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ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *Laetiporus sulphurous* (Bull.) Murrill

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Abstract

Laetiporus sulphureus (Bull.) Murrill is an edible wood-rotting Basidiomycete widely consumed as a nutritional food. These mushrooms were found to be medically active in some therapies, such as: antimicrobial, antitumor, antiviral, and immunomodulating treatments. In this work, antioxidant and antimicrobial potentials of L. sulphureus alcoholic extracts from dried fruiting bodies, dried mycelia broth and mycelia-free broth submerged cultures were investigated. For determination of potential antioxidant activity of thedried fruiting bodies and mycelia-free broth methanol extracts, the total phenols amount and scavenging capacity on 2.2-diphenyl-1-picrylhydrazyl (DPPH) radicals have been analyzed. The results showed that the highest total phenols amount (283.9 mg GAE/100g) was found in fruiting bodies extract. Radical scavenging activity was found higher for fruiting bodies extract (EC_{50}=35.45 mg/ml) followed by mycelia-free broth extract (EC_{50}=46.67 mg/ml). The antimicrobial effects of the ethanol extracts were analyzed against Candida albicans ATCC10321, Candida parapsilopsis CBS604, Escherichia coli ATCC8739, Staphylococcus aureus ATCC6538, Enterococcus faecalis and Staphylococcus epidermidis ATCC12228. Two extracts presented a wide antimicrobial spectrum and were active against both yeast and bacteria tested: fruit bodies extract and dried biomass extract.

Key words: *Laetiporus sulphureus, submerged culture, antioxidant activity, antimicrobial activity.*

INTRODUCTION

In the last decades a significant number of people are involved in various forms of alternative medicine. Medicinal use of mushrooms, with an ancient tradition in the Asia, has been slightly increased in Europe since the past decades (Lindequist et al., 2005). Excessive use of antibiotics in the treatment of infectious diseases caused by human pathogenic microorganisms can led to multiple drug resistance. Thus, the scientists search for new substances with antimicrobial activity in natural products which could substitute the synthetic antibiotics. A number of various compounds that have been proved to posses significant antimicrobial activities were isolated from polypore fungi such as: *Laetiporus sulphureus, Ganoderma* sp. and *Trametes versicolor*. *Laetiporus sulphurous* (*Polyporaceae*, Fungi) is a wood-rotting basidiomycete mushroom that causes heart-rot disease in deciduous trees and conifers (Imazeki and Hongo, 1998; Rogers et al., 1999).The fruiting bodies of *Laetiporus* species mushrooms contain a number of lanostane triterpenoids, laetiporic acids and other compounds (Weber et al., 2004; Davoli et al., 2005; Radic et al., 2009). Mushrooms can accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Among the antioxidant compounds, polyphenols have gained importance due to their large array of biological actions that include free radical scavenging, metal chelation enzyme modulation activities and inhibition of Low-Density Lipoprotein (LDL) oxidation, among others (Teissedre and Landrault, 2000; Rodrigo and Bosco, 2006). Therefore, the objective of this study was to

evaluate antimicrobial and antioxidant properties of various extracts of *Laetiporus sulphurous* fruit bodies or mycelia cultivated *in vitro*.

MATERIALS AND METHODS

Mushroom samples

Samples of the fruiting bodies of *Laetiporus sulphureus,* collected from Sinaia woods, were surface sterilizing and cutting out a piece of trama using a sterile scalpel. The pieces were placed in Petri dishes on PDA (potato-dextrose-agar) medium and incubated at 25^0C for a week, in the dark. After the mycelium growing on the medium surface, mycelia agar segments (10 x 10 mm) obtained from the active growth areas were placed in 500 ml Erlenmeyer flasks, with 200 ml PD (potato-dextrose) broth. Then, the samples were incubated at 25^0C with a rotary shaker at 148 rpm for 14 days and in static condition for another 14 days. In the mean time, some fresh mushrooms were left to completely dry at room temperature. The biomass developed in submerged culture and the dried mushrooms were used for the extracts preparation with various solvents (Table 1).

Extracts preparation

Mushroom extracts were prepared depending on the analysis which carried out. The filtrated mycelia mass and the fine dried mushroom powder were extracted with different solvents for 24h at 4^0C (Table 1). Then, the mixtures were centrifuged at 10000 rpm for 10 min. and the resulted supernatant was kept at 4^0C and used to determine: total phenolic content, antioxidant capacity, total carbohydrate content and antimicrobial activities.

Table 1. Variants of different mushroom extracts used for analysis

Analysis	Extract types
Total phenolic content	1g of mycelia mass and 1g of dried mushroom powder extracted in 80% of methanol (10 ml)
Antioxidant capacity	1g of mycelia mass and 1g of dried mushroom powder extracted in 80% of methanol (10 ml)
Total carbohydrate content	100mg mycelia mass and 100 mg dried mushroom powder extracted in 1 ml distillated water
Antimicrobial activity	100mgcrude mycelia mass, 100mg dried mycelia mass and 100 mg dried mushroom powder extracted in 1 ml 70% ethyl alcohol

Determination of total carbohydrate content

The carbohydrates content were determined by the slightly modified Phenol-sulfuric acid method according to DuBois (DuBois et al., 1956). 100 mg wet mycelia mass and 100 mg dried mushroom powder were extracted in 1 ml distillated water than 50 µl from each sample was mixed with 50 µl of 5% phenol and 1.5 ml concentrated sulphuric acid. The reaction mixture was kept at 90^0C for 10 min. The absorbance of the mixture after cooling to room temperature was measured at 490 nm. The total carbohydrate content was calculated using a standard curve of D-glucose.

Total phenolic content

Total phenolic content determination was performed according to the modified Folin-Ciocalteu assay (Singleton, 1999). The method consists in chemical reduction of Folin-Ciocalteu reagent (a mixture of tungsten and molybdenum oxides) and measuring the absorbance at 750 nm. Total phenols values were expressed as mg gallic acid (GAE) equivalent per 100g of mushroom. The measurements were achieved with a UV/Visible Thermo-Spectronic Helios spectrophotometer. All analyses were performed in triplicate.

Antioxidant activity - DPPH assay

The free radical scavenging activity of mushroom extracts was determined according to the method of Blois (1958) with some modifications reported by Brand-Williams (1995). The mushroom extract was mixed with 100 µM methanol 80% solution of DPPH to give a final concentration of extract between (4-80) mg/ml. After 30 min of incubation in the dark at room temperature, the change in color from deep violet to light yellow was measured at 515 nm on a spectrophotometer and converted into percentage of the radical scavenging activity (RSA%). Radical scavenging activity was calculated as follows:

$$RSA\% = (1-[A_{sample}/ A_{blank\ t=0}]) \times 100$$

Were:

A sample - is absorbance of sample solution and A blank - is absorbance of blank sample.

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The RSA% was plotted against the sample concentrations and a linear regression curve was established in order to calculate the

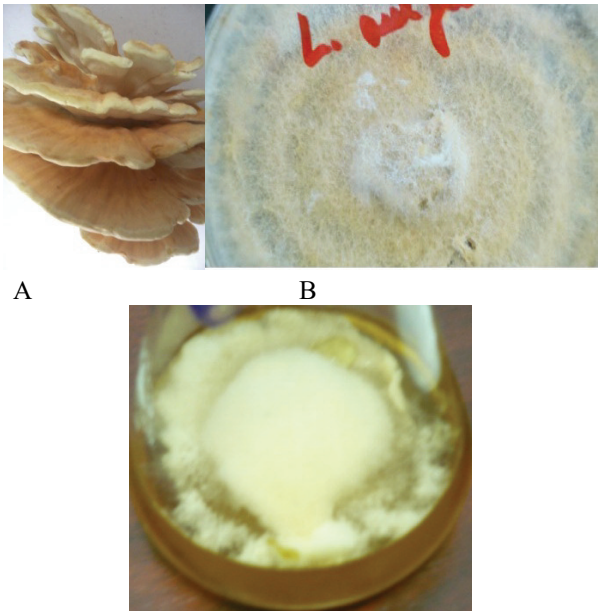
EC_{50} value (mg/ml) which is the concentration of the sample required to give 50% of decrease in absorbance compared to the blank sample. Glutathione was used as antioxidant standard at various concentrations (50-300 µg/ml).

Antimicrobial activity

The antimicrobial activities of *L. sulphurous* extracts were evaluated on several microorganisms of medicinal importance. 70% ethanol extracts from fruit bodies and submerged mycelium developed in liquid media were analyzed against microorganism strains of *Candida albicans* ATCC10321, *Candida parapsilopsis* CBS604, *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* and *Staphylococcus epidermidis* ATCC12228. The microorganisms were obtained from National Research Institute of Chemical - Pharmaceutical Development of Bucharest. Antimicrobial activities of the extracts were screened by the agar disk diffusion method. 1 ml from each bacterial and yeasts suspensions were inoculated in Petri dishes on Luria Broth and YPG media respectively. After removing the excess suspension using a micropipette, sterile filter paper discs (5 mm diameter) soaked in alcoholic extracts were placed on the surface of the inoculated medium. 24 hours after incubation at 37^0C for bacteria and 30^0C for yeasts, occurrence of inhibition halos around each disk was observed.Ethanol (70%) was used as negative control.

RESULTS AND DISCUSSIONS

Fragments of fruit bodies of *L. sulphureus* were cultivated on PDA medium. Five days old mycelium grown on the solid medium surface (Figure 1A) was used for inoculation of PD broth medium. After 28 days of cultivation on PD broth the fungus has developed a rich mycelia biomass. Cultivation under static condition, for the last period of incubation, led to rapid development at the medium surface of a dense layer of fungal culture.After incubation time we observed, in both pure culture of *Laetiporus* isolate, a pigmentation which the color ranging from white, yellow to orange (Figure 1-A, B, C). These results are similar to those from Davoli et al, (2005) which found that this pigment is a non-isoprenoid polyene laetiporic acid A from *L. sulphurous* fruit-bodies (Figure 1A). This orange pigment we found to be major in mycelium grown either in solid or in liquid cultures (Figure 1-B, C).



A B C

Figure 1. Carpophores aspects of *L. sulphureus* (A) and mycelia growth on PDAmedium (B) and PD broth (C)

Total carbohydrate content

Mushrooms are known to contain high quantities of carbohydrate. In this study, quantitative examination of carbohydrate in *L. sulphurous* was carried out according to Dubois et al. (1956) method, using glucose as standard. The concentration of carbohydrate in the *L. sulphureus* aqueous extracts of dried fruiting body and crude biomass (from liquid cultures) was calculated from the standard graph (Figure 2).

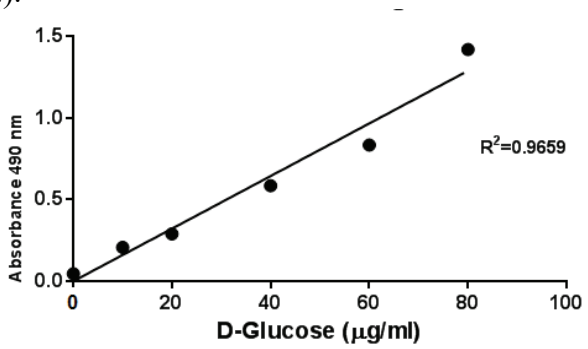


Figure 2. The concentration of carbohydrate in the *L. sulphureus* aqueous extracts

In dried fruit body extract the total carbohydrate contentwere found to be 40.556 µg/ml and 88.019 µg/ml in crude biomass, respectively. This result indicates that aqueous

extract of cultured mycelia contains higher carbohydrate content than dried fruit body.

Scavenging effect on DPPH radicals

DPPH method is usually used to evaluate antioxidant activity of various natural compounds by reducing stable DPPH radicals. DPPH radical scavenging ability is responsible for hydrogen-donating efficiency of antioxidants (Lung and Huang, 2012). In this study we tested antioxidant capacity of biomass developed in submerged culture and dried mushroom extracted inmethanol. Extracts in different concentrations exhibited high antioxidant activity, expressed as percentage of DPPH reduction. DPPH free radical-scavenging activity of dried fruit bodies extract was found to exhibit 40.84%, 44.39%, 49.53% and 54.59% inhibition, respectively, at concentrations of 28, 32, 36 and 40 mg/ml (Figure 3).

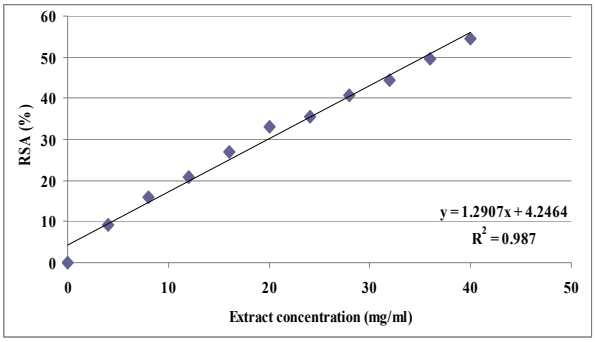


Figure 3. Radical scavenging activity (%) of dried fruiting body extracts at different concentrations

DPPH free radical-scavenging activity of mycelia extracts was found to exhibit 52.67%, 56.58%, 62.39% and 69.99% inhibition, respectively, at concentrations of 49, 56, 63 and 70 mg/ml (Figure 4). Radical scavenging activity of the samples extracts increased with the increase in concentration.

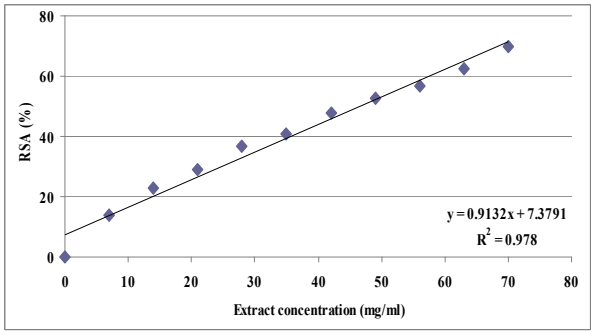


Figure 4. Radical scavenging activity (%) of mycelia mass extracts at various concentrations

These results expressed as EC_{50} values show that dried fruiting bodies extract have the highest scavenging activity (35.45 mg/ml). High EC_{50} values correspond to weak antioxidant properties and values lower than 10 mg/ml stand for effective antioxidant activities (Liang et al., 2009). Figure 5 represents the antioxidant activity of glutathione with EC_{50}=139.16 µg/ml. The outcomes indicated a significant radical scavenging potential for the samples extracts compared with the reference standard glutathione.

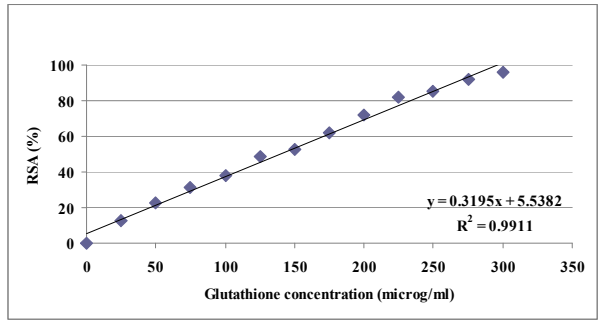


Figure 5. Radical scavenging activity (%) of glutathione at different concentrations

Total polyphenols were the major naturally occurring antioxidant components found in the methanolic extracts from wild edible mushrooms (Keleş et al., 2011). The total polyphenolic content, expressed as mg of GAE/ 100 g of mycelia and dried mushroom, is showed in Table 2 in correlation with radical scavenging activity (expressed as EC_{50}), in order to highlight the influence of these bioactive compounds on antioxidant activity of tested samples.

Table 2. Total polyphenolic content correlated with radical scavenging activity expressed as EC_{50}

Samples	Total polyphenolic content (mg GAE/100 g)*	EC_{50} (mg/ml)
Dried mushroom extract	283.90 ± 8.94	35.45
Mycelia mass extract	79.39 ± 2.62	46.67

*The results represent mean ± S.D of 3 separate experiments

As it shown from data Table 2, the amount of phenolic compounds extracted from dried mushrooms (283.90 ± 8.94 mg GAE/100 g) was higher than that obtained from mycelia mass (79.39± 2.62 mg GAE/100 g) which is in

concordance with the scavenging effects of methanolic extracts. The phenolic compounds may contribute directly to the antioxidant activity (Duh et al., 1999).

Phenols are important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). In addition, it was reported that phenolic compounds could be associated with antioxidant activity to play an important role in stabilizing lipid peroxidation (Halliwell and Gutteridge, 2003). Antioxidant activity is one of the important bioactivities revealed in higher Basidiomycetes.

Although many researchers have investigated antioxidant properties of a wide spectrum of mushroom fruiting bodies, little attention has been paid to antioxidant production by submerged cultures of medicinal mushrooms (Elisashvili, 2012).

Antimicrobial activity

Ethanol extracts obtained from dried mushroom fruit bodies, wet biomass developed in submerged culture and dried biomass were tested against pathogenic strains of *Candida albicans* ATCC10321, *Candida parapsilopsis* CBS604, *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* and *Staphylococcus epidermidis* ATCC12228.

The data relating to the antimicrobial activities of extract samples is summarized in Table 3. The results shown that the antimicrobial activities of the extracts are influenced by biological material used (Table 3).

Table 3. Antimicrobial activities of *L. sulphurous* ethanol extracts

Samples	Solvent	Inhibition zone (mm)					
		A	B	C	D	E	F
1	70% ethanol	-	-	++	+	-	++++
2	70% ethanol	-	-	+	+	-	-
3	70% ethanol	+	-	+	++	+	+++

Samples: 1-Dried fruit bodies; 2-Crude mycelia mass; 3-Dried mycelia mass: A. *Escherichia coli* ATCC8739, B. *Staphylococcus aureus* ATCC6538, C. *Enterococcus faecalis,* D. *Staphylococcus epidermidis* ATCC12228, E. *Candida albicans* ATCC10321, F. *Candida parapsilopsis* CBS604. *Activities were classified according to the diameter of the inhibition zones around the disks containing 10 µl/disk extract or control: +, <10 mm; ++, 10–15 mm; +++, 15–20 mm, ++++, >20 mm; -, without activity.

Among the tested yeast strains, *L. sulphureus* extracts of fruit bodies and dried biomass strongly inhibited *Candida parapsilopsis*. A poorlyinhibitory effect of the extracts was observed against *C. albicans.* As for the remaining bacterial strains crude mycelia mass extract showed activity only against *Enterococcus faecalis* and *Staphylococcus epidermidis*, but insufficient to be considered as inhibitory. The bacteria most the most resistant to the effect of the extracts were proved to be *S. aureus* followed by *E. coli*. Our results reveal that two extracts resulted from dried fruit bodies and dried biomass presented a wide antimicrobial spectrum and were active against both yeast and bacteria tested. The reduced antimicrobial activities detected in crude mycelium could be explained by the dilution of the compound in the extracts. Reports on mushroom antimicrobial activities has shown that some compounds like terpenes, lectins or polysaccharides have an effect on bacterial cytoplasmatic membrane, making it vulnerable (Lin and Chou, 1984; Yang et al., 2002). *L. sulphurous* is a rich source of these compounds, which may be making responsible for its antimicrobial activity.

CONCLUSIONS

Dried mycelia broth, mycelia-free broth submerged cultures and dried fruiting bodies of *Laetiporus sulphureus* were extracted with alcoholic solvents and investigated for their antioxidant and antimicrobial properties. The results of the current study suggest that submerged cultivation of this edible/medicinal mushroom is an appropriate approach to obtain significant antioxidant compounds from the submerged mycelium and culture filtrate. The biological activity of the mushrooms can be directly correlated with the chemical constituents present in them. For this reason, the submerged cultivation of medicinal mushrooms has received a great attention as a promising and reproducible alternative for the efficient production of mushroom mycelium and metabolites. Hence, bioactive and structurally diverse fungal metabolites could be used for the development of valuable pharmaceuticals.

Laetiporus sulphurous (Bull. Fr.) Murr. can be easy found in nature and also can be easy cultivated. Therefore, this edible mushroom could be considered as a promising candidate for biotechnology studies.

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