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RESEARCH ARTICLE

Evaluation of Antioxidant Activities of 3 Edible Mushrooms: *Ramaria flava* (Schaef.: Fr.) Quél., *Rhizopogon roseolus* (Corda) T.M. Fries., and *Russula delica* Fr.

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Abstract The methanolic extracts of *Ramaria flava, Rhizopogon roseolus*, and *Russula delica* were analyzed for their antioxidant activities in different test systems including β-carotene/linoleic acid, 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, reducing power, and metal chelating activities in addition to their total phenolic and flavonoid contents. In the first case, methanol extract of *R. roseolus* showed the strongest activity. In DPPH system, the scavenging effects increased with the concentration. The reducing power of the mushroom also increased with concentration. Chelating effect was 96.75±0.28% for *R. flava*. In the case of total phenolic and flavonoid assays, *R. flava* found to have the highest phenolic content. Total flavonoid content of *R. flava* again found the superior to the other mushrooms. Experimental results indicate that the mushroom species evaluated here can be consumed safely. On the other hand, knowing the biological activity of these mushrooms will contribute to the establishment of conscious consumption.

Keywords: *Ramaria flava, Rhizopogon roseolus, Russula delica,* antioxidant activity, edible mushroom

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Introduction

Reactive oxygen species are formed during normal cellular metabolism, but when present in high concentration they become toxic. Mammalian cells possess intracellular defences such as superoxide dismutase, catalase, or glutathione peroxidase, in order to protect the cells against excessive levels of free radicals. Also exogenous addition of compounds such as vitamins (A, E, β-carotene), minerals (selenium, zinc), or proteins (transferrin, ceruloplasmin, albumin) can provide additional protection (1). These natural antioxidants or other compounds that can neutralize free radicals may be of central importance in the prevention of vascular diseases, some forms of cancer (2,3), and oxidative stress responsible for DNA, protein, and membrane damage. Superoxide, hydrogen peroxide, and hydroxyl radicals, which are mutagens produced by radiation, are also by-products of normal metabolism. Lipid peroxidation is also a major cause of food deterioration, affecting color, flavor, texture, and nutritional value (4). Even though it is unclear whether active compounds are active against free radicals after being absorbed and metabolized by cells in the body, radical scavenging assays have gained acceptance for their capacity to rapidly screen materials of interest.

Mushrooms have been used for traditional foods and medicines in Asia (5). Generally, mushrooms are rich in dietary fiber, minerals, vitamins, and low in fat (6,7). Moreover, mushrooms contain various polyphenolic compounds recognized as an excellent antioxidant (8). Several important compounds including bioactive polysaccharides (lentinan), dietary fiber, ergosterol, vitamin B_1, B_2, C, and minerals have been isolated from the fruiting body, mycelia, and culture medium of the mushrooms. Recent numerous studies have shown their

medicinal attributes including anti-tumor, antimicrobial, liver function improving, and cholesterol lowering activities (9-11).

In recent years, several undesirable disorders have developed, due to the side effects of the use of synthetic antioxidants commonly used in the food and food-flavoring industries. This situation has forced scientists to search for new antioxidant substances from various plants which are good sources of novel antioxidant agents.

The mushroom species evaluated here have been collected by the local people and they are often offered for sale in public market. A large part of the people consumes these mushrooms with pleasure. Knowing the biological activity of these mushrooms will contribute to the establishment of conscious consumption.

The reason for this study is that antioxidant activities of the mushrooms given here have not previously been reported in the literature although Anatolian people have been using them as food for a long time. Therefore, the aim of present work is to evaluate the antioxidant potentials of methanol extracts of *Ramaria flava, Rhizopogon roseolus,* and *Russula delica* by 4 different antioxidant test systems namely β-carotene/linoleic acid, DPPH, reducing power, and chelating effect, in addition to their total phenolic and flavonoid contents.

Materials and Methods

Materials Potassium ferricyanide, ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), Folin-Ciocalteu's reagent, methanol, and trichloroacetic acid (TCA) were obtained from E. Merck (Darmstadt, Germany). 1.1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), and α-tocopherol were obtained from Sigma-Aldrich (Sternheim. Germany). All other chemicals and solvents are of analytical grade.

Mushrooms Fruiting bodies of mushrooms were collected in 2004 from Mugla, Turkey and were authenticated based on their microscopic and macroscopic characteristics by Dr. M. Halil Solak, Program of Fungi, Ula Ali Kocman Vocational School, Mugla University, Mugla, Turkey. The voucher specimens have been deposited at the Mugla University Ula Ali Kocman Vocational School Herbarium Laboratory (Voucher No: *Ramaria flava* MHS 1651, *Rhizopogon roseolus* MHS 1756, *Russula delica* MHS 2573).

Preparation of methanolic extract The air-dried fruiting bodies of mushroom samples (5 g) were extracted by stirring them with 100 mL of methanol at 25°C at 150 rpm for 24 hr and filtering through filter paper. The residue was then extracted with 2 additional 100 mL of methanol as described above. The combined methanol extracts were then rotary evaporated at 40°C to dryness and kept in the dark at 4°C until tested. Extract yields of the mushroom samples were 18.09, 32.50, and 24.13%(w/w), respectively.

Total antioxidant activity by β-carotene/linoleic acid method 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 (12). A stock solution of β-carotene/linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1 mL of chloroform (high performance liquid chromatography, HPLC grade). Twenty-five μL linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL of oxygenated distilled water was added with vigorous shaking; 2.5 mL of this reaction mixture was dispersed to test tubes and 350 mL portions of the extracts prepared in methanol at 20 mg/mL concentrations were added and the emulsion system was incubated for up to 2.5 hr at 50°C. The same procedure was repeated with the positive control BHT, and a blank. After this incubation period, absorbance of the mixtures was measured at 490 nm. Measurement of absorbance was continued until the color of β-carotene disappeared. The bleaching rate (R) of β-carotene was calculated according to Eq. 1.

$$R = ln(a/b)/t \quad (1)$$

Where, ln=natural log, a=absorbance at time t (0), b=absorbance at time t (150 min) (13). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Eq. 2.

$$AA = [(R_{Control} - R_{Sample})/R_{Control}] \times 100 \quad (2)$$

Antioxidant activities of the extracts were compared with those of BHT at 2 mg/mL and blank consisting of only 0.35 mL methanol.

Scavenging effect on DPPH The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds were measured from the bleaching of purple colored methanol solution of DPPH. The effect of methanolic extracts on DPPH radical was estimated according to Kirby and Scmidt (14). Briefly, a 0.004% solution of DPPH radical solution in methanol was prepared and then, 4 mL of this solution was mixed with 1 mL of various concentrations (2-12 mg/mL) of the extracts in methanol. Finally, the samples were incubated for 30 min in the dark at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm using a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan). Inhibition of free radical DPPH in percent (I %) was calculated in following way:

$$I\ \% = 100 \times (A_{Control} - A_{Sample})/A_{Control}$$

Where, $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound. BHT, quercetin, and α-tocopherol were used as a control.

Reducing power The reducing power was determined according to the method of Oyaizu (15). Each extract (1-6 mg/mL) in methanol (1 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricynide and the mixture was incubated at 50°C for 20 min. Then, 2.5 mL of 10% TCA were added, and the mixture was centrifuged at 200×g (Mistral 2000; MSE, London, UK) for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% $FeCl_3$. Finally the absorbance was measured at 700 nm against a blank. BHT, quercetin, ascorbic acid, and α-tocopherol were used as the controls.

Chelating effects on ferrous ions The chelating effect was determined according to the method of Decker and Welch (16). Briefly, 2 mL of various concentrations (0.5-2.0 mg/mL) of the extracts in methanol was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Total volume was adjusted to 5 mL with methanol and then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrica at 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Metal chelating effect } (\%) = [(A_{Control}/A_{Sample})/A_{Control}] \times 100$$

Where, $A_{Control}$ is the absorbance of control (The control contains $FeCl_2$ and ferrozine complex formation molecules) and A_{Sample} is the absorbance of the test compound. BHT, quercetin, and α-tocopherol were used as the controls.

Assay for total phenolics Total phenolic constituent of the methanol extracts were determined by employing the methods given in the literature (17,18) involving Folin-Ciocalteu reagent and gallic acid as standard. One mL of extract solution containing 2,000 μg extract was added to a volumetric flask. A 45 mL distilled water and 1 mL Folin-Ciocalteu reagent was added and flask was shaken vigorously. After 3 min, a 3 mL of Na_2CO_3 (2%) solution was added and the mixture was allowed to stand for 2 hr by intermittent shaking. Absorbance was measured at 760 nm. The concentrations of phenolic compounds were calculated according to the following equation that was obtained from the standard gallic acid graph:

$$\text{Absorbance} = 0.003044 \text{ gallic acid } (\mu g) + 0.0030676 \quad (R^2 = 0.999297)$$

Assay for total flavonoids Total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand *et al.* (19). Briefly, 1 mL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the methanolic extracts (2,000 μg). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 mL extract solution with 1 mL methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph:

$$\text{Absorbance} = 0.030166 \text{ quercetin } (\mu g) - 0.014785 \quad (R^2 = 0.99858)$$

Results and Discussion

Antioxidant activity in β-carotene/linoleic acid system Polyunsaturated fatty acids, such as linoleic acid, are easily oxidized by the oxygen in the air. This auto-oxidation leads to the occurrence of chain reactions with the formation of coupled double bonds, and at a later stage also to obtaining secondary products, such as aldehydes, ketones, and alcohols. Using the β-carotene/linoleic acid method, methanolic extracts of 3 edible mushroom species showed different patterns of antioxidant activities (Fig. 1). As can be seen from the figure, methanol extract of *R. roseolus* showed the strongest linoleic acid inhibition capacity (96.09±0.38%) at 20 mg/mL concentration, which is almost equal to the synthetic antioxidant BHT (96.17±0.50%) at 2 mg/mL concentration. This activity was followed by *R. flava* and *R. delica* with the inhibition percentages of 95.02±0.29 and 80.97±1.05%, respectively.

In previous studies, the antioxidant activities of methanolic

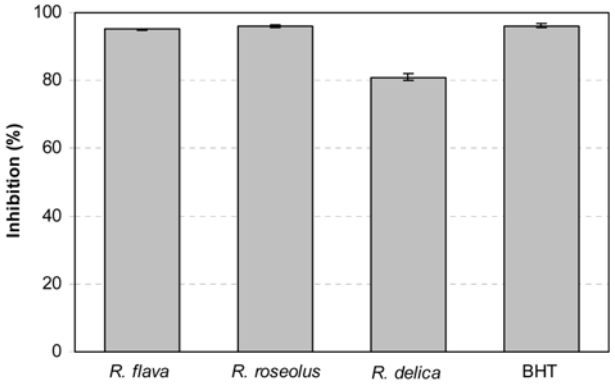

Fig. 1. Antioxidant activity (%) of the methanolic extracts of mushrooms and BHT measured by β-carotene/linoleic acid method. Values expressed are mean±SD of 3 parallel measurements.

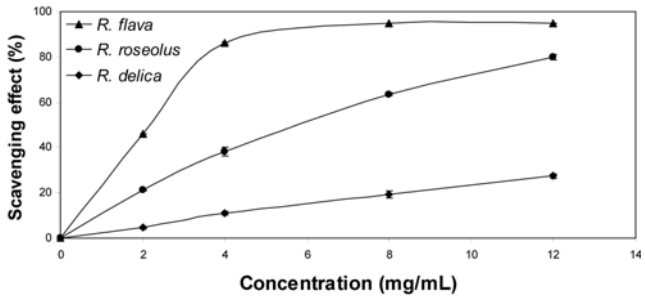

Fig. 2. Scavenging effect of the methanolic extracts of mushrooms on DPPH at different concentrations. Values expressed are mean±SD of 3 parallel measurements.

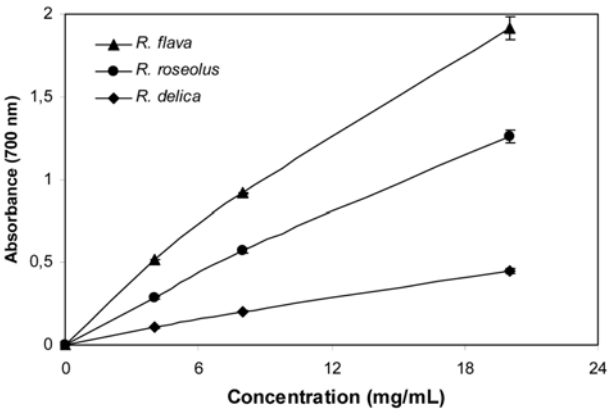

Fig. 3. Reducing power of the methanolic extracts of mushrooms at different concentrations. Values expressed are mean±SD of 3 parallel measurements.

extracts of several commercial and medicinal mushrooms have been reported (20,21). Those studies claimed that the methanolic extracts of mushrooms species showed high antioxidant activity on the lipid peroxidation.

Scavenging effect on DPPH The radical scavenging of mushrooms extracts was tested using a methanolic solution of the 'stable' free radical, DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (22). A freshly prepared DPPH solution exhibits a deep purple color with absorption maximum at 517 nm. This purple color generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless/bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time scale.

The radical scavenging activity values of methanolic extracts from the species evaluated here were examined and compared against one another (Fig. 2). From the analysis of Fig. 2, we can conclude that the scavenging effects of mushrooms methanolic extracts on DPPH radicals increased with the concentration increase and were high (94.78±0.06% at 12 mg/mL) for *R. flava*. Methanolic extract from *R. delica* presented moderate radical scavenging values (27.43±0.96% at 12 mg/mL) and lower than the other species, *R. roseouls* (79.98±1.30% at the same concentration). However, the scavenging effects of BHT, α-tocopherol, and quercetin (at 0.3 mg/mL) were 16.66±0.23, 30.41±0.37, and 92.04±1.13%, respectively.

Reducing power In the present study, assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (15).

The reducing power of mushrooms methanolic extracts as a function of their concentration is shown in Fig. 3. The reducing power of the mushroom methanolic extracts increased with concentration. The reducing power of the all species was excellent. At the concentration of 20 mg/mL, the reducing power was higher than 4 mg/mL and in the order *R. flava*>*R. roseolus*>*R. delica*. At the concentration of 20 mg/mL, the strongest reducer was determined as *R. flava* with a value of 1.915±0.072. Reducing power of BHT, α-tocopherol, quercetin, and ascorbic acid at 0.4 mg/mL were 1.267±0.083, 0.695±0.047, 2.713±0.012, and 2.598±0.026, respectively. The lowest reducing power was exhibited by the methanolic extract of *R. delica*. According to these results, *R. flava* was found as the better radical reducer for this system.

It was reported that the reducing power of mushrooms might be due to their hydrogen donating ability (23). Accordingly, the mushroom species evaluated here might contain higher amounts of reductone, which could react with free radicals to stabilize and block radical chain reactions.

Chelating effects on ferrous ions Metal ions can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food (24). The catalysis of metal ions also correlates with incidents of cancer and arthritis (25). Ferrous ions, the most effective pro-oxidants, are commonly

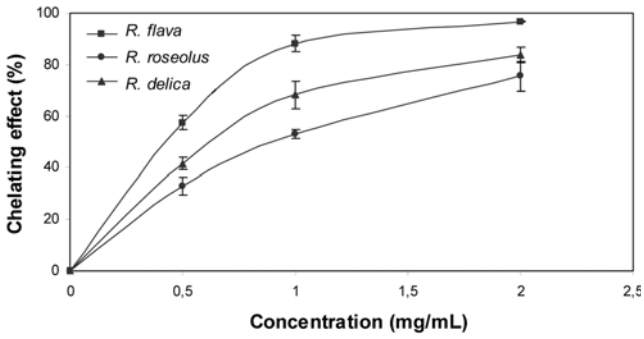

Fig. 4. Chelating effect of the methanolic extracts of mushrooms at different concentrations. Values expressed are mean±SD of 3 parallel measurements.

found in food systems (26). In the present study, the chelating ability of the mushroom extracts toward ferrous ions was investigated.

Figure 4 shows the chelating effects of the methanolic extract of 3 edible mushroom species. In this study, BHT, α-tocopherol, and quercetin were used as standards on ferrous ions. As can be seen from the Fig. 4, chelating capacity of the extracts was increased with the increasing concentration. Chelating effect was 96.75±0.28% for the methanol extract of *R. flava* at a concentration of 2.0 mg/mL. This is also the strongest chelating effect obtained from the extracts in this study. At this concentration, the lowest chelating effect was exhibited by *R. roseolus* extract (75.43±6.10%). All of the extracts evaluated here showed significantly higher chelating effects on ferrous ions than those of the standards BHT, α-tocopherol, and quercetin (68.20±1.25, 56.55± 0.66, and 29.98±0.99%, respectively) at the concentration of 0.50 mg/mL.

Ferrous ions could stimulate lipid peroxidation by Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (27). Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions (24). Accordingly, it is suggested that the low-to-moderate ferrous ions chelating effects of these extracts would be somewhat beneficial to protect against oxidative damage.

Assay for total phenolics and flavonoids Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic, and anti-carcinogenic activities. These activities may be related to their antioxidant activity (28). Thus, the total phenolic content of the edible mushrooms was also evaluated, using the Folin-Ciocalteu method.

Table 1. Total phenolic and flavonoid contents of the methanolic extracts of mushrooms

Mushroom	Phenolic content (μg GAE/mg extract)[1)]	Flavonoid content (μg QE/mg extract)[2)]
Ramaria flava	10.51±0.47[3)]	0.50±0.01
Rhizopogon roseolus	6.65±0.11	0.48±0.09
Russula delica	2.09±0.01	0.16±0.03

[1)]GAE, gallic acid equivalent
[2)]QE, quercetin equivalent
[3)]Values expressed are mean±SD of 3 parallel measurements.

As can be seen from the Table 1, *R. flava* found to have the highest phenolic content (10.51±0.47 mg GAE/mg extract) among the mushroom species evaluated. This is followed by *R. roseolus* with a value of 6.65±0.11 mg/mg. On the other hand, total flavonoid content of *R. flava* again found superior to the other mushrooms (0.50±0.01 mg QE/mg extract). The lowest flavonoid content was exhibited by the methanol extract of *R. delica* (0.16±0.03 mg/mg extract).

When the results obtained from the total phenolic assay is compared with those of 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 (29,30). The phenolic compounds may contribute directly to antioxidative action (31). 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 (32).

In conclusion, searching wild sources may bring new natural products into the food industry with safer and better antioxidants that provide good protection against the oxidative damage, which occurs both in the body and our daily foods. Therefore, new wild edible mushrooms, as natural sources, could be introduced for this purpose. As far as our literature survey could ascertain, there is no information about the mushroom species presented here. From this point of view, this study could be assumed as the first report on these species.

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