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Full Length Research Paper

Free-radical scavenging capacity and antimicrobial activity of wild edible mushroom from Turkey

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Antioxidant capacity and antimicrobial activities of *Ramaria flava* (Schaeff) Quél. (RF) 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. Inhibition values of *R. flava* extracts, BHA and α-tocopherol standards were found to be 94.7, 98.9 and 99.2%, respectively, at 160µg/ml. When compared the inhibition levels of ethanol extract of *R. flava* and standards in linoleic acid system, it was observed that the higher the concentration of both RF ethanol extract and the standards the higher the inhibition effect. Total flavonoid amount was 8.27±0.28 µg mg^{-1} quercetin equivalent while the total phenolic compound amount was 39.83±0.32 µg mg^{-1} pyrocatechol equivalent in the ethanolic extract. The ethanol extract of *R. flava* inhibited the growth of Gram-positive bacteria better than Gram-negative bacteria and yeast. The crude extract showed no antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Morganella morganii* and *Proteus vulgaris*. The antimicrobial activity profile of *R. flava* against tested strains indicated that *Micrococcus flavus, Micrococcus luteus* and *Yersinia enterocolitica* was the most susceptible bacteria of all the test strains. *R. flava* was found to be inactive against *Candida albicans*.

Key words: *Ramaria flava,* mushroom*,* antioxidant activity, antimicrobial activity, DPPH.

INTRODUCTION

The degenerative diseases associated with aging include cancer, cardiovascular disease, immune-system decline, brain dysfunction and cataracts (Ames et al., 1993). They are also associated with free radicals because oxidative damage to DNA, proteins and other macromolecules accumulates with age and has been postulated to be a major type of endogenous damage leading to aging (Fraga et al., 1990; Harman, 1981). Superoxide, hydrogen peroxide and hydroxyl radicals, which are mutagens produced by radiation, are also by-products of normal metabolism (Sies, 1986; Wagner et al., 1992). Besides giving rise to mutagenic lipid epoxides, hydroperoxides, alkoxyl and peroxyl radicals, lipid peroxidation is also a major cause of food deterioration, affecting colour, flavour, texture and nutritional value (Halliwell and Gutteridge, 1999). Vegetables and fruits are rich sources of antioxidants such as vitamin C, vitamin E and beta-carotene, which are suggested to be antiatherogenic in epidemiological studies (Enstrom et al., 1992; Rimm et al., 1993; Stampfer et al., 1993). Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular diseases, especially atherosclerosis. Phenolic compounds are other type of antioxidant that possesses a strong inhibition effect against lipid oxidation through radical scavenging (Frankel et al., 1993).

Mushrooms have long been appreciated for their flavour and texture. They are now recognized as a nutriatious food as well as an impotent source of biologically active compounds of medicinal value (Breene, 1990). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroides. Also, a mushroom phenolic compound

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has been found to be an excellent antioxidant and synergist that is not mutagenic (Ishikawa et al., 2001).

Ramaria flava is a well known and extraordinary mushroom species found in Turkey. It grows on soil in hardwood and conifer forests, as well as at forests. To the best of our knowledge, no research has available on chemical composition and biological activities of *R. flava* extract in literature. Therefore, the aim of the present work is to evaluate the antioxidant and antimicrobial potential of ethanol extract of the *R. flava* extract on several microorganisms that are medical importance.

MATERIALS AND METHODS

Mushroom

R. flava samples were collected from Kayseri, located in the middle Anatolia Region 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 *R. flava* representing a combination of young and old basidiocarps, were collected in the area in the spring in 2002. Fresh mushroom were randomly selected into three samples, 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 30oC 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 4oC for further use.

Chemicals

β-carotene, linoleic acid, 1,1-Diphenly-2-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), buthylated hydroxyanisol (BHA) and α-tocopherol were purchased from Sigma (Germany). Pyrocatechole, Tween-20, Folin-Ciocalteu's phenol reagent (FCR), sodium carbonate, ethanol, chloroform and the other chemicals and reagents were purchased from Merck (Germany). 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 1,1-diphenly-2-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits and Bucar, 2000; Cuendet et al., 1997). 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 Ablank is the absorbance of the control reaction (containing all reagents except the test compound), and Asample is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC50) 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 volatile organic compounds and 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 20 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen (30 min 100 ml/min) was 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 antioxidant 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.

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 and Singleton, 1977) using pyrocatechol as a standard. Briefly, 1 ml of extract solution (contains 2000µg/ml) 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 Na2CO3 (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:

Absorbance = 0.00246 µg pyrocatechol + 0.00325 (R2: 0.9996)

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 0.1 ml of 10% aluminum nitrate and 0.1 ml of 1 M aqueous potassium acetate were added. 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 (R2: 0.9999)

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, *Candida albicans* (clinical isolate) were used as test microorganisms. The bacteria were obtained from the culture collection of the Microbiology Department of Pamukkale Uni-

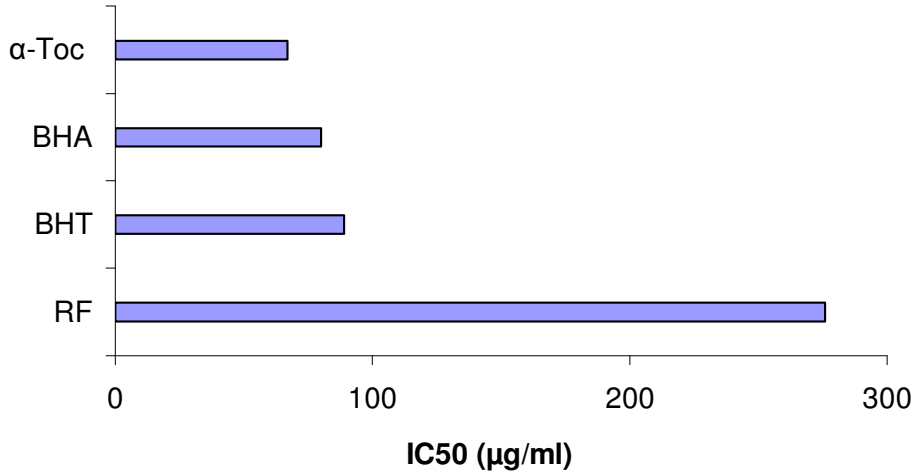

Figure 1. Free radical scavenging capacities of the extracts measured in DPPH assay.

versity and Ankara University.

Screening of antimicrobial activity of mushroom samples

Antimicrobial activity of ethyl alcohol extract of *R. flava* 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 broth at 28±0.1°C for 48 h. The culture suspensions were prepared and adjusted by comparing against 0.4-0.5 McFarland 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 (Ali-Shtayeh et al., 1998; Tepe et al., 2005). Each sample (100 □l) 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 incubated at 3°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 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) and Gentamicin (10 µg). All determinations were done duplicate.

RESULTS AND DISCUSSION

Antioxidant activity of extracts

The ethanolic extract 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 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 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 Figure 1. All concentration studied showed free radical scavenging activity. The 50% of inhibition value for RF ethanol extract seems to be fairly significant when compared to commonly used synthetic antioxidant BHA and α-tocopherol. (IC50= 276 µg/ml ethanolic extract was necessary to obtain 50% of DPPH degradation).

160 µg of *R. flava* ethanol extract has an equivalent inhibition value of 80 µg BHA. The inhibition value increases with concentration. Linoleic acid oxidation was compared with those of *R. flava* ethanol extract, α-tocopherol and BHA. It was found that inhibition values of both *R. flava* ethanol extract and the standards increased with concentration. For example, in 80 µg/ml concentration, *R. flava* extract, BHA and α-tocopherol showed 73.3, 96.4 and 98.6% of inhibition, respectively, whereas in 160 µg/ml concentrations the values were 94.7, 98.9 and 99.2% of inhibition, respectively. According to this, it is possible that the high inhibition value of *R. flava* extract is due to the high concentration of phenolic compounds.

The total phenolic compound amount was calculated as quite high for *R. flava* ethanol extracts (39.83±0.32 µg mg-1 pyrocatechol equivalent). In contrast to this, the total flavonoid compound concentration was measured as 8.27±0.28 µg mg-1 quercetin equivalent. The key role of phenolic compounds as scavengers of free radicals is emphasised in several reports (Komali et al., 1999; Moller et al., 1999). Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen et al., 1993; Gulcin et al., 2003). (Figure 2). 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,

Table 1. Antimicrobial activity of ethyl alcohol extract of *R. flava* and antibiotic sensitivity of microorganisms (zone size, mm)

Test bacteria	RF	N	A	P	G	O	T
Pseudomonas aeruginosa NRRL B-23	-	NT	NT	NT	16	NT	8
Salmonella enteritidis RSKK 171	4 ± 0	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	11± 1	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	8 ± 0	NT	NT	31	NT	21	20
Staphylococcus aureus Cowan I	4 ± 0	NT	NT	28	NT	18	21
Micrococcus luteus NRRL B-4375	13 ± 1	NT	30	31	NT	22	19
Micrococcus flavus	20 ± 2	NT	29	31	NT	24	20
Bacillus subtilis ATCC 6633	7 ± 1	NT	NT	12	NT	8	17
Bacillus cereus RSKK 863	8 ± 0	NT	NT	22	NT	14	19
Candida albicans	-	19	NT	NT	NT	NT	NT

RF: *Ramaria flava,* 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.

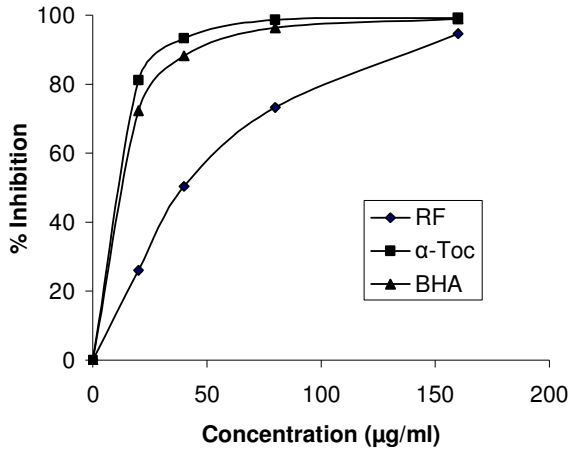

Figure 2. Total antioxidant activity of BHA, α-tocopherol and different doses of ethanolic extract mushroom the linoleic acid emulsion.

when up to 1.0 g daily ingested from a diet rich in fruits and vegetables (Tanaka et al., 1998). The results indicate that this mushroom extract compete with BHA and α-tocopherol in β-carotenlinoleic acid system used to determine the antioxidant capacity of *R. flava* ethanol extract.

Antimicrobial activity of extracts

To determine antimicrobial activity, *R. flava* were tested against Gram-negative (*Pseudomonas aeruginosa, Salmonella enteritidis, Escherichia coli, Morganella morganii, Yersinia enterecolitica, Klebsiella pnuemoniae, Proteus vulgaris*) bacteria, Gram-positive (*Staphylococcus aureus, Micrococcus luteus, Micrococcus flavus, Bacillus subtilis, Bacillus cereus*) bacteria and yeast (*Candida albicans*). The results of the antimicrobial screening assay of the ethyl alcohol extract of *R. flava* are shown in Table 1. Among the selected bacteria studied, extract inhibited the growth of Gram-positive bacteria better than Gram-negative bacteria and yeast. The result of a previous study (Turkoglu et al., 2007) on the antimicrobial activity of *Laetiporus sulphureus* on some bacteria showed that Gram-negative bacteria were less susceptible activity than Gram-positive strains. As can be seen from the results, ethanol extract of *R. flava* showed no antibacterial activity against *P. aeruginosa*, E. coli, *M. morganii* and *P. vulgaris* at the concentration used. The antimicrobial activity profile of *R. flava* against tested strains indicated that *M. flavus, M. luteus* and *Y. enterocolitica* was the most susceptible bacterium of all the bacterial test strains (20, 13 and 11 mm diameter, respectively). *R. flava* was found to be inactive against *C. albicans*. Dulger et al. (2002) reported that *Candida albicans* is resistant to the action of the methanolic extract of *Lepista nuda*. The culture fluid of Lentinus edodes showed poor activity against *C. albicans* (Hatvani, 2001).

Conclusion

In this study, the antimicrobial properties of *R. flava* were not as effective as the commercial drugs. However, microorganisms become resistant to antibiotics after some

time. *R. flava* inhibited the growth of some bacteria. In the future, *R. flava* may constitute an alternative for treating different strains of bacteria if strongly antibacterial concentrations can be prepared.

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