Antioxidant activity of ethanolic extract of *Penicillium chrysogenum* and *Penicillium fumiculosum*

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Abstract

The aim of this study was to investigate the biological and chemical activity of the two fungi species, genus *Penicillium*, isolated from wastewater. For the selected species of fungi, different antioxidant activity assays were used: DPPH free-radical scavenging activity, total antioxidant activity, Fe^{2+}-chelating ability and Fe^{3+}-reducing power. Total phenolic content was also determined for ethanolic extract of mycelia. *Penicillium chrysogenum* ethanolic extract contained higher total phenolic content and better total antioxidant capacity as well as ferrous ion chelating ability. *Penicillium fumiculosum* ethanolic extract showed higher DPPH free-radical scavenging activity, as well as reducing power. Based on the obtained results it can be concluded that two types of fungi are potential new sources of natural antioxidants.

Keywords: DPPH free-radical scavenging, ferrous ion chelating ability, reducing power, total antioxidant activity, total phenols, *Penicillium*.

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Numerous pharmaceutical properties of medicinal mushrooms that have been used in the traditional oriental medicine are known, including anticancer, antimicrobial, anti-inflammatory and anti-atherosclerotic. In western civilization, the research on medicinal properties of fungi and yeast are relatively new as well as their use for therapeutic purposes. These fungi are a significant source of natural antioxidants due to their production of secondary metabolites. These are compounds such as polysaccharides, triterpenes, and triterpenoids, various acids (*e.g.*, ganodermic acid), β-glucan, vitamins and alkaloids. Phenolics or polyphenols, including flavonoids are the main secondary metabolites of medicinal plants, mushrooms and fungi, responsible for their antioxidant, antimutagenic and antitumor activity [1,2]. Screening of biological activity of endophytic fungi showed that they represent a significant source of new bioactive agents with potential use in medicine, agriculture and industry area. Biologically active ingredients are synthesized in the apex tissue of the hyphal strand of fungi and their extractions are carried out by solvents with different polarity. The biological activity of these substances depends on their chemical structure so that different extraction solvents resulted in various biologically active substances, with different levels of bioactivity. Ethyl acetate extract of endophytic fungi *Xylariaceae sp., Tolypocladium sp., Chaetomium glotosum, Chaetomium sp., Creosphaeria sp.*, contain an extraordinary antioxidant activity [3]. The aqueous extract of mycelium *Tolypocladium sp*. Ts-1 isolated from the fruiting body of a wild *Cordyceps sinensis,* has strong antioxidant activity and is a potential source of natural antioxidants [4]. The methanolic extracts of the fungi *Fusarium, Aspergillus, Penicillium* and *Mucor* species isolated from *L. nicotianifolia* showed significant antioxidant potential and the antioxidant nature of the extracts depended on the concentration [5]. Many species of fungi isolated from the soil, such as *Aspergillus fumigatus*, represent a potential source of natural antioxidants [6]. As active participants in the degradation of organic materials, primarily wood waste, fungi are exposed to large amounts of free radicals in nature. In order to survive and perform their task as scavengers in nature, fungi have developed specific defense mechanisms against a variety of toxins and free radicals.

The fungi species of genus *Penicillium* are very attractive organisms for production of useful protein and biologically active secondary metabolites. It was found that fungi produce pigments that inhibit the cholesterol biosynthesis by binding to the catalytic site of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis [7] and scavenged DPPH radicals [8–10]. Penicillenols secreted by *Penicillium* sp showed biological activity against HL-60 cell lines [11]. Atrovenetin was isolated as a potential antioxidant in some species of *Penicillium* [12]. Different active substances were

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isolated from *P. chrysogenum,* like alkaloids, carbohydrates, tannins and terpenoids by using different solvents.

The potential antioxidant activity of two fungal species: *Penicillium chrysogenum* and *Penicillium fumiculosum* was investigated in this study. The fungi were isolated from wastewater of the river basin of Lepenica and Western Morava, Serbia. Total phenolic content was determined by using Folin–Ciocalteu method. The antioxidant activity of alcoholic extract of fermentation broth was carried out by four assays: DPPH free - radical scavenging activity, total antioxidant activity, Fe^{2+}-chelating ability and Fe^{3+}-reducing power.

EXPERIMENTAL

Cultivation and extraction of tested fungal mycelia

The fungal species used in our study were isolated from the wastewaters originating from households, that were flowed directly into the riverbed of the Lepenica and Western Morava River (Serbia). The identification of fungi was carried out at the Institute of Biology and Ecology of Kragujevac, and later became part of our laboratory collection. Fungi were grown on potato dextrose agar at room temperature (28±1 °C) for 7 days. The 250 mL erlenmeyer flask, containing 100 mL of liquid PDB medium was sterilized for 15 min at 121 °C. The media was inoculated with 1 mL of spore suspension to yield specific density and incubated at room temperature for 5 days, with occasional stirring. After the completion of incubation, mycelia were separated from the liquid medium by filtration and drying at 50 °C. Dried mycelium was pulverized and extracted with ethanol (1:1, v/v) three times. The supernatant was separated by centrifugation at 5000 rpm for 10 min, fractions were pooled and ethanolic extract was concentrated under reduced pressure conditions to yield the final extract. Alcoholic extracts of all tested fungal species were stored in dark at 4 °C before being used for the bioactivity test.

DPPH radical scavenging assay

1,1-Diphenyl-2-picrylhydracyl (DPPH) radical scavenging activity was done according to the method by Takao *et al.,* with slight modification [13]. Working solution of extracts was made by diluting stock solution (1 mg/mL). DPPH was dissolved in methanol to obtain the concentration of 8 μg/mL. To 1 mL of DPPH solution, 1 mL of various concentrations of the extracts or the standard (ascorbic acid) solution were added separately. The reaction mixtures were incubated at 37 °C for 30 min, followed by measuring absorbance at 517 nm using pure methanol as blank reference. The DPPH scavenging activity (%) of the standard and extracts was determined using following equation:

$$\text{Inhibition} = [(Ac–As)/Ac]\times 100 \quad (1)$$

where: *Ac* is absorbance of the control sample and As is the absorbance of the tested sample.

Total antioxidant activity

The total antioxidant activity was determined by phosphomolybdenum method according to Prieto *et. al.,* [14]. To 1 mL of samples or standard 2 mL of reagent solution (ammonium molybdate, 4 mM, sodium phosphate ,28 mM and sulphuric acid, 0.6 M) was added and mixed vigorously. All the reaction tubes were incubated at 95 °C for 90 min. The absorbance was measured at 695 nm against blank (methanol) after cooling to room temperature. Ascorbic acid was used as standard. Reducing capacity of the extract has been expressed as the ascorbic acid equivalents.

Total phenolic contents

The total phenolic contents in the extract were determined according to the Folin–Ciocalteu method of Singleton and Rossi with some modifications [15,16]. To 1 mL of ethanolic extracts, 2 mL of 7.5% (w/v) sodium carbonate solution was added and vortexed vigorously. After 5 min, 1 mL of 1:10 diluted Folin––Ciocalteu's phenolic reagent was added and vortexed again. Same procedure was followed for the standard solution of gallic acid. All the tubes were incubated at room temperature for 30 min and the absorbance was measured at 765 nm. The total phenolic content of the extracts was expressed as gallic acid equivalent in mg/g (GAE mg/g extract).

Measurement of ferrous ion chelating ability

The ferrous ion chelating activity of the extracts was measured by the decrease in absorbance at 562 nm of the iron (II)–ferrozine complex according to Carter *et al.* [17] and Yan *et al.* [18]. To 1 mL sample (with different dilution), 1 mL of 0.125 mM $FeSO_4$ solution was added, followed by 1 mL of 0.3125 mM ferrozine. The test tubes were allowed to equilibrate at room temperature for 10 min. The absorbance was measured at 562 nm against blank. EDTA was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the expression on the right of Eq. (1).

Reducing power assay

The reducing power assay was conducted as described by Oyaizu [19]. To 1 mL sample extract at different concentration, 2.5 mL of 0.2 M phosphate buffer, pH 6.6 and 2.5 mL of 1 % potassium ferricyanide were added and mixed vigorously. After incubation at 50 °C for 20 min, 2.5 mL of 10 % trichloroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of upper layer of mixture was added to 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride, and the absorbance

of resulting solution was read at 700 nm against blank. Ascorbic acid was used as positive control.

RESULTS

The results of DPPH scavenger activity investigations of ethanolic extracts of tested fungi, showed that the maximum decolorization has *Penicillium fumiculosum* (51.34 %), followed by *P. chrysogenum* (37.42%) at the maximum concentration of 1000 μg/mL. The IC_{50} value against DPPH radical was found to be 974 μg/mL for *P. fumiculosum* and 1336 μg/mL for *P. chrysogenum* (Figure 1). These results indicate that ethanolic extract of tested fungi may serve as effective radical scavenging with DPPH free radical, converting them to stabile products.

The results of examinations of the total antioxidant activity of ethanolic extract of tested fungi are shown in Figure 2. By increasing the concentrations of ethanolic extract from 0.0156 to 1 mg/mL, the total antioxidant activity of the tested fungi also increased. The total antioxidant activity of *P. chrysogenum* (3.874 μg AA/g) was slightly higher in comparison to fungus *P. fumiculosum* (3.171 μg AA/g).

The results of ferrous ion chelating activity of alcoholic extract are shown in Figure 3. Ethanolic extract of *P. chrysogenum* showed better chelating activity than *P. fumiculosum* at concentrations ranging from 0.0125 to 0.500 mg/mL. By increasing the concentration of the extract from 0.5 to 1 mg/mL, the absorbance increased too, but the absorbance values were much higher compared to the blank absorbance. Therefore, this method cannot be successfully applied for higher concentrations of fungi extract, since the results of chelating capacity may not be valid. However, ethanolic extract of fungus *P. chrysogenum* showed a better chelating ability compared to standard EDTA solutions at concentrations from 0.0125 to 0.625 mg/mL.

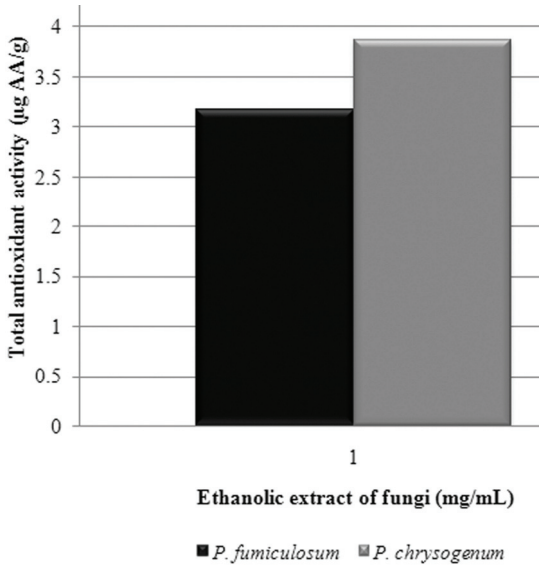

Figure 2. Total antioxidative activity of the ethanolic extract of mycelia.

The results of the total phenolic content in ethanolic extract of *P. chrysogenum* and *P. fumiculosum* are presented in Figure 4. The total phenolic content in the extract of mycelia is slightly different, but fungus *P. chrysogenum* (2.859 mg GAE/g) showed better yield compared to *P. fumiculosum* (2.109 mg GAE/g).

The reducing power of the ethanolic extract of mycelia for tested fungi is shown in Figure 5. The

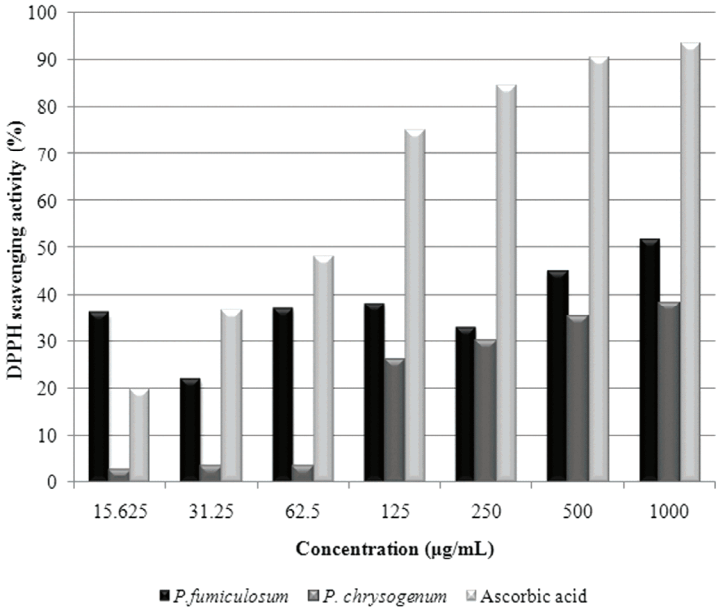

Figure1. DPPH scavenging activity of the ethanolic extract of mycelia against ascorbic acid.

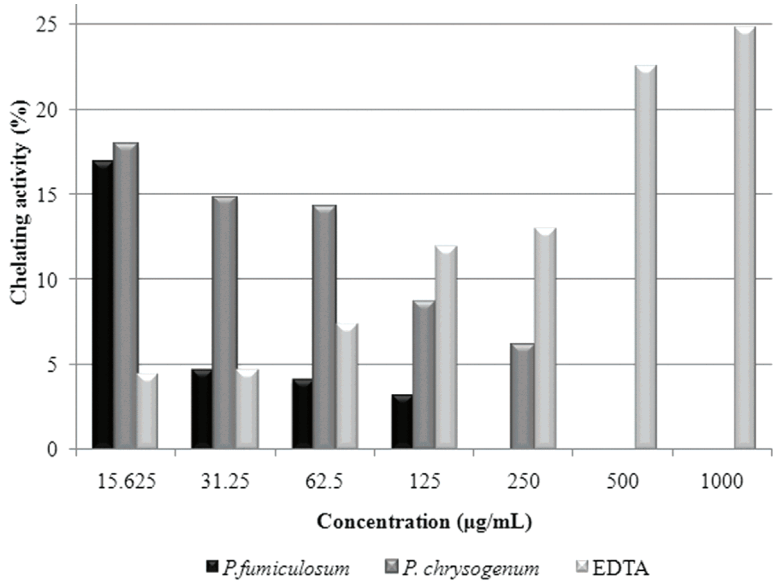

Figure 3. Chelating inhibition of the ethanolic extract of mycelia against EDTA.

reduction potential of the extracts exhibited a dose--dependent activity within a concentration range of 0.015625 to 1 mg/mL. Slightly better reducing ability has fungus *P. fumiculosum* than *P. chrysogenum* for all concentrations of the extract, but it is far less compared to synthetic antioxidant (ascorbic acid).

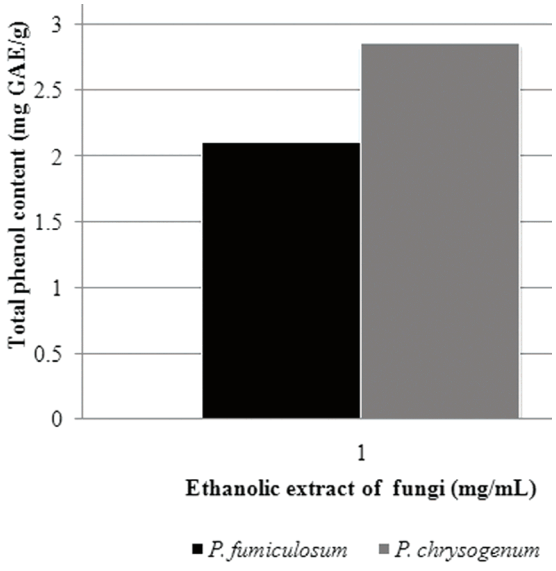

Figure 4. Total phenol content of the ethanolic extract of tested fungi.

DISCUSSION

The need for discovery and development of effective and safe antioxidants from natural sources has prompted scientists to search for sources of these bioactive resources among filamentous fungi. Antioxidant activity, which was examined using four different assays, was confirmed for both tested species of *Penicillium*.

Although DPPH scavenger activity of mycelia extract for tested fungi was lower in comparison to commercial antioxidant (ascorbic acid), activity of 1 mg/mL concentration extracts was between 37.42 and 51.34%. IC_{50} values for these two extracts were still higher than those of tested fungi from genus *Penicillium*. It was found that the percentage inhibition of DPPH free radical ranges from 72 to 88% for *Penicillium sp*. NIOMI-02 [20] to 91.1% in *P. citrinum* [21]. These differences may be attributed to various conditions in which the fungi are grown.

The results suggest that the extract from *P. fumiculosum* mycelium is a promising resource of natural antioxidants.

Alcoholic extract of *P. chrysogenum* showed higher total antioxidant capacity than *P. fumiculosum* at the concentration of 1 mg/mL.

Phenolic compounds have been associated with antioxidative action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [22]. Extract of *P. chrysogenum* mycelium had higher content of total phenol (2.859 mg GAE/g) than extract of *P. fumiculosum* (2.109 mg GAE/g). However, total phenol content found in some fungi of genus *Penicillium* was much higher than in these two species. It was found that total phenolic content of *P. granulatum* was 7.01 mg GAE/g [23], while in endophytic *Penicillium* species was only 1 mg GAE /g [24].

The obtained results indicate that the total phenolic content correlated with the total antioxidant activity.

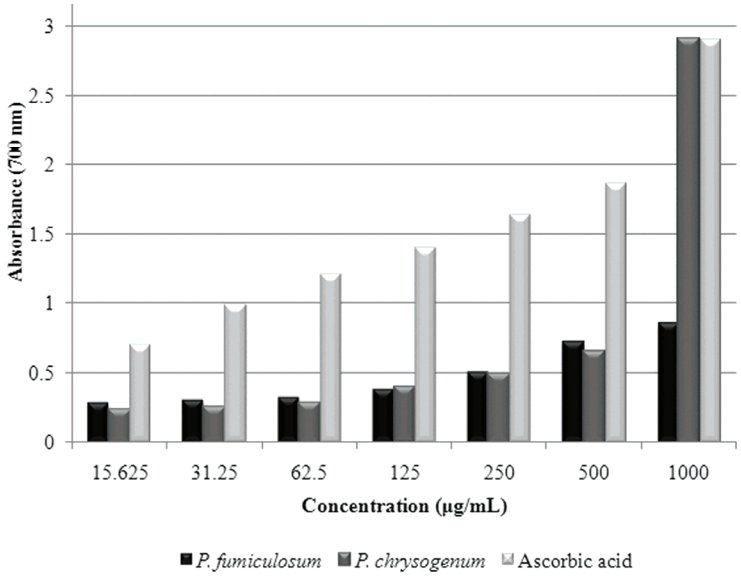

Figure 5. Reducing power (absorbance at 700 nm) of the ethanolic extract of mycelia.

Ferrous ions are most effective pro-oxidants and since they are commonly found in food, they can initiate lipid peroxidation and start a chain reaction that leads to food deterioration [25]. Their interaction with hydrogen peroxide in biological systems leads to formation of highly reactive hydroxyl radicals [26].

The mycelia extracts of tested fungi showed better chelating capacity compared to standard EDTA at concentrations of 0.00156 to 0.0625 mg/mL. Among two tested fungi, the extract of *P. chrysogenum* showed better ferrous ion chelating capacity. There was positive correlation between chelating activity and total phenol content. The results suggest that *P. chrysogenum* contains phenolic compounds/ligands that are the most effective in sequestering ferrous ions by intercepting all coordination sites of metal ions.

The presence of reductants (antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form [27]. The extract of *P. fumiculosum* mycelium has better reduction potential than *P. chrysogenum*, as confirmed by reducing power assay. In this case, there is no correlation between total phenolic content and reducing power. These results indicate that the presence of some other compounds in extract, instead of phenol, act as reduktones and inhibit lipid peroxidation by donating a hydrogen atom, thereby terminating the free radical chain reaction [28]. These results are consistent with the results of the antioxidant activity obtained for other fungi, such as *Aspergillus candidus*, *A. fumigates*, *Cladosporium sp*, *Chaetomium sp*, and many mushrooms [29,30], lichens and medicinal plants [31–33].

CONCLUSION

The aim of this study was preliminary examination of whether the selected fungi species could be considered as source of potential natural antioxidants. The results show antioxidative activity of two species of fungi. The highest DPPH free-radical scavenging activity as well as reducing power was shown by *Penicillium fumiculosum.* On the other hand, ethanolic extract of *Penicillium chrysogenum* showed higher total antioxidant capacity, as well as chelating activity and total phenolic content. These results represent a good basis for further analysis of bioactive substances synthesized in fungi that exhibit different effects on antioxidant activity, which would be beneficial for their selective application in biotechnological processes in the future.

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IZVOD

Antioksidativna aktivnost etanolnog ekstrakta *Penicillium chrysogenum* i *Penicillium fumiculosum*

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(Naučni rad)

Cilj našeg rada bio je ispitivanje biološke i hemijske aktivnosti dve vrste gljiva iz roda *Penicillium* koje su izolovane iz otpadnih voda. Na odabranim vrstama gljiva *Penicillium chrysogenum* i *Penicillium fumiculosum* ispitivana je potencijalna antioksidativna aktivnost. Na etanolnom ekstraktu micelije testiranih gljiva, primenjene su četiri različite antioksidativne metode: sposobnost hvatanja DPPH slobodnih radikala, ukupni antioksidativni kapacitet, Fe^{2+}-helataciona aktivnost i Fe^{3+}-redukujući kapacitet. Ukupan sadržaj fenolnih jedinjenja u etanolnom ekstraktu micelija određen je metodom po Folin–Ciocalteu. Veća količina ukupnih fenola izmerena je u etanolnom ekstraktu micelije *P. chrysogenum* koji je ispoljio i veću ukupnu antioksidativnu i fero-helatacionu aktivnost. Sa druge strane, etanolni ekstrakt micelije *P. fumiculosum* imao je znatno veći procenat inhibicije DPPH slobodnih radikala i neznatno veći redukcioni kapacitet. Na osnovu dobijenih rezultata može se zaključiti da testirane vrste gljiva sintetišu različite sekundarne metabolite koji se mogu primeniti kao prirodni antioksidanti u prehrambenoj i farmaceutskoj industriji.

Ključne reči: Aktivnost hvatanja DPPH slobodnih radikala • Helataciona aktivnost • Redukujući kapacitet • Ukupan antioksidativni kapacitet • Ukupna količina fenola • *Penicillium*