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Biological prospecting of endophytic fungi from the stem bark of *Oroxylum indicum* (L.) Kurz with antioxidant efficacy

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ABSTRACT

Reactive oxygen species (ROS) require careful regulation in living organisms, as growing scientific evidence suggests their continuous production occurs even during typical cellular processes. Excessive production of ROS can lead to systemic oxidative stress, harming biological macromolecules and accelerating the aging process, diabetes, cancer, and other severe illnesses. This study investigated the presence of fungal endophytes associated with the stem bark of *Oroxylum indicum* (OI), and their phytochemical profile and antioxidant efficacy. We identified three fungal endophytes from two distinct genera. Three fungal endophytes namely, *Simplicillium obclavatum* (SO), *Neopestalotiopsis clavispora* (NC), and *Trametes polyzona* (TP) are the first to be documented from OI stem bark. The ethyl acetate extract of NC shown notable antioxidant activity against superoxide anion radical and (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) free radical among the identified endophytes with the scavenging percentage value of 33.54 ± 0.4, 57.03 ± 0.69, and 37.41 ± 0.32, respectively, at the concentration of 100 µg/ml of the extract. The significant antioxidant activity of 24.07 ± 0.88 ascorbic acid equivalents was observed for NC extract. The mean total phenolic and total flavonoid content of NC extract was found to be 33.33 ± 0.22 and 38.90 ±1.17 gallic acid and quercetin equivalents, respectively, which was significantly higher compared to SC and TP isolates. Our study has provided insights on the presence of phytochemicals and their antioxidative potency in the isolated endophytic fungi from the stem bark of OI.

1. INTRODUCTION

Plants are the source of several medicinally important compounds, including alkaloids, phenolics, terpenoids, and flavonoids. Polyphenols and flavonoids are two of these, which possess extremely important functions in pharmacological activities such as free radical scavenging and antioxidant [1]. Accordingly, medicinal plants that produce polyphenols have long time satisfied the requirements of the pharmaceutical industry [2].

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Oroxylum indicum (OI), (L.) Kurz belongs to the family Bignoniaceae. It is a medicinally important plant that was used traditionally in the ancient era. This plant is commonly known as the trumpet tree and as Shyonaka in Sanskrit. This plant is found in India, Malaysia, Indonesia, Thailand, China, Vietnam, the Philippines, and Japan. It has a thick bark and usually reaches a height of 8–15 m. This medicinal plant has several uses, including as a food source, mostly from its fruits and seeds, and as a medicine using components of the plant. According to reports, the plant's seeds, bark, fruits, and leaves have a variety of biological functions and have already been utilized in alternative medicine to cure human illnesses [3]. As per the available literature, a paste made by grinding the seeds with fire soot is applied to the neck to treat tonsillitis. A poultice prepared from the bark is used to address inflammations, skin

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conditions, sprains, and rheumatism. In addition, powdered bark is applied to burns, while a paste is used on wounds [4–6]. The stem bark is also utilized in the treatment of stomach disorders [7]. Among the Halam tribe of northeast India, a decoction made from the bark, mixed with two teaspoons of sugar, is commonly administered to treat jaundice [8].

The leaves are traditionally used to treat snakebites, ulcers, headaches, and an enlarged spleen [9,10]. Sonapatha fruits, known for their sweet and slightly acrid taste, possess anthelmintic, stomachic, and expectorant properties. They are used to stimulate appetite and treat conditions such as throat infections, bronchitis, heart ailments, leukoderma, and piles [11,12]. Both the seeds and bark are also administered by various tribal communities across India for respiratory issues, pneumonia, and fever [13,14]. This plant exerts anti-inflammatory, anti-bacterial, cardioprotective, anti-hyperglycemic, and anti-cancer properties [15].

Fungal endophytes are investigated as a potential substitute for the extraction of biologically active principles in order to protect the ethnopharmacological relevance and biodiversity of medicinal plants. A growing body of research has revealed that secondary metabolites from common fungal genera like *Cladosporium*, *Alternaria, Colletotrichum, Aspergillus, Curvularia, Penicillium, Fusarium,* and *Diaporthe* have significant antioxidative property and have been shown to induce cancer cell death [16,17]. Determining the biological characteristics of substances generated from fungal endophytes and isolating them from therapeutic plants is thus a forward-thinking strategy.

Endophytic organisms are usually fungal or bacterial species, which exhibit symbiotic interaction by colonizing into intra and intercellular tissues of the host plant [18,19]. Most of the fungal endophytes have been reported to be accompanying different plant tissues in natural ecosystems. The host-endophyte relationship has resulted in adaptation to the extreme environmental conditions [20,21]. In contrast to other endophytic microbial species, fungi show asymptomatic relationships with plants and have the capacity to replicate the bioactive chemicals present in the host plant. The discovery and development of new drugs, as well as the food, agricultural, and pharmaceutical industries, all heavily rely on bioactive chemicals produced by fungal endophytes.

With this background, in the present investigation, we evaluated the antioxidant activity of fungal endophytes isolated from OI stem bark.

2. METHODOLOGY

2.1. Plant sample collection

OI stem bark (Fig. 1) was collected in the month of August 2023 at Ira village, Bantwal taluk, Mangaluru, Karnataka, India. The plant specimen was identified and authenticated by a Taxonomist. The specimen was submitted with voucher specimen No. 83951 for future reference. The herbarium specimen was deposited at the Central Research Laboratory, K.S Hegde Medical Academy.

2.2. Isolation and morphological identification of fungal endophytes

The isolation of endophytic fungi was carried out within 48 hours of plant specimen collection. To eliminate the soil particles, the stem bark of OI was first cleaned with running tap water followed by distilled water. After being divided into tiny (0.6–0.8 cm) pieces, the samples were cleaned with 70% alcohol. The parts that had been alcohol-washed were then cleaned once again with 0.5% sodium hypochlorite and three times with sterile distilled water. The sterile samples were aseptically transferred to Potato Dextrose Agar (PDA) supplemented with 150 mg/l of chloramphenicol. The Petri dishes were incubated at 27°C ± 2°C in a biological oxygen demand incubator for 7–8 days. Mycelial growth was closely observed, and the hyphal tips emerging from each fungus were sub-cultured onto fresh PDA plates, then incubated for 9–10 days at 27°C ± 2°C [22]. The resulting endophytic fungal strains were stained with cotton blue under a bright-field microscope (Olympus CH20i, India) to examine their microscopic features [23]. Fungal colonies' front and reverse sides were distinguished morphologically using the reference manual [24,25].

2.3. Genetic characterization of isolated fungal endophytes

2.3.1. Extraction of DNA from the isolated fungal endophytes

The Kenjar *et al.* [26] method was followed for this experiment. The lyticase extraction buffer containing 50-

Figure 1. OI tree and stem bark.

mM Tris HCL, 10-mMEDTA, 28-mM β-mercaptoethanol, 10-U/µl lyticase was incubated for 30 minutes at 37°C to disrupt the fungal mycelia. After this step, they were incubated for 60 minutes at 60°C with lysis buffer consisting 10-mM Tris (Himedia), 10-mM KCl, 10-mM $MgCl_2$, 500-mM NaCl, 2-mM EDTA, 0.5% SDS, and proteinase K (20 mg/ml). After incubation period, extraction was done with phenol: chloroform: isoamyl alcohol (25:24:1) and finally precipitated with isopropanol (Rankhem, India). 30 µl Water free of nuclease (Lal reagent water) was used to dissolve the DNA. A nanodrop spectrophotometer (Thermoscientific, USA) was used to measure the amount and quality of isolated DNA.

2.3.2. Amplification of the isolated DNA by PCR (polymerase chain reaction)

In order to assess the quality of the isolated DNA, fungal Internal Transcribed Spacer (ITS) region was amplified using the DNA as template and primers (Bioserve, India) that target the universal barcode internal transcribed region. The following was the setup for the reaction mixtures (30 µl) - 3.0µl of 10× buffer (1.5 mM $MgCl_2$, 100 mM Tris HCl), Deoxyribonucleotide phosphates (dATP, dTTP, dGTP, and dCTP) at 2.5 nM concentrations each; 10 picomoles of primers ITS 1 (F-5′-TCCGTAGGTGAACCTTGCGG-3′) and ITS 4 (R-5′-TCCTCCGCTTATTGATATGC-3′); 1-U of Taq DNA polymerase (Himedia, India);~100 ng of template DNA; volume was increased to 30 µl with nuclease-free water. An Eppendorf Nexus GX2 thermocycler was used to conduct the reactions. The initial setting was 95°C, for 5 minutes then 35 cycles at 95°C for 30 seconds, 47°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 10 minutes at 72°C. The PCR results underwent electrophoresis on a 1.5% agarose gel. Bromophenol staining was applied for resolution, and a gel documentation system (Bio-rad, India) was utilised to visualise the bands.

2.3.3. Sequence alignment and phylogenetic analysis

For the molecular identification of the isolated endophytic fungi, consensus DNA sequences were used, whereas raw sequences (obtained after sequencing) of ITS region of fungal isolates were utilized to construct a phylogenetic tree [27]. For each isolate, identification of the overlapping common sequence was done through the sequence alignment between FP-ITS and RP-ITS regions. The NCBI—BLAST (Basic Local Alignment Search Tool – http://www.ncbi.nlm.nih.gov/BLAST/) query was created using the common sequences constructed for every isolate. The query sequence in NCBI-BLAST was compared with the previously submitted sequences in GenBank and assigned an accession number in order to identify the fungal endophytes and their consensus DNA sequences. If a common overlapping sequence was not available for an isolate, both the FP-ITS and RP-ITS sequences were utilized individually. These consensus DNA sequences were used in carrying out phylogenetic analysis, which was done using the MEGA 11.0 phylogenetic analysis tool by the neighbor-joining method. A bootstrap of 1,000 replications was used to determine the nodes tree's reliability.

2.4. Setting up a fungal mat culture

The endophytic fungal isolate was cultivated in a 1,000 ml conical flask containing 350 ml of potato dextrose broth. After inoculation, the isolates were incubated in a shaker incubator at 100 rpm for 3 days at 28°C ± 1°C, followed by 18 days of incubation under static conditions at the same temperature [28].

2.5. Extraction of secondary metabolites

Slight modifications were made to the protocol described by Higginbotham *et al.* [22] in order to extract secondary metabolites successfully. The fermentative broth and fungal biomass were separated using Grade 1 Whatman qualitative filter paper. The filtered broth underwent three extractions with an equal volume of ethyl acetate. The organic phase that was produced after three extraction cycles was concentrated in a rotary evaporator (Superfit, India), The crude secondary metabolite extract that was obtained was kept for further use at −20°C Deep freezer.

2.6. Preliminary phytochemical analysis

The method of Raman [29] and Harborne [30] with slight modifications was followed for the screening of phytochemical compounds present in the isolated endophytic fungal extract. A small amount of the crude ethyl acetate extract was used to identify various phytoconstituents, namely, steroids, triterpenoids, glycosides, saponins, alkaloids, flavonoids, tannins, proteins, free amino acids, carbohydrates, and vitamin C.

2.7. Antioxidant assays

The antioxidant potential of endophytic fungal crude extracts was assessed using four different assays: (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS) assay, superoxide anion scavenging assay, ferric reducing antioxidant power (FRAP) assay, and total antioxidant capacity (TAC) assay. All experiments were performed in triplicate, and the average values were calculated.

2.7.1. ABTS radical scavenging assay

The ABTS assay was conducted with slight modifications based on the method described by Du and Vuong [31]. A 7.4 mM solution of ABTS was prepared in distilled water. To generate the ABTS•+ free radical cation, 2 ml of the ABTS solution was mixed with 2 ml of 2.6 mM potassium persulfate. The mixture was then kept in the dark for 12 hours. The resulting solution was diluted with distilled water to achieve an optical density (OD) of 1 ± 0.1 at 734 nm. Various concentrations of the sample solution (ranging from 0 to 100 µg/ml) were combined with 1 ml of the diluted ABTS•+ solution and incubated in the dark for 20 minutes. The OD of the samples was measured over time using a UV-vis spectrophotometer (Genesys, Thermofisher Scientific) set at 734 nm. The free radical scavenging efficiency was

calculated using the following formula. Radical scavenging activity (%) = (A control-A test) / A control × 100.

2.7.2. Superoxide anion scavenging assay

Lalhminghlui and Jagetia [32] method was used to test the scavenging of the superoxide (O2•-) anion radical. The reaction mixture had a final volume of 2.8 ml and included 0.2 ml of Nitroblue Tetrazolium (1 mg/ml of solution in Dimethyl Sulfoxide (DMSO), 0.6 ml of ethyl acetate extract of the endophytic fungi (EAEEF) at various concentration (0–100 µg/ml), and 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml distilled water). Using a UV–VIS spectrophotometer, the absorbance was measured at 560 nm. DMSO was taken as blank. As a standard, ascorbic acid equivalent (AAE) has been utilised to express the results. The percentage of Superoxide ($O2^{\cdot}-$) free radical scavenging activity was calculated using the following formula:

Radical scavenging activity (%) = (A control-A test) / A control × 100.

2.7.3. FRAP assay

Sample solutions containing varying concentrations (0–100 µg/ml) of EAEEF were prepared, and their volume was made up to 1 ml using distilled water. 0.5 ml of 1%, potassium hexacyanoferrate (potassium ferricyanide) was added and the mixture was incubated for 20 minutes at 50°C in a water bath. A control tube was maintained without adding the extract. The extract was not added to the control tube. To stop the reaction, 0.5 ml of 10% Tri-chloroacetic acetic acid was added after incubation. 1 ml of distilled water and Ferric chloride ($FeCl_3$), (SRL, India) solution (0.1%) were added. The reaction mixture was kept at room temperature for 10 minutes, after which the OD was recorded at 700 nm using an appropriate blank solution. The reference standard employed was ascorbic acid (AA). Every test was run in triplicates [33,34].

2.7.4. Total antioxidant capacity

The TAC was evaluated according to Prieto *et al.* [35] method. A volume of 100 µl of the EAEEF at different concentrations (0–100 µg/ml) was pipetted into sterile test tubes. To each tube, 1 ml of the TAC reagent – containing 0.6 M sulfuric acid, 28 mM sodium dihydrogen phosphate, and 4 mM ammonium heptamolybdate was added. The mixture was then incubated at 90°C for 90 minutes in a water bath. A blank sample was prepared simultaneously by replacing the EAEEF with 100 µl of distilled water. Following incubation, the mixtures were allowed to cool, and the OD of the resulting greenish-bluish color was measured at 695 nm, against blank solution. The TAC of the endophytic fungal extracts was expressed in terms of AAE.

2.7.5. Estimation of total phenolic content

For this assay, Folin-Ciocalteu (FC) reagent was used, as per Yadav *et al.* [36]. Briefly, 2.5 ml of sodium carbonate (20%) and 0.5 ml of FC reagent were added to a tube containing standard gallic acid (1 mg/ml) and EAEEF (1 mg/ml). The final volume was made upto 10 ml using distilled water. The absorbance of the reaction mixture was measured at 760 nm in a UV–VIS spectrophotometer. Gallic acid was used as standard, and the results were represented in µg/ml gallic acid equivalent.

2.7.6. Estimation total flavonoid content

A spectrophotometric method, as described by Qui *et al.* [37], was used to measure the total flavonoid concentration in the extracts. Standard curve was constructed using Quercetin equivalent. A 2% methanolic solution of aluminium chloride was added to 1 ml of the samples. The mixtures were incubated at room temperature for 15 minutes, and then the absorbance of the reaction mixture was measured at 430 nm. The total flavonoids content was expressed in µg/ml quercetin equivalent.

2.8. Statistical analysis

All the experiments were conducted in triplicate. Statistical analyses were performed using GraphPad Prism version 10.2.3 (Boston, MA). Antioxidant assay data are presented as mean ± standard deviation (Mean ± SD) and graphically represented in a histogram. One-way ANOVA, followed by Tukey's multiple comparison test, was employed to evaluate the statistical significance of the results. Jamovi software 2.6.26 solid version was used to determine Pearson's correlation between TAC, free radical scavenging and reducing power activity of the three endophytic fungal extracts. In all the assays, $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Morphological and molecular identification of the isolated endophytic fungal strain

Three fungal endophytes were obtained from the stem bark of OI using a culture-dependent method. They were encoded as endophytic fungi – 1, EF-2, and EF-3. The isolated strains were identified using a light microscope, focusing on their characteristics such as colony color, morphological features of the hyphae, seta, conidia, and spores [38–40] (Fig. 2).

The EF-1 strain was identified as a *Simplicillium* species, a filamentous fungus characterized by white, floss-like morphology (Fig. 2 EF-1(a)), with a citron-yellow reverse after 10 days of cultivation at 25°C (Fig. 2 EF-1(b)). The colonies appeared dense, and under an optical microscope, the hyphae were septate, while the conidia were predominantly obclavate to ellipsoidal in shape and dark taupe in color (Fig. 2 EF-1(c)).

The EF-2 strain was identified as a *Neopestalotiopsis s*pecies, characterized by white, cottony, and flocculent growth with undulate edges and dense aerial mycelium on its surface. Superficial black conidiomata were scattered over the PDA after 10 days of incubation at ambient temperature, containing a slimy black conidial mass (Fig. 2 EF-2(a), EF-2(b), and EF-2(c)). The conidia were fusoid to ellipsoidal in shape and slightly curved.

The mature fruiting body of the EF-3 strain exhibited distinct morphological features, including a basidiocarp that was reniform to sub-circular in shape. Its upper surface appeared creamy, characterized by radial furrows, fine velvety hairs, and the absence of a stipe (Fig. 2 EF-3 (a and b). The basidiospores were curved, cylindrical, and sausage-like, with smooth walls

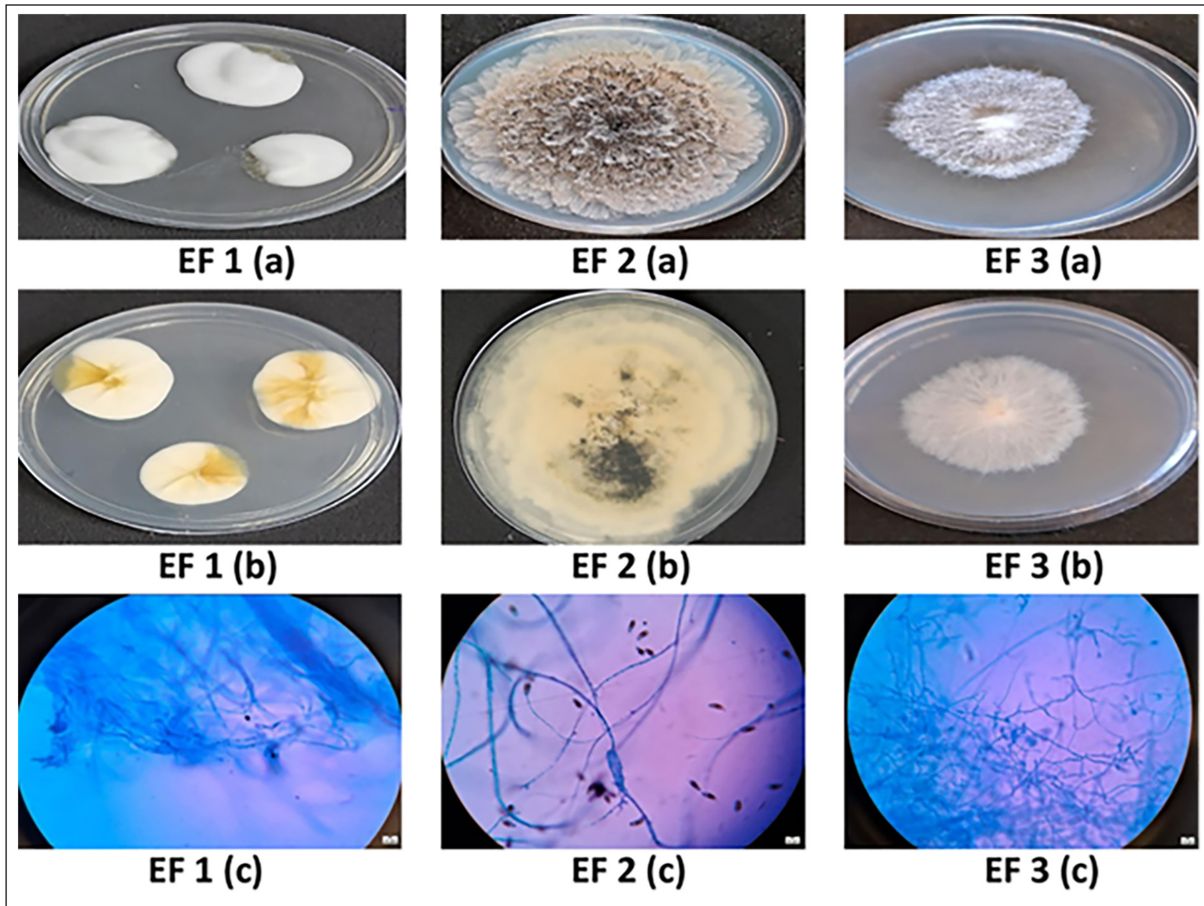

Figure 2. The morphology (colony appearance, hypha and conidia) of endophytic fungi from stem bark of OI (L.) kurz. Fungal isolates - EF1, EF2 and EF3. Colonies on PDA after 7 days of each isolate, a - upper side, b- reverse side and c - generative hyphae and conidia of each isolate.

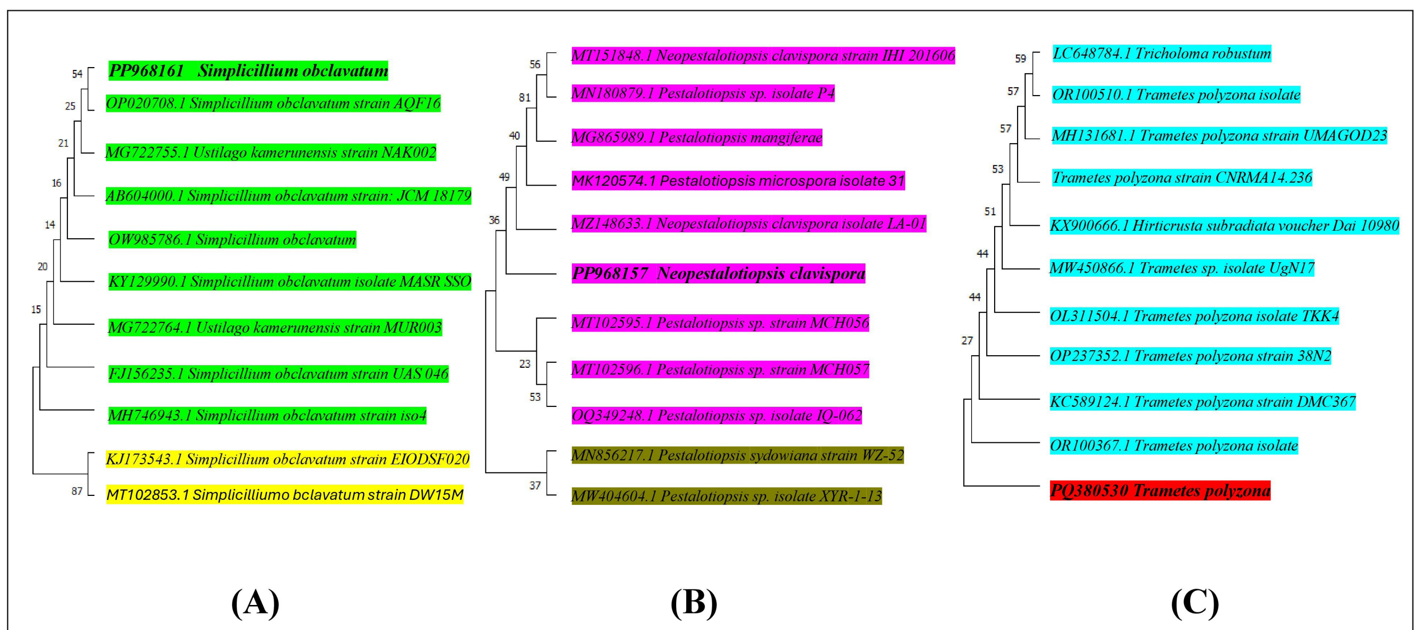

Figure 3. Neighbor-joining phylogenetic tree analysis of fungal endophyte sp.

and produced a white spore print (Fig. 2 EF-3(c)). These traits indicated that the EF3 isolate belongs to the genus *Trametes.*

The ITS1 and ITS4 primers were utilised for PCR on the high-quality DNA that was isolated from endophytic fungus. The isolates of EF-1, EF-2, and EF-3 had 500–600 bp of PCR fragments (Fig. 3). Porter and Collins [41] stated that the average length of the ITS region to Ascomycetes and Basidiomycetes in the fungus kingdom was 500–600 bp. We identified the fungal strains using ITS sequences as a query in a BLAST search (Table 1). The identified fungal strains were *Simplicillium obclavatum* (SO), *Neopestalotiopsis clavispora* (NC), and *Trametes polyzona* (TP) (named as EF-1, EF-2, and EF-3, respectively). EF1 Isolate was 97.72% identical to a SC strain AQF16 (OP020708.1), EF-2 isolate was 96.21% identical to a NC isolate LA-01(MZ148633.1), and EF3 isolate was 99.41% identical to TP isolate (OR100367.1).

Table 1. NCBI blast results of the endophytic fungal isolates.

SL.No	Isolates code	Accession ID with organism name	Identity (%)
01.	EF1	OP020708.1 SO strain AQF16	97.92
02.	EF2	MZ148633.1 NC isolate LA-01	96.21
03.	EF3	OR100367.1 TP isolate	99.41

Table 2. Phytochemical profile of crude endophytic fungal extract (EF1, EF2 and EF3) isolated from OI stem bark.

SL.NO.	Phytochemical test	SO (EF -1)	NC (EF-2)	TP (EF-3)
1.	Liebermann Burchard test for triterpenoids and steroids	-	-	-
2.	Keller Killiani test	-	+	-
	Bromine water test	-	+	-
	For glycosides			
3.	Foam test for saponins	-	+	+
4.	Hager's test	+	+	+
	Wagner's test	+	+	+
	For alkaloids			
5.	$FeCl_3$ test	+	+	+
	Alkaline reagent test	+	+	+
	Lead acetate solution test	+	+	+
	Shinoda test	+	+	+
	For flavonoids			
6.	Gelatin test for tannins	-	-	+
7.	Biuret test for proteins	-	-	-
8.	Ninhydrin test for amino acids	-	-	-
9.	Benedict's test for carbohydrates	-	-	-
10.	DNPH test for Vitamin C	-	-	-

EAEEF showed the presence of glycosides, alkaloids, flavonoids, and saponins. Table 2 displays the findings of phytochemical screening. The symbols "+" and "-" stand for the presence and absence of phytochemical, respectively.

We generated the phylogenetic tree from these sequences to analyze fungal evolution and their taxonomic relationships. It was observed that the phylogenetic tree EF–1 isolate consisted of 10 fungal strains (Fig. 3). The details of the strains are mentioned in the supplementary file.

3.2. Phytochemical screening of EAEEF

The isolated fungal crude extracts were analyzed for phytochemical components, which may serve as potential sources for medicinal and industrial applications [42] (Table 2). The detection of these components suggests their potential as precursors in the development and advancement of synthetic drugs.

Bioactive phytoconstituents can serve as precursors in the development and advancement of synthetic drugs. Key active compounds include glycosides, phenols, flavonoids, terpenoids, alkaloids, tannins, carbohydrates, and saponins. Phytochemical analysis of the crude ethyl acetate extract from the isolated endophytic fungus SO revealed the presence of alkaloids and flavonoids. Similarly, the crude extract of NC contained glycosides, saponins, alkaloids, and flavonoids, while the extract of TP showed the presence of saponins, alkaloids, flavonoids, and tannins. According to Selim [43], endophytes have the capacity to produce certain metabolites but not others. The endophytes residing within a host plant may generate different bioactive compounds, contributing to the plant's diverse functions. Thus, the total number of metabolites in a plant extract may result from the combined activity of all endophytes present in the host plant [43]. The synthesis and quality of these bioactive constituents from endophytic fungi are influenced by the natural environment of their association and the composition of the artificial growth medium [44]. Strategies can be developed to harness these fungi for large-scale production of bioactive compounds.

3.3. Antioxidant activity against ABTS free radicals

Oxidative stress primarily results from free radicals, while antioxidants act as reducing agents that minimize oxidative damage to biological structures by donating electrons to free radicals, thereby neutralizing them. Free radicals are produced when oxygen interacts with specific molecules and can cause harm by reacting with critical cellular components such as DNA, proteins, and cell membranes. When these free radicals interact with antioxidants, they are neutralized before any damage occurs. Endophytic fungi, which live within host plants, produce various secondary metabolites with antioxidant properties. Hence, this study was conducted to evaluate the free-radical scavenging potential of endophytic fungal extracts from the stem bark of OI.

Free radicals, along with reactive oxygen (ROS) and nitrogen species, play a crucial role in diseases caused by oxidative stress, including cancer. However, phenolic and flavonoid compounds effectively scavenge these free radicals, thereby protecting cells from oxidative damage [45]. The high content of phenolic and flavonoid compounds in NC likely contributes to its free radical scavenging activity, as confirmed

by the analysis of total phenolics and flavonoids. The results demonstrate that the EF-2 extract exhibits superior antioxidant activity, with bioactive compounds from NC showing significant ABTS free radical scavenging effects compared to EF-1 and EF-3.

The ABTS-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay determines the efficacy of antioxidative compounds to scavenge ABTS radicals, which are produced by the oxidation of ABTS by potassium persulfate, which is a strong oxidizing agent, that results in a deep green colored solution [46]. Samples rich with antioxidants, scavenge these radicals, leading to a loss or reduction of green coloration, which can be measured spectrophotometrically.

In the present assay, a maximal scavenging activity of EF extracts was observed at a concentration of 100 μg/ml. Among the three endophytic fungal extracts, EF-2 (NC) showed the highest ABTS radical scavenging activity with a percentage of 57.03 ± 0.69, EF-1 (SC*)* showed 44.46% ± 0.52% of ABTS radical scavenging activity, and EF-3 (TP) showed 47.35% ± 2.02% radical scavenging potential. AA was used as the standard antioxidant that showed 88.39% ± 0.58% of ABTS radical scavenging efficacy (Fig. 4A). The NC extract at a concentration of 100 μg/ml showed a statistically significant difference ($p < 0.001$) compared to SO and TP extracts. The IC50 value for EF-1, EF-2, and EF-3 extracts was found to be 109.57, 80.27, and 111.07 μg/ml, respectively, which was calculated using the linear graph, whereas standard AA showed IC50 value of 29.79 μg/ml.

The synergistic effects between antioxidants in the EF-2 extract indicate that antioxidant activity depends not only on their concentration but also on their molecular structure and interactions. This explains why extract with comparable total phenolic content can exhibit differing radical scavenging activity, as measured by the ABTS assay. Since AA is a pure standard compound, it outperforms the crude extract in scavenging the free radicals.

3.4. Antioxidant activity against superoxide anion

In the present assay, among the three endophytic fungal extracts, EF-2 (NC) showed the highest radical activity against superoxide anions with the percentage of 37.41 ± 0.32, EF- 1 (SC*)* showed 32.02% ± 1.83% of superoxide radical scavenging activity and EF-3 (TP) showed 36.0% ± 0.51% radical scavenging potential. A statistically significant difference ($p < 0.05$) was observed for the NC extract at 100 μg/ml when compared to SO and TP extracts. The standard AA showed 77.60% ± 0.23% of radical scavenging efficacy (Fig. 4B). This can be correlated to the presence of phytochemicals, phenolic, and flavonoid content.

Superoxide production was inhibited by EAEEF in a concentration-dependent manner, with the maximum scavenging activity observed at 100 μg/ml (Fig. 5B). Superoxide anions (O_2^{-}•) are generated in biological systems and, while they are relatively less toxic during cellular respiration, they can become highly reactive when in the presence of iron, leading to the formation of hydroxyl radicals (•OH). In addition, incomplete oxygen metabolism results in the production of superoxide anions, which can cause damage to biomolecules either directly or indirectly by forming reactive species such as •OH, peroxynitrite, hydrogen peroxide, and singlet oxygen [47–50]. Therefore, neutralizing or removing superoxide radicals is crucial for protecting cells from their harmful effects. The EAEEF extracted from the stem bark of OI demonstrated a concentration-dependent inhibition of O_2^{-}• formation. The enhanced superoxide free radical scavenging activity observed in NC may be attributed to its higher phenolic and flavonoid content, as confirmed by the analysis of total phenolic and flavonoid compounds. These findings suggest that the EF-2 extract exhibits superior antioxidant activity, with bioactive compounds from NC showing significant superoxide free radical scavenging potential compared to EF-1 and EF-3 extracts.

Kaur *et al.* [51], in their study, showed that the chloroformic fungal extract of *Chaetomium sp*. demonstrated notable dose-dependent scavenging activity, ranging from

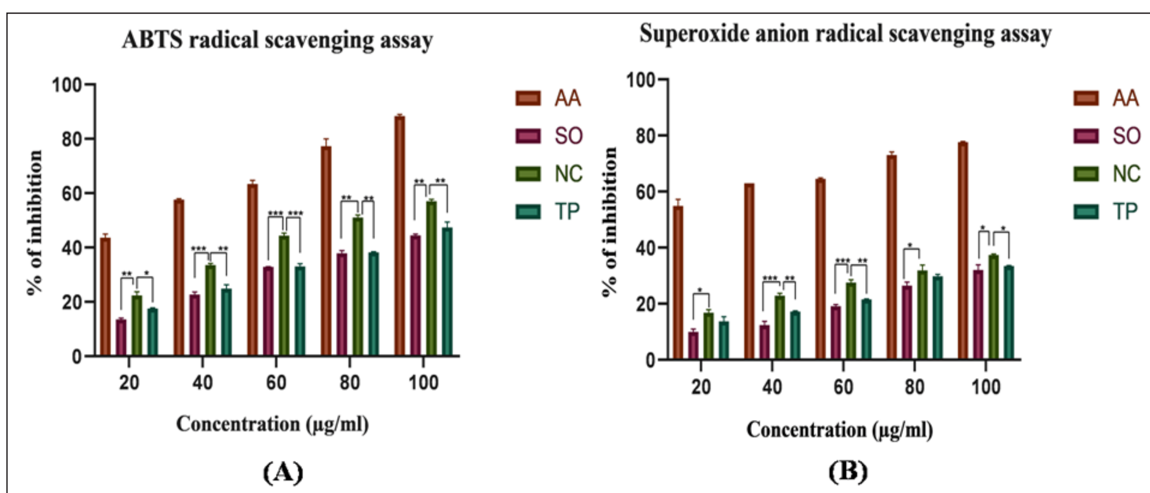

Figure 4. The free-radical scavenging activity of EAEEF. (A) ABTS and (B) superoxide radicals. AA: Ascorbic acid, SO - *Simplicillium obclavatum* extract; NC - *Neopestalotiopsis clavispora* extract and TP – *Trametes polyzona* extract. The data are expressed as mean ± standard deviation; $n = 3$.

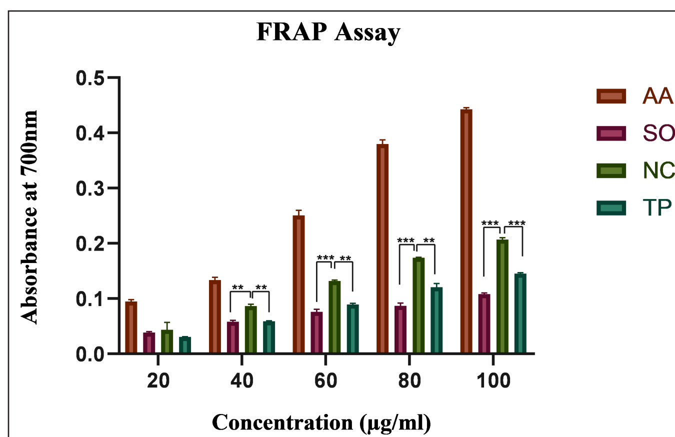

Figure 5. FRAP. AA: Ascorbic acid, SO - *Simplicillium obclavatum* extract; NC - *Neopestalotiopsis clavispora* extract and TP – *Trametes polyzona* extract. The data are expressed as mean ± standard deviation; *n* = 3.

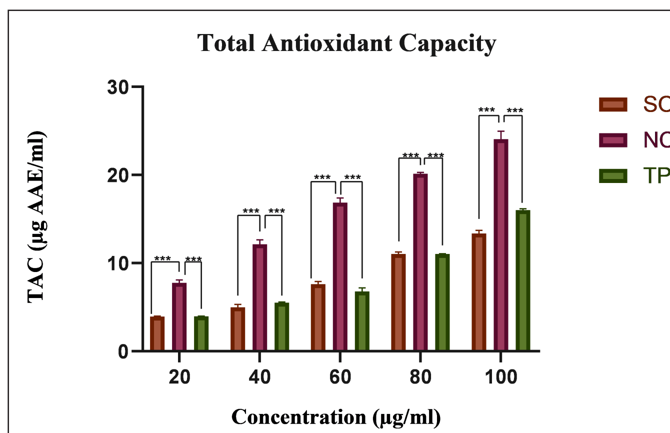

Figure 6. TAC activity of EAEEF. AA: Ascorbic acid, SO - *Simplicillium obclavatum* extract; NC - *Neopestalotiopsis clavispora* extract and TP – *Trametes polyzona* extract. The data are expressed as mean ± standard deviation; *n* = 3.

20.6% to 90.5% across a concentration range of 20–100 µg/ml. At the lowest tested concentration of 20 µg/ml, the extract exhibited 20.6% inhibition, which increased to 41% at 40 µg/ml and reached a maximum of 90.5% at 100 µg/ml [51]. In our study, EF-2 (NC) extract exhibited the highest antioxidant capacity at 100 µg/ml with a free radical inhibition percentage of 57.03 ± 0.69.

The IC50 value for EF-1, EF-2, and EF-3 extracts against superoxide free radicals was found to be 158.64, 150.20, and 151.99 µg/ml, respectively. While standard AA showed IC50 value of 4.62 µg/ml.

3.5. FRAP of the fungal endophytes

The FRAP value of EAEEF showed a concentration-dependent increase, the highest being at 100 µg/ml concentration. NC extracts were more effective in reducing ferric iron to ferrous form (Fig. 5). The FRAP values of AA, EF-1, EF-2, and EF-3 were 0.44 ± 0.002, 0.10 ± 0.002, 0.206 ± 0.003, and 0.145 ± 0.001, respectively, at the highest concentration tested. NC extract at 100 µg/ml exhibited a statistically significant difference ($p < 0.001$) compared to SO and TP extracts. The highest FRAP value observed for EF-2 (NC) may be due to the highest phenolic content and flavonoid content in the isolate.

The FRAP assay is a straight forward and rapid technique commonly used to assess antioxidant activity [52,53]. A concentration-dependent increase in FRAP values was observed in the three isolated endophytic fungal extracts. Previous studies have reported that various plant extracts demonstrate antioxidant activity through elevated FRAP values *in vitro*. The FRAP assay results indicate that the isolated fungal endophytes exhibit reducing potential, reflecting their antioxidative properties.

3.6. TAC of the fungal endophytes

In this assay, the significant antioxidant activity of 24.07 ± 0.88 AAE (µg/ml) was observed for EF-2 (NC) isolate at 100 µg/ml extract. The EF-1 (SC*)* showed 13.35 ± 0.36 and EF-3 (TP) showed 16.02 ± 0.13 AAE µg/ml of TAC, respectively (Fig. 6). The NC extract at 100 µg/ml showed a statistically significant difference, with $p < 0.001$ compared to SO extract and $p < 0.01$ compared to TP extract. This could be attributed to the higher concentration of phytoconstituents, total phenolics, and total flavonoids in the EF-3 extract compared to EF-1 and EF-2 extracts. The TAC assay, conducted using the phosphomolybdenum method, relies on the reduction of Mo (VI) to Mo (V) by the sample analyte, resulting in the formation of a green phosphate/Mo (V) complex under acidic conditions. This method is typically used to detect antioxidant compounds such as phenolics, AA, α-tocopherol, and carotenoids. Phenolic compounds play a major role in absorbing and neutralizing free radicals, quenching ROS, and decomposing peroxides due to their redox properties [54].

3.7. Total phenolic and total flavonoid content of the fungal endophytes

A dose-dependent increase in total phenolic content was seen in EAEEF, reaching a value of 100 µg/ml (Fig. 7A). The EAEEF exhibited an increase in the total flavonoid contents in a concentration-dependent manner. The maximum quantity of flavonoids was estimated for 100 µg/ml of the extracts (Fig. 7B).

In our present study, we found that the total phenolic content ranged from 12.23 ± 0.44 to 33.33 ± 0.22 µg GAE/ml (Gallic Acid Equivalents per milli litre) at 100 ug/ ml concentration of the crude Endophytic fungal extracts. The mean phenolic content of EF-1, EF-2 and EF-3 was found to be 23. 23 ± 0.255, 33.33 ± 0.22, and 26.41 ± 0.36 µg GAE/ml, respectively, at 100 ug/ml. The NC extract at 100 µg/ml showed a highly significant difference ($p < 0.001$) compared to SO and TP extracts. These result show that the EF-2 (NC) extract possess highest phenolic content compared to EF-1 (SC*)* and EF-3 (TP) extracts. The standard graph plotted for Gallic acid represents linear relationship with correlation coefficient, $R^2 = 0.9905$ between concentration and absorbance.

Total flavonoid content in the three isolates was expressed in terms of Quercetin Equivalents (QE in µg/ml)

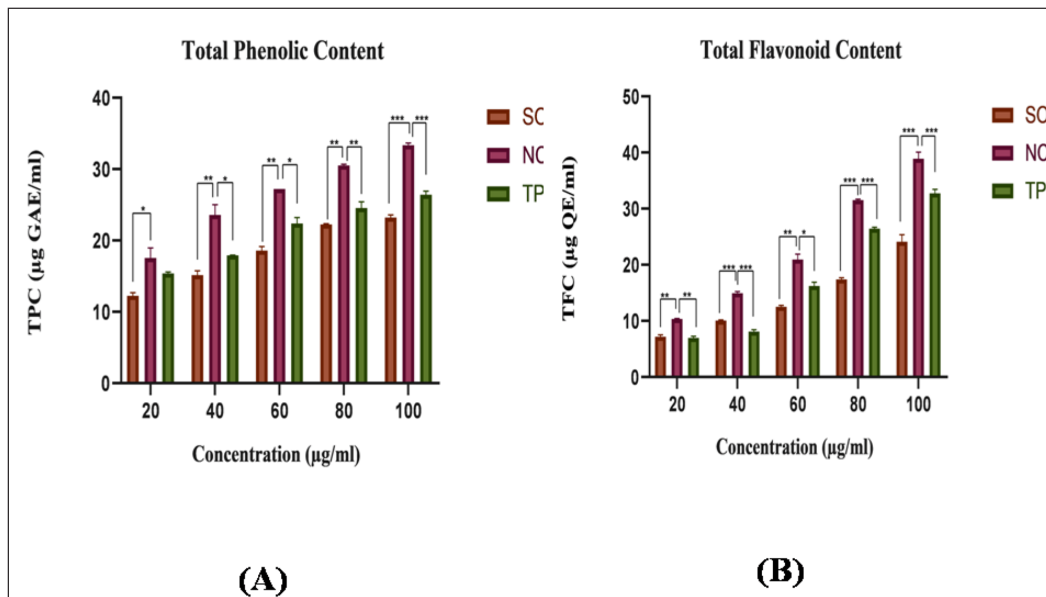

Figure 7. The total phenol and flavonoid contents of EAEEF. (A) Total phenols, (B) flavonoids. AA: Ascorbic acid, SO - *Simplicillium obclavatum* extract; NC - *Neopestalotiopsis clavispora* extract and TP – *Trametes polyzona* extract. The data are expressed as mean ± standard deviation, n = 3.

(Fig. 7B). The highest value for flavonoid content was exhibited by EF-2 (NC) extract that is, 38.90 ± 1.17 QE in µg/ml and also showed highest antioxidant activity, whereas EF-1 (SC) possessed lowest flavonoid content with the value of 24.11± 1.22 µg of QE. EF-3 (TP) extract showed 32.72 ± 0.71 µg of QE. The NC extract at 100 µg/ml demonstrated a significant difference, with $p < 0.01$ compared to SO extract and $p < 0.05$ compared to the TP extract. The standard graph plotted for Quercetin represents linear relationship with correlation coefficient, R^2 = 0.9914 between concentration and absorbance.

Plant Phenolics are important compounds exhibiting redox properties, thus responsible for antioxidant activity since hydroxyl groups in these compounds facilitate free radical scavenging property [55]. Phenolic compounds in medicinal plants, plays a major role in imparting antioxidant capacity [56]. Variation in the amount of phenolic and flavonoid content in the ethyl acetate extract of isolated fungal endophytes were observed in TPC and TFC experiments. The highest phenolic and flavonoid compounds were observed in the fungal endophytic extract, EF-2 (NC), compared to EF-1 and EF-3 isolate. Research on plant phenolic compounds has shown that they help prevent oxidative stress caused by free radicals in biological systems. This is achieved through mechanisms such as free radical scavenging, metal ion inactivation, oxygen removal, and the breakdown of peroxides [57]. In a report, increased production of phenolics (4.577 µg RE) and flavonoids (4.111 µg RE) were observed for the fungal isolates which showed better agreement with the increased antioxidative efficacy. Similarly, 80% antioxidant activity was exhibited by *Chaetomium sp.* with high phenolic value, i.e., 60.13 ± 0.41 mg/GAE [55]. Similarly, our results align with the established understanding that phenolic and flavonoid content is linked to antioxidant activity. This further indicates that the EF extract of OI stem bark holds potential as a promising candidate for combating various oxidative stress-related diseases. This finding may be attributed to the synergistic and antagonistic interactions among different phenolic compounds with antioxidant properties [58].

3.8. Correlation between TAC, radical scavenging activity, and reducing capacity

The TAC of NC extract correlated positively and significantly with the reducing antioxidant capacity (FRAP) assay (Table 3). The ABTS and Superoxide radical scavenging activity of NC extract showed positive but no significant correlation with its total antioxidant activity. Whereas, the TAC of SO extract showed negative correlation with no statistical significance with the radical scavenging and reducing capacity assays. The TAC of TP extract showed negative correlation with ABTS radical scavenging assay but exhibited positive correlation with superoxide radical scavenging and reducing capacity. In all the correlation analysis TP showed no statistically significant difference when correlated its radical scavenging and reducing power with its TAC.

To the best of our knowledge, this is the first study investigating the correlation between TAC with free radical scavenging activity and reducing power of SO, NC, and TP endophytic fungal extracts. In this study, borderline statistically significant positive correlation (p = 0.05) was found between the TAC of NC extract with the reducing antioxidant capacity (FRAP) assay. The observed correlation suggests a potential association between radical scavenging activity and antioxidative properties, likely due to the presence of secondary metabolites in endophytic fungi [59]. These bioactivities can be exploited in the development of therapeutic agents targeting oxidative stress-mediated conditions, including cancer and age-associated disorders.

Table 3. Pearson's correlation coefficient of TAC, radical scavenging activity and reducing capacity of SO, NC and TP.

	ABTS		$O2^{\cdot-}$		FRAP	
	Pearson	Sig	Pearson	Sig	Pearson	Sig
TAC - SO	−0.265	0.666	−0.221	0.721	−0.493	0.399
TAC - NC	0.150	0.810	0.078	0.901	**0.868**	**0.056**
TAC - TP	−0.611	0.273	0.222	0.719	0.202	0.745

TAC–NC correlation with FRAP ($r = 0.8678$ $p = 0.056$) is considered borderline significant at the 5% level (bold).

Antioxidant agents can exert their effects through various molecular mechanisms, such as scavenging free radicals, chelating metal ions, and enhancing the activity of endogenous antioxidant enzymes. Different assays offer indirect insights into these mechanisms [60,61]. For instance, high ABTS radical scavenging activities indicate a substance's ability to donate hydrogen atoms or electrons, thereby stabilizing free radicals [62]. Likewise, strong reducing power observed in the FRAP assay reflects a compound's electron transfer capacity, which is essential for neutralizing oxidative species [63]. From our study results, the highest antioxidant potential of NC extract can be attributed to the presence of more Phyto-constituents and better phenolic and flavonoid content compared to SO and TP extract.

The overall results of our study showed that EF-2 outperforms EF-1 and EF-3 in all the analysis performed. This can be correlated to the presence of phytochemicals, phenolic, and flavonoid content. NC (EF-2) extract contained glycosides, saponins, alkaloids, and flavonoids. Glycosides were present only in EF-2 extract compared to EF-1 and EF-3 extracts. The TPC and TFC value of EF-2 was higher compared to EF-1 and EF-3 extracts. This may be the reason for EF-2 extract showing better antioxidative activity compared to EF-1 and EF-3 extracts.

In conclusion, Endophytic fungi, which are present ubiquitously in all plants hold significant potential for biological and health applications due to the presence of promising sources of secondary metabolites with potent antioxidant efficacy. However, endophytic fungi remain largely underexplored despite their documented importance. The prevalent issue of oxidative stress-mediated disease pathogenesis and metabolic dysfunctions has urged the quest for novel free radical scavenging and antioxidant compounds. In this framework, fungal endophytes have garnered attention as a treasure house of such bioactive metabolites.

Overall, the findings of this study highlight the significant potential of NC extract to produce biologically active compounds. Its rapid and straightforward cultivation further enhances the value for large-scale production. Future research can focus on strategies to enhance metabolite and enzyme production through optimization of growth conditions, co-cultivation techniques, and gene overexpression. In addition, exploring the application of NC extract in fields such as agriculture, biofuels, as therapeutics in anti-ageing formulations, and other related sectors holds considerable promise.

While *in vitro* results are valuable, they do not always translate directly to *in vivo* outcomes. Although *in vitro* studies are useful for preliminary screening and understanding mechanisms of action, *in vivo* studies are crucial for assessing the actual efficacy and safety of a substance within a living organism. As a part of extending this study, we intend to explore the bioactive secondary metabolites present in the NC extract through Liquid Chromatography–Mass Spectrometry studies and further carry out a pre-clinical study in an animal model, as our major aim is to determine the radioprotective potential of this endophytic fungus against ionizing radiation-induced damages.

4. CONCLUSION

In this study, we could isolate and identify three novel endophytic fungi, namely SO, NC, and TP. To the best of our knowledge, and based on scientific literature review, this is the first study on the isolation and identification of endophytic fungi from OI stem bark, a traditionally important medicinal plant. Furthermore, our study has provided insights on the presence of phytochemicals, its antioxidative potency and also the presence of bioactive metabolites. Even though the antioxidant potential of endophytic fungi has been largely explored, there are still a lot of unexplored possibilities. Research in this area has the potential to produce new understandings and uses that have a significant influence on human health and well-being.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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