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Endophytic Fungi from Asparagus Racemosus: A New Source of Antimicrobial and Antioxidant Compounds

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Asparagus racemosus, Endophytic fungi, *Aspergillus terreus*, *Aspergillus oryzae*, Antimicrobial, Antioxidant, HPLC, Saponin

ABSTRACT:

The current study explores endophytic fungi isolated from *Asparagus racemosus* roots, focusing on its antimicrobial and antioxidant activity. A total of twenty four fungal strains were isolated and screened against seven pathogenic microbes (*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Streptococcus entericus, Escherichia coli, Candida albicans*, and *Aspergillus niger*). The endophytic fungus, S-32 exhibited the highest antimicrobial activity, against all tested pathogens. The DPPH assay revealed that endophytic fungus S-5, had the strongest effect with an IC50 value of 4.4 (µg/µl) and the highest antioxidant activity index (AAI) of 1.7. High-performance liquid chromatography (HPLC) was used to analyze the saponin concentration in the extracts of endophytic fungi S-32 (2174.95 ng/mg) and S-5 (1244.558 ng/mg). The analysis of 18S rRNA sequencing revealed that S-32 and S-5 were identified as *Aspergillus terreus* and *Aspergillus oryzae* respectively.

Introduction

Asparagus racemosus, also referred to as Shatavari, is a widely recognized plant in traditional Indian medicine owing to its therapeutic properties. Native to tropical and subtropical parts of India, this plant's roots possess numerous pharmacological properties, such as anticancer and antioxidant effects (Parihar et al. 2004), antitussive, antihyperlipidemic, antistress, antidepressant, antianxiety, immunomodulatory, anti-inflammatory, anti-urolithiatic, antibacterial, and antidiarrheal properties (Mandal et al. 2000). These bioactivities are attributed to the plant's rich profile of secondary metabolites, including lignin, acemannan, oligosaccharides, quercetin, rutin, racemoside, diosgenin, hyperoside, sarsasapogenin, steroidal saponins, and triterpene saponins. Despite its widespread use, it faces threats from unsustainable harvesting practices and habitat destruction, making it an endangered species (Bopana et al. 2008; Pise et al. 2015).

Endophytic fungi, living within plant tissues without causing visible symptoms, offer a promising source of secondary metabolites to address the growing demand for medicinal plants.

Most endophytic fungi belong to the Ascomycota division, while some are from the Basidiomycota, Mucoromycota, and other divisions. They exhibit a wide biological diversity that has drawn significant interest due to their potential to synthesize various secondary metabolites, including various structural classes such as alkaloids, peptides, steroids, terpenoids, phenols, quinones, saponins, and flavonoids (Suryanarayana et al. 2009, Gunasekaran et al. 2017). Researchers have identified endophytic fungi as a source of novel natural products with significant biological properties, including antimicrobial, antioxidant, anti-inflammatory, anticancer, and antiviral activities (Guo et al. 2008). Keeping this in view, the goal of the present study was to isolate

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endophytic fungi from *A. racemosus* and evaluate their antimicrobial and antioxidant activities along with subsequent quantification of saponin.

1. Materials and Methods

2.1 Plant Material Collection

The roots of *A. racemosus* were collected from several locations included Chaudhary Charan Singh Haryana Agricultural University (CCS HAU), Hisar, Haryana, India; ICAR-National Bureau of Plant Genetic Resources New Delhi, India; Haryana Forest Department, Rohtak, Haryana, India; Chaudary Devi Lal Herbal Nature Park, Chuharpur, Yamunanagar, Haryana, India and Guru Jambheswar University Science and Technology (GJUS&T), Hisar, Haryana, India.

2.2 Isolation of Endophytic Fungi

The isolation of endophytic fungi from *A. racemosus* roots was done by following a modified method from Arnold et al. (2000). The roots were rinsed with sterile water, sterilized by immersing them in 70% ethanol and 0.1% mercuric chloride solution, and subsequently thoroughly rinsed with demineralized water. The inner tissue near the pith was dissected into small pieces and placed on Potato Dextrose Agar petri dishes supplemented with streptomycin to inhibit bacterial growth. The petri dishes were incubated at 28°C for 1-2 weeks to promote fungal growth (Lu et al. 2012). Each sample is processed in triplicate for consistency.

2.3 Preparation of Crude Extract

Crude extracts from endophytic fungi were prepared using a modified method based on Sharma et al. (2016) and McCloud (2010). Endophytic fungi were cultured in potato dextrose broth (PDB) at 28ºC, 180 rpm for 7 days, then lysed and filtered. To isolate compounds, ethyl acetate and methanol were added to the filtered lysate. The upper organic layer, containing the compounds, was collected and dried to obtain a crude extract. The crude extract was analysed for antimicrobial and antioxidant activity, and the saponin was quantified using High-Pressure Liquid Chromatography (HPLC).

2.4 Antimicrobial activity

The antimicrobial assay involved a range of gram-positive and gram-negative bacteria, as well as certain fungi. Gram-positive bacteria included *Streptococcus entericus, Staphylococcus aureus* (MTCC 3160), and *Bacillus subtilis* (MTCC 441), while gram-negative bacteria were *Escherichia coli* (MTCC 16521) and *Pseudomonas aeruginosa* (MTCC 647). The fungi strains that were analyzed consisted of *Candida albicans* (MTCC 183) and *Aspergillus niger* (MTCC 280). Stock cultures were prepared from 1-day-old bacterial and 7-day-old fungal colonies. Bacterial suspensions were standardized to optical densities between 0.09 and 0.11, achieving a concentration of 10^8 CFU/ml according to the 0.5 McFarland standards (Gupta et al. 2015). Petri dishes were filled with 25 ml of agar medium. Then, 100 µl of extract at varying concentrations (16, 32, 64, and 128 µg/ml) were added to each well. The dishes were then incubated at 37ºC for 24 h for bacterial testing and at 28ºC for 48 h for fungal tests. (Cao et al. 2004). The zones of inhibition were measured using a zone reader (Hi Antibiotic zone scale). These measurements indicated the antimicrobial activity of endophytic fungi extracts and provided insights into their potential applications in antimicrobial product development.

2.5 Determination of IC_{50} of endophytic fungi extracts

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging assay was used to evaluate the antioxidant activity of crude extracts from 21 endophytic fungi strains. In its oxidized state, DPPH is purple, but it becomes yellow when reduced by antioxidants. A 0.2 mM DPPH solution was prepared in methanol. Crude extracts was prepared at a concentration of 1 mg/ml. 100 µl of each extract was combined with 100 µl of a DPPH solution (7.88µg/100µl) in a 96-well microplate and was then homogenized. The plate was incubated in dark at room temperature for 30 minutes to allow the reaction to occur. Absorbance at 517 nm was measured with a spectrophotometer to assess DPPH reduction. A blank control, consisting of 100 µl methanol and 100 µl DPPH, was used to set the baseline. A positive control was prepared using 100 µl ascorbic acid and 100 µl methanol, serving as a standard antioxidant

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(Gadamsetty et al. 2013; Praptiwi et al. 2018). All measurements were conducted in triplicate for consistency. The half maximal inhibitory concentration (IC50) was calculated for each extract, indicating its effectiveness as an antioxidant. Antioxidant activity index (AAI) was calculated as follows:

$$\mathbf{AAI} = \mathbf{final\ concentration\ of\ DPPH\ in\ the\ reaction\ /IC50}$$

IC_{50} = the concentration of 50% inhibition was calculated by linear regression equation.

2.6 Standard and Sample Preparation for Saponin Analysis by HPLC

A saponin standard (SIGMA BioChemika) was prepared by dissolving 5 mg in HPLC-grade water and vortexed for 30 seconds. The solution was then filtered through a 0.45 µm syringe filter to remove particulate matter. This standard solution was used to generate a calibration curve in the RP-HPLC. The sample preparation procedure involves mixing 1 mg of crude extract with 1 ml of HPLC-grade water. The mixture was then heating it in a water bath for 5 minutes, cooled and subjected to vortexing for 2 minutes. To eliminate any residual particulates, the solution is filtering through a 0.45 µm syringe filter and finally injected into the HPLC system for saponin quantification (Haghi et al., 2012).

2.7 Saponin Quantification

Saponin was quantified using Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) on an Agilent HPLC model 1260 Infinity with a diode array detector and an Eclipse plus C18 reversed-phase column. The mobile phase was a mixture of water and acetonitrile (30:70 v/v), with a flow rate of 1 ml/min and an injection volume of 10 µl. The column temperature was maintained at 22ºC and the detection wavelength was set to 231 nm (Rani et al. 2022). A calibration curve for saponin was constructed, demonstrating linearity across a concentration range of 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1,000 ppm. Samples were injected into the column in triplicate for accuracy. The regression equation was used to calculate saponin concentration in crude extracts of endophytic fungi strains S-32 and S-5.

The total saponin content in the crude extracts was calculated using this formula:

$$\frac{\mathbf{Area\ of\ sample} \times \mathbf{R^2} \times \mathbf{D.F}}{\mathbf{Wt.\ of\ sample(mg)} \times \mathbf{S}}$$

Where R^2 represents the regression coefficient, S indicates the slope of the calibration curve and D.F is the dilution factor.

2.8 Molecular Characterization and Phylogenetic Analysis

Molecular identification of the isolates was performed by sequencing the 18S rRNA gene. The 18S rRNA gene was amplified using universal primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3') (White et al. 1990). A phylogenetic analysis was performed to understand the evolutionary relationships between these endophytic fungi. A phylogenetic tree was constructed using MEGA X software, applying the maximum likelihood method with the Tamura-Nei model (Tamura et al. 1993). Bootstrap analysis with 1,000 replicates provided statistical support for the phylogenetic branches.

3 Results and Discussion

3.1 Isolation of endophytic fungi

A total of twenty four endophytic fungi strains were isolated from *A. racemosus* roots, with eight from CCS HAU, Hisar, eleven from Haryana Forest Department in Rohtak, three from ICAR-NBPGR, New Delhi, one from Chaudhary Devi Lal Herbal Nature Park, Yamuna nagar, Haryana and one from GJUS&T, Hisar, Haryana. Details of the genotypes described in Table 1. Previous research on the isolation of endophytic fungi isolated 60 asymptomatic fungi representing different genera such as *Penicillium*, *Aspergillus oryzae*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Fusarium oxysporum* from *A. racemosus* (Chowdhary & Kaushik 2019). Another study isolated fifteen fungal endophytes from healthy leaves of *Ephedra pachyclada*, belonging to the genera *Penicillium*, *Alternaria*, and *Aspergillus* (Khalil et al. 2021). Furthermore, thirteen

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endophytic fungi were isolated from the roots, bark, and leaves of the rose myrtle (*Rhodomyrtus tomentosa* (Ait) Hassk) plant, which included species such as *Aspergillus heteromorphus*, *Aspergillus niger*, and *Paecilomyces subglobosus* (Handayani et al. 2023). These findings provide a foundation to study the diversity of endophytic fungi and their potential applications in various fields.

Table1. Illustration of the accession number and locations of respective isolated strains.

Sr.No.	Code of endophytic fungus	Accession number	Location
1.	E.F-2	GP-HAR-2	CCS HAU
2.	E.F-3	GP-HAR-3	CCS HAU
3.	E.F-4	GP-HAR-4	CCS HAU
4.	E.F-5	GP-HAR-5	CCS HAU
5.	E.F-6	GP-HAR-6	CCS HAU
6.	E.F-10	GP-HAR-10	CCS HAU
7.	E.F-13	GP-HAR-13	CCS HAU
8.	E.F-16	GP-HAR-16	CCS HAU
9.	S-1	Wild variety	Haryana Forest Department in Rohtak
10.	S-3	Wild variety	Haryana Forest Department in Rohtak
11.	S-4	Wild variety	Haryana Forest Department in Rohtak
12.	S-5	Wild variety	Haryana Forest Department in Rohtak
13.	S-19	Wild variety	Haryana Forest Department in Rohtak
14.	S-20	Wild variety	Haryana Forest Department in Rohtak
15.	S-27	Wild variety	Haryana Forest Department in Rohtak
16.	S-28	Wild variety	Haryana Forest Department in Rohtak
17.	S-32	Wild variety	Haryana Forest Department in Rohtak
18.	S-33	Wild variety	Haryana Forest Department in Rohtak
19.	S-34	Wild variety	Haryana Forest Department in Rohtak
20.	ND-5	IC471905	ICAR-NBPGR
21.	ND-13	IC471903	ICAR-NBPGR
22.	ND-17	IC471897	ICAR-NBPGR
23.	Y-1	Wild variety	Chaudhary Devi Lal Herbal Nature Park ,Yammuna nagar

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24.	M-1	Wild variety	GJUS&T

3.2 Antimicrobial Activity

The antimicrobial activity of crude extracts from twenty four endophytic fungi strains was analysed using minimum inhibitory concentration (MIC) values. Strain S-32 showed strong antimicrobial activity with MIC values of 32 µg/ml inhibiting gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). It also inhibited *Streptococcus entericus* at 64 µg/ml and showed antifungal activity against *Candida albicans* and *Aspergillus niger* at 128 µg/ml. Strain S-34 exhibited MIC values of 32 µg/ml against *S. aureus*, *B. subtilis*, and *P. aeruginosa*, and 64 µg/ml against *S. entericus*. Strain S-4 showed a broader antimicrobial range, inhibiting *S. aureus, B. subtilis, P. aeruginosa, S. entericus*, and *E. coli.* Strain S-19 had limited activity, inhibiting *B. subtilis* and *P. aeruginosa*, whereas strains E.F-13 and ND-13 showed no detectable antimicrobial activity at the tested concentrations (Table 2).

The minimum inhibitory concentration (MIC) values of strains S-32, S-34, and S-4 suggest that they possess compounds with wide-ranging antimicrobial activities, which aligns with earlier research revealing potent antibacterial activity (Pessini et al. 2003). These results align with previous studies highlighting the antimicrobial potential of endophytic fungi. The endophytic fungus PAL-07B1 from *Piper peltatum* demonstrated significant antibacterial activity against *S. aureus* and *E. coli* with MIC values of 8 µg/ml (Praptiwi et al. 2018). Other research has reported antimicrobial activity against *B. subtilis, S. agalactiae, S. aureus, P. aeruginosa,* and *E. coli* (Yang et al. 2015; Dissanayake et al. 2016). Jin et al. (2017) identified two novel saponin-producing endophytic fungi, *Fusarium sp*. (PN8) and *Aspergillus sp.* (PN17), from *Panax notoginseng* that showed antimicrobial activity against five pathogenic bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341 and two yeasts *Candida albicans* ATCC 90028 and *Saccharomyces cerevisiae* ATCC Y139. These studies suggest that endophytic fungi are a promising source of antimicrobial compounds, with potential applications in combating multidrug-resistant bacterial and fungal pathogens (Elkady et al. 2022).

Table 2. Minimum inhibitory concentrations (MICs, µg/ml) of the extracts of endophytic fungi from *Asparagus racemosus* that presented antimicrobial activity.

Endophytic Fungi Code	MIC (µg/ml)						
	SA	BS	PA	SE	EC	CA	AN
E.F-2	-	128	64	128	128	-	-
E.F-3	-	64	64	64	128	-	-
E.F-4	128	64	64	128	64	-	-
E.F-5	64	64	128	64	128	-	-
E.F-6	64	128	128	-	-	64	-

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E.F-10	64	64	64	128	32	-	-
E.F-13	-	-	-	-	-	-	-
E.F-16	64	32	128	64	128	-	-
S-1	64	64	64	128	64	-	-
S-3	64	64	64	128	64	-	-
S-4	64	64	64	64	64	128	-
S-5	128	64	64	128	32	-	-
S-19	-	128	128	-	-	-	-
S-20	-	64	128	128	64	-	-
S-27	64	32	64	64	32		
S-28	64	32	32	32	64	-	-
S-32	32	32	32	64	32	128	128
S-33	64	128	64	64	64	-	-
S-34	32	32	32	64	32	128	-
ND-5	64	64	64	64	32	-	-
ND-13	-	-	-	-	-	-	-
ND-17	64	32	64	64	64	-	-
Y-1	128	128	128	32	128	-	-
M-1	-	-	-	-	-	64	-

SA = *Staphylococcus aureus*; BS = *Bacillus subtilis*; PA = *Pseudomonas aeruginosa*; SE = *Streptococcus entericus*; EC = *Escherichia coli*; CA = *Candida albicans*; AN = *Aspergillus niger*, (-) No significant MIC.

3.3 Antioxidant Activity

The antioxidant activity of crude extracts from twenty four endophytic fungi strains using the DPPH radical scavenging assay was evaluated. All strains exhibited antioxidant activity to varying degrees, strain S-5 showed the strongest antioxidant capacity with an IC50 of 4.4 ug/ml. Other strains, such as ND-5, ND-13, and ND-17, also showed significant antioxidant activity. The Antioxidant Activity Index (AAI), which reflects overall antioxidant strength, supported these results, with S-5 having an AAI of 1.7, while ND-5, ND-13, and ND-17 exhibited AAIs of 1.1, 0.9, and 0.9, respectively (Table 3). The antioxidant activity observed in the current study supported the results showed by the previous research which obtained significant antioxidant activity against *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Aspergillus flavus* (Pan et al. 2017). Saponin and flavonoid-producing fungi exhibited particularly strong antioxidant properties (Gunasekaran et al. 2017). Specifically, *Fusarium proliferatum* SaR-2 and *Alternaria alternata* SaF-2 demonstrated notable DPPH (83.25%) scavenging activities due to phytochemicals like saponins, phenols, and flavonoids (Li et al. 2015). Further research is needed to isolate and characterize

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other compounds responsible for these antioxidant effects and explore potential applications in health, food preservation, and related industries.

Table 3. IC_{50} and antioxidant activity index of endophytic fungi extract

Extracts	**IC_{50} (ug/ml)**	**AAI**	**Activity**
Standard Ascorbic acid	1.61	4.9	Very strong
E.F-2	40.33	0.197	Moderate
E.F-3	9.37	0.847	Moderate
E.F-4	42.17	0.18	Moderate
E.F-5	38.23	0.2	Moderate
E.F-6	49.82	0.15	Moderate
E.F-10	60	0.13	Moderate
E.F-13	94.64	0.08	Moderate
E.F-16	17.29	0.45	Moderate
S-1	32	0.24	Moderate
S-3	12.88	0.61	Moderate
S-4	49	0.16	Moderate
S-5	4.4	1.7	Very strong
S-19	71	0.11	Moderate
S-20	56.30	0.13	Moderate
S-27	41	0.19	Moderate
S-28	17	0.4	Moderate
S-32	22.30	0.3	Moderate
S-33	179	0.04	Moderate
S-34	62.18	0.12	Moderate
ND-5	7.14	1.1	Strong
ND-13	8.33	0.9	Strong
ND-17	8.75	0.9	Strong
Y-1	217.98	0.035	Low

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M-1	214.87	0.036	Low

3.4 HPLC Analysis for quantification of saponin

Crude extracts from endophytic fungi strains S-32 and S-5 were selected for saponin quantification following initial screening. As indicated in Table 4, strain S-32 had the highest saponin concentration at 2174.95 ng/mg, while strain S-5 also demonstrated a significant saponin content of 1244.55 ng/mg. The quantification was supported by chromatographic analysis, with Figures 1 and 2 illustrating the chromatogram for the saponin standard and the calibration curve used to ensure accuracy. Figures 3 and 4 present the chromatograms for S-32 and S-5, clearly showing saponin peaks. Saponin peaks observed at 1.8 minutes retention time. These results are consistent with earlier studies, such as Wu et al. (2012), which found a high saponin concentration of 2.049 mg/ml in endophytic fungus G22 (*Penicillium sp*.) isolated from *Aralia elata* in Northeast China. This alignment suggests that some endophytic fungi are capable of producing significant saponin levels, with potential applications in pharmaceuticals, cosmetics, and agriculture. The observations of Wu et al. (2013) regarding the antioxidant activity of endophytic fungus Pg27 (*Fusarium sp*.) also point to the potential medicinal application of saponins produced by these fungi.

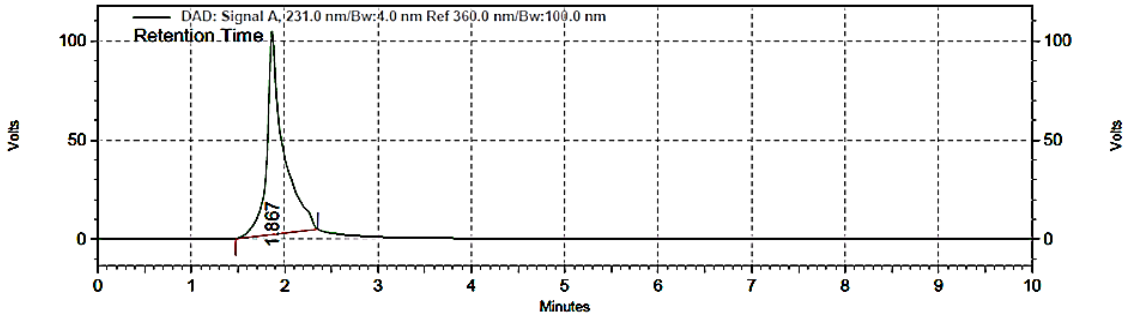

Figure 1. Chromatogram of saponin standard

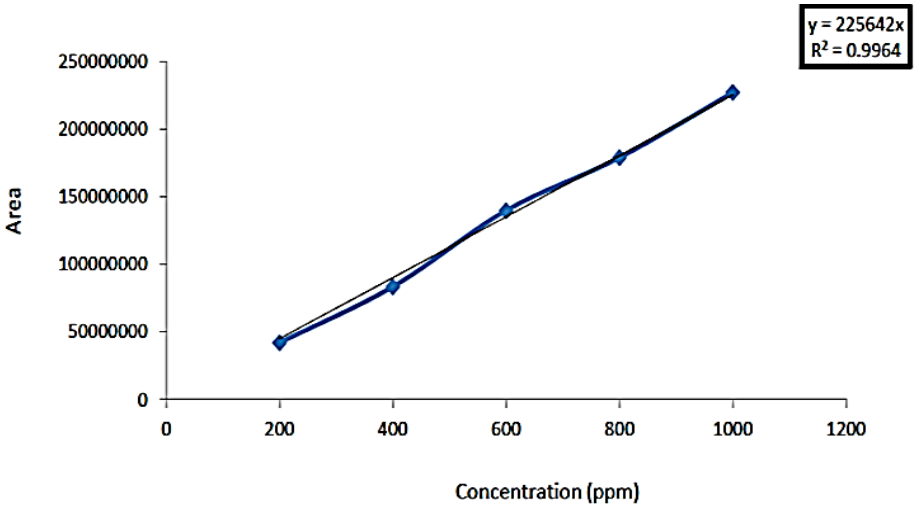

Figure 2. Calibration curve of saponin standard

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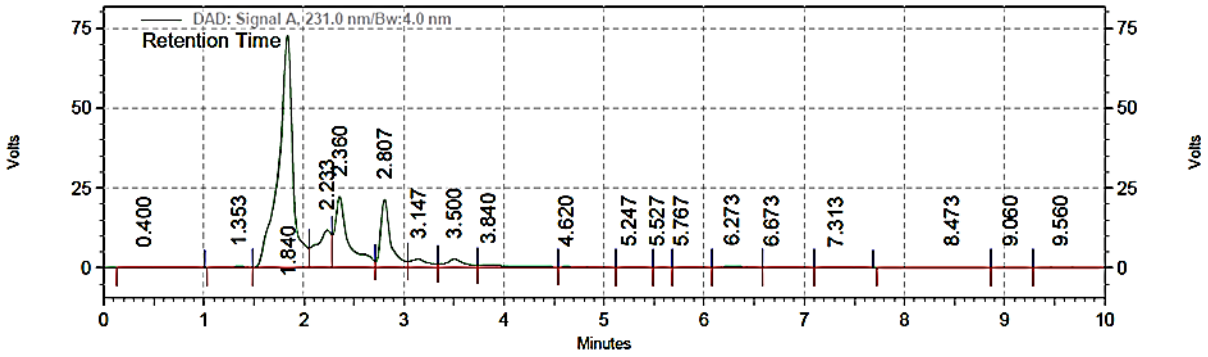

Figure 3. Chromatogram of extract of S-32 endophytic fungus

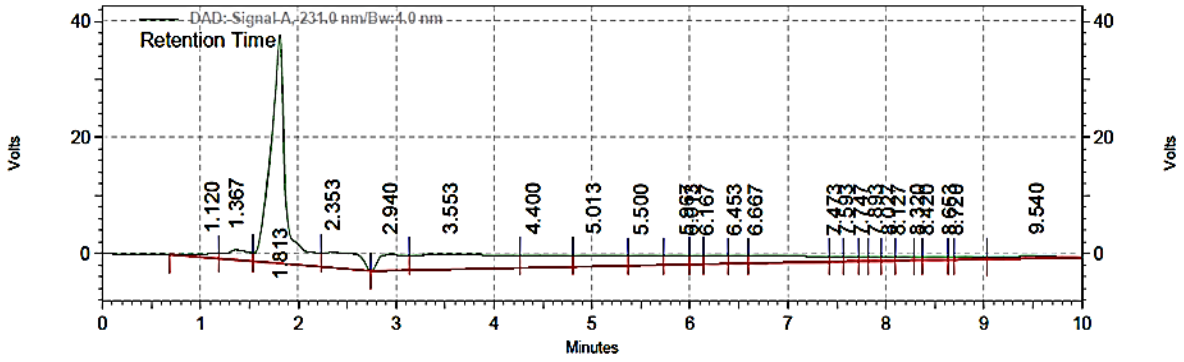

Figure 4. Chromatogram of extract of S-5 endophytic fungus

Table 4. Concentration of saponin in S-32 and S-5 endophytic fungi extracts

Strains	Retention Time(RT)	Mean Area of triplicates	Saponin Concentration (ng/mg)
S-32	1.8	98507083	2174.95
S-5	1.8	56367838	1244.558

3.5 Molecular characterization of endophytic fungi

The phylogenetic analysis of the 18S rRNA sequences revealed that strains S-32 and S-5 exhibited 100% similarity with their closest relatives. Strain S-32 showed a complete match with *Aspergillus terreus* (GU227345), while strain S-5 matched with *Aspergillus oryzae* (XR_002735719.1). Figures 5 and 6 illustrate the evolutionary trees, confirming the identification of S-32 as *Aspergillus terreus* and S-5 as *Aspergillus oryzae.* In previous study, PN8 and PN17 endophytic fungi were isolated from *Panax notoginseng*, as *Fusarium sp.* and

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Aspergillus sp. with 99.9% and 97.7% similarity with sequence (Jin et al. 2017). Similarly, saponin-producing fungal endophytes Pg27, Pg30, and Pg42-1 from *Panax ginseng* were classified as species of *Fusarium*, *Aspergillus*, and *Verticillium*, respectively (Wu et al. 2013). Moreover, strains AK1, AK3, and AK4 from *Artemisia sieberi* identified them as *Aspergillus oryzae*, while AK6 was identified as *Aspergillus niger* (Ababutain et al. 2021). Additionally, *Aspergillus niger* and *Aspergillus flavus* were isolated from the roots of *A. mexicana*, showing 98% and 100% similarity, respectively. These results add to the growing evidence on the diversity and potential applications of endophytic fungi as sources of bioactive compounds.

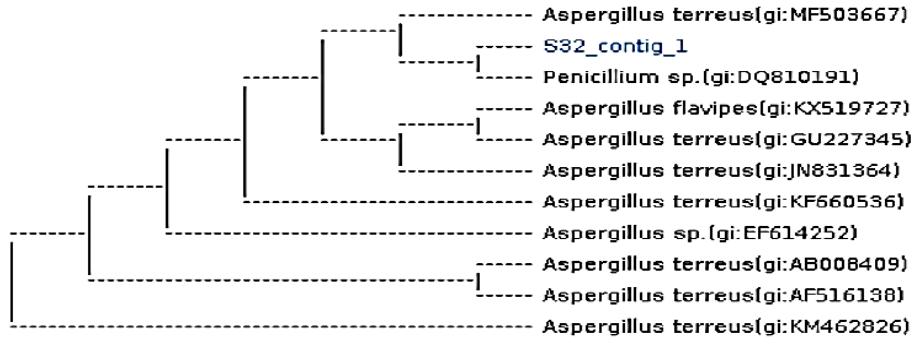

Figure 5. Phylogenetic tree of S-32 endophytic fungus isolated from *A.racemosus*

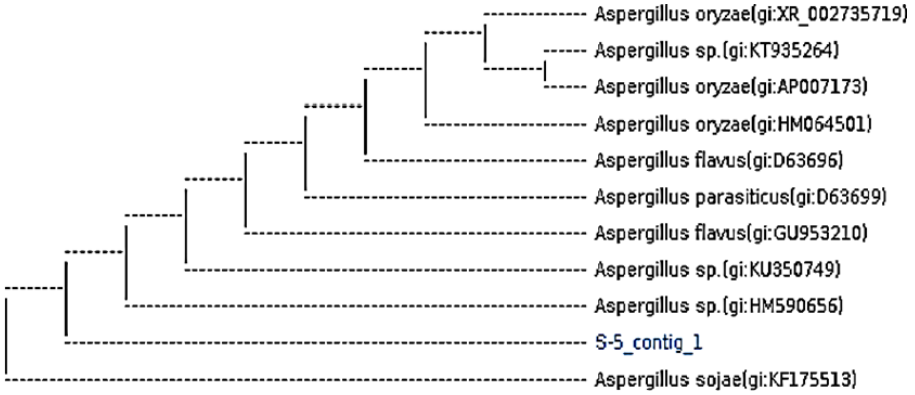

Figure 6. Phylogenetic tree of S-5 endophytic fungus isolated from *A.racemosus*

4. Conclusion

This study underscores the potential of endophytic fungi as a valuable source of bioactive compounds with significant applications in pharmaceuticals, agriculture, and cosmetics. The antimicrobial and antioxidant activities exhibited by the crude extracts suggest that these fungi harbor compounds with beneficial properties. Additionally, the high saponin content found in strains S-32 and S-5 points to further research opportunities. Saponins, known for their diverse applications, could have significant implications for drug development, natural pesticides, and skincare products. By isolating and characterizing these saponins, researchers can explore their potential bioactivities and broader uses. The findings indicate that endophytic fungi offer a promising avenue for the development of natural antioxidants and other bioactive compounds. Further studies should aim to delve deeper into these strains' unique properties, unlocking new pathways for sustainable and innovative applications.

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Author contributions

VC and SJ conceptualized the research study and prepared the technical materials for the experiment. MR conducted the experiment and performed the experimental analysis. MR drafted the manuscript with support from VB. VC and VB reviewed and edited the manuscript for finalization.

Declaration

All Authors declare no conflict of interest is involved in the present manuscript.

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