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Antioxidant and Antimicrobial Activity of *Russula delica* Fr: An Edidle Wild Mushroom

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Abstract

Antioxidant capacity and antimicrobial activities of *Russula delica* Fr. (RD) extracts obtained with ethanol were investigated in this study. Four complementary test systems, namely DPPH free radical scavenging, β-carotene/linoleic acid systems, total phenolic compounds and total flavonoid concentration, have been used. It was observed that inhibition values of both RD ethanolic extract and the standards (BHA and α-tocopherol) increased in parallel with the elevation of concentration in linoleic acid system. Total flavonoid amount was 8.71 ± 0.56 μg mg^{-1} quercetin equivalents while the phenolic compound amount was 47.01 ± 0.29 μg mg^{-1} pyrocatechol equivalents in the extract. The antimicrobial activity of RD extract was tested in vitro by using the agar-well diffusion method. In our study, the RD extract showed antibacterial activity against. The RD extract did not exhibit anticandidal activity against *Candida albicans*. Therefore, the extracts could be suitable as antimicrobial and antioxidative agents in the food industry.

Keywords: *Russula delica*, Mushroom, phenolic compounds, Antioxidant and antimicrobial activity, DPPH

1. Introduction

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 [1]. 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 [2, 3]. It has been known for many years that

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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 [4]. 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 [5]. 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. However, the antioxidants present in human diet are of great interest as possible protective agents to help the human bodies reduce oxidative damage.

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Since ancient times, spices in different types of food to improve favours are well known for their antioxidant capacities [6]. In recent decades, the essential oils and various extracts of plants have been of great interest as they have been the sources of natural products. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. But according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms [7, 8]. For this reason, governmental authorities and consumers are concerned about the safety of the food and also about the potential effects of synthetic additives on health [9]. The many pharmacological effects of phenolic compounds and flavonoids are linked to their ability to act as strong antioxidants and freeradical scavengers, to chelate metals, and to interact with enzymes, adenosine receptors, and biomembranes [10]. It was reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds [11, 12]. Phenolic compounds, especially phenolic acids and flavonoids, are ubiquitously present in vegetables, fruits, seeds, tea, wines and juices; thus they are an integral part of the human diet. Recently, they have received much attention since many epidemiological studies suggest that consumption of polyphenol-rich foods and beverages is associated with a reduced risk of cardiovascular diseases, stroke and certain forms of cancer. These protective effects have partly been ascribed to the antioxidant properties especially of flavonoids [11, 13, 14, 15, 10, 16, 17, 18]. Among flavanone aglycons, naringenin, hesperetin, eriodictyol and isosakuranetin are the most common, but they are present in much smaller quantities than are glycosides. Citrus flavonoids, especially

hesperidin, have shown a wide range of therapeutical properties such as anti-inflammatory, antihypertensive, diuretic, analgesic and hypolipidemic activities [19, 20, 21, 14, 22].

Researchers showed antimicrobial activity of several mushrooms [23, 24, 25, 26, 27, 28, 29]. The chloroform and ethyl acetate extracts of the dried mushroom have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* [30]. Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity [31]. 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 for searching new antimicrobial substances from various sources which are the good sources of novel antimicrobial chemotherapeutic agents [32]. Infusion of this macrofungus is used to prevent beriberi. In addition, the decoction is used for the treatment of abscesses and wounds [33].

Russula delica is a well known and used as food in Turkey. The cap is domed at first, later expands and flattens. Mature specimens become funnel shaped with inrolled margin. The cap is whitish. The gills are decurrent and white. Spores white. The stem is white and tough. It grows in wooded habitats under both coniferous and deciduous trees. *Russula delica* is picked up every year if the weather condition is suitable to grow in Turkey.

To the best of our knowledge, no research has been available on antioxidant activities of the RD extract in literature. Therefore, the aim of the present work is to evaluate the antioxidant and antimicrobial potential of ethanol extracts of *Russula delica* on several microorganisms of medicinal importance.

2. Materials and Methods

2.1. Mushroom

Russula delica samples were collected from Uşak, located in the western part of Turkey in the autumn of 2006. Identification and classification of macrofungus were carried out by mycologist, Dr Aziz Turkoglu and all specimens were deposited at the laboratory of Department of Science Education, Pamukkale University, Denizli, Turkey. Fresh mushroom were randomly divided into three samples of 150 g and airdried in an oven at 40 ^{o}C before analysis. Dried mushroom sample (50 g) was extracted by stirring with 500 mL of ethanol at 30 ^{o}C at 150 rpm for 24 h and filtering through Whatman No. 4 filter paper. The residue was then, extracted with two additional 500 mL of ethanol as described above. The combined ethanolic extract were then

rotary evaporated at 40 ^{o}C to dryness, redissolved in ethanol to a concentration of 10 mg mL^{-1} and stored at 4 ^{o}C for further use.

2.2. Antioxidant activity

2.2.1. Chemicals

β-carotene, linoleic acid, 1,1-Diphenly-2-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), buthylated hydroxyanisol (BHA) and α-tocopherol were purchased from Sigma (Sigma, Aldrich GmbH, Sternheim, GERMANY). Pyrocatechole, Tween-20, Folin-ciocalteu's phenol reagent (FCR), sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck (Darmstat, GERMANY). All other unlabeled chemicals and reagents were 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^{1} diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [34, 35]. One thousand microlitres 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:

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

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.

2.2.3. β-Carotene-linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [36]. 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 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 50^0C temperature. The same procedure was repeated with synthetic antioxidant, BHT, BHA, α-tocoferol , 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-tocoferol and blank.

2.2.4. 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 [37] using pyrocatechol as a standard. Briefly, 1 mL of extract solution (contains 2000µg) in a volumetric flask diluted glass-distilled water (46 mL). Folin-Ciocalteu reagent (1 mL) was added and the contents of flask were mixed thoroughly. After 3 min, 3mL 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 standart pyrocatechol graph is given as:

Absorbance = 0.00246 µg pyrocatechol + 0.00325 (R^2: 0.9996)

2.2.5. Determination of total flavonoid concentration

Flavonoid concentration was determined as follows: mushroom ethanolic extracts solution (1 mL) was diluted with 4.3 mL of 80% aqueous ethanol and test tubes was added to containing 0.1 mL of 10% aluminum 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 standart [38].

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

2.3. Antimicrobial activity

2.3.1. 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* 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.

2.3.2. Screening of antimicrobial activity of mushroom extract

Antimicrobial activity of ethyl alcohol extract of *Laetiporus sulphureus* was determined by the agar-well diffusion method. All the microorganisms mentioned above were incubated at 37±0.1 ^{o}C (30±0.1 ^{o}C for only *M. luteus* NRRL B-4375 and *M. flavus*) for 24 h by inoculation into Nutrient broth. *C. albicans* was incubated YEPD broth at 28±0.1 ^{o}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 (10x100 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 extract were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 20% and sterilized by filtration through a 0.22 μm membrane filter [39, 40, 28, 29]. Each sample (100 μL) was filled into the wells of agar plates directly. Plates injected with the yeast cultures were incubated at 28 ^{o}C for 48 h, and the bacteria were incubated at 37 ^{o}C (30 ^{o}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 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 are as follows: Nystatin (100 U), Ketoconazole (50 μg), Tetracycline (30 μg), Ampicillin (10 μg), Penicillin (10 U), Oxacillin (1 μg), Tetracycline (30 μg) and Gentamicin (10 μg). All determinations were done duplicate.

3. Results and discussion

3.1. Antioxidant activity of extracts

The ethanolic extract of mushroom was 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, total flavonoid concentration have been 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 absorption decreases. 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. All concentration studied showed free radical scavenging activity. The 50% of inhibition value for RD ethanol extract seems to be fairly significant when compared to commonly used synthetic antioxidant BHT, BHA and α-tocopherol. (IC_{50}= 207.09 µg/mL ethanolic extract was necessary to obtain 50% of DPPH degradation).

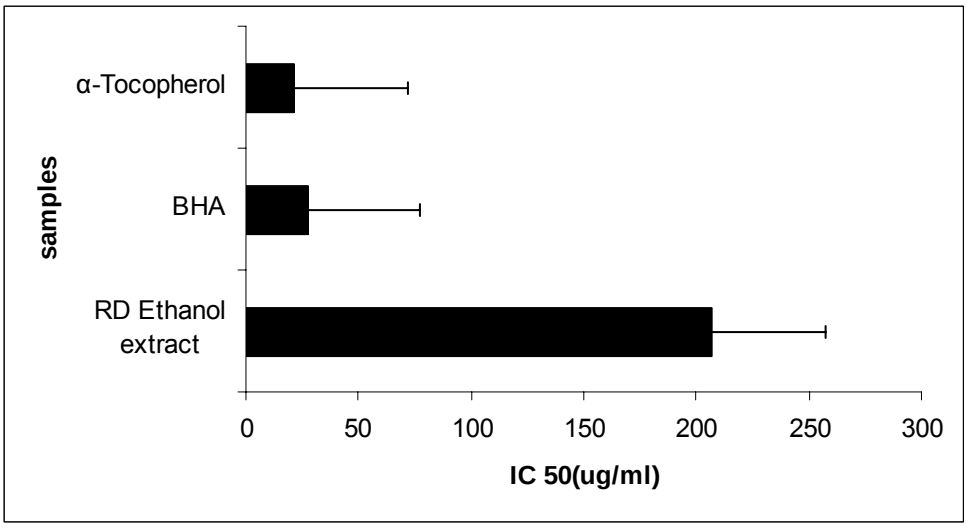

Fig 1. Free radical scavenging capacities of the ethanolic extract of *R. delica* measured in DPPH assay.

Linoleic acid oxidation was compared with those of RD ethanol extract, α-tocopherol and BHA. It was found that inhibition values of RD ethanol extract and the standards increased with concentration IC_{50}= 207.09 µg/mL., Fig. 2). 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 RD ethanol extract.

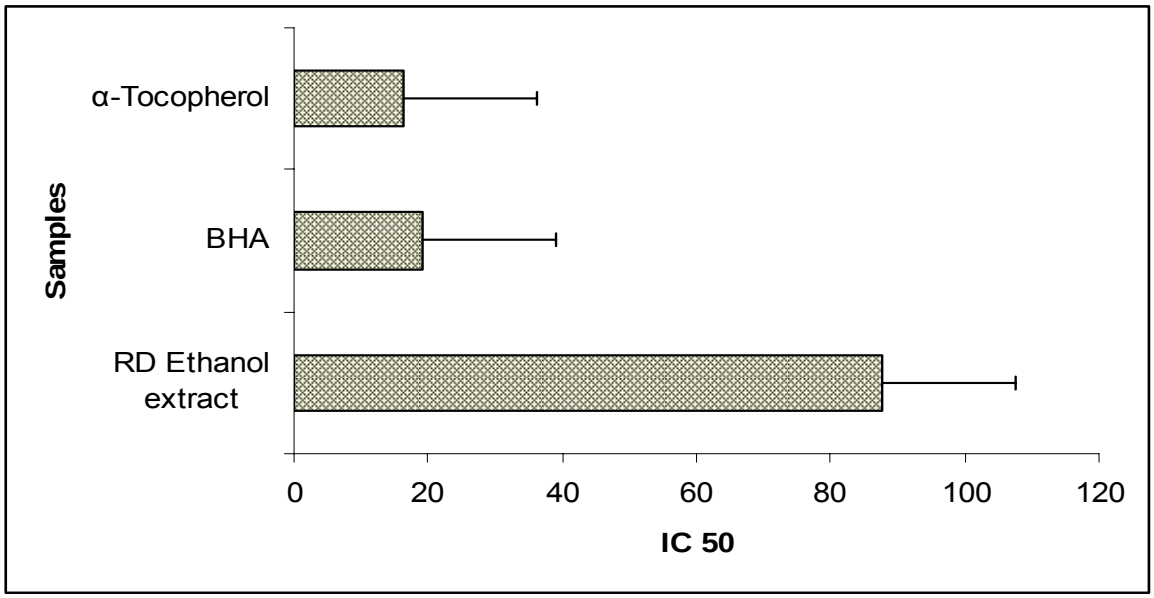

Fig 2. Total antioxidant activity of BHA, α-tocopherol and different doses of *R. delica* ethanolic extract in the linoleic acid emulsion.

The total phenolic compound amount was calculated as quite high RD ethanol extract (47.01 ± 0.29 μg mg^{-1} pyrocatechol equivalent). According to this, it is possible that the high inhibition value of RD extract is due to the high concentration of phenolic compounds. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports [41, 42]. Phenols are important components of plants. They were reported to eliminate radicals due to their hydroxyl groups [43], and they contribute directly to antioxidant effect of system [44]. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity [11, 45]. The phenolic compounds may contribute directly to antioxidative action [44]. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables [46]. In contrast to this, the total flavonoid compound concentration was measured as 8.71 ± 0.56 μg mg^{-1} quercetin equivalent. Like phenol compounds, the contribution of flavonoids to antioxidant activity was known. It has been reported that as compared to BHT I3, II8-biapigenin and hypericine which have the structure of biflavanoid have a very high antioxidant effect. This effect was proposed to stem from hydroxyl groups in the structure of flavonoids [11].

Table 1. Amounts of total flavonoid and total phenolic compounds in *Russula delica* ethanolic extracts.

Extracts	Total phenolic compounds [pyrocatechol equivalent (μg mg^{-1})]	Total flavonoid content [quercetin equivalent (μg mg^{-1})]
Control	-	-
Ethanol extract	47.01 ± 0.29	8.71 ± 0.56 [a]

Data expressed as mean ± s.e.m. of three samples analysed separately.

[a]Standard deviation

3.2. Antimicrobial activity of extracts

The antimicrobial effect of ethanol extracts of *R. delica* was tested against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and one species of yeast (Table 2). As can be seen from the results, *R. delica* had a narrow antibacterial spectrum against microorganisms. The test extract showed more potent activity against Gram-positive than Gram-negative bacteria. The antimicrobial activities were comparable with those of commonly used antibiotics and antifungal against these microorganisms. In general, reference discs are more active than ethyl alcohol extract of *R. delica*. But, microorganisms get resistant to the antibiotics after sometime. Therefore, *R. delica* may be used as source of antimicrobial agents.

Table 2. Antimicrobial activity of ethyl alcohol extract of *R. delica* and antibiotic sensitivity of microorganisms (zone size, mm)

Test bacteria	**RD**	**N**	**A**	**P**	**G**	**O**	**T**
Pseudomonas aeruginosa NRRL B-23	-	NT	NT	NT	16	NT	8
Salmonella enteritidis RSKK 171	5 ± 1	NT	-	NT	NT	NT	12
Escherichia coli ATCC 35218	-	NT	10	11	NT	NT	8
Morganella morganii	-	NT	NT	NT	-	NT	-
Yersinia enterecolitica RSKK 1501	4 ± 0	NT	20	18	NT	NT	7
Klebsiella pneumoniae ATCC 27736	4 ± 0	NT	-	NT	NT	NT	5
Proteus vulgaris RSKK 96026	-	NT	-	NT	NT	NT	16
Staphylococcus aureus ATCC 25923	-	NT	NT	31	NT	21	20
Staphylococcus aureus Cowan I	8 ± 0	NT	NT	28	NT	18	21
Micrococcus luteus NRRL B-4375	15.5 ± 0	NT	30	31	NT	22	19
Micrococcus flavus	20 ± 0	NT	29	31	NT	24	20
Bacillus subtilis ATCC 6633	5 ± 1	NT	NT	12	NT	8	17
Bacillus cereus RSKK 863	9 ± 1	NT	NT	22	NT	14	19
Candida albicans	8 ± 0	19	NT	NT	NT	NT	NT

RD: *Russula delica*, N: Nystatin (100 U), A: Ampicillin (10 μg), P: Penicillin (10 U), G: Gentamicin (10 μg), O: Oxacillin (1 μg), T: Tetracycline (30 μg), NT: Not tested, (-): No inhibition

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