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Antioxidant and antimicrobial properties of ethanolic extract from *Lepista nuda* (Bull.) Cooke

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Abstract - Antioxidant capacity and antimicrobial activities of *Lepista nuda* (Bull.) Cooke extracts obtained with ethanol were investigated. Four complementary test systems, namely DPPH free radical scavenging, β-carotene/linoleic acid systems, total phenolic compounds and total flavonoid concentration, have been used. Linoleic acid inhibition values of *L. nuda* ethanolic extract, BHA and α-tocopherol standards were found to be 84.3%, 98.9% and 99.2% respectively in the concentration of 160 µg/ml. Total flavonoid amount was 8.21 ± 0.56 µg mg^{-1} quercetin equivalent while the phenolic compound amount was 48.01 ± 0.29 µg mg^{-1} pyrocatechol equivalent in the extract. The antimicrobial activity of *L. nuda* extract was tested *in vitro* by using the agar-well diffusion method. The *L. nuda* extract showed antibacterial activity against *Micrococcus flavus*, *Micrococcus luteus*, *Bacillus cereus*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*. The *L. nuda* extract did not exhibit anticandidal activity against *Candida albicans*. The extracts could be suitable as antimicrobial and antioxidative agents in the food industry.

Keywords: *Lepista nuda*, mushroom, antioxidant and antimicrobial activity, DPPH.

INTRODUCTION

Lepista nuda (Bull.) Cooke is a well-known and widespread species found of in Turkey. The cap is convex when young, but it expands and flattens sometimes becoming depressed in centre. The cap colour is brown, sometimes tinged with red or violet when young. The gills are an extremely attractive, intense bluish-purple colour. The stem is bluish-white, and has a slightly bulbous base. It is edible and delicious although causes stomach upsets in some people. The flesh is delicate lilac colour. It grows in deciduous woodlands and gardens among fallen leaves.

Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Japan, China, Korea, and other Asian countries continue to rely on mushroom-derived preparations. Mushrooms have been used for many years in oriental culture as tea and nutritional food because of their special fragrance and texture (Manzi *et al*., 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, immuno-suppressor and antibiotic, among others (Asfors and Ley, 1993; Longvah and Deosthale, 1998). It has been known for many years that selected mushrooms of higher *Basidiomycetes* origin are effective against cancer. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis, and atherosclerosis as well as in degenerative processes associated with aging (Halliwell and 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, tocopherol and glutathione (Mau *et al*., 2002). When the mechanism of antioxidant protection becomes unbalanced by factors such as aging, deterioration of physiological functions may occur, resulting in diseases and accelerating aging.

Flavonoids have been proven to display a wide range of pharmacological and biochemical actions such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities (Cook and Samman, 1996; Kandaswami and Middleton, 1997; Sahu and 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.

It was reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioğlu *et al*., 1998). Therefore, 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 com-

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ponents that have been associated with the inhibition of atherosclerosis and cancer (Williams and Iatropoulos, 1997). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997).

Researchers showed antimicrobial activity of several mushrooms (Lee *et al*., 1999; Kim and Fung, 2004; Gao *et al*., 2005). The chloroform extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* (Hirasawa *et al*., 1999). Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity (Jong and Birmingham, 1993). The mycelium-free culture fluid was bacteriostatic against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Bacillus megaterium* (Hatvani, 2001). 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 forced scientists to search new antimicrobial substances from various sources, which are the good sources of novel antimicrobial chemotherapeutic agents (Karaman *et al*., 2003).

In a previous research, it was established that the sporophore of *L. nuda* contained Vit B_1. Infusion of this macrofungus is used to prevent beriberi. In addition, the decoction is used for the treatment of abscesses and wounds (Dulger *et al*., 2002). On the other hand, to the best of our knowledge, no research has been available on antioxidant activities of the *L. nuda* extract in literature. Therefore, the aim of the present work is to evaluate the antimicrobial potential of the *L. nuda* extract on several microorganisms.

MATERIALS AND METHODS

Mushroom. *Lepista nuda* (Bull.) Cooke samples were collected from Denizli, located in the western part of Turkey. Identification and classification of macrofungus were carried out by mycologist, Dr. Aziz Türkoğlu and all specimens were deposited at the laboratory of Department of Science Education, Pamukkale University, Denizli, Turkey. Specimens of *L. nuda* representing a combination of young and old basidiocarps, were collected in the conifer forest and gardens among fallen leaves in the spring of 2004. Fresh mushroom 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 of ethanol as described above. The combined ethanolic extract were then rotary 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.

Chemicals. β-carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), buthylated hydroxyanisol (BHA) and α-tocopherol were purchased from Sigma. Pyrocatechole, Tween-40, Folin-Ciocalteau's phenol reagent (FCR), sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck. All other unlabeled chemicals and reagents were analytical grade.

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 DPPH. This spectrophotometric assay uses the stable radical DPPH as a reagent (Cuendet *et al*., 1997; Burits and Bucar, 2000). Various concentrations of the extracts (1 ml) in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After 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:

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

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. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate.

β-Carotene-linoleic acid assay. In this assay, antioxidant capacity was determined by measuring the inhibition of the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius *et al*., 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 linoleic acid and 200 mg 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. This reaction mixture (4 ml) 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 ^{0}C. The same procedure was repeated with synthetic antioxidant, 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, BHT, α-tocopherol and blank. Tests were carried out in triplicate.

Determination of total phenolic compounds. Total soluble phenolics in the mushroom ethanolic extracts were determined with Folin-Ciocalteau reagent according to the method of Slinkard and Singleton (1977) using pyrocatechol as a standard. Briefly, 1 ml from extract solution (2000 ppm) was transferred into a volumetric flask of 50 ml, and made up to 46 ml with distilled water. Folin-Ciocalteau reagent (1 ml) was added and the contents of 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 extracts determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph is given as:

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

Determination of total flavonoid concentration. Flavonoid concentration was determined as follows: 1 ml mushroom ethanolic extracts solution (2 mg/ml) was diluted with 4.3 ml of 80% aqueous ethanol and test tubes was

added to 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 *et al*., 1997):

Absorbance = 0.002108 µg quercetin – 0.01089
(R^2: 0.9999)

Screening of antimicrobial activity of mushroom samples. 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 enterocolitica* RSKK 1501, *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* RSKK 96026, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 12598, *Micrococcus luteus* NRRL B-4375, *Micrococcus flavus, Bacillus subtilis* ATCC 6633, *Bacillus cereus* RSKK 863, *Candida albicans* (clinical isolate). The bacteria were obtained from the culture collection of the Microbiology Department of Pamukkale University and Ankara University. All pathogenic bacteria used as test organisms were inoculated in Nutrient broth (Acumedia Manufacturers, Inc., Maryland) and *Candida albicans* was incubated in Yeast Extract Peptone Dextrose (YEPD) broth (Difco). *Micrococcus luteus* NRRL B-4375 and *Micrococcus flavus* were incubated at 28 ± 0.1 °C for 24 h; the others, which are pathogen bacteria, were incubated at 30 ± 0.1 °C for 24 h; the yeast *Candida albicans* was incubated at 28 ± 0.1 °C for 48 h. Final concentration of the cells varies from 10^{-7} to 10^{-8} CFU ml^{-1}. The agar-well diffusion method was used to detect the antimicrobial activity of extract. Cultures of bacteria and yeast (100 ml) were poured into sterilised Petri dishes (10 x 100 mm diameter) and then Nutrient agar (NA) and YEPD agar (20 ml) were distributed homogeneously. For the investigation of the antibacterial and anticandidal activity, the dried mushroom extract were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 20 mg/100 ml and sterilised by filtration through a 0.22 mm membrane filter. Each sample (100 ml) was filled into the wells of agar plates (Ali-Shtayeh *et al*., 1998; Tepe *et al*., 2005). After incubation, the plates were examined for the presence and diameter (mm) of inhibition zones around the wells. Reference discs used for control were as follows: nystatin (100 U), ampicillin (10 mg), penicillin (10 U), gentamicin (10 mg) and oxacillin (1 mg). All determinations were done in duplicate and inhibitory activity of DMSO was also tested.

RESULTS AND DISCUSSION

Antioxidant assay. The ethanolic extract was investigated 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 amount. DPPH a stable free radical with a characteristic absorption at 517 nm, was used to study the radical-scavenging effects of ethanolic extract. As antioxidants donate protons to these radicals, the absorption decreases. Free radical DPPH may gain an electron or hydrogen radical to get stable state (Soares *et al*., 1997). It is supposed that the effects of antioxidants to eradicate DPPH radicals stem from their hydrogen giving ability. The decrease in absorption 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. Since the reaction followed a concentration dependent pattern, only concentrations of active extracts providing 50% inhibition were included. The free radical scavenging activity of ethanol extract was (IC_{50}: 212 µg/ml). The ethanol extract of *L. nuda* showed an inhibition similar to that of synthetic antioxidants such as BHT, BHA and α-tocopherol (Fig. 1).

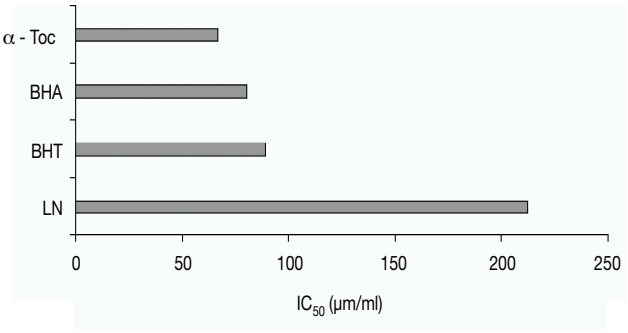

FIG. 1 – Free radical scavenging capacities of the extracts measured in DPPH assay.

The presence of antioxidant compounds in the system or their addition into system enables neutralization of peroxide compounds that are oxidation products of linoleic acid. Consequently, colour of β-carotene is preserved. The higher absorbance of the samples shows the higher oxidant activity (Dapkevicius *et al*., 1998). Linoleic acid oxidation was compared with those of *L. nuda* ethanol extract, α-tocopherol and BHA. It was found that inhibition values of both *L. nuda* ethanol extract and the standards increased with concentration. For example; in 80 µg/ml concentration, *L. nuda* extract, BHA and α-tocopherol showed 55.4%, 96.4%, 98.6% of inhibition where as in 160 µg/ml concentrations those were 84.3%, 98.9%, 99.2% of inhibition (Fig. 2).

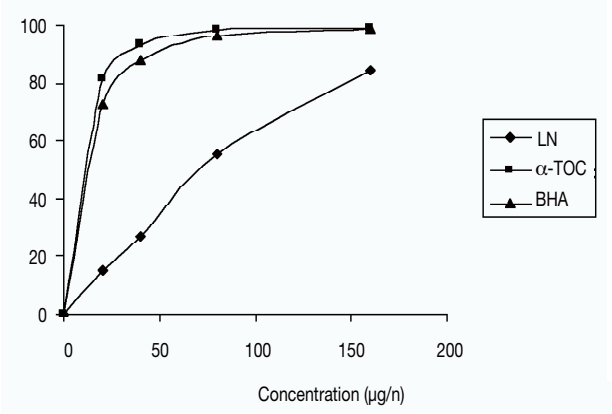

FIG. 2 – Total antioxidant activity of BHA, α-tocopherol and different doses of ethanolic extract mushroom the linoleic acid emulsion.

As a conclusion, we can say that this mushroom extract compete with BHA and α-tocopherol method in β-caroten-linoleic acid system used to determine the antioxidant capacity of *L. nuda* ethanol extract. According to this, it is possible that the high inhibition value of *L. nuda* extract is due to the high concentration of phenolic compounds.

TABLE 1 – Amounts of total flavonoid and total phenolic compounds in *Lepista nuda* ethanolic extracts

Sample	Total phenolic compounds (pyrocatechol equivalent, µg mg^{-1})	Total flavonoid content (quercetin equivalent, µg mg^{-1})
Control (Ethanol)	–	-
Ethanol extract	48.01 ± 0.29	8.21 ± 0.56

Data expressed as mean ± S. D. of three samples analysed separately.

Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen *et al*., 1993; Gülçin *et al*., 2003). The phenolic compounds may contribute directly to antioxidative action (Duh *et al*., 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1 g daily ingested from a diet rich in fruits and vegetables (Tanaka *et al*., 1998). It was reported that polyphenolic compounds are of significance to prevent lipid oxidation, and with increased *L. nuda* ethanolic extract amount of which the antioxidation effect also increased (Ramarathnam *et al*., 1986; Velioğlu *et al*., 1998). Therefore, in determination of antioxidant effect of a substance, determination of amount of phenolic compounds contents is of great significance based on the absorbance values of the various extract solutions, reacting with Folin-Ciocalteau reagent and compared with the standard solutions of pyrocatechol equivalents, as described above. The results of the colorimetric analysis of total phenolics are given in Table 1. Total phenolic content of *L. nuda* ethanol extract (1 mg) was found as 48.01 ± 0.29 µg pyrocatechol equivalent. The phenolic compounds may contribute directly to antioxidative action (Duh *et al*., 1999; Tepe *et al*., 2004).

The development of the antioxidant supplement industry has created a need for reliable antioxidant assays to measure the real antioxidant activity of a product. Some compounds with flavonoid structure have recently been used to help hearth health, and as an antioxidant compound (Hunter and Fletcher, 2002). It is also known that flavonoids increases blood flow into brain, arms and legs, which is beneficial for the old people.

In contrast to this, the total flavonoid compound concentration was measured as 8.21 ± 0.56 µg mg^{-1} quercetin equivalent (Table 1). The key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Komali *et al*., 1999; Moller *et al*., 1999). 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 *L. nuda*, it is thought that high level of free radical scavenging activity and total antioxidant activity may result from the existence of phenolic and flavonoid-type compounds.

Antimicrobial activity of extracts. The antimicrobial activity of *L. nuda* extract was tested *in vitro* by using the agar-well diffusion method with the indicator microorganisms reported in Table 2.

It was observed that *L. nuda* extract did not demonstrate antibacterial activity against *Pseudomonas aeruginosa*, *Morganella morganii, Klebsiella pneumoniae*, *Proteus vulgaris* and *Bacillus subtilis* as the *L. nuda* extract showed antibacterial activity against *Micrococcus luteus*, *Micrococcus flavus*, *Staphylococcus aureus* ATCC 12598 and ATCC 25923, *Bacillus cereus* RSKK 863, *Yersinia enterocolitica* RSKK 1501, *Salmonella enteritidis*, *Escherichia coli*. The highest activity of *L. nuda* extract was observed against *Micrococcus luteus* and *Micrococcus flavus*. The diameters of growth inhibition zones in this extract varies from 4 to 20 mm against the medically important pathogens such as *Staphylococcus aureus* ATCC 12598 (10 mm), *Staphylococcus aureus* ATCC 25923 (6 mm), *Yersinia enterocolitica* (10 mm), *Escherichia coli* (4 mm) and *Salmonella enteritidis* (5 mm).

The ethanol extract of *Morchella conica* was found active on *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 12598. The *Morchella conica* ethanol extract did

TABLE 2 – Antimicrobial activities (zone size, mm) of the *Lepista nuda* extract and antibiotic sensitivity of microorganisms

Test bacteria	LN	N	A	P	G	O
Pseudomonas aeruginosa NRRL B-23	-	nt	nt	nt	16	nt
Salmonella enteritidis RSKK 171	5 ± 1	nt	-	nt	nt	nt
Escherichia coli ATCC 35218	4 ± 0	nt	10	11	nt	nt
Morganella morganii	-	nt	nt	nt	-	nt
Yersinia enterecolitica RSKK 1501	10 ± 0	nt	20	18	nt	nt
Klebsiella pneumoniae ATCC 27736	-	nt	-	nt	nt	nt
Proteus vulgaris RSKK 96026	-	nt	-	nt	nt	nt
Staphylococcus aureus ATCC 25923	6 ± 2	nt	nt	31	nt	21
Staphylococcus aureus ATCC 12598	10 ± 0	nt	nt	28	nt	18
Micrococcus luteus NRRLB-4375	13 ± 1	nt	30	31	nt	22
Micrococcus flavus	20 ± 2	nt	29	31	nt	24
Bacillus subtilis ATCC 6633	-	nt	nt	12	nt	8
Bacillus cereus RSKK 863	11 ± 1	nt	nt	22	nt	14
Candida albicans	-	19	nt	nt	nt	nt

LN: *Lepista nuda* extract; N: nystatin (100 U), A: ampicillin (10 mg), P: penicillin (10 U), G: gentamicin (10 mg), O: oxacillin (1 mg), nt: not tested, (-): no inhibition.

not exhibit anticandidal activity against *Candida albicans* (Turkoglu *et al*., 2006a). The ethanol extract of *Laetiporus sulphureus* showed narrow antibacterial activity against Gram-negative bacteria and strongly inhibited the growth of the Gram-positive bacteria (Turkoglu *et al*., 2006b).

Dulger *et al*. (2002) reported that *Candida albicans* was resistant to the action of the methanolic extract of *Lepista nuda*, while the culture fluid of *Lentinus edodes* showed poor activity against *Candida albicans* (Hatvani, 2001). In our study, *L. nuda* extract did not show anticandidal activity against *Candida albicans* and this result was in agreement with those of Dulger *et al*. (2002).

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

In conclusion, the results of this study indicated that the extracts of *L. nuda* have a capacity to scavenge free radicals, and to inhibit the growth of pathogenic microorganisms. Therefore, they could be used as antimicrobial and antioxidative agents in the food industry.

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