vitro experimental study was conducted in the Department of Microbiology, SRM Medical College Hospital and Research Centre, Kattankulathur, Tamil Nadu, India, from January 2023 to December 2023. The study was approved by the Institutional Ethical Committee of SRM Medical College Hospital and Research Centre.

A total of 25 *E.coli* and 25 *S.aureus* isolates were obtained from various clinical samples, including urine, pus, sputum, blood, ear swabs, wound swabs, tracheal aspirates, and tissue samples.

Sample size calculation:

Taking p=62 and q=34 where p (62) represents the expected prevalence or proportion (percentage) of antimicrobial activity among the isolates, based on findings from previous studies [14] and q (34) represents the complementary proportion, calculated as 100-p, indicating the percentage not expected to show antimicrobial activity.

$$=\Sigma_{\alpha/2}p.\ q\ /d^2$$
$$=1.96)^2.\ 64.\ 36/12.8^2$$
$$=3.841\times2108/163.84$$
$$=50$$
$$n=50$$

E.coli=25 and *S.aureus*=25.

Inclusion criteria: The current study included the isolation of *A.flavus* from soil samples collected from dump yards, as well as the isolation of *E.coli* and *S.aureus* from clinical samples.

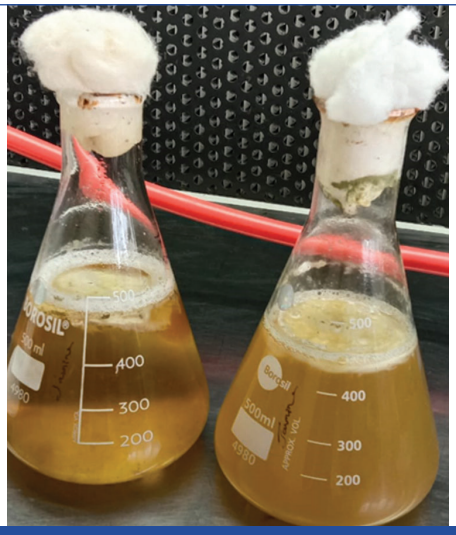
Exclusion criteria: Other fungal isolates obtained from soil samples and bacterial organisms other than *E.coli* and *S.aureus* isolated from clinical samples were excluded from the study.

Study Procedure

Sample collection and identification: Soil samples were collected from dump yard sites. *A.flavus* was isolated from solid waste, and approximately 200 g of soil was collected from the ground surface at depths of 15-20 cm. Each sample was properly sealed and stored at 4°C until further processing. Bacterial strains, including *E.coli* and *S.aureus*, were obtained from the culture collection of the Department of Microbiology, SRM Medical College Hospital and Research Centre, Kattankulathur, Tamil Nadu, India. Identification of organisms was performed using conventional microbiological techniques. Direct Gram staining was used for initial morphological classification, followed by culture on selective and differential media such as nutrient agar, MacConkey agar (for *E.coli*), blood agar (for haemolysis), and Urinary Tract Infection (UTI) chromogenic agar (for urine samples). Phenotypic characterisation was carried out using standard biochemical tests, including the indole test, methyl red test, Voges-Proskauer test, citrate utilisation test, catalase test, coagulase test (for *S.aureus*), and sugar fermentation tests (e.g., lactose, glucose, and mannitol). Antibiotic Susceptibility Testing (AST) using the agar well diffusion method was performed according to Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines [15].

The confirmed isolates of *E.coli* and *S.aureus* were preserved in peptone glycerol broth at -70°C for further analysis. Various clinical samples, including urine, pus, sputum, ear swabs, wound swabs, tracheal aspirates, and tissue samples, were processed. Direct Gram staining was performed on all samples. The samples were streaked onto nutrient agar, MacConkey agar, and blood agar, except urine samples, which were streaked only on UTI chromogenic agar and blood agar. After incubation at 37°C for 18-24 hours, colony morphology was examined. Further phenotypic characterisation of *E.coli* and *S.aureus* was based on biochemical reactions, with species confirmation achieved through sugar fermentation tests and antibiotic susceptibility testing in accordance with CLSI 2023 guidelines [15]. The isolates were stored in peptone glycerol broth at -70°C.

Isolation of *Aspergillus flavus*: Soil samples were collected from four different dump yard locations [12]. One gram of soil from each sample was suspended in 10 mL of phosphate-buffered saline and centrifuged at 2,500 rpm for 10 minutes. After centrifugation, 10 µL of the supernatant was inoculated onto glycerol casein agar plates and incubated at 37°C for 48 hours [2]. Based on CLSI 2023 guidelines, the spot method was employed for the isolation of *A.flavus* [15]. Using sterile toothpicks, a single colony was picked and inoculated onto Sabouraud Dextrose Agar (SDA) plates, followed by incubation at 37°C for 48 hours. The isolates were confirmed as *A.flavus* based on macroscopic and microscopic morphological characteristics after the incubation period [Table/Fig-1]. Distinct colonies were maintained on SDA plates and stored at 4°C for future use [16].



[Table/Fig-1]: Isolation of *Aspergillus flavus* from soil sample using Sabouraud dextrose broth.

Determination of soil temperature: The soil temperature at four different locations was measured using a thermometer by inserting it into the soil at a depth of 15 cm and allowing it to remain in place for 15 minutes [Table/Fig-2].

Determination of soil pH: The soil pH at four different locations was measured using pH indicator (litmus paper). One gram of soil was dissolved in distilled water and allowed to settle. The pH paper was then immersed in the supernatant, and the pH was recorded based on the observed colour change [Table/Fig-2].

Location	pH	Temperature (°C)
Sample 1	6.5±0.2	27±0.5
Sample 2	7.0±0.2	24±0.5
Sample 3	8.5±0.2	27±0.5
Sample 4	6.9±0.2	25±0.5

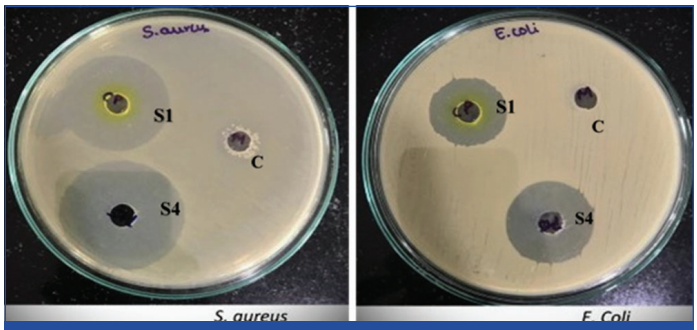
[Table/Fig-2]: Physical and chemical properties of soil (pH and temperature).

Primary screening: Primary screening for antimicrobial activity was performed against two test organisms, *E.coli* and *S.aureus*, using the perpendicular cross-streaking method [Table/Fig-3]. Mueller-Hinton agar plates were inoculated with fungal isolates by streaking a straight line at the centre of the plate and incubated at 37°C for 48 hours.

A 24-hour-old culture of the test organism was prepared, and the inoculum density was adjusted to a 0.5 McFarland standard. The test organisms were then streaked perpendicular to the fungal growth. Mueller-Hinton agar plates streaked only with the test organisms served as negative controls, while plates containing fungal extracts served as positive controls [Table/Fig-4].

All plates were incubated at 37°C, and ZOI were observed. Fungal isolates demonstrating inhibition of test organism growth were collected and stored at -20°C in 60% glycerol. During secondary

[Table/Fig-3]: Primary screening for antimicrobial activity using the cross-streaking method.



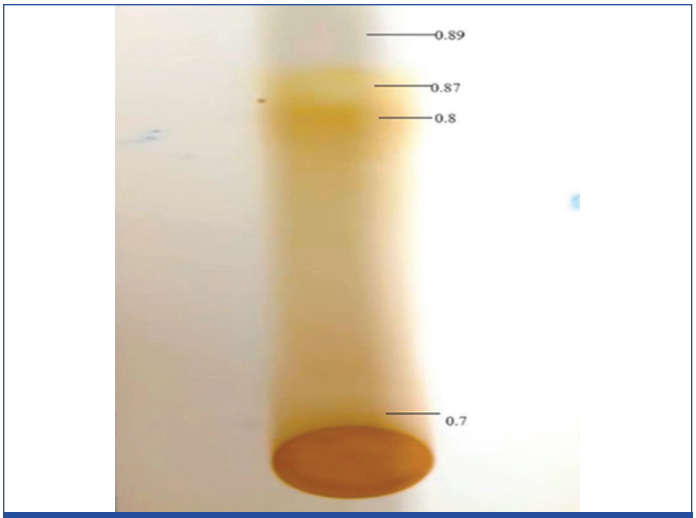
[Table/Fig-4]: The antimicrobial activity of *Aspergillus flavus* showing Zone Of Inhibition (ZOI) against pathogens *S.aureus* and *E.coli* using agar well diffusion method.

screening, fungal isolates that showed positive results in primary screening were cultivated in Sabouraud dextrose broth and incubated at 28°C for seven days. After incubation, the broth was filtered, and the mycelium was collected and washed with distilled water to remove residual media components. Approximately 8 g of fungal biomass was spread onto sterile Petri plates and dried in a hot air oven at 37-40°C for three days. The dried biomass was then powdered using a mortar and pestle and suspended in methanol in a 1:9 ratio for further analysis.

Bio activity assays: The organic solvent extract was subjected to antimicrobial testing using the agar well diffusion method, following CLSI 2023 guidelines [15]. A 100 µL volume of bacterial inoculum was spread evenly onto Mueller-Hinton agar plates using the lawn culture technique. Two wells of 6 mm diameter were created in each plate, and 200 µL of the fungal extract dissolved in methanol was added to each well. The plates were incubated at 37°C for 24 hours and examined for the presence of ZOI around the wells containing the fungal extract [Table/Fig-4].

Thin Layer Chromatography (TLC): Separation of the extracted bioactive compounds was performed using Thin-Layer Chromatography (TLC). The antimicrobial extract of *A.flavus* was dissolved in acetone and applied onto silica gel pre-coated TLC plates using capillary tubes. The plates were developed in a solvent system consisting of methanol, chloroform, and hexane in the ratio of 1:1.2:0.8 to separate the various constituents of the extract. After development, the plates were air-dried and observed under visible light. Multiple distinct spots were observed, and their Retention factor (Rf) values were calculated and recorded [Table/Fig-5].

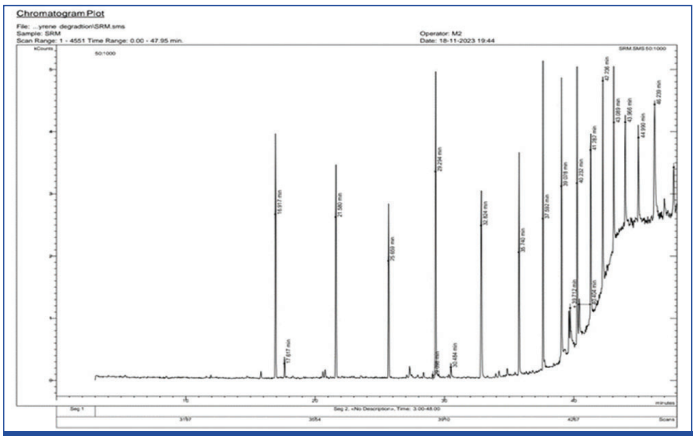
Gas Chromatography-Mass Spectrometry (GC-MS): The bioactive compounds were characterised using GC-MS equipped with an HP-5MS column (30 m length, 0.25 mm internal diameter) on an Agilent 7980A GC-MS system (Agilent Technologies India Pvt. Ltd.). ChemStation software from Agilent Technologies was used for system control and data processing.



[Table/Fig-5]: Thin Liquid Chromatography (TLC) analysis of bioactive compounds extracted from *A.flavus* with different bands and Rf values (0.7, 0.8, 0.87, 0.89).

Helium was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 µL, and a splitless injection mode was employed. The temperature of the Mass Selective Detector (MSD) ion source was set at 170°C, the mass filter at 150°C, the GC-MS interface at 280°C, and the inlet temperature at 270°C.

The column temperature programme was as follows: an initial temperature of 60°C held for 2 minutes, followed by an increase at 25°C/min to 240°C, and a final ramp of 5°C/min to 300°C. Electron Ionisation (EI) was carried out at 70 Electron Volt (eV), and spectra were monitored using Selected Ion Monitoring (SIM) mode [Table/Fig-6].



[Table/Fig-6]: GC-MS Chromatogram of *A.flavus* extracted bioactive compounds.

STATISTICAL ANALYSIS

Microsoft Excel and SPSS version 25.0 were used for data compilation and analysis of the GC-MS results. The relative abundance of detected compounds was expressed as a percentage of the total ion count. Peak areas of major compounds were measured in triplicate, and the mean values with standard deviations were calculated. Descriptive statistics were used to compare peak intensities and retention times to ensure the accuracy and reliability of compound identification.

RESULTS

The fungal isolates obtained from dump yard soil demonstrated antibacterial activity against both *E.coli* and *S.aureus*. TLC analysis revealed the presence of four bioactive compounds with Rf values of 0.70, 0.80, 0.87, and 0.89, indicating chemical diversity within the metabolites extracted from *A.flavus* [Table/Fig-5].

GC-MS analysis of the methanolic extract of *A.flavus* identified a total of 23 volatile compounds. These compounds represented diverse functional groups and molecular weights, suggesting the complex bioactive potential of *A.flavus* metabolites [Table/Fig-7].

S. No.	RT	Peak Name	Res Type	Area	Amount	R.Match
1	16.917	Cyclotetrasiloxane, octameth	TIC	13256	6.94	885
2	17.617	Benzene, (1-((1-(1-methylethyl)	TIC	1378	0.721	880
3	21.58	Cyclooctasiloxane, hexadecimal	TIC	12554	6.572	734
4	25.659	Cyclononasiloxane, octadecam	TIC	9800	5.131	671
5	29.098	Benzene, (3-nitropropyl)-	TIC	195	0.102	465
6	29.294	Cyclotetrasiloxane, octameth	TIC	17674	9.252	873
7	30.484	Acetic acid,1,4-dihydroxy -9	TIC	848	0.444	715
8	32.824	Ethylphosphonic acid, bis (te	TIC	14030	7.345	826
9	35.74	Ethylphosphonic acid, bis (te	TIC	10928	5.721	805
10	37.592	Cyclotetrasiloxane, octameth	TIC	14096	7.379	837
11	39.028	Ethyl(5-hydroxy-1-(6-method	TIC	13580	7.11	953
12	39.6	2-Thiophenecarboxylic acid,	TIC	1990	1.042	590
13	39.712	2-(3-thenoylamino) propanamide	TIC	4102	2.147	560
14	40.232	Ethyl (5-hydroxy-1-(6-methoxy-	TIC	12386	6.484	945
15	40.404	1-(4-hydroxy-3-isopropenyl-4	TIC	3515	1.84	517
16	41.282	Ethyl (5-hydroxy-1-(6-methox	TIC	11918	6.239	955
17	42.236	Ethyl (5-hydroxy-1-(6-methox	TIC	9871	5.14	945
18	43.089	Ethyl (5-hydroxy-1-(6-methox	TIC	7917	4.145	931
19	43.966	Cyclotetrasiloxane, octameth	TIC	6657	3.485	838
20	44.99	Cyclotetrasiloxane, octameth	TIC	5702	2.985	868
21	46.239	[2] (1,4)Naphthalene [2] (2,6)p	TIC	13204	6.912	555
22	47.011	16-hydroxyimino-5.beta.-and	TIC	1473	0.771	598
23	47.72	2,4-dihydroxybenzophenone,	TIC	3998	2.093	703

[Table/Fig-7]: GC-MS results showing 23 volatile compounds from *A.flavus* methonolic extract.
RT- Retention Time; Res Type - Resolution Type; R. Match - Reverse Match factor

The fungal extract exhibited promising antibacterial activity against the clinical pathogens *E.coli* and *S.aureus*. The ZOI against *S.aureus* measured 24 mm and 23 mm for two different fungal samples, while the ZOI against *E.coli* measured 16 mm and 18 mm, respectively. These findings indicate that the fungal extract was more effective against the Gram-positive bacterium *S.aureus* than against the Gram-negative bacterium *E.coli* [Table/Fig-8], supporting the potential of *A.flavus*-derived metabolites as effective antimicrobial agents.

Test Organisms	Sample 1	Resistant/Sensitive	Sample 4	Resistant/ Sensitive
E.coli	16mm	Sensitive	18mm	Sensitive
S.aureus	23mm	Sensitive	24mm	Sensitive

[Table/Fig-8]: Zone of Inhibition values for *E.coli* and *S.aureus* (Sample 1 and 4).

DISCUSSION

In the current study, soil samples were collected from four different dump yard sites surrounding the SRM University campus in

Kattankulathur. A total of 50 clinical isolates, comprising 25 *E. coli* and 25 *S.aureus*, were evaluated for antimicrobial activity against *A.flavus* isolated from these soil samples. Notably, soil samples from sites 1 and 4 yielded *A.flavus* isolates exhibiting antibacterial activity, with isolates from site 4 demonstrating greater sensitivity. The *A.flavus* isolate obtained from site 4 showed inhibitory activity against both *E.coli* and *S.aureus*.

These findings are consistent with the study by Devadass BJ et al., who investigated the antimicrobial activity of soil actinomycetes from the Western Ghats of Tamil Nadu, India. Actinomycetes isolated from soil samples collected across five different districts were tested against a variety of pathogens and demonstrated significant antimicrobial activity [17]. Similarly, the present study focused on *A.flavus* isolates from soil, which exhibited antibacterial effects against *E.coli* and *S.aureus*.

Additionally, Azish M et al., (2021) optimised the production of antifungal metabolites from *Streptomyces libani* isolated from northern forests in Iran [18]. They identified bacterial strains with strong antifungal activity, including *Streptomyces libani*, *Streptomyces angustmyceticus*, *Bacillus subtilis*, and *Sphingopyxis* spp. This research complements the present study by further highlighting the potential of soil microorganisms as sources of antimicrobial compounds.

In the present study, *A.flavus* extracts exhibited significantly larger ZOI against *S.aureus* than against *E.coli*. The TLC analysis revealed four bioactive compounds, which were further characterised by GC-MS, identifying a total of 23 volatile compounds. These findings are in agreement with the study by Dudeja S et al., who explored the antimicrobial properties of *A.flavus* MTCC 13062 isolated from solid waste and demonstrated notable inhibitory activity against various pathogens, including *S.aureus* and *E.coli* [3].

The results also correlate with the work of Helen PAM et al., who screened antibiotic-producing fungi from soil and identified bioactive compounds using GC-MS analysis [19]. The effectiveness of fungal extracts against pathogenic bacteria and fungi underscores the potential of soil-derived fungi in the development of novel antimicrobial agents. Overall, this research adds to the growing body of evidence supporting the antimicrobial potential of soil microorganisms, particularly *A.flavus*, against clinically relevant pathogens such as *E.coli* and *S.aureus*.

Limitation(s)

The present study has several limitations. The bioactive compounds extracted were in crude form and require further purification. Additionally, the fermentation process is expensive, prone to contamination, and may produce variable yields; therefore, it was not employed for large-scale production of fungal metabolites. Techniques such as GC-MS and TLC also have limitations in distinguishing structural isomers or identifying unknown compounds. Furthermore, the present study was limited to *E.coli* and *S.aureus*, and the absence of *in vivo* studies restricts conclusions regarding the therapeutic applicability of the identified antimicrobial agents.

CONCLUSION(S)

The bioactive compounds extracted from *Aspergillus flavus* demonstrated greater antibacterial activity against Gram-positive bacteria (*S.aureus*), indicating strong potential as antimicrobial agents. In the context of rising microbial resistance, these compounds may offer alternative therapeutic options with potentially lower toxicity. The present study identifies soil-derived *A.flavus* as a promising source of novel antibacterial compounds. Future studies should focus on large-scale production, detailed metabolite characterisation, and broader antibacterial evaluation. Such efforts may lead to the development of safe, effective, and natural antimicrobial agents.

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Authors' contribution: JB: Conducted the fieldwork, collected primary data, developed the methodology, and contributed to the theoretical framework; JM: Provided guidance on experimental techniques, ensured adherence to ethical standards, and contributed to manuscript revision; R: Drafted the manuscript, including the Introduction and Discussion sections; LKV: Oversaw ethical compliance and ensured the study adhered to institutional guidelines.

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