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In Vitro Antioxidant Potential of Some Soil Fungi: Screening of Functional Compounds and their Purification from *Penicillium citrinum*

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Abstract Fungal isolates (*Aspergillus wentii* 1, *A. wentii* 2, *Penicillium citrinum*, *Penicillium granulatum*) 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, *P. citrinum* was the best followed by *P. granulatum*, *A. wentii* 1, and *A. wentii* 2. The chromatographic analyses showed several compounds possessing antioxidant activity in the fungal extracts. Two such compounds were partially purified from *P. citrinum* which demonstrated potent antioxidant activity, equally effective or better than some of the standard antioxidants.

Keywords Antioxidant activity · Dot blot assay · Fungi · HPLC · *Aspergillus wentii* · *Penicillium citrinum* · *Penicillium granulatum*

Introduction

Free radicals are highly reactive, short-lived, toxic molecules that have one or more unpaired electrons and can damage DNA, proteins, lipids, and carbohydrates within the tissue, leading to many common diseases like atherosclerosis, cancer, and many others [1]. Examples of oxygen-centered free radicals, known as reactive oxygen species (ROS), include superoxide (O_2^{-}), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), hydroxyl ($HO^{\cdot}$), and nitric oxide ($NO^{\cdot}$). The hydroxyl (half-life of 10^{-9} s) and the alkoxyl (half-life of seconds) free radicals are very reactive and rapidly attack the molecules in nearby cells, and probably the damage caused by them is unavoidable and is dealt with by repair processes. On the other hand, the superoxide anion, lipid hydroperoxides, and nitric oxide react with free radicals to form other ROS nonradicals, such as the singlet oxygen ($_1O^2$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) [2].

Antioxidants are the molecules, which prevent cellular damage by reducing the oxidative stress and therefore have a beneficial effect on human health [3]. The main cause of

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mortality and morbidity in the world is atherosclerosis, the accumulation of oxysterol, cholesterol, and peroxide lipids in arteries, generated by free radicals which lead to heart attack. Hence, there has been an increased interest in the application of antioxidants [4]. The effect of dietary antioxidants on the development of human atherosclerosis is also controversial and a number of contradictory examples have been published. Most of the research on the role of antioxidants in cardiovascular diseases has focused on testing pure compound to prevent lipid peroxidation by examining its ability to scavenge free radicals. However, in vivo, the contribution of antioxidants goes far beyond scavenging free radicals. Moreover, antioxidants are usually not present alone in biological systems but act in combination with other antioxidants. Hence, the protective effect of a diet is not equivalent to the protective effect of antioxidants in it [5].

Natural antioxidants, i.e., flavonoids, tannins, coumarins, curcuminoids, xanthones, phenolics, and terpenoids are found in various fungi and plant products; such as fruits, leaves, seeds, and oils. Phenolics belong to a heterogeneous group of compounds with a great variety of biological effects including anti-inflammatory, anti-microbial, and antioxidant properties [6].

The objective of the study was to evaluate the antioxidant potential of different soil fungi, to find out the best and to further screen out the compound/s responsible for the antioxidant activity. 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 tested 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, and 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 MTCC, IMTECH, Chandigarh and 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%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05%, $FeSO_4$ 0.001%; pH 7.0). The medium was inoculated with two disks (8 mm) of fungal mycelia obtained from 6 to 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 used for the analysis of antioxidant potential by different assay procedures and extracellular total phenolic content was estimated by Folin–Ciocalteau (FC) method.

Selection of Different Media

At initial screening to work out the best suitable media, all the four fungi were grown on Czapek–Dox's medium, 2% malt extract, potato dextrose broth, and yeast extract glucose broth and their culture filtrate were tested to assay their antioxidant potential.

Estimation of Half Maximal Effective Concentration Value

On the basis of above experimentation, all the four fungi were grown on Czapek–Dox's medium and to estimate half maximal effective concentration (EC_{50}) value the extracellular

fungal extracts were lyophilized. The lyophilized extracts were weighed and stock solutions of 2 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. EC_{50} represents the amount of sample (milligram extract per milliliter) necessary to scavenge free radicals by 50%. EC_{50} value is also the effective concentration at which the absorbance for reducing power is 0.5. Such EC_{50} 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 methanol 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 best antioxidant activity as obtained above was loaded onto thin layer chromatography (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 evaluated for their Rf values [8]. All detected active antioxidants constituents were noted according to their Rf values.

Extraction and purification of active components from ethyl acetate extract of *Penicillium citrinum*

For the extraction and purification of active antioxidant components from *P. citrinum*, eight Erlenmeyer flasks (1,000 ml), each containing 400 ml medium were inoculated with 16 disks (8 mm) of fungal mycelia obtained from 6 to 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 liters of the culture broth was extracted thrice with equal volume of ethyl acetate (1:1). The ethyl acetate fraction was treated with Na_2SO_4 and then evaporated to dryness in vacuo and the resulting solids (9.7 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 (100:0, 90:10, 80:20, 70:30, 60:40,50:50, 40:60, 30:70, 20:80, 10:90, 0:100) 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 (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) 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 other quantitative assay procedures.

HPLC Analysis

The partially purified fractions obtained from *P. citrinum* were evaporated to dryness using a rotary evaporator and dissolved in high-pressure liquid chromatography (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 0.3 ml/min and injection volume was 20 μl at a column temperature 25 °C. 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, rutin, and catechin).

Thermostability of Antioxidant Bioactivity

To check the temperature sensitivity of the isolated compounds for antioxidant activity, the compounds were subjected to 40, 60, 80, 100 °C for 2 h and the heat-treated isolated compounds were then assayed for the residual antioxidant activity.

Antioxidant Activity

Quantitative Assay for DPPH Free-Radical Scavenging Activity

The scavenging activity for DPPH free radicals was measured according to Zhao et al. [9]. An aliquot of 1 ml of 0.1 mM DPPH solution in ethanol and 0.5 ml of extract was mixed. The reaction mixture was shaken vigorously and allowed to reach a steady state at 37 °C for 30 min. Decolorization of DPPH was determined by measuring the decrease in absorbance at 517 nm and the DPPH radical scavenging effect was calculated according to the following equation:

$$\%\ \text{scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100$$

where, A_0 is the absorbance of the control, A_1 is the absorbance of extract, and A_2 is the absorbance without DPPH.

Determination of Antioxidant Activity by Reducing Power Measurement

The reducing power of the extracts was determined according to Chang et al. [10]. An aliquot of 0.5 ml extract was added to 0.1 ml of 1% (*w*/*v*) potassium ferricyanide. After incubating the mixture at 50 °C for 30 min, it was supplemented with 0.1 ml of 1% (*w*/*v*) trichloroacetic acid and 0.1% (*w*/*v*) $FeCl_3$, and left for 20 min. Absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicates higher reducing power of the sample.

Determination of Antioxidant Activity by FRAP Assay

FRAP assay was carried out according to Othman et al. [11]. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM ferric chloride in a ratio of 10:1:1. For FRAP assay, the reaction mixture containing 2 ml of FRAP reagent, 0.5 ml of extract, and 1 ml of distilled water was incubated for 10 min and the absorbance measured at 593 nm. Antioxidant potential of the sample was compared with the activity of 0.5 ml stock solution of 1 mg/ml $FeSO_4$.

Determination of Ferrous Ion Scavenging (Metal Chelating) Activity

The chelating activity of the extracts for ferrous ions was measured according to Zhao et al. [9]. The reaction mixture containing 0.5 ml of extract, 1.6 ml of deionized water, 0.05 ml of $FeCl_2$ (2 mM), and 0.1 ml of ferrozine (5 mM) was incubated at 40 °C for 10 min and the absorbance measured at 562 nm. The chelating activity was calculated as

$$\text{Chelating rate} = [1 - (A_1 - A_2)/A_0] \times 10$$

where, A_0 represents the absorbance of the control, A_1 represents the absorbance of extract, and A_2 represents the absorbance without $FeCl_2$.

Determination of NO Scavenging Activity

Nitric oxide production from sodium nitroprusside was measured according to Kang et al. [12]. An equal amount of sodium nitroprusside (5 mM) solution and extract was mixed and incubated at 25 °C for 2.5 h. After every 0.5 h, 0.5 ml of the reaction mixture was mixed with an equal amount of Griess reagent (1% (*w*/*v*) sulfanilamide, 2% (*w*/*v*) phosphoric acid, and 0.1% (*w*/*v*) naphthylethylene diamine dihydrochloride), and absorbance was taken at 546 nm and compared with absorbance of standard solution (1 mg/ml sodium nitrite) treated in the same way with Griess reagent.

Determination of Total Phenolic Contents

The total phenolic contents (TPC) were determined colorimetrically using the FC method according to Singleton et al. [13] with slight modifications. Test sample (0.5 ml) was mixed with 0.2 ml of FC reagent and allowed to stand for 10 min to which 0.6 ml of 20% (*w*/*v*) sodium carbonate was added and mixed completely. The reaction mixture was incubated at 40 °C for 30 min. Absorbance of the reaction mixture was measured at 765 nm. Gallic acid was used as standard.

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 *Salmonella typhimurium* [14]. 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 1×10^5 RBCs/well in 96-well ELISA plates for 24 h. 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 [15].

Results

Antioxidant Activity Assayed by Different Procedures in Different Growth Media

Various basal media were screened to find out their suitability for the fungal growth and best expression of antioxidant activity. Czapek–Dox's medium proved to be the most suitable for antioxidant activity for all the fungi. *P. citrinum* gave the highest antioxidant activity as compared to *Penicillium granulatum*, *Aspergillus wentii* 1, and *A. wentii* 2 (Fig. 1). The DPPH scavenging rate was 78.5%, 72.1%, 72.01%, and 62.9% for *P. citrinum*, *P. granulatum*, *A. wentii* 1, and *A. wentii* 2, respectively. The reducing potential also showed the same profile. Scavenging effect for ferrous (60.2%) and NO ion (64.2%) remained the best for *P. citrinum* followed by *P. granulatum*, *A. wentii* 1, and *A. wentii* 2. Czapek–Dox's medium remained best for all the 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 20.6, 12.1, 12.03, and 7.2 mg/ml in *P. citrinum*, *A. wentii* 1, *A. wentii* 2, and *P. granulatum*, respectively (Fig. 2). Based on the above observations, Czapek–Dox's medium was used for all further studies.

Antioxidant Activity of Lyophilized Extracellular 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 80.1% for *P. citrinum* followed by *P. granulatum* (74.8%), *A. wentii* 1 (72.1%), and *A. wentii* 2 (70.4%). The reducing potential of 2.0, 1.1, 1.04, and 1.02 was observed in *P. citrinum*, *P. granulatum*, *A. wentii* 1, and *A. wentii* 2, respectively. The results for FRAP assay revealed that ferric ion reduction capacity was highest (73.1%) in *P. citrinum* followed by *P. granulatum* (65.1%), *A. wentii* 1 (62.7%), and *A. wentii* 2 (61.8%). The scavenging effect for ferrous (73.1%) and NO ion (75.2%) was again best in the extract of *P. citrinum*.

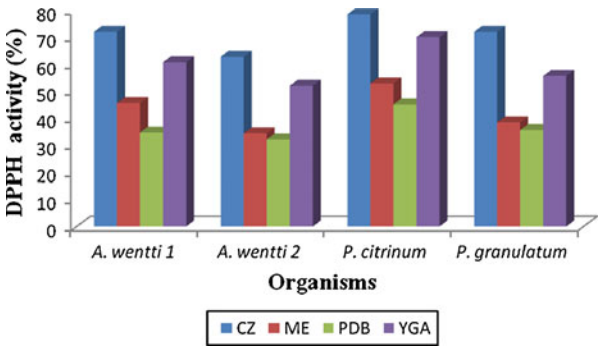

Fig. 1 DPPH scavenging activity of fungi grown on different media; *CZ* Czapek–Dox's, *ME* malt extract, *PDB* potato dextrose broth medium, *YGA* yeast extract glucose medium

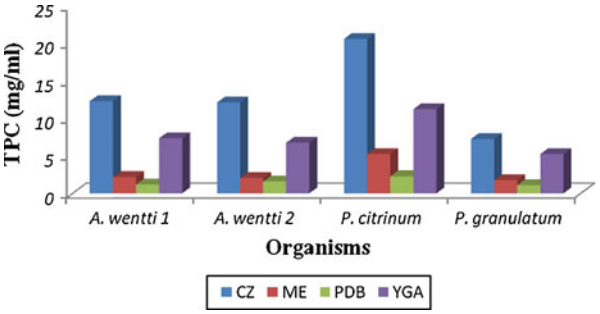

Fig. 2 Total phenolic content of fungi produced extracellularly in different media; *CZ* Czapek–Dox's, *ME* malt extract, *PDB* potato dextrose broth medium, *YGA* yeast extract glucose medium

Total Phenolic Contents

The total phenolic contents of the lyophilized extracellular fungal extracts have been expressed as gallic acid equivalent, i.e., milligram gallic acid/gram lyophilized culture. *P. citrinum* produced maximum TPC (28 mg/g) followed by *A. wentii* 1 (18.6 mg/g), *A. wentii* 2 (18.1 mg/g), and *P. granulatum* (15.3 mg/g). The TPC of different fungi positively correlated with their antioxidant potential assayed through various procedures.

EC_{50} Value of Lyophilized Extracellular 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. The results indicate the increase in activity with the increase in concentration of the extract irrespective of the assay procedure used (Fig. 3, 4, 5, 6, and 7). The EC_{50} values of extracts of *P. citrinum*, *P. granulatum*, *A. wentii* 1, and *A. wentii* 2 were 0.1, 0.12, 0.3, and 0.32 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, 0.4, 0.8, and 0.8 mg/ml, respectively. The EC_{50} values of extracts of *P. citrinum*, *P. granulatum*, *A. wentii* 1, and *A. wentii* 2 against nitric oxide ion were 0.08, 0.32, 0.8, and 0.8 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.

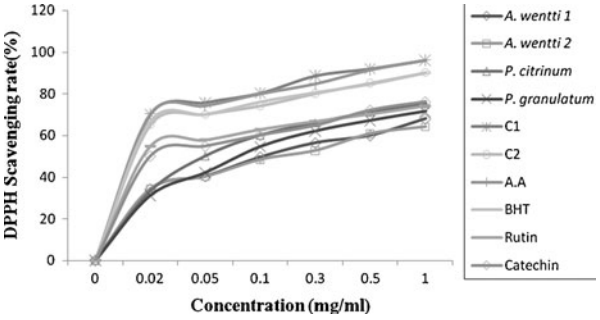

Fig. 3 DPPH scavenging activity shown by fungal lyophilized extract, purified compounds and standard antioxidants at different concentrations (*C1* compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene)

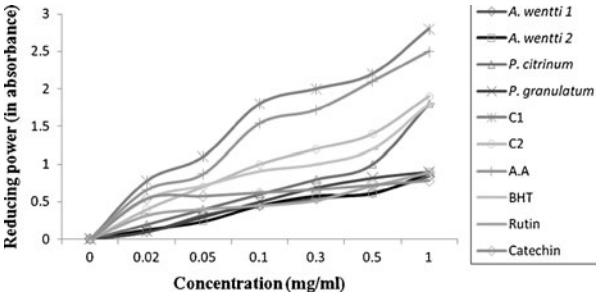

Fig. 4 Reducing potential shown by fungal lyophilized extract, purified compounds and standard antioxidants at different concentrations (*C1* compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene)

Thin Layer Chromatographic Analysis and Screening of Functional Compounds in the Fungal Extracts Using Dot Blot Assay

Ethyl acetate extracts of all the fungi showing the best result were subjected to TLC and the dot blot assay. The chromatograms of *P. citrinum* resolved into three bands with Rf value of 0.25, 0.40, and 0.55 among which Rf value of 0.25 and 0.55 bands showed positive antioxidant activity screened on the basis of dot blot assay. Seven different bands were observed in the chromatograms of *A. wentii* 1 out of which five showed positive results for antioxidant activity with respective Rf value of 0.22, 0.42, 0.68, 0.78, and 0.84. Similarly, all the three bands observed in the chromatograms of *P. granulatum* were positive for antioxidant activity while only two out of five were positive chromatograms of *A. wentii* 2.

Purification of the Active Compounds

P. citrinum showed the best antioxidant activity among all the fungi; hence, subjected to column chromatography for the purification of compounds responsible for the activity. 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 (58 mg) and compound with Rf value 0.53 was named as C2 (42 mg). Both the fractions 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 2.0 and 4.5 min, respectively. The partially purified compounds obtained were subjected to various assay procedures and the results revealed both the compounds to be potent antioxidants.

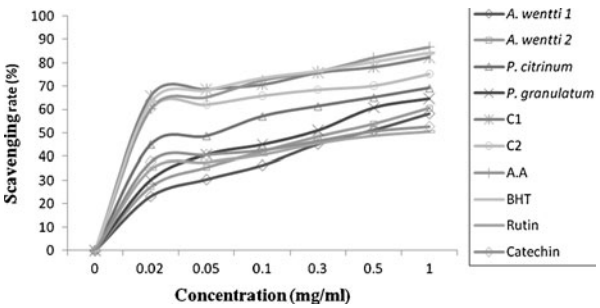

Fig. 5 Ferrous ion scavenging activity shown by fungal lyophilized extract, purified compounds and standard antioxidants at different concentrations (*C1* compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene)

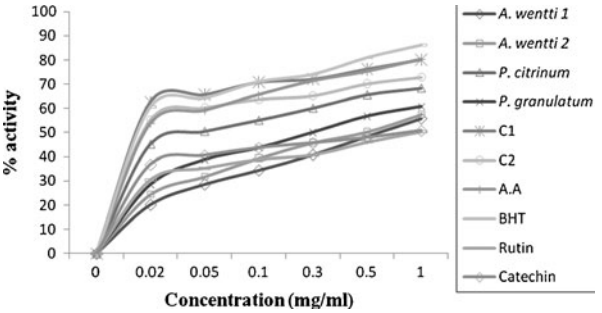

Fig. 6 Results of FRAP assay shown by fungal lyophilized extract, purified compounds and standard antioxidants at different concentrations (*C1* compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene)

Antioxidant Activity of Isolated Compounds (C1 and C2) and their Comparison with Known Antioxidants

The isolated compounds from *P. citrinum* at a concentration of 0.5 mg/ml demonstrated equal or higher activity than standard antioxidants tested at the same concentration (Fig. 3, 4, 5, 6, and 7). The compound C1 was better than all the standard antioxidants. 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 with standard antioxidants, such as ascorbic acid (91.6%), butylated hydroxytoluene (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 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. C1 again retained the supremacy even against nitric oxide ion.

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 h at 40°C the activity decreased by only 5%, while at 100°C it suffered a maximum loss of 35% in their activity.

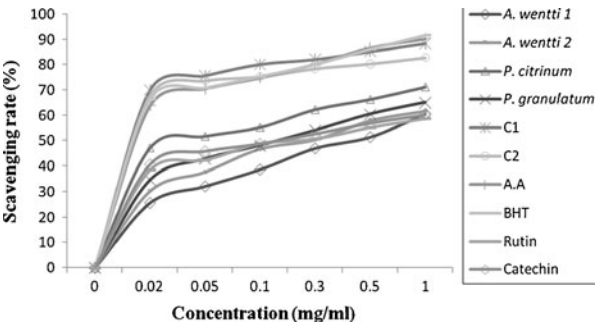

Fig. 7 NO scavenging activity shown by fungal lyophilized extract, purified compounds and standard antioxidants at different concentrations (*C1* compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene)

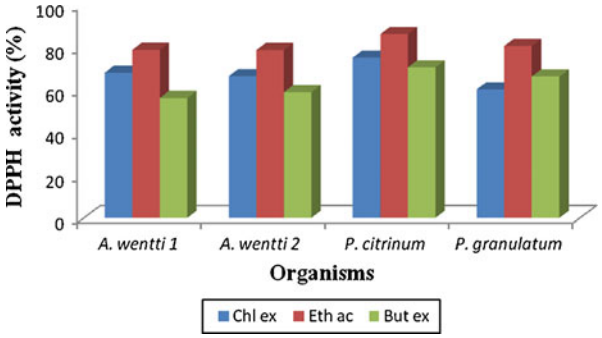

Fig. 8 DPPH scavenging activity of fungal extracts in different solvents. *Chl ex* chloroform extract, *Eth ac* ethyl acetate extract, *But ex* butanol extract

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 1,000 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 noncytotoxic.

Discussion

Number of antioxidants is 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 [16]. 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 [17–19]. They are known to produce several novel metabolites possessing antioxidant activity and are equally potent as synthetic antioxidants and phytochemicals. *Chaetomium* sp., *Cladosporium* sp., *Torula* sp., *Phoma* sp., and *Penicillium roquefortti* produce various secondary metabolites like phenolic acid derivatives, terpenoids, benzoic acid, rutin with antioxidant activity, and also a wide range

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

	C1	C2	AA	BHT	Rutin	Catechin
DPPH assay	96.1	90.1	91.6	84.8	72.8	70.3
Reducing power	2.5	1.9	2.1	1.2	0.714	0.72
Fe^{2+} scavenging activity	82.3	75.2	82.1	80.41	50.8	48.9
FRAP assay	80.9	72.8	75.3	75.2	47.9	45.9
NO scavenging activity	88.4	82.7	86.6	86.2	56.9	55.2

C1 compound 1, *C2* compound 2, *AA* ascorbic acid, *BHT* butylated hydroxytoluene

of other biological activities such as antibacterial, antiviral, antimutagenic, and immunomodulatory [18]. Gebhardt et al. [20] 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 are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging [21]. The results obtained 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 *P. citrinum*. The activity was higher than many other already reported fungi, plants, and mushrooms [18, 19, 22].

Antioxidant activity of the fungi towards DPPH free radicals may be attributed to their hydrogen-donating ability. DPPH is an unstable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [23]. The extracts and purified compounds showed good scavenging activity against DPPH radicals. 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 [24]. Ferrozine can quantitatively form complexes with Fe^{2+}. 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 peroxyl and alkoxyl radicals that can themselves abstract hydrogen and stop the chain reaction of lipid peroxidation [25].

Phenolic compounds are well-known antioxidant constituents because of their high ability to scavenge toxic free radicals and reactive oxygen species [26]. 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 [27]. The TLC analyses demonstrated the presence of different compounds possessing antioxidant activity which is based on developing of TLC plates stained with DPPH solution. This procedure is used for the rapid preliminary observation of antioxidant constituents present in any extract. Using this procedure, compounds with antioxidant activity turned out to be yellow in the purple background of the plate. The intensity of the yellow color depends on the amount and nature of radical scavengers present in the extract [28].

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 the fungi to be the good sources of antioxidants [29, 30]. Various compounds with antioxidant potential isolated from *Mycelia sterilia*, *Colletotrichum gloesosporioides*, *Pestalotiopsis microspora*, and *Aspergillus candidus* showed comparable/higher activity than various known antioxidants [31–33]. The present study get further credence as the isolated compounds

did not show any cytotoxicity or mutagenicity in consonance with earlier studies where some of the isolated compounds were neither cytotoxic nor genotoxic towards human intestine 407 cells and nonmutagenic towards *S. typhimurium* TA98 and TA100 [34, 35]. The results of the present study thus endorse the future prospects for the commercial production of natural and safer antioxidant compounds from such fungi. Further, easier downstream processing of the fungal compounds as compared to phytochemicals offers a ray of hope for further development of chemotherapeutic agents as antioxidants are used as protective measure 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 the further studies to gain better understanding of the production of bioactive metabolites in fungi, which may be helpful in their biotechnological mass production in 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 positive physiological properties and the nontoxicity of the antioxidant compounds of the fungi is 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.

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