Phylogenetic Diversity and Antioxidant Activity of Selected Fungi From Ethno Medicinal Plants & Soil

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Abstract

Endophytic fungi are prolific generators of bioactive metabolites with natural antioxidant properties that have a range of potential uses. In the present work, the antioxidant activity and phylogenetic diversity of endophytic fungi obtained from semi-arid terrestrial plants and the fungi obtained from the mangrove soil samples is investigated. The fungal isolates were identified by employing morphological features and phylogenetic characterization by internal transcribed spacer (ITS) sequences. Six taxonomic orders of Ascomycota associated with nine genera were identified, these being Diaporthales, Eurotiales, Hypocreales, Onygenales, Pleosporales and Xylariales. The antioxidant activity methanolic extracts of the fungal isolates were determined using modified 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) methods. Among the fungal isolates, *Aspergillus* sp. AREF023 and *Fusarium* sp. AREF014, obtained from different parts of *Datura metel,* displayed the highest level of radical inhibition (80.76% and 70.62% respectively). GC/-MS analysis of the active isolates confirmed the presence of known antioxidant phenolic compounds, including 2-tert-Butyl-5-hydroxymethyl-5-methyl-[1,3]dioxolan-4-one and Phenol, 2,5-bis(1,1-dimethylethyl), in the their crude extracts. Our findings suggest that fungal isolates from *D. metel* can be a sustainable resource for natural antioxidants.

Introduction

Fungal endophytes are a group of diverse filamentous fungi belonging to ascomyta that grows within plant tissues without causing any immediate harmful effects (Hyde and Soytong 2008; Debbab et al. 2013). These endophytic fungi are prolific producers of secondary metabolites and can serve as a valuable reservoir of lead molecules in the hunt for drug candidates against infectious diseases, cancer and many other disorders (Rana et al. 2020; Mishra et al. 2021). Among various important leads obtained from endophytic fungi, cholesterol lowering drugs (statins), immunosuppressant drugs (cyclosporins), anti - cancer drug taxol and penicillin are well known for their pharmacological actions (El-Bondkly et al. 2020; Sharmila and Kalaiselvam 2014; El-Sayed et al. 2020). In addition, phomoterpene A, phomoisocoumarin D isolated from *Diaporthe prunorum*, an endophytic fungi isolated from *Hypericum ascyron,* has potential antimicrobial activity (Qu et al. 2020), while *Curvularia* sp. G6-32, an endophytic isolate of medicinal plant *Sapindus saponaria,* produces Asperpentyn, an epoxyquinone compound, which is a potential antioxidant (Polli et al. 2020).

Indian has a large biodiversity and diverse ecosystems of the landforms that are an excellent source of bioactive secondary metabolites. The present study screened endohpytic fungi isolated from medicinal plants and soil from two diverse regions for antioxidant activity. Firstly, the semi-arid region characterized by the intermediate climatic conditions between the desert and humid regions. Semi-arid land forms are comprised of succulent, non- succulent and xerophytic perennials (Yadav et al. 2020). Secondly, mangroves sediments which are complex as compared to terrestrial ecosystems in terms of a characteristic marine biome. The vegetation is well equipped to survive saline stress (Guillén-Navarro et al. 2015). There is no up to date published study that describes the phylogenetic diversity of endophytic fungi and soil fungi obtained from these two sources. In the current study we have identify, establish the genetic relationship, and screened the antioxidant activity of endophytic fungi isolated from some semi-arid terrestrial plants and the fungi obtained from the soil of the mangrove region of a marine area. These endophytic and soil fungal isolates were found to be significantly active by inhibiting the DPPH, ABTS radicals and showed active spots on the DPPH bio – autography assay. These bioactive endophytic and soil fungi isolates were further subjected to molecular characterization and were their chemical constituents investigated using GC- MS.

Materials And Methods

Sampling sites and collection of samples –

1. Terrestrial plants:

Healthy terrestrial plant parts such as stem, leaves and roots were collected from Aravalli Bio-diversity Park (28°28'59.9"N 77°06'34.7"E) Gurugram, Haryana, India. The identification and validation of plant species were done as previously described (Gamble, J.S. 1925), and samples were preserved in our laboratory. Healthy plant parts were collected by sampling from different parts of the plant. Plant parts such as leaf, stem and roots were randomly cut with an ethanol-disinfected sickle and placed independently in sterile polythene bags. Endophytic fungi were isolated within 48 hours of collecting the samples.

2. Soil samples:

The mangrove soil samples were collected randomly in July 2018 from the marine area of Alibaugh (18°39' 23.9544"N, 72°52'47.5248"E) Maharashtra, India. 09 soil samples each with 1 km distance from the sea bed were randomly collected and the soil from the surface to a depth of 5-10 cm was collected in sterile zip a lock bag which is placed in an ice box and taken to the laboratory. The samples were then stored in the refrigerator (4 °C) for fungal isolation.

Isolation of fungal samples –

1. Endophytic fungi:

Isolation of the fungal isolates was done using a previous method of Qadri et al with some modifications (Qadri et al. 2013). Briefly, mature and excided small *Bacopa monnieri, Cymbopogon citratus, Datura metel* and *Ocimum tenuiflorum* plant parts (leaves, stems, roots) were used for isolating the fungal endophytes. The samples were washed for 1 min with 70% ethanol (v/v) and disinfected for 2 min using 2% sodium hypochlorite solution (v/v) followed by 20s of brief rinsing with 70% (v/v) ethanol. Later they were rinsed with sterile distilled water. These plant parts were placed on potato dextrose agar (PDA) plate amended with 50µg/ml chloramphenicol. PDA plates containing three pieces of plant material were incubated for 7–10 days at 28 °C. Plating was done in triplicates.

2. Fungi from soil:

Endophytic fungi were isolated on water agar (WA) by employing soil plate method, incubation was done at 28°C for 3-5 days, and checked for fungal colonies appearing in culture, then a single colony was transferred on PDA plate and further incubated for growing pure culture. The cultures in petri dishes were incubated at 28°C for 7 – 10 days for observation and further identification was done on the basis of morphological characteristics. All the isolates were deposited in the TERI Deakin Nano-biotechnology culture collection.

Growth kinetic analysis of potential bioactive fungal isolate

Ergosterol estimation and analysis methods were adapted from Axelsson et al. (Axelsson et al. 1995). 50 mg of dried fungal mycelium (collected regularly at 24 hr interval) was mixed with 1 ml ethanol. Sterol was extracted and pooled twice (30 s agitation, 1 hr incubation and centrifugation at 14,000 rpm for 5 mins), The pooled and ethanol partitioned sterol was filtered using 0.22 mm nitrocellulose filter and 10 µL injection volume was injected into a high performance liquid chromatography (Shimadzu, Japan). A five point calibration curve was plotted with the standard in concentration range (10–50 ppm) in ethanol employing an absorption detection at 282 nm (Total run time was 20 min). Variation of ergosterol content was compared by referring to the standard graph (R^2 – 0.97).

Preparation of methanolic extract:

Methanolic extracts of all the fungal isolates were prepared using a Diaion HP20 column (Merck). Three fungal plugs (8-9 mm in diameter) from 10 day old culture (in PDB medium) were inoculated in 1000 ml Erlenmeyer flasks containing 250 ml of the medium. The flasks were incubated at 28º C for 8 - 10 days at 150 rpm. For preparation of the extract, methanol was added slowly in the grown culture (1:1) (to avoid over heating), for metabolite extraction and kept at shaking for 6 hrs. The media was then filtered thorough sterile muslin cloth to separate mycelia. The filtrate obtained was then concentrated to 1/5 volume using rotary evaporator and diluted to 100 mL with distilled water. The diluted solution was then passed through Dianion HP20 column and was eluted using 70% methanol. The obtained elute was concentrated in a peer shape flasks, diluted with distilled water and lyophilized to obtain a powdered extract.

Thin layer chromatography (TLC) bio - autography:

Each fungal extract was spotted and eluted with $CHCl_3$: MeOH (85: 15 v/v) on silica 60 F_{254} TLC plate (Merck), followed by spraying with 0.2 % DPPH solution in methanol and incubated for 30 minutes in dark. Active compounds appeared as yellow spots against a purple background.

Total antioxidant capacity assay:

DPPH assay –

DPPH radical scavenging assay was conducted according to the previous method of Blois (Blois 1958) with modifications. DPPH (Sigma) was dissolved in 100 mL of MeOH. The investigated samples were prepared by dissolving 1mL of 0.2 mM 1,1- diphenyl -2- picrylhydrazyl (DPPH) in methanol and 0.5mL of test sample at different concentrations (5 µg/mL to 100 µg/mL) at room temperature. The absorbance at 517 nm was measured after 30 min versus the blank (0.5 mL of methanol instead of test sample in 1mL DPPH solution). Positive controls ascorbic acid, tert-Butylhydroquinone (TBHQ) and Trolox were also subjected to the same procedure for comparison. The analysis was carried out in triplicate, and the percentage (%) of inhibition was calculated by the following formula:

% inhibition = absorbance of control – absorbance of sample x 100 / absorbance of control

The DPPH radical scavenging activity of each sample was expressed as Trolox equivalent antioxidant capacity (TEAC) (Shimamura et al. 2007). TEAC was calculated as follows:

TEAC = IC_{50} of Trolox (µg ml^{-1})/ IC_{50} of sample (µg ml^{-1})

ABTS assay –

The ABTS radical scavenging assay of the fungal crude extracts performed in accordance with method reported by Re et al (Re et al. 1999). Briefly, 2.45 mM potassium persulfate and 7 mM ABTS aqueous solution was mixed to generate $ABTS^+$ radical. The initial absorbance mixture solution was adjusted to 0.70 ± 0.02 at 745 nm. Different sample concentrations (5 µg/mL to 100 µg/mL) and standard Trolox (1 µM to 50 µM) with $ABTS^+$ working solution was adjusted to a final volume of 1 mL and reacted at room temperature (25º C) for 6 min. The decrease in absorbance at 745nm was recorded and calculated as half maximal inhibitory concentration (IC_{50}) for test samples and trolox was calculated by plotting the scavenging capacity against the concentration. Trolox standard solution (final concentration 0 –50 µM) was used. Results were expressed in terms of TEAC (Shimamura et al. 2007). TEAC was calculated as follows:

TEAC = IC_{50} of Trolox (µg ml^{-1})/ IC_{50} of sample (µg ml^{-1})

Molecular identification of the fungal isolates:

Genomic DNA Extraction

The pure culture obtained after isolation of the fungal isolates were grown in 250 ml Erlenmeyer flasks containing 50 ml of the medium, incubated at 28º C for 8 - 10 days at 150 rpm . The fungal mycelia obtained were freeze-dried and the genomic DNA was extracted by the CTAB (Cetyl trimethylammonium bromide) method (Ausubel et al. 1999).

Phylogenetic analyses by partial ITS1-5.8S-ITS2 ribosomal gene sequencing:

Phylogenetic analyses of the endophytes were carried out by the acquisition of the ITS1-5.8s ITS 2 ribosomal gene sequencing. ITS region of rDNA was amplified and sequenced with universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS1 (5'-CCGTAGGTGAACCTGCGG-3'). PCR amplification was performed according to the methods of Reajeendran et al (Rajeendran et al., 2017). The amplicons obtained were gel purified by amicon ultra columns (Millipore, USA) and 20–40 ng were used for sequencing at Eurofins Laboratory Pvt, Ltd (Bengaluru, India). Finch TV software (http://www.geospiza.com/Products/finchtv.shtml) was used for assembling sequences and homology was determined using BLASTn against the NCBI Gen Bank database (Altschul et al. 1997). A phylogenetic tree was constructed using the sequences displaying maximum homology. CLUSTAL W was employed for multiple sequence alignment and MEGA X was used for phylogenetic and molecular evolutionary analyses (Kumar et al. 2018). Following the Tamura - Nei model and Maximum Likelihood algorithm phylogenetic reconstruction was done with bootstrap values calculated from 1000 replicate runs (Tamura and Nei 1993).

GC/MS analysis

The volatiles and major chemical components of the fungal extracts were analysed using Agilent 6890 GC-MS coupled to Agilent 5873 (EI mode, 70 eV). The GC conditions were: 1 min split less time, helium carrier at 1.0 ml/min, oven temperature from 70^oC to 135^oC at 2^oC/min, for 10 min; then to 220^oC at 4^oC/min, 10 min; and finally to 270^oC at 3.5^oC /min, for 20 min. For analysis HP-5MS capillary column (0.32 mm x 30 m x 0.25 µm), GC injector with temperature set at 280^oC and MS transfer line temperature at 290^oC was used. The detected compounds were compared with the mass spectra from the NIST library for their identification (Proestos et al. 2006).

Results

Isolation of endophytic fungi:

In total 43 fungal isolates were obtained from different parts of the plant and soil. The sources were given lab codes and a portion was used for isolation. Ethno-medicinal values of the sources (wherever applicable) of 17 antioxidant related endophytic fungi (AREF) isolates (10 cultivable endophytic fungi and 07 soil fungi) obtained after initial screening of the pure isolates are illustrated in **Table S1.** Subsequently, these sub-cultured pure isolates were further used for preparation of extracts for bioactivity guided screening. Ergosterol content was estimated using standardised control. The inoculated medium displayed significant growth and continual increase in ergosterol content from the 7th to 10th day. However, growth saturation was observed after the 10th day. The growth kinetics of the fungal isolate and standard graph are shown in **Figure S1.**

Screening of isolated endophytic fungi for antioxidant activity:

Methanol extracts of the cultivable fungal isolates were screened for antioxidant potential using two assays. Extract antioxidant activity was tested at a concentration of 1000 µg/ml.

TLC bio-autography analysis - Among the various solvent elution tested, chloroform/methanol/acetic acid (8.5:1.5 by volume) gave the best separation of compounds from the 70% methanol extracts with retention factor values (R_f) ranging from 0.15 to 0.87. The existence of strong adsorbing sites, as depicted on TLC plate (**Figure S2**), confirm the suitability of this solvent system for separation and mobility of the fungal extracts. The baseline of all the methanolic fractions indicated a range of deep to light yellow colour, indicating the presence of antioxidant constituents in varying amounts. The spots of R_f 0.87 _ 0.71 displayed bright yellow spot on the TLC plate confirming the presence of antioxidant compounds in these extracts. Radical scavenging activity of methanol extracts from all fungal isolates against DPPH, $ABTS^+$ radical are shown in **Table 1**. The IC_{50} values were obtained in the range of 33 µg/ml - 616 µg/ml for DPPH assay and 14 µg/ml - 167µg/ml for ABTS assay. The lower the IC_{50} and the higher the TEAC value, the greater is the antioxidant activity.

Amongst all the 17 fungal crude extracts, isolate AERF023 identified as *Aspergillus sp.* obtained from the internal tissues of roots of *D. metel,* exhibited promising DPPH scavenging activity (IC_{50} 33.49 µg/ml and TEAC of 0.3) and also against ABTS (IC_{50} 14.33 µg/ml). The highest DPPH scavenging activity was from AERF023, which exhibited 80.76% of DPPH radical inhibition. The result of $ABTS^+$ scavenging activity was also maximum from AERF023, which exhibited 75.18 % of $ABTS^+$ radical inhibition. The second most potent isolate was AREF014 which showed moderate antioxidant activity (IC_{50} 35.39 µg/ml against DPPH and TEAC of 0.28; and IC_{50} 33.50 µg/ml against $ABTS^+$).

Phylogenetic diversity of cultivable fungi

Out of the 17 fungal isolates, the 11 isolates **(Figure 1)** exhibiting the highest antioxidant activities were subjected to sequencing for their molecular characterization. A further categorization of these isolates were carried out into 9 genera, namely *Alternaria, Curvularia, Fusarium, Monascus, Diaporthe, Talaromyces, Aspergillus, Auxarthron* and *Xylaria* which belongs to six orders Pleosporales, Hypocreales, Diaporthales, Eurotiales, Onygenales and Xylariales, found in phylum Ascomycota. Among isolated endophytic fungi *Alternaria* and *Aspergillus* were the predominant genus, with relative frequency of 18.18%, while the remaining genera *Auxarthron, Fusarium, Monascus, Talaromyces, Xylaria, Curvularia, Phomopsis* were found with relative frequency of 9.99%.

The phylogenetic tree (**Figure 2**) showed four large clades contained a few sub clades. The first clade divided into two sub-clades, one of them containing two endophyte sequences, which cluster with sequences from *Aspergillus niger*, *Aspergillus nidulans and Aspergillus terreus,* acquired from NCBI (ID: LT745387, GQ461904, MG459155, JQ361749, MN592939, MN592937) with 70% bootstrapping. The second sub-clade confined one endophyte sequence grouped with 93% bootstrapping to two *Talaromyces assiutensis* and one *Talaromyces trachyspermus* sequence picked up from NCBI (ID: JN899320, KR909179 and MH856401). The second clade consist of two endophyte sequences which were grouped with six sequences from *Auxarthron umbrinum* MH424172, *Auxarthron conjugatum* HM036583, *Auxarthron ostraviense* NR_121474, *Xylaria palmicola* GU322436*, Xylaria psidii* MF773655 *and Xylaria oxyacanthae* MF773432 selected from NCBI with 99% bootstrapping. The third clade was again divided into two sub-clades, the first sub clade contained two endophyte sequence that grouped to *Fusarium falciforme, Fusarium solani, Monascus kaoliang, Monascus purpureus,* obtained from NCBI (NCBI (ID: KY318489, MF401578, KP132214, AB477250, AB477247, AB477252), with 98% bootstrapping. The second sub clade consisted of three endophyte grouped with nine sequences of *Curvularia sp.*, *Curvularia lunata, Alternaria spp., Alternaria alternata, Alternaria brassicae* obtained from NCBI (ID: MN215713, NR_138223, HF934911, MG228423, MG228422, MN615420, MF356599, MG722823, MF356599). The last clade was the smallest with one endophyte sequence clustered with two sequences of *Diaporthe detrusa* and *Dipothe sp.* picked with NCBI (ID: KC343061, MH267914) with 71% bootstrapping, however, one sequence of *Diaporthe eres,* NCBI ID: MH003633, fall apart with 54% bootstrap value.

Detection of bioactive metabolites of the methanol extracts by GC-MS analysis:

Considering the highest antioxidant activity obtained from the methanol extracts of *Aspergillus sp.* AREF023 and *Fusarium* sp. AREF014, a tentative identification of the metabolites were carried out using GC-MS. Twenty metabolites were found in the extract of AREF023 and seven were identified in the extract of AREF014. The identified myco-constituents belonged to diverse classes of alkenes, di-ethers, esters, fatty acids, phenols, polysaccharides, organic acids and hydrocarbons. Chemical diversity was observed in the constituents of both the extracts. A total of 20 compounds constituting 96.3% of the relative area in the methanol extract and 95% similarity with the standard mass spectra were revealed from the GC/-MS analysis of the AREF023 extract **(Figure 3a) (Table 4a)**. The methanol extract of AREF023 indicated the presence of phenolics, including 2-tert-Butyl-5-hydroxymethyl-5-methyl-[1,3]dioxolan-4-one (8.97%); a common antioxidant molecule which is used in a range of applications. Similarly, analysis of AREF014 showed the presence of 7 compounds, having resemblance almost 97% with the standard masses in the library and representing 100% of the relative area in the extract **(Figure 3b) (Table 4b)**. The extract clearly showed the presence of the phenolic compound, Phenol, 2, 5-bis (1, 1-dimethylethyl) (36.45 %), which is widely used as a dietary antioxidant compound.

Discussion

Endophytic fungi are a novel source of natural bio actives which can sustainably help to overcome the negative impact of diminishing plant diversity on the discovery of bioactive natural products. Moreover, plants with the history of use as traditional medicine can become excellent scaffolds for growing and isolating endophytic fungi that can produce similar bioactive metabolites to those of the host plant (Strobel 2002). With this rationale, the ethno-medicinal plants, *D. metel, B. monnirei, C. citratus, O. tenuiflorum and* soil sediments from marine ecosystem were selected for the isolation of endophytic fungi. The methanolic extracts of these isolated fungi were subjected to two antioxidant assays. Among all the isolates *Aspergillus* sp. AREF023 displayed highest antioxidant activity; (IC_{50} 33.49 µg ml^{-1} and 14.33 µg ml^{-1}) followed by *Fusarium* sp. AREF014 with IC_{50} 35.36 µg ml^{-1} and 33.50 µg ml^{-1} in DPPH and ABTS assays. Both isolates AREF023 and AREF014 showed excellent TEAC values, i.e. 0.3 and 0.28 respectively, compared to the positive controls (Ascorbic acid – 1.9; Trolox – 1.0 and TBHQ – 1.01) (Table 1). The antioxidant activities of the methanol extracts were comparable to that of the standards used, Trolox, TBHQ and Ascorbic acid. Varying activities between these isolates is due to varying metabolite content, as indicated by Scavenging actvity being directly proportional to the extract's concentration. This is the first report of the antioxidant activities of endophytic fungi identified as Aspergillus sp.AREF023 obtained from *D. metel*.

Selected for their higher antioxidant capacity, 11 fungal isolates (08 endophytic and 03 soil fungi) representing the phylum *Ascomycota,* were identified. It is the largest phylum of fungi with over 64,000 species reported (Crozier et al. 2006; He et al. 2012; Koukol et al. 2012). *Alternaria, Aspergillus and Fusarium sp.* are the common endophytes reported from medicinal plants (El-Said et al. 2020; Kamel et al. 2020). The phylogenetic tree developed in this study represents the relations amongst the isolated fungal samples (Figure 2) (Table 3). Characterisation data from this study have been submitted in the NCBI GenBank, accession codes MT013399 to MT013409 (Table 2).

To assess the bio-active constituents responsible for antioxidant activities of the fungal isolates, the methanolic extracts were subjected to GC-MS analysis. Different bioactive compounds with various pharmaceutical applications, such as antibacterial, antioxidant, anticancer activities, including some used industrially, were detected in the present study. For example, the compound diethyl phthalate is known to have antimicrobial, acetylcholinesterase and neurotoxic activity (Velanganni et al. 2011). Phthalates are known to have antagonism against pathogens and other pharmacological activities. The active isolates *Aspergillus sp.* AREF023 and *Fusarium sp.* AREF014 confirms the presence of phenolic compounds 2-tert-Butyl-5-hydroxymethyl-5-methyl-[1,3]dioxolan-4-one and Phenol, 2,5-bis(1,1-dimethylethyl), respectively. Plant phenolic compounds are well known to have potent antioxidant activity (Adhami et al. 2020; Heendeniya et al. 2020; Khalil et al. 2020; Xiu-Qin et al. 2009). In another study carried out on fungal species Xylaria isolated from *Gingko biloba*, several phenolic compounds, including 2-hydrazino-8-hydroxy-4-phenylquinoline, 3, 4-dimethoxy-phenol, 2,4-bis(1,1-dimethylethyl)-phenol, 3,4-dihydro-8-hydroxy-3-methyl-isocoumarin and ferruginol were reported. Antioxidant compounds have previously been shown to be present in some endophytic fungal. Luteolin was obtained from the ethyl acetate fractions of the endophytic fungus *Aspergillus fumigates* obtained from the roots of Pigeon pea (*Cajanus cajan*). It displayed a scavenging potential of IC_{50} - 16.38µg/mL against the DPPH radical, (standard luteolin - IC_{50} - 10.03µg/mL), in comparison to BHT – 29.94 µg/mL (Zhao et al. 2014). A compound 4,5-dihydroxy-3-(1-propenyl)-2-cyclopenten-1-one was obtained from the extract of endophyte *Aspergillus terreus* JAS-2 isolated from the terrestrial plant *Achyranthus aspera*. The compound exhibited an antioxidant activity with an IC_{50} value of 112µg/mL, in comparison to the positive control (Goutam et al. 2017). Shikonin (5,8-Dihydroxy-2-[(1R)-1-hydroxy-4-methyl-pent-3-enyl]naphthalene-1,4-dione) was isolated from endophytic fungi *Fusarium tricinctuman*, displaying scavenging activity in the DPPH radical scavenger assay with an IC50 value of 56.3 µg/mL, whereas it showed an IC50 value of 1.93 µg/ mL against the ABTS radical (Moussa et al. 2019).

Conclusion

Discovering and developing new antioxidant compounds of natural origin that can be used safely in pharmaceutical as well as food industry is an important area of research. The unique phenomenon of cross talk existing between the colonizing endophytic fungi and the plant host results in the production of related and often the same bioactives as originally produced by the host plant. This symbiosis is harnessed as a useful reservoir of lead compounds for multiple applications. The most significant advantage is the relative ease of mass cultivation of endophytic fungi that can potentially replicate valuable metabolites of their hosts, without diminishing the plant biodiversity in natural ecosystems.

The results in this study have established that the endophytic fungal isolates, *Aspergillus sp.* AREF023 and Fusarium *sp.* AREF014, contain metabolites rich in phenolics and flavonoids which are the major contributors to antioxidant activities in extracts from these strains. The extract with the highest phenolic content displayed the highest antioxidant capacity. The endophytic fungi *Aspergillus terreus* and *Fusarium solani* are excellent sources of naturally derived antioxidants. However, the toxicity of these fungal extracts having antioxidant activity needs further investigation, particularly in regard to their applicability as neutraceutics and pharmaceutics. In addition, the mechanisms of antioxidant activity of these extracts required investigation in order to gain more insight into their scavenging behaviour in food and biological systems.

Declarations

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Conflicts of Interest: The authors declare no conflict of interest.

Data availability statement

All data generated or analysed during this study are included in this article (and its supplementary information files). The names of the repository/repositories and accession number(s) can be found in the article/ supplementary material.

Author's Contribution

MG conceptualized and designed the work. RCM performed the experiments, carried out data analysis and initial manuscript drafting. SKD and ND helped in the identification and phylogenetic analysis of the fungal strains. MG and CJB contributed toward the critical revision and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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Tables

Table 1: Antioxidant activities of extracts of AREF isolates, and standards (Trolox, Ascorbic acid, TBHQ) against ABTS and DPPH radical.

Sample	DPPH (IC_{50} µg ml^{-1})	ABTS (IC_{50} µg ml^{-1})	TEAC
Ascorbic Acid	5.33	4.37	1.90
TBHQ	9.95	3.83	1.01
Trolox	10.13	7.42	1.00
AREF001	273.96	144.28	0.03
AREF004	171.6	18.51	0.05
AREF007	210.35	26.15	0.04
AREF010	71.2	33.90	0.14
AREF011	171.7	63.24	0.05
AREF014	35.36	33.50	0.28
AREF015	97.4	55.95	0.10
AREF016	183	59.10	0.05
AREF017	302.37	125.11	0.03
AREF018	357.05	167.63	0.02
AREF019	149.44	63.47	0.06
AREF020	222.89	116.72	0.04
AREF021	80.36	51.71	0.12
AREF023	33.49	14.33	0.30
AREF026	124.26	36.70	0.08
AREF029	134.26	29.59	0.07
AREF030	616	39.53	0.01

Table 2. Isolated and identified endophytes, including genus or species, and their identity percentage found in the NCBI (National Centre for Biotechnology Information) website

Sl. No	Endophytic isolate	Phylum	Order	Genera	Description	Accession	Identity (%)
1.	AREF001	Ascomycota	Onygenales	*Auxarthron*	*Auxarthron sp.*	MT013399	98.28
1.	AREF004	Ascomycota	Pleosporales	*Alternaria*	*Alternaria* sp.	MT013400	98.93
1.	AREF007	Ascomycota	Pleosporales	*Alternaria*	*Alternaria sp.*	MT013401	96.27
1.	AREF010	Ascomycota	Eurotiales	*Aspergillus*	*Aspergillus sp.*	MT013402	99.62
1.	AREF014	Ascomycota	Hypocreales	*Fusarium*	*Fusarium sp.*	MT013403	99.81
1.	AREF019	Ascomycota	Hypocreales	*Monascus*	*Monascus sp.*	MT013404	98.48
1.	AREF020	Ascomycota	Eurotiales	*Talaromyces*	*Talaromyces sp.*	MT013405	99.62
1.	AREF021	Ascomycota	Xylariales	*Xylaria*	*Xylaria sp.*	MT013406	99.52
1.	AREF023	Ascomycota	Eurotiales	*Aspergillus*	*Aspergillus sp.*	MT013407	99.82
1.	AREF029	Ascomycota	Pleosporales	*Curvularia*	*Curvularia sp.*	MT013408	100
1.	AREF030	Ascomycota	Diaporthales	*Phomopsis*	*Phomopsis sp.*	MT013409	99.44

Table 3: DNA reference sequences used in this study, including original references (where applicable) and GenBank accession numbers

Species	Strain	GenBank accession numbers - ITS	References
Auxarthron ostraviense	CCF 4241	NR_121474	(Schoch et al. 2014)
Auxarthron umbrinum	AU-BRK4	MH424172	(Angelone and Bidochka 2018)
Auxarthron conjugatum	KRP24-4	HM036583	(Menkis and Vasaitis 2011)
Alternaria brassicae	M1	JN108903	(Sharma et al. 2013)
Alternaria brassicae	W8	MF356599	(Zhao et al. 2018)
Alternaria alternata	Cc-01	MG722823	(Zhang et al. 2018)
Alternaria alternata	YZU 191238	MN615420	(Zhang et al. 2020)
Alternaria alternata	ZmH31	MG228423	(Xing et al. 2018)
Alternaria alternata	ZmH30	MG228422	(Xing et al. 2018)
Aspergillus nidulans	NA	MG459155	(Jedidi et al. 2018)
Aspergillus niger	DF1	LT745387	(Sivaraman et al. 2018)
Aspergillus nidulans	UOA/HCPF 10647	GQ461904	(Arabatzis et al. 2011)
Fusarium falciforme	UOA/HCPF 14802	KP132214	(Irinyi et al. 2015)
Fusarium solani	F174	MF401578	(Moumni et al. 2020)
Fusarium solani	28	KY318489	(Manganyi et al. 2018)
Monascus kaoliang	NA	AB477250	(Shinzato et al. 2009)
Monascus purpureus	NA	AB477247	(Shinzato et al. 2009)
Monascus kaoliang	NA	AB477252	(Shinzato et al. 2009)
Talaromyces assiutensis	KK	KR909179	(Travadon et al. 2015)
Talaromyces assiutensis	CBS 118440	JN899320	(Samson et al. 2011)
Talaromyces trachyspermus	CBS 373.48	MH856401	(Vu et al. 2019)
Xylaria palmicola	604	GU322436	(Hsieh et al. 2010)
Xylaria psidii MF773655	AT_L13_E2	MF773655	(Maduranga et al. 2018)
Xylaria oxyacanthae	YMJ 1551	MF773432	(Ju et al. 2018)
Aspergillus terreus	JAS 1	JQ361749	(Silambarasan and Abraham 2013)
Aspergillus terreus	NTOUTL11	MN592939	(Pang et al. 2019)
Aspergillus terreus	NTOU4989	MN592937	(Pang et al. 2019)
Curvularia sp.	MR-2019p	MN215713	(Raza et al. 2019)
Curvularia lunata	CBS 730.96	NR_138223	(Amaradasa et al. 2014)
Curvularia lunata	NA	HF934911	(Amaradasa et al. 2014)
Diaporthe detrusa	CBS 109770	KC343061	(Gomes et al. 2013)
Diaporthe eres	FPH2017229	MH003633	(Lin et al. 2018)
Diaporthe sp.	AHGB02_1A	MH267914	(Skaltsas et al. 2019)

Table 4: (a) Chemical constituents and their % content from the methanol extract of *Aspergillus sp.* AREF023 as determined using GC/MS

Peak No.	Identified Compound Name (IUPAC)	Compound Class	CAS No.	PubChem CID	Molecular weight	Molecular Formula	Area %	RT	% Identity
1	Methyl 3,3-dimethoxypropionate	Methyl ester	7424-91-1	81924	148.16	$C_6H_{12}O_4$	12.09	5.316	90
2	Pentanoic acid, 4-oxo-, methyl ester	Methyl ester	0624-45-3	69354	130.14	$C_6H_{10}O_3$	3.38	5.649	94
3	1-(1-Butoxypropan-2-yloxy)propan-2-yl acetate	Acetates	1000367-08-4	21226488	232.32	$C_{12}H_{24}O_4$	1.39	6.09	38
4	Butanedioic acid, dimethyl ester	Methyl esters	106-65-0	7820	146.14	$C_6H_{10}O_4$	21.7	6.887	90
5	2-Pentene, 2-methoxy-	Alkenes	061142-47-0	5363459	100.16	$C_6H_{12}O$	3.1	7.081	47
6	Hexanoic acid, 4-oxo-, methyl ester	Carboxylic esters	2955-62-6	546293	144.17	$C_7H_{12}O_3$	1.16	8.597	94
7	2-t-Butyl-5-hydroxymethyl-5-methyl-[1,3]dioxolan-4-one	Oxalidinone	1000187-75-0	554337	188.22	$C_9H_{16}O_4$	8.97	8.67	43
8	Hexanoic acid, 5-oxo-, methyl ester	Methyl ester	13984-50-4	84129	144.17	$C_7H_{12}O_3$	10.93	8.966	80
9	Monomethyl malonate	Carboxylic esters	16695-14-0	538366	118.09	$C_4H_6O_4$	1.54	10.18	25
10	Pentanedioic acid, dimethyl ester	Methyl esters	1119 – 40-0	14242	160.17	$C_7H_{12}O_4$	5.99	10.718	59
11	Octanal dimethyl acetal	Di-ethers	10022-28-3	61431	174.28	$C_{10}H_{22}O_2$	7.43	11.636	53
12	(1-Ethylvinyloxy)trimethylsilane	Organosilicon	6651-40-7	554663	144.29	$C_7H_{16}OSi$	2.02	12.736	25
13	Pentanoic acid, 5,5-dimethoxy-, methyl ester	Methyl esters	23068-91-9	551444	176.21	$C_8H_{16}O_4$	2.69	13.388	37
14	Hexanedioic acid, dimethyl ester	Methyl esters	627-93-0	12329	174.19	$C_8H_{14}O_4$	3.37	15.95	91
15	Nonanal dimethyl acetal	Di-ethers	18824-63-0	87813	188.31	$C_{11}H_{24}O_2$	2.08	18.808	27
16	Heptanedioic acid, dimethyl ester	Methyl esters	1732-08-7	74416	188.22	$C_9H_{16}O_4$	1.95	21.557	95
17	Octanedioic acid, dimethyl ester	Methyl esters	1732-09-8	15611	202.25	$C_{10}H_{18}O_4$	1.34	27.388	96
18	Diethyl Phthalate	Esters	84-66-2	6781	222.24	$C_{12}H_{14}O_4$	3.97	35.871	97
19	Hexadecanoic acid, methyl ester	Methyl ester	112-39-0	8181	270.5	$C_{17}H_{34}O_2$	3.04	55.308	98
20	Methyl stearate	Fatty acid	112-61-8	8201	298.5	$C_{19}H_{38}O_2$	1.85	60.921	99

Table 4: (b) Chemical constituents and their % content from the methanol extract of *Fusarium sp.* AREF014 as determined using GC/MS

Peak No	Identified Compound Name (IUPAC)	Compound Class	CAS No.	PubChem CID	Molecular weight	Molecular Formula	Area %	RT	% Identity
1	1,2,4 - Butanetriol	polysaccharide	42890-76-6	640997	106.12	$C_4H_{10}O_3$	3.14	5.274	33
2	Benzoic acid, methyl ester	di-ester	93-58-3	7150	136.15	$C_8H_8O_2$	13.1	8.978	68
3	1-Tridecene	Alkenes	2437-56-1	17095	182.35	$C_{13}H_{26}$	10.2	24.095	52
4	Phenol, 2,5-bis(1,1-dimethylethyl)	Phenol	111342-06-4	14028656	240.77	$C_{14}H_{21}ClO$	12.62	31.001	90
5	Pentadecanoic acid, 14-methyl-, methyl ester	Ester	5129-60-2	21205	270.5	$C_{17}H_{34}O_2$	36.45	55.314	97
6	Methyl stearate	Fatty acid	112-61-8	8201	298.5	$C_{19}H_{38}O_2$	13.62	60.921	99
7	Bis(2-ethylhexyl) phthalate	Esters	117-81-7	8343	390.6	$C_{24}H_{38}O_4$	10.89	73.554	72

Figures

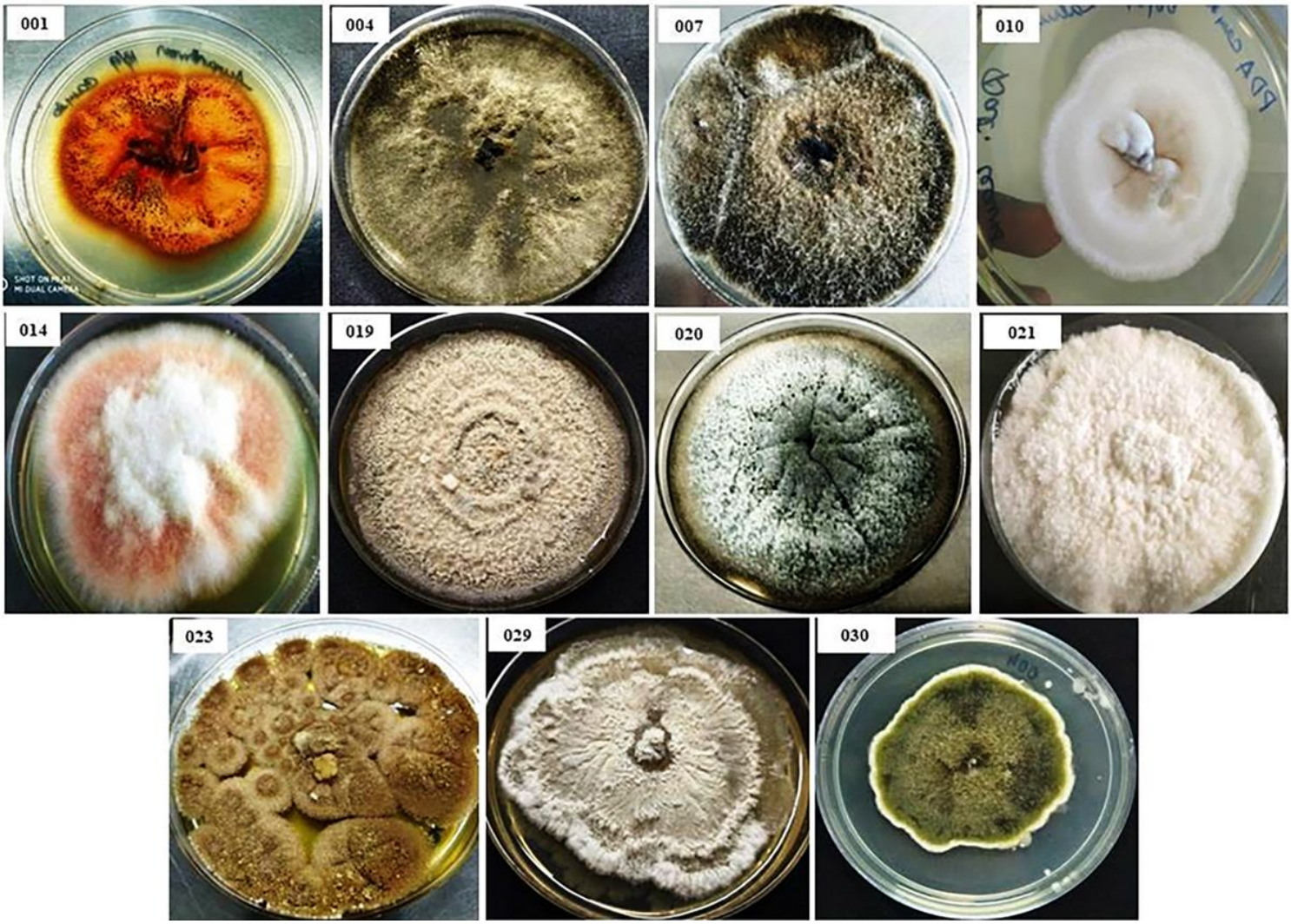

Figure 1

Some of fungal isolates obtained from plants of Aravalli Bio-diversity Park, Gurugram, Haryana and mangroves of Alibaugh coastal area, Maharashtra, India

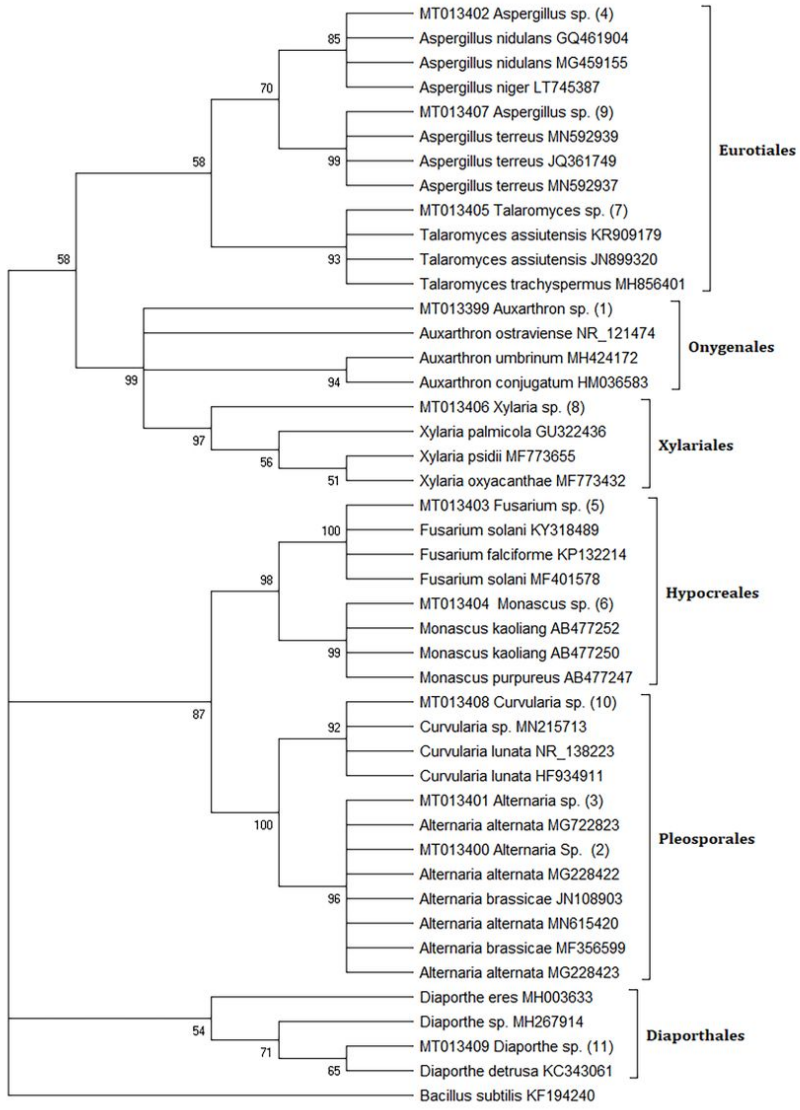

Figure 2

Phylogenetic relationships for eleven fungal isolates based on ITS1, 5.8S rDNA, and ITS2 regions of DNA sequences. The Maximum composite likelihood substitution model was used for phylogenetic reconstruction. Numbers on tree branches indicate the percentage of bootstrap replications derived from 1000 replications.

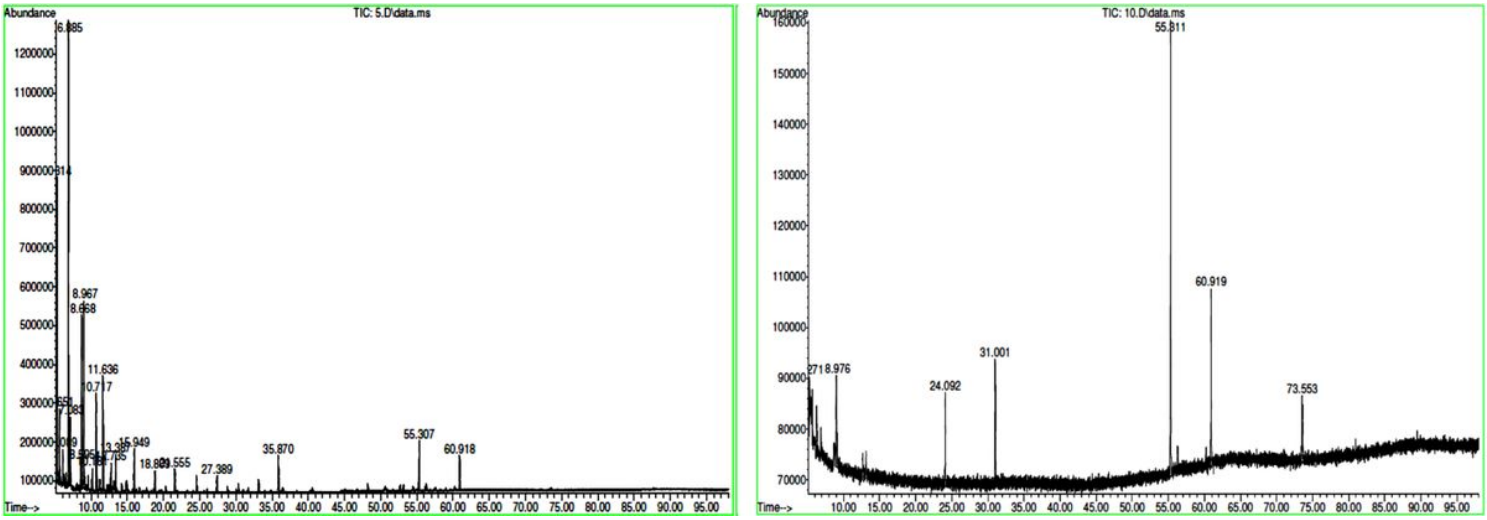

Figure 3

(a) GC-MS chromatogram of Aspergillus sp. AREF023 methanol extract. (b) GC-MS chromatogram of Fusarium sp. AREF014 methanol extract

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