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Evaluation of Antibiofilm, Antimicrobial, Cytotoxic and Antioxidant Effects of Some Wild Mushroom Species

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Abstract: In this study, ethanol extracts of some wild mushroom species, in the phylum *Basidiomycota*, (*Armillaria mellea* (Vahl) P. Kumm., *Infundibulicybe geotropa* (Bull.) Harmaja, *Leucopaxillus gentianeus* (Quél.) Kotl. and *Trametes versicolor* (L.) Lloyd) were tested for their antioxidant, antimicrobial, antibiofilm, and cytotoxic activities. Mushroom samples showed low antimicrobial activity on *Enterococcus faecalis* and *Staphylococcus aureus*, while biofilm inhibitory activity on test microorganisms ranged from 24.6% to 80.5%. At the end of the antioxidant activity studies, *A. mellea* was the mushroom sample having the highest DPPH radical scavenging capacity (0.105±0.001 mg/mL) whereas *T. versicolor* was the one having the highest iron (III) ion reducing power (40.709±0.003 µg TE/100g). The highest polyphenol content was observed in *T. versicolor* (29.916±0.002 mg GAE/100g) samples, and the lowest in *A. mellea* (9.5±0.006 mg GAE/100g) samples. The cytotoxic effects of the samples were tested on MCF-7, MDA-MB-231 (breast cancer) and L929 (mouse fibroblast) cell lines using the MTT method. As a result, it was observed that *A. mellea* and *T. versicolor* samples were more effective on the MCF-7 cell line, and *A. mellea* on MDA-MB-231 cell line.

Keywords: *Basidiomycota*, Bioactivity, Nutraceutical foods, Medicinal mushrooms

Bazı Doğal Mantar Türlerinin Antibiyofilm, Antimikrobiyal, Sitotoksik ve Antioksidan Etkilerinin Değerlendirilmesi

Öz: Bu çalışmada *Basidiomycota* şubesinde yer alan bazı doğal mantar türlerinin (*Armillaria mellea* (Vahl) P. Kumm., *Infundibulicybe geotropa* (Bull.) Harmaja, *Leucopaxillus gentianeus* (Quél.) Kotl. ve *Trametes versicolor* (L.) Lloyd) etanol ekstraktları, antioksidan, antimikrobiyal, antibiyofilm ve sitotoksik aktiviteleri açısından test edildi. Mantar örnekleri *Enterococcus faecalis* ve *Staphylococcus aureus* üzerinde düşük antimikrobiyal aktivite gösterirken, test mikroorganizmaları üzerinde %24.6 ile %80.5 arasında değişen biyofilm önleyici aktivite gösterdi. Antioksidan aktivite çalışmaları sonucunda, *A. mellea* en yüksek DPPH radikal temizleme kapasitesine (0.105±0.001 mg/mL), *T. versicolor* ise en yüksek demir (III) iyonu indirgeme gücüne (40.709± 0.003 µg TE/100g) sahip mantar türü olmuştur . En yüksek polifenol içeriği *T. versicolor* (29,916±0,002 mg GAE/100g) örneklerinde, en düşük ise *A. mellea* (9,5±0,006 mg GAE/100g) örneklerinde gözlendi. Örneklerin sitotoksik etkileri MCF-7, MDA MB-

231 (meme kanseri) ve L929 (fare fibroblast) hücre hatlarında MTT yöntemi kullanılarak test edildi. Sonuç olarak *A. mellea* ve *T. versicolor* örneklerinin MCF-7 hücre hattında, *A. mellea*'nın MDA-MB-231 hücre hattında daha etkili olduğu görüldü.

Anahtar kelimeler: *Basidiomycota*, Biyoaktivite, Nutrasötik gıdalar, Tıbbi mantarlar

Introduction

Medicinal mushrooms are macroscopic fungal organisms used in different ways (such as powder and extract) for various purposes, not only as natural food sources but also for various purposes such as protection from diseases, alleviation of disease process or healing of diseases and taxonomically found in *Basidiomycota* and *Ascomycota* divisions (Chang and Miles, 1992; Phillips 1981; Lindequist et al., 2014). In the past, mushrooms were widely used in Asian countries (such as China, and Japan) for their various medicinal effects, but today it has spread all over the world and their importance has gradually increased especially in Western countries (Lindequist et al., 2005). These organisms, which are not delicious and nutritious food, have also been a significant resource of medicine for the people who collect and consume them (Eraslan et al., 2021).

Mushrooms have been preferred as nutraceutical dietary promoters or functional foods due to their high protein, vitamin, fibre, mineral content and, on the contrary, low calorie and cholesterol content in recent years (Lindequist et al., 2014; Waktola and Temesgen, 2018). The results of the studies have shown that about 200 mushroom species have medicinal properties (Chang, 1996). It is estimated that primary and secondary metabolites (such as polysaccharides, polysaccharide-protein complexes, proteoglycans, peptides, phenolic compounds, lectins, steroids and terpenes) obtained from medicinal mushrooms have approximately 130 medicinal activities (Alves et al., 2012; Eraslan et al., 2021; Chugh et al., 2022). Antidiabetic, anti-inflammatory, antioxidant, antiviral, antibacterial, antifungal, cholesterol and lipid reducing, wound healing, hepatoprotective, immunomodulatory and antitumor can be given as examples for these biological activities that metabolites of fungal origin show (Wasser, 2010; Eraslan et al., 2021; Chugh et al., 2022).

Armillaria mellea (Vahl) P. Kumm. is a plant pathogen that is commonly seen in coniferous, deciduous and mixed forests and infects many plant species causing root rot in them. Basidiocarps are edible, but they can cause stomach problems after eating (Muszyńska et al., 2011; Kalu et al., 2022). Researchers have reported that some water-soluble polysaccharides (AMP) and some other substances (such as armillarikin, and armillaridine) obtained from this fungus have cytotoxic effects on different cancer cells (Wu et al., 2012; Chen et al., 2016). In addition, it has been detected that some sesquiterpene-class compounds (4-O-methylmelleolide, judeol, melleolide) and armillaric acid, and it has antimicrobial effects (Donnelly et al., 1985; Obuchi et al., 1990). *Infundibulicybe geotropa* (Bull.) Harmaja, an edible macrofungus species, develops by forming ring-shaped associations in grassy areas of deciduous or mixed forests usually in autumn (Phillips, 1981). It has been determined by studies that it has antioxidant, antigenotoxic, neuroprotective, antimicrobial (Altınsoy et al., 2017; Kosanić et al., 2020; Sevindik et al., 2020). L-amino acid oxidases obtained from this mushroom have been shown to have cytotoxic activity (Pišlar et al., 2016). *Leucopaxillus gentianeus* (Quél.) Kotl. is a bitter-tasting inedible macrofungus grown under trees in coniferous or deciduous forests (Moser, 1983). Cucurbitacin, a triterpene isolated from this mushroom, has many bioactive properties such as antimicrobial, antioxidant and antitumor (Kanani and Pandya, 2023; Delgado-Tiburcio et al., 2022). Medicinal mushroom *Trametes versicolor* (L.) Lloyd is an inedible macrofungus with wavy margins and fan-shaped sessile basidiocarps that grow in rows on dead stumps, trunks and branches of broadleaf rarely coniferous trees. There are concentric zones of different colours on the basidiocarps and the lower surface is porous (Jordan, 1995; Cui and Chisti, 2003). The high bioactivity of the polysaccharides (polysaccharide krestin, exopolysaccharides, polysaccharide peptide) obtained from this mushroom has attracted the attention of researchers and has been proven to have antiviral antimicrobial, antioxidant, anti-inflammatory and antitumor properties (Akagi and Baba, 2010; Sun et al., 2014; Duvnjak et al. 2016). Our aim in the present study is to reveal in vitro some biological activities of *A*. *mellea*, *I*. *geotropa*, *L*. *gentianeus* and *T*. *versicolor* species collected from their natural habitats and to contribute to the studies on this subject.

Material and Metod

Mushroom samples

Mushroom samples that constitute our study material [*A. mellea* (Turkish name: Bal mantarı), *I. geotropa* (Turkish name: Etçe), *L. gentianeus* (Turkish name: Boz hunişapka) and *T. versicolor* (Turkish name: Hindikuyruğu)] were collected while in regular field trips in Tokat province (Türkiye) (Sesli et al., 2020). The

mushroom samples were photographed in the field, then they were brought to the laboratory environment by wrapping them in separate paper packages without being damaged. Using data collected from the field and microscopic features revealed as a result of laboratory studies, the samples were diagnosed by Dr. Hakan Işık. The samples were dried with the help of a fan heater (Kumtel LX-6335 2000 W) at approximately 50°C and stored in polyethene bags for further studies.

Extraction procedure

In this study, the methods applied by Barros et al. (2007) and Redfern et al. (2014) was changed and used in the extraction processes. The identified dry samples were powdered separately and the extraction process of the samples was performed for 24 hours in 400 mL absolute ethyl alcohol using the soxhlet. Then, the solvent was removed at 40°C with the help of an evaporator and a dry-pure substance was obtained. To prepare the main stock, 0.2 g of dry pure substance was taken and redissolved in ethanol (10 mL). The prepared solution was sterilized using a 0.22 µm diameter syringe filter and kept at -20°C until the bioactivity analysis. The rest of the pure substance was stored at +4°C.

Antimicrobial Activity

In-vitro antimicrobial activity analyses of ethanol extracts of mushroom samples were carried out making use of the micro-broth dilution method. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (two gram-negative bacteria), *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 (two gram-positive bacteria) and *Candida albicans* ATCC 10231 (a yeast fungus) were selected as indicator microorganisms. Serial dilutions of sample extracts varying in the density range of 1000 – 31.25 µg/mL were made in MHB (Müller Hilton Broth). Then, 100 µL was taken from each of them and transferred to ELISA multi-well plates. The density of indicator microorganisms grown in 10 mL MHB medium for 16-24 hours in a shaker incubator was adjusted to 1.5 x 108 CFU/mL according to McFarland standard (No: 0.5). After dilution of 1/100 of the prepared bacterial and yeast suspensions, 100 µL was transferred to the wells. Plates were kept in an incubator at 30°C for 48 h for indicator yeasts and at 37 °C for 24 h for indicator bacteria. At the end of the incubation, measuring the absorbance value was performed at 600 nm by BMG LABTECH's ultra-fast UV/vis spectrophotometer (SPECTROstar Nano, Germany) and the minimum inhibitory concentrations (MIC) values were determined. In the analyses positive control dilutions were prepared of antibiotics (Fluconazole and Ciprofloxacin) whose effective on indicator microorganisms was utilized, and as negative control 200 µL of indicator bacteria and yeast solution was utilized. All analyses were performed according to CLSI guidelines (The Clinical & Laboratory Standards Institute) (Balouiri et al. 2016).

Antibiofilm activity

For biofilm analysis in the present study, the method proposed by Christensen et al. (1982, 1985) was used with slight modification. As indicator pathogen microorganisms *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 10231 were used. Firstly, pathogenic microorganisms were grown in MHB medium, then taken into Tryptic Soy Broth (TSB) medium including 2% dextrose (glucose) and incubated overnight at 37°C. In microplate wells, 100 µL of pathogenic microorganism suspension whose densities were adjusted by comparing with 0.5 McFarland standard and 100 µL of extracts prepared in six several densities in the range of 500-15.62 µg/mL in TSB was added. Following 24 hours of incubation at 37°C, the medium in the wells was poured, and sterile PBS (phosphate-buffered saline) solution was used to clear the planktonic cells from the plates. Then the plates were left at room temperature for 2 hours to dry and painted for 15 minutes with 200 µL crystal violet solution (1%). Following they were washed 3 times with PBS solution to clean the paint that did not adhere to the analysis. At last, methanol/acetone (80/20) solution was transferred to the microplates to dissolve the crystal violet. OD (optical density) was measured at 595 nm using ELISA microplate reader BMG LABTECH's ultra-fast UV/vis spectrophotometer (SPECTROstar Nano, Germany). In the analyses, negative control microorganism suspensions (200 µL) and positive control TSB including 2% glucose were used. The analyses were performed 3 times and the mean of the values were calculated. % BIC (biofilm inhibition concentration) was computed using the formula [BIC: $OD_{\text{negative control}} - OD_{\text{experimental group}} \times 100 / OD_{\text{negative control}}$].

Cytotoxic Activity

Cell Culture

In our study, human breast cancer cell lines (MDA-MB-231, MCF-7) and healthy mouse fibroblast cell lines (L929) were used. Cell lines in the study were purchased from ATCC (American Type Culture Collection). DMEM (Dulbecco's Modified Eagle Medium High Glucose-Gibco) with L-glutamine was used as the medium for cell proliferation. Then, penicillin-streptomycin (1%) and 10% FBS (fetal bovine serum) (Sigma) were added to the medium. Cells were grown in this prepared medium in an incubation medium containing 95% humidity, 5% CO_2 and 37°C.

MTT Analysis

The cytotoxic effect of mushroom extracts was determined using the MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-Diphenyltatrazilium bromide) method (Danışman Kalındemirtaş et al., 2021). For this purpose, serial dilutions of the extracts at seven various concentrations (2.5, 5, 10, 20, 40, 80, 100 µg/mL) were prepared. MTT is the formazone salt used for the determination of cell viability. Cells were seeded into the wells of ELISA plates at 1 x 10^5/mL cells. After the mushroom extract was applied to the cells, it was kept in the incubator for 24 hours. After incubation, 50 µL MTT solution (5 mg MTT/1 mL PBS) was added to each well. After the incubation period was completed, the MTT solution was removed by aspiration method. Dimethyl sulfoxide (DMSO) was transferred to the wells. Then plates were kept in incubation for 2 hours to dissolve the formazan crystals formed. Cell viability analysis was measured at a wavelength of 545 nm. Each experimental group was tested three times and the average value was determined. As a positive control, Cis-platinum was used and the selectivity index was determined with the formula: IC_{50} of healthy cell line/IC_{50} of cancer cell line (IC_{50}: the half-maximal inhibitory concentration).

Antioxidant Activity Tests

Total Polyphenolic Content (TPC)

Total Polyphenolic Content (TPC) of sample extracts was determined by changing the Folin-Ciocalteu method (Singleton and Rossi, 1965). This method is based on phosphotungstic acid reduction reaction in the basic solution. To determine the total amount of phenolic substances, 623.5 µL of 1:10 Folin & Ciocalteu reagent (phosphomolybdic-phosphotungstic acid reagents) and 12.5 µL of the mushroom samples (concentration 1 mg/mL) were transferred to the tubes and mixed by vortex. After waiting for a few minutes, 125 µL of 20% Na_2CO_3 solution was added to the mixture and vortexed again. The final mixture obtained was kept for 30 minutes in a dark environment at room temperature and absorbance was read at 700 nm wavelength using BMG LABTECH's ultra-fast UV/vis spectrophotometer (SPECTROstar Nano, Germany). A gallic acid (used as a positive control) standard graph was created according to the absorbances obtained. The results in the study were given as mg gallic acid equivalents (GAE)/100g dried sample.

Determination of Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method is the most widely used method among the antioxidant activity determination methods. In this study, the DPPH radical scavenging capacity of mushroom extracts was tested by changing the method proposed by Blois (1958). 3.94 mg DPPH was prepared in ethyl alcohol/100 mL. A determined amount (1-1000 µg/mL) was taken from the stock solutions of the samples, 1 millilitre of DPPH was added, and ethyl alcohol was added until the volume of the sample in the tube was 4 mL. After vortexing, it was left to incubate for 50 minutes. After incubation, the absorbance value of each sample was measured at 517 nanometers with a spectrophotometer. Ascorbic acid, a natural antioxidant, was used as a positive control to compare the results. Free radical scavenging activities (RSA) by determining the absorbance values of the extracts

[(OD $_{\text{negative control}}$ − OD $_{\text{experimental group}}$) / OD $_{\text{negative control}}$] x 100 calculated with the formula.

From the graph drawn with the % inhibition values calculated against the antioxidant concentrations fifty antioxidant concentrations causing inhibition (IC_{50}) were calculated.

Ferric Reducing Antioxidant Power (FRAP)

Ferric Reducing Antioxidant Power (FRAP) analyzes of ethanolic extracts of the mushrooms were made by changing the method of Benzie and Strain (1996), and (Harris, 1992). Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was utilised as a positive control. 100 µL of stock solution was taken and the volume was completed to 1.25 mL with phosphate buffer (0.2M, pH=6.6), then 1.25 mL of potassium ferric cyanide [$K_3Fe(CN)_6$] (1%) was transferred. This mixture was incubated at 50°C for 20 minutes. After incubation, TCA (1.25 mL, 10%) and $FeCl_3$ (0.25 mL, 0.1%) were added to this mixture. The absorbance of the resulting mixture was recorded at 700 nm. The results were given as µg Trolox equivalents (TE)/100g dried sample.

Results and Discussions

Antimicrobial Activity

In the study, antimicrobial activities of the extracts obtained from wild mushroom species were analyzed against 5 different microorganisms with the broth microdilution method. It was observed that none of the extracts showed effectiveness on *P. aeruginosa* and *E. coli*. On the other hand, it was observed that the extracts obtained from *A. mellea*, *I. geotropa* and *L. gentianeus* had a low effect on *S. aureus*. In addition, *A. mellea* and *T. versicolor* extracts were found to be slightly effective on *C. albicans* (Table1).

Table 1. MIC values of macrofungi samples against indicator microorganisms (µg/mL)

	E.coli ATCC 25922	*E. faecalis* ATCC 29212	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213	*C. albicans* ATCC 10231
A. mellea	500	250	500	125	250
I. geotropa	500	250	500	125	500
L. gentianeus	500	250	500	125	500
T. versicolor	500	250	500	250	250
Ciprofloxasin	< 1.9	< 1.9	< 1.9	< 1.9	
Fluconazole					< 1.9

The literature and our results show similarity Yamaç and Bilgili (2006) determined that *A. mellea* and *I. geotropa* extracts were less effective on *S. aureus*.

Dundar et al. (2016) determined that methanol extracts of *A. mellea* were not effective on *S. aureus* and *P. aeruginosa*, but showed low activity on *E. coli*. Giri et al. (2012) reported that methanol extracts of *A. mellea* had little effect on *E. coli* and *S. aureus*, but were ineffective against *P. aeruginosa* and *C. albicans*.In another study, the extract obtained from the mycelia of *A. mellea* showed antimicrobial effect on *E. coli* and *S. aureus*, but it did not show sufficient efficacy on *C. albicans* (Barranco et al., 2010). Previous studies have shown that some sesquiterpene-class compounds (such as 4-O-methylmelleolide, judeol, melleolide) and armillaric acid isolated from *A. mellea* have antibacterial and antifungal effects (Donnelly et al., 1985; Obuchi et al., 1990). Dizeci et al. (2021) determined that *I. geotropa* extract was effective on *E. faecalis*. Altuner and Akata (2010) found that *I. geotropa* extract was effective against *P. aeruginosa*. However, it had no effect on *C. albicans* and *E. coli*. Sterniša et al. (2022) reported that the L-amino acid oxidase activity in *I. geotropa* has antimicrobial and antibiofilm activity. In a different study, methanol and dichloromethane extracts prepared from *L. gentianeus* showed varying degrees of activity on some bacteria and yeasts (Sevindik, 2021). In a way that supports this, some researchers have reported various biological activity of Cucurbitacin isolated from *L. gentianeus* (Clericuzio et al., 2004; Kanani and Pandya, 2023). In our study, ethanol extracts obtained from *T. versicolor* showed little effect on *E. faecalis*, *S. aureus* and *C. albicans*, but did not show antimicrobial effect on *P. aeruginosa* and *E. coli*. Ozgor et al. (2016) determined that different extracts of *T. versicolor* were effective on *P. aeruginosa*, *E. coli*, *E. faecalis*, *S. aureus* and *C. albicans* with zone diameters ranging from 5 to 10.33 mm. In addition, another study shows that *T. versicolor* has different degrees of antimicrobial activity on *P. aeruginosa*, *S. aureus*, *E. faecalis* and *C. albicans* strains (Kaplan et al., 2021).

Polysaccharide-peptide complexes, polysaccharide crestin (PSK), polyphenols and terpenoids produced by *T. versicolor* have been revealed to have antimicrobial activity on various pathogens (Matijašević et al., 2016).

Antibiofilm activities

In this study, the biofilm inhibitory effects of macrofungi collected from their natural habitats on some gram positive (*S. aureus*, *E. faecalis*) bacteria and gram negative (*E. coli*, *P. aeruginosa*) and *C. albicans* were tested. According to the results of the study, it was observed that the macrofungi samples had varying degrees of antibiofilm effects on microorganisms. *L. gentianeus* extract had the lowest activity on *E. faecalis* (24.6%), while *I. geotropa* extract had the highest activity on *E. coli* (80.5%).

In addition, it was observed that *A mellea* and *T. versicolor* were not effective on *C. albicans* and *E. faecalis*, and *I. geotropa* on *E. faecalis*, *P. aeruginosa* and *C. albicans*. *L. gentianeus* was not effective only on *C. albicans* strains (Table 2).

Table 2. Anti-biofilm activity of mushroom ethanolic extracts [concentration (µg/ml, A) and biofilm inhibition concentration (%, BIC)]

	E. coli ATCC 25922		*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 29213		*E. faecalis* ATCC 29212		*C. albicans* ATCC 10231	
	A (µg/mL)	BIC %	A (µg/mL)	BIC %	A (µg/mL)	BIC %	A (µg/mL)	BIC %	A (µg/mL)	BIC %
A. mellea	125	75.6	125	78.04	500	70.7	-	-	-	-
I. geotropa	500	80.5	-	-	250	54.2	-	-	-	-
L. gentianeus	250	79.7	250	71.1	250	67.5	250	24.6	-	-
T. versicolor	250	46.8	250	78.1	500	27.1	-	-	-	-

Studies by other researchers on the antibiofilm activities of mushrooms are very limited. In recent years, some researchers have reported that mushrooms have a significant antibiofilm activity. Dizeci et al. (2021), similar to our study, found that extracts of *I. geotropa* exhibited biofilm inhibitory activity against *E. coli*. However, while their study detected activity against *P. aeruginosa*, our study did not observe this effect. Sterniša et al. (2022) determined that *I. geotropa* extracts had antibiofilm activity on *Salmonella infantis*.

Antioxidant Activity

Antioxidant activities of macrofungi samples were determined using the methods of TPC, DPPH radical scavenging activity and FRAP analysis. The results are shown in Table 3.

In the FRAP analysis of mushroom extracts, the graph equation obtained according to the calibration curve was found as ($y = 0.0031x-0.0012$, $R^2=0.9999$) and the amount of iron reducing power in the extracts was calculated as equivalent to trolox. As a result of the analysis of mushroom samples, the ferric reducing power was observed to be the highest in *T versicolor* extracts. When the results obtained in the study were compared with the literature, it was seen that the reducing power of *I. geotropa* and *L. gentianeus* was compatible with the literature (Kolaylı et al., 2012; Barros et al., 2007), while the reducing power of *A. mellea* was lower than expected (Dündar et al., 2016; Popescu et al., 2016). When the antioxidant properties of the mushroom samples used in the study were assessed in terms of their radical scavenging effects, it was determined that the species with the strongest antioxidant properties was *A. mellea* with an IC_{50} value of 0.105 mg/mL. When compared with different studies, it was observed that the radical scavenging effect of *A. mellea* samples was stronger than the samples in the literature (Lung and Chang, 2011; Dündar et al., 2016; Aytar et al., 2020). The results obtained for *L. gentianeus* and *T. versicolor* species were found to be compatible with the literature (Tel et al., 2013; Herawati et al., 2021). Phenolic compounds that have antioxidant properties with metal chelating and free radical capture properties are considered to be the most important group of water-soluble antioxidants. In this sense, the total phenolic content of mushroom samples was determined in this study. In our study, the graph equation obtained according to the calibration curve was found as ($y = 0.0012x+0.0726$, $R^2=0.9997$) and the total quantity of phenolic substances contained in the mushroom samples was calculated in terms of gallic acid. As a result, it was observed that the species with the highest polyphenol content was *T. versicolor.* While the total phenolic content of *A. mellea* species was found to be quite low (Aytar et al., 2020; Lung and Chang, 2011), it was seen to support the literature (Wang and Xu, 2014). In the study, the total amount of phenolic substances determined for *L. gentianeus* and *T. versicolor* was found to be compatible with the literature.

Table 3. Antioxidant activity values of mushroom ethanolic extracts

	TPC (mg GAE/100g)	DPPH (IC_{50} mg/mL)	FRAP (µg TE/100g)
1.	9.5 ± 0.006	0.105±0.001	21.032 ± 0.005
2.	13.666 ± 0.001	0.170±0.002	12.967 ± 0.003
3.	4.083 ± 0.002	0.237±0.001	23.72 ± 0.006
4.	29.916 ± 0.002	0.363±0.004	40.709 ± 0.003
5.		0.004±0.000	

1. *A. mellea* 2. *I. geotropa* 3. *L. gentianeus* 4. *T. versicolor* 5. Ascorbic acid

Antioxidant activity is one of the most important activities that mushrooms have shown. The content of secondary metabolites in the extracts and environmental conditions affecting the manufacture of secondary metabolites determine the power of antioxidant activity. For example, stressful environments increase antioxidant activity (Wardani et al., 2020; Lung and Chang, 2011).

Ascorbic acid, *β*-carotene, flavonoids and phenolic acids obtained from *A. mella* are chemicals with antioxidant properties (Lung and Chang, 2011). Phenolic substances (for example catechin, coumaric acid, chlorogenic acid etc.) obtained from *I. geotropa* and phenolic compounds (for example phenolic acids, gallic acid, catechin, chlorogenic acid etc.) obtained from *L. gentianeus* have antioxidant activity (Sevindik et al. 2020; Sevindik, 2021). Substances such as flavonoids, triterpenoids, saponins, tannins, and coumarin isolated by *T. versicolor* show antioxidant activity (Herawati et al. 2021).

Cytotoxic Analysis

In our study, the antiproliferative properties of mushroom extracts on breast cancer cell lines were investigated (Table 4). Cell viability was determined by MTT analysis. The second most common cancer in the world is breast cancer. It ranks first among cancer types seen in women with 25% in terms of incidence (Ferlay et al., 2015).

Table 4. Cytotoxic effect values of mushroom extracts on cell lines in vitro (IC_{50})

	MDA-MB-231	MCF-7	L929	Selectivity Index/MCF	Selectivity Index/MDA
A. mellea	24.56±1.85	20.73±0.56	51.93±1.93	2.11	2.51
I. geotropa	22.75±1.86	33.74±1.05	36.84±2.02	1.62	1.09
L. gentianeus	26.25±1.56	26.96±1.15	54.02±1.83	2.06	2.00
T. versicolor	23.73±0.47	24.50±0.67	50.03±1.78	2.11	2.04
cisplatin	10.39±0.64	8.03±0.23	22.87±0.38	2.20	2.85

While MCF-7 cell line has estrogen receptors, MDA-MB-231 cell line does not estrogen receptors. Therefore, these two cancer cell lines were preferred in this study. Since many polysaccharides, extracted from fungi show antitumor and immunostimulatory activities, research continues to explore the antitumor potential of various mushroom species (De Silva et al., 2013; Poyraz et al., 2015).

Wu et al. (2012) according to the study; they isolated a polysaccharide called AMP from *Armillaria mellea*. They demonstrated that AMP showed antiproliferative properties in A549 cells by activating the pathways of caspase-3 and caspase-9. Chen et al. (2016) found that the armillarikin substance isolated from *A. mellea* had a cytotoxic effect on human hepatocellular carcinoma. In our study, although the selectivity indexes of *A. mellea* were not very different, it was found to have high antiproliferative properties on breast cancer cell lines.

Altinsoy et al. (2017) reported that while the methanolic extract of *C. geotropa* did not show any cytotoxic effect on the MDA-MB-231 cell line, a cytotoxic effect was observed in ethanol. Toxicity varied depending on the concentration. In our present study, we determined that the ethanolic extracts of *C. geotropa* had cytotoxic activity in the MDA-MB-231 cell line.

In the study conducted by Sevindik (2021), it was observed that *L. gentianeus* extracts had a cytotoxic effect on the A549 (lung cancer) cell line. In our study, we observed a cytotoxic effect on the breast cancer cell line. The selectivity indexes were found to be high, around 2.

In our literature study, it was seen that the antitumor activity of *T. versicolor* mushroom extract was studied the most among our samples. It has been observed that the antitumor effect of polysaccharides in this mushroom species is high. In general, this mushroom extract inhibits p53 protein expression which is is a breast cancer-associated protein. Therefore, the extracts of *T. versicolor* have a high cytotoxic effect on breast cancer cell lines (Chauhan et al., 2019). In a study on human breast cancer cell lines, it was determined that *T. versicolor* extracts had antitumor activity on cell lines without estrogen receptors (BT-20 and MDA-MB-231) and on cell lines with estrogen receptors (MCF-7 and T-47D). It was determined that the extracts significantly inhibited the proliferation of MDA-MB-231, MCF-7 and T47D cells depending on the dose, but there was no significant growth suppression in BT-20 cells (Ho et al., 2005). Our study also supported the literature.

While *A. mellea* showed selectivity on MCF-7 cells, *I. geotropa* and *T. versicolor* on MDA-MB-231 cells, *L. gentianeus* did not show selectivity on either cell line. The selectivity index is calculated by dividing the healthy cell IC_{50} value by the cancer cell IC_{50} value. A high selectivity index (especially >3) is a very important feature for molecules that can be drug candidates. Because one of the most important side effects of chemotherapeutic agents used today is to kill healthy cells as well as cancerous cells (Nurgali et al., 2018). A low IC_{50} value indicates high cytotoxic activity. The lowest IC_{50} value was found in *A. mellea* on the MCF-7 cell line. All mushroom extracts showed lower cytotoxic activity than cisplatin after 24 hours of incubation. However, it is known that plant extracts show high cytotoxic activity when the IC_{50} value is below IC_{50}≤30 (Shawkey et al., 2013). Therefore, all fungi used in our study showed high cytotoxic activity.

Conclusions

Today natural mushrooms seem to be an important alternative functional foodstuff to increase the diversity of nutrients at the point of meeting the increasing nutritional needs with the rapid increase in the human population. Mushrooms were used for medicinal purposes only in narrow areas (such as far eastern countries) apart from using them widely as food in ancient times. Recent studies on the detection of bioactive compounds produced by mushrooms have increased. Thanks to the protective, supportive or therapeutic effects of these discovered chemicals, they attract attention in the field of medicine and biopharmaceuticals and bioactivity studies conducted thanks to newly discovered species, which continue to increase. As in the studies in the literature, the results of our study demonstrated that the mushrooms we tested (*A. mellea*, *I. geotropa*, *L. gentianeus* and *T. versicolor*) have various bioactivity at different degrees too. Edible mushrooms such as *A. mellea* and *I. geotropa* can be consumed directly while inedible mushrooms such

as *T. versicolor* can be consumed in forms such as extracts, capsules or tea. Primary and secondary metabolites obtained from these organisms can be a solution to problems such as the resistance of pathogenic microorganisms to semi-synthetic or synthetic drugs and the high costs of drugs used in cancer treatment. There is a need for extensive studies in terms of subjects such as the discovery of new metabolites with bioactive properties, their isolation, extraction of their chemical structures and their mechanisms of action.

Author contributions

All authors have equal contribution.

Conflicts of interest

The authors declare no competing interests.

Ethical Statement: It is declared that scientific and ethical principles have been followed while carrying out and writing this study and that all the sources used have been properly cited (Hakan IŞIK, Ceylan HEPOKUR, Uğur TUTAR, Emine DİNÇER)

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