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SELECTED MUSHROOMS AS POTENTIAL SOURCES OF ANTIMICROBIALS AND ANTIOXIDANTS

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ABSTRACT. The objective of the research is to investigate the antimicrobial and antioxidant properties of acetone extracts of the mushrooms *Macrolepiota mastoidea*, *Clitocybe nebularis*, *Lepista nuda*, *Chroogomphus helveticus*, *Lactarius deliciosus*, *Russula atropurpurea*, and *Ramaria stricta.* The microdilution method was used to determine the minimal inhibitory concentration (MIC) and assess the antimicrobial effects against five bacterial and ten fungal species. Extract of *C. helveticus* exhibited more powerful antimicrobial properties, with ranged MIC values from 0.16 mg/mL to 10 mg/mL. The assessment of the antioxidant activity involved: measuring the ability to scavenge DPPH radicals, conducting a reducing power assay, and determining the quantities of total phenolics and flavonoids in extracts. The findings of the research indicate that the extract from *C. helveticus* exhibited greater antioxidant potency (IC_{50} = 395.15 μg/mL). The strongest effect of reducing power showed extract of *C. helveticus.* The highest content of total phenols and flavonoids was detected in the extract of *C. helveticus*.

Keywords: mushrooms, acetone extract, antimicrobial activity, antioxidant activity.

INTRODUCTION

Fungi are among the most widely distributed organisms on Earth. Over the past decade, the use of novel DNA sequencing technologies has transformed how fungal taxonomy and diversity are investigated. This has resulted in a shift in estimations of the number of fungal species present, which is now ranging from 2.2 to 3.8 based on host association, and from 11.7 to 13.2 million species using high-throughput sequencing. Despite this, the number of formally described fungal species is relatively small, with only around 150.000 known species. Therefore, it is widely believed that fungi exhibit far greater diversity than is currently recognized (HYDE, 2022). Fungi range from simple forms like thread fungi to complex forms like mushrooms. A macroscopic fungus with a unique fruiting structure that is visible to the naked eye and can

be manually harvested is known as a mushroom. The fruiting body may be either hypogeous or epigeous (CHANG and MILES, 2004).

Many mushrooms are highly esteemed for their distinct taste and texture, as well as their nutritional benefits. The edible mushrooms are a valuable source of essential nutrients, such as proteins (containing all the essential amino acids), dietary fiber, essential oils, minerals, and vitamins. Furthermore, they are low in calories, fat, and cholesterol, making them a highly significant addition to the diet (OUZOUNI *et al*., 2009; BOONSONG *et al*., 2016).

Apart from their nutritional value, many edible mushrooms have potential therapeutic applications due to their capacity to produce diverse secondary metabolites, which may include organic acids, alkaloids, terpenoids, steroids, and phenolic compounds (JAYAKUMAR, 2011; KOSANIĆ *et al*., 2016; KOSANIĆ *et al*., 2020b). Repeatedly, the intake of mushrooms has been linked to positive impacts on human health, owing to the immunological and anticancer qualities of specific mushrooms, as well as their antihypertensive and cholesterol-lowering benefits, liver protection, anti-inflammatory, antidiabetic, antiviral, and antimicrobial attributes (KOSANIĆ *et al*., 2016). Mushrooms possess high contents of qualitative antioxidant components which neutralize free radicals by donating one of their electrons (HALLIWELL and WHITEMAN, 2004). Mushrooms are also a rich source of phenolic and flavonoid contents as major antioxidant compounds. Most edible mushrooms produce significant antimicrobial compounds and they could be used as a natural source of antibiotics (ALVES *et al*., 2012; STAJIĆ, 2015).

Because of all that, this research aims to evaluate *in vitro* antioxidant and antimicrobial potential of the acetone extracts derived from the mushrooms *Macrolepiota mastoidea*, *Clitocybe nebularis*, *Lepista nuda*, *Chroogomphus helveticus*, *Lactarius deliciosus*, *Russula atropurpurea*, and *Ramaria stricta.* In the literature, a few studies describe the bioactive properties of mushroom species which has been tested in this study. It has been found that some investigators have described antimicrobial and antioxidant activities for *Lactarius deliciosus* and *Clitocybe nebularis* (KOSANIĆ *et al*., 2016; 2020a). As well similar results for antimicrobial and antioxidant activities were found for species *Macrolepiota mastoidea* and *Lepista nuda* (DULGER *et al*., 2002; YANG-SUK *et al*. 2006; AKATA *et al*., 2018; ĆIRIĆ *et al*., 2019). Related to previous studies, our study can be considered different because of the difference in applied methods and solvents used. In addition, species *Russula atropurpurea*, *Ramaria stricta* and *Chroogomphus helveticus* have been investigated for the first time in this study.

MATERIALS AND METHODS

Fungal samples of *Macrolepiota mastoidea* (Fr.) Singer, *Clitocybe nebularis* (Batsch), P. Kumm, *Lepista nuda*., (Bull.), H.E. Bigelow & A.H.Sm., *Chroogomphus helveticus* (Schaeff.), O.K.Mill, *Lactarius deliciosus* (L.) Gray, *Russula atropurpurea* (Krombh.) Britzelm and *Ramaria stricta* (Pers.) Quél., were collected from Vitanovac, Kraljevo, Serbia, on Kotlenik mountain (43º44'19.1" N 20º47'02.6" E), in October of 2021. The demonstration samples are preserved in the facilities of the Department of Biology and Ecology of Kragujevac, Faculty of Science. The identification of mushrooms was done using standard keys (BESSETTE *et al*., 2000; UZELAC, 2009).

Extraction

Dry fungal material was powdered by an electrical mill. Finely ground mushrooms were extracted using acetone for 48 h. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator at 40°C. The dry extracts were stored at -18ºC until used in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO). DMSO was dissolved in sterile distilled water to the desired concentration.

Antimicrobial activity

Microorganisms and media

The following bacteria were used as test organisms in this study: *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 12453) and *Klebsiella oxytoca* (ATCC 13182). All the used bacteria were obtained from the American Type Culture Collection (ATCC). Their identification was confirmed at the Microbiological Laboratory of Kragujevac, Department of Biology, University of Kragujevac. The fungi used as test organisms were: *Trichophyton mentagrophytes* (ATCC 9533), *Fusarium solani* (ATCC 11712), *Cladosporium cladosporioides* (ATCC 11680), *Aspergillus niger* (ATCC 16888), *Aspergillus fumigatus* (ATCC 13073), *Penicillium italicum* (ATCC 10454), *Mucor mucedo* (ATCC 20094), *Trichoderma viride* (ATCC 13233), *Geotrichum candidum* (ATCC 34614) and *Paecilomyces variotii* (ATCC 18502). They were from the American Type Culture Collection (ATCC). Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose (PD) agar and Sabourad dextrose (SD) agar (Torlak, Belgrade). All cultures were stored at 4ºC and subcultured every 15 days. Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37ºC on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/mL. Suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures that grew at 27°C on a PD agar substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS (National Committee for Clinical Laboratory Standards M 38 - P, 1998).

Minimal inhibitory concentration

The antimicrobial activity of investigated mushrooms was tested by determining the minimal inhibitory concentration (MIC) by the broth microdilution method using 96-well microtiter plates (SARKER *et al*., 2007). A series of dilutions with concentrations ranging from 40 mg/mL to 0.0097 mg/mL for extracts were used in the experiment against every microorganism tested. The starting solutions of extracts were obtained by measuring off a certain quantity of extract and dissolving it in 5% DMSO. Two-fold dilutions of test samples were prepared in Müller-Hinton broth for bacterial cultures and Sabourad dextrose broth for fungal cultures. The MIC for antibacterial activity was determined with resazurin. Resazurin is an oxidation-reduction indicator used for the evaluation of bacterial growth. It is a blue nonfluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin or visible fungi growth was defined as the MIC for the tested microorganism at the given concentration. The MIC for antifungal activity was determined by establishing the visible growth of microorganisms. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as a positive control. A solvent control test was performed to study the effect of 5% DMSO on the growth of a microorganism.

Antioxidant activity

Scavenging DPPH radicals

The free radical scavenging activity of mushroom extracts was measured by using 1,1-diphenyl-picryl-hydrazil (DPPH). The method used was modified in detail, but similar to some other authors (IBANEZ *et al*., 2003; DORMAN *et al*., 2004). Two milliliters of methanol solution

of DPPH radical in the concentration of 0.05 mg/mL and 1 mL of extract were placed in cuvettes. The mixture was shaken vigorously and left to stay at room temperature for 30 min. After that, the absorbance was measured at 517 nm in a spectrophotometer ("Jenway" UK). Ascorbic acid was used as the positive control while the negative control consisted of all the reaction agents without extract. The DPPH radical concentration was calculated using the following equation (1):

$$\text{DPPH scavenging (\%)} = \frac{[\text{A0} - \text{A1}] \text{ x } 100}{\text{A0}} \tag{1}$$

where A0 is the absorbance of the negative control and A1 is the absorbance of the reaction mixture or standards.

Reducing power

The reducing power of extracts was determined according to the method of OYAIZU (1986). One milliliter of extracts was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixtures were incubated at 50ºC for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. Finally, the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance of the solution was measured at 700 nm in a spectrophotometer. Blank was prepared with all the reaction agents without extract. Higher absorbance of the reaction mixture indicated that the reducing power is increased. Ascorbic acid was used as a positive control.

Total phenolic and flavonoid content

Total phenolic compounds in the mushroom extracts were determined with Folin-Ciocalteu reagent according to the method of SLINKARD and SINGLETON (1997) using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the extract (1 mg/mL) in a volumetric flask diluted with distilled water (46 mL). One milliliter of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of 2% sodium carbonate (Na_2CO_3) was added and then was left to stay for 2h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The total concentration of phenolic compounds in the extract was determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extracts by using an equation (2) that was obtained from a standard pyrocatechol graph as:

$$\text{Absorbance} = 1{,}745 \text{ x total phenols } \left[\mu\text{g} \frac{\text{PE}}{\text{mg}} \text{extracts}\right] + 0{,}0001 \tag{2}$$

The total flavonoid content was determined by using the Dowd method (MEDA *et al.*, 2005). Two mL of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (1 mg/mL). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in a spectrophotometer against blank samples. The total flavonoid content was determined as microgram of rutin equivalent (RE) per milligram of dry extracts by using an equation (3) that was obtained from a standard rutin graph as:

$$\text{Absorbance} = 0{,}014165 \text{ x total flavonoid } \left[\mu\text{g} \frac{\text{PE}}{\text{mg}} \text{extracts}\right] + 0{,}1045 \tag{3}$$

Statistical analyses

Data analyses were performed with EXCEL package (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Antimicrobial activity

The acetone extracts of the tested mushrooms exhibited fairly potent antimicrobial activity against the tested bacteria and fungi.

The antibacterial activity of the tested mushroom extracts against the tested microorganisms was presented in Table 1. The antibacterial activity of the tested mushroom extracts against the tested microorganisms was shown in Table 1. The MIC of the extracts against the tested bacteria ranged from 0.16 mg/mL to 10 mg/mL. The maximum antibacterial activity was detected in the acetone extract of the mushroom *C. helveticus* against *B. cereus* (MIC = 0.16 mg/mL). The measured MIC values for *M. mastoidea* and *L. deliciosus* against bacteria was 0.62 mg/mL against *B. cereus* and 10 mg/ml against against all other bacteria. The acetone extracts of *R. stricta, C. nebularis*, and *L. nuda* had nearly equivalent antimicrobial activity, hindering the growth of the tested bacteria at concentrations of 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL. Conversely, the antimicrobial potency of the acetone extract of the mushroom *R. atropurpurea* was the weakest, inhibiting the tested bacteria only at concentrations of 5 mg/mL and 10 mg/mL.

Table 1. Antibacterial activity of acetone extracts of mushroom species.

Tested organisms	*B. cereus*	*S. aureus*	*K. oxytoca*	*E. coli*	*P. mirabilis*
Mushroom species	**MIC (mg/mL)**				
R. atropurpurea	5	10	10	10	10
R. stricta	2.5	5	10	10	10
L. deliciosus	0.62	10	10	10	10
C. nebularis	1.25	10	10	10	10
M. mastoidea	0.62	10	10	10	10
L. nuda	2.5	10	10	10	10
C. helveticus	0.16	0.62	2.5	5	0.62
Streptomycin	0.03	0.02	0.02	0.06	0.06

Table 2. displays the exhibition of the antifungal influence of the analyzed mushroom extracts against the tested microorganisms. The MIC of the extracts against the scrutinized bacteria ranged from 0.31 to 10 mg/mL. The highest antifungal efficacy with MIC values of 0.31 mg/mL was found in the acetone extract of the mushroom *C. helveticus* against *T. mentagrophytes* and *M. mastoidea* against *F. solani*. The rest acetone extracts of mushrooms have similar antifungal effects.

In this experiment, the tested mushroom extracts exhibit moderate antimicrobial activity. The examined mushrooms exhibited more powerful antibacterial than antifungal potency at the same concentrations. These outcomes were predictable since multiple tests have demonstrated that bacteria are more sensitive to antibiotics compared to fungi (HELENO *et al.,* 2015). Antimicrobial activity depends on the species of microorganism and the fungus used. The dissimilarities in the antimicrobial potential of tested mushrooms are likely due to the presence of distinct constituents with antimicrobial properties. Nevertheless, it is essential to comprehend that extracts are blends of natural compounds, and their antimicrobial activity is not only a consequence of the diverse actions of individual constituents but can also result from their interactions, which may produce a distinct impact on the general activity of extracts (KOSANIĆ *et al*., 2016). The potential mechanisms of the antimicrobial activity of the tested mushrooms include obstructing cell wall synthesis, hindering protein synthesis, modifying cell membranes, and impeding nucleic acid synthesis. In all instances, the cells lose their capacity to function

normally (MATIJAŠEVIĆ *et al*., 2016). The most sensitive bacterium was *B. cereus*, whereas *E. coli* exhibited the highest degree of resistance. Among the tested fungi, *T. viride* was the most sensitive, whereas *A. fumigatus, A. niger*, and *P. italicum* were the most resistant. Streptomycin and ketoconazole, utilized as reference standards, demonstrated greater antimicrobial potency than the tested samples.

Table 2. Antifungal activity of acetone extracts of mushroom species.

Tested organisms / Mushroom species	*T. mentagrophytes*	*F. solani*	*C. cladosporioides*	*A. niger*	*A. fumigatus*	*P. italicum*	*M. mucedo*	*T. viride*	*G. candidum*	*P. variotii*
	MIC (mg/mL)									
R. atropurpurea	2.5	2.5	10	10	10	10	5	2.5	2.5	10
R. stricta	2.5	5	10	10	10	10	10	2.5	5	10
L. deliciosus	2.5	10	10	10	10	10	5	1.25	10	5
C. nebularis	2.5	1.25	10	10	10	10	5	0.62	1.25	5
M. mastoidea	1.25	0.31	10	10	10	10	5	0.62	0.62	5
L. nuda	2.5	2.5	10	10	10	10	5	0.62	2.5	5
C. helveticus	0.31	5	5	5	5	5	5	1.25	10	2.5
Ketoconazole	0.16	0.08	0.08	0.04	0.16	0.16	0.08	0.08	0.04	0.16

Comparable to our acquired data, some researchers discovered relatively potent antimicrobial efficacy for *L. deliciosus* and *C. nebularis* (KOSANIĆ *et al*., 2016; 2020a), but against other species of microorganisms. As well similar results for antimicrobial activity were found for species *M. mastoidea* and *L. nuda* (DULGER *et al*., 2002; ĆIRIĆ *et al*., 2019). Species *R. atropurpurea*, *R. stricta* and *C. helveticus* have been investigated for the first time with this study.

Antioxidant activity

Table 3 illustrates the results of the DPPH radical scavenging assay conducted on the examined extracts. Acetone extracts of the tested mushrooms exhibited moderate scavenging activity against DPPH radicals. The most active species, *C. helveticus*, recorded the highest DPPH radical scavenging activity among the acetone extracts. The extract of *C. helveticus* has neutralized over 50% DPPH radicals at concentrations of 1 mg/mL and 0.5 mg/mL, which resulted in calculation of IC_{50} value (395.15 μg/mL). For the rest of the extracts, the percentage of radical neutralization was less than 50% at the tested concentrations, so IC_{50} values were not calculated. The lowest DPPH radical scavenging showed acetone extract of *R. atropurpurea*.

The outcomes of the investigation on the reducing power assay of the examined extracts are shown in Figure 1. High levels of absorbance demonstrate strong reducing power. The values of absorbance ranged from 0.018 to 0.224, and they increased as the concentration of the extracts increased. The acetone extracts of *C. helveticus* demonstrated the most substantial reducing power among the examined extracts, followed by the acetone extract of *R. stricta*. In contrast, other extracts exhibited less potent reducing power. The lowest reducing power confirmed the acetone extract of *R. atropurpurea*.

The total phenolic compounds of tested extracts are presented in Figure 2. The pyrocatechol equivalent was used to determine the amount of total phenolic compounds by applying the equation obtained from the standard pyrocatechol graph. According to the findings of the study, the phenolic compounds in the tested extracts ranged from 6.25 to 18.85 µg PE/mg. Acetone extract of *C. helveticus* had the highest amount of phenolic compounds at 18.85 µg PE/mg, while *R. stricta* had 11.4 µg PE/mg. On the other hand, *R. atropurpurea* had the lowest amount of phenolic compounds, which is 6.25 µg PE/mg.

Table 3. DPPH radical scavenging activity of acetone extracts of mushroom species.

Mushroom species	1 mg/mL	0.5 mg/mL	0.25 mg/mL	0.125 mg/mL
	% DPPH radical scavenging			
R. atropurpurea	19.39	18.18	16.77	14.57
R. stricta	24.53	21.28	16.35	14.99
L. deliciosus	20.19	19.50	17.38	16.25
C. nebularis	19.29	24.84	17.29	18.55
M. mastoidea	26.41	22.43	16.56	18.87
L. nuda	16.77	19.39	19.71	17.61
C. helveticus	76.52	68.76	42.14	28.20

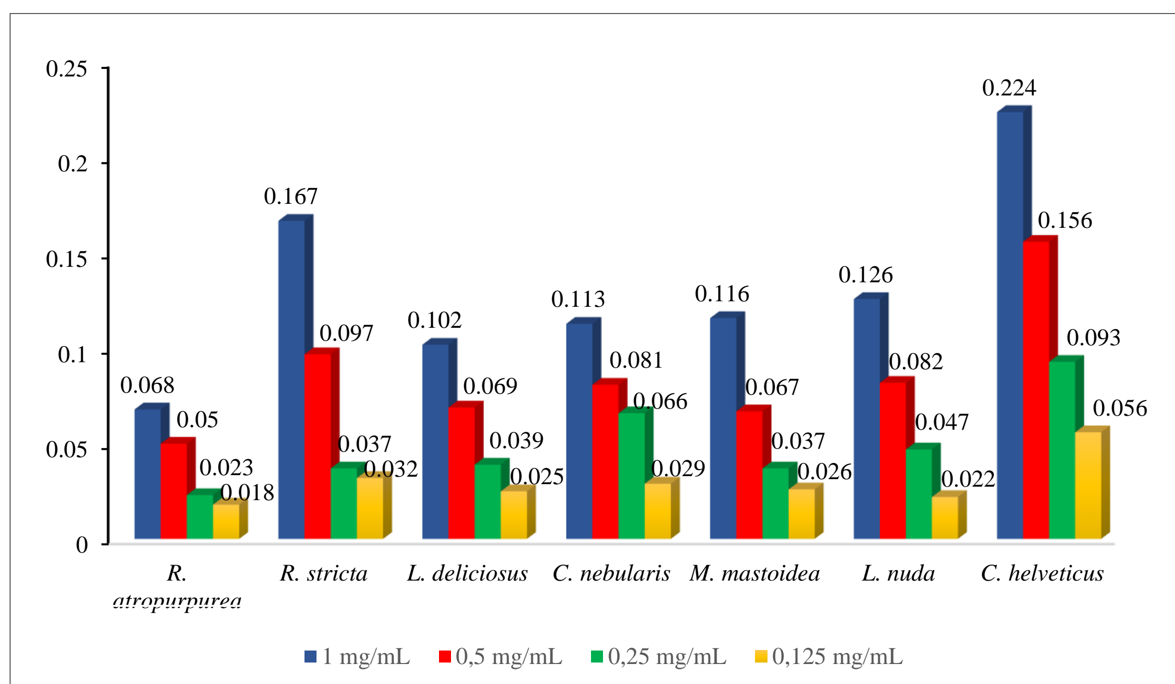

Figure 1. Reducing power of acetone extracts of mushroom species (Absorbance at 700 nm).

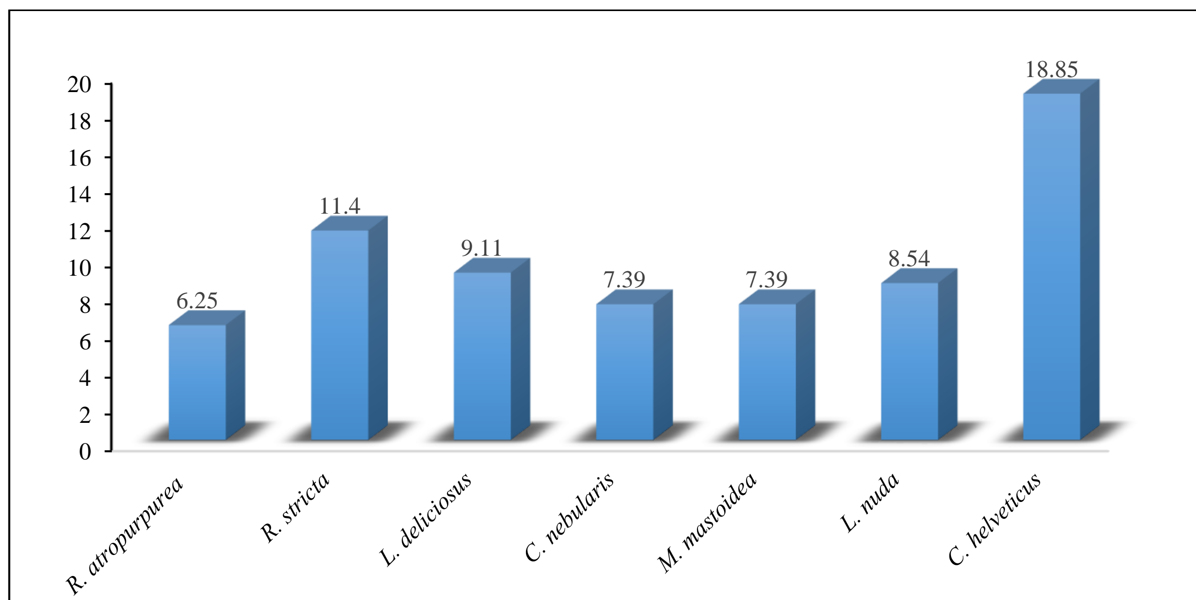

Figure 2. Total phenolic content of acetone extracts of mushroom species (μg PE/mg extracts).

The total flavonoid components found in the tested extracts are shown in Figure 3. The quantity of flavonoids was measured using an equation derived from a standard rutin graph and expressed in terms of rutin equivalents. The flavonoid compound of the tested extracts varied from 16.77 to 119.06 μg RE/mg extracts. The highest amount of flavonoid compounds were identified in the acetone extract of *C. helveticus*, with a value of 119.06 μg RE/mg, while the lowest flavonoid compounds were identified in the acetone extract of *M. mastoidea* (16.77 μg RE/mg extract).

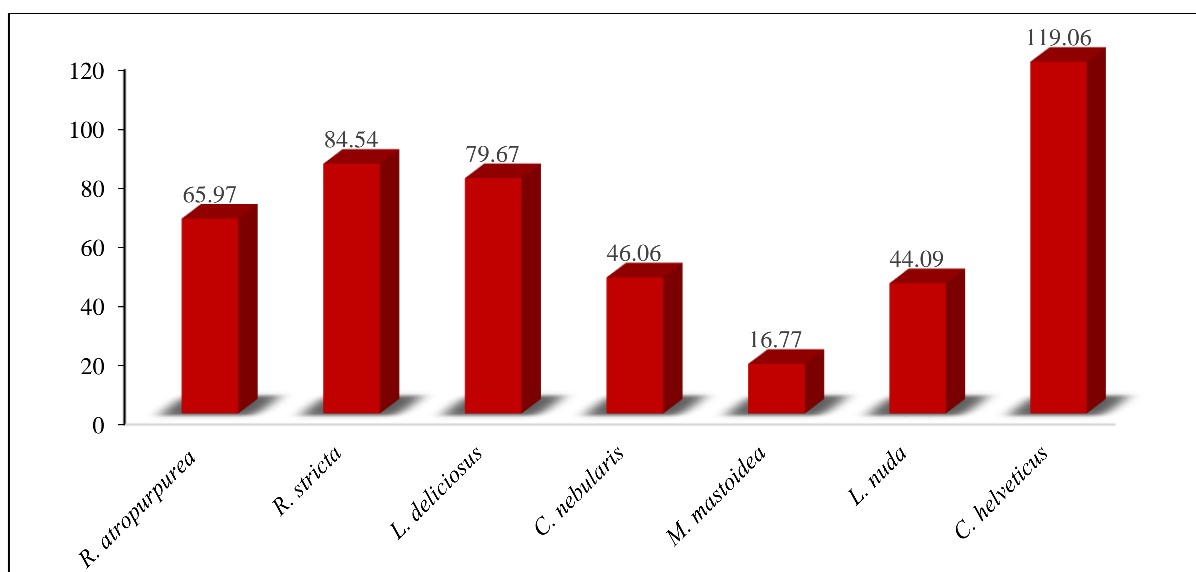

Figure 3. Total flavonoid content of acetone extracts of mushroom species (μg RE/mg extracts).

Evaluation of DPPH radical scavenging activity, reducing power, and determination of total phenolic and flavonoid compound content of the acetone extracts of the mushrooms were examined in this study. The antiradical activity of mushroom extracts was assessed by measuring their ability to decolorize the stable DPPH radical, which is a commonly used method for the evaluation of antioxidant potential (KOLEVA *et al*., 2002; ANANDJIWALA *et al*., 2008). The reducing power of a component can be indicative of its antioxidant properties, and reductones

are often associated with this characteristic (BECKER *et al*. 2004). Phenolic compounds are known to possess antioxidant activity by donating hydrogen to free radicals, which inhibits the chain reaction of lipid oxidation at the initial stage. Flavonoids are a diverse class of natural compounds and are among the most significant natural phenolics. They possess numerous biological and chemical functions, including the ability to scavenge free radicals. Earlier research has demonstrated a strong correlation between the antioxidative properties of mushrooms and their phenolic content (AZIEANA *et al.,* 2017; PODKOWA *et al*., 2021; BRISTY *et al.,* 2022). The significant correlation between the total phenolic content of mushroom extracts and their antioxidative activity indicates that phenolics could be the primary antioxidant compounds in mushrooms. Consistent with this, our study found that the extract of *C. helveticus* had the highest levels of phenols and flavonoids, as well as the most potent reducing power and DPPH radical scavenging activity, which confirms that phenols and flavonoids are probably responsible for the detected antioxidant potential of this mushroom.

With the exception of *C. helveticus,* remaining tested mushrooms have a moderate antioxidant activity. The decreasing order of inhibition of the tested extracts on DPPH radicals was as follows *C. helveticus* > *M. mastoidea* > *C. nebularis* > *R. stricta* > *L. nuda* > *L. Deliciosus* > *R. atropurpurea.* The values of total reducing power decreased in the following order: *C. helveticus* > *R. stricta* > *L. nuda* > *M. mastoidea* > *C. nebularis* > *L. deliciosus* > *R. Atropurpurea.* The values of total phenols decreased in the following order: *C. helveticus* > *R. Stricta* > *L. deliciosus* > *M. mastoidea* > *C. nebularis* > *L. nuda* > *R. atropurpurea*. The values of total flavonoids decreased in the following order: *C. helveticus* > *R. stricta* > *L. deliciosus* > *R. atropurpurea* > *L. nuda* > *C. nebularis* > *M. mastoidea*.

There are numerous records in the literature concerning the antioxidative properties of mushroom species that have been examined. Methanolic extracts of *L. deliciosus* and *C. nebularis* were previously investigated by KOSANIĆ *et al*. (2016; 2020a). It was discovered that the examined extracts displayed relatively elevated abilities in scavenging DPPH radicals, superoxide anion radicals, and in reducing power. Similarly, AKATA *et al*. (2018) found high antioxidant activity for the methanol extract of *M. mastoidea*. Results concerning the antioxidant capacity for ethanol extract of *Lepista nuda* also have appeared in the literature (YANG-SUK *et al*. 2006). The dissimilarities in our results, when compared to theirs, can be attributed to the disparity in the extraction technique and extraction solvent. Depending on the polarity of the extraction solvent, distinct compounds may be extracted that could potentially impact the antioxidative potential of the extracts. Despite the absence of research on the antioxidative effectiveness of *R. atropurpurea*, *R. stricta,* and *C. helveticus*, our outcomes indicate the positive potential for these mushrooms to be utilized as natural antioxidant agents, especially *C. helveticus*.

CONCLUSION

Based on the results presented in this study, the tested mushrooms manifested moderate antibacterial and antioxidant activities. The present study represents *C. helveticus* as a promising mushroom with great potential for serving as an alternative to synthetic antioxidants and antimicrobials. These results can have great medical and pharmaceutical importance. Further investigations should be focused on the separation and identification of the bioactive components present in *C. helveticus.* Also, it is imperative to conduct more comprehensive *in vitro* and *in vivo* investigations to confirm the possible bioactive characteristics of this mushroom.

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