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Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill

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Abstract

Antioxidant capacity and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. extracts obtained with ethanol were investigated in this study. The study was aimed at determining the antioxidant activity (DPPH free radical-scavenging, β-carotene/linoleic acid systems), total phenolic content and total flavonoid concentration of *L. sulphureus*. Inhibition values both of *L. sulphureus* ethanol and the standards increased parallel with the elevation of concentration in the linoleic acid system. Inhibition values of *L. sulphureus* (LS) extract, BHA and α-tocopherol standards were found to be 82.2%, 96.4% and 98.6%, respectively, at a concentration of 160 μg/ml. DPPH free radical-scavenging activity was found to exhibit 14%, 26%, 55% and 86% inhibition, respectively, at concentrations of 100, 200, 400 and 800 μg/ml. Total flavanoids were 14.2 ± 0.12 μg mg^{-1} (quercetin equivalent) while the phenolics were 63.8 ± 0.25 μg mg^{-1} (pyrocatechol equivalent) in the extract. Positive correlations were found between total phenolic content in the mushroom extracts and their antioxidant activities. Edible mushrooms may have potential as natural antioxidants. *L. sulphureus* showed narrow antibacterial activity against Gram-negative bacteria and strongly inhibited the growth of the Gram-positive bacteria tested. The crude extract exhibited high anticandidal activity on *Candida albicans*. Therefore, the extracts could be suitable as antimicrobial and antioxidative agents in the food industry.

Keywords: *Laetiporus sulphureus*; Mushroom; Antioxidant and antimicrobial activity

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species, that are continuously produced in vivo, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell & Gutteridge, 1984). Almost all organisms are well protected against free radical damage by enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione (Mau, Lin, & Song, 2002; Niki, Shimaski, & Mino, 1994). Although almost all organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988). However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage (Yanga, Linb, & Maub, 2002).

Synthetic antioxidants have been used in stabilization of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene

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(BHT), and *tert*-butylated hydroxyquinone (TBHQ), that are applied in fat and oily foods to prevent oxidative deterioration (Löliger, 1991). However, BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck, Verhagen, Goldbohm, Kleinjans, & Brandt, 2000).

Laetiporus sulphureus (Bull.) Murrill. (Polyporales, Fungi) is a wood-rotting basidiomycete, growing on several tree species and producing shelf-shaped fruit-bodies of pink-orange colour, except for the fleshy margin which is bright yellow (Weber, Mucci, & Davoli, 2004). *Laetiporus* species contain *N*-methylated tyramine derivatives (Lee et al., 1975; List, 1958; Rapior, Konska, Guillot, Andory, & Bessiere, 2000), polysaccharides, a number of lanostane triterpenoids, laetiporic acids and other metabolites (Davoli, Mucci, Schenetti, & Weber, 2005; Weber et al., 2004). Davoli et al. (2005) have reported that Laetiporic acids might have potential as food colorants.

The nutritional values and taste components of these commercial mushrooms have been thoroughly studied (Yang, Lin, & Mau, 2001). Recently, these commercial mushrooms were found to be medically active in several therapies, such as antitumor, antiviral, and immunomodulating treatments (Wasser & Weis, 1999). Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Japan, China, Korea, and other Asian countries continues to rely on mushroom-derived preparations. Mushrooms have been used for many years in oriental culture as tea and nutritional food and because of their special fragrance and texture (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). The scientific community, in searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found variable therapeutic activity such as anticarcinogenic, anti-inflammatory, immunosuppressor and antibiotic, effects (Asfors & Ley, 1993; Longvah & Deosthale, 1998).

Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolic compounds were found to have antioxidant activity in the inhibition of LDL oxidation (Teissedre & Landrault, 2000). Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity, which is well correlated with their total phenolic content. Recently, mushrooms are considered to be a good source of protein and phenolic antioxidants, such as variegatic acid and dibooviquinone, which have been found in mushrooms (Cheung, Cheung, & Ooi, 2003).

It was reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioglu, Mazza, Gao, & Oomah, 1998). So, it is important to consider the effect of the total phenolic content on the antioxidant activity of mushroom extracts. Phenolics are one of the major groups of non-essential dietary components that have been associated with the inhibition of atherosclerosis and cancer (Williams & Iatropoulos, 1997). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997).

Flavonoids have been proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities (Cook & Samman, 1996; Kandaswami & Middleton, 1997; Sahu & Green, 1997). In food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides. Therefore, they present antioxidative efficiency in oils, fats and emulsions (Das & Pereira, 1990; Madhavi, Singhal, & Kulkarni, 1996; Nieto et al., 1993; Roedig-Penman & Gordon, 1998). A method widely used to predict the ability of flavonoids to transfer H atoms to radicals is based on the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998).

Researchers have reported antimicrobial activity of several mushrooms (Gao et al., 2005; Kim & Fung, 2004; Lee, Yeo, Yun, & Yoo, 1999). The chloroform and ethyl acetate extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* (Hirasawa, Shouji, Neta, Fukushima, & Takada, 1999). Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity.

In recent years, multiple drug resistance in human pathogenic microorganisms has developed, due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various plants which are good sources of novel antimicrobial chemotherapeutic agents (Karaman et al., 2003). The reason for this study is that antimicrobial activities of LS have not been reported in the literature although Anatolian people have been using it as food for a long time. Therefore, the aim of the present work is to evaluate the antioxidant and antimicrobial potentials of ethanol extracts of *L. sulphureus* on several microorganisms of medicinal importance.

2. Material and methods

2.1. Mushroom

L. sulphureus was collected from Denizli, in the western part of Turkey. Identification and classification of macrofungus were carried out and all specimens are deposited at the laboratory of the Department of Science Education, Pamukkale University, Denizli, Turkey. Specimens of *L. sulphureus* representing a combination of young and old basidiocarps, were collected in *Salix alba* in the spring and autumn of 2004. Fresh mushrooms were randomly

divided into three samples of 150 g and air-dried in an oven at 40 °C before analysis. Dried mushroom sample (20 g) was extracted by stirring with 200 ml of ethanol at 30 °C at 150 rpm for 24 h and filtering through Whatman No. 4 filter paper. The residue was then extracted with two additional 200 ml portions of ethanol as described above. The combined ethanolic extract was then evaporated at 40 °C to dryness, redissolved in ethanol to a concentration of 10 mg ml^{-1} and stored at 4 °C for further use.

2.2. Antioxidant activity

2.2.1. Chemicals

β-Carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and α-tocopherol were purchased from Sigma (Sigma, Aldrich). Pyrocatechol, Tween 20, Folin–Ciocalteu's phenol reagent (FCR), sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck (Darmstadt, Germany). All other unlabelled chemicals and reagents were of analytical grade.

2.2.2. DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical, DPPH· as a reagent (Burits & Bucar, 2000; Cuendet, Hostettmann, & Potterat, 1997). One thousand microlitre of various concentrations of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH·. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent ($I\%$) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of LS ethanolic extract. Tests were carried out in triplicate.

2.2.3. β-Carotene–linoleic acid assay

The antioxidant activity of LS extract was determined, according to the β-carotene bleaching method described by Dapkevicius, Venskutonis, Van Beek, and Linssen (1998). A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 μl of linoleic acid and 200 mg of Tween 40 were added. Chloroform was completely evaporated, using a vacuum evaporator. Then, 100 ml of distilled water, saturated with oxygen (30 min 100 ml/min), were added with vigorous shaking. Four thousand microlitres of this reaction mixture were dispensed into test tubes and 200 μl portions of the extracts, prepared at 2 mg/l concentrations, were added and the emulsion system was incubated for 2 h at 50 °C temperature. The same procedure was repeated with synthetic antioxidants, BHT, BHA, α-tocopherol, as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those of BHA, α-tocopherol and blank. Tests were carried out in triplicate.

2.3. Determination of total phenolic compounds

Total soluble phenolics in the mushroom ethanolic extracts were determined with Folin–Ciocalteu reagent, according to the method of Slinkard (Slinkard & Singleton, 1977), using pyrocatechol as a standard. Briefly, 1 ml of extract solution (contains 2000 μg) in a volumetric flask was diluted glass-distilled water (46 ml). Folin–Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the mushroom ethanolic extract, determined as microgrammes of pyrocatechol equivalents, by using an equation that was obtained from the standard pyrocatechol graph, is given as:

$$\text{Absorbance} = 0.00246\ \mu\text{g pyrocatechol} + 0.00325 \times (R^2 = 0.9996)$$

2.4. Determination of total flavonoid concentration

Flavonoid concentration was determined as follows: mushroom ethanolic extract solution (1 ml) was diluted with 4.3 ml of 80% aqueous ethanol containing 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1 M aqueous potassium acetate. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard (Park, Koo, Ikegaki, & Contado, 1997):

$$\text{Absorbance} = 0.002108\ \mu\text{g quercetin} - 0.01089 \times (R^2 : 0.9999)$$

2.5. Antimicrobial activity

2.5.1. Microorganisms

The following strains of bacteria were used: *Pseudomonas aeruginosa* NRRL B-23, *Salmonella enteritidis* RSKK 171, *Escherichia coli* ATCC 35218, *Morganella morganii* (clinical isolate), *Yersinia enterecolitica* RSKK 1501, *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* RSKK 96026, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* Cowan I, *Micrococcus luteus* NRRL B-4375, *Micrococcus flavus*, *Bacillus subtilis* ATCC 6633, *Bacillus cereus*

RSKK 863, and *Candida albicans* (clinical isolate). The bacteria were obtained from the culture collection of the Microbiology Department of Pamukkale University and Ankara University.

2.5.2. Screening of antimicrobial activity of mushroom samples

Antimicrobial activity of ethyl alcohol extract of *L. sulphureus* was determined by the agar-well diffusion method. All the microorganisms mentioned above were incubated at 37 ± 0.1 °C (30 ± 0.1 °C for only *M. luteus* NRRL B-4375 and *M. flavus*) for 24 h by inoculation into Nutrient broth. *C. albicans* was incubated YEPD in broth at 28 ± 0.1 °C for 48 h. The culture suspensions were prepared and adjusted by comparing against 0.4–0.5 Mc Farland turbidity standard tubes. Nutrient Agar (NA) and YEPD Agar (20 ml) were poured into each sterilized Petri dish (10 × 100 mm diameter) after injecting cultures (100 μl) of bacteria and yeast and distributing medium in Petri dishes homogeneously. For the investigation of the antibacterial and anticandidal activity, the dried mushroom extracts were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 20% and sterilized by filtration through a 0.22 μm membrane filter (Ali-Shtayeh, Yaghmour Reem, Faidi, Salem, & AlNuri, 1998; Tepe, Daferera, Sokmen, Sokmen, & Polissiou, 2005). Each sample (100 μl) was filled into the wells of agar plates directly. Plates injected with the yeast cultures were incubated at 28 °C for 48 h, and the bacteria were incubation at 37 °C (30 °C for only *M. luteus* NRRL B-4375 and *M. flavus*) for 24 h. At the end of the incubated period, inhibition zones formed on the medium were evaluated in mm. Studies were performed in duplicate and the inhibition zones were compared with those of reference discs. Inhibitory activity of DMSO was also tested. Reference discs used for control were as follows: nystatin (100 U), ketoconazole (50 μg), tetracycline (30 μg), ampicillin (10 μg), penicillin (10 U), oxacillin (1 μg), tetracycline (30 μg) and gentamycin (10 μg). All determinations were done duplicate.

3. Results and discussion

3.1. Antioxidant activity of extracts

The ethanolic extracts were subjected to screening for their possible antioxidant activity. Four complementary test systems, namely DPPH free radical-scavenging, β-carotene/linoleic acid systems, total phenolic compounds, and total flavonoid concentration, were used for the analysis.

DPPH·, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to these radicals, the absorbance decreases. The decrease in absorbance is taken as a measure of the extent of radical-scavenging. Free radical-scavenging capacities of the extracts, measured by DPPH· assay, are shown in Fig. 1. It was observed, that in line with the increase seen in the amount of LS ethanol extract, an increase in DPPH free radical-scavenging occurred. Inhibition values in the concentrations of 100, 200, 400 and 800 μg/ml were, respectively, 14%, 26%, 55% and 86%.

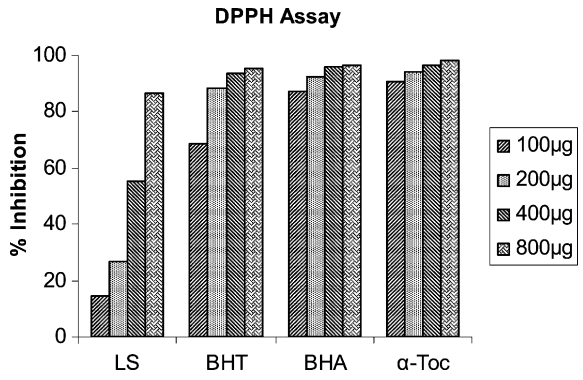

Fig. 1. Free radical-scavenging capacities of the extract measured in DPPH assay.

Three hundred and twenty micrograms of LS ethanol extract has an inhibition value equivalent to 40 μg α-tocopherol. The inhibition value increases with concentration. Linoleic acid oxidation was compared with those of LS ethanol extract, α-tocopherol and BHA. It was found that inhibition values of both LS ethanol extract and the standards increased with concentration. For example; at 80 μg/ml concentration, LS extract, BHA and α-tocopherol showed 57.4%, 88.2%, 93.3% of inhibition whereas, at 160 μg/ml concentrations, these were 82.2%, 96.4%, 98.6%. According to this, it is possible that the high inhibi-

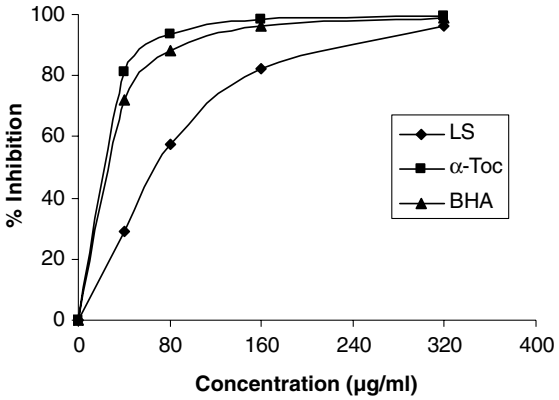

Fig. 2. Total antioxidant activities of BHA, α-tocopherol and different doses of ethanolic extract mushroom in the linoleic acid emulsion.

Table 1
Amounts of total flavonoid and total phenolic compounds in LS ethanolic extract

Extract	Total phenolic compounds [pyrocatechol equivalents (μg mg^{-1})]	Total flavonoid content [quercetin equivalents (μg mg^{-1})]
Control	–	–
Ethanol extract	63.8 ± 0.25[a]	14.2 ± 0.12

Data expressed as means ± s.e.m. of three samples analysed separately.
[a] Standard deviation.

Table 2
Antimicrobial activity of ethyl alcohol extract of *Laetiporus sulphureus* and antibiotic sensitivity of microorganisms (zone size, mm)

Test bacteria	*L. sulphureus*	N	A	P	G	O	T
Pseudomonas aeruginosa NRRL B-23	6 ± 0	NT	NT	NT	16	NT	8
Salmonella enteritidis RSKK 171	5 ± 1	NT	–	NT	NT	NT	12
Escherichia coli ATCC 35218	10 ± 0	NT	10	11	NT	NT	8
Morganella morganii	4.5 ± 0.5	NT	NT	NT	–	NT	–
Yersinia enterecolitica RSKK 1501	6 ± 0	NT	20	18	NT	NT	7
Klebsiella pneumoniae ATCC 27736	–	NT	–	NT	NT	NT	5
Proteus vulgaris RSKK 96026	5.5 ± 0.5	NT	–	NT	NT	NT	16
Staphylococcus aureus ATCC 25923	9 ± 1	NT	NT	31	NT	21	20
Staphylococcus aureus Cowan I	7 ± 1	NT	NT	28	NT	18	21
Micrococcus luteus NRRL B-4375	15 ± 3	NT	30	31	NT	22	19
Micrococcus flavus	23 ± 1	NT	29	31	NT	24	20
Bacillus subtilis ATCC 6633	8 ± 0	NT	NT	12	NT	8	17
Bacillus cereus RSKK 863	12 ± 1	NT	NT	22	NT	14	19
Candida albicans	21 ± 1	19	NT	NT	NT	NT	NT

N, nystatin (100 U); A, ampicillin (10 μg); P, penicillin (10 U); G, gentamycin (10 μg); O, oxacillin (1 μg); T, tetracycline (30 μg); NT, not tested; (–) No inhibition.

tion value of the LS extract is due to the high concentration of phenolic compounds (see Fig. 2).

In conclusion, we can infer that this mushroom extract competes with BHA and α-tocopherol.

3.2. Amount of total phenolics and flavonoids

The key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Komali, Zheng, & Shetty, 1999; Moller, Madsen, Altonen, & Skibsted, 1999). Amounts of phenolic and flavonoid compenents of LS ethanol extract were, respectively, found to be 63.8 ± 0.25 μg mg^{-1} pyrocatechol equivalents and 14.2 ± 0.12 μg mg^{-1} quercetin equivalents. The amount of phenolic compound was calculated as pyrocatechol equivalents. When this amount is compared to those found in other studies in the literature, polyphenolic compounds seem to have important role in stabilizing lipid oxidation and to be associated with antioxidant activity (Gülçin, Büyükokuroglu, Oktay, & Küfrevioglu, 2003; Yen, Duh, & Tsai, 1993). The phenolic compounds may contribute directly to antioxidative action (Duh, Tu, & Yen, 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka, Kuei, Nagashima, & Taguchi, 1998) (Table 1).

Previously, in a study performed with *Hypericum hyssopifolium*, it was found that antioxidant activity was based on flavonoid-type compounds (Cakir et al., 2003). In this study performed with LS, it is thought that the high free radical-scavenging activity and total antioxidant activity may result from the existence of phenolic and flavonoid-type compounds.

3.3. Antimicrobial activity of extract

The antimicrobial effect of ethanol extracts of *L. sulphureus* was tested against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and one species of yeast. As summarized in Table 2, *L. sulphureus* had a narrow antibacterial spectrum against Gram-negative bacteria and strongly inhibited the growth of the Gram-positive bacteria tested, including *Bacillus subtilis*, *B. cereus*, *M. luteus* and *M. flavus*. The maximal zones of inhibition ranged from 10 to 23 mm. The most susceptible bacterium was *M. flavus* (23 ± 1 mm diameter). The ethanol extract of *L. sulphureus* showed no antibacterial activity against *Klebsiella pneumoniae* at the concentration used. The culture fluid of *Lentinus edodes* showed poor activity against *C. albicans* (Hatvani, 2001). Dulger, Ergul, and Gucin (2002) reported that *C. albicans* and *Rhodotorula rubra* are resistant to the action of the methanolic extract of *Lepista nuda*. In the present study, the ethanol extract of *L. sulphureus* exhibited high anticandidal activity on *C. albicans*.

In this study, the antibacterial properties of *L. sulphureus* were not as effective as the commercial drugs. But, microorganisms become resistant to antibiotics overtime. Previously become it has been demonstrated that mushrooms show antimicrobial effects (Hur et al., 2004; Ishikawa, Kasuya, & Vanetti, 2001; Sheena, Ajith, Mathew, & Janardhanan, 2003). Similarly, in our survey, *L. sulphureus* was found to inhibit the growth of microorganisms that cause infectious diseases. In conclusion, the ethanolic extract of the LS investigated possessed activity against one strain of fungus and some bacteria. The antimicrobial activities of LS against different strains of bacteria and fungi, which are known to be responsible for causing various diseases, could also be tested in future studies.

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