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# The Chemical Content, Antioxidant and Antimicrobial Assays of *Lactarius controversus* and *Lactarius musteus*: Two Edible Wild Mushrooms from Giresun Province of Turkey

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- Radical scavenging
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**Abstract**

This investigation was evaluation of the chemical content, antioxidant and antimicrobial activity of *Lactarius controversus* and *Lactarius musteus*. Antioxidant activities were evaluated by reduction of Mo(VI) to Mo(V), linoleic acid peroxidation inhibition, reducing power, metal chelating, superoxide anion scavenging, free radical scavenging, hydrogen peroxide scavenging and peroxide scavenging activity. In addition, the known antioxidant phenolics were determined as components of the methanolic extracts, spectrophotometrically. Results demonstrated that the graded-dose [50-500 µg/mL] of methanolic extracts markedly scavenged $O_2^{\cdot}$ (superoxide) and DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals and, exhibited metal chelating ability as well as reducing power. Extracts were found to show total antioxidant activity, to scavenge hydrogen peroxide and peroxide scavenging activity, and to inhibit lipid peroxidation in linoleic acid system at 100 µg/mL. The extracts of *L. controversus* and *L. musteus* displayed higher total antioxidant activity than BHT and trolox because of amounts of total polyphenol, flavonoid, anthocyanosides, ascorbic acid, β-carotene and lycopene content. It seems that *L. controversus* and *L. musteus* exerted peroxide scavenging and metal chelating activity, and also they are close the other activities being probably due to secondary metabolits. Microorganisms (some wild type and clinical isolates) were used for evaluating antimicrobial activity of the mushrooms. Methanol extracts of *L. controversus* and *L. musteus* showed high antimicrobial activity ( ≥ 15 mm) on some studied microorganisms. The content of secondary metabolites were found higher in *L. controversus*.

## INTRODUCTION

Many different types mushrooms in nature are used as edibles by human. Asian countries traditionally and commonly use wild edible mushrooms as nutritional foods and medicine. Wildly grown edible mushrooms are regarded not only for their texture and flavor but also for the chemical contents and nutritional properties [1]. They are becoming more important in our diet than cultivated mushrooms for their nutritional and pharmacological characteristics. Wild edible mushrooms have used effectively as a functional food and as a source for the development of drugs, and nutraceuticals [2]. Nowadays, the uses of their traditional medicine are widespread and tent to development of novel drugs. Mushrooms can be applied as neuroprotective [3], anti-inflammatory [4], hepatoprotective [5], cholesterol lowering [6], hypertension [7], diabetes [8], hypercholesterolemia [9], anticancer [10], antimicrobial [11], digestive [12], antinecrotic drugs [13], Some species of mushrooms have recently been exhibited to have an effective antioxidant properties, superoxide and antiradical scavenging mechanism [14-17]. Research has shown that mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. The quality of a nutraceutical is dependent on the chemical composition of the fruiting body, particularly in relation to the phenols and flavonoids content, hydroxyl benzoic acids and hydroxycinnamic acids, stilbenes, lignans, tannins, and oxidised polyphenols, displaying a great diversity of structures [18,19]. A combination of mushroom chemicals such as flavanoids and polyphenols are reffered to be the major bioactive

compounds for the real health benefits. The phenolic contents and their antioxidant activities in mushrooms are one of the groups of non essential dietary components that have been associated with the inhibitory effect of atherosclerosis and cancer. The powerful phenolic antioxidants may be related to their ability to chelate metals, inhibit lipid oxidation, possess healthy properties and scavenge free radicals [15,20]. Mushrooms provide a wealth of protein, fiber, vitamins (A, B, C, D, and K vitamins), and minerals [21,22].

Synthetic antioxidants such as butylatedhydroxyanisole [BHA] and butylatedhydroxytoluene (BHT) and tert-butylatedhydroxyquinine (TBHQ) at higher levels have been used as food additives to prevent rancidification, owing to their high performance, low cost and wide availability [23,24]. The wide-spread use of synthetic antioxidants can result in potential health risks associated with their intake [25]. However, restrictions on the use of synthetic antioxidant compounds such as carcinogenicity cause an increased interest towards natural antioxidants and extracted materials. Thus, the research for natural antioxidants has increased notably in recent years due to their health benefits and pharmaceutical properties [26-29]. Wild and cultivated mushrooms are well known to contain various polyphenolic compounds which are recognized as excellent antioxidants due to their ability to scavenge free radicals by acting as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers [2,14,15,30,31]. Anatolian people have been using wild grown edible mushrooms as food for a long time [14,32]. Climate, vegetation and moisture especially in Northern Region in Turkey are suitable for the growing of wild edible mushroom. Giresun is located in northeastern part of Anatolia (Turkey) where the consumption of wild edible mushrooms is common, is as a part of the local traditional applications. The humid climate dominates hot and dry in the summers and tepid and rainy in the winters [33]. The different climate around Giresun is enables to the widest distribution of plant species and includes ranging from coniferous forest to broad leaves [34]. The inhabitants in Giresun consume the aerial parts of various wild grown edible mushrooms [32]. *L. controversus* and *L. musteus* are the most commonly wild edible mushroom species. Several studies have shown correlation related to the mushrooms antioxidant capacity and their phenolic compound contents were described by us [32,35], and by other authors [2,14,15]. Also many scientific papers have exhibited that edible mushrooms have antimicrobial activity [15,36-38]. The reason for this study is that antioxidant activities of the *L. controversus* and *L. musteus* given here have not previously been reported in literature although Northern Anatolian people have been using them as food for a long time. The aim of this study is to investigate the evolution of antioxidant components, antioxidant and antimicrobial activity of *L. controversus* and *L. musteus*. The antioxidant properties were investigated though inhibition of lipid peroxidation, total antioxidant activity, reducing power, metal chelating, superoxide anion, DPPH·, hydrogen peroxide, peroxide scavenging activities of *L. controversus* and *L. musteus*. The main goal of this research was to investigate *in vitro* antioxidant abilities of *L. controversus* and *L. musteus* compared with those of standard antioxidants such as α-tocopherol, BHA, BHT and trolox commonly used by the pharmaceutical and food industry. All these antioxidant activity parameters were correlated to the phenolics, flavonoids, anthocyanins, and ascorbic acid, β-carotene and lycopene contents. The methanol extract of mushrooms were tested for antimicrobial activity against both wild type and clinic isolate microorganisms with disc diffusion method, correlated to the bioactive compounds present in the extracts.

## MATERIALS AND METHODS

### Chemicals and reagents

All the reagents and solvents were in analytical grade. 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), trichloroacetic acid (TCA), Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2-picryl-hydrazl (DPPH), ferrous chloride, nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), gallic acid, α-tocopherol, $KH_2PO_4$, $KHPO_4$, potassium ferricyanide and linoleic acid were purchased from Sigma-Aldrich (St. Louris, USA). The other chemicals and reagents used were obtained from local medical market and were of analytical grade.

### Sample preparation and extraction

The Wild fruiting bodies of *L. controversus* (Vouncher Number: 2161) and *L. musteus* (Vouncher Number: 2417) were collected in spring 2010 from Giresun (Yavuz Kemal Plato) Province, Turkey. Taxonomic identification was made by Dr. İbrahim Türkekul, Department of Biology, Faculty of Arts and Sciences, Gaziosmanpaşa University, Tokat, Turkey. The mushrooms were deposited at the our research laboratory. Fresh mushrooms were washed to remove surface soil and air-dried in an oven at 40 ºC, gradually. After drying, the samples were ground into a fine powder and then stored at room temperature in desiccators until further analysis. The samples of dried sample (10 g) were extracted by stirring with 500 mL of methanol at 25 ºC at 200 rpm for 24 h and filtered with Whatman No 1 filter paper. The residue was then extracted with two additional 500 mL portions of methanol [32]. The combined methanolic extracts were then evaporated at 40 ºC and lyophilized in a Christ Alpha 1-2 LD Plus lyophilizer (CHRIST Model; Martin-Christ, Osterode, Germany). The extracts were stored at 4 ºC for further use.

### Total phenolic contents

Phenolic compounds in the mushroom extract were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications [39]. 0.1 mL extract (contains 0.1 mg extract) was mixed with water (46 Ml). 1 mL of Folin-Ciocalteu reagent and 3 mL of $Na_2CO_3$ (2%) were added, consequently. After incubation for 2 h at 25 °C, The absorbance was measured at 760 nm. The standard curve was prepared by 0-100 µg/mL solutions of gallic acid in ethanol (y=0681+0.0096, $R^2$=0.9968). The concentration of total phenolic compounds in extracts was determined as µg of gallic acid equivalent using an equation obtained from the standard curve and expressed as mg gallic acid/g dry weight (DW) of the plant material. The results were calculated into mg gallic acid equivalents (GAEs) per g of extract.

## Total flavonoid contents

Flavonoid contents in the extracts were determined by a colorimetric method described by Chang et al., (2002) with some modifications [40]. Briefly, each mushroom extracts (0.1 g) were dissolved in 1 mL methanol. This solution (0.1 mL) was mixed with 10% $AlCl_3.6H_2O$ and 0.1 mL of 1 M potassium acetate ($CH_3COOK$). It was kept at 25 °C for 30 min and the absorbance of reaction mixture was measured at 415 nm. Quercetin was chosen as a standard. Using standard curve (0-100 µg/mL; y=0.7727x-0.002, $R^2$=0.9992), the levels of total flavonoid contents in sample extract were determined in triplicate, respectively. The results were calculated into mg quercetin equivalents/g dried mushroom materials.

## Ascorbic acid contents

Ascorbic acid was determined according to the method in ref [41]. Methanolic extract from mushrooms (20 mg) was extracted with 10 mL of 1% metaphosphoric acid for 45 min at room temperature and filtered through what man No. 4 filter paper. The filtrate (1 mL) was mixed with 9 mL of 2, 6-dichloroindophenol and the absorbance was measured in 15 s at 515 nm. The content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (100-1000 ppm; y=0.0149x-0.0071, $R^2$=0.9975).

## β-Carotene and lycopene contents

β-Carotene and lycopene were determined according to the method in ref [42]. The dried methanolic extract (100 mg) was vigorously shaken with 10 mL of acetone-hexane mixture (4:6) for 1 min and filtered through what man No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645, and 663 nm, respectively. Contents of β-carotene and lycopene were calculated according to the following equations:

Lycopene (mg/100 mL) = $-0.0458 \times A_{663} + 0.372 \times A_{505} - 0.0806 \times A_{453}$ and

β-carotene (mg/100 mL) = $0.216 \times A_{663} - 0.304 \times A_{505} + 0.452 \times A_{453}$.

The results expressed as mean values ± standard deviations. The β-carotene and lycopene value were expressed as mg of β-carotene/g of extract, and mg of carotenoid/g of extract, respectively.

## Determination of total anthocyanins

Total anthocyanins were measured according to the methods described earlier with slight modification in ref [43]. The dried material was mixed with acidified methanol (1% HCl/methanol) for 2 h at 25°C, and then centrifuged at 2000 x g. The supernatant was measured at 520 and 700 nm, respectively. The absorbance values were measured at 520 and 700 nm, respectively. Two different dilutions of the mushroom samples were prepared, one for pH 1.0 using 0.03 M KCl buffer and the other for pH 4.5 using 0.4 M $CH_3CO_2Na$ buffer. The absorbance of each sample was measured at 520 nm. The concentration (mg/L) of each anthocyanin was calculated according to the following formula and expressed as cy-3-glc (cyanidin-3-glucoside) equivalents:

Total anthocyanins (mg/mL, cy-3-glc): $(A \times MW \times DF \times 103)/\varepsilon \times L$.

Where A is the absorbance $A = [A_{520}\ nm - A_{700}\ nm]$ pH 1.0 - $[A_{520}\ nm - A_{700}\ nm]$ pH 4.5, MW is the Cy-3-glc molecular weight: 449.2 g/mol, DF is the dilution factor [0.2 mL sample is diluted to 2 mL, DF = 10], ε is the extinction coefficient [$L^{-1}cm^{-1} \times mol^{-1}$, 26,900 for Cy-3-glc and L =1 cm

## UV-Vis analysis

UV–Vis Analysis was measured on a Thermo Scientific Evolution Array UV-Vis spectrophotometer [36]. The UV–Vis absorption (200-800 nm) spectra of the mushrooms extracts were assessed for the characterization of phenolic compounds, flavonoids and anthocyanins.

## Determination of total antioxidant activity

The total antioxidant capacity of the crude methanolic extracts of mushroom materials was evaluated by the method in ref [44]. The antioxidant capacity of the extracts was measured spectrophotometrically using a phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of specific green phosphate/Mo(V) compounds. A 0.3 mL aliquot of sample solution (100 µg/mL) was combined with 2.7 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). All samples were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm. For the blank, 0.3 mL ethanol was mixed with 2.7 mL of the reagent. A typical blank solution contained 2.7 mL of reagent solution and the appropriate volume of methanol used for the dissolution of the samples and it was incubated under the same conditions as the rest of the samples. Stock solutions of α-tocopherol were prepared in methanol. The antioxidant activity of extracts was expressed as equivalents of α-tocopherol using extinction coefficient of $4x10^3$ 1/M. cm. The total antioxidant activity was expressed as equivalents of α-tocopherol (µmol α -tocopherol/g of DW).

## The Inhibition of linoleic acid peroxidation

The procedure was performed according to modified method in ref [45]. The extracts at the concentration of 100 µg/mL were mixed with 550 µL linoleic acid solution (0.28 mg linoleic acid and 0.28 mg tween-20 in 100 µM phosphate buffer,pH 7.4), 500 µL of phosphate buffer (100 µM, pH 7.4) and 150 µl of ascorbic acid (10 µM). The linoleic acid peroxidation was initiated by the addition of 0.1 mL $FeSO_4$ (10 µM) and incubated at 37 °C for 60 min. The mixture of reaction was cooled and added 1.5 mL trichloroacetic acid (10% in 0.5% HCl). Then, 3 mL TBA (1%, in 50 mM NaOH) was added. The reaction mixture and TBA solution were heated in the water bath at 90 °C for 60 min. After cooling down, 2 mL aliquots were taken from each sample and vortexed with 2 mL butanol and centrifuged at 1000 x g for 30 min. The upper layer solution was separated for the spectrophotometrically measurement. The absorbance of solution was read at 532 nm and calculated the percentage of linoleic acid peroxidation inhibition in the following equation:

Linoleic acid peroxidation inhibition (%) = $((A_{control} - A_{sample})/A_{control}) \times 100$

Where $A_{control}$ is the absorbance of control including methanol instead of extracts or standards and $A_{sample}$ the absorbance extracts and standards.

## Reducing power

The reducing power of extracts was determined according to the method in ref [46]. All extracts in 1 mL of distilled water and standard antioxidants (50-500 µg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL; 10 g/L). The mixtures were incubated at 50 °C for 20 min. Then, a portion of TCA (10%; 2.5 mL) was added to the each mixture and centrifuged at 5000 x g for 20 min. Finally, the supernatants (2.5 mL) were mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL; 0.1%). The absorbance of the solution was measured by at 700 nm. It was indicated that high absorbance of sample has good reducing power in the reaction condition.

## Metal chelating activity

The chelating of ferrous ions by extracts was determined by the method in ref [47]. Briefly, the samples (extracts or standard antioxidants; 100 µg/mL) were 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) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the resulting solution was then measured at 562 nm. The metal chelating activities were calculated by the given formula:

Metal chelating activity (%) = $((A_{control}-A_{sample})/A_{control}) \times 100$

Where $A_{control}$ is the absorbance of control and $A_{sample}$ is the absorbance of extracts or standards. The control contains $FeCl_2$ and ferrozine.

## Superoxide anion scavenging activity

The determination of superoxide anion scavenging activity of extracts was measured according to slightly modified in ref [48]. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. One milliter of all species and standard antioxidants (100 µg/mL), 1.0 mL of NBT solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4) and 1.0 mL of NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) were mixed. The reaction was started by adding 100 µL of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixture was incubated at 25°C for 5 min, and its absorbance was measured at 532 nm wavelength against blank samples. The decrease of absorbance of the mixtures indicates an increasing superoxide anion scavenging activity.

The percentage inhibition of superoxide anion generation was calculated using the following formula: Inhibition of superoxide anion generation (%) = $((A_{control}-A_{sample}) / A_{control}) \times 100$ Where $A_{control}$ is the absorbance of control, and $A_{sample}$ is the absorbance of mushroom or standards.

## Free radical scavenging activity

The effect of all species on DPPH was estimated according to the method in ref [49]. Wherein the bleaching rate of a stable free radical, DPPH· is monitored at a characteristic wavelength in the presence of samples. An amount of 0.5 mL of 0.1 mM ethanolic solution of DPPH· was added to 3.0 mL of all species extracts or standard antioxidants solution (100 µg/mL). The mixture was shaken vigorously and waited at room temperature for 30 min. Then the absorbance was measured at 517 nm. The decrease in the absorbance of the DPPH· solution indicates an increasing of DPPH-scavenging activity. This activity was calculated by the following equation:

DPPH scavenging effect (%) = $((A_{control}-A_{sample}) / A_{ontrolc}) \times 100$, Where $A_{control}$ is the absorbance of control and $A_{sample}$ is the absorbance of mushrooms or standards.

## Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured according to slightly modified method in ref [50]. Briefly, 1 mL of $H_2O_2$ (0.1 mM) and 1 mL of 100 µg/mL concentration of the extract or standard antioxidants were mixed with 100 µL ammonium molybdate (3%), 10 mL of $H_2SO_4$ (2 M) and 7 mL of KI (1.8 M). The mixed solution was titrated with $Na_2S_2O_3$ (5 mM) until disappearing yellow color. The percentage scavenging effect was calculated as follows: Hydrogen peroxide scavenging activity (%)= $(V_o-V_1)/ V_o \times 100$), where $V_0$ is volume of $Na_2S_2O_3$ solution hydrogen peroxide (without extract), $V_1$ is the volume of $Na_2S_2O_3$ solution mixed with the extracts of mashrooms or standard antioxidants.

## Peroxide scavenging activity

Peroxide scavenging activity was measured according to slightly modified method in ref [51]. Peroxide radicals were generated from the mixture of $FeSO_4$ and $H_2O_2$. The reaction mixture composed of 1 mL $FeSO_4$ (1.5 mM), 0.7 mL $H_2O_2$ (6 mM), 0.3 mL sodium salicylate (20 mM) and samples (100 µg/mL). After incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as using the following formula: The peroxide scavenging activity (%) = $(1- (A_1 - A_2) / A_o) \times 100$ where $A_0$ is the absorbance of the control (without extract or standards) and $A_1$ is the absorbance including the extract or standard, $A_2$ was the absorbance without sodium salicylate.

## Antimicrobial activities

The methanol extracts of *L. musteus* and *L. controversus* were individually tested against a panel of microorganisms including three gram positive (*Staphylococcus aureus* ATCC 6535, *Bacillus cereus* ATCC 7064, *Staphylococcus epidermidis* ATCC 12228), three gram negative (*Escherichia coli* W3110, *P. aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048), one yeast (*Candida albicans* ATCC 10231) and clinical isolates (*E. coli, P. aeruginosa, E. aerogenes*, *Acinetobacter baumanii, Morganella morganii*, Methicillin resistant *S. aureus*-MRSA, Methicillin resistant koagulase (-) *Staphylococcus* –MRKNS). The stock cultures of microbial strains were kept on nutrient agar at 4 °C. The antimicrobial activity of the methanol extract of *L. musteus* and *L controversus* was studied by disc diffusion methods according to Ozen et al., 2011[32,52]. The diameter of the inhibition zones were measured in millimeters. Inhibition zones were measured in four repeated. Standard deviation was calculated by taking average of four experiments.

### Statistical analysis

Data were presented as mean ± standard deviation (S.D.). Statistical analysis for antioxidant activities was analyzed using one-way ANOVA followed by Tukey's HSD test with α= 0.05. This assessment was carried out using SPSS (17.0) software.

## RESULTS AND DISCUSSION

*L. controversus and L. musteus* From the north east of Turkey were evaluated for their content in total phenols; flavonoids, ascorbic acid, anthocyanins, β-carotene and lycopene, and the methanolic extract were screened for its antioxidant and antimicrobial activity. Antioxidant asays are performed using several methods in the literature. Because of the different chemical content of extracts, a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour could lead to scattered results, and related to the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extract would be more informative and even necessary. Therefore, the antioxidant activities and content of extracts of *L. controversus* and *L. musteus* were screened using eight complementary test systems, and also evaluated for their antimicrobial activities.

### Extractable yield, phenolic compounds, flavonoids, ascorbic acid, β-carotene and lycopene content

The results showed that extractable yield, phenol, flavonoid, ascorbic acid, β-carotene and lycopene were the major naturally occurring antioxidant components found in the methanolic extracts from two wild edible mushrooms (Table 1). Whereas phenolics and flavonoids compounds were the major in the methanolic extracts, ascorbic acid, β-carotene and lycopene were found in small amounts, which is in agreement with other investigations related to ascorbic acid and β-carotene quantification in different *Lactarius* species [30,32,53]. Furthermore, Yim et al., 2011 [54] and Yim et al., 2012 [55] found a direct correlation between mushrooms antioxidant activity and total phenolic content, although the antioxidant action is raised by other substances such as ascorbic acid, β-carotene and lycopene. Phenolic compounds are widely distributed in wild edible mushrooms and are very important diet in human. The results of phenolic contents were comparable with the results described in the literature for the other extracts of mushroom products and in agreement with the reports [14,32,35] (Table 1). The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports [56-58]. The higher content of total phenols in the *L. controversus* and *L. musteus* extracts might account for the better results found in its antioxidant assays. The bioactivity of phenolics may be related to their ability to chelate metals, lipid per oxidation, reducing power, free radicals and peroxide scavenge [59]. Flavonoids are an important group of natural compounds, which can prevent human disease and have an effective antioxidant properties. Most of them belong to the Mediterranean food culture. During the last few decades, they attracted great interest because of their beneficial effects on human health [60]. In food systems, flavonoids and related compounds can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides [61]. Flavonoids occur throughout the mushroom kingdom. Many flavonoids and related compounds are reported to possess strong antioxidant properties [62]. The content flavonoid of *L. controversus* and *L. musteus* are very important mushroom constituents is in agreement with the reports [30,32,63] (Table 1). The contents of total anthocyanosides in *L. controversus* and *L. musteus* showed in (Table 1). The methanolic extracts of *L. controversus* and *L. musteus* were most abundant in phenolics, and also most abundant in anthocyanins which contribute to the total phenolic levels. It has been found that polyphenolic compounds are one of the most effective antioxidant constituents in will grown edible mushrooms [32,34,35] (Table 1). β-carotene is a natural, fat-soluble pigment found mainly in mushrooms. It acts as a powerful antioxidant and is known to have immunomodulatory effect. Lycopene also appears to exhibit strong antioxidant capability A number of studies suggested that a diet high in lycopene might be associated with a lower risk of prostate cancer and cardiovascular disease [64]. The contents of β-carotene and lycopene in the methanolic extracts were found in valuable amounts, which were in agreement with other reports concerning ascorbic acid, β-carotene and lycopene quantification in different wild edible mushrooms [32] (Table 1).

**Table 1**: The yield of extractable compounds, total phenolic, flavonoid, anthocyanoside, ascorbic acid, β-carotene and lycopene contents of methanol extract.

| Chemical content | *L.controverss* | *L. musteus* |
|---|---|---|
| Yield, g/100 g DW (Dried Weight) | 32.50 | 20.63 |
| Phenolics, mg GAEs /g DW | 24.78 ± 0.49 | 8.84 ± 0.51 |
| Flavonoids, mg quercetin/g of DW | 0.176 ± 0.026 | 0.157 ± 0.005 |
| Anthocyanosides, mg cyanidin 3-glucoside/g DW | 1.157 ± 0.120 | 0.527 ± 0.080 |
| Ascorbic acid, mg ascorbic acid/g DW | 0.071 ± 0.002 | 0.057 ± 0.001 |
| β-carotene, mg β-carotene / g DW | 1.49 ± 0.14 | 1.36 ± 0.28 |
| Lycopene, mg carotenoid / g DW | 3.03 0.14 | 1.08 ± 0.09 |

The UV–Vis absorption (200-800 nm) spectra of the methanolic extracts of *L. controversus* and *L. musteus* were assessed for the characterization of phenolic compounds and flavonoid (Figure 1). Phenolic compounds which are a first band in the range between 300-390 nm and a second band in the 230 to 280 nm range showed two major absorption bands in the UV/Vis regions at 260-360 ($\lambda_{max}$) [34,65]. $\lambda_{max}$ of methanolic extract 400-450 nm may be due to the presence of favonoids. It was found that favonoids exhibits comparable special bands to the values reported in the literature [40,66]. These results indicate that the highest content of that phenolic compounds and, favonoids in the mushrooms might contribute to the better results found in their antioxidant activity and found a direct correlation between antioxidant activity *L. controversus* and *L. musteus* extracts and chemical contents [14,67] (Figure 1).

### Total antioxidant activity

The phosphomolybdate method has been used routinely to evaluate the total antioxidant capacity of extract. In the presence of methanolic extract, Mo (VI) is reduced to Mo (V) and forms a green colored phosphomolybdenum V complex, which shows a maximum absorbance at 695 nm. The total antioxidant activity

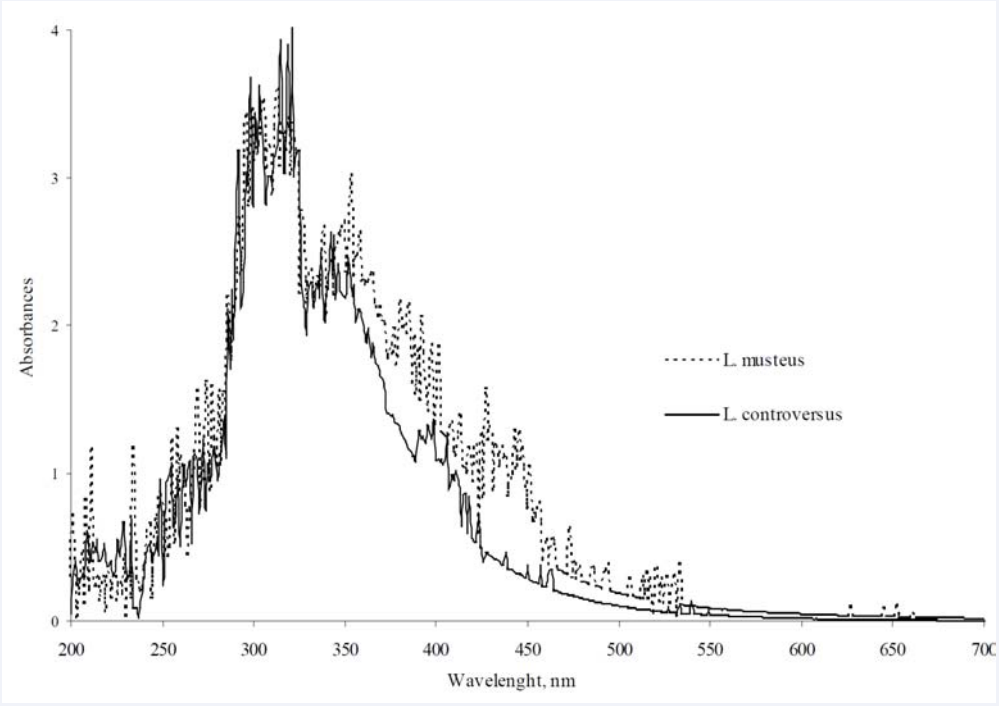


**Figure 1** UV-Vis spectra of methanol extracts from L. controversus and L. Musteus.

**Table 2**: Reducing power activity (700 nm, absorption) of the methanol extract of *L. controversus* and *L. musteus* at the different concentrations (50–500 µg/ml).

| Samples | Concentration (µg/mL) | | | |
|---|---|---|---|---|
| | 50 | 100 | 250 | 500 |
| *L. controversus* | 0.195 ± 0.024 | 0.254 ± 0.010 | 0.310 ± 0.032 | 1.076 ± 0.024 |
| *L. musteus* | 0.187 ± 0.006 | 0.270 ± 0.019 | 0.520 ± 0.046 | 0.983 ± 0.109 |
| BHA | 0.273 ± 0.001 | 0.576 ± 0.016 | 1.152 ± 0.018 | 1.811 ± 0.022 |
| BHT | 0.258 ± 0.016 | 0.411 ± 0.024 | 0.866 ± 0.004 | 1.681 ± 0.054 |
| α-tocopherol | 0.184 ± 0.012 | 0.304 ± 0.050 | 0.670 ± 0.025 | 1.189 ± 0.040 |
| Trolox | 0.323 ± 0.001 | 0.468 ± 0.014 | 0.727 ± 0.028 | 1.465 ± 0.029 |

of *L. controversus* and *L. musteus* has been screened. Total antioxidant activity of mushrooms and standards exhibited the following order: BHA > *L. controversus* > *L. musteus* > BHT > trolox (Figure 2). These results were not statistically significant. The results are considered to be noteworthy when compared to the findings of other studies of ours concerning mushrooms in Turkey [32]. The antioxidant activity of mushroom seems to be due to the presence of phenolic compounds, flavonoids and anthocyanosides that may be acted by donating electrons and free radicals [2,21,30,68].

## Reducing power

Table 2 depicts the reducing properties of methanolic extracts of *L. controversus* and *L. musteus* as a function of different concentration (50-500 µg/mL). In this assay, the yellow colour of the assay solution changes to various shades of green and blue, depending on the reducing power of extract including phenolics.

$K_3(Fe^{3+}(CN)_6)$ + Ph-OH → $K_x(Fe_n{}^{3+}(Fe^{2+}(CN)_6)_3)$ + Ph-O + $H^+$

Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the $Fe^{2+}$ concentration [69]. The results suggest that as electron donors, each extract could convert free radicals into more stable products, leading to the termination of radical chain reactions. The reducing powers of extracts and standard antioxidats were BHA > BHT > trolox > α-tocopherol > *L. controversus* > *L. musteus* at 500 µg/mL. The reducing power of the methanolic extracts of *L. controversus* and *L. musteus* were lower than that of the stantard antioxidants. The mushroom extracts showed some degree of electron donating capacities in a concentration-dependent manner. But, the reducing power of the extract increased with concentration significantly, $P<0.01$. It was reported that the reducing power of mushrooms might be due to their hydrogen-donating ability [32,70].

## Metal chelating activity

Iron has the most important lipid pro-oxidant. It is known that the $Fe^{+2}$ accelerates lipid peroxidation by breaking down hydrogen and lipid peroxides forms by Fenton free radicallic reaction [71].

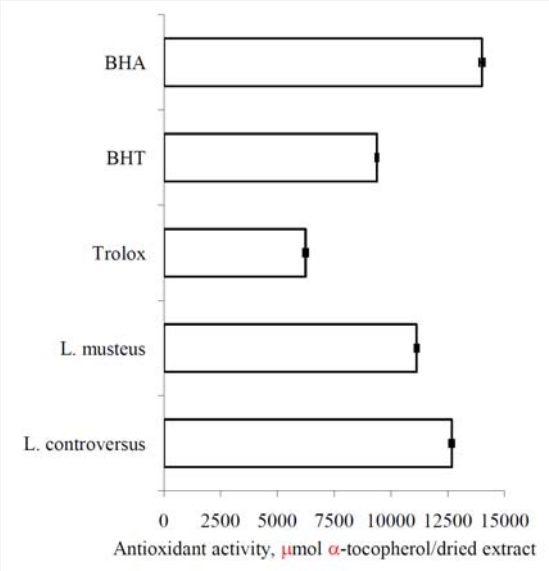


**Figure 2** Antioxidant activities of methanol extract of L. controversus and L. musteus at concentration of 100 µg/ml.

$(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-)$

Ferrozine can quantitatively form complexes with $Fe^{2+}$. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction, therefore, allows estimation of the chelating activity of the coexisting chelator [72]. As shown in (Table 3), the formation of the $Fe^{2+}$-ferrozine complex was prevented by methanolic extracts of the mushroom. The activity of metal chelating was increased in a linear dose-dependent manner (50-500 µg/mL). All of the extracts evaluated here showed significantly higher chelating effects on ferrous ions than those of the standards, $P < 0.05$. At 500 mg/mL, the percentage of chelating capacities of EDTA, *L. controversus*, *L. musteus*, BHT, BHA, α-tocopherol trolox were found to be 98.90 ± 3.01, 95.91 ± 0.16, 95.72 ± 3.25, 80.00 ± 4.32, 75.05 ± 1.15, 69.50 ± 2.46, and 64.02 ± 3.17%, respectively. The data obtained from Table 3 revealed that extracts of the wild edible mushrooms in this study demonstrated a marked capacity for iron binding, suggesting that their action as a peroxidation protector may be related to its iron binding capacity.

## Superoxide anion scavenging activity

The superoxide anions are well-known free radical species, and they are produced continuously and regularly by several cellular processes, including mitochondrial electron transport systems [73]. Although the superoxide anion is limited in activity, its combination with other reactive species might suply more reactive species such as singlet oxygen and hydroxy radicals [74]. The results showed that the methanolic extracts had a superoxide radical scavenging ability, significantly ($P < 0.05$). The superoxide anion scavenging activities of *L. controversus* and *L. musteus* were increased in a linear dose-dependent manner (50-500 µg/mL) (Table 4). At 500 mg/mL, the percentage of scavenging capacities of trolox, BHA, BHT, *L. musteus*, *L. controversus*, α-tocopherol were found to be 97.04 ± 0.04, 82.51 ± 0.79, 76.62 ± 0.82, 62.23 ± 3.25, 61.65 ± 0.97, 54.78 ± 1.97 %, respectively. A similar report [32]. found that the methanolic extracts of *L. controversus* and *L. musteus* strongly inhibits chemically generated superoxide radical. These results idicated that the superoxide anion scavenging activity could be due to the action of a free hydroxyl group.

## Free radical scavenging activity

In mushroom samples, free radical scavenging activities were evaluated as % inhibition in the extracted samples using 50-500 µg/mL and tested using a ethanolic solution of DPPH (Table 5) summarizes the effective concentrations of each extract and reference compound required to scavenge DPPH, the scavenging values as percentage. The free radical scavenging activities of extracts and standard antioxidants were trolox > BHA > BHT > *L. musteus* > *L. controversus* > α-tocopherol at 500 µg/mL. *L. musteus* is better DPPH scavenger than BHA, BHT, α-tocopherol at concentration of 50, 100, 250 and 500 µg/mL, and also is higher

**Table 3**: Metal chelating activity, (%) of the methanol extract of *L. controversus* and *L. musteus* at the different concentrations (50–500 µg/mL).

| Samples | Concentration (µg/mL) | | | |
|---|---|---|---|---|
| | 50 | 100 | 250 | 500 |
| *L. controversus* | 80.72 ± 2.86 | 85.02 ± 2.50 | 92.89 ± 2.88 | 95.72 ± 3.25 |
| *L. musteus* | 89.91 ± 0.78 | 94.02 ± 0.56 | 95.17 ± 0.67 | 95.91 ± 0.16 |
| BHA | 65.90 ± 2.54 | 67.85 ± 4.16 | 71.43 ± 3.69 | 75.00 ± 1.15 |
| BHT | 65.10 ± 0.78 | 69.94 ± 1.23 | 71.04 ± 0.70 | 80.37 ± 4.32 |
| α-tocopherol | 66.60 ± 1.88 | 69.63 ± 2.23 | 68.39 ± 2.76 | |
| Trolox | 58.52 ± 3.78 | 60.00 ± 2.54 | 59.40 ± 3.03 | 64.00 ± 3.17 |
| EDTA | 97.16 ± 0.36 | 97.18 ± 2.04 | 97.26 ± 2.07 | 98.90 ± 3.01 |

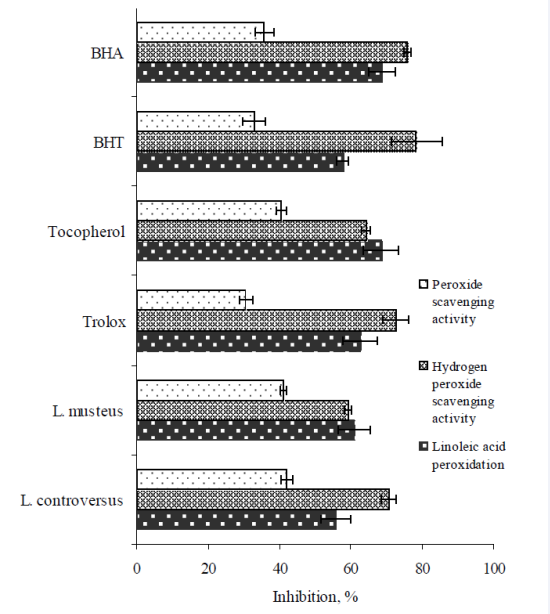


**Figure 3** Linoleic acid peroxidation, hydrogen peroxide and peroxide scavenging activity of L. controversus and L. musteus by the sample at 100 µg/ml.

DPPH scavenging activity than α-tocopherol at concentration of 500 μg/mL. The results were significantly different from others ($p < 0.01$). The methanolic extracts from other mushrooms showed a moderate increase in DPPH˙ free radical scavenging activities. In the previous studies, the same situations were reported for the methanolic extracts of wild grown mushrooms [68]. DPPH reacts with different antioxidants at different molar ratios [75]. More quenching of DPPH, in molar basis, takes place with phenolics and flavonoids possessing hydroxyl groups in ortho positions in the aromatic rings or having higher numbers of hydroxyl groups. As a natural product, variation in chemical composition of mushrooms due to geographical and climatic variables is expected. Furthermore, The antioxidant and antiradical properties of flavonoids are attributed to their ability to act as transient metals chelaters, radical scavengers, and their involvement in electron and hydrogen atom transfer [76].

## The Inhibition of linoleic acid peroxidation

Lipid peroxidation is a major cause of food deterioration, affecting color, flavor, texture, and nutritional value [77]. The oxidative modification depends on a common initiating steps the peroxidation of polyunsaturated fatty acid components in the low-density lipoproteins, and also the oxidative modification of low-density lipoproteins may be related to a role in the development of atherosclerosis [78]. The generated lipid peroxides further act on the cell/ cellular components, leading to both structural and functional damage of the biomolecules as well as the cellular structures. Edible mushrooms may be considered to be good phenolic natural antioxidants to inhibit lipid peroxidation [79]. Malondialdehyde (MDA) is the secondary byproduct, which is released during the lipid peroxidation. A decrease in the production of MDA symbolizes the inhibition of lipid peroxidation [36]. (Figure 3) shows that the extract of *L. controversus* and *L. musteus* inhibited linoleic acid peroxidation. The results obtained for *L. musteus* extract was higher than BHT, not significantly. In conclusion, we can infer that the extract competes with BHT. In previous studies, the inhibition of linoleic acid of methanolic extraction of several commercial and medicinal mushrooms have been reported [14,80].

## Hydrogen peroxide scavenging activity

$H_2O_2$ can be formed in vivo by many oxidizing enzymes such as superoxide dismutase [81]. It can cross membranes and might slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive and it might be converted into more reactive species such as singlet oxygen and hydroxyl radicals [82]. The hydrogen peroxide scavenging activity of *L. controversus* and *L. musteus* extracts was compared to BHA, BHT, trolox and α-tocopherol as standards. *L. musteus* extract showed a low scavenging activity at 100 μg/mL concentration. But, the scavenging effect of *L. controversus* was higher than α-tocopherol. The $H_2O_2$ scavenging activity were in the following order: BHT > BHA > Trolox > *L. controversus* > α-tocopherol > *L. musteus* (Figure 3). Results were found to be statistically significant ($p < 0.05$) when compared with the control. These results showed that *L. controversus* and *L. musteus* extracts can moderate $H_2O_2$ scavenging activity. This result was in agreement with our previous reports [21,32,36].

## Peroxide scavenging activity

The peroxide radical scavenging activity of *L. controversus* and *L. musteus* extracts was assessed by its ability to compete with salicylic acid for OH radicals in the ˙OH generating / detecting system [79]. As shown in (Figure 3), the methanolic extracts of *L. controversus* are higher than that of BHA, BHT, trolox and α-tocopherol as standards. The $H_2O_2$ scavenging activity was in the following order: *L. musteus* > *L. controversus* > α-tocopherol > BHA > BHT > trolox, statistically significant ($p < 0.05$). The results showed that the scavenging effects of methanolic extracts from *L. musteus* and *L. controversus* might moderate on hydroxyl free radicals at 100 μg/mL. Previous studies had reported two types of antioxidant mechanism: suppression against hydroxyl radical generation, and cleaning hydroxyl radical generated [83]. The former mechanism was related to the transition of metal ions. In the absence of transition of metal ions, hydrogen peroxide is fairly stable. However, hydroxyl radicals act in superoxidation by hydrogen peroxide with metal ions, usually ferrous or cupper. The molecules that could chelate iron, and render them inactive of poorly active fenton reaction might have scavenging ability on hydroxyl radical [84].

## Antimicrobial activity

Table 6 shows the results of antimicrobial activity of *L. controversus* and *L. musteus* methanol extracts on selected microorganisms. *L. controversus* and *L. musteus* methanol extracts showed good antimicrobial activity (≥ 15 mm) against 3 and 4 bacteria between studied wild type microorganisms, respectively. These bacteria were *E. coli* (15.5 and 16.5 mm), *P. aeruginosa* (17.5 and 15.5 mm), *B. cereus* (16.2 and 15.5 mm) and *S. aureus* (16 mm for *L. musteus*) as wild type. *L. controversus* showed also antimicrobial activiy on *P. vulgaris* (16.75 mm), *M. morganii* (15 mm), *P. aeruginosa* (15.5 mm) as clinical isolate while *L. musteus* did not show a activity on same microorganisms. *L. musteus* methanol extract showed good antimicrobial activity (≥15 mm) 1yeast, but *L. controversus* did not show activity on *C. albicans*. There were no observable differences in the susceptibility from in point of cell wall as gram (+) and gram (-) bacteria at the antimicrobial effect of mushroom extracts. Also there has not got different effect on prokaryote and eukaryote in antimicrobial activity of *L. musteus.* But *L. controversus* effective only on bacteria. The *L. controversus* and *L. musteus* extracts also has been a more effective wild type than clinical isolates of microorganisms (for example wild type *E. coli* 15,5, clinic isolate *E. coli* 13,7 mm) in (Table 6).

According to literature, there are a number of studies on antimicrobial activity of Lactarius sp (*L. deterrimus, L. sanguifluus, L. semisangiufluus, L. piperatus, L. delicious, L. salmonicolor, L. rufus, L. vellereus* etc) [2,15,32,37, 39,85,86]. There is only one study showed that the extract of *L. controversus* had an antibacterial activity against *P. aeruginosa* [87]. In our study, it was determined that the extract of *L. controversus* showed higher activity both on wild type and on clinical isolate *P. aeruginosa* between the microorganisms studied. In literature, *L. controversus* was studied in terms of its effect on insects [88]. Dulger et al., (2002) [86] showed that *Lactarius* species revealed antimicrobial activity against some gram (+) and gram (-) bacteria, but showed

**Table 4**: Superoxide scavenging activity, (%) of the methanol extract of *L. controversus* and *L. musteus* at the different concentrations (50–500 µg/mL).

| Samples | Concentration (µg/mL) | | | |
|---|---|---|---|---|
| | 50 | 100 | 250 | 500 |
| *L. controversus* | 53.11 ± 0.32 | 52.96 ± 0.46 | 53.34 ± 0.21 | 54.78 ± 1.97 |
| *L. musteus* | 37.13 ± 5.82 | 49.24 ± 1.14 | 56.27 ± 3.07 | 62.23 ± 3.25 |
| BHA | 54.78 ± 0.18 | 59.25 ± 0.71 | 64.96 ± 1.07 | 82.51 ± 0.79 |
| BHT | 52.76 ± 0.11 | 56.75 ± 1.11 | 60.39 ± 1.75 | 76.62 ± 0.82 |
| α-tocopherol | 50.61 ± 0.07 | 52.65 ± 0.18 | 57.40 ± 0.57 | 61.65 ± 0.97 |
| Trolox | 67.34 ± 0.79 | 92.77 ± 1.72 | 96.99 ± 0.04 | 97.04 ± 0.04 |

**Table 5**: Free radical scavenging activity, (%) of the methanol extract of *L. controversus* and *L. musteus* at the different concentrations (50–500 µg/mL).

| Samples | Concentration (µg/mL) | | | |
|---|---|---|---|---|
| | 50 | 100 | 250 | 500 |
| *L. controversus* | 53.32 ± 0.01 | 53.17 ±0.01 | 58.60 ± 1.08 | 66.50 ± 0.04 |
| *L. musteus* | 66.08 ± 0.24 | 67.71 ± 0.15 | 67.71 ± 0.12 | 68.01 ± 0.10 |
| BHA | 54.78 ± 7.89 | 59.25 ± 5.81 | 70.80 ± 4.69 | 86.00 ± 2.35 |
| BHT | 52.76 ± 5.93 | 56.75 ± 0.86 | 60.39 ± 2.78 | 71.30 ± 0.46 |
| α-tocopherol | 50.61 ± 3.98 | 52.65 ± 6.94 | 55.66 ± 4.88 | 61.65 ± 0.76 |
| Trolox | 60.80 ± 1.65 | 68.30 ± 2.14 | 82.40 ± 2.06 | 98.80 ± 3.06 |

**Table 6**: Inhibition zone (mm) of crude extracts from *Lactarius controversus* and *Lactarius musteus* against wild type and clinical microorganisms (1.2 mg/disc).

| Assays (Microorganisms) | | L. controversus* | L. musteus* |
|---|---|---|---|
| Prokaryote: Gram (-) | *E. coli* W3110 | 15.50 ± 0.58 | 16.50 ± 0.58 |
| | *P. aeruginosa* ATCC 27853 | 17.50 ± 0.58 | 15.50 ± 0.58 |
| | *S. typhimurium* LT2 | 13.00 ± 0.82 | NT |
| | *E. aerogenes* ATCC *13048* | 8.75 ± 0.50 | 13.75 ± 0.50 |
| Prokaryote: | *S. epidermidis* **ATCC 12228** | 14.75 ± 0.96 | 14.75 ± 0.96 |
| Gram (+) | *B. cereus* **ATCC 7064** | 16.25 ± 0.50 | 15.50 ± 0.50 |
| | *E. faecalis* **ATCC 29212** | 14.75 ± 0.50 | NT |
| | *S. aureus* **ATCC 6535** | 12.75 ± 0.96 | 16.00 ± 0.82 |
| Prokaryote Clinical isolates | *E. coli* | 13.75 ± 1.50 | 14.75 ± 1.50 |
| | *P. aeruginosa* | 15.50 ± 1.00 | 13.50 ± 0.96 |
| | *E. aerogenes* | 11.50 ± 0.58 | 12.50 ± 0.58 |
| | MRKNS- Methicillin resistant koagulase (-) | 10.75 ± 0.96 | 11.50 ± 0.58 |
| | MRSA-Methicillin resistant *S. aureus* | 11.75 ± 0.50 | 12.50 ± 0.50 |
| | *M. morganii* | 15.00 ± 0.82 | 13.00 ± 0.58 |
| | *P. vulgaris* | 16.75 ± 0.50 | NT |
| | *A.baumania* | 12.00 ± 0.82 | 12.00 ± 0.96 |
| Eukaryote | *C. albicans* ATCC 10231 | 12.00 ± 0.58 | 15.50 ± 1.00 |

Inactive (-), Lower active (10 ≤). Moderately active (10-15). Higher active ≥ 15 mm active
*Zones of inhibition (mm ± SD), NT: Not tested

no antimicrobial effect against yeast. Methanol extracts from *L. piperatus* revealed antimicrobial activity against *E. coli, Proteus vulgaris*, and *M. smegmatis*, but did not show any effect against *C. albicans* [86]. Dulger et al., (2002) [86] showed that *L. delicious* was more effective than *L. piperatus*. But Barros et al., (2007) [15] showed that *L. piperatus* was more effective on microorganism than *L. delicious*. Moreover, the extract of *L. delicious* was found to be particularly effective against *Mycobacterium smegmats* and *M. tuberculosis* [89]. *Lactarius* is a large genus containing in g over 200 species of milky caps, and Turkey has got 40 lactarius species

which have been determined until now [90].

The majority of *Lactarius* species has yet to be determined in terms of its antimicrobial activity. Despite some similarities in the composition of mushroom samples, it is known that the chemical compositions of mushrooms are affected by a number of factors. In this situation, there is diversity in the amount of chemical substances of mushrooms at different cultivation status of same species. Therefore, chemical contents and antimicrobial substances of species of mushrooms naturally grown in different geographic locations in the world must be analyzed and the comparison of this analysis is very important [21]. It is a well-known fact that resistant bacteria are quite important for public health, and there is an urgent demand for new antibiotics and new classes of chemical formula that will efficiently inhibit the growth of pathogenic microorganism. Isolation and identification of specific antimicrobial agents from *L. controversus* can be investigated for its comparison with infections.

## CONCLUSION

*L. controversus* and *L. musteus* have been used as a natural food. In this study we report for the first time, the antioxidant and antimicrobial activities of methanol extracts of these mushrooms originating from Giresun Province of Turkey. The methanolic extract of *L. musteus* and *L. controversus*, that was exhibited to contain phenolic compounds and especially flavonoid, anthocyanin, ascorbic acid, β-carotene and lycopene, shown effective antioxidant, peroxide scavenging, reducing power, metal chelating, free radical scavenging and superoxide scavenging activity. These extracts of mushrooms exhibited also noteworthy antimicrobial activity against *P. aeruginosa* (*E. coli*), *B. cereus, P. vulgaris, M. morganii, P. aeruginosa* and *P. aeruginosa*.

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