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Total Phenolics, Antioxidative and Antimicrobial Activities of Some Edible Ascomycota Collected from Niğde

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A B S T R A C T

Mushrooms have been used as food and medicinal purposes for centuries. In this study, some edible Ascomycota were collected in 2018 and 2019 from Niğde, Turkey. Molecular identifications of the samples were performed afterward. PCR products were sent for sequence analysis after ITS fragments were amplified with suitable primers concerning DNA isolation of samples. The obtained data were compared with the Genbank database for samples' determination. The mushroom samples were determined to be *Terfezia claveryi* Chatin and *Morchella esculenta* (L.) Pers. Samples were extracted by ethanol and methanol to determine antimicrobial activity using the disc diffusion method. Antimicrobial zones against microorganisms were measured. Then DPPH radical scavenging effects and total phenolic content of *T. claveryi* and *M. esculenta* mushroom samples were studied. The highest antimicrobial activity was observed in ethanol extracts of *M. esculenta*. When the DPPH radical scavenging activities are taken into account, the activity is increased depending on the concentration. The highest DPPH scavenging activity was found in the extract of *M. esculenta* with 40.86%. It was determined that the total phenolic substances in methanol extracts of mushroom samples varies between 533.28 ± 1,15-537.34 ±2.20 µg GAE / mg extract.

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Introduction

The traditional identification of the edible Ascomycota is based on the characteristics of morphological structures such as shape, size and color of the ascocarp, gleba, and ascospores. Since these characteristics are highly dependent on the environmental conditions identification by morphological structures may be misleading at the 'species' level. Thus, DNA-based methods are widely used for avoiding such problems. The Internal Transcribed Spacer (ITS), which is an approximately 700bp long and highly conserved region of the nuclear DNA is generally used as a molecular marker for the identification of the fungal species due to its high inter-species variation (Giusti et al., 2021).

Edible Ascomycota have long been consumed by humans both for food and medicinal purposes since they have high digestible protein, essential fatty acids content (Doğan and Aydın, 2013) and bioactive compounds that have antioxidant, anti-inflammatory, antitumoral, antimicrobial, antidiabetic, antiallergic and immune system boosting effects (Janakat et al., 2004; Badalyan, 2012; Akyüz et al., 2019; Sevindik, 2020), as well as their unique taste and flavor.

Oxidative stress due to the accumulation of the reactive oxygen species (ROS) produced in living organisms, as a result of energy-producing reactions, may lead to serious diseases such as Parkinson's, Alzheimer's, cancer and cardiovascular disorders (Sevindik et al., 2018). In some cases, supplemental antioxidants are needed when endogenous antioxidants produced by the organisms to suppress ROS are inadequate (Bal et al., 2019). Extracts of Ascomycota fungi are known to have high antioxidant activity (Sevindik and Akata, 2019; Sarıdoğan et al., 2021) due to the high phenolic compounds and other secondary metabolite content of the fungal species (Taşkın et al.,

2021). Moreover, fungal extracts show various levels of inhibition against pathogen microorganisms (Gücin and Dülger, 1997; Duman et al., 2003; Akpi et al., 2017; İşlek et al., 2021). Since antibiotic resistance developed by pathogen microorganisms is a global concern, studies on searching novel antimicrobial agents have indicated that fungal species have a great potential in this era (Lindequist et al., 2005; Doğan et al., 2013; Sevindik et al., 2016; Badalyan et al., 2019).

In this study it was aimed to determine the content of total phenolic compounds, antioxidative and antimicrobial activity of the crude extracts of some edible Ascomycota collected in 2018 and 2019 from Niğde, Turkey.

Materials and Methods

Materials

Macrofungi samples were collected from different locations in Niğde between the years 2018 and 2019. All samples were photographed in their natural habitats. Environmental conditions and altitudes were noted. All samples were identified by Associate Professor Dr. Ilgaz AKATA, Ankara University. Identified samples were then freeze-dried and stored at the laboratory in the Biotechnology Department of Niğde Ömer Halisdemir University.

DNA Extraction and Molecular Identification

For supporting morphological identification and eliminate possible confusions at a species level, molecular identification based on Internal Transcribed Spacer (ITS) regions of the rDNA, which is widely used for discriminating fungal species, was also performed (White et al., 1990; Schoch et. al., 2012; Vu et al., 2019).

DNA extractions from samples were performed by the Macherey-Nagel™ Nucleospin Plant II DNA extraction kit with slight modifications in the supplier instructions. ITS regions were amplified by Polymerase Chain Reaction (PCR) with ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers which are forward and reverse, respectively. After agarose gel electrophoresis PCR products were sent to BM Labosis (Ankara, Turkey) for sequence analysis. Macrofungi samples were identified by comparing the data obtained from sequence analysis with National Center for Biotechnology Information (NCBI) GenBank database via Basic Local Alignment Search Tool (BLAST).

Preparation of Fungal Extracts

Dried macrofungi samples were weighed (10g), ground into a powder with mortar and pestle, mixed with 100mL solvent (ethanol and methanol separately for each sample), and incubated at 30°C for 48 hours at 150 rpm. After incubation extracts were filtered through Whatman No:1 filter papers and solvents were removed by a rotary evaporator. Obtained extracts were stored at +4°C until use.

Determination of Antioxidant Activity

The antioxidant potential of fungal extracts was evaluated by the DPPH scavenging method. DPPH (2,2 diphenyl-1-picrylhydrazyl) is a dark-colored powder comprised of stable free radicals showing maximum absorbance at 517 nm (Shimada et al., 1992). 0,1 mM DPPH solution and a serial dilution of fungal extracts-5 grade with a volume of 500 µL- were prepared with ethanol. 100µL extract was added onto 2.9 mL DPPH solution to form a mixture with a final volume of 3mL. The mixture was then incubated at room temperature for 15 minutes and the absorbance was measured by a spectrophotometer at 517nm. DPPH scavenging activity of the extracts was evaluated by the formula below where % SA is % DPPH scavenging activity of extracts, ADPPH is the absorbance of DPPH at 517nm and ASample is the absorbance of the sample at 517nm.

$$\% \; SA = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \times 100$$

Determination of Total Phenolic Compounds

Total phenolic compound analysis was conducted using Folin–Ciocalteu's reagent, which is also known as the Gallic acid equivalence (GAE) method. Gallic acid was used to establish the standard curve for the total phenolic content. 1mL of Folin–Ciocalteu's phenol reagent was added to 100µL of each sample and shaken. After 5 min, 1mL of (6%) Na_2CO_3 solution was added to the mixture and incubated at room temperature for 90 minutes. The absorbance of the samples was measured at 765nm by a spectrophotometer. Total phenolic compounds were expressed as µg GAE/g (Singleton et al., 1999).

Antimicrobial Activity Test

Disc diffusion method was applied to evaluate the antimicrobial activity of the fungal extracts against selected microorganisms. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* DSMZ 50071, *Salmonella typhimurium* SL1344, *Bacillus subtilis* DSMZ 1971 strains were used as test microorganisms. Microorganisms were inoculated in Luria-Bertani (LB) medium and incubated at 37°C for 24 hours. The turbidity of the microbial cultures was in accordance with the 0.5 McFarland standard.

100µL of microorganisms were plated on LB agar medium with a sterile drigalski spatula. 15, 30 and 60µL of fungal extracts were applied on sterile paper discs with a diameter of 6mm. Discs were then placed on an agar medium and incubated at 37°C for 24 hours. Antibiotic discs containing gentamycin (10µg/disc) and vancomycin (30µg/disc) were used as positive control and solvents (ethanol or methanol) were used as a negative control. After incubation, diameters of the inhibition zones against test microorganisms were measured and expressed in mm.

Results and Discussion

Identification of Samples

The fruiting bodies that constitute the material of this study were identified both morphologically according to the current literature (Breitenbach and Kränzlin, 1984) and molecularly by comparing the data obtained from sequence analysis with the NCBI GenBank database. GenBank is the most comprehensive and widely used database for researchers and most of the identifications of fungal species deposited in the GenBank were performed using the ITS region of the nuclear DNA as a standard marker

(Badotti et al., 2017). In this context, investigated fungal samples of this study were identified as *T. claveryi* and *M. esculenta* with a similarity rate of 100 and 99.2%, respectively.

Antimicrobial Activity

In our study, the antimicrobial activities of ethanol and methanol extracts of *T. claveryi* and *M. esculenta* at 15, 30 and 60 mg/mL concentrations against *E. coli, P. aeruginosa, S. aureus, B. subtilis, S. typhimurium* were investigated. The results are shown in tables 1, 2 and 3.

The inhibition zones against test organisms of the antibiotic discs containing gentamycin and vancomycin were used as reference (Table 1) to determine the antimicrobial activity of the fungal extracts.

While the highest antimicrobial activity was obtained from *T. claveryi* at 60 mg/mL ethanol concentration (27 ± 0.6 mm) against *E. coli* ATCC 25922 bacteria, the lowest antimicrobial activity was against *S. typhimurium* SL1344 bacteria at 15 mg/mL methanol concentration (8.7 ± 0.6 mm) was determined in *T. claveryi* (Table 2).

Janakat et al. (2004) investigated the in vitro antimicrobial activities of aqueous and methanolic extracts of *T. claveryi* against *S. aureus*. They showed that 5% aqueous extracts inhibited the growth of *S. aureus* by 66.4%, but methanolic extracts did not have a significant effect. It has been determined that methanolic extracts of *Terfezia* species have antimicrobial effects especially against *B. subtilis* and *S. aureus* (Hussan and Al-Ruqaie, 1999).

In a study, they investigated the in vitro antimicrobial activities of aqueous and methanolic extracts of *T. claveryi* against *P. aeruginosa.* The aqueous extract of the mushroom inhibited the growth of *P. aeruginosa* by 40.9%, while the methanolic extract was found to be ineffective.

It has been emphasized that the antimicrobial activities of *T. claveryi* may vary depending on the test method used, the microorganism species and the chemicals used (İnci and Kırbağ, 2018).

Ethanol extracts of *M. esculenta* did not show any antimicrobial effect on *B. subtilis DSMZ 1971* bacteria (Table 3). Eraslan et al., 2021 found that ethanol extract of *M. esculenta* showed the highest antimicrobial activity against *S. aureus*, *E. faecalis* and *E. coli* at a concentration of 50 µg/mL. The mushroom extract was also effective against *P. aeruginosa* at 50 µg/mL concentration.

In a study in which the antimicrobial activity of *M. esculenta* ethanol extract against 11 microorganisms was analyzed, low antimicrobial activity was determined against *S. aureus*, *S. lutea*, *S. typhimurium* and *C. albicans* (Kalyoncu et al., 2010). When these results are compared with our study, it is thought that the reason for the difference is the collection of samples from different localities and the variability of the strains.

In their study, researchers collected *M. esculenta* and *T. versicolor* mushroom samples from Bolu, Abant region. The antimicrobial activity of the ethanol extracts of the samples against Gram-positive, Gram-negative bacteria and fungi was investigated. No inhibition was detected in 40 and 15 µL against *E. coli* strain (Canlı et al., 2019). In our study, *M. esculenta* ethanol extracts (60 µL) showed an inhibition zone of 25± 0.6 mm against *E. coli* bacteria. In our study, the inhibition zones obtained against the bacteria *P. aeruginosa* DSMZ 50071, *S. aureus* ATCC 25923 and *B. subtilis* DSMZ 1971 were much higher than the inhibition obtained in this study.

Table 1. Inhibition zones of antibiotics Gentamycin and Vancomycin were used as a positive control.

Tested Organism	Inhibition zone (mm)	
	Gentamycin (CN)	Vancomycin (VA)
Escherichia coli ATCC 25922	20	12
Pseudomonas aeruginosa DSMZ 50071	20	-
Staphylococcus aureus ATCC 25923	22	17
Bacillus subtilis DSMZ 1971	21	8
Salmonella typhimurium SL1344	21	8

Table 2. Antimicrobial activities of crude extracts of *T. claveryi* collected from Niğde

Tested organisms	Inhibition zone (mm)					
	Ethanol extract			Methanol extract		
	15	30	60	15	30	60
Escherichia coli ATCC 25922	12± 0.6	20± 1.0	27± 0.6	9.7± 0.6	13± 0.6	16± 0.6
Pseudomonas aeruginosa DSMZ 50071	12± 3.2	16± 1.5	23± 3.1	10±0.0	13± 0.6	17± 0.0
Staphylococcus aureus ATCC 25923	12±0.6	17± 0.6	19± 0.0	10±1.2	13± 0.6	16± 0.6
Bacillus subtilis DSMZ 1971	12±0.6	16± 0.6	21± 1.7	9.3±0.6	13± 0.6	15± 0.6
Salmonella typhimurium SL1344	12±0.0	16± 0.6	22± 1.0	8.7±0.6	14± 0.6	17± 0.6

Table 3. Antimicrobial activities of extracts of *M. esculenta* collected from Niğde

Tested organisms	Inhibition zone (mm)					
	Ethanol extract			Methanol extract		
	15	30	60	15	30	60
Escherichia coli ATCC 25922	9.7± 0.6	18± 1.0	25± 0.6	10± 0.6	14± 0.0	17± 0.6
Pseudomonas aeruginosa DSMZ 50071	14± 1.2	19± 1.5	25± 0.6	11±0.6	13± 0.6	17± 0.6
Staphylococcus aureus ATCC 25923	14±0.6	19± 0.6	23± 0.6	11±0.6	13± 0.6	16± 1.2
Bacillus subtilis DSMZ 1971	00±0.0	00±0.0	00±0.0	11±1.2	13± 0.6	15± 1.0
Salmonella typhimurium SL1344	9.3±0.6	12± 0.6	15± 1.2	11±0.6	15± 1.2	19± 1.5

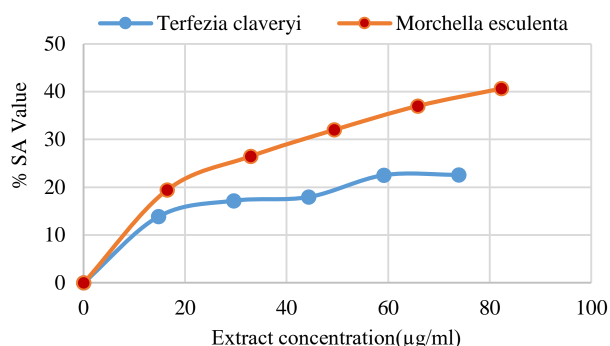

Figure 1. DPPH Scavenging Activities of Fungal Extracts

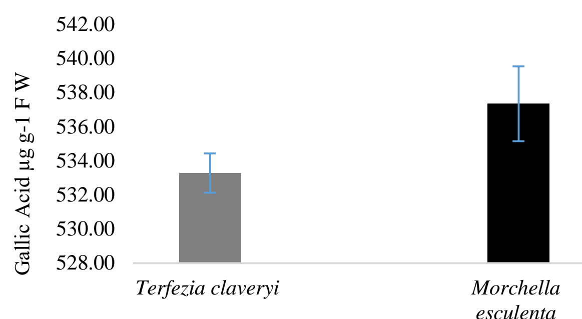

Figure 2. The total phenolic content of Fungi extracts

In the study, it was determined that *M. esculenta* samples did not form an inhibition zone against *E. coli* and *B. subtilis* strains in hexane, methyl alcohol and ethyl alcohol solvents. It was determined that it formed an inhibition zone against *P. aeruginosa* and *S. aureus* strain (Acay, 2018). The results obtained with 15 mg/mL methanol extract against these two bacterial strains were similar to the results of this study.

In our study, it was determined that mushroom extracts prepared with different solvents showed different antimicrobial effects against various microorganisms. The results are partially consistent with the findings of previous studies. As a result, it was determined that *T. claveryi* and *M. esculenta* can be a natural antimicrobial agents against the tested bacteria.

Determination of Antioxidant Activities

It has been stated in many studies that edible mushrooms have powerful antioxidants (Kozarski et al., 2015; Boonsong et al., 2016; Krupodorova and Sevindik, 2020; Roman et al., 2020). In most of the studies, it was stated that there is a positive correlation between the total phenolic content in mushroom extracts and their antioxidative properties, which confirms that edible mushrooms have the potential as natural antioxidants due to the ability of phenolics to inhibit lipid oxidation (Rathee et al., 2012).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is one of the most widely used spectrophotometric methods for the measurement of antioxidant activity (Akar et al., 2017; Szabo et al., 2007). In this study, the antioxidant potential of mushroom extracts was evaluated by the DPPH scavenging method. Free radical scavengers are one of the known mechanisms of antioxidants that inhibit lipid oxidation (Bajpai et al., 2017). The effect of scavenging potential of methanol extracts from fungi on DPPH radicals increased with increasing concentration (Figure 1)

According to Figure 1, among the fungi samples, *M. esculenta* (82.25 µg/mL) had the highest free radical scavenging effect with 40.86 %, while *T. claveryi* (14.78 µg /mL) mushroom showed the lowest free radical scavenging effect with 13.89 %.

Kıvrak (2015) found that methanol extracts of *T. claveryi* exhibited DPPH with an IC50 of 159.97 µg/mL. Saddiq and Danial (2012) found that *T. claveryi* extract has a DPPH activity of 72.6%. Akyuz (2013) found that *T. boudieri* has a DPPH activity of 22.24%. DPPH activity was affected greatly by the extraction solvents (Danham et al., 2018).

When the DPPH scavenging activities we obtained in our study were evaluated, lower results were obtained than in previous studies. It is thought that this difference between the results is due to the difference in the methods applied for extraction, the number of extracts used and the solvents.

Phenolic compounds draw attention with their potential to be beneficial to human health with their functions such as antioxidant, antitumor and antimutagen (Govindarajan et al., 2005). It has been shown in many studies that the antioxidant activity of mushrooms is proportional to the phenolic compounds in their content (Kim et al., 2008; Azieana et al., 2017; Vamanu and Nita, 2013; Vamanu 2014).

As a result of the studies conducted with *T. claveryi*, it has been reported that it has high antioxidant properties, total phenolic content and antimicrobial activity (Saddiq and Danial, 2012). In our study, the total phenolic content of the *T. claveryi* methanol extract was calculated as 533.28±1.15 µg GAE/mg (Figure 2). Wahabi et al. (2016), determined the polyphenol content of *T. claveryi* as (15.4±0.11 mg GAE/g).

The cap structure of *M. esculenta* has a wide variety of active ingredients, including tocopherols, carotenoids, organic acids, and phenolic compounds (Sevim and Sevim, 2021).

In one study, they determined the total amount of phenolic compounds in *M. esculenta* methanol extract as 47.01±0.89 (Wagay et al., 2019). In another study, the total phenolic content of the *M. esculenta* var. species was determined as 21.33 ± 1.40 µg GAEs/ mg extract. (Gürsoy et al., 2009).

In our study, the phenolic compound content of the methanol extract of *M. esculenta* was determined as 537.34 ±2.20 µg GAE/mg (Figure 2).

In this study, the extracts of *T. claveryi* and *M. esculenta* were found to have potent antibacterial and antioxidant properties. Our country is potentially rich in terms of edible macrofungi species. The amount of substance contained in macrofungi varies according to the geographical conditions of the region, genetic factors and collection time. Extracts from macrofungi prepared with various solvents or compounds isolated from them are used in biological activity studies. It is thought that biological activity studies using various extraction methods with mushrooms collected from different regions will play an important role in adding new information to the literature.

For fungi to be used as medicine or food support, they must be produced in large quantities and of standard quality.

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