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Endophytic Fungi from *Evolvulus alsinoides* Linn.: Molecular Insights and AChE Inhibition Potential

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ABSTRACT

The isolation of endophytic fungi from the stem of *Evolvulus alsinoides* Linn. revealed two distinct fungal strains: *Penicillium citrinum* NRRL 1841 (EASF-1) and *Aspergillus flavus* ATCC 16883 (EASF-2). Two fractions, EASF-2-EA and EASF-2-NB, extracted from *Aspergillus flavus* ATCC 16883 were evaluated for their potential therapeutic effects. Phytochemical analysis of the bioactive compounds in both fractions unveiled the presence of alkaloids, flavonoids, terpenoids, saponins, coumarins, glycosides and tannins. Both fractions demonstrated *in-vitro* antioxidant activity against DPPH and hydroxyl radicals, with EASF- 2-NB exhibiting superior efficacy. These compounds also exhibited anti-cholinesterase activity, with EASF-2-NB being more potent. The results underscore the antioxidative properties and AChE inhibitory effects observed in the EASF-2-EA and EASF-2-NB fractions derived from *Aspergillus flavus* ATCC 16883, indicating their potential in addressing memory-related conditions, notably Alzheimer's disease.

KEYWORDS – Anticholinesterase activity, *Aspergillus flavus,* Endophytic fungi, *Evolvulus alsinoides* Linn., *Penicillium citrinum.*

INTRODUCTION

Endophytic fungi, residing asymptomatically within plant tissues, have gained recognition as prolific producers of bioactive metabolites that often mimic or enhance the metabolic profiles of their host plants[1,2] (Leifert et al. 1991; Li et al. 1998). Medicinal plants like *Evolvulus alsinoides* are particularly rich reservoirs of these endophytes, which offer a diverse array of secondary metabolites with therapeutic potential [3,4] (Strobel 2002; Pathak et al. 2022). *E. alsinoides*, known for its neuroprotective and adaptogenic properties, contains various phytochemicals such as alkaloids, flavonoids, and phenolic compounds [5] (Kim et al. 2010). Endophytes isolated from *E. alsinoides* have been found to produce secondary metabolites that contribute to its medicinal value, particularly in neuroprotective and enzyme inhibitory activities.

Studies have shown that endophytes associated with *E. alsinoides* can produce bioactive compounds, including alkaloids, flavonoids, and phenolic acids, which exhibit antioxidant, antimicrobial, and acetylcholinesterase (AChE) inhibitory activities [6,7] (Na et al. 2016; Garyali et al. 2013). These microorganisms are recognized for their role in the production of novel drug leads, contributing to the development of therapeutic agents for conditions like Alzheimer's disease [8,9] (Devi et al. 2023; Bondi et al. 2017). The isolation and characterization of endophytes involve culturing fungal strains from the plant tissues, followed by molecular identification through techniques such as DNA sequencing and bioactivity screening [10] (Devi et al. 2023). Given the limited efficacy of current Alzheimer's treatments, investigating endophytes from plants like *E. alsinoides* offers a promising alternative for drug discovery. Hence, this study sought to isolate endophytic fungi dwelling within both the leaves and stems of *E. prostrata* Linn. to screen potential fungal fractions for quantitative phytochemical investigations for *in vitro* antioxidant, acetylcholinesterase inhibitory and further exploration for nootropic activities.

MATERIALS AND METHODS

Sampling of plant materials

Recently, harvested and dried twigs constituting the foliage and stalks of *E. alsinoides* Linn. were collected from Nipani, District Belgavi, Karnataka. This collected sample was authenticated by a taxonomist associated with the Botany Department at Karnataka Science College, Dharwad, Karnataka, India. Following the validation of the plant material, an herbarium specimen (SETCPD/Ph.cog/Herb/2023/02) was meticulously prepared and archived at the College of Pharmacy, Dharwad, in the Pharmacognosy division under SET's umbrella.

Isolation of endophytic fungi

The authenticated botanical specimens underwent a meticulous cleaning process using running tap water to remove any clinging dust particles, followed by air-drying. Prior to sterilization, the leaves and stems were separated. The leaf and stem fragments were subjected to surface sterilization through a series of steps, including treatment with a 4% bleach solution for 5 minutes, then with 70% ethanol for 1 minute, and finally with aseptic distilled water for 1 minute. This process was repeated 2–3 times. Additionally, certain surface-sanitized fragments were transferred to an alcohol-sterilized mortar and crushed individually into suspensions using distilled water, followed by serial dilutions. Both the surface-sterilized plant fragments and diluted suspension aliquots were inoculated into sterile petri dishes filled with potato dextrose agar (PDA) supplemented with 150 mg/L streptomycin to hinder microbial proliferation[11] (Dhankhar et al. 2012). After an incubation at 23-25°C for 7-14 days, prevalent fungal strains were identified, selected and refined based on their colony morphology to ensure culture purity. Three distinct, genuine endophytic mycelia, namely, EASF-1 and EASF-2. EASF-2 (endophytic mycota of *E. alsinoides* Linn.), were identified for further investigation [12] (Wiyakrutta et al. 2004, Silva et al. 2005).

Molecular identification of endophytic fungi

Endophytic fungal identification of *Penicillium citrinum* NRRL 1841 and *Aspergillus flavus* ATCC 16883 involved extracting genomic DNA using standard methods, involving cell breakdown at 68°C for 30 minutes[13] (Doty et al. 2005). Successful amplification of approximately 550 base pairs of ribosomal DNA fragments was achieved with ubiquitous primers. PCR sequencing was conducted using the ABI-Big Dye® Terminatorv3.1 Cycle Sequencing Kit. The resulting sequence data were aligned with publicly accessible genetic sequences and analysed to similarity. PCR targeting universal rDNA was carried out with the previously reported ITS primers ITS1F TCCGTAGGTGAACCTGCGG and ITS4R TCCTCCGCTTATTGATATGC. The thermocycler program comprised stages set at 94°C for 10 minutes, 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes and was cycled 35 times. Following PCR, the samples were subjected to electrophoresis, using a 1.2% agarose matrix within a horizontal electrophoresis apparatus by Tarsonand documented using the BiorAD gel documentation sy1stem. Purification of PCR products was conducted using an Oxygen PCR Purification kit, and then DNA quantification was performed using a NanoDrop spectrophotometer. Afterward, sequencing PCR was conducted using the ITS4R primer from the Applied Biosystems sequencing kit using Big Dye Terminator technology. Clean-up of the products was achieved through the EDTA method, followed by analysis on an automated DNA sequencer (ABI-Applied Biosystems) model Prism 3100. Manual editing of the obtained sequences was carried out to rectify inconsistencies, and identity was determined through an NCBI BLAST search [14,15] (Saitou et.al. 1987, Kim et.al.1993).

Fermentation and preparation of endophytic extracts

Two distinct endophytic fungal isolates, EASF-1 and EASF-2 were obtained and subsequently purified. The fungi were individually inoculated and fermented in 100ml Potato- Dextrose-Broth (PDB). Upon observation of enhanced growth in EASF-2, this particular isolate was chosen for further fermentation. EASF-2 was transferred to a 3000 ml conical laboratory flask containing 3L of PDB (comprising 72 gm of potato dextrose broth and 0.5 mg of streptomycin adjusted to a pH of 5.1± 0.2). The inoculated flask with media was incubated at 23°C-27°C for a minimum of 28 days under stationary conditions devoid of light. Following a one-month incubation period, 300 ml of ethyl acetate/n-butanol was added to each flask, the mixture was thoroughly mixed, and the mixture was left to stand overnight. The fungus culture treated with ethyl acetate was filtered through four layers of cheesecloth and blended at 4000 rpm to separate the fungal filaments from the broth. The filtrate underwent a triple extraction process using 300 ml of ethyl acetate/n-butanol each time. The resultant non-aqueous phase was subjected to evaporation under reduced pressure employing a rotary evaporator, culminating in the determination of the weight to establish the composition of the unrefined extract[16]. (Puri et al. 2021). The fractions obtained from the endophytic sources were labelled EASF-2-EA and EASF-2-NB for the ethyl acetate and n-butanol fractions, respectively.

Analysis of the unpurified endophytic fractions EASF-2-EA and EASF-2-NB: A qualitative and quantitative approach

Preliminary phytochemical screening of endophytic extracts

The qualitative analysis of EASF-2-EA and EASF-2-NB was performed following standard procedures to detect the various classes of secondary metabolites [17,18]. (Khandelwal K 2008, Kokate CK 2005).

Quantification of Overall Phenolic Levels

The Folin–Ciocalteu method was used to assess the total phenolic content within the endophytic fractions. By measuring the intensity of the blue colour resulting from the presence of polyphenols across a range of concentrations (10-1000 µg/mL) in trial samples at 760 nm using an ultraviolet– visible spectrometer, the analysis was conducted. A standard curve was established using gallic acid at different concentrations(10-100µg/mL). The quantification of total phenolic content within the endophytic fungal fractions (EASF-2-EA and EASF-2-NB) was denoted as milligrams of GAE (Gallic Acid Equivalents) per gram of sample [19] (Mendonça et al. 2021).

Quantification of overall flavonoid levels:

The aluminum chloride colorimetric method was used to determine the total flavonoid content present in the endophytic fractions. Analysing the reactions of different strengths (10 - 1000 µg/mL) of the trial samples at an absorbance of 415 nm using a UV–VIS spectrometer facilitated this assessment. A standard graph was generated using a range of concentrations (10-100 µg/mL) of quercetin on the X-axis against the corresponding absorbance on the Y-axis. The quantification of total flavonoid content within the endophytic fungal fractions (EASF-2-EA and EASF-2-NB) was derived from this standard graph, denoted as milligrams of quercetin equivalents per gram of sample.

Investigation of antioxidant potential: Assessing DPPH radical scavenging activity

The scavenging activity of EASF-2-EA and EASF-2-NB (at concentrations of 20–200 µg/ml) was assessed in the presence of the stable radical DPPH, employing ascorbic acid at concentrations ranging from 1–5 µg/ml as the standard reference. The establishment of the standard curve was accomplished by plotting the percentage of DPPH scavenging activity against the concentration of ascorbic acid. This process aimed to gauge the antioxidative potential of the tested endophytic fractions in comparison to the known standard, ascorbic acid [20,21] (Vijayaraghavan et al. 2013, Raut et al. 2022).

Assessment of antioxidant capacity: Hydroxyl radical elimination activity

This study involved evaluating the steady-state scavenging activity of EASF-2-EA and EASF-2-NB against hydroxyl radicals (*****OH) at various concentrations (05–450 µg/ml). This assessment was conducted using the deoxy-D-ribose degradation method. For comparative analysis, mannitol served as the standard reference. Various concentrations (ranging from 0.5–5.0 µg/ml) of mannitol were combined as described previously. A standard curve was generated by graphing the percentage of *****OH scavenging inhibition relative to that of the various concentrations of mannitol. This process aimed to establish the antioxidative potential of the tested endophytic fractions in comparison to the standard compound mannitol [22]. (Tong et al 2021).

Assessment of AChE inhibitory potential in endophytic fractions employing the Ellman's reagent technique

The reduction in acetylcholinesterase (AChE) activity was evaluated by employing a Microplate spectrophotometer (BioTek, USA) following the principles of Ellman's method. This method relies on the enzymatic action of Acetylcholinesterase (AChE) on Acetylthiocholineiodide substrate, leading to the production of acetylthiocholine. Thiocholine subsequently reacts with Ellman's reagent (DTNB) to yield a yellow-tinted product, the intensity of which, measured at 405 nm, is indicative of enzymatic performance [23] (Ali-Shtayeh et al. 2014). For the assay, 25 µl of 15 mM Acetylthiocholineiodide in water, 125 µl of 3 mM Ellman's reagent (DTNB) in buffer B, and 25 µl of the fungal extract were combined in 96-well plates. The absorbance was recorded at 405 nm. Subsequently, 25 microliters of AChE solution at a concentration of 0.22 units per milliliter was dispensed into the wells, and readings were taken at the same wavelength at one-minute intervals for ten readings.

Galantamine, dissolved in methanol at a concentration of 1 mg/ml, was used as the standard drug. A control solution consisting of methanol within a 50 mM Tris-HCl buffer at pH 8 was utilized as a blank. The percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 1 - (\text{Abs of control}/\text{Abs of sample}) \times 100$$

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RESULTS

Fresh and dry twigs of *Evolvulus alsinoides* Linn. (leaves and stems) were collected from Nipani, Belgavi district, Karnataka. Endophytic fungi were observed in the stem of *E. alsinoides* Linn. The isolated endophytic fungi were designated EASF-1(Fig.1) and EPSF-2(Fig.2).

Fig 1. Colony morphology of endophytic fungus *Penicillium citrinum* NRRL 1841

Fig 2. Colony morphology of endophytic fungus *Aspergillus flavus* ATCC 16883

Morphological identification of EASF-1

The colonies of EASF-1, as observed in the image, display a compact and well-defined circular morphology with dark pigmentation, indicative of spore development. The surface of the colonies is rough and wrinkled, especially toward the central region, giving them a distinctive texture. The color of the colonies is predominantly dark, almost black, which is characteristic of certain filamentous fungi like *Aspergillus* or *Penicillium* species. Multiple distinct colonies are present, suggesting robust and rapid growth. Additionally, a slight zonation can be observed, with a central dark area transitioning gradually toward the edges, though the overall colony color remains relatively uniform. These features align with typical fungal growth in nutrient-rich conditions.

The colony morphology of EASF-2, based on the provided image and its resemblance to *Aspergillus flavus* ATCC 16883, exhibits a velvety or powdery texture typical of *Aspergillus* species, with a dense, greenish surface, which is consistent with the spore production seen in *Aspergillus flavus*. The colonies show a radial growth pattern with clearly defined circular zones of different densities. The color is predominantly green, indicating the presence of conidia, which is characteristic of *A. flavus*. The outer edges of the colony appear slightly lighter, suggesting active sporulation. The colony surface seems fluffy and raised, particularly at the center, while the outer regions spread out more thinly across the agar surface.

These features closely resemble those of *Aspergillus flavus*, which is known for producing green conidia, a powdery texture, and zonal colony morphology. The robust and rapid growth of EASF-2 further suggests that it shares many traits with *A. flavus*.

Molecular identification of EASF-1

The identity of EASF-1 was confirmed via nucleotide sequence investigation utilizing the mega Basic Local Alignment Search Tool (BLAST) housed within the National Center for Biotechnology Information (NCBI). High-quality DNA isolated from EPSF-1 was subjected to PCR using the ITS1F and ITS4R primers. The rDNA genes of EASF-1 exhibited 98.79% similarity with those of other *Penicillium citrinum* NRRL 1841. Specifically, EASF-1 was conclusively identified as *Penicillium citrinum* NRRL 1841. Detailed information regarding the GeneBank accession number of EASF-1 and its similarity with closely related fungal strains and the resulting phylogenetic tree can be found in Table:1 and Figure 3.

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Table:1 NCBI BLAST Findings for EASF-1 Fungal Isolates Extracted from *E. alsinoides* Linn.: Identification and Similarity Analysis

ID	Description	% Similarity
NR_121224.1	*Penicillium citrinum* NRRL 1841	98.79%
NR_111482.1	*Penicillium hetheringtonii* CBS 122392	98.15%
NR_111485.1	*Penicillium tropicum* CBS 112584	95.54%

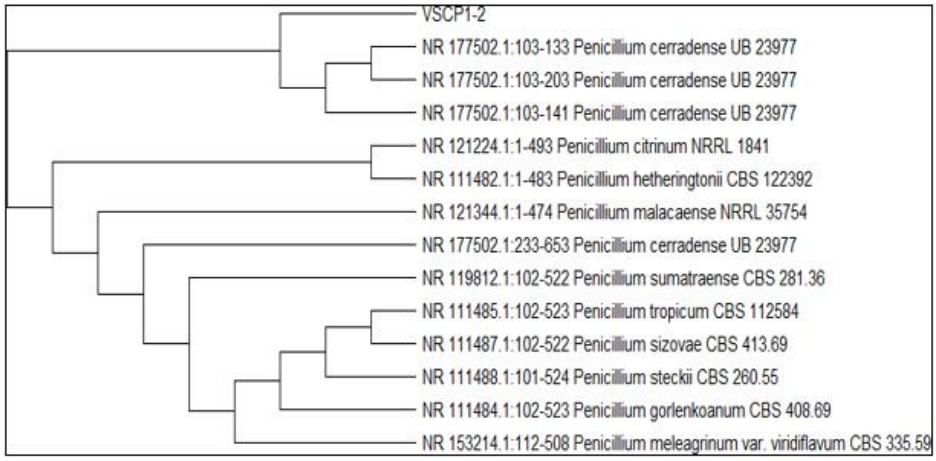

Fig 3. Phylogenetic tree of EASF-1

Molecular identification of EASF-2

The molecular analysis of the strain EASF-2 was performed using the ITS gene sequence and compared against the NCBI database using the BLASTN program. The DNA sequence of EASF-2 matched most closely with *Aspergillus flavus* ATCC 16883, showing 99.64% similarity.

Individual colonies were selected for direct colony PCR. The 600 bp amplicons were cut from the agarose gel, purified, and sequenced. Sequence analysis of EASF-2 confirmed a high degree of homology with strains in the *Aspergillus* genus, specifically *A. flavus*.

The ITS gene sequence of EASF-2 was aligned with 10 matching sequences using the Maximum Likelihood method under the Tamura-Nei model. The tree with the highest log likelihood (-967.95) was constructed based on Neighbor-Join and BioNJ algorithms. Branch lengths represent substitutions per site across 638 positions, with codon positions 1st, 2nd, 3rd, and noncoding regions considered. The analysis, involving 11 sequences, was conducted using MEGA X software. Detailed information regarding the GeneBank accession number of EASF-2 and its similarity with closely related fungal strains and the resulting phylogenetic tree can be found in Table-2 and Figure 4.

Table:2 NCBI BLAST Findings for EASF-2 Fungal Isolates Extracted from *E. alsinoides* Linn.: Identification and Similarity Analysis

ID	Description	% Similarity
NR_111041.1	*Aspergillus flavus* ATCC 16883	99.64%
NR_135395.1	*Aspergillus oryzae* NRRL 447	99.64%
NR_172033.1	*Aspergillus sergii* MUM 10.219	98.75%

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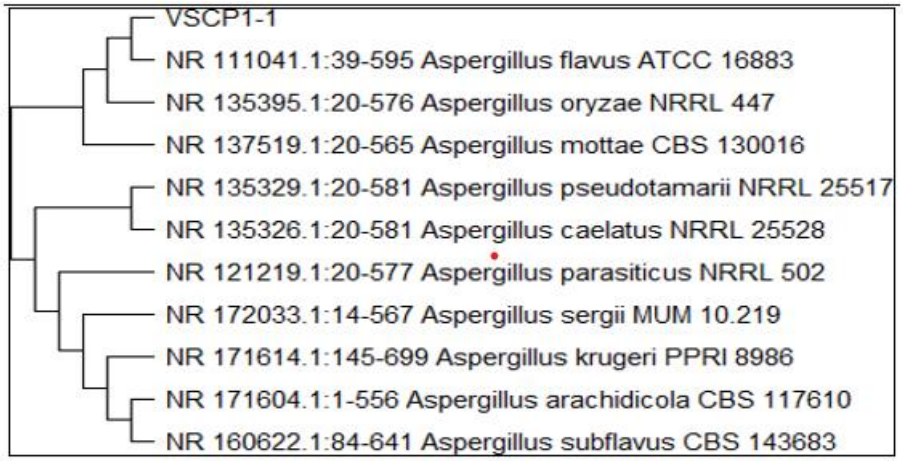

Fig 4. Phylogenetic tree of EASF-2

The DNA sequence of EASF-2 shows the highest similarity with *Aspergillus flavus* ATCC 16883.

Fermentation and preparation of endophytic extracts

EASF-2 exhibited maximum growth and was fermented in PDB to prepare crude endophytic fractions. The % yield for the EASF-2 fraction was found to be 0.5 g/L for ethyl acetate (EPSF-1- EA) and 0.7 g/L for n-butanol (EPSF-1-NB).

Qualitative and quantitative determination of secondary metabolites

Both EASF-2-EA and EASF-2-NB were found to contain coumarins, flavonoids, phenolic compounds, saponins, tannins and steroids in qualitative phytochemical investigations. According to our quantitative methods, the total phenolic content in EASF-2-EA and EASF-2-NB was 30.97 mg and 43.56 mg GAE/gm, respectively, of the extract. The total flavonoid contents in EASF-2-EA and EASF-2-NB were found to be 22.11 mg and 39.42 mg QUE/gm of the extract, respectively.

Assessment of DPPH radical scavenging activity

The DPPH assay relies on the reaction between a methanolic solution containing the colored free radical DPPH and compounds that act as free radical scavengers. DPPH's absorbance decreases specifically at the maximum absorption point at a wavelength of 516 nm, which is directly correlated with the concentration of the added antioxidant in the DPPH solution. The ability of EASF-2-EA and EASF-2-NB to scavenge DPPH radicals was evaluated using a 96-well method in an ELISA reader. The determined IC_{50} values for EASF-2-EA and EASF-2-NB were 83.8 micrograms per milliliter and 45.5 micrograms per milliliter, respectively. Ascorbic acid, utilized as the reference or control substance, demonstrated an IC_{50} value of 3.44 micrograms per milliliter. as illustrated in Figure 5.

Assessment of *OH radical neutralization capability

The hydroxyl radical elimination/scavenging assay involves the *in-vitro* creation of hydroxyl radicals utilizing the ascorbate-Fe-EDTA-H2O2 system through Fenton chemistry. The resulting yellow color intensity was spectrophotometrically measured at 412 nm against a solvent control. The data presented demonstrate the generation of *OH radicals in solution by phenyl hydrazine, as measured through the 2-Deoxyribose degradation assay.

Notably, the present study revealed the *OH radical scavenging activity of EASF-2-EA and EASF-2-NB, which exhibited median inhibitory concentrations of 44.60 µg/ml and 38.21 µg/ml, respectively. In contrast, mannitol had an IC_{50} of 4.67 µg/ml. The findings revealed the potent scavenging ability of EASF-2-EA and EASF-2-NB against hydroxyl radicals, albeit at higher concentrations than mannitol. as depicted in Figure 6, illustrating the impact of EASF-2-EA and EASF-2-NB on hydroxyl radical activity.

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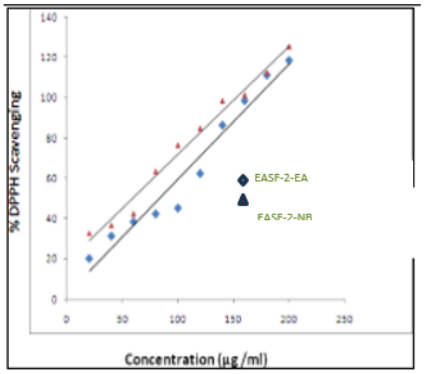

Fig 5. Impact of EASF-**2**-EA and EASF-**2**-NB on the DPPH radical

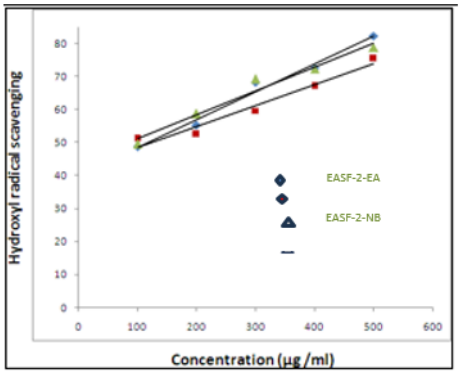

Fig 6. Impact of E2SF-1-EA and EASF-**2**-NB on hydroxyl radical

Assessment of Acetylcholinesterase inhibition using Ellman's assay

The results on the AChE inhibitory potential of EASF-2-EA and EASF-2-NB are shown in Figure 7. The results are expressed as IC_{50} values derived from the equations obtained through regression analysis of the different concentrations of the samples. Both EASF-2-EA and EASF-2-NB showed strong inhibitory activity, with half-maximal inhibitory concentrations of 11.36 µg/mL and 9.35 µg/mL, respectively. Galantamine was used as a standard inhibitor.

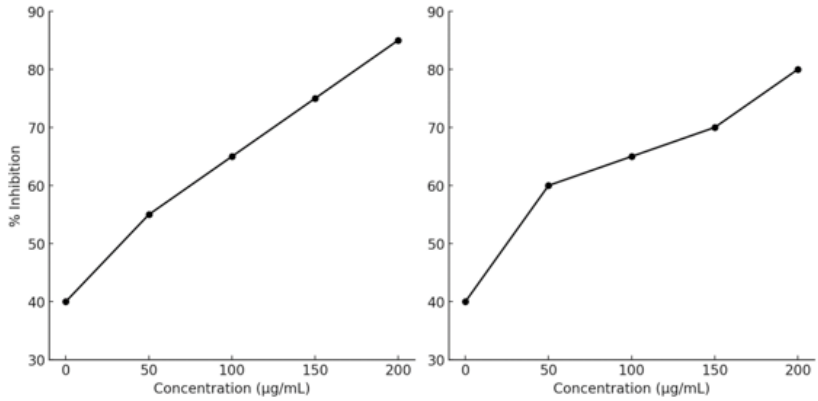

Fig 7. AChE Inhibitory effects of EASF-2-EA and EASF-2-NB

DISCUSSION

The isolation and characterization of endophytic fungi from *Evolvulus alsinoides Linn.* led to the identification of two significant strains: *Penicillium citrinum NRRL 1841* (EASF-1) and *Aspergillus flavus ATCC 16883* (EASF-2). The phytochemical analysis of the ethyl acetate (EASF-2-EA) and n-butanol (EASF-2-NB) fractions extracted from *Aspergillus flavus* highlighted the presence of bioactive compounds, including alkaloids, flavonoids, terpenoids, saponins, coumarins, glycosides, and tannins. These metabolites are known to possess diverse pharmacological properties. The study revealed potent antioxidant activities in both fractions, with EASF-2-NB demonstrating superior efficacy in scavenging free radicals, particularly DPPH and hydroxyl radicals. Additionally, both fractions exhibited significant acetylcholinesterase (AChE) inhibitory activity, with EASF-2-NB showing greater potency. These findings suggest that the secondary metabolites produced by *Aspergillus flavus* endophytes contribute to their antioxidative and neuroprotective potential, particularly in combating oxidative stress and inhibiting AChE, which is critical in the management of neurodegenerative diseases like Alzheimer's. The promising results from the *in vitro* assays underline the therapeutic potential of these fungal extracts in treating memory-related conditions, warranting further investigation into their bioactive components and mechanisms of action.

CONCLUSION

The study successfully isolated two endophytic fungi from *Evolvulus alsinoides*, with *Aspergillus flavus ATCC 16883* showing significant antioxidant and acetylcholinesterase inhibitory activities. Both the EASF-2-EA and EASF-2-NB fractions demonstrated potential for addressing oxidative stress and neurodegenerative conditions, particularly Alzheimer's disease. These findings suggest that the bioactive compounds present in the fungal extracts hold promise for therapeutic applications and warrant further investigation.

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