



Biocontrol Potential of Rhizospheric Soil Bacteria, Including *Streptomyces* and *Comamonas* Species, Against Aflatoxigenic *Aspergillus flavus*

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ABSTRACT

After decades of extensive exploration of bioactive compounds from *Streptomyces* spp., the discovery of new bioactive chemicals from routinely studied strains has been declining progressively. To tackle this challenge, recent studies have focused on *Actinobacteria* from under-explored ecological niches with significant microbial diversity. The present study aimed to assess the possible antifungal activity of *Streptomyces* spp. isolated from rhizospheric soils of Karura Forest, Nairobi, Kenya, against *Aspergillus flavus* (*A. flavus*), a fungal species known to produce aflatoxin. A total of 236 bacteria were isolated through *in vitro* cultures and characterized using macroscopic, microscopic, and biochemical tests to identify *Streptomyces*-like isolates, which were identified by dry, powdery colonies, filamentous branching hyphae, aerial mycelia, and the formation of spore chains, with Gram staining carried out later on isolates advanced through dual culture screening for antifungal activity. Dual culture assays, with *A. flavus* grown alone as the negative control, were performed to select the promising candidates for further extraction, gas chromatography-mass spectrometry (GC-MS) metabolite profiling, and antifungal testing by the disc diffusion method against PBS and nystatin as the negative and positive controls, respectively. The selected isolates also underwent molecular identification by PCR amplification and Sanger sequencing of the 16S rRNA gene. The analysis revealed that 190 isolates exhibited *Streptomyces*-like characteristics. Dual culture assays revealed that certain isolates inhibited the mycelial growth of *A. flavus*, and GC-MS profiling of the corresponding extracts confirmed the presence of bioactive metabolite classes. The pellet crude extracts of the six selected isolates (SA18, SD102, SD109, SD110, SE143, and SE194), exhibited the highest antifungal efficacy, with inhibitory zones of up to 31.60 ± 2.80 mm, relative to the positive control (nystatin); five of the isolates belonged to the genus *Streptomyces*, while SE143 was identified as *Comamonas*. Supernatant extracts and crude filtrates revealed lower and more variable inhibition across most of the isolates. The present findings indicated that rhizosphere-derived *Streptomyces* and *Comamonas* isolates from Karura Forest are potential natural candidates for biological control of *A. flavus*.

Keywords: Antifungal activity, *Aspergillus flavus*, Biocontrol, *Comamonas* sp., *Streptomyces* spp.

INTRODUCTION

Poverty and food insecurity remain major, interconnected problems in many nations, affecting millions of households and communities (FAO et al., 2025). Food insecurity, characterized by unstable access to sufficient, safe, and nutritious food, has directly motivated large-scale crop intensification projects worldwide (FAO, 2010; Godfray et al., 2010). The objective of the crop intensification project was to enhance the production rate of multiple agricultural crops (FAO, 2011; Pretty et al., 2018), including wheat, corn, and rice (FAO, 2016); cassava (FAO, 2013); bananas (Ronner et al., 2023); and soybeans and beans (Nassary et al., 2020). In food crop production, mycotoxin emergence poses a significant health risk to consumers, surpassing the dangers associated with pesticide and synthetic residue presence, in addition to yield losses from fungal infections (Oliveira et al., 2013). The concept of mycotoxin was initially utilized in the 1960s during the investigation of the fatalities of many turkeys (genus *Meleagris*) in England due to the ingestion of groundnut meal (Bennett and Klich, 2003). The harmful compound was subsequently recognized as B1 aflatoxin (McCormick, 2013). Aflatoxin B1 (AFB1) is the most potent and toxic aflatoxin, produced mainly by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (Frisvad et al., 2019). AFB1 is carcinogenic, mutagenic, and suppressive of immunity, regarded as the most deleterious mycotoxin (Riba et al., 2010; Yu, 2012).

Mycotoxins, including aflatoxins, infect a substantial amount of the world's food supply, including cereals such as maize, groundnuts, and tree nuts (Eskola et al., 2020). Regulations have been put in place in different countries to shield consumers from the adverse effects of mycotoxins (Van Egmond et al., 2007). Aflatoxins are complex aromatic

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compounds that contaminate a range of staple foods such as groundnuts and maize (Payne and Brown, 1998; Wild and Gong, 2010). In low-income countries, aflatoxins (AFB1, AFB2, AFG1, and AFG2) are naturally occurring compounds and have been shown to be strong carcinogens in humans (WHO, 2023). AFB1 is the predominant secondary metabolic form of aflatoxin (Ismail et al., 2018; Frisvad et al., 2019).

Aflatoxin contamination of food products contributes markedly to the global risk of hepatocellular carcinoma (HCC), one of the most common causes of cancer-related death worldwide (Liu and Wu, 2010; McGlynn et al., 2021). Reducing this risk requires effective control of the fungi responsible for aflatoxin contamination in food crops.

Biocontrol is one of the options to restrict disease spread because it utilizes naturally occurring antagonistic microbes (Khan et al., 2023). Soil-dwelling actinomycetes such as *Streptomyces* are of interest as biocontrol agents because they produce a variety of antifungal metabolites, including lytic enzymes, chitinases, and antibiotics (Shakeel et al., 2018). Antimicrobial resistance has become a major global public-health concern (Murray et al., 2022), intensifying interest in discovering novel bioactive compounds from environmental microorganisms. The increasing occurrence of antifungal resistance and difficult-to-treat invasive fungal infections further demonstrates the need to identify new antifungal compounds, particularly for the protection of vulnerable and immunocompromised populations (Fisher et al., 2022). Only a few antifungal agents are commercially available compared with antibacterial agents because the similarity in cellular characteristics between fungi and human cells complicates the creation of selective antifungal agents (Perfect, 2017; Fisher et al., 2022). The limited availability of antifungal agents has stimulated the exploration of novel bioactive chemicals across diverse biological systems through various methodologies, for instance, microbial genome mining as well as metagenomics (Mendes et al., 2013).

The bulk of *Actinobacteria* species that have been researched commercially were isolated from soil (Barka et al., 2015). After decades of bioprospecting, novel bioactive chemicals from soil *Actinobacteria* are getting more difficult to strain-isolate (Goodfellow and Fiedler, 2010). Therefore, many researchers have turned their search for microbial sources of putative antifungal chemicals to a variety of biological habitats, such as tropical rainforests, marine environments, hydrothermal vents, and microbiological symbionts associated with animal and plant species (Kim et al., 2006; Choi et al., 2009).

Actinobacteria are a cost-effective and promising natural method for efficient detoxification of mycotoxins (Oliveira et al., 2013). *Actinobacteria* are Gram-positive, filamentous bacteria found in a wide range of aquatic and terrestrial settings (Barka et al., 2015). *Streptomyces* are well studied for their ability to produce different useful compounds, such as antifungal drugs, antibiotics, and enzymes like chitinases, which give considerable antagonistic capabilities against the growth of fungi (Barka et al., 2015). The majority of natural-based antimicrobial agents used in veterinary and human medicine are produced by *Streptomyces* spp. (Barka et al., 2015). For instance, strains like *Streptomyces yanglinensis* 3-10, *roseolus*, *philanthi* RL-1-178, and *alboflavus* TD-1 were found to produce antifungal agents against *Aspergillus flavus* (Caceres et al., 2018; Shakeel et al., 2018; Yang et al., 2019; Boukaew and Prasertsan, 2020). Microbes in the soil were studied to identify beneficial microbes to control aflatoxin production in food and animal feed (Uasontornnop et al., 2022). Current strategies used to combat AFB1 include both chemical and physical methods (Sipos et al., 2021; Hamad et al., 2023). Their practical use is, however, constrained by nutrient loss, reduced sensory quality, and higher equipment costs (Sipos et al., 2021; Hamad et al., 2023). Synthetic fungicides such as triazoles and acylalanines are commonly used in agriculture to control fungal diseases (Pánek et al., 2022; Szczygieł et al., 2024), yet their prolonged use has driven the emergence of fungicide-resistant fungal strains and raised concerns about harm to human health and beneficial microbiota (Emmert and Handelsman, 1999; Verweij et al., 2009). The standard fungicide Bavistin, for example, indicated markedly lower activity against agriculturally important fungi than antifungal compounds isolated from bacteria such as *Bacillus cereus* subsp. *thuringiensis* and *Streptomyces* sp. (Kumar et al., 2014; Kaur et al., 2019; Uasontornnop et al., 2022). Biological control has therefore become a priority as a dependable and secure technique that reduces the need for chemical pesticides (Khan et al., 2023).

Kenyan bioprospecting for antifungal *Actinobacteria* has so far centred on the country's national parks (Nonoh et al., 2010); Karura Forest, one of the largest remaining tracts of indigenous forest in greater Nairobi, has not been examined for this purpose (Njeru, 2010). Its rhizospheric *Actinobacteria* remain untested against *Aspergillus flavus* (*A. flavus*), making it a promising site for identifying locally adapted antifungal isolates.

While *Streptomyces* is the most extensively documented genus for this purpose, antifungal activity among soil bacteria is not restricted to this genus alone. Other genera such as *Comamonas*, though less commonly studied for this purpose, have also demonstrated antifungal activity through mechanisms such as competition for nutrients and space, production of iron-chelating siderophores, and release of antifungal volatile compounds (El-Banna, 2007; Timofeeva et al., 2022; Zhao et al., 2022). Given this broader potential, the present study set out to isolate and characterize bioactive soil bacterial isolates, with *Streptomyces* spp. as the primary target genus, from rhizospheric soil collected in Karura Forest, Kenya, and to evaluate their antifungal activity as candidate biocontrol agents against *A. flavus*.

MATERIALS AND METHODS

Ethical considerations

Prior to conducting field sampling, authorization was sought from the National Commission for Science, Technology and Innovation (NACOSTI) under license Number NACOSTI/P/25/4177513.

Samples source

Bacterial isolates used in this study were obtained from soil collected at a depth of 5-10 cm and from the plant rhizosphere (root zone) in Karura Forest in Northern Central Nairobi, Kenya, a forest known for its diverse indigenous vegetation (Nyambane et al., 2016). After purification and identification, the active isolates were found to belong to two genera, *Streptomyces* and *Comamonas*. The *Aspergillus flavus* strain used in the present study was provided by the Kenya Seed Company, from an infected maize sample exhibiting visible mould growth. The maize had not been treated with chemical pesticides and was sourced from Kitale County, Kenya's maize-growing region.

Field sampling

Rhizosphere soil samples were collected from the root zones of plants at a depth of 5-10 cm from 10 sampling sites within Karura Forest. Approximately 50 g of soil was collected in duplicate from each site, placed in sterile polyethylene bags, properly labelled, and tightly sealed to prevent spillage, before being transported to the laboratory in a cool box containing ice packs at approximately 4°C. Samples were then air-dried at room temperature 25°C for 7 days, following an established protocol for selective *Streptomyces* isolation (Antido and Climacosa, 2022); this step exploits the greater desiccation resistance of actinomycete spores relative to vegetative bacterial cells, reducing non-spore-forming bacteria and other microorganisms that were not *Streptomyces* spp. (Antido and Climacosa, 2022).

Large debris was then removed by manually crushing and sieving the air-dried soil with a sterile mortar and pestle and passing it through a 0.8-mm stainless-steel mesh sieve, sterilized by autoclaving at 121°C for 20 minutes before use and between samples to minimize cross-contamination (Njenga et al., 2017; Khadayat et al., 2020).

Soil from each predefined pair of sampling sites was then pooled to produce five composite samples representing the ten original sites, in order to maximize the recovery of potentially useful *Streptomyces*-like isolates across the sampled area. Sites KE1 and KE2 were combined to form composite sample SA; sites KE3 and KE4 to form SB; sites KA1 and KA2 to form SC; sites KA3 and KA4 to form SD; and sites KA5 and KA6 to form SE; and these SA to SE, KE1 to KE4 and KA1 to KA6 labels are used throughout the present study to refer to the pooled samples. The locations of all ten individual sampling sites within Karura Forest are shown in Figure 1.

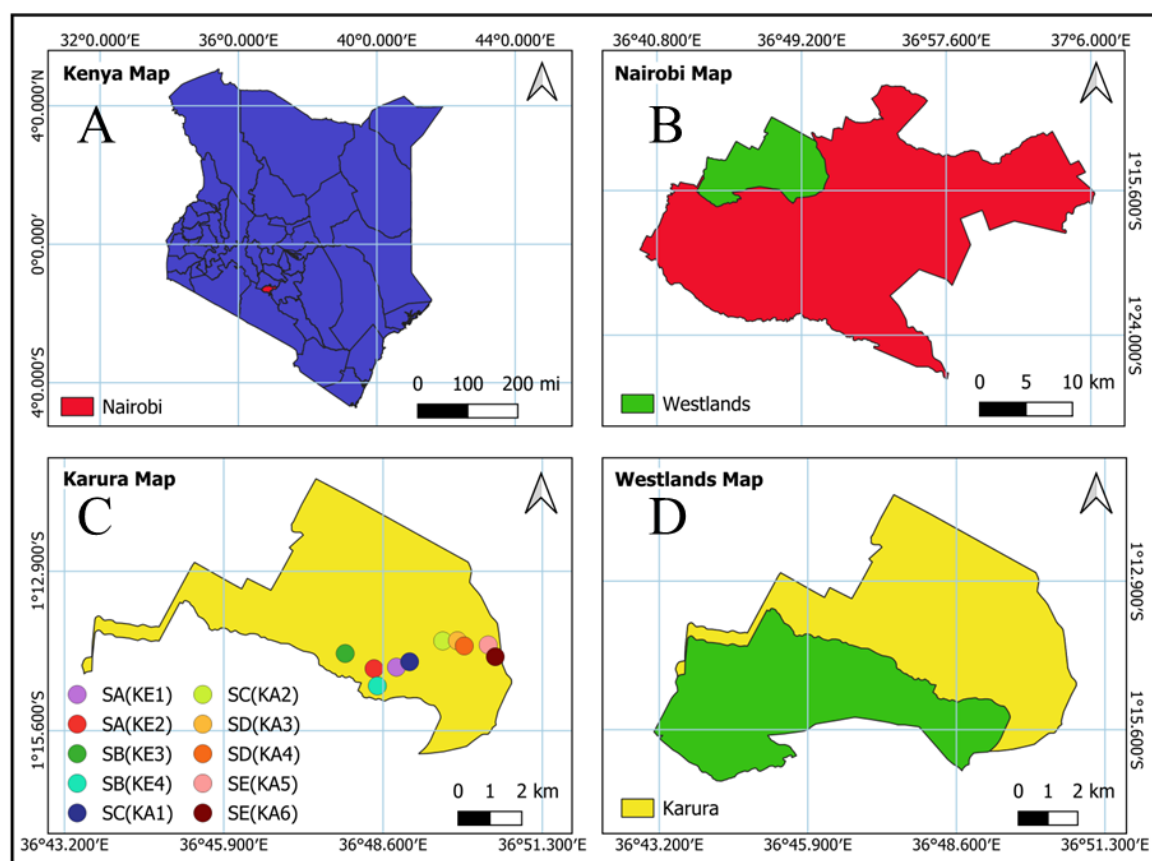


Figure 1. Location of the study area. **A:** Kenya, showing Nairobi County, **B:** Nairobi County, showing Westlands sub-county, **C:** Karura Forest, showing the ten sampling sites (KE1-KE4, KA1-KA6) color-coded by composite sample (SA-SE), **D:** Westlands sub-county, showing the boundary of Karura Forest.

Isolation of *Streptomyces*-like bacteria

Streptomyces-like bacteria were isolated from soil samples using a conventional serial dilution plate method on Yeast Extract-Malt Extract Agar (ISP2), prepared according to the formulation described by [Shirling and Gottlieb \(1966\)](#), including 4 g/L yeast extract (Loba Chemie Pvt. Ltd., India), 10 g/L malt extract (Loba Chemie Pvt. Ltd., India), 4 g/L dextrose (Loba Chemie Pvt. Ltd., India), and 20 g/L agar (Griffchem Fine Chemicals, India), with the pH adjusted to 7.2. No antifungal or antibacterial supplements were added to the medium, in order to avoid suppressing antibiotic-sensitive *Streptomyces* morphotypes; selectivity was instead achieved through soil pretreatment, morphological screening, and repeated purification. 3 g of soil was taken and subsequently mixed with 27 mL of sterile distilled water and vortex-mixed until visibly homogeneous (approximately 1 minute) to prepare a 10^{-1} suspension, with each subsequent tenfold dilution vortex-mixed in turn up to 10^{-6} . An aliquot of 0.1 mL from each dilution (10^{-1} to 10^{-6}) was plated in triplicate onto ISP2 agar plates ([Kharel et al., 2010](#); [Caceres et al., 2018](#)). Plates were checked daily for the appearance of chalky, powdery, or leathery colonies typical of *Streptomyces* spp. and incubated for 5-7 days at 28°C ([Saraswathi et al., 2020](#); [Kazi et al., 2022](#)). Between 2 and 8 presumptive colonies were selected from each plate, depending on colony abundance, and purified by repeated streaking onto fresh ISP2 plates until visually pure cultures were obtained. The purified isolates were subsequently used for characterization and co-culture assays. Working cultures were maintained on ISP2 slants at 4°C for use throughout the study, while backup stocks were preserved in 20% glycerol at -20°C or -80°C for future use ([Njenga et al., 2017](#)).

Characterization of *Streptomyces*-like isolates

Identification of *Streptomyces*-like isolates relied on cultural and morphological features, assessed according to the *Actinobacteria* criteria outlined in *Bergey's Manual of Systematic Bacteriology* ([Kämpfer, 2012](#)). Colony color and texture were recorded for each isolate, with color classified according to the scheme described by [Uasoontornnop et al. \(2022\)](#); isolates displaying colony morphology characteristic of *Streptomyces* spp., such as chalky, powdery, or leathery texture, were retained for further study. Gram reaction, along with catalase and indole tests as supportive biochemical checks, was determined afterward, on isolates carried forward through dual culture screening, following standard procedures ([MacFaddin, 2000](#); [Njenga et al., 2017](#)).

Initial screening of *Aspergillus flavus*-inhibiting bacterial isolates through co-culture

The possible antagonistic effects of *Streptomyces*-like species against *A. flavus* were assessed using dual culture. All isolates displaying *Streptomyces*-like colony morphology, regardless of Gram reaction, were screened for antagonistic activity against *A. flavus* by dual culture. A loopful of the bacterial isolate was streaked onto one side of the Potato Dextrose Agar (PDA; Oxoid, United Kingdom) plate, no less than 3 cm from the center, and pre-incubated for 3-5 days at 28°C to allow the streak to establish before the fungus was introduced ([Sangdee et al., 2016](#); [Campos-Avelar et al., 2021](#)). A 6-mm-diameter agar plug was then removed from the leading edge of a 7-day-old *A. flavus* culture and positioned on the opposite side of the plate, no more than 3 cm from the center; the dual-culture plates were incubated for a further 5 days at 28°C, with three replicates per isolate. Negative-control plates contained only the *A. flavus* plug on PDA, with no bacterial streak, and were incubated under the same conditions. At this stage, inhibition was assessed qualitatively, based on the presence or absence of a visible zone of reduced fungal growth; isolates showing clear inhibition were selected as candidate antagonists for further investigation ([Sangdee et al., 2016](#); [Shakeel et al., 2018](#)).

Extraction of the bioactive compounds from the bacterial isolates

A loopful of each bacterial isolate was inoculated into 250 mL of ISP2 broth and incubated at 28°C for 7 days on a rotary shaker at 150 rpm to promote production of antifungal substances ([Shakeel et al., 2018](#)). Following incubation, the fermentation broth was centrifuged at 2,000 rpm for 5 minutes at 20°C to separate the bacterial biomass from the culture supernatant. The supernatant was passed through a 0.22 µm membrane filter and designated as the crude filtrate (CF), used directly for antifungal activity testing. The remaining supernatant was extracted with an equal volume of ethyl acetate in a separating funnel, shaken overnight at 28°C, and left to separate into two distinct phases; the upper ethyl acetate layer was collected as the supernatant crude extract (SCE). The biomass pellet was likewise extracted overnight at 28°C, using 150 mL of methanol (Laboklin Chimique, France), to yield the pellet crude extract (PCE). Both extracts were concentrated under reduced pressure using a rotary evaporator (Labtech EV400-L, Italy) at 45°C, and the final crude extracts (SCE and PCE) were stored at 4°C until use in antifungal activity assays ([Shakeel et al., 2018](#)).

In vitro antifungal activity using the disc diffusion assay

The crude extracts (PCE, SCE, and CF) were dissolved in dimethyl sulfoxide (DMSO; Griffchem, India) to a stock concentration of 15 mg/mL (w/v) and stored at 4°C until required. Working solutions at a final concentration of 1.5

mg/mL were prepared by diluting the stock in sterile distilled water. Antifungal activity was evaluated by disc diffusion, including sterile paper discs (Whatman, 12.5 cm, U.K.), 6 mm in diameter, were placed onto PDA plates inoculated with a fungal spore suspension (10^6 spores/mL) using a sterile cotton swab, in line with the concentration used by [Abdel-Nasser et al. \(2024\)](#), followed by application of a 20 μ L aliquot of each extract (PCE, SCE, or CF) to the discs, alongside a positive control disc (Nystatin, SD025-1CT, HiMedia Laboratories, India) at 100 μ g/mL and phosphate-buffered saline (PBS) as the negative control. Plates were incubated at 28°C for 72 hours, after which inhibition zones were measured, with the 6 mm disc diameter subtracted from each reading to obtain the true zone of inhibition. The assay was conducted in three replicates ([Shakeel et al., 2018](#)).

Chemical profiling using gas chromatography-mass spectrometry

The crude extracts of the six active bacterial isolates were analyzed by gas chromatography-mass spectrometry (GC-MS), a technique suitable for the detection of volatile and semi-volatile organic compounds in complex biological samples, to identify bioactive metabolites potentially responsible for the antifungal activity observed in the *in vitro* screening assay. The pellet crude extract (PCE) of each isolate was analyzed directly in absolute methanol, prior to injection. Profiling of SA18, SD102, SD109, SD110, SE143, and SE194 was performed on a Shimadzu GC-MS (QP-2010SE system, Japan), and detected compounds were tentatively identified by comparing their mass spectra against the NIST 14 Mass Spectral Library.

Molecular characterization based on 16S rRNA sequencing

DNA was extracted from isolates showing activity in the *in vitro* antifungal assays, using the Quick-DNA Fungal/Bacterial Miniprep kit (D6005, Zymo Research, USA) according to ZYMO Research's protocol. The 16S rRNA gene was then amplified by PCR in a Biometra TRIO Thermal Cycler (Analytik Jena, Germany), using the universal primers fD1 (27F: AGAGTTTGATCMTGGCTCAG) and rP3 (1492R: TACGGYTACCTTGTTACGACTT; [Weisburg et al., 1991](#)). The cycling program comprised an initial denaturation at 94°C for 5 minutes, followed by 31 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 90 seconds, and extension at 72°C for 120 seconds, with a final extension step at 72°C for 5 minutes. Under these conditions, the primer pair yields a product of approximately 1.4 to 1.5 kb ([Frank et al., 2008](#)). Amplicons were resolved on a 1% (w/v) agarose gel in 1× TAE buffer, alongside an Accuris SmartCheck 50 bp DNA Ladder (Accuris Instruments, USA), stained with SafeView (ABM G108), and visualized on an E-Box Gel Documentation System (Vilber, France). The products were subsequently purified and sent for Sanger sequencing at MacroGen, Europe. Background noise was removed from the resulting sequences, which were then edited in BioEdit software version 7.0.5.2. The edited sequences were compared against the NCBI database using BLASTn for identification ([Abiola and Oyetayo, 2016](#); [Antido and Climacosa, 2022](#); [Seidu et al., 2024](#)). The sequences were then aligned using MUSCLE in MEGA11, and a phylogenetic tree was inferred by the Neighbor-Joining method, with node support assessed through 1,000 bootstrap replicates ([Tamura et al., 2021](#)).

Statistical analysis

OriginPro® (2024b) software was utilized for graphical analysis and data processing, with each experiment performed at least three times. Data were expressed as the mean $\pm$ standard deviation and analysed using one-way analysis of variance (ANOVA) with Tukey's test. Statistically significant differences across different groups were identified at $p < 0.05$.

Accession numbers

The bacterial isolates identified in the present study have been deposited in NCBI GenBank under the following accession numbers, including PZ675387, PZ675345, PZ675385, PZ675346, PZ675374, and PZ675373.

RESULTS AND DISCUSSION

Streptomyces-like isolate characterization

After subculturing, presumptive *Streptomyces*-like colonies collectively displayed aerial mycelia ranging from white to grey, brown, and black, with dry, chalky, raised, and wrinkled colony surfaces (Figure 2). A total of 190 isolates displaying *Streptomyces*-like colony morphology were selected for a dual-culture screening assay against *A. flavus*, with Gram staining and biochemical characterization carried out afterward, including on isolates that indicated antifungal activity in the screening. All six active isolates, SA18, SD102, SD109, SD110, SE143, and SE194, were catalase-positive and indole-negative. Five active isolates, SA18, SD102, SD109, SD110, and SE194, were Gram-positive and filamentous, consistent with the phenotypic characteristics of *Streptomyces* spp., in agreement with previous studies

(Law et al., 2017; Khan et al., 2023). *Streptomyces* species have also been reported to produce secondary metabolites capable of inhibiting *A. flavus* (Managamuri et al., 2017; Alam et al., 2022). Isolate SE143 was Gram-negative and was subsequently identified by molecular analysis as *Comamonas* sp. rather than *Streptomyces*.

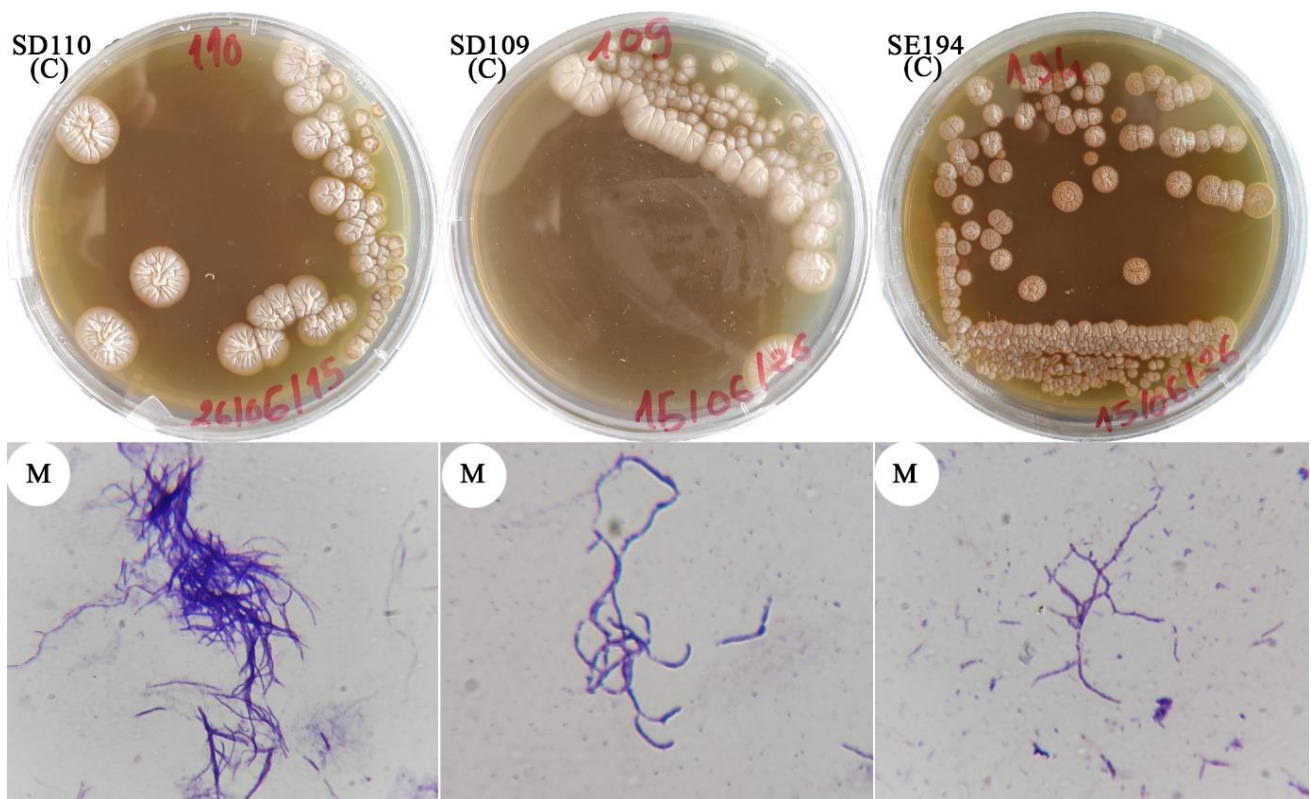


Figure 2. Morphological characterization of representative *Streptomyces* isolates SD110, SD109, and SE194. The upper panels correspond to the macroscopic colony (C) morphology, such as dry, raised, wrinkled, and chalky colonies, which are typical of *Streptomyces* spp. On Yeast Extract-Malt Extract Agar plates at 7 days. The lower panels correspond to the microscopic morphology (M), showing Gram-positive, filamentous, and branching structures, consistent with Actinobacterial characteristics.

Characterization of *Aspergillus flavus*

The *A. flavus* strain was examined macroscopically and microscopically on PDA. The gross colony morphology, including its yellowish-green to olive-green color, and the microscopic features in lactophenol cotton blue-stained wet mounts were consistent with *A. flavus* morphology (Hedayati et al., 2007; Leema et al., 2010), as shown in Figure 3.

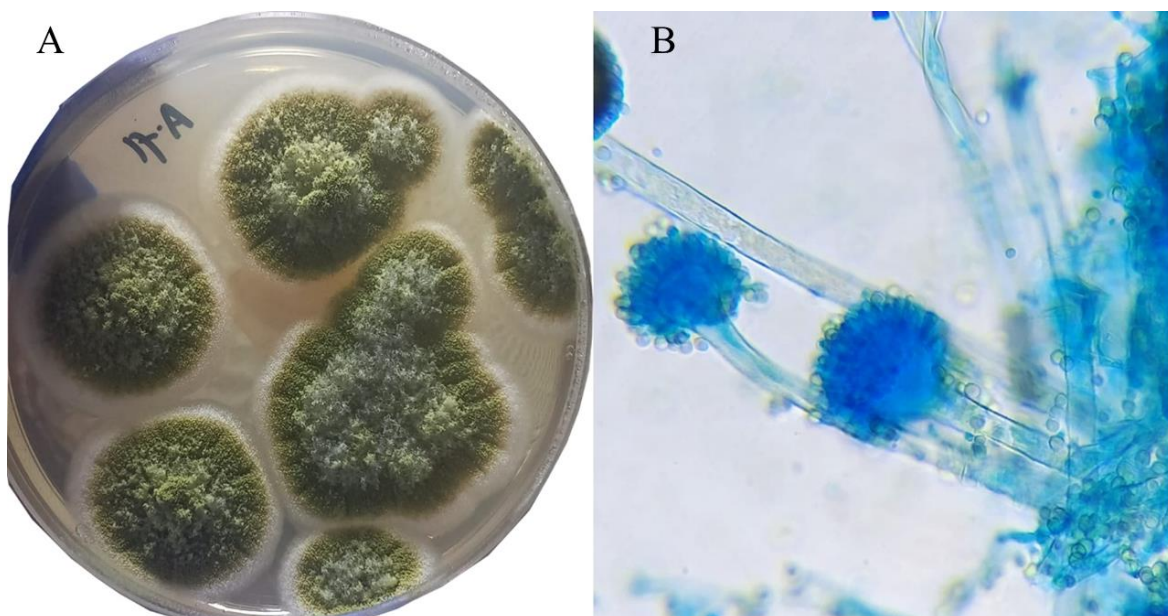


Figure 3. The colony and microscopic features of *Aspergillus flavus*. **A:** The 7-day-old colonies on Potato Dextrose Agar appear powdery, yellowish-green to olive-green with white margins, indicating active growth and spore production. **B:** Microscopically, septate hyphae, upright conidiophores, swollen vesicles, and chains of round conidia are observed, which support the identification of the isolate as *Aspergillus flavus*.

Initial screening of *Aspergillus flavus*-inhibiting bacterial isolates through co-culture

Six of the isolates tested in the initial dual-culture screening, SA18, SD102, SD109, SD110, SE143, and SE194, revealed potential antagonistic activity against *A. flavus*. All six isolates produced large inhibition zones, while the negative control indicated no inhibition. The isolates were therefore selected for extraction and the subsequent antifungal assay (Figure 4). The pattern of inhibition zones observed in the co-culture screening, contrasted with the lack of inhibition in the negative control, suggested that the isolates possess the ability to produce secondary metabolites capable of inhibiting *A. flavus*, consistent with antagonistic behavior reported for other soil bacterial isolates against *A. flavus* in co-culture (Sangdee et al., 2016; Shakeel et al., 2018; Campos-Avelar et al., 2021).

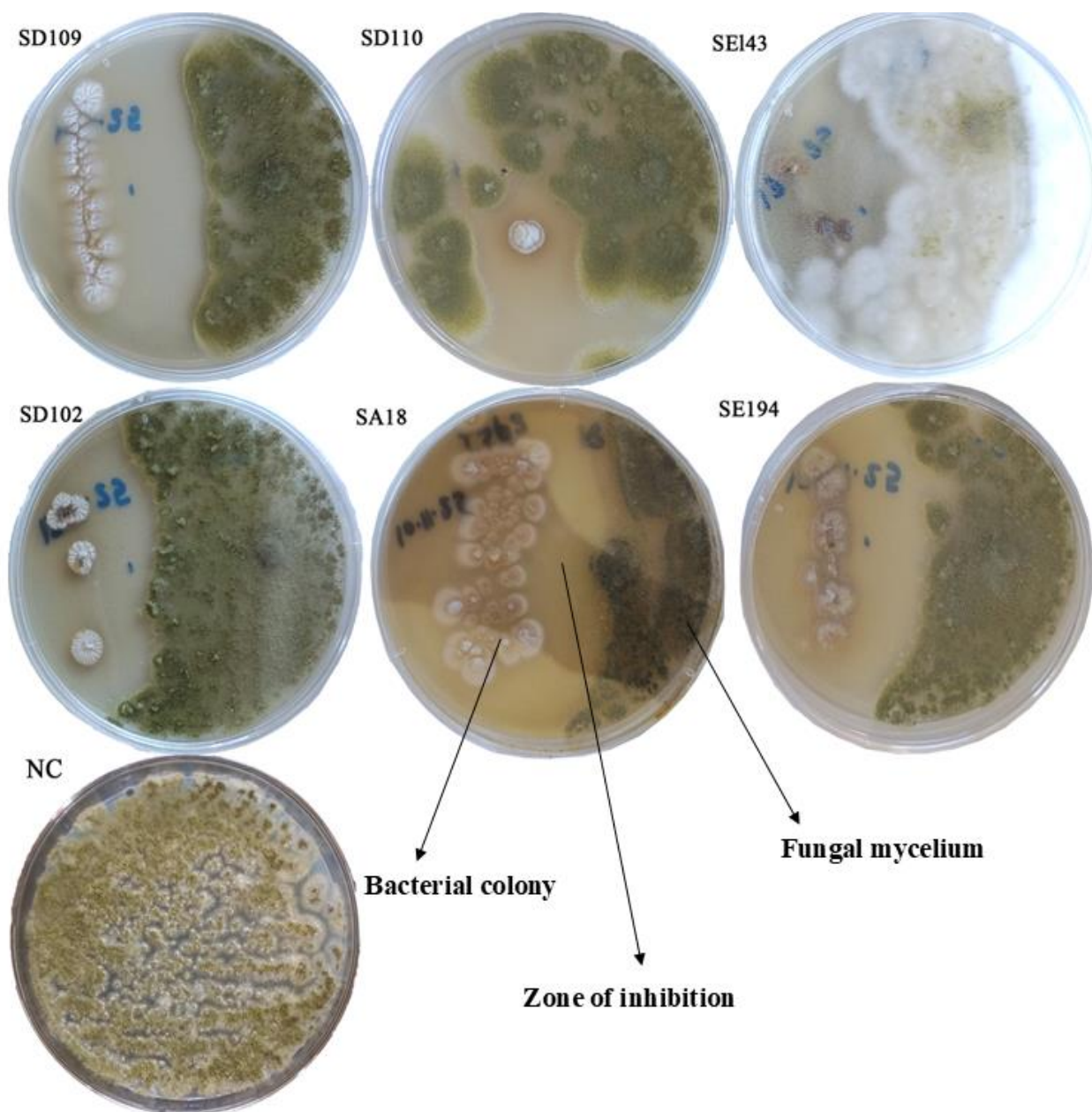


Figure 4. Dual culture assay revealing the antagonistic activity of selected *Streptomyces*-like isolates against *Aspergillus flavus* on Potato Dextrose Agar plates. The isolates SA18, SD102, SD109, SD110, SE143, and SE194 indicated inhibition of growth of *A. flavus*, as evidenced by the clear zone of inhibition observed between the bacterial colonies and the fungal mycelium.

In vitro antifungal assay

As illustrated in Figure 5A, the negative control (PBS) indicated no inhibition of *A. flavus*; however, the inhibitory zone was clearly observable across all isolates subjected to a concentration of 1.5 mg/mL CE for each of the PCE, SCE, and CF.

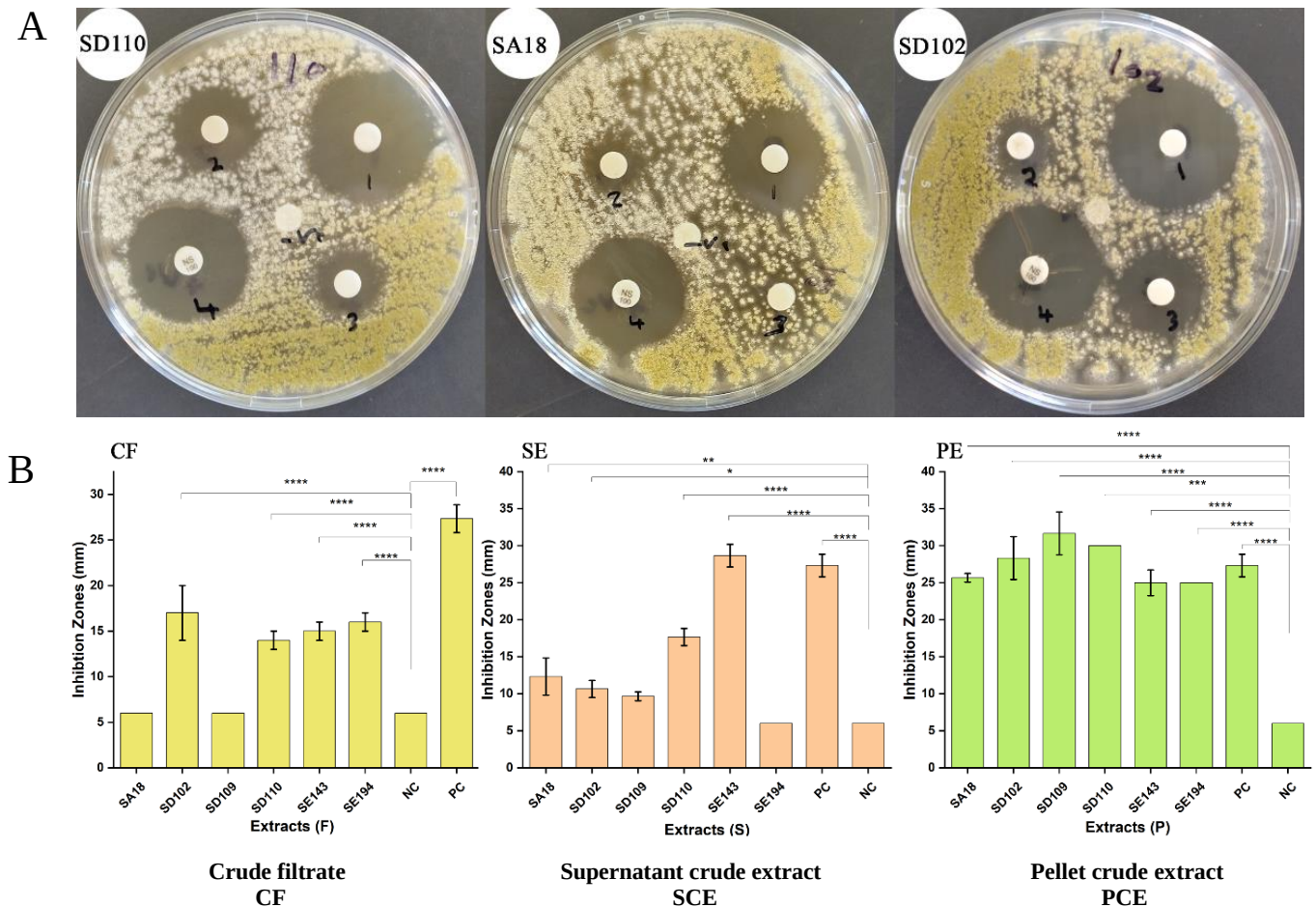


Figure 5. Antifungal activity of bacterial crude extracts against *A. flavus* in the disc diffusion assay. Inhibition zones produced by the extracts and controls for three of the six selected isolates against *A. flavus*. Discs 1, 2, 3, and 4 represent the pellet extract, supernatant extract, crude filtrate, and positive control, respectively. **A:** The negative control, PBS, was located in the center of each plate. **B:** Statistical analysis of the zones' diameters of the extracts against the negative control at the 0.05 level. The extracts significantly inhibited the growth of *A. flavus* compared with the negative control ($p < 0.05$).

The PCE indicated inhibition zones of 25.60 ± 0.50 , 28.30 ± 2.80 , 31.60 ± 2.80 , 30.00 ± 0.00 , 25.00 ± 1.70 , and 25.00 ± 0.00 mm, corresponding to SA18, SD102, SD109, SD110, SE143, and SE194, respectively. The SCE, tested at the same concentration, indicated inhibition zones of 12.30 ± 2.50 , 10.60 ± 1.10 , 9.60 ± 0.50 , 17.60 ± 1.10 , 28.60 ± 1.50 , and 0.00 mm for the same six isolates, respectively. Notably, SE143 was the one isolate for which the SCE (28.60 ± 1.50 mm) outperformed the PCE (25.00 ± 1.70 mm), the reverse of the pattern seen in the other five isolates, indicating that its antifungal metabolites were released into the culture supernatant rather than remaining concentrated in the biomass. The CF produced inhibition zones of 0.00 , 17.00 ± 3.00 , 0.00 , 14.00 ± 1.00 , 15.00 ± 1.00 , and 16.00 ± 1.00 mm for SA18, SD102, SD109, SD110, SE143, and SE194, respectively, while the positive control produced an inhibition zone of (27.30 ± 1.50) mm (Figure 5B). Several PCE values, most notably SD109 at 31.60 ± 2.80 mm, exceeded the positive control, while others, including SA18, SD102, and SE143, remained below it. The inhibition zones of all PCE at the tested concentration were significantly different compared to the negative control ($P < 0.05$); meanwhile, the zones of inhibition for the SCE for all isolates were significantly different except for SD109 and SE194 isolates (Figure 5B). Furthermore, the CF indicated significant differences in most of the isolates, including SD102, SD110, SE143, and SE194, without any difference in the SA18 and SD109 isolates (Figure 5B).

Taken together, the data indicated that the PCE consistently outperformed the supernatant extract and crude filtrate across most isolates, a pattern consistent with reports that many *Streptomyces* secondary metabolites remain associated with the mycelium or adsorbed onto the cell surface rather than being released into the surrounding medium, generally yielding more bioactive material from biomass extracts than from extracellular fractions (Manteca et al., 2008; Van Wezel and McDowall, 2011; Barka et al., 2015). Extracellular activity was not entirely absent, however: the supernatant crude extract inhibited *A. flavus* growth for five of the six isolates, and the crude filtrate inhibited growth for four of the six isolates, generally at lower potency than the pellet extract. Only SE194's supernatant extract, along with the crude filtrates of SA18 and SD109, revealed no detectable inhibition, which may reflect that extracellular metabolites were not produced at that growth stage, were synthesized below the inhibitory threshold, or were degraded during extraction and cultivation (Djinni et al., 2014). Similar observations have been reported for other *Streptomyces* species, in which antifungal activity was mostly shown in mycelial extracts, indicating the relevance of intracellular or cell-related metabolites in fungal proliferation suppression (Manteca et al., 2008; Prapagdee et al., 2008).

Molecular identification of isolates using 16S rRNA sequencing

Molecular analysis confirmed the morphological identification of five of the six soil isolates from Karura Forest as *Streptomyces*, while SE143 was reclassified as *Comamonas*, correcting its earlier morphology-based identification as a *Streptomyces*-like colony. Isolates were originally selected based on colony traits typical of *Streptomyces*. Amplification was clearly visible in all six lanes on the agarose gel, including SA18, SD102, SD109, SD110, SE143, and SE194, with PCR products of approximately 1.5 kb (Figure 6).

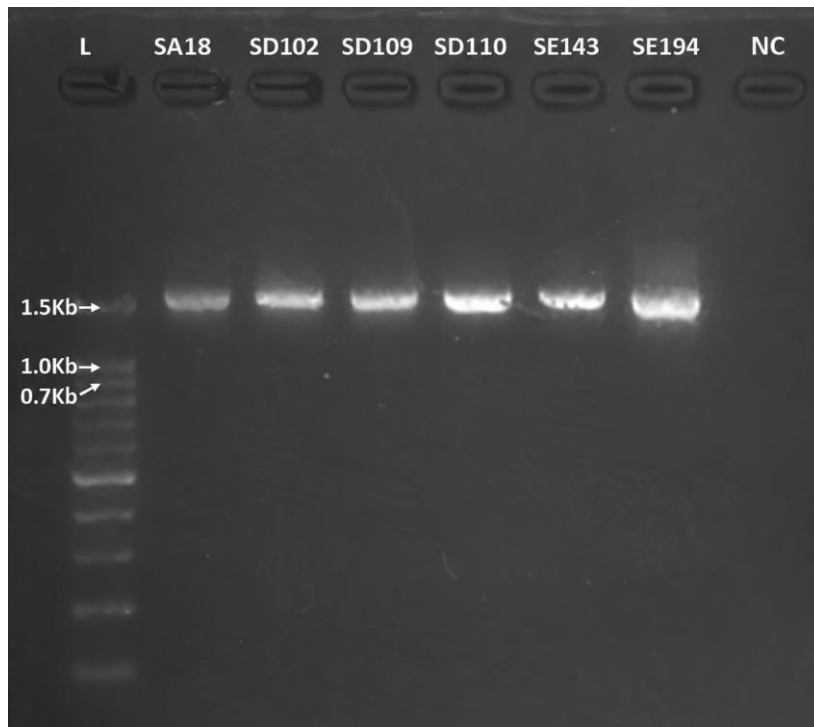


Figure 6. Electrophoresis of 16S rRNA PCR amplicons from antifungal bacterial isolates. Lane L represents the 50 bp DNA ladder, while lanes SA18, SD102, SD109, SD110, SE143, and SE194 indicate PCR products from isolates that exhibited antifungal activity, and lane NC represents the no-template negative control. The discrete bands observed at approximately 1.5 kb confirm successful amplification of the bacterial 16S rRNA gene, with no amplification in the negative control.

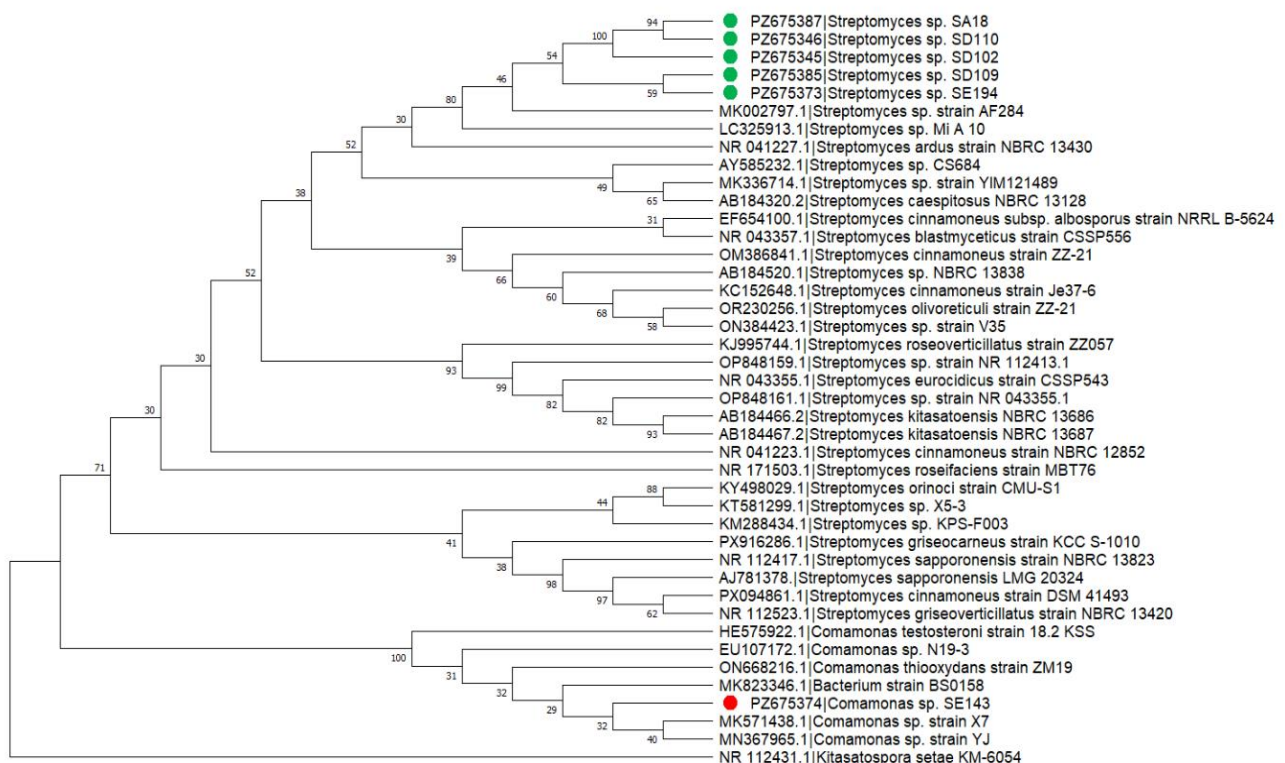


Figure 7. Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between the selected isolates SA18, SD110, SD102, SD109, SE194, and SE143 and closely related reference strains obtained from GenBank. The clustering pattern places SA18, SD102, SD109, SD110, and SE194 within the genus *Streptomyces*, while SE143 clusters separately within the genus *Comamonas*.

Analysis of 1,634 aligned 16S rRNA nucleotide positions enabled the resolution of six bioactive Karura Forest soil isolates into two independent bacterial lineages; a dominant *Streptomyces* lineage and a separate branch of *Comamonas*-like bacteria. Five of the isolates (SA18, SD102, SD109, SD110, and SE194) were definitively clustered within the *Streptomyces* genus. The five *Streptomyces* isolates are indicated by green markers, while SE143 is shown by a red marker. Bootstrap values are displayed at the internal nodes, and *Kitasatospora setae* KM-6054 was included as the outgroup (Figure 7).

The phylogenetic placement demonstrated that most of the active antifungal strains belong to the phylum *Actinomycetota*, specifically the genus *Streptomyces*. The predominance of *Streptomyces* among the antifungal-active isolates aligned with earlier reports describing *Streptomyces* as a ubiquitous and readily culturable soil inhabitant capable of producing a wide range of bioactive secondary metabolites, a combination of traits that likely explains why *Streptomyces* dominated the pool of antifungal isolates recovered from Karura Forest soil in the present study (Dow et al., 2023; Manhas et al., 2023).

Further analysis of the topology of this genus identified a sibling-taxa pair (94% bootstrap value) between isolates SA18 and SD110, suggesting a close phylogenetic relationship in the forest ecosystem. SD102 diverged closest to this pair with strong bootstrap support of 100%, and SD109 and SE194 formed a neighboring sister subclade (Figure 7). All five isolates cluster closely within the same clade, but their different branch lines revealed that they are distinct wild-type strains. However, the 16S rRNA gene is highly conserved, and single-gene alignments generally lack sufficient resolution to confidently resolve *Streptomyces* to the species level. Consistent with a recent study by Kiepas et al. (2024) that 16S rRNA identity is a poor surrogate for multi-locus or whole-genome species classifications in *Streptomyces* genus, and the isolates have therefore been cautiously categorized as *Streptomyces* sp; the clear isolation of these bacteria from the standard database reference strains clearly suggests that Karura Forest harbors a may harbor locally adapted *Streptomyces* varieties of locally adapted *Streptomyces* varieties.

The *in vitro* inhibitory activity of the six indigenous isolates against *A. flavus* growth points to a potential tool for addressing a key ecological and agricultural concern in Kenya, aflatoxin contamination, although the present results reflect growth inhibition under laboratory conditions rather than confirmed suppression of aflatoxin biosynthesis or performance in field or storage settings. The growth inhibition of *A. flavus* observed here is consistent with earlier reports indicating that environmental *Streptomyces* strains can suppress *A. flavus* mycelial growth, with some also shown to reduce aflatoxin B1 biosynthesis, supporting the broader potential of *Streptomyces* as a biocontrol resource (Shakeel et al., 2018; Campos-Avelar et al., 2021).

Interestingly, Isolate SE143 was completely different from the actinobacterial clusters and clustered closely within the genus *Comamonas* with the reference strains X7 and YJ. The divergent placement of SE143 raises the possibility that antifungal potential within the Karura Forest soil microbiome is not limited to *Actinobacteria*, though that pattern rests on a single non-*Streptomyces* isolate and would need confirmation from additional *Comamonas* or other non-actinobacterial isolates before it can be generalised. *Comamonas* species are not as commonly used as *Streptomyces* for direct antifungal uses, but their opportunistic metabolic flexibility is gaining recognition (El-Banna, 2007). Other *Comamonas* strains have been reported to inhibit fungal pathogens through mechanisms such as competition for space and nutrients, iron-chelating siderophore production, and the release of antifungal volatile organic compounds (El-Banna, 2007; Timofeeva et al., 2022; Zhao et al., 2022). None of the proposed mechanisms were investigated for SE143 in the present study, so their relevance to SE143's antifungal activity remains to be tested.

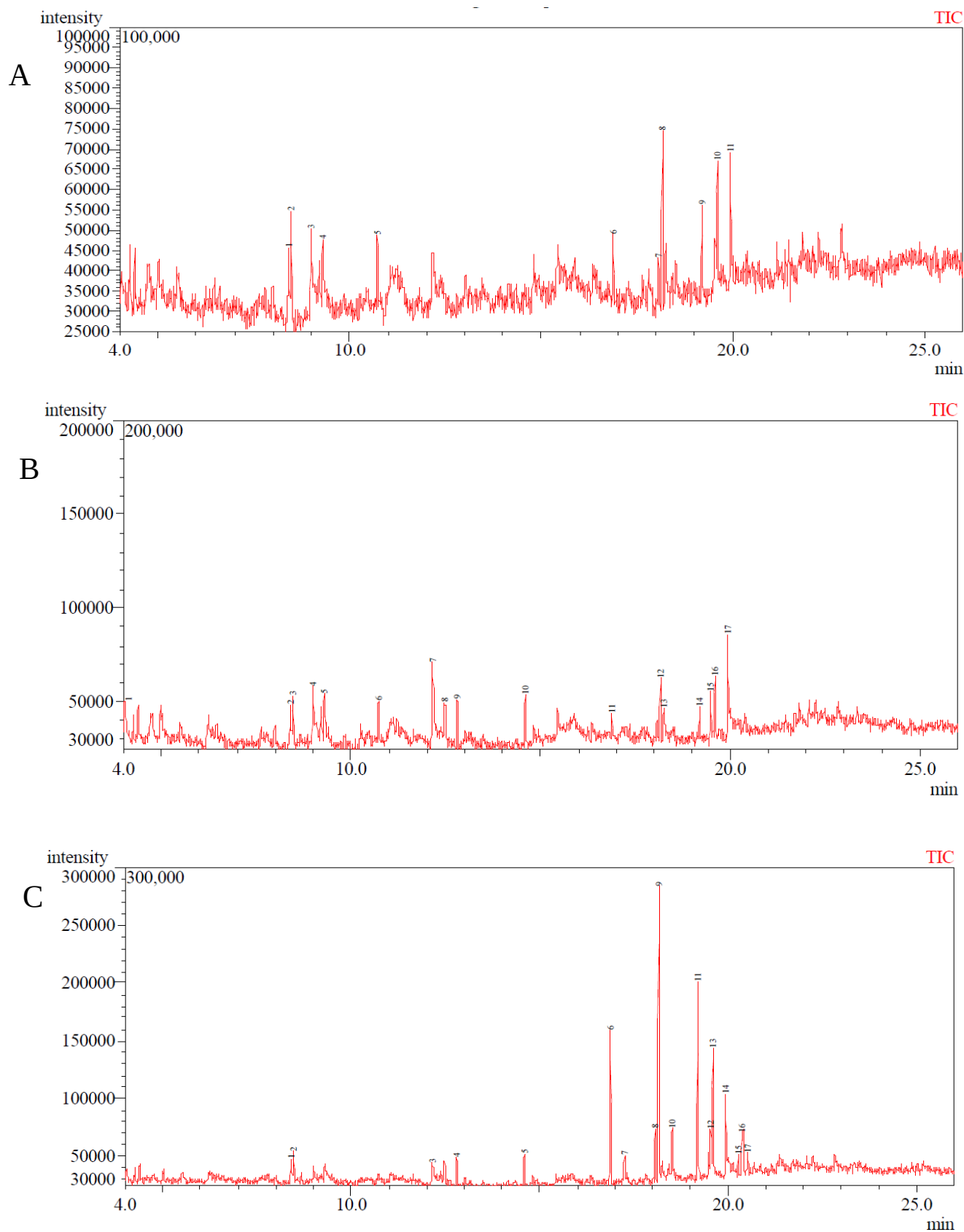
Gas chromatography-coupled mass spectrometry analysis

Chemical components of the PCE of the bacterial isolates SA18, SD102, SD109, SD110, SE143, and SE194, as determined by GC-MS, are shown in Figure 8A-F and Tables 1 to 6.

GC-MS analysis of the six bioactive isolates produced chromatograms of varying complexity, with the number of peaks ranging from 11 (SD102 and SE194) to 19 (SD110) across an overlapping retention window of roughly 4.0 to 21.4 minutes (Figure 8A-F, Tables 1 to 6). Fatty acids and their methyl esters, particularly tetradecanoic, pentadecanoic, and hexadecanoic acid derivatives, formed the leading peak class in SD102, SD109, and SE143. SA18, SD110, and SE194 instead revealed a more oxygenated profile, led by butanedioic acid derivatives, dl-glyceraldehyde dimer, and 2(3H)-furanone, dihydro-4-hydroxy-. Notably, SE143, which was identified as *Comamonas* rather than *Streptomyces*, still produced a lipid-rich profile comparable to the *Streptomyces* isolates, dominated by pentadecanoic acid, 14-methyl-, methyl ester and related fatty acid methyl esters.

Across all six isolates, the chromatograms converged on a shared core of chemical classes fatty acids and fatty acid methyl esters; organic acid derivatives, such as substituted butanedioic acids; furanone derivatives; and glyceraldehyde-related oxygenated compounds. Two isolates, SD110 and SE194, additionally carried the nitrogen-containing heterocycle pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, a metabolite previously reported from *Streptomyces* extracts

(Ser *et al.*, 2015), while the phenolic compound 2,4-di-*tert*-butylphenol was restricted to SA18 and SD109. Minor peaks were recorded for every isolate, including cyclopropyl carbinol, short-chain hydrocarbons, and additional fatty acid methyl ester isomers. The isolates therefore differed less in the broad chemical classes present than in the relative abundance and the specific fatty acid or organic acid derivatives that dominated each extract.



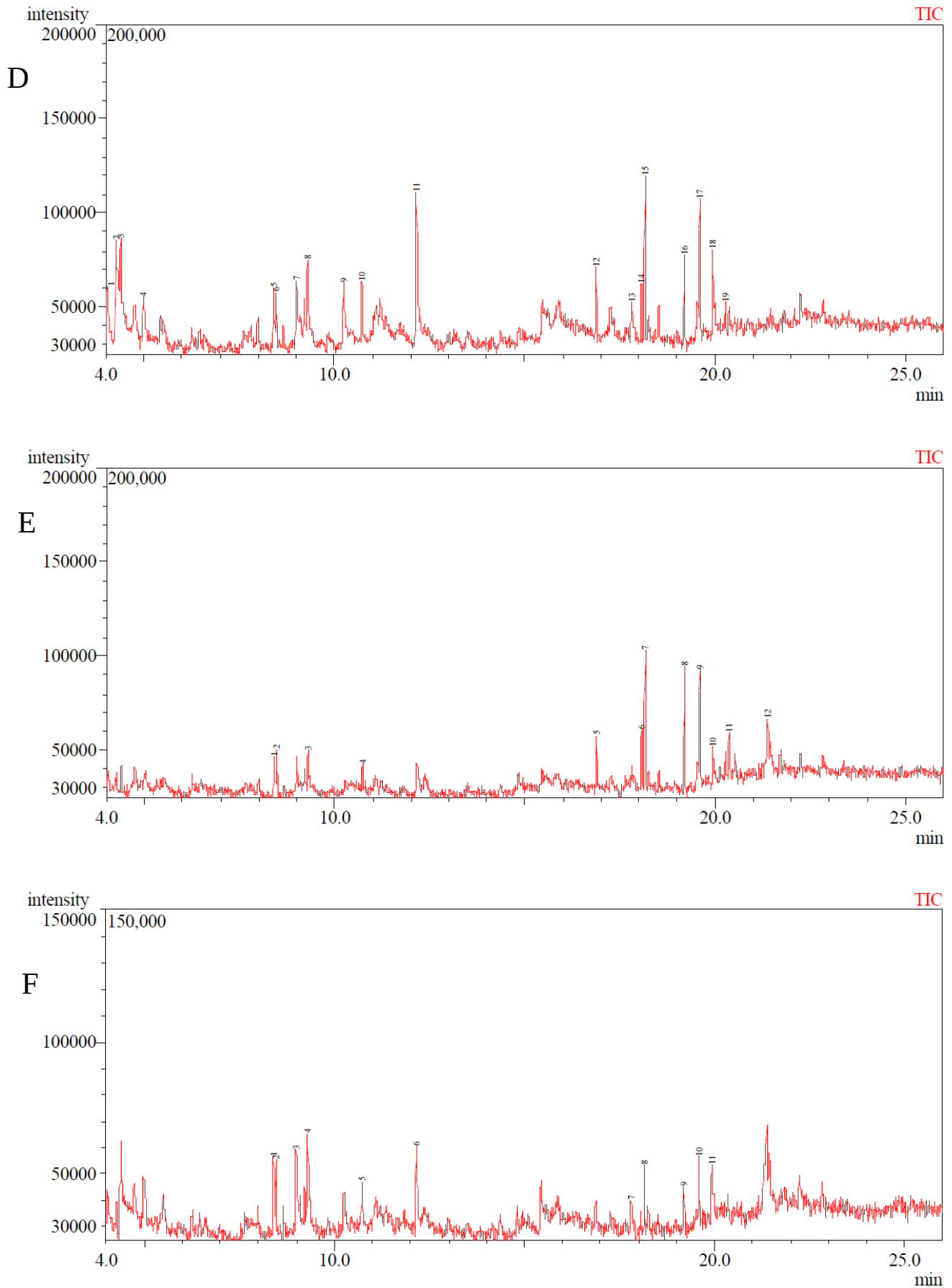


Figure 8. Gas chromatography-mass spectrometry total ion chromatogram of isolates; **A:** SA18, **B:** SD102, **C:** SD109, **D:** SD110, **E:** SE143, and **F:** SE194.

Table 1. Gas chromatography-mass spectrometry profile of bioactive compounds identified in SA18 isolate

SA18 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	4.035	4.006	4.37	Glyceraldehyde	90.08	C ₃ H ₆ O ₃	751
2	8.413	8.346	5.56	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
3	8.472	8.445	4.81	Tridecane, 2-methyl-	198.39	C ₁₄ H ₃₀	15269
4	9.000	8.958	8.65	Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*,R*)]-	234.16	C ₈ H ₁₀ O ₈	103496
5	9.297	9.262	7.75	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	C ₄ H ₆ O ₃	95652
6	10.729	10.691	4.89	1,2,3-Propanetriol, 1-acetate	102.09	C ₄ H ₆ O ₃	33510
7	12.160	12.114	12.44	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	C ₅ H ₈ O ₅	441696
8	12.472	12.447	4.43	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	216.32	C ₁₂ H ₂₄ O ₃	6490
9	12.796	12.756	4.98	Butanoic acid, butyl ester	144.21	C ₈ H ₁₆ O ₂	7983
10	14.601	14.564	5.09	2,4-Di-tert-butylphenol	206.32	C ₁₄ H ₂₂ O	7311
11	16.880	16.857	1.79	Tridecanoic acid, 12-methyl-, methyl ester	242.40	C ₁₅ H ₃₀ O ₂	21204
12	18.159	18.128	5.26	Tetradecanoic acid, 12-methyl-, methyl ester; (S)-	256.42	C ₁₆ H ₃₂ O ₂	23618376
13	18.249	18.210	3.27	3-Hydroxy-4,5-dimethoxybenzoic acid	198.17	C ₉ H ₁₀ O ₅	74709
14	19.193	19.167	2.41	Hexadecanoic acid, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	8181
15	19.476	19.447	4.91	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	282.33	C ₁₇ H ₂₄ O ₃	545303
16	19.597	19.534	6.29	Methyl tetradecanoate	242.40	C ₁₅ H ₃₀ O ₂	31284
17	19.932	19.896	13.07	Pentadecanoic acid	242.40	C ₁₅ H ₃₀ O ₂	13849

Table 2. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD102

SD102 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.414	8.369	6.75	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
2	8.470	8.439	8.80	Tridecane, 2-methyl-	198.39	C ₁₄ H ₃₀	15269
3	8.994	8.958	9.23	dl-Glyceraldehyde dimer	180.16	C ₆ H ₁₂ O ₆	251615
4	9.299	9.262	9.22	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	C ₄ H ₆ O ₃	95652
5	10.726	10.697	6.62	1,2,3-Propanetriol, 1-acetate	134.13	C ₅ H ₁₀ O ₄	33510
6	16.878	16.810	6.20	Tridecanoic acid, 12-methyl-, methyl ester	242.40	C ₁₅ H ₃₀ O ₂	21204
7	18.059	18.047	3.59	Pentadecanoic acid, methyl ester	256.42	C ₁₆ H ₃₂ O ₂	23518
8	18.157	18.087	14.07	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	C ₁₆ H ₃₂ O ₂	23618376
9	19.196	19.143	8.39	Methyl tetradecanoate	242.40	C ₁₅ H ₃₀ O ₂	31284
10	19.597	19.558	11.43	Hexadecanoic acid, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	8181
11	19.932	19.896	15.71	Tetradecanoic acid	228.37	C ₁₄ H ₂₈ O ₂	11005

Table 3. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD109

SD109 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.411	8.375	2.34	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
2	8.470	8.439	2.69	Tridecane	184.36	C ₁₃ H ₂₈	12388
3	12.160	12.114	2.38	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	C ₅ H ₈ O ₅	441696
4	12.796	12.768	2.69	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	216.32	C ₁₂ H ₂₄ O ₃	6490
5	14.602	14.570	2.36	2,4-Di-tert-butylphenol	206.32	C ₁₄ H ₂₂ O	7311
6	16.878	16.839	11.97	Tridecanoic acid, 12-methyl-, methyl ester	242.40	C ₁₅ H ₃₀ O ₂	21204
7	17.254	17.230	1.64	Tetradecanoic acid	228.37	C ₁₄ H ₂₈ O ₂	11005
8	18.065	18.029	4.06	Pentadecanoic acid, methyl ester	256.42	C ₁₆ H ₃₂ O ₂	23518
9	18.159	18.105	22.70	Methyl tetradecanoate	242.40	C ₁₅ H ₃₀ O ₂	31284
10	18.517	18.484	3.78	Eicosanoic acid	312.53	C ₂₀ H ₄₀ O ₂	10467
11	19.195	19.161	15.21	Hexadecanoic acid, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	8181
12	19.538	19.505	3.78	Eicosanoic acid	312.53	C ₂₀ H ₄₀ O ₂	10467
13	19.597	19.563	10.26	Nonadecanoic acid, methyl ester	312.53	C ₂₀ H ₄₀ O ₂	15610
14	19.935	19.896	7.92	Octadecanoic acid	284.48	C ₁₈ H ₃₆ O ₂	5281
15	20.270	20.252	1.26	Hexadecanoic acid, 14-methyl-, methyl ester	284.48	C ₁₈ H ₃₆ O ₂	520159
16	20.363	20.328	3.40	Heptadecanoic acid, methyl ester	284.48	C ₁₈ H ₃₆ O ₂	15609
17	20.514	20.485	1.57	10-Octadecenoic acid, methyl est	296.49	C ₁₉ H ₃₆ O ₂	12830109

Table 4. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD110

SD110 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	4.035	4.012	4.24	Glyceraldehyde	90.08	C ₃ H ₆ O ₃	751
2	4.277	4.251	8.43	Dimethyl Sulfoxide	78.13	C ₂ H ₆ OS	679
3	4.391	4.362	4.48	2-Furanmethanol	98.10	C ₅ H ₆ O ₂	7361
4	4.985	4.957	5.41	Dihydroxyacetone	90.08	C ₃ H ₆ O ₃	670
5	8.411	8.375	3.72	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
6	8.470	8.445	2.43	Undecane	156.31	C ₁₁ H ₂₄	14257
7	9.008	8.958	8.11	dl-Glyceraldehyde dimer	180.16	C ₆ H ₁₂ O ₆	251615
8	9.300	9.256	4.41	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	C ₄ H ₆ O ₃	95652
9	10.251	10.218	4.81	Tetrahydrofuran-5-on-2-methanol, .alpha.- [.alpha.-methoxy-(tetrahydrofuran-5-on-2- ylmethoxy)]-	260.24	C ₁₁ H ₁₆ O ₇	558876
10	10.727	10.691	4.01	1,2,3-Propanetriol, 1-acetate	134.13	C ₅ H ₁₀ O ₄	33510
11	12.158	12.114	12.88	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	C ₅ H ₈ O ₅	441696
12	16.876	16.845	3.78	Methyl 9-methyltetradecanoate	256.42	C ₁₆ H ₃₂ O ₂	554137
13	17.815	17.790	1.29	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	154.17	C ₇ H ₁₀ N ₂ O ₂	193540
14	18.061	18.029	3.61	Pentadecanoic acid, methyl ester	256.42	C ₁₆ H ₃₂ O ₂	23518
15	18.159	18.099	9.31	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	C ₁₆ H ₃₂ O ₂	23618376
16	19.194	19.167	4.52	Nonadecanoic acid, methyl ester	312.53	C ₂₀ H ₄₀ O ₂	15610
17	19.597	19.563	7.04	Methyl tetradecanoate	242.40	C ₁₅ H ₃₀ O ₂	31284
18	19.934	19.896	5.92	Tridecanoic acid	214.34	C ₁₃ H ₂₆ O ₂	12530
19	20.269	20.240	1.60	Heptadecanoic acid, methyl ester	284.48	C ₁₈ H ₃₆ O ₂	15609

Table 5. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SE143

SE143 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.409	8.381	4.36	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
2	8.470	8.445	4.89	Undecane	156.31	C ₁₁ H ₂₄	14257
3	9.302	9.268	5.26	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	C ₄ H ₆ O ₃	95652
4	10.737	10.702	3.50	1,2,3-Propanetriol, 1-acetate	134.13	C ₅ H ₁₀ O ₄	33510
5	16.876	16.851	5.81	Tridecanoic acid, 12-methyl-, methyl ester	242.40	C ₁₅ H ₃₀ O ₂	21204
6	18.064	18.035	5.79	Pentadecanoic acid, methyl ester	256.42	C ₁₆ H ₃₂ O ₂	23518
7	18.157	18.122	14.94	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	C ₁₆ H ₃₂ O ₂	23618376
8	19.194	19.155	13.40	Hexadecanoic acid, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	8181
9	19.595	19.563	16.23	Pentadecanoic acid, 14-methyl-, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	21205
10	19.932	19.902	5.55	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652.94	C ₃₈ H ₆₈ O ₈	54722209
11	20.358	20.293	6.57	Methyl 18-methylnonadecanoate	326.56	C ₂₁ H ₄₂ O ₂	530340
12	21.375	21.272	13.69	9,12-Octadecadienoic acid, methyl ester	294.47	C ₁₉ H ₃₄ O ₂	5284421

Table 6. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SE194

SE194 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.406	8.375	10.05	Cyclopropyl carbinol	72.11	C ₄ H ₈ O	75644
2	8.469	8.445	7.61	Undecane	156.31	C ₁₁ H ₂₄	14257
3	8.994	8.958	16.39	dl-Glyceraldehyde dimer	180.16	C ₆ H ₁₂ O ₆	251615
4	9.298	9.268	8.88	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	C ₄ H ₆ O ₃	95652
5	10.728	10.697	5.42	1,2,3-Propanetriol, 1-acetate	134.13	C ₅ H ₁₀ O ₄	33510
6	12.161	12.120	13.36	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	C ₅ H ₈ O ₅	441696
7	17.811	17.772	7.12	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	154.17	C ₇ H ₁₀ N ₂ O ₂	193540
8	18.156	18.134	8.28	Tridecanoic acid, 12-methyl-, methyl ester	242.40	C ₁₅ H ₃₀ O ₂	21204
9	19.193	19.161	5.31	Pentadecanoic acid, 14-methyl-, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	21205
10	19.593	19.558	7.55	Hexadecanoic acid, methyl ester	270.45	C ₁₇ H ₃₄ O ₂	8181
11	19.937	19.902	10.04	Tridecanoic acid	214.34	C ₁₃ H ₂₆ O ₂	12530

The GC-MS profiling of the selected bioactive isolates revealed a diverse metabolite pattern, reinforcing the antifungal activity observed in the *in vitro* disc diffusion assay. In general, the chromatograms were mainly characterized by fatty acids, fatty acid methyl esters, derivatives of organic acids, oxygenated metabolites, phenolic compounds, compounds related to furanone, and nitrogen-containing heterocyclic metabolites. The presence of such chemical compounds is common in bacterial extracts, as fatty acids and derived compounds are known to impede the propagation of fungi by affecting cellular integrity, altering permeability properties, and disrupting fungal growth (Zhao et al., 2020; Guimarães and Venâncio, 2022). Consequently, the presence of fatty acid, organic acid, and furanone-related compounds is consistent with the antifungal effects observed. In particular, the GC-MS profile of SA18 (Figure 8A, Table 1) indicated a broad chemical composition dominated by organic acids, furanone derivatives, fatty acid derivatives, and phenolic compounds. The major compound was pentadecanoic acid, followed by butanedioic acid, 2-hydroxy-2-methyl-, (S)-, butanedioic acid, 2,3-bis(acetyloxy)-, and 2(3H)-furanone, dihydro-4-hydroxy-. The co-occurrence of 2,4-di-tert-butylphenol, methyl tetradecanoate, and hexadecanoic acid methyl ester in the SA18 extract points to metabolite classes that other studies have linked to membrane-active and oxidative stress-related antifungal mechanisms (Zhao et al., 2020; Guimarães and Venâncio, 2022), although neither mechanism was tested directly in the present study and no single compound from the extract was isolated for individual bioassay against *A. flavus*. Bioactive metabolites such as phenolic compounds, e.g., 2,4-di-tert-butylphenol, were reported from different biological sources, while fatty acid derivatives may destabilize fungal lipid membranes and increase cellular leakage (Zhao et al., 2020; Guimarães and Venâncio, 2022). The mixed metabolite profile most likely contributed to the antifungal inhibition recorded for SA18.

SD102 (Figure 8B, Table 2) exhibited a more lipid-related chemical composition, with tetradecanoic acid, 12-methyl-, methyl ester, and hexadecanoic acid methyl ester as the main components. Other identified metabolites, such as dl-glyceraldehyde dimer, 2(3H)-furanone, dihydro-4-hydroxy-, and methyl tetradecanoate, also imply that the isolate produced oxygenated and furanone-type compounds. Fatty acids and fatty acid methyl esters were present, indicating that the main mechanism of antifungal activity of SD102 could be the disruption of the membrane and alteration of permeability (Bhattacharyya et al., 2020). Fungal cell membranes are very sensitive to chemicals interacting with lipids, and this has been associated with antifungal action in similar fatty acid profiles (Guimarães and Venâncio, 2022). In the case of SD109, the GC-MS profile (Figure 8C, Table 3) was dominated by lipid-derived metabolites. The main compounds were methyl tetradecanoate, hexadecanoic acid methyl ester, tridecanoic acid, 12-methyl-, methyl ester, nonadecanoic acid methyl ester, and octadecanoic acid. Medium- and long-chain fatty acids and their methyl esters made up most of the detected peaks in the SD109 extract, a pattern consistent with membrane-targeting antifungal activity reported in other fatty acid-rich extracts (Prasath et al., 2019; Bhattacharyya et al., 2020; Lee et al., 2021). The additional detection of 2,4-di-tert-butylphenol adds further strength to the bioactive profile of SD109, as phenolic metabolites can also increase antimicrobial action through the induction of oxidative stress, interference with enzymatic activity, and membrane damage (Zhao et al., 2020). Thus, the antifungal activity of SD109 is probably due to synergistic effects of lipid-derived metabolites and phenolic compounds. The GC-MS profile of SD110 (Figure 8D, Table 4) revealed a more balanced chemical composition with oxygenated metabolites, organic acid derivatives, fatty acid methyl esters, furanone-related compounds, and nitrogen-containing heterocycles. The major compounds were butanedioic acid, 2-hydroxy-2-methyl-, (S)-, tetradecanoic acid, 12-methyl-, methyl ester, dimethyl sulfoxide, dl-glyceraldehyde dimer, and methyl tetradecanoate. SD110 additionally contained the heterocyclic metabolite pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, a compound previously reported from *Streptomyces* extracts and linked to bioactive secondary metabolism (Ser et al., 2015). The combined presence of fatty acid methyl esters, organic acid derivatives, and the heterocyclic compound suggested that SD110 may function via more than one antifungal pathway, potentially including membrane destabilization (Bhattacharyya et al., 2020; Guimarães and Venâncio, 2022) as well as metabolic interference and oxidative stress-related effects (Yu et al., 2025).

Although SE143 has been identified as a *Comamonas* and not a *Streptomyces*, its GC-MS profile (Table 5) also supports its antifungal activity. The chromatogram (Figure 8E) was dominated by fatty acid methyl esters, especially pentadecanoic acid, 14-methyl-, methyl ester, tetradecanoic acid, 12-methyl-, methyl ester, 9,12-octadecadienoic acid methyl ester, and hexadecanoic acid methyl ester. Both saturated and unsaturated fatty acid methyl esters were present, a combination linked in other bacterial extracts to lipid membrane interaction as a possible antifungal route (Bhattacharyya et al., 2020). The presence of 9,12-octadecadienoic acid methyl ester, a linoleic acid methyl ester derivative, is especially relevant because unsaturated fatty acids and their derivatives are often discussed as antifungal compounds, capable of altering membrane function and fungal development (Prasath et al., 2019; Bhattacharyya et al., 2020; Guimarães and Venâncio, 2022). Thus, the activity of SE143 appears to be explained more by its fatty acid-rich chemical profile than by taxonomic identity.

The GC-MS profile of SE194 (Figure 8F, Table 6) differed from the more lipid-dominant isolates by revealing a stronger contribution from oxygenated and heterocyclic compounds. The major compounds included dl-glyceraldehyde

dimer, butanedioic acid, 2-hydroxy-2-methyl-, (S)-, cyclopropyl carbinol, tridecanoic acid, and 2(3H)-furanone, dihydro-4-hydroxy-. Hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione was detected at a higher relative abundance in SE194 than in SD110, the only other isolate in which the compound occurred; it has been reported from *Streptomyces* extracts and associated with biologically active secondary metabolism (Ser et al., 2015). The antifungal activity observed for SE194 can be attributed to the presence of a heterocyclic molecule, organic acids, and furanone derivatives, a combination consistent with the broader capacity of *Streptomyces* species to generate diverse secondary metabolites (Alam et al., 2022). Overall, the GC-MS data revealed that the active isolates contain a variety of metabolite classes that have been reported. The chemical profiles of the *Streptomyces* isolates SA18, SD102, SD109, SD110, and SE194 were in agreement with the ability of *Streptomyces* to produce a wide range of beneficial secondary metabolites (Alam et al., 2022). SE143, identified taxonomically as *Comamonas* rather than *Streptomyces*, nonetheless indicated antifungal activity in the disc diffusion assay, consistent with its high abundance of fatty acid methyl esters and with reports of antifungal activity in *Comamonas* species (El-Banna, 2007; Guimarães and Venâncio, 2022). The observed *in vitro* antifungal activity is therefore unlikely to result from a single compound. A more plausible explanation is the combined presence of several metabolite classes, fatty acid derivatives, phenolic compounds, organic acids, furanone derivatives, and nitrogen-containing heterocyclic metabolites, acting together rather than individually, a pattern consistent with reports that environmental bacteria, including *Streptomyces*, commonly co-produce multiple antimicrobial compounds that function synergistically (Meyer and Nodwell, 2021).

CONCLUSION

The current findings demonstrated that Karura Forest soil is a valuable source of bioactive bacterial isolates with antifungal potential. Selective isolation, preliminary characterization, 16S rRNA gene amplification, and antifungal screening resulted in the successful isolation and confirmation of five active *Streptomyces* isolates (SA18, SD102, SD109, SD110, and SE194) and one active *Comamonas* isolate (SE143) as promising candidates for further biological evaluation. The isolates demonstrated consistent antifungal activity in the *in vitro* disc diffusion assay, thus suggesting that their inhibitory effects were associated with the biologically active metabolites produced by the bacteria. GC-MS profiling of the bioactive extracts revealed diverse metabolite classes, including fatty acids, fatty acid methyl esters, organic acid derivatives, furanone-related compounds, phenolic metabolites, and nitrogen-containing heterocyclic compounds such as 2,4-di-tert-butylphenol and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro. The presence of the compound classes across both *Streptomyces* and *Comamonas* extracts indicates that useful antifungal metabolites in the collection were not limited to *Actinobacteria*. Overall, Karura Forest soil represents a promising reservoir of indigenous, beneficial microorganisms for biocontrol development. The six isolates identified here are attractive candidates for bio-inoculant development, with potential to help manage *A. flavus* contamination and support food safety in Kenyan agricultural systems, pending further evaluation under crop-based, storage, and field conditions as candidates for future biocontrol against *A. flavus* and other postharvest fungal pathogens. Different limitations should be addressed in future work. Quantitative measurement of aflatoxin B1 levels, by high-performance liquid chromatography (HPLC) or ELISA, following treatment with the bioactive extracts is needed to confirm whether the observed suppression of *A. flavus* growth translates into reduced aflatoxin production, since only fungal growth inhibition was assessed in the present study. *In vivo* validation on crop and stored-grain substrates under realistic storage and field conditions is also required before the isolates can be considered practical biocontrol candidates, given that efficacy was demonstrated only under controlled *in vitro* conditions.

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Authors' contributions

Esraa Mohammady Abdelzaher Elsemin conceived the study idea, designed the methodology, carried out the data collection and statistical analysis, and composed the initial draft of the manuscript. Andrew Nyerere Kimang'a proposed Karura Forest as the soil sampling location and, together with Joel Ltilitan Bargul, contributed to the critical revision and editing of the manuscript. All authors reviewed and approved the final version of the manuscript prior to its publication in the current journal.

Availability of data and materials

All data generated and analyzed in the current study are incorporated within the publication, with no additional files provided. Inquiries for supplementary data produced in this work should be addressed to the corresponding author.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declared that the current study is original, has not been submitted for publication or under review elsewhere, and complies with ethical norms concerning data integrity and the prevention of plagiarism. The authors confirmed that no artificial intelligence tools were employed in the composition and production of the manuscript. The authors confirm that the results and underlying data presented in this manuscript are original and have been independently verified by the authors for accuracy and integrity prior to submission and acceptance for publication.

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