Original Research Article

Chemical Composition, Antimicrobial and Antioxidant Activities of *Lactifluusvellereus*

ABSTRACT

The consumption of cultivated or wild mushrooms has increased significantly in recent yearsThis is because of their beneficial features, particularly their nutritional value and antioxidant capacity. Vitamins, fiber, protein, amino acids, and phenolic compounds are all abundant in mushrooms. Belonging to the family Russulaceae (Russulales), *Lactifluus* (Pers.) Roussel is a genus of milkcaps, which is predominantly represented in subtropical and tropical regions in the World. In this study it was aimed to determine the chemical composition (ash, total protein, total fat, and fatty acid composition), total amount of phenolic compounds, antioxidant capability and antimicrobial activity of *Lactifluus vellereus* (Fr.) Kuntze (1891) provided from Kastamonu, Türkiye. According to the existing literature, mushroom samples were identified based on their morphological characteristics. Dried mushroom samples were extracted with two different solvents, methanol and acetone, to determine antimicrobial activity against test microorganisms by disk diffusion method. The highest inhibition was recorded against *Staphylococcus aureus* ATCC 25923 strain with 16±0.81 mm zone diameter for acetone extract while it was 15.33±0.47 mm against *Salmonella typhimurium* SL1344 strain for methanol extract. The amount of total phenolic compounds and antioxidant capability of *L. vellereus* were also determined using the methanol extract via Folin-Ciocalteu phenol reagent method and DPPH radical scavenging activity and Pfrap methods, respectively. The amount of total phenolic compounds was found as 3.58±0.12 mg GAE/g DW while DPPH scavenging activity and PFRAP values were 334.88±0.29 mg TE/g DW and 381.17±0.99 mg AAE/g DW, respectively. The ash, total protein and total fat content of *L. vellereus* were 8.47±0.17 %, 20.48±0.07 % and 2.28±0.04 %, respectively. The fatty acid composition of the mushroom sample was also analyzed by GC-MS and oleic acid was found to be the predominant fatty acid with a concentration of 18.95 % followed by elaidic acid (9.30%) and linoleic acid (5.81%).

Keywords: Lactifluusvellereus,antioxidant activity,antimicrobial activity, chemical composition, GC-MS

1. INTRODUCTION

In recent times, there has been a notable surge in the consumption of both wild and cultivated mushrooms. This is because of their advantageous qualities, particularly

their nutritional value and antioxidant content. Mushrooms are a rich source of protein, amino acids, fiber, phenolic compounds, and vitamins [1]. Since they include various levels of minerals and trace elements required for specific biochemical reactions and cellular functions, they are regarded as one of the key foods that promote health [2].

A large number of edible and medicinal mushrooms represent an under-utilised source of substances that can be used in the treatment of a variety of diseases. Mushrooms are mainly used to improve the immune response to various diseases due to their high content of proteins and secondary metabolites. Although items made from mushrooms cannot totally replace prescription medications, using them can help the patient's overall health [3,4]. The cultivation and commercialisation of naturally growing mushrooms that can serve as nutraceutical, food and/or pharmaceutical ingredients is a promising area of research for scientists. This is because factors such as nutrition, healthcare and socio-economic changes can be supplemented with existing foods and medicines by considering these mushrooms. It is clear that native mushroom species represent an important and largely unexplored source of biologically active compounds with a wide range of potential applications in medicine, pharmacy and the food industry [3, 5]. Mushrooms are natural foods that can combat bacterial resistance thanks to their natural compounds with different mechanisms of action [6]. Mushroom extracts inhibit pathogenic microorganisms. It is advantageous over the use of synthetic antimicrobial compounds, especially because it has fewer unwanted side effects and can fight microbial resistance [7].

Lactifluus (Pers) is a genus of milkcaps that is mainly found in tropical and subtropical areas and is a member of the Russulaceae (Russulales) family [8]. It is one of the wild edible mushroom genus collected from rural areas. It is of economic importance for many local markets. More than 600 species of the genus *Lactifluus* Pers. (Russulales) have been described worldwide [9]. A considerable number of novel Lactifluus species have been identified in the last decade [10,11]. They are generally known for their medicinal and nutritional properties and are considered promising fungi in the medical industry [12]. Since Lactarius species are ectomycorrhizal species, they are associated with a variety of plants and hence have an ecological role in terrestrial ecosystems [9]. For example, *Lactarius deliciosus* (L.) Grey is a species widely consumed by rural populations and commercialized in some countries. It is also widely consumed in some parts of Türkiye [13]. Several research have been conducted on the mineral and chemical composition of *Lactarius vellereus* (Fr.) Fr. and *Lactarius piperatus* (L.) Pers., including analyses of their nutritional value, phenolic acid, fatty acids, protein and tocopherol contents [14-16]. Furthermore, some studies have investigated the antioxidant activity and antimicrobial activity of these species against different ATCC strains [17-19]. The purpose of this study was to determine the chemical composition, antioxidant and antimicrobial effects of *L. vellereus* mushroom.

2. MATERIAL AND METHODS

2.1 Material

The mushroom samples used in this study were obtained from a local bazaar in Kastamonu Province, Türkiye in 2019. Identification of the samples was performed based on morphological traits, such as size, shape and color of the cap, stipe and spores, according to current literature [20, 21]. The mushroom samples were dried using a conventional vegetable drier at 40 °C until the samples reached a constant weight. After that, the dried samples were pulverized and kept in laboratory conditions away from direct sunlight.

2.2 Preliminary Analyses

The chemical composition of *L. vellereus* was ascertained in terms of ash, total protein and total fat content for the evaluation of nutritional value. The ash content was determined using a Protherm PC442T furnace at 550 °C for 5 h. The total protein content was measured by Kjeldahl method via full automated OpsisKjelROC KD310 vehicle following the digestion of the sample with H_2SO_4 and titanium tablets at 400 °C. Using an Ankom XT15 device the total fat content was determined. The results were presented as % for all three parameters.

2.3 Extract Preparation

Two g of powdered sample was mixed with 30 mL of acetone or methanol, homogenized by Daihan HG-15D homogenizer at 9000 rpm for 3 min and incubated for 24 h at 30 ℃ 150 rpm by a shaking incubator. Following that, extracts were filtered through Whatman No:1 filter papers. A rotary evaporator was used to remove solvents until the material was dry. The residues were recovered with the same solvents to obtain solutions with a concentration of 200 mg/mL. The methanol extract was used to assess the total phenolic compound and antioxidant capability analyses. Both extracts were used to perform the antimicrobial activity assay. The extracts were kept at +4 ℃ until use.

2.4. Total Phenolic Compounds and Antioxidant Activity

Determination of the amount of total phenolics was performed by the Folin-Ciocalteu's phenol reagent method [22]. An aliquot of 100 µL methanolic extract was added to 1 mL Folin-Ciocalteu's phenol reagent and incubated at ambient temperature for 5 min. Then 1 mL 7.5% Na_2CO_3 solution was added to the mixture and incubated for an additional 90 min at ambient temperature in the dark. Following the incubation the absorbance of the sample tubes was measured by a spectrophotometer at 760 nm against blank. The amount of total phenolic compounds was calculated as mg GAE/g DW.

A commonly employed technique to examine the fungal samples' antioxidant activity is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH radical scavenging activity assay was conducted as reported by Blois [23] (1958). The absorbance of test tubes including the DPPH solution and the extract was measured by a spectrophotometer at 517 nm against a blank. A standard curve was

established using the DPPH scavenging values of Trolox solutions with various concentrations and the obtained data were presented in mg TE/g DW.

The antioxidative activity of the sample can be related to an increase in the absorbance of ferric ferrocyanide, a blue-colored complex with a maximum absorbance at 700 nm, which is formed by potassium ferricyanide reacting to potassium ferrocyanide. The antioxidant capability of *L. vellereus* methanol extract was also evaluated using the PFRAP method according to current literature. The ascorbic acid standard curve was used for data evaluation and the results were expressed as mg AAE/g DW [24].

2.5Antimicrobial Activity

Acetone and methanol extracts of *L. vellereus* were tested against pathogen microorganisms to evaluate the antimicrobial activity of the mushroom sample. *Escherichia coli* ATCC25922*, Salmonella typhimurium* SL1344, *Salmonella kentucky*, *Salmonella infantis*, *Pseudomonas aeruginosa* DSMZ50071, *Staphylococcus aureus* ATCC25923, *S. epidermidis* DSMZ20044, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC29212 and *Candida albicans* ATCC10231 strains were used as test microorganisms. The Strains were regularly cultured at 36.5 °C in Luria-Bertani (LB) agar. Each microorganism-bearing mixture was used to inoculate a single colony, which was then incubated at 36.5 °C for 18-24 h. The test microorganisms' turbidity complied with the 0.5 McFarland standard.

The antimicrobial activity of *L. vellereus* extracts was assessed using the disc diffusion method. By a sterile Drigalski spatula, 100 µL of each microbial culture in broth media was plated on agar media. Sterile discs of 6 mm in diameter were covered with 25 µL aliquots of extracts and placed on agar media. Subsequently, the plates were incubated for 24 hours at 36.5 °C. Discs containing gentamycin (10 µg) and vancomycin (30 µg) served as the positive control, while the negative control consisted of empty discs, acetone, and methanol. After the incubation period, the sizes of the inhibitory zones were measured. Every experiment was performed as three replicates, and the mean and standard deviations in mm were reported as results.

2.6Fatty Acid Composition

The fatty acid composition of *L. vellereus* was determined using gas chromatography-mass spectrometry (GC-MS). In this context, 1 g of powdered sample was mixed with 20 mL n-hexane and homogenized. The mixture was then incubated in an ultrasonic bath (Sonorex, Bandelin) for 30 min at 40 °C. After filtering the homogenate through a Whatman No:1 filter paper. A rotary evaporator was used to remove the solvent.The residue was resuspended with 5 mL of n-hexane. Methyl esters of the fatty acids in hexane extract were derived by 2M KOH in methanol and 1N HCl. After the phase separation, the clear upper layer was dried with anhydrous Na2SO4 and passed through a 0.45 µm syringe filter. Using a

Restek Rxi-5MS column, a Shimadzu QP2010 Ultra GC-MS system examined an aliquot of 1 µL of sample. The carrier gas, helium, was employed at a 1 mL/min flow rate. The fatty acids were characterized by comparing the obtained spectra with those from the Wiley (W9N11) mass spectra library and Flavor and Fragrance Natural and Synthetic Compounds (FFNSC 1.2) library [25].

3. RESULTS AND DISCUSSION

3.1 Identification of the Mushroom

Edible mushrooms are a great resource for both culinary and therapeutic uses. As a result, research into creating viable cultivation methods and reproducing wild species keeps growing. The samples of mushrooms used in the present study were purchased from a local bazaar in Kastamonu province, Türkiye. The current literature was followed in the identification process, which was based on the morphological characteristics of the mushroom samples. The mushroom samples were identified as *Lactifluusvellereus* (Fr) Kuntze (1891).

3.2. Preliminary Analysis

The mushroom samples were analyzed in terms of nutritional value including the ash, total protein and total fat content. The results are shown in Table 1.

Table 1:Ash, total protein and total fat content of *L. vellereus*

Ash (%)	Total protein (%)	Total fat (%)
8.4717±0.173	20.4837±0.074	2.28±0.041

According to the results *L. vellereus* contains 8.4717±0.173 % ash, while the total protein and total fat contents were determined as 20.4837±0.074 and 2.28±0.041 %, respectively. Consuming 200 g of dried mushrooms per day in place of meat has the effect of substituting the meat and maintaining a balanced protein intake. One kilogram of dried mushrooms is equivalent to two times the amount of albumin found in beef and eleven times the amount found in milk. The studies on the nutritional value of Lactarius species revealed that the content of ash, total protein and total fat might vary between 5.1-8.3 %, 13.06-31.81 and 2-2.69 %, respectively [12, 14, 26]. The findings of our study are in accordance with the current literature in terms of ash, total protein and total fat content of the *L. vellereus*.

3.3. Total Phenolic Compounds and Antioxidant Capability

L. vellereus was found to exhibit strong antioxidant activity in numerous investigations examining the antioxidant capacity of mushroom extracts using a variety of methodologies. Studies looking at the overall amount of the antioxidant and phenolic compounds potential of mushroom extracts all agree that there is a positive relationship between phenolic compounds and antioxidative activity [5, 15, 17, 27].

The amount of total phenolic compounds (TPC) and the antioxidant capability via DPPH radical scavenging activity and PFRAP assays of *L. vellereus* methanol extract were demonstrated in Table 2.

Table 2: Amount of total phenolic compounds and antioxidant capability of *L. vellereus* methanol extract

TPC (mg GAE/g DW)	DPPH (mg TE/g DW)	PFRAP (mg AAE/g DW)
3.5804±0.117	334.8812±0.286	381.1705±0.989

According to the results of individual experiments conducted as triplicates, the methanol extract of L. vellereus exhibited 334.8812±0.286 mg TE/g DW DPPH radical scavenging activity and 381.1705±0.989 mg AAE/g DW PFRAP capacity with a TPC value of 3.5804±0.117 mg GAE/g DW.

Determination of the phenolic content of natural extracts provides a first insight into the potential of the extracts. In a study, total phenolic contents of 3 different *Lactarius* species were found between 6.55-9.64 mg GAE/g. It has been observed in the literature that the total phenolic contents of Lactarius species have different values [28-35]. In a study using L. deliciosus and L. salmonicolor mushroom extracts, total phenolic compound amounts were calculated as 6.281±0.0006 and 8.615±0.0008 mg GAE/100 g dry material, respectively [36]. These results can be explained by differences in the place and time of collection or the methods used in extraction procedures [37-39].

Synthetic antioxidants should be replaced with safer antioxidants derived from natural sources. In the present study, the antioxidant properties of Lactariusvellereus were determined using different test systems and in this way, it was aimed to reach a more precise conclusion about the antioxidant properties of the extracts studied. DPPH is the most commonly used radical in antioxidant capacity studies and provides the evaluation of radical scavenging properties of extracts. Plant antioxidants neutralize radicals by donating electrons or hydrogen. The reducing power of Lactarius extract was also determined by FRAP test. There is a positive correlation between total phenolic content and radical scavenging and reducing power. Confirming these correlation results, many studies have reported the existence of a positive relationship between total phenolic content and radical scavenging and reducing power [40,41]. There is a consensus that phenolic compounds, especially phenolic acids, rank first among the phytochemicals responsible for the antioxidant activity of fungi. This is because these structures contain one or more aromatic rings and one or more hydroxyl (-OH) groups. Thanks to these properties, they have the potential to scavenge free radicals [42,43]. Antioxidant activity of Lactarius species indicates that these species can be used as a source of natural antioxidants due to concerns about the use of synthetic ones.

3.4. Antimicrobial Activity

In this study, *in vitro* antimicrobial activities of 30 µL aliquots of *L. vellereus* acetone and methanol extracts with a concentration of 200 mg/mL were investigated against

Escherichia coli ATCC25922, *Salmonella typhimurium* SL1344, *S. kentucky*, *S. infantis*, *Pseudomonas aeruginosa* DSMZ50071, *Staphylococcus aureus* ATCC25923, *S. epidermidis* DSMZ20044, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC29212 and *Candida albicans* ATCC10231 strains as test microorganisms. The results are shown in Table 3.

According to the results, the highest inhibition was observed against *S. aureus* with a zone diameter of 16.33±0.94 mm by *L. vellereus* acetone extract. Interestingly, the acetone extract did not show any effect against other pathogen strains. On the other hand, the methanol extract exhibited low to moderate inhibition against all pathogen microorganisms. The highest inhibition zone diameter was measured as 15.66±0.94 mm against *S. typhimurium* while the lowest was 9.33±0.47 mm against *E. coli* for the methanol extract.

Table 3: Antimicrobial activity of *L. vellereus* methanol and acetone extracts against test microorganisms

	Inhibition Zone Diameters (mm)			
Test Microorganisms	**VA**	**CN**	**Acetone**	**Methanol**
Salmonella typhimurium SL1344	17	22	nd	15.66±0.94
Pseudomonas aeuruginosa DSMZ 50071	8	18	nd	12.33±1.25
Salmonella kentucky	7	14	nd	12±0.82
Salmonella infantis	8	21	nd	10.33±0.47
S. epidermidis DSMZ 20044	nd	20	nd	11.66±0.47
Staphylococcus aureus ATCC 25923	7	10	16.33±0.94	10.33±0.47
Enterococcus faecalis ATCC 29212	nd	nd	nd	13.66±0.47
Escherichia coli ATCC 25922	12	20	nd	9.33±0.47
Listeria monocytogenes ATCC 7644	9	19	nd	13.5±1.47
Candida albicans ATCC 10231	nd	nd	nd	14.66±0.47

*VA: Vancomycin (30 µg), *CN: Gentamycin (10 µg)

According to the literature, there are many studies on the antimicrobial activity of *Lactarius sp.* (*L. deterrimus, L. deliciosus, L. sanguifluus, L. piperatus, L. semisangiufluus, L. vellereus*, *L. salmonicolor, L. rufus*) [44-48].

Lactariusdeliciosus showed inhibitory effect against the growth of *Klebsiella pneumoniae* and *Escherichia coli* under in vitro conditions. It was reported that *Lactariusdeliciosus* showed antimicrobial activity against *Staphylococcus aureus*, *Proteus vulgaris*, and *Mycobacterium smegmatis* [49], *Bacillus cereus*, *Bacillus subtilis* [14], *Candida albicans* [50]. It was stated that antimicrobial properties may be affected by factors such as solvent type, duration and concentration of extracts [14].

The antibacterial properties of L. controversus methanol and ethanol extracts against a variety of microorganisms (*Pseudomonas aeruginosa* DMS50071, *Bacillus*

megaterium DSM32, *Klebsiella pneumoniae* ATCC700603, *Escherichia coli* ATCC25922, *Staphylococcus aureus* COWAN1, *Candida glabrata* ATCC 66032, *Candida albicans* FMC17, and *Trichophyton* sp.) were examined in a study. The methanol and ethanol extracts of *L. controversus* were reported to have an antibacterial (8.3-25.3 mm) effect against the microorganisms utilized, based on the disk diffusion method [51].

The methanol extract obtained from the maceration method of *L. vellereus*, which has the highest antimicrobial activity among all the extracts we used in the study, showed high activity especially against *S. pyogenes, K. pneumonia, P. aeruginosa* and *S. enteriditis* at low concentrations (0.0048-0.0024-0.0097 mg/ml) [52].

In another study, it was reported that this species had no antimicrobial effect against *E. coli, C. albicans*, *P. aeruginosa, S. enterica* but formed an 8 mm inhibition zone against *S. flexneri* [53]. Methanol extracts of the same species were reported to have antimicrobial effects (12.00-17.50 mm) against *S. aureus,* P. aeruginosa, *E. coli*, and *C. albicans* [18]. Dulger et al. [49] reported that L. controversus has antimicrobial effect against Gram (+) and Gram (-) bacteria but not against yeasts. MIC values of some *Lactarius* species against *P. aeruginosa, E. coli, S. aureus*were found to be in the range of 9.38-37.50 mg/mL [54]. Methanol and ethanol extracts of *L. piperatus*, *L. quietus,* and *L. vellereus* species were reported to have MIC values in the range of 12.5-25 mg/mL against *S. aureus* [5].

The difference in antimicrobial activity results may vary depending on the species of the fungus, the place of collection, the components it contains, the solvent used in extraction, and the type of pathogenic microorganism. Therefore, it is important to analyze the chemical contents and antimicrobial substances of mushroom species growing naturally in different geographical regions and to compare these analyses [5, 18, 54-57].Using the data obtained from the studies, the active ingredients in the extracts with known antimicrobial activity can be determined in future studies. The obtained compounds can be isolated from fungi and used in diseases caused by pathogenic microorganisms after their effects on living tissues are tested.

3.5. Chemical Composition

In this study, the chemical composition of *L. vellereus* n-hexane extract was also investigated by GC-MS technique and the results are shown in Table 4.

According to the results oleic acid (18.95%, RT: 42.956), elaidic acid (9.3%, RT: 41.621), linoleic acid (5.81%, RT: 44.507), stearic acid (4.61%, RT: 42.132) and palmitic acid (1.72%, RT: 37.795) were found as predominant fatty acids existing in *L. vellereus* n-hexane extract. These findings support that edible mushrooms can be considered a rich source of dietary unsaturated fatty acids, which are valuable secondary metabolites with key roles in human metabolism. Hexacontane (7.34%, RT: 46.474), hexatriacontane (16.1%, RT: 46.649) and hexadecanal (11.57 %, RT: 53.342) were also determined in *L. vellereus*.

Table 4:Chemical constituents derived from L. vellereus n-hexane extract determined by GC-MS

No.	Compound	RT	MW+CF	RC	SI
1	Palmitic acid methyl ester	37.795	270 ($C_{17}H_{34}O_2$)	1.72	91
2	Elaidic acid methyl ester	41.621	270 ($C_{19}H_{36}O_2$)	9.3	94
3	Stearic acid methyl ester	42.132	298 ($C_{19}H_{38}O_2$)	4.61	93
4	Oleic acid ethyl ester	42.956	310 ($C_{20}H_{38}O_2$)	18.95	95
5	Linoleic acid methyl ester	44.507	298 ($C_{19}H_{34}O_2$)	5.81	97
6	Hexacontane	46.474	842 ($C_{60}H_{122}$)	7.34	82
7	Hexatriacontane	46.649	506 ($C_{36}H_{74}$)	16.1	92
8	Hexadecanal	53.342	240 ($C_{16}H_{32}O$)	11.57	84

*RT: Retention time (min), MW: Molecular weight, CF: Chemical formula, RC: Relative concentration, SI: Similarity index (%)

Dietary fatty acids, which are essential for human metabolism, are abundant in mushrooms. Lactarius species are known to have high concentrations of Myristic, Stearic, Palmitic, Linoleic and Oleic fatty acids. It was reported that Lactarius species are rich in organic components and include significant concentrations of sugars, fatty acids, tannins, flavonoids, ascorbic acid, and phenolic acids. According to the common statement of the researchers, the considerable biological activities reported may be related to the high concentrations of these chemical compounds in mushroom extracts [57-59].

4. CONCLUSION

Mushrooms consumed as food are known to be an ideal food because of their rich phytochemical content, low sugar and fat content and especially because they are good dietary products. In addition, edible mushrooms are recognized as a good food source for cardiovascular disease due to their protein and mineral content. Furthermore, these mushrooms are important for their antibacterial, antifungal, antiparasitic, detoxification and antidiabetic properties. The results of the study show that *L. vellereus* has important nutritional components, bioactive chemicals, antioxidant, and antibacterial properties. It may be used as a natural agent for these uses. It is advised that more investigation be done on the therapeutic and medical properties of L. vellereus. The results obtained should be confirmed by *in vivo* tests and the mechanisms of action of specific bioactive compounds found in this species should be studied.

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