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Metabolomic Insights into the Endophytic *Aspergillus flavus* through GC-MS/MS and its Antifungal Activity against *Fusarium oxysporum* and *Rhizoctonia solani*

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ABSTRACT

Isolated from the medicinal plant *Dalbergia sissoo*, the endophytic fungus *Aspergillus flavus* was subjected to their in-depth investigation integrating both the antifungal and chemical analysis. The antifungal activity was validated through a series of methodologies, including dual culture assays, poisoned food techniques, and the disc diffusion assays. Advanced metabolite profiling using Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS) was also carried out. The mycoendophyte exhibited potent antifungal activity against the pathogens *Rhizoctonia solani* and *Fusarium oxysporum.* GC-MS/MS led to the identification of seventeen key metabolites, with 9-Hexadecanoic acid, 9-hexadecenyl ester, and lidocaine standing out for their significant abundance, suggesting their critical contribution to the bioactivity observed. This comprehensive study accentuates the immense biotechnological potential of *A. flavus* as a reservoir of pharmacologically active compounds with promising antifungal applications, thus offering a promising alternative to minimize reliance on chemical fungicides for the pathogen control.

Keywords: Antifungal compounds, 9-Hexadecanoic acid, Chromatography, Mycoendophytes, Pathogens

INTRODUCTION

Medicinal plants have emerged as vital reservoirs of fungal endophytes, renowned for their ability to produce unique and bioactive metabolites with profound pharmaceutical significance (Strobel *et al.*, 2004). Intriguingly, certain plants are believed to host rare and specialized microbes that possess the potential to generate identical bioactive compounds, or even more potent derivatives with enhanced therapeutic efficacy, than those naturally synthesized by the host organism itself (Radu and Kqueen, 2002; Wang *et al.*, 2007). Residing silently within the internal tissues of plants, endophytes are microorganisms that typically cause no harm to their hosts (Wiyakrutta *et al.*, 2004). These often-symbiotic partners can bolster plant health and adaptability, though environmental stressors may trigger a shift towards pathogenicity (Christel *et al.*, 2000; Tejesvi *et al.*, 2007). Their composition and function are closely influenced by the host's surroundings and geographic conditions (Hata *et al.*, 1998). A diverse array of medicinal plants has been found to harbor endophytic fungi with significant antimicrobial potential. Xu *et al.* (2007) highlighted the antibacterial activity of endophytes from *Dioscorea zingiberensis*, while Liu *et al.* (2005) reported similar findings from the aerial tissues of *Argyrosomus argentatus*. In the same vein, Sette *et al.* (2006) isolated 25 fungal strains from *Coffea arabica* and *C. robusta*, and Li et al. (2005) identified 32 endophytes from *Cynodon dactylon*, all exhibiting varying degrees of antimicrobial efficacy, exemplifying the rich microbial reservoir within medicinal microflora. Mycoendophtyes, potentially acquiring biosynthetic genes from host plants via horizontal gene transfer (Strobel *et al.*, 2004; Wu *et al.*, 2001), are emerging as prolific sources of novel bioactive metabolites with vast medicinal, industrial, and agricultural applications (Yuna et *al.*, 2012).

Comprising a vast array of micro-fungal species, the genus *Aspergillus* is celebrated for its exceptional biosynthetic abilities, acting as a powerful reservoir that produces an extensive range of primary and secondary metabolites (Andersen, 2014; Frisvad, 2015). Among them, *Aspergillus flavus* stands out as a significant producer of alfatoxin, a highly potent carcinogen. These metabolites are not only synthesized for internal processes but are also externalized either as extrolites or as volatile organic compounds (VOCs), which play crucial roles in ecological interactions, including pathogenicity, competitive

inhibition, and self-preservation (Hung *et al.*, 2015). Pertaining to self-improvement, some of the compounds produced contribute towards increasing biological fitness or facilitate mutually beneficial associations, such as symbiosis, with other microorganisms (Cary *et al.*, 2018; Keller, 2019; Macheleidt *et al.*, 2016). The dynamic interplay between *A. flavus* and its microenvironment is increasingly evident, as unidentified volatiles are now believed to modify aflatoxin biosynthesis in response to the neighbouring colonies (Sweany and Damann, 2020), an idea reinfoeced by earlier observations of substrate-driven VOC variability among the alfatoxigenc and non-aflatoxigenic strains (Zeringue *et al.*, 1993).

Pathogens like *Rhizoctonia solani* inflict substantial global economic losses, with existing control measures offering only limited success (Anon, 2001). On the other hand, *Fusarium oxysporum* is also responsible for causing wilt diseases and crown and root rot of the plants (McGovern, 2015). In recent years, the alarming rise in antimicrobial resistance and the emergence of novel fungal pathogens have intensified the global search for therapeutic agents such as bioactive compounds. While soil has long been a traditional source for isolating fungi with medicinal properties, medicinal plants have emerged as a promising alternative, offering a rich niche for diverse endophytic fungi that have been known to possess certain pharmacological benefits (Strobel *et al.*, 2004). These endophytes residing asymptomatically within plant tissues have been increasingly recognized for their ability to synthesize a wide spectrum of bioactive secondary metabolites. It is proposed that such symbiotic microorganisms may not only mimic the host plant's chemistry but could also generate more effective derivatives with enhanced biological activity (Radu and Kqueen, 2002; Wang *et al.*, 2007). Among the compounds reported from these fungal endophytes are valuable phytochemicals such as flavonoids (Chen *et al.*, 2007) and terpenoids (Kumar *et al.*, 2005), known for their therapeutic relevance.

Particularly, endophytic strains of *Aspergillus flavus* and *A. fumigatus* have demonstrated notable antioxidant, antibacterial, and antifungal properties (Ravindran *et al.*, 2012; da Silva *et al.*, 2017; Sornakili *et al.*, 2020), highlighting the immense pharmaceutical potential of exploring plant-fungus associations in the quest of new bioactive molecules. *A. terreus* has shown a strong potential in suppressing *R. solani* in legumes like *Phaseolus vulgaris* and *Vicia faba*. The ethyl acetate extract of endophytic *A. terreus* offers a safe, eco-friendly, and promising biofungicidal alternative to chemical pesticides for managing damping-off disease in legume crops (Abdelaziz *et al.*, 2023).

The present study aims at isolating *Aspergillus flavus* from the leaves of *Dalbergia sissoo*, evaluating its antifungal efficacy against two commonly occurring pathogens, namely, *Rhizoctonia solani* and *Fusarium oxysporum* and detecting the compounds in endophytic extract through Gas Chromatography-Tendem Mass Spectrometry (GC-MS/MS) analysis.

MATERIALS & METHODS

Collection of samples

Mature, diseas-free leaves of *Dalbergia sissoo* were systemically collected from Bir Gurdialpura Wildlife Sanctuary (76°10'E and 76°15'E; 30°00'N) during the month of January 2025. Immediately after collection, the leaves were placed in sterile, sealed polythene bags and transported to the laboratory under controlled conditions. To preserve their biochemical integrity, the samples were stored at sub-zero temperatures and subsequently processed within 24 hours of collection.

Isolating the mycoendophyte

To eliminate the residual contaminants and the surface-dwelling microflora, the collected leaf samples underwent a thorough surface sterilization protocol. Initially, the samples were immersed in 70% ethanol for 1 minute, followed by a 2-minute treatment in 3% NaOCl (sodium hypochlorite) solution. This was succeeded by another 2 min wash in 70 % ethanol and a final rinse in sterile distilled water for 5 min, following the procedure outlined by Larran *et al.*, 2007. Once air-dried under sterile conditions, the leaves were aseptically cut into 10 mm segments and inoculated onto Potato Dextrose Agar (PDA) plates. A separate, untreated rinsed sample served as a negative control to validate the sterilization process. All inoculated plates were incubated in complete darkness at 25ºC for 9 days, with daily monitoring for fungal growth. Emerging mycelia were immediately transferred to fresh PDA, MEA (Malt Extract Agar), and YEA (Yeast Extract

Agar) and maintained under optimal conditions for further morphological and molecular characterization.

Assessing the antifungal potency of *Aspergillus flavus*

Dual culture According to the methodology outlined by Luo *et al.,* 2015, a dual culture assay was conducted to evaluate the antifungal activity of the endophytic fungus, *Aspergillus flavus*, against the *Rhizoctonia solani* (MTCC 4633) and *Fusarium oxysporum* (MTCC 284, procured from CSIR-IMTECH, Chandigarh, India). The two fungal isolates were inoculated on opposite sides of the PDA plate, maintaining a 4 cm separation between them, and incubated at 25ºC for a duration of 3 to 7 days. Antagonistic interaction was assessed by measuring the width of the inhibition zone formed between the two colonies, recorded in millimeters. The degree of antifungal activity was classified into four categories based on inhibition zone size: no inhibition (−), weak inhibition (+), moderate inhibition (++), and strong inhibition (+++). Furthermore, the inhibition rate of *R. solani* and *F. oxysporum* was quantified using the formula described by Liu *et al.* (2025).

Inhibition rate (%) = 100 − (a/b × 100)

a Colony growth of the pathogen with endophytic fungus, and

b Colony growth of the pathogen without the endophyte.

Food Poisoning Technique In the present investigation, two mycelial agar plugs (6 mm in diameter) were aseptically excised and inoculated into 100 mL of Potato Dextrose Broth (PDB) contained in a sterile conical flask. The cultures were incubated for 7 days at 25ºC under constant agitation of 200 rpm. One flask served as an uninoculated control. Upon completion of the incubation period, the cultures were subjected to centrifugation at 4000 rpm for 15 minutes to facilitate the removal of fungal mycelium. The resulting supernatant, enriched with extracellular fungal metabolites, was carefully filtered through a sterile syringe filter to obtain clear culture filtrate. For the antifungal bioassay, 15 mL of molten PDA was mixed with 1 mL of sterile culture filtrate and poured into petri dishes designated for treatment. Control plates received an equivalent volume of sterile PDB in place of the culture filtrate. Once the media got solidified, a mycelial disc of the pathogen was plated at the center of the plate. The radial growth of the pathogen was monitored to evaluate the inhibitory potential of the endophytic fungal isolate (Luo *et al.,* 2015; Balouiri *et al.,* 2016).

The inhibition of the mycelial growth on the treatment plates as compared to the control will be assessed and will be calculated as

Percentage of mycelial growth inhibition = (dc − dt)/dc × 100,

Where, dc = the average diameter (in mm) of fungal colonies in the control,

dt = the average diameter (in mm) of fungal colonies in the treatment groups.

Disc diffusion assay

To obtain the crude extract of the endophytic fungus, fermentation was carried out in accordance with the method described by Yehia *et al.,* 2020, with slight modifications. A pure culture of the fungus, previously cultivated on PDA, was used to inoculate 500 mL of PDB in a 1-liter Erlenmeyer flask by transferring a 5 mm agar plug containing actively growing mycelium. The inoculated broth was incubated for 21 days at 25ºC under static conditions. Following incubation, the fungal culture was filtered through Whatman filter paper no. 1, and the resulting filtrate was sequentially extracted three times with 100 mL of each methanol, acetone, and distilled water. The pooled extract was then concentrated using a rotatory vacuum evaporator at 40 ºC. The dried residues were reconstituted in 1 mL of 50 % DMSO (dimethyl sulfoxide) to obtain final concentrations of 25 mg/mL, 50 mg/mL, and 75 mg/mL for subsequent bioassays. For antifungal evaluation, the inoculum of the respective pathogens was prepared and uniformly spread on assay plates prior to the placement of treatment disks. Fluconazole (2 mg/mL; Pfizer Pharmaceuticals India) served as the positive control. Sterile 50 µg disks were prepared by impregnating each with 25 µL of the fluconazole solution (Kirkpatrick *et al.,* 1998).

Gas Chromatography-Tendem Mass Spectrometry

The chemical profile of *Aspergillus flavus* was characterized using GC-MS/MS, employing an Rxi-5 capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness) interfaced with an SSQ 7000 quadrupole mass spectrometer. The injector temperature was precisely maintained at 260 ºC. The oven temperature was programmed to initiate at 40 ºC with a 5-minute isothermal hold, followed by a linear increase at 6 ºC per minute to reach 260 ºC, where it was held for a final 15 minutes, culminating in a total analytical run time of 60 minutes. The MS transfer line was set at 280 ºC to ensure optimal analyte transmission. The sample, solubilized in methanol, was induced via split injection at a ratio of 1:15. High-purity helium was employed as a carrier gas at a constant flow rate of 1 mL/min. Mass spectrometric detection was conducted at electron ionization at 70 eV, with a filament emission current of 60 mA and an ion source temperature maintained at 200 ºC. Compound identification was accomplished through spectral matching with the NIST database, supplemented by comprehensive analysis of mass fragmentation patterns.

Statistical analysis

To evaluate the statistical differences, the data expressed as mean values with corresponding standard errors were analyzed using one-way ANOVA, followed by Tukey's HSD post hoc test in SPSS. A significant threshold of $p \leq 0.05$ was applied to determine significant differences among groups.

RESULTS

Antifungal activity

Aspergillus flavus (MTCC accession no. 13763) (Figure 1a), isolated from the leaf of *Dalbergia sissoo*, exhibited strong inhibition (+++) towards both the pathogens, namely *Rhizoctonia solani* and *Fusarium oxysporum*. The rate of colony growth inhibition was found to be 75 % and 72.36 % in the case of dual culture (Figure 1b,c) for *R. solani* and *F. oxysporum*, respectively. The mycoendophytic cultural filtrate against the *R. solani* and *F. oxysporum* using the food poisoning technique along with their controls has been depicted in Figure 1d,e and Figure 1f,g, respectively. The number of sclerotial bodies decreased when 1 mL of the endophytic extract was supplemented to the 15 mL of the culture media, thus suggesting high antifungal potency of the endophyte towards *R. solani*. The percentage of the mycelial growth inhibition was about 60 % in the case of *F. oxysporum*. Pertaining to the disc diffusion assay, the antifungal efficacy of the various extracts (methanol, acetone, and aqueous) with distinct concentrations (25 mg/mL, 50 mg/mL, and 75 mg/mL) was assessed against *R. solani* and *F. oxysporum* along with their zones of inhibition measured in mm in Figure 2h and Figure 2i, respectively.

The maximum average inhibition zone observed in the case of fluconazole (positive control) was 15.83 ± 0.166 mm, while there was no clear zone of inhibition seen around DMSO (negative control for the study). The average zones (in mm) along with the standard error of the methanolic (7.833±0.167, 9.1667±0.166, 11.4±0.1), acetone (6.833±0.166, 7.833±0.67, 10.333±0.333), and aqueous extract (7.333±0.166, 7.833±0.167, 11.167±0.166) showed a significant increase with the increase in the concentration of the extracts (25 mg/mL, 50 mg/mL, and 75 mg/mL) in the case of *R. solani*. On the other hand, the ZOI with respect to *F. oxysporum* were 9.833±0.166, 10.667±0.333, 11.833±0.167 (methanolic extract with increasing concentrations), 6.833±0.166, 8.833±0.167, 9.833±0.166 (acetone), and 6.833±0.167, 9.833±0.166, 10.667±0.333 (aqueous extract). Fluconazole (Flu) exhibited the highest antifungal potency, followed by methanolic extract (ME), then aqueous extract (AqE), and lastly the acetone extract in both cases, whereas there was no clear measurable zone of inhibition seen around the disc of dimethyl sulfoxide (DMSO). The comparative analysis among these extracts has been shown in Figure 3j and Figure 3k against *R. solani* and *F. oxysporum*, respectively. Distinct alterations in the solvent variations and the concentrations exerted a significant impact upon the observed response.

Table 1 display the ANOVA results, revealing significant variations in the treatment efficacy across different concentrations. Distinct superscripts (a, b, and c) indicate the statistically significant differences among the groups mentioned ($p < 0.05$, Turkey HSD), underscoring the differential effectiveness of the treatments.

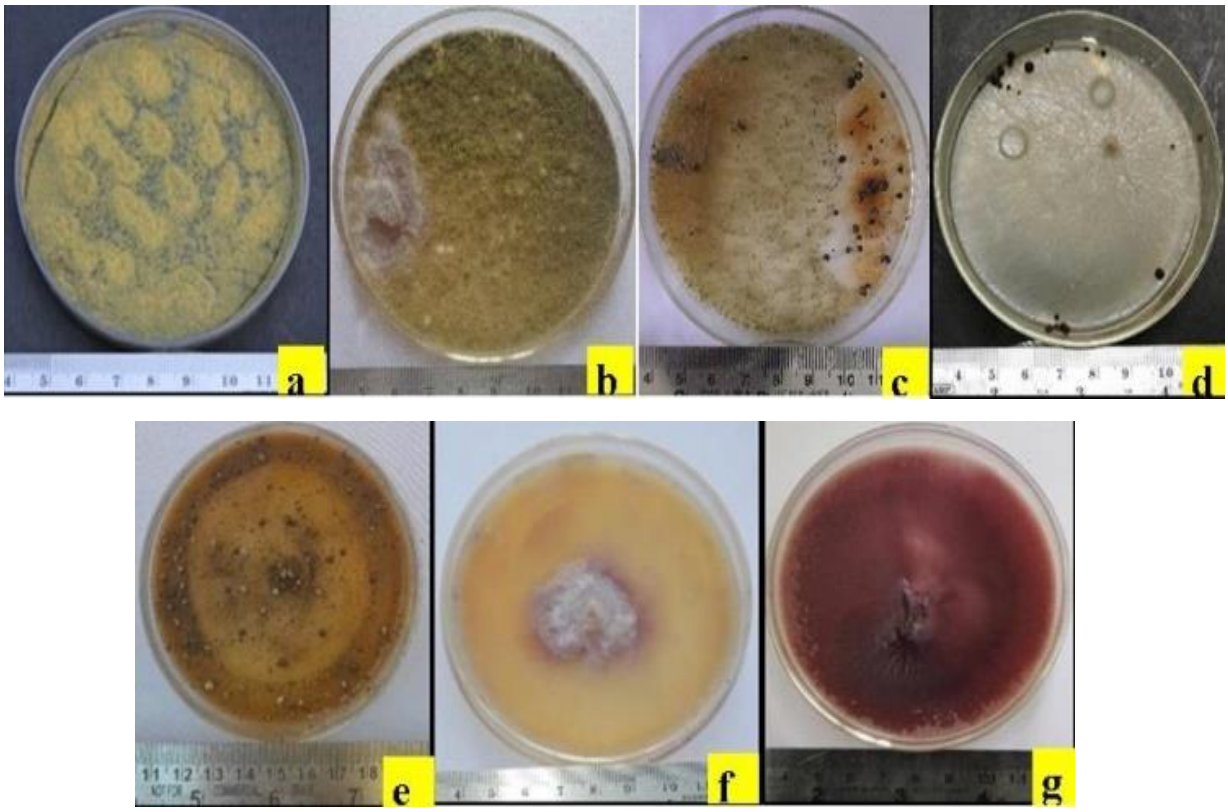


Figure 1: a, Colony of *Aspergillus flavus* on potato dextrose agar (PDA); **b-c,** Dual culture of *A. flavus* along with *F. oxysporum* (b) and *R. solani* (c); **d-e,** Growth inhibition of *Rhizoctonia solani* (the fungal pathogen) upon addition of the culture filtrate of *Aspergillus flavus* (d) with control (e); **f-g,** Growth inhibition of *Fusarium oxysporum* (the fungal pathogen) upon addition of the culture filterate of *Aspergillus flavus* (f) with control (g).

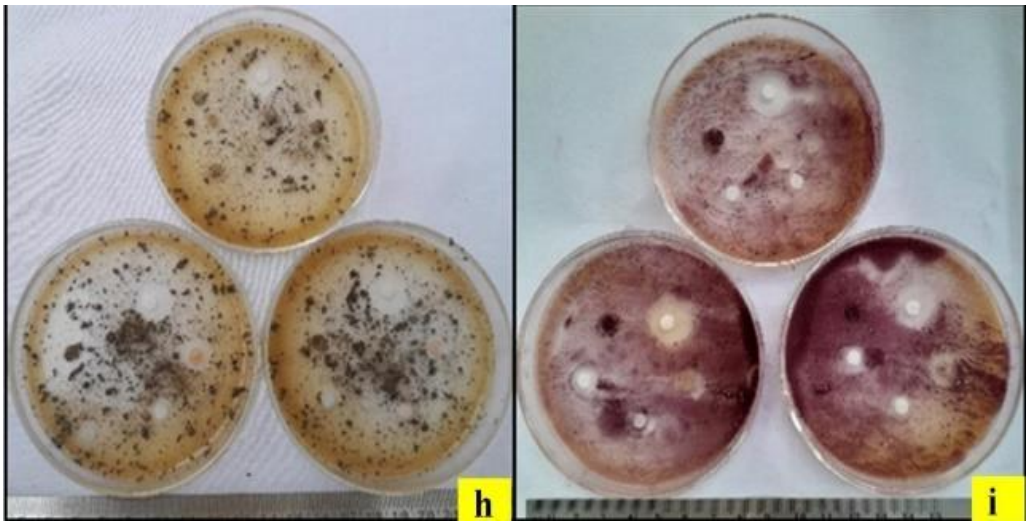


Figure 2: Disc diffusion assay representing the antifungal potential of the extracts [25 mg/mL, 50 mg/mL, and 75 mg/mL (in the clockwise direction)] of *Aspergillus flavus* against *Rhizoctonia solani* **(h)** and against *Fusarium solani* **(i)** with the fluconazole disc placed at the top center of each plate that served as the control (2 mg/mL) followed by methanol, acetone, aqueous extract, and DMSO (negative control for the study) kept in the clockwise direction as well, corresponding to every plate.

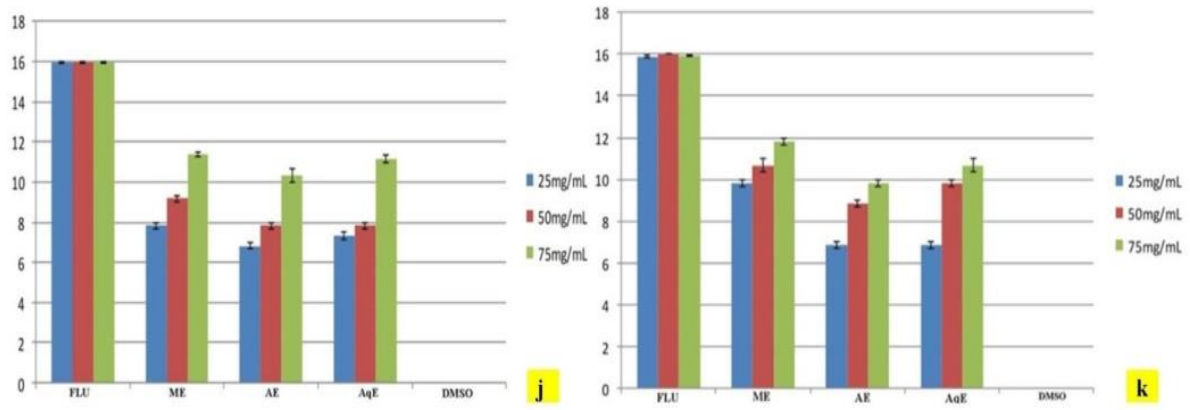


Figure 3: The average zone of inhibitions along with the standard errors of the treatment type (Flu: Fluconazole, ME: Methanolic extract, AE: Acetone extract, AqE: Aqueous extract, DMSO: dimethyl sulfoxide) and the varying concentration of the mycoendophytic extract, highlighting the antifungal activity of *A. flavus* against *R. solani* **(j)**, and against *F. oxysporum* **(k)**.

Table 1: The mean Zone of Inhibition (ZOI) of the endophytic extract against *R. solani* followed by *F. oxysporum* respectively are expressed with the standard error (±) and the significance level (ANOVA, $p < 0.05$, Turkey HSD post hoc). Values in the respective columns with different superscript letters differ statistically (a, b, c, d). Flu: Fluconazole (2 mg/mL). Mean values followed by the same letters (a, b, c) are not significantly different.

Treatment	25 mg/mL	50 mg/mL	75 mg/mL
Flu (+ control)	15.83^{a} ± 0.440, 15.83^{a} ±0.166	15.83^{a} ±0.166, 15.66^{a}±0.333	15.66^{a}±0.333, 15.66^{a}±0.333
ME	7.833^{b}±0.167, 9.833^{b}±0.166	9.166^{b}±0.166, 10.667^{b}±0.333	11.4^{b}±0.1, 11.833^{b}±0.167
AE	6.833^{c}±0.166, 6.833^{c}±0.166	7.833^{c}±0.67, 8.833^{c}±0.167	10.333^{c}±0.333, 9.833^{c}±0.166
AqE	7.333^{b}±0.166, 6.833^{b}±0.167	7.833^{b}±0.167, 9.833^{b}±0.166	11.167^{b}±0.166, 10.667^{b}±0.333
DMSO (-control)	0^{c}	0^{d}	0^{d}

GC-MS/MS analysis

The GC-MS/MS GC-MS/MS (Figure 4) reveals a diverse array of bioactive compounds (Table 2) characterized by varied chemical structures along with their compound spectra and chromatogram (Figure 5-8). Notably, high molecular weight constituents such as 2,2-Bis[4-[[4-chloro-6-(3ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane and 4a-Phorbol 12,13-didecanoate highlight the presence of complex, potentially pharmacologically active molecules. Fatty acid esters like hexadecanoic acid, 14-methyl-, methyl ester, and linoleic acid ethyl ester exhibit significant presence and abundance in the sample. Lidocaine stood out with the highest peak intensity, thus demonstrating its abundance in the extract. Alkaloid deravatives such as papaveroline and calycotomine, alongside the structurally intricate esters like 3-Pyridinecarboxylic acid tris (acetyloxy) ester, illustrate a wide spectrum of chemical diversity. The profile reflects a complex chemical landscape rich in esters, alkaloids, fatty acid derivatives, and heterocyclic compounds with substantial analytical significance.

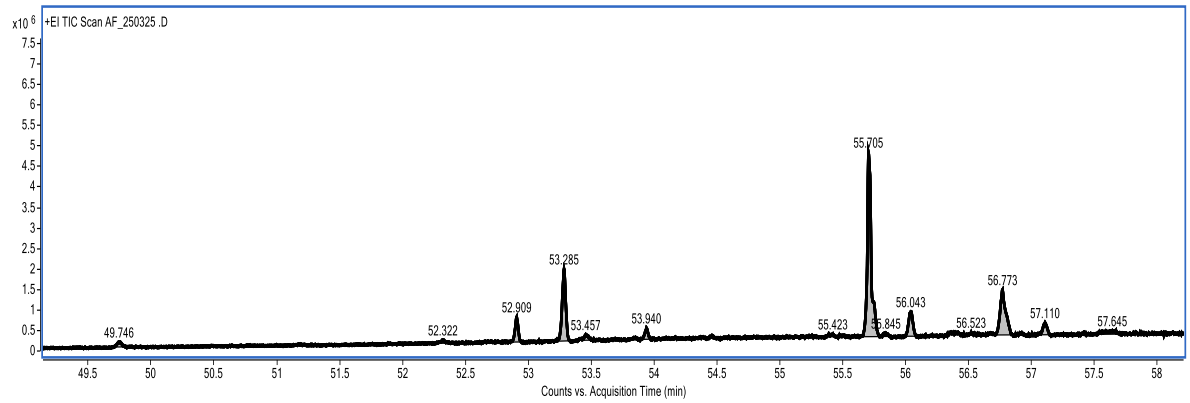


Figure 4: GC-MS/MS chromatogram of the methanolic extract of *Aspergillus flavus*.

Table 2. List of compounds identified in the extract of *Aspergillus flavus*.

Compound names	Chemical formula	Molecular weight (gms/mol)	Retention time (min)	Height	Area %
2,2-Bis[4-[[4-chloro-6-(3ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane	$C_{37}H_{24}C_{l2}N_6O_4$	636.272	24.703	29473	1.69
4a-Phorbol 12,13-didecanoate	$C_{40}H_{64}O_8$	672.64	49.746	115810	3.21
Papaveroline, 2'-bromo-2-methyl-, tetramethyl(ester)	$C_{21}H_{26}BrNO_4$	436.38	52.322	75583	2.81
Hexadecanoic acid, 14-methyl-, methyl ester	$C_{18}H_{36}O_2$	284.48	52.909	609177	10.64
Lidocaine	$C_{14}H_{22}N_2O$	234.34	53.285	1753001	35.0
3-Pyridinecarboxylic acid, 2,7,10 tris(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-1,1,3,6,9-pentamethyl-4-oxo-4a,7a-epoxy-5H-cyclopenta[a]cyclopropa[f]cycloundecen-11-yl ester	$C_{32}H_{39}NO_{10}$	597.661	53.457	132510	3.70
Hexadecanoic acid, 1a,2,5,5a,6,9,10,10 a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl ester	$C_{36}H_{58}O_6$	564.9	53.940	272739	5.02
Papaveroline, 2'-bromo-2-methyl-, tetramethyl(ester)	$C_{21}H_{26}BrNO_4$	436.38	55.423	65169	1.02

Compound names	Chemical formula	Molecular weight (gms/mol)	Retention time (min)	Height	Area %
9-Hexadecenoic acid, 9-hexadecenyl ester, (Z,Z)	$C_{32}H_{60}O_2$	476.92	55.705	4495952	100
Normorphine, bis(trimethylsilyl) ether	$C_{22}H_{33}NO_3Si_2$	415.74	55.845	76004	1.79
Isoquinoline, 1-[(3,4-dimethoxyphenyl) methyl]-1,2,3,4-tetrahydro-6,7-methoxy	$C_{20}H_{25}NO_4$	343	56.523	63491	1.08
Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308.508	56.773	1100614	33.90
N-(3-Hydroxyphenyl)-2- (2,4,5-trichlorophenoxy)acetamide	$C_{20}H_{24}C_{l3}NO_3Si$	510.66	57.645	81315	4.47
1,2,3,4- Tetrahydroisoquinolin, 2-acetyl-6,7-dimethoxy-1-phenmethylene	$C_{20}H_{21}NO_3$	323.42	61.172	159882	5.18
3, 3', 4, 4' tetrahydro-1,1',2,2'-tetrahydro-1-hydroxy-1'-methoxy (Carotene)	$C_{41}H_{58}O_2$	582.99	64.241	129918	5.68
Calycotomine, N-methyl	$C_{13}H_{19}NO_3$	237.33	65.478	225113	10.70
2,2-Dimethyl-2-[2,3,5,6-tetramethyl]ethanol	$C_{14}H_{22}O$	206.33	66.603	903447	48.23

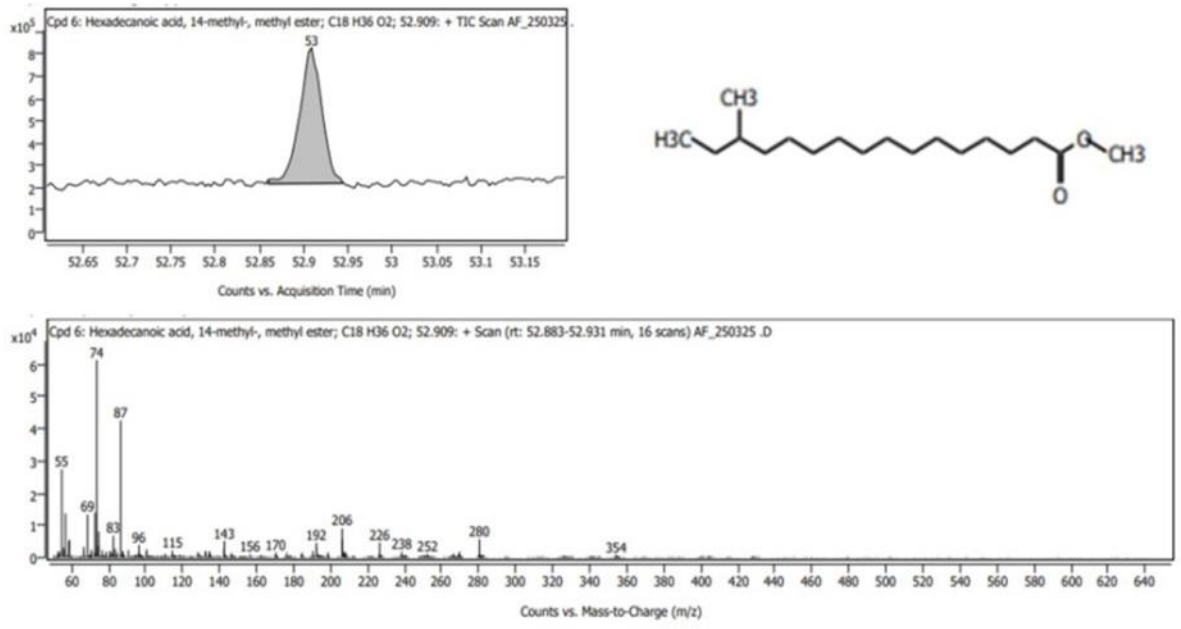


Figure 5: Chromatogram, structure and spectra of the compound Hexadecanoic acid, 14-methyl-, methyl ester.

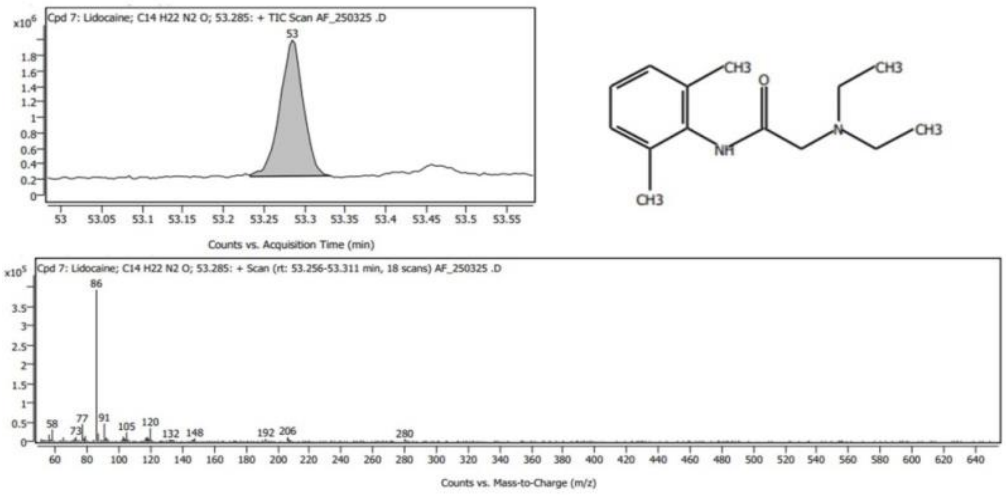


Figure 6: Chromatogram, structure and spectra of the compound Lidocaine.

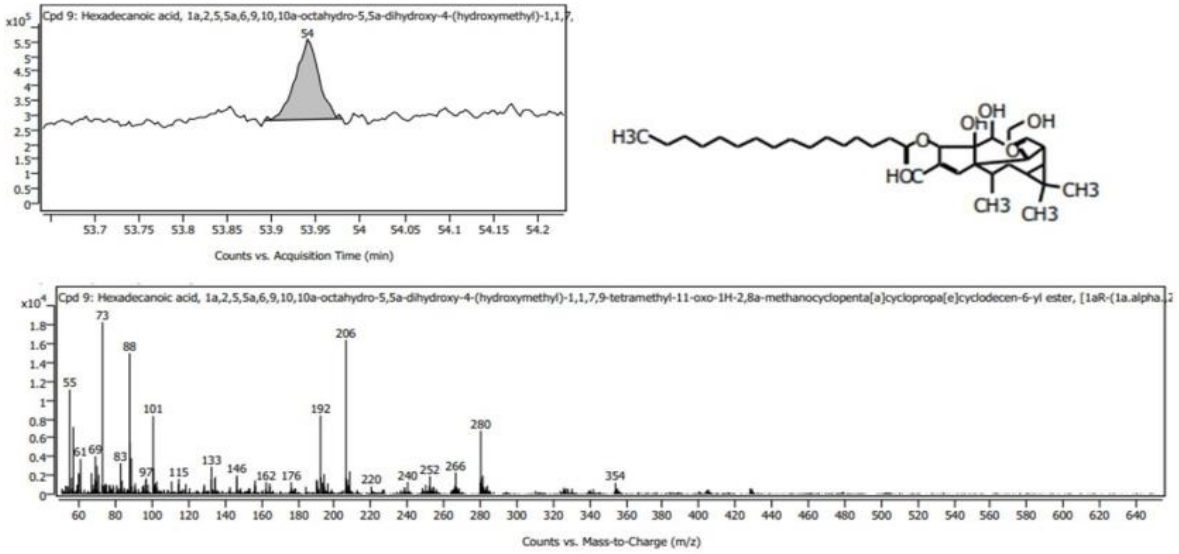


Figure 7: Chromatogram, structure and spectra of the compound Hexadecanoic acid, 1a,2,5,5a,6,9,10,10 a octahydro-5,5a- dihydroxy-4- (hydroxymethyl)-1,1,7,9- tetramethyl-11-oxo-1H- 2,8a-methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl ester

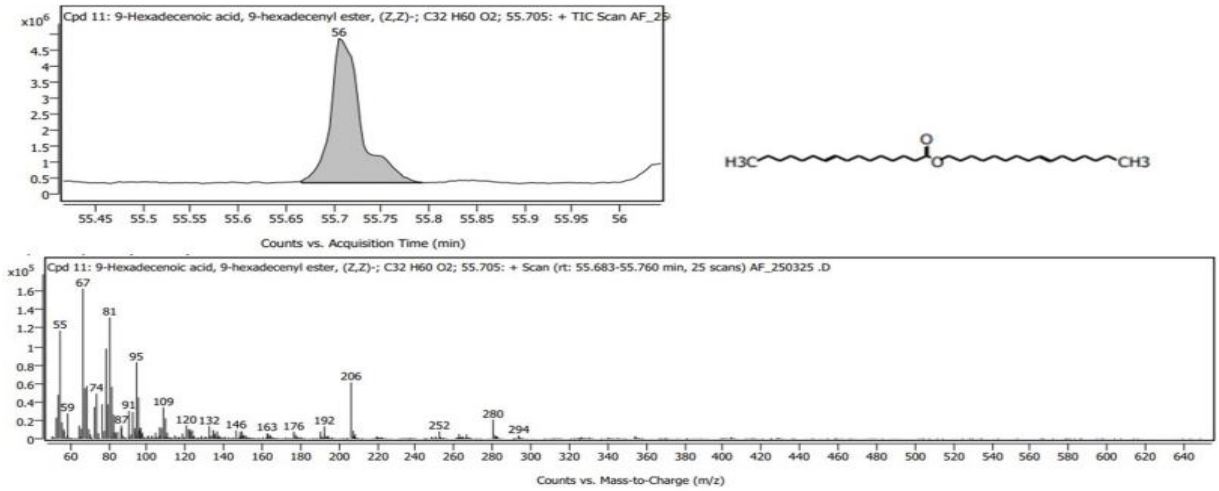


Figure 8: Chromatogram, structure and spectra of the compound 9-Hexadecenoic acid, 9-hexadecenyl ester, (Z,Z).

DISCUSSION

Volatile organic compounds (VOCs) are chemically diverse gases, including acids, aromatics, aldehydes, alcohols, esters, ketones, heterocycles, and terpenes (Hung *et al.,* 2014), with complex structures spanning multiple chemical families, including esters, hydrocarbons, ethers, alcohols, lactones, ketones, and glycol ethers, leading to varied properties (Korpi *et al.,* 2009; Schnurer *et al.,* 1999). Astonishingly, endophytes have the intrinsic capacity to biosynthesize the bioactive molecules that define the therapeutic or chemical identity of their host plants (Tan and Zou, 2001). Volatile organic compounds emitted by *Aspergillus flavus* have been utilized to refine detection methods, enable accurate species identification (Borjesson *et al.,* 1992; Gao *et al.,* 2002; Sun *et al.,* 2014; Sun *et al.,* 2016), and discriminate between the aflatoxigenic and non-aflatoxigenic strains with greater precision (De lucca *et al.,* 2010; De lucca *et al.,* 2012).

Hexadecanoic acid methyl ester, which is found in the endophytic *Aspergillus flavus* extract in the present study, is a prominent fatty acid derivative. This compound has previously been reported (Adekunle *et al.,* 2015) as a major metabolite produced by *A. flavus* associated with microbial biosurfactants, reinforcing its relevance in fungal secondary metabolism. Moreover, methyl esters of hexadecenoic acids are one of the most prevalent constituents of biodiesels (Hoekman *et al.,* 2012), highlighting their industrial significance. The frequent occurrence of hexadecenoic acid derivatives in marine-derived *Aspergillus* sp. suggests a conserved biosynthetic trend across diverse ecological niches. Similarly, Siddiquee et al. (Siddiquee *et al.,* 2015) reported the presence of these compounds in *A. niger* using GC-MS-based profiling with different capillary columns and solvents.

Our GC-MS/MS analysis identified lidocaine as a prominent bioactive, which might be responsible for possessing the antifungal activity against *Rhizoctonia solani* and *Fusarium oxysporum*, beyond its well-established role as a local anesthetic. Lidocaine exhibits potent, dose-dependent fungicidal activity, primarily through disruption of the fungal cytoplasmic membrane, a mechanism characterized by significant potassium ion leakage and membrane destabilization (Pina-Vaz *et al.,* 2000). Effective concentrations typically range from 15 to 30 mg/mL exceeding minimum inhibitory concentrations (MIC), observed in strains of *Candida albicans* and *C. krusei*, which exhibited MIC values near 10 mg/mL (Palmeira-de-Oliveira *et al.,* 2012). Its hydrochloride form (LDCH), while primarily used for analgesia, also provides symptomatic relief cutaneous candidiasis (Pina-Vaz *et al.,* 2000; Palmeira-de-Oliveira *et al.,* 2012), alleviating pain, burning, and pruritus (Tejada *et al.,* 2020). Notably, lidocaine demonstrates synergistic antimicrobial properties when combined with agents like propofol and miconazole, enhancing efficacy against *E. coli* (Gajraj *et al.,* 1998) and *C. albicans* (dos Santos *et al.,* 2017), respectively. Despite its reduced permeability on palatal mucosa compared to alveolar tissue, lidocaine remains a key therapeutic agent in magic mouthwash for managing mucositis in cancer patients (Kirk *et al.,* 2017). Furthermore, its antifungal activity extends to *Cryptococcus* sp. (Schmidt and Rosenkranz, 1970) and includes inhibition of germ tube formation, underscoring its broad-spectrum potential and membrane-targeting mechanisms (Rodrigues *et al.,* 1994; Rodrigues *et al.,* 2000).

Several classes of bioactive compounds detected in the current study, including isoquinoline alkaloids, papaveroline, and tetrahydroisoquinoline derivatives, as well as the carotenoids, have been previously reported to exhibit significant antifungal properties. Isoquinoline derivatives have demonstrated antifungal properties (Qing *et al.,* 2017), while papaveroline from *Moringa oleifera* leaves further supports this potential (Abdulhafedh *et al.,* 2023). Oceanalin B, a tetrahydroisoquinoline-based glycoside from the marine sponge *Oceanapia* sp., exhibited strong in vitro activity against *Candida glabrata* with MIC 25 µg/mL (Makarieva *et al.,* 2021). Additionally, carotenoids, especially beta carotenoids from microalgae, cyanobacteria, and chlorophyta, have shown significant antibacterial and antifungal effects (Vargas-Sinisterra and Ramirez-Castrillon, 2021). Fungal beta carotene has also proven effective against dermatomycotic pathogens (Abdelaziz *et al.,* 2023). The presence of these compounds in our findings underscores their relevance and supports their potential as natural antifungal agents.

GC-MS analysis of the crude methanolic leaf extract of *Macrosolen* revealed the presence of 4

alpha phorbol, 12, 13-didecanoate and hexadecanoic acid, and 1-(hydroxymethyl)- 1,2-ethanediyl ester. Notably, 4 alpha phorbol, 12, 13-didecanoate, previously detected in *Mimordica* sp. from Nigeria, is known to undergo enzymatic hydrolysis, releasing decanoic acid, a potent medium-chain fatty acid with broad spectrum anti-microbial properties, including antibacterial, antiviral, antifungal, antiparasitic, and antiprotozoal effects (Oragwa *et al.,* 2013). The detection of these compounds in our extracts reinforces their potential as antimicrobial agents, aligning with earlier reports (Kadhim *et al.,* 2017).

In vitro studies have shown that fatty acids such as linolenic acid, linoleic acid, and erucic acid possess antifungal activity against major plant pathogens such as *Rhizoctonia solani*, *Pythium ultimum*, *Pyrenophora avenae*, and *Crinipellis perniciosa*. Linolenic acid and linoleic acid were particularly effective, with linolenic acid notably reducing the mycelial growth of *R. solani* and *C. perniciosa* (Walters *et al.,* 2004). These fatty acids, especially linoleic acid, also produced by *A. flavus*, serve as key precursors in the biosynthesis of antifungal trihydroxy oxylipins in plants (Hamberg, 1999). Linoleic acid, also detected in our study, suggests a broader ecological role for this fatty acid in fungal interactions and defense mechanisms.

Aspergillus flavus has emerged as a promising endophytic fungus with multiple beneficial traits. Notably, it produces bioactive metabolites such as the antifungal aspirochlorines (Klausmeyer *et al.*, 2005), and certain isolates, such as those cultured on coconut under UV light, have demonstrated an absence of aflatoxin production, ensuring their safety for agricultural applications. When used as plant growth-promoting fungus (PGPF) in tomatoes, *A. flavus* significantly enhanced a plant growth and secondary metabolite production (Lin and Xu, 2013), leading to increased resistance against aggressive pathogens like *Alternaria phragmospora* (Abdel-Motaal *et al.,* 2020). In fact, complete protection of tomato leaves from early blight was observed following *A. flavus* inoculation. This protection effect is attributed to physiological and biochemical shifts in the host plant's defense system. Additionally, *A. flavus* isolated from *Eucheuma geniculate* exhibited strong antagonistic activity against *A. phragmospora*, reinforcing its potential as a biocontrol agent (Kamel *et al.,* 2020). Furthermore, its extract has been successfully used in the photobiosynthesis of silver nanoparticles, which showed potent antifungal activity against *Sclerotium rolfsii* and *Rhizoctonia solani* (Kanaujiya *et al.,* 2020). These findings suggest the multifaceted role of *A. flavus* as a safe, effective plant growth promoter and bioprotective endophyte with broad-spectrum antifungal capabilities.

CONCLUSION

Dalbergia sissoo leaves harbor certain fungal endophytes that demonstrate exceptional potential as a source of bioactive compounds with great pharmaceutical significance. The endophyte studied, exhibits strong antifungal activity against *Rhizoctonia solani* and *Fusarium oxysporum*, suggesting that the therapeutic qualities attributed to the host may have been derived from the symbiotic microbes producing complex and potent biochemical agents. Unlocking this hidden reservoir by isolating and thoroughly characterizing these bioactive substances is essential. Such effects could lead to the antimicrobial drug development and offer valuable insights into the underlying mechanisms, ultimately advancing the frontier of natural product-based therapeutics.

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Author contributions All authors contributed to the study conceptualization, organization, and scrutinization of the manuscript. Methodology and drafting were done by Tanya Joshi; material preparation and sample collection were made by Avneet Pal Singh; and analysis of the data and validation of the data was done by Rajnish Kumar Verma. All the authors have read and approved the final version of manuscript.

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