Antioxidant potential of fungal isolates assayed through various procedures, screening of functional compounds and their purification from Aspergillus terreus

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**ABSTRACT**

Three fungal isolates (Aspergillus terreus 1, Aspergillus terreus 2 and Aspergillus fumigatus) were selected, to study their in vitro antioxidant potential by various assay procedures. Czapek dox’s medium was selected for the growth of fungi as it supported the best antioxidant activity based on their $EC_{50}$ values, Aspergillus terreus 1 was the best followed by Aspergillus terreus 2 and Aspergillus fumigatus. The chromatographic analyses showed several compounds possessing antioxidant activity in the fungal extracts. Two such compounds were partially purified from Aspergillus terreus 1 which demonstrated potent antioxidant activity, equally effective or better than some of the standard antioxidants.

**Keywords**, Antioxidant activity, Aspergillus fumigatus, Aspergillus terreus, Dot blot assay, fungi, HPLC

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**INTRODUCTION**

It is an undeniable fact that, worldwide, people are being threatened by more and more advent diseases such as cancer, cardiovascular disease, diabetes etc. Therefore, it is necessary to search new and beneficial compounds for better health care. Natural products with diverse chemical structures still remain the most important source for discovery of new and potent drug molecules [1]. Microorganisms including bacteria, fungi and actinomycetes are the potential sources of novel bioactive compounds for exploitation in medicine, agriculture, and industry. The fungal kingdom offers enormous biodiversity with an estimated 1.5 million species and of these over seventy thousand are known [2]. Fungi have contributed a lot for the welfare of mankind, since the beginning of civilization and are recognized as both beneficial and harmful to humans although the former is predominant. Recent studies have reported hundreds of natural products including alkaloids, terpenoids, flavonoids, steroids, etc. from fungi. Up to now, most of the natural products obtained from fungi are antibiotics, anticancer agents, biological control agents, and other bioactive compounds with different functional roles [3].

Natural products with antioxidant activity are used to provide protective system against free radicals [4]. Free radicals produced as a result of normal physiological processes in the body, cause oxidative damage, are implicated in contributing to cancer, atherosclerosis, aging, immunosuppression, inflammation, ischemic heart disease, diabetes, and neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease [5]. The antioxidants in human diets are of great interest as possible protective agents to help human body reduce oxidative damage. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [6]. Fungi isolated from soil were screened for antioxidant...
activity (data not shown) and some of these (Aspergillus terreus 1, Aspergillus terreus 2 and Aspergillus fumigatus) showing best results were selected to study their in vitro antioxidant potential.

Numerous techniques are available to evaluate the antioxidant activities of compounds and just one procedure cannot identify all possible mechanisms characterizing an antioxidant. Therefore, fungi were screened for their possible antioxidant potential by different complementary test systems, namely 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay, reducing power, ferrous ion and nitric oxide ion scavenging activity, ferric reducing antioxidant power (FRAP) assay.

MATERIALS AND METHODS

The fungal cultures were isolated from soil of different areas of Amritsar, Punjab, India (31° 37’ 59” North, 74° 51’ 56” East) and identified on the basis of standard protocols and the identity was confirmed by National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India. To study the antioxidant potential, the fungi were grown on 50 ml Czapek dox’s broth (sucrose 3%, NaNO₃ 0.2%, K₂HPO₄ 0.1%, MgSO₄ 0.05%, KCl 0.05%, FeSO₄ 0.001%; pH 7.0). The medium was inoculated with two discs (8mm) of fungal mycelia obtained from 6-7 days grown culture on Yeast extract glucose agar plates. After incubation under stationary conditions at 25°C for 10 days, the culture broth was filtered through Whatman filter paper no.1 and the filtrate so obtained was analyzed for antioxidant potential by different assay procedures and extracellular total phenolic content was estimated by Folin-Ciocalteau (FC) method.

Selection of different media
Different media viz Czapek dox’s medium, 2% malt extract, potato dextrose broth and yeast extract glucose broth were screened to work out the most suitable medium for the best antioxidant activity.

Estimation of half maximal effective concentration (EC₅₀) value
The extracellular fungal extracts were lyophilized. The lyophilized extracts were weighed and stock solutions of 4 mg/ml were made for each fungal extract which were further diluted to obtain 0.02, 0.05, 0.1, 0.3, 0.5, 1 mg/ml of concentrations. EC₅₀ represents the amount of sample (mg extract/ml) necessary to scavenge free radicals by 50%. EC₅₀ value is also the effective concentration at which the absorbance for reducing power is 0.5. Such EC₅₀ value was calculated from the graph plotting inhibition percentage against extract concentration [7].

Extraction with different organic solvents
To work out the best organic solvent for extraction of bioactive component, the culture broth was treated with different solvents viz petroleum ether, chloroform, ethyl acetate and butanol. Solvent extracted components were then evaporated to dryness in vacuo and the resulting solids were reconstituted in DMSO to get five times concentrated stock preparations which were then checked for their antioxidant potential by various assays.

Thin layer chromatographic analysis and screening of functional compounds in the ethyl acetate fungal extracts using Dot blot assay
Concentrated ethyl acetate extract (10 µl) showing the best antioxidant activity, was loaded onto TLC plates and dried at 80 °C for 10 min. which were then developed in the solvent system of chloroform and ethyl acetate (1:1). Chromatograms so developed were observed under UV light 254 and 365 nm and also observed in iodine chamber. Various separated spots were noted as their Rf values. The developed TLC plates were sprayed with the ethanolic solution of 0.4 mM DPPH. The active antioxidants compounds were detected as yellowish white bands produced with the conversion of unstable di phenyl hydrazine to stable di phenyl hydrazyl [8]. All detected active antioxidants constituents were noted according to their Rf values.

Extraction and purification of active components from ethyl acetate extract of Aspergillus terreus 1 showing antioxidant potential
For the extraction and purification of active antioxidant components from A. terreus 1, eight Erlenmeyer flasks (1000 ml), each containing 400 ml medium were inoculated with 16 discs (8mm) of fungal mycelia obtained from 6-7 days grown culture on Yeast extract glucose agar plates. After incubating these flasks for 10 days, under stationary conditions at 25° C, the culture broth was filtered through Whatman filter paper no.1 and the filtrate so obtained was pooled and analyzed for the antioxidant potential. Three litres of the culture broth was extracted thrice with equal volume of ethyl acetate (1:1). The ethyl acetate fraction was treated with Na₂SO₄ and then evaporated to dryness in
vaccuo and the resulting solids (10.7 g) were subjected to column chromatography using silica gel packed and pre-equilibrated with chloroform. The column was first eluted with equilibration solvent i.e. chloroform followed by linear gradients of chloroform to ethyl acetate at a flow rate of 1 ml/min. A total of 55 fractions were collected and the fraction size kept to 20 ml. Each fraction after concentration was subjected to thin layer chromatography and dot blot assay. Ethyl acetate, chloroform (1:1) was used as screening system to develop the chromatograms. Fractions which showed similar TLC pattern were pooled, concentrated and again loaded onto silica gel packed and pre-equilibrated with petroleum ether. The column after elution with petroleum ether was eluted with linear gradients of petroleum ether, Ethyl acetate at flow rate 1 ml/min and fraction size reduced to 5 ml each. The collected fractions were subjected to thin layer chromatography and antioxidant activity testing using dot blot and different assay procedures.

HPLC analysis
The partially purified fractions obtained from A. terreus 1 were evaporated to dryness using rotatory evaporator and dissolved in HPLC grade methanol. High-pressure liquid chromatography analyses were performed using a Dionex P680 HPLC. Acetonitrile (75% aqueous solution) was used as mobile phase at a flow rate of 0.3 ml/min and injection volume was 20 µl at a column temperature 25ºC. The detections were monitored at different wavelengths (λmax) i.e. 225, 250, 275 and 300 nm.

Comparison of antioxidant potential of isolated compounds with some of the known antioxidants
Antioxidant activity of isolated compounds at the same concentration (0.02, 0.05, 0.1, 0.3, 0.5, 1 mg/ml) was compared with the activity of known antioxidants (ascorbic acid and BHA) and some of their potent antioxidant phytochemicals (rutin and catechin). The data was analyzed by ANOVA.

Thermostability of antioxidant bioactivity
To check the temperature sensitivity of the isolated compounds for antioxidant activity, the compounds were subjected to 40°C, 60°C, 80°C, 100 ºC for two hour and were then assayed for the residual antioxidant activity.

Antioxidant activity
Assay procedures for antioxidant activity and determination of total phenolic content (TPC)
Different assay procedures for antioxidant activity was used as described earlier [9]. The total phenolic content was determined colorimetrically using the Folin-Ciocalt eau (FC) method according to Arora & Chandra, 2010 [9].

Toxicity tests
The extracts were tested for their mutagenic effect by using standard method of Ames test by using Salmonella reverse mutation test based on histidine dependence and mutations in S. typhimurium [10]. Cytotoxicity was tested by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The extracts (100 µl) were incubated with 1×10⁸ RBCs/well in 96-well ELISA plates for 24 hours. Then 100 µl MTT solution (0.5 %, w/v) was added to each well and incubated further for 4 h. After incubation, the supernatant was removed and 100 µl DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 590 nm using an automated microplate reader. The wells with untreated cells served as control [11].

RESULTS

Various basal media were screened to find out their suitability for the best expression of antioxidant activity. Czapek dox’s medium proved to be the most suitable for antioxidant activity for all the three fungi. Similar profile for their antioxidant activity was observed as assayed by different procedures. A. terreus 1 gave the highest activity as compared to A. terreus 2 and A. fumigatus (Fig.1). The DPPH scavenging rate was 85.2%, 68.2% and 68.9% for A. terreus 1, A. terreus 2 and A. fumigatus, respectively. The reducing potential also showed the same profile. Scavenging effect for ferrous (68.3%) and NO ion (64.2%) remained best for A. terreus 1 followed by A. terreus 2 and A. fumigatus. Czapek dox’s medium remained best for the three fungi to produce high content of extracellular TPC followed by yeast extract broth, malt extract and potato dextrose broth. The extracellular TPC obtained in Czapek dox’s medium was 16.75, 13.05 and 5.68 mg/ml in A. terreus 1, A. terreus 2 and A. fumigatus, respectively (Fig.2). Based on above observations, Czapek dox’s medium was used for all the further studies.
Antioxidant activity of lyophilized fungal extracts
The stock solution of lyophilized extracellular extracts at a concentration of 2 mg/ml of all the fungi was assayed for antioxidant activity. The maximum inhibition for DPPH radical was 88.0 % for *A. terreus* 1, closely followed by *A. terreus* 2 (74.6%) and *A. fumigatus* (65.4 %). The reducing potential of 2.1, 1.3 and 0.75 was observed in *A. terreus* 1, *A. terreus* 2 and *A. fumigatus*, respectively. The results for FRAP assay revealed that ferric ion reduction capacity was highest (72.3%) in *A. terreus* 1 followed by *A. terreus* 2 (61.2%) and *A. fumigatus* (58.4%). The scavenging effect for ferrous (76.3%) and NO ion (78.2%) was again best in the extract of *A. terreus* 1.

Total phenolic contents (TPC)
The total phenolic contents of the lyophilized extracellular fungal extracts have been expressed as gallic acid equivalent (GAE) i.e. mg gallic acid /g lyophilized culture. *A. terreus* 1 possessed highest TPC (25 mg/g) followed by *A. terreus* 2 (18 mg/g) and *A. fumigatus* (10 mg/g). The TPC positively correlated with their antioxidant potential assayed through various procedures.

EC$_{50}$ value of lyophilized fungal extracts against different free radicals
In order to calculate the EC$_{50}$ value against different free radicals, antioxidant activity was estimated at different concentrations. An increase in activity was observed with the increase in concentration of the extract irrespective of the assay procedure used (Fig. 3-7). The EC$_{50}$ values of extracts of *A. terreus* 1, *A. terreus* 2 and *A. fumigatus* were 0.08, 0.28, 0.9 mg/ml for both DPPH ion scavenging activity and reducing potential. Again, their corresponding EC$_{50}$ values were similar for ferric ion reduction capacity and ferrous ion scavenging potential and are 0.1, 1.0, 1.0 mg/ml, respectively. The EC$_{50}$ values of extracts of *A. terreus* 1, *A. terreus* 2 and *A. fumigatus* against nitric oxide ion were 0.06, 0.4, 1.0 mg/ml, respectively.

Effect of different organic solvents
The extraction of culture broth with different solvents revealed ethyl acetate to be the best to elute the components responsible for antioxidant potential that was followed by chloroform and butanol extract (Fig 8). Extracts obtained from petroleum ether did not show any activity.

Thin layer chromatographic analysis and screening of functional compounds in the fungal extracts using Dot blot assay
Ethyl acetate extracts of all the three fungi showing the best result were subjected to TLC and the dot blot assay. The TLC of the extract of *A. terreus* 1 resolved into 5 bands with Rf value of 0.16, 0.34, 0.48, 0.53 and 0.8 among which all the bands showed positive antioxidant activity, except one with Rf value 0.48. All the four bands in the TLC of the extract of *A. terreus* 2 showed positive results for antioxidant activity with respective Rf value of 0.25, 0.30, 0.41 and 0.68. Similarly, all the five bands observed in the TLC of the extract of *A. fumigatus* were positive for antioxidant activity.

Purification of the active compounds
The fractions obtained from the column chromatography were subjected to TLC and the fractions having same bands were pooled. Two compounds were obtained, one with the Rf value 0.34 was named as C1 (64 mg) and compound with Rf value 0.53 was named as C2 (40 mg). Both the fractions of C1 and C2 were subjected to HPLC analysis. The single peak of the C1 and C2 indicates the purification of the compounds. The retention time for C1 and C2 were 2.1 and 4.2 min, respectively. The partially purified compounds obtained were analyzed for their antioxidant potential by various assay procedures and the results revealed both the compounds to be potent antioxidants.

Antioxidant activity of isolated compounds (C1 and C2) and their comparison with known antioxidants
The isolated compounds from *A. terreus* 1 at a concentration of 0.5 mg/ml demonstrated equal or higher activity than standard antioxidants tested at the same concentration (Fig. 3-7). The compound C1 was better than standard antioxidants except ferrous ion scavenging rate where ascorbic acid was better. On the other hand, C2 showed comparable activity with standard antioxidants (Table 1).

Both the purified compounds showed a good DPPH scavenging effect. C1 (95.4 %) was significantly ($p <$0.05) stronger than C2 (90.1%). Activity shown by the compounds was better/comparable to standard antioxidants, such as ascorbic acid (91.6%); BHT (84.8%); catechin (72.8%) and rutin (70.3%). C1 (2.7) and C2 (1.9) exhibited strong reducing potential than standard antioxidants. Similarly, both the compounds demonstrated effective ferric ion scavenging effect.
reduction than standard antioxidants. C1 was more effective than C2 and gave reduction rate of 78.6% and 75.1%, respectively and C1 remained best among all other standard antioxidants.

The chelating activity for ferrous ion was also good with 80.2% and 78.2% for C1 and C2, respectively but was lesser than ascorbic acid and significantly equal to BHT ($p < 0.05$). C1 again retained the supremacy even against nitric oxide ion. The activity of ascorbic acid and C2 was significantly equal while other standard antioxidant demonstrated less activity (Table 1).

**Thermostability of the isolated compounds**

The isolated compounds were found to be relatively thermostable as they suffered a slight loss in their antioxidant activity with increase in temperature. After 2 hrs at 40°C the activity decreased by only 6%, while at 100°C it suffered a maximum loss of 38% in their activity.

**Toxicity testing**

The cell free fungal extracts and isolated compounds showed no mutagenicity as no bacterial colony was observed on agar plates containing fungal extracts, while more than 1000 colonies were observed on positive control (sodium azide) containing plate. Similarly, results obtained from MTT assay revealed that the cell free extracts and isolated compounds were non cytotoxic.

**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 [12]. 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 [13]. They are known to produce several novel metabolites possessing antioxidant activity which are equally potent as synthetic antioxidants and phytochemicals. *Chaetomium sp.*, *Cladosporium sp.*, *Torula sp.*, *Phoma sp* and *Penicillium roqueforti* produces 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 [14,15]. Gebhardt et al. [16] reported anti-inflammatory and antioxidant activity of quercinol obtained from *Daedalea quercina*.

In the present study, all the three isolates showed good antioxidant activity against various free radicals. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging [17]. The results obtained from various assay procedures prove the potent broad spectrum antioxidant activity of the extracts obtained from the three *Aspergillus* spp. and the compounds purified from *A. terreus* 1. The activity was higher than many other fungi, plants and mushrooms [14,15,18].
Figure 4. Reducing potential of lyophilized fungal extracts, purified compounds and standard antioxidants at different concentrations.

(C1, compound 1; C2, compound 2; A.A, ascorbic acid; BHT, butylated hydroxytoluene)

Figure 5. Ferrous ion scavenging activity of lyophilized fungal extracts, purified compounds and standard antioxidants at different concentrations.

(C1, compound 1; C2, compound 2; A.A, ascorbic acid; BHT, butylated hydroxytoluene)
Figure 6. Ferric ion reducing antioxidant power of lyophilized fungal extracts, purified compounds and standard antioxidants at different concentrations.
(C1, compound 1; C2, compound 2; A.A, ascorbic acid; BHT, butylated hydroxytoluene)

Figure 7. Nitric oxide ion (NO) scavenging activity of lyophilized fungal extracts, purified compounds and standard antioxidants at different concentrations.
(C1, compound 1; C2, compound 2; A.A, ascorbic acid; BHT, butylated hydroxytoluene)
Figure 8. DPPH free radical scavenging activity in different solvent extracts of all fungi.
*Chl ex., chloroform extract; Eth ac, ethyl acetate extract; But ex, butanol extract*

Table 1. Antioxidant activity of purified compounds and their comparison with standard antioxidants at concentration of 0.5 mg/ml.

Superscript (a,b,c,d,e) shows statistical significant difference (p<0.05)

(C1, compound 1; C2, compound 2; AA, ascorbic acid; BHT, butylated hydroxytoluene)

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