



Phytochemical Screening and Antioxidant Potential of Endophytic Fungi Isolated From *Hibiscus sabdariffa*

Doaa M.A. Khalil^{1*}, Soad A. El-Zayat¹, Magdi A. El-Sayed^{1,2*}

¹Botany Department, Faculty of Sciences, Aswan University, Aswan 81528, Egypt

²Unit of Environmental Studies and Development, Aswan University, Aswan 81528, Egypt

Corresponding Author: 1. Doaa M.A. Khalil, PhD Candidate, Botany Department, Faculty of Sciences, Aswan University, Aswan 81528, Egypt. Tel: +20-1100824842, Email: doaa.khalil30@sci.aswu.edu.eg
2. Magdi A. El-Sayed, PhD, Professor, Unit of Environmental Studies and Development, Aswan University, Aswan 81528, Egypt. Tel: +20-1124807000, Email: magdiel_sayed@aswu.edu.eg

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Abstract

Introduction: Fungal endophytes are friendly microbes colonizing in plants and play an effective role in plant-environment interactions. They produce valuable secondary metabolites that both plants and human beings can benefit from such products. Diverse compounds are biosynthesized by endophytes to promote plant growth and prevent pathogen attacks. Some of these compounds can be used for human drugs as antibiotics.

Materials and Methods: Thirteen endophytic fungal species were isolated from root, stem and leaves of *Hibiscus sabdariffa*. The assessment of the antioxidants activity, total phenolic content (TPC), total flavonoids content and total antioxidant capacity (TAC), hydrogen peroxide scavenging and reducing power assays of 26 ethyl acetate and 13 methanol crude fungal extracts has been carried out. Qualitative assay of bioactive compounds for 39 endophytic fungal extracts have been checked by standard procedures. Quantitative estimation of TPC and flavonoids content was done. The DPPH free radical scavenging was estimated using DPPH (1,1-diphenyl-2-picrylhydrazyl), and hydrogen peroxide scavenging and reducing power assays were used to evaluate the antioxidants activity.

Results: Qualitative detection of secondary metabolites revealed that endophytic fungal extracts from *Aspergillus terreus* isolated from the leaves of *Hibiscus sabdariffa* was found to be able to produce all the tested functional metabolites with the highest total phenolic contents (204.5±0.4 mg GAE/g) and antioxidant activity for DPPH radical scavenging assay (91.88±0.17 µg/mL).

Conclusions: The present study revealed that metabolites produced by some endophytic fungi isolated from the medicinal plant, *Hibiscus sabdariffa* could be a potential source of novel natural antioxidant compounds.

Keywords: Fungal Endophytes, Antioxidant, Secondary Metabolites

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Introduction

Fungi have proven to be a rich source of bioactive and novel organic compounds with interesting biological activities and a high level of biodiversity.^{1,2} Fungi produce a diverse array of secondary metabolites. Secondary metabolites have a tremendous impact on society and are exploited for their antibiotics and pharmaceuticals activities such as anticancer, antitumor, immuno-stimulatory, and antioxidants.³ It is clear that, fungi represent a largely untapped source of potentially powerful new pharmaceutical products.^{4,5}

The need for new bioactive compounds used in medicine, industry and agriculture has increased. While plants have been a major source of new compounds for drug discovery, attention has more recently turned to endophytes as these microorganisms demonstrate great potential sources for new bioactive compounds.⁶ Endophytes are microbes which colonize living internal tissues of plants without causing any harm to their host.⁷ Almost all groups of microorganisms have been found in endophytic association with plants such

as fungi, bacteria or actinomycetes. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites.⁸ Many researchers have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture, industry,⁹ antiviral, anticancer, antidiabetic and antimicrobial effects, but very little is known about their antioxidant capacity.⁶ A lot of work has been done on the bioactive potential of endophytes, such as endophytic fungi which are fungi that colonize living, internal tissues of various parts of the plants without harming them.¹⁰ All higher plants are hosts to one or more endophytic microbes on this earth. Recently, endophytes are viewed as outstanding sources of secondary metabolites and bioactive antimicrobial natural products.¹¹ Antioxidants are the molecules, which prevent cellular damage by reducing the oxidative stress and therefore have a beneficial effect on human health.¹² Antioxidants may be characterized by their mode of action in preventing oxidative damage, being classified as preventative, scavenging,

and repair or *de novo* antioxidants.¹³ Antioxidants prevent the formation of reactive oxygen and nitrogen species ROS/RNS by reducing hydrogen peroxide and lipid hydro peroxidases, respectively, or by sequestering metal ions such as iron and copper.¹⁴

The objective of the present study was to screen the phytochemical classes of 13 endophytic fungal extracts isolated from *Hibiscus sabdariffa* and to assay their antioxidant activities.

Materials and Methods

Isolation of Endophytic Fungi

Healthy plant materials of *H. sabdariffa* was collected from a natural habitat in the desert of Aswan University campus, located at latitude: 24° 5' 15" N 32° and 53' 56" E. The climate in this region ranged between moderately cold dry winter, average temperature 25°C (maximum), 8°C (minimum) to very hot summer, 50°C (maximum) and 25°C (minimum). The collected sample (root, stem and leaf) were completely rinsed in running tap water, followed roots were initially rinsed in tap water to eliminate surface soils and then surface-sterilized by successive immersion in 95% ethanol (10 seconds), 2% sodium hypochlorite (3 minutes) and 70% ethanol (2 minutes). Surface-sterilized roots, stem and leaves were cut into approximately ~5 mm pieces under sterile conditions placed on petri dishes containing potato dextrose agar supplemented with 100 U/mL chloramphenicol to suppress bacterial growth.¹⁵ Plates were incubated at 28°C until formed colonies.¹⁶ The growing tips of mycelia were transferred to new PDA plates for pure culture and maintained by continuous subculturing.¹⁷ All isolated fungal colonies were identified according to microscopic observation of mycelia, asexual/sexual spores and colony morphology, cultural characteristics (color, texture, and pigmentation), and spores and spore-bearing structure using standard identification manuals.¹⁸⁻²¹

Preparation of Extracts

The fungal inoculums obtained from pure cultures were inoculated into 1000 Erlenmeyer flasks containing 750 mL potato dextrose broth media. All cultures were incubated at 28±1°C on a rotary shaker at 160 rpm for 15 days. The fermentation broth of each endophyte was filtered to remove the mycelia. The fermentation broth was extracted with 250 mL of ethyl acetate overnight. The water fraction (lower layer) was removed by using a separation funnel and the ethyl acetate fraction (upper layer) was collected and evaporated using a rotary evaporator. The Mycelia of each fungal strain were grinded and extracted with 75 mL ethyl acetate and were left overnight. Then, filtered and the filtrates were evaporated using a rotary evaporator to get the ethyl acetate extract of mycelia fraction. The residue mycelia were extracted with 50 mL methanol then filtrate and the filtrate were evaporated using a rotary evaporator to get mycelia fraction with methanol.^{22,23}

Qualitative Detection of Bioactive Compounds in Endophytic Fungal Extracts

Thirty-nine crude endophytic fungal extracts were used

for phytochemical analysis for detecting the presence of secondary metabolites like alkaloids, tannins, phenols, flavonoids and terpenes according to standard procedure.^{24,25}

Quantitative Determination of Total Phenolic Contents

Total phenolic content (TPC) was determined according to the Folin-Ciocalteu (F-C) colorimetric method.²⁶ Briefly, 50 µL of sample and 50 µL of F-C reagent were pipetted into a tube. The contents were shaken for 10 seconds and were then left to stand at room temperature for 2 minutes and stopped the reaction by adding 500 µL of 5% (w/v) sodium carbonate solution and 400 µL of distilled water, and the volume was adjusted to 1 mL. The mixture was incubated at 45°C for 30 minutes. The absorbance of the solution was measured at 760 nm. The samples were prepared in triplicate for each analysis to obtain the mean value of absorbance. Gallic acid concentrations ranging from 10 to 300 µg/mL were prepared and a calibration curve was obtained using a linear fit. The phenol content in the fungal extracts was derived from the standard curve. The results were expressed as mg of gallic acid eq. per ml of extracts. Also, the values were expressed as mean ± SD.

Quantitative Determination of Flavonoid Contents

Total flavonoid content was determined according to a standard protocol of aluminum chloride method.²⁷ Briefly, 50 µL of each endophyte extract and 300 µL of NaNO₂ (1:20 w/v) were pipetted into a test tube and the contents were shaken for 10 seconds and were then left to stand at room temperature for 5 minutes. After standing, 300 µL of AlCl₃ (1:10 w/v), 2 mL of NaOH (1 M) and 1.9 ml of distilled water were added to the reaction mixture, which was then vortexed for 10 seconds, and the absorbance was measured at 510 nm. Naringenin concentrations ranging from 10 to 800 µg/mL were prepared and a standard calibration curve was obtained using a linear fit. The samples were prepared in triplicate for each analysis to obtain the mean value of absorbance. The flavonoid content in the fungal extracts was derived from the standard curve. The results were expressed as mg of naringenin eq. per g dry weight of the fungal extracts. Also, the values were expressed as mean ± SD.

Antioxidant Assays

Four different assays including free radical scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, hydrogen peroxide scavenging assay, total antioxidants capacity and reducing power assay were used to evaluate the antioxidant potential of endophytic fungal crude extracts. Each experiment was done in triplicate and the mean values were taken.

DPPH Free Radical Scavenging Activity

To check the antioxidant activity through free radical scavenging by the test samples, the change in optical density of DPPH radicals was monitored. Endophytic fungal extracts at 1 mg/mL concentration were used. The DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. When a substrate that can donate a hydrogen atom was added in the DPPH solution, it was reduced to a

yellow colored product, diphenylpicryl hydrazine. The DPPH solution (0.5 mmol/L) was prepared in 95% methanol. The sample extract (0.1 mL) was diluted with methanol. A total of 2 mL of DPPH solution (0.5 mmol/L) was added in the test sample and incubated for 30 minutes at room temperature in darkness. After 30 minutes, the absorbance was measured at 517 nm.²⁸ Free radical scavenging activity was expressed as a percentage. The percentage of the DPPH radical scavenging was calculated as:

Inhibition of DPPH radical (%) = [(control absorbance - extract absorbance)/(control absorbance)] × 100

Inhibition (%) = $(A_i - A_t)/A_i \times 100\%$

which A_i is the absorbance of the DPPH free radical and A_t is the absorbance of DPPH free radical+ sample/standard.

Determination of Total Antioxidant Capacity

The assay was done according to Prieto and colleagues' study in 1999. An aliquot of 50 µL of each extract was combined in a tube with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 minutes. After the samples had cooled at room temperature, the absorbance of the aqueous solution of each sample was measured at 695 nm against a blank. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. The antioxidants capacity was expressed as ascorbic acid equivalent.²⁹

Hydrogen Peroxide Scavenging (H₂O₂) Assay

The ability of fungal extracts to scavenge hydrogen peroxide was estimated by following the method of Ruch et al that a solution of hydrogen peroxide (40 mmol/L) was prepared in phosphate buffer (50 mmol/L, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm. Endophytic fungal extracts (1 mg/mL) in distilled water was added to hydrogen peroxide and absorbance at 230 nm by using a spectrophotometer and was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide.³⁰ The percentage of hydrogen peroxide scavenging was calculated as follows:

Scavenged H₂O₂ (%) = $[(A_i - A_t)/A_i] \times 100$

Where A_i is the absorbance of control and A_t is the absorbance of test samples.

Determination of Antioxidant Activity by Reducing Power Measurement

The reducing power of the extract was determined according to Chang and colleagues' method with slight modification as follows: an aliquot of 50 µL extract was added to 0.1 mL of 1% (w/v) potassium ferricyanide. After incubating the mixture at 50°C for 30 minutes, during which the ferricyanide was reduced to ferrocyanide, it was supplemented with 0.1 mL of 1% (w/v) trichloroacetic acid and 0.1% FeCl₃ and left for 20 minutes. Absorbance was read at 700 nm to determine

the amount of ferric ferrocyanide (Prussian blue) formed.³¹ Higher absorbance of the reaction mixture indicates higher reducing power of the sample. Ascorbic acid concentrated of 10 to 200 µg/mL was used as standard.

Results

Qualitative Detection of Secondary Metabolites

Screening various secondary metabolites of crude extracts revealed a good concentration of alkaloids, phenols, tannins, terpenes, flavonoids and sterols in crude extracts of endophytic fungi as shown in Table 1. These chemical compounds are responsible for different medicinal properties of extracts. It has revealed that *Aspergillus terreus* was found to be able to produce all the functional metabolites so far tested while *Aspergillus niger*, *Aspergillus ustus* and *Torula ramosa* produced all secondary metabolites except tannins. The production of tannins was only in *A. terreus* and *A. oryzae*. All the isolates showed more or less efficient (as observed from intensity of color) for the production of alkaloids, flavonoids, tannins, terpenoids and sterols.

Total Phenolics Contents

There was a wide range of total phenolic concentrations (TPC) in the endophytic fungal extracts of ethyl acetate and methanolic extracts as shown in Figure 1. The highest phenolic producers (formed ≥100 mg/mL) were 9 fungal crude extract while there were seven fungal crude extract produced in moderate concentration of phenolic content and 5 fungal crude extract which showed least concentration of phenols. The values of total phenolic concentrations varied from 15.5 to 204.5 mg/mL. It is evident that the highest concentration of phenols was estimated in both extracts of ethyl acetate from filtrate and methanolic mycelia extract of *A. terreus* (204.5 mg/mL, 191.85 mg/mL) respectively, followed by culture filtrate of *Fusarium solani* (177.62 mg/mL). Whereas culture filtrate of *A. chlamydozoa* contained considerably least concentration of phenols (15.5 mg/g). It is evident that *A. terreus* with its 3 extracts proved to be the best

Table 1. Qualitative Analysis of the Phytochemical Components of Endophytic Fungal Extracts

| Fungal Endophyte | Phytochemical Present |
|-----------------------------------|--|
| <i>Aspergillus flavus</i> | Alkaloids, phenols, terpenes |
| <i>Aspergillus fumigatus</i> | Alkaloids, terpenes, phenols, flavonoids |
| <i>Aspergillus niger</i> | Alkaloids, flavonoids, phenols, terpenes, sterols |
| <i>Aspergillus oryzae</i> | Alkaloids, flavonoids, phenols, tannins |
| <i>Aspergillus terreus</i> | Alkaloids, flavonoids, phenols, tannins, terpenes, sterols |
| <i>Aspergillus ustus</i> | Alkaloids, flavonoids, phenols, terpenes, sterols |
| <i>Alternaria chlamydozoa</i> | Alkaloids, phenols, terpenes |
| <i>Cochliobolus australiensis</i> | Alkaloids, flavonoids, phenols |
| <i>Drechslera bioseptata</i> | Alkaloids, phenols, terpenes |
| <i>Helicosporium</i> | Alkaloids, flavonoids, phenols, terpenes |
| <i>Fusarium solani</i> | Alkaloids, flavonoids, phenols, terpenes |
| <i>Mucor racemosus</i> | Alkaloids, flavonoids, phenols, terpenes |
| <i>Torula ramosa</i> | Alkaloids, flavonoids, phenols, terpenes, sterols |

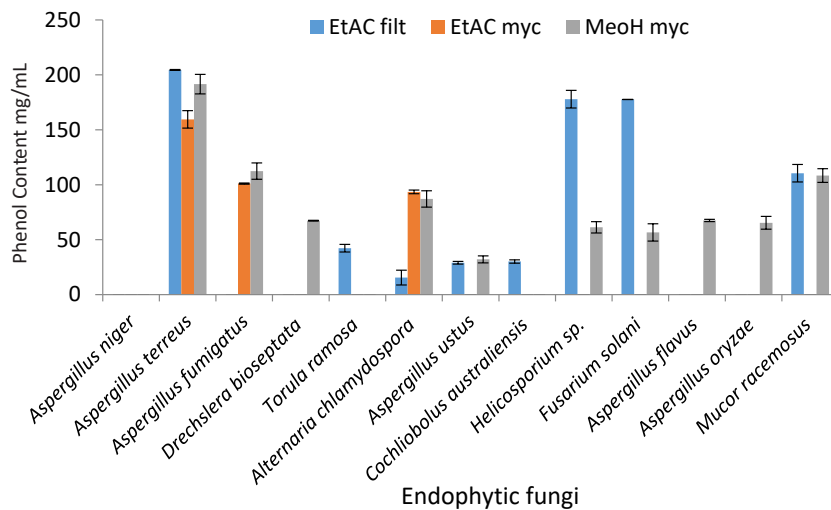


Figure 1. Graphical Representation of the Phenol Content of the Culture Filtrate and Mycelium. All assays carried out in triplicate; mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (\pm SD).

in phenol production as shown in Figure 1.

Total Flavonoids Contents

The amount of total flavonoids content was determined as the naringenin equivalent using an equation obtained from a standard naringenin graph. According to the results of this study it appears that the tested fungal strains have the ability to produce flavonoids with levels of 12.75 to 240.43 mg/g (Figure 2). The highest flavonoid producers (formed ≥ 90 mg/g) were only two fungal crude extracts of ethyl acetate culture filtrate and mycelia of *A. terreus*. Out of 26 ethyl acetate and 13 methanol crude fungal extracts of endophytic fungi, ethyl acetate extract filtrate of *A. terreus* was recorded as a high producer strain in flavonoids content and formed 263.66 ± 5.75 mg/g followed by the ethyl acetate extract from mycelia of *A. terreus* which formed 151.63 ± 3.34 mg/g. This

is while its methanolic extract recorded 37.34 ± 5.15 mg/g that showed low amount of flavonoids content in contrast of its ethyl acetate mycelial extracts. Ethyl acetate of filtrate of *Torula ramosa* also recorded high levels of flavonoids content (98.36 ± 6.6 mg/g) followed by methanolic extract of *Helicosporium* and extract of ethyl acetate mycelia of *Torula ramosa* (77.41 ± 5.61 , 64.66 ± 14.16) mg/g respectively. Ethyl acetate fungal crude extract from filtrate of *Cochliobolus australiensis* formed moderate flavonoids content (73.77 ± 13.38) mg/g followed by ethyl acetate fungal crude extract from *F. solani* filtrate that recorded 42.34 ± 5.57 mg/g. However, the other mycelia extract of *F. solani* with ethyl acetate and methanol showed no flavonoids content. Ethyl acetate extract of mycelia of *Aspergillus fumigatus* showed low flavonoids content (20.49 ± 1.11) mg/g while its filtrate ethyl acetate extract and methanolic extract showed no

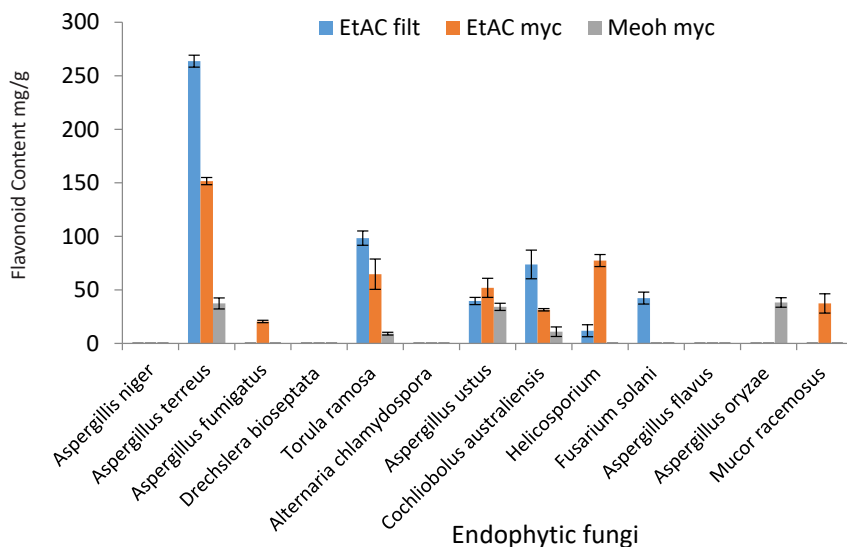


Figure 2. Graphical Representation of the Flavonoid Content of the Fungal Culture Filtrate and Mycelium. All assays carried out in triplicate; mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (\pm SD).

flavonoids content. *A. ustus* showed different concentration in flavonoids content in its 3 extracts. *Drechslera bioseptata*, *Alternaria chlamydospora*, *Aspergillus flavus*, *Aspergillus oryzae* ethyl acetate extracts' for filtrate and mycelia, ethyl acetate extract for filtrate and methanolic extract of *Mucor racemosus* and Methanolic extract of *Helicosporium* had no flavonoids content.

Antioxidant Activity

In the present study, 26 ethyl acetate and 13 methanol crude fungal extracts of endophytic fungi isolated from *H. sabdariffa* were investigated for their antioxidant potential by using 4 different methods. All 21 extracts showed antioxidant activity up to varying extent. Five (13%) fungal extracts showed significant antioxidant activity ranging from 50% to 80%. The remaining extracts showed a narrow spectrum of activity ranging from 3% to 40%.

DPPH Radical Scavenging Activity

Antioxidant capacity of fungal extracts was detected semi quantitatively by a rapid DPPH staining method. This method is typically based on the inhibition of the accumulation of oxidized products, since the generation of free radicals is inhibited by the addition of antioxidants and scavenging of the free radicals shifts to the end point. In the present study, crude extract of ethyl acetate filtrate and mycelia for *A. terreus* showed a high scavenging activity of 80% while its methanolic crude extract showed 65%, the ethyl acetate extract from filtrate of *F. solani* recorded 71% antioxidant activity but their extracts of mycelia by ethyl acetate and methanol did not show any antioxidant activity. On the other side, methanolic extract of *A. flavus*'s mycelia showed 55% antioxidant activity whereas ethyl acetate filtrate extract of *D. bioseptata* showed the least antioxidant activity (3.6%). The percentage of DPPH radical scavenging activity of the endophytic fungi and ascorbic acid are shown in Figure 3.

Total Antioxidant Capacity

The total antioxidant capacity (TAC) of ethyl acetate crude

fungal extract from filtrate of *F. solani* was evaluated and found to be 7.81 mg/mL concentration while the methanolic extract of *A. chlamydospora* showed high TAC (2.89 ± 0.66 mg/mL). Also, ethyl acetate crude fungal extract from filtrate of *A. flavus* was 2.71 ± 0.12 mg/mL. Methanolic and ethyl acetate crude fungal extract from mycelia of *A. terreus* also showed high TAC (2.46 ± 0.74 and 2.33 ± 0.70 mg/mL). Ethyl acetate crude fungal filtrate extract of *Aspergillus fumigatus* was 2.09 ± 0.70 mg/mL. Methanolic extract of *A. chlamydospora* recorded the highest TAC while methanolic crude extract of *A. oryzae* showed the lowest TAC (0.013 ± 0.001 mg/mL). This is while filtrate ethyl acetate extract and methanolic extract from mycelia of *C. australiensis* showed no antioxidant capacity. All other fungal crude extracts showed low TAC as shown in Table 2.

Hydrogen Peroxide Scavenging (H₂O₂) Assay

As shown in Figure 4, reduction potential of fungal extracts in the H₂O₂ scavenging assay ranged from 93% to 99%.

Reducing Power Assay

The reducing power evaluation of the fungal extracts is an important parameter related to assessing the antioxidant activity. Reducing power measures the ability of a sample to act as an electron donor and therefore, reacts with free radicals converting them to more stable products and thereby terminate radical chain reaction. In order to examine the reducing power of fungal extracts, the reaction of Fe³⁺ was observed. The reducing power of the extracts was determined according to Change et al.²³ An increase in the absorbance of the reaction mixture indicated increased reducing power of the sample. Absorbance was read to determine the amount of ferric Ferro cyanide (Prussian blue) formed. Ascorbic acid was taken as the standard. The results revealed that *A. terreus* mycelia extracted with methanol and ethyl acetate, respectively had high absorbance values that indicated their greater reductive potential and electron donor ability for stabilizing free radicals. This is while the filtrate extract with ethyl acetate showed moderate antioxidant activity. *C.*

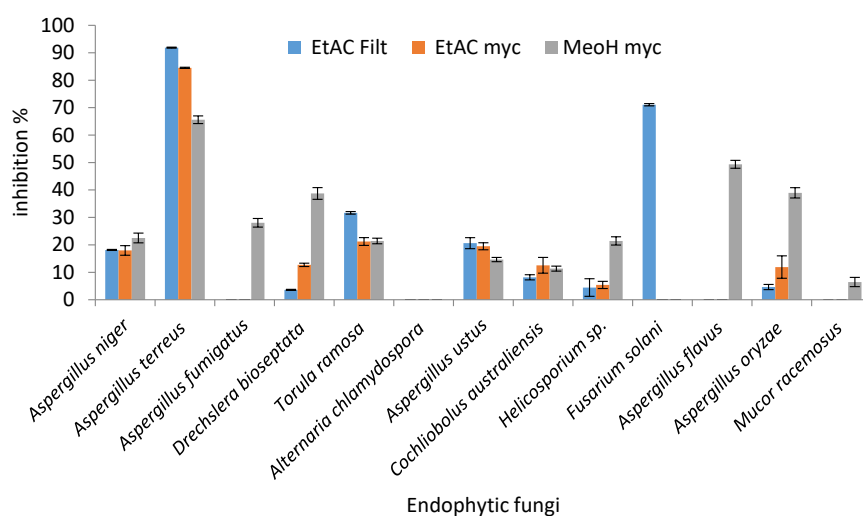


Figure 3. Graphical Representation of % Inhibition of DPPH Radical Activity of Fungal Culture Filtrate and Mycelium by Free Radical Scavenging Assay. All assays carried out in triplicate, mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (\pm SD)

Table 2. Total Antioxidants Capacity and Reducing Power as Antioxidant Activities of Some Endophytic Fungi Recorded as mg/mL Fungal Extracts

| Endophytic Fungi | Antioxidants Capacity | | | Reducing Power | | |
|-----------------------------------|--------------------------------|-------------------------------|--------------------------|--------------------------------|-------------------------------|--------------------------|
| | Ethyl Acetate Filtrate Extract | Ethyl Acetate Mycelia Extract | Methanol Mycelia Extract | Ethyl Acetate Filtrate Extract | Ethyl Acetate Mycelia Extract | Methanol Mycelia Extract |
| <i>Aspergillus niger</i> | 0.07±0.01 | 0.89±1.14 | 0.18±0.07 | 0 | 0 | 0 |
| <i>Aspergillus terreus</i> | 1.25±0.05 | 2.33±0.7 | 2.46±0.74 | 3.47±2.39 | 7.52±4.2 | 12.13±0.14 |
| <i>Aspergillus fumigatus</i> | 2.09±0.71 | 1.05±0.22 | 1.94±0.66 | 0 | 0 | 0 |
| <i>Drechslera bioseptata</i> | 1.02±0.28 | 1.14±0.35 | 0.18±0.04 | 0 | 2.35±0.96 | 0 |
| <i>Torula ramosa</i> | 0.61±0.17 | 0.33±0.08 | 0.2±0.06 | 1.32±0.01 | 0 | 1.11±0.04 |
| <i>Alternaria chlamydozpora</i> | 0.33±0.12 | 0.74±0.23 | 2.9±0.66 | 0.25±0.11 | 2.36±0.05 | 0.14±0.05 |
| <i>Aspergillus ustus</i> | 0 | 0.1±0.02 | 0.26±0.08 | 0.39±0.24 | 1.37±0.24 | 0 |
| <i>Cochliobolus australiensis</i> | 0 | 0.07±0.05 | 0 | 2.03±0.13 | 3.19±0.82 | 0 |
| <i>Helicosporium sp.</i> | 0.77±0.04 | 0.75±0.01 | 0.93±0.04 | 0.6±0.03 | 0.32±0.25 | 2.19±0.21 |
| <i>Fusarium solani</i> | 7.81±0.07 | 1.81±0.07 | 2.02±0.16 | 1.47±0.17 | 0.46±0.28 | 0.32±0.07 |
| <i>Aspergillus flavus</i> | 2.71±0.13 | 1.86±0.14 | 0.57±0.13 | 4.04±0.11 | 10.97±1.45 | 2.01±0.07 |
| <i>Aspergillus oryzae</i> | 0.16±0.09 | 0.97±0.01 | 0.01±0.001 | 2.89±0.47 | 0.25±0.03 | 1.57±0.26 |
| <i>Mucor racemosus</i> | 0.25±0.01 | 2.02±0.15 | 0.47±0.19 | 4.07±0.04 | 0 | 0 |

australiensis' mycelia and filtrate extracted with ethyl acetate, ethyl acetate filtrate extract of *A. oryzae*, ethyl acetate mycelia extract of *A. chlamydozpora*, ethyl acetate mycelia extract of *D. bioseptata*, methanolic extract of *Helicosporium*, ethyl acetate filtrate extract of *C. australiensis* and methanolic extract of *A. flavus* had moderate antioxidant activity values. Other fungal extracts showed least antioxidant activity in all assays. The activity of all fungal extracts and ascorbic acid with respect to their absorbance values are represented in Table 2. All assays revealed that two crude extracts of mycelia with methanol and ethyl acetate of *A. terreus* possessed maximum antioxidant potential while filtrate extract with ethyl acetate of *A. chlamydozpora* showed the least antioxidant activity.

Discussion

A number of antioxidants are known to provide protection against several diseases. Epidemiological studies have demonstrated that higher intake of antioxidants results in reduced risk of heart disease and many other diseases. This is the reason for the strong interest in natural antioxidants and their role in human health and nutrition.³² Several medicinal plants, spices, vegetables, fruits, and fungi have been researched as sources of potentially safe natural antioxidants. Various compounds have been isolated and many of these are polyphenols. Recently, various fungi, endophytes, and mushrooms have been reported to produce antioxidant activity.^{33,34} They are known to produce several

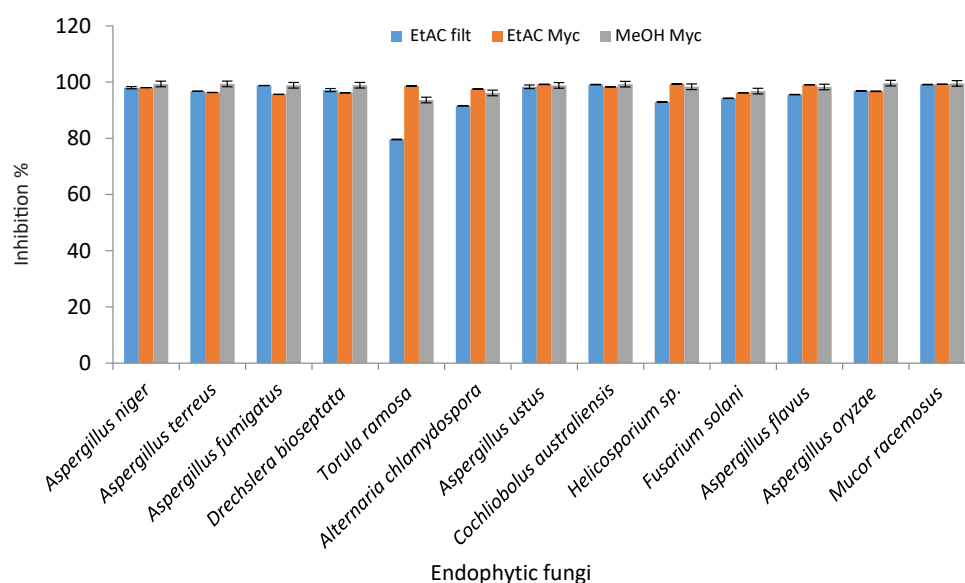


Figure 4. Graphical Representation of Hydrogen Peroxide Scavenging Activity of Fungal Culture Filtrate and Mycelium by Free Radical Scavenging Assay. All assays carried out in triplicate, mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (±SD).

novel metabolites possessing antioxidant activity and are equally potent as synthetic antioxidants and phytochemicals. *Chaetomium* sp., *Cladosporium* sp., *Torula* sp., *Phoma* sp., and *Penicillium roqueforti* produce various secondary metabolites like phenolic acid derivatives, terpenoids, benzoic acid, rutin with antioxidant activity, and also a wide range of other biological activities such as antibacterial, antiviral, antimutagenic, and immunomodulatory.³⁵ Gebhardt et al³⁶ reported anti-inflammatory and antioxidant activity of quercinol obtained from *Daedalea quercina*. In the present study, all the isolates showed good antioxidant activity against various free radicals. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which include the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging.³⁷ The obtained results from various assay procedures prove the potent wide ranging antioxidant activity of the extracts obtained from all the fungal spp. and the compounds purified from *Penicillium citrinum*. The activity was higher than many other already reported fungi, plants, and mushrooms.^{34,35,38} Antioxidant activity of the fungi towards DPPH free radicals may be attributed to their hydrogen-donating ability. The DPPH is an unstable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.³⁹ The extracts and purified compounds showed good scavenging activity against DPPH radicals specially in fungal crude extracts of *A. terreus* that showed a high antioxidant activity value of 80%. Its methanolic extract showed 65% followed by ethyl acetate filtrate extract of *F. solani* that showed 71% antioxidant activity. This finding is in contrast to the antioxidant activity study of endophytic fungi isolated from *Mussaenda luteola* by Gunasekaran et al⁴⁰ which showed that the EA extract of *Alternaria* sp. (ML4) showed a high scavenging activity of 85.20%. Reducing power assay proves the potential of the compounds and extracts to act as reductones that inhibit lipid peroxidation by donating a hydrogen atom thereby terminating the free radical chain reaction. Moreover, this reducing potential may be due to the di- or monohydroxy substitution in the aromatic rings that possess potent hydrogen-donating ability.⁴¹ Ferrozine can quantitatively form complexes with Fe²⁺. In the presence of chelating agents, the complex formation is disrupted resulting in decreased red color of the complex. Measurement of color reduction therefore allows estimation of the chelating activity of the coexisting chelator. In this assay, fungal extracts and compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals that can themselves abstract hydrogen and stop the chain reaction of lipid peroxidation.⁴² In the present study, the results revealed that a total of 29 crude extracts out of 13 endophytic fungi had a reducing power activity and formed ferric ferro cyanide with activities ranging between 0.14 and 12.13 mg/mL fungal extract. In a study of antioxidant potentialities of some strains belonging

to endophytic, entomopathogenic and saprophytic fungi, it revealed that a total of 21, 35 and 19 out of 26, 42 and 32 strains had a reducing power activity between 0.01 and 1.116 mg/mL fungal extract indicating that our extracts have higher reducing power activity than this study.⁴³

Hydrogen peroxide itself is not very reactive but sometimes rapidly decomposes into oxygen and water producing hydroxyl radicals (OH⁻) that causes DNA damage.⁴⁴ These results indicated that scavenging activity by H₂O₂ was correlated with the scavenging activity by DPPH and reducing power assay. In the study of antioxidant activity and TPC of endophytic fungi isolated from *Eugenia jambolana* Lam showed that the reduction potential of fungal extracts in H₂O₂ assay ranged from 75% to 10% while in the present study the reduction potential of fungal extracts in H₂O₂ assay ranged from 99% to 93%.⁴⁵

Phenolic compounds are well-known antioxidant constituents because of their high ability to scavenge toxic free radicals and reactive oxygen species.⁴⁶ Interestingly, the extracellular phenolic compounds produced by these fungi were found to be much higher than other medicinal plants. Such experimental data after comparison with previously published reports clearly indicate that soil fungal isolates might contain various types of phenolic compounds with higher antioxidant activities.⁴⁷ The actual significance of the present study is that the isolated compounds were potent antioxidants and showed diverse activity against different free radicals as supported by the results of different assay procedures. Moreover, the activity shown by the compounds was higher/comparable with the activity of known antioxidants such as ascorbic acid and other phytochemicals like rutin and catechin. Earlier studies have also demonstrated that the fungi are good sources of antioxidants.^{48,49} Various compounds with antioxidant potential are isolated from *Mycelia sterilia*, *Colletotrichum gloeosporioides*, *Pestalotiopsis microspora*, and *Aspergillus candidus* showed comparable/higher activity compared to various known antioxidants.^{50,51} There are several studies on the detection of the total phenolic compounds produced by endophytic fungi such as the study by Yadav et al⁴⁵ who isolated 21 endophytic fungal isolates from *Eugenia jambolana* Lam in India and screened their ability to produce TPC.⁴⁵ They found that their TPC varied from 4.20 to 60.13 mg/g of dry weight. Also, they observed that the highest level of TPC was in the extract of *Chaetomium* sp. (60.13 mg) followed by *Aspergillus niger* (58.46 mg).⁴⁵ On the other side, in the present study the values of total phenolic concentrations varied from 15.5 to 204.5 mg/mL. Also, the highest level of TPC was in the ethyl acetate extract of *A. terreus* (204.5 mg/mL) followed by the ethyl extract of *F. solani* (177.66 mg/mL).

Total Flavonoid Content

The most important and widespread polyphenolic secondary metabolite in the plant kingdom is the flavonoids. The quantitative estimation of the total flavonoid revealed that all the selected endophytic fungi were known to produce flavonoid in varied quantities. In the present study, only 18 of the test crude fungal extracts from the 39 extracts had the

ability to produce flavonoids. While the results that obtained by several researchers such as Kumaresan et al⁵² reported that all tested endophytic fungi had the ability to produce flavonoids in varied quantity also Smith et al⁵³ examined 10 species of filamentous fungi for their antioxidant capacity and their ability for producing flavonoid compounds and Zohri et al⁴³ reported that The highest flavonoid producers (formed ≥ 35 mg/mL) were only 5 of tested fungal strains of endophytes (1), entomopathogens (1) and saprophytes (3). Finally, phenolics and flavonoids play an important role in stabilizing lipid peroxidation by their antioxidant activity.⁴⁸ The results of the present study thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. Furthermore, easier downstream processing of the fungal compounds as compared to phytochemicals offers hope for further development of chemotherapeutic agents as antioxidants are used as protective measures in various diseases. The study thus demonstrates that not only plants but also the fungi may be a good source of compounds having bioactive potential and these findings will facilitate further studies to gain better understanding of the production of bioactive metabolites in fungi, which may be helpful in their biotechnological mass production in the near future. The presence of such compounds with antioxidant activity could be useful in the prevention of diseases in which free radicals are implicated. If the physiological properties and the non-toxicity of the antioxidant compounds of the fungi are proven in vivo, these could be suggested as possible natural sources of antioxidants to prevent many free radical-mediated diseases and the health of consumers.

Conclusions

The present study revealed that endophyte fungi isolated from plants growing in extreme hot desert conditions are able to produce compounds having significant antioxidant activities. Phenolic and flavonoid compounds were recorded in the extracts of most tested fungal strains. Production of these compounds by fungi will be helpful in the biotechnological mass production of safe alternative sources of antioxidants. Furthermore, active crude extracts are being subjected to purification process for the identification of active compounds which may provide a better source for developing new pharmacological agents. These active natural compounds have a potential application as antioxidants and antimicrobial agents in many pharmaceutical products.

Authors' Contributions

MA, SE supervised the project, designed the research, wrote and revised the manuscript. DKh did experimental work, figures and shared writing the first draft. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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