



Bjerkandera adusta Collected from Niğde: Analysis of Total Phenolic Compound, Antioxidant, vnd Antimicrobial Properties[#]

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ABSTRACT

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Macrofungi have long been used as food and medicinal purposes by humankind. This study aimed to identify a macrofungus sample collected from Niğde, Türkiye in 2019 via molecular techniques and determine the content of total phenolic compounds, antioxidant and antimicrobial activities. According to data obtained from the sequence analysis of ITS fragment of rDNA the macrofungus sample was identified as *Bjerkandera adusta* (Willd.) P. Karst (1880). The sample was extracted with ethanol and methanol. Total phenolic compound content, antioxidant activity via DPPH scavenging method and antimicrobial activity via disc diffusion method of *B. adusta* was determined by using these extracts. The amount of total phenolic compounds was found as 772.28 µg GAE/mL for a methanolic extract of *B. adusta*. The ethanol extract of *B. adusta* showed 79.66% scavenging activity of 0.1 mM DPPH solution. The highest inhibition zone diameter was measured as 28±1 mm against *P. aeruginosa* by ethanolic extract, while the lowest antimicrobial activity was found in 15 µL methanol extract against *S. typhimurium* with an inhibition zone diameter of 8.7±1.2 mm.

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Introduction

In general, wild macrofungi grow naturally on the trunks of trees and/or in decaying woody locations with high humidity. They are considered as valuable food and therapeutic sources (Chugun et al., 2008; Zhang et al., 2013; Selamoglu et al., 2020) and perfect decomposers, thus having great importance in the natural material cycle. Macrofungi are cosmopolitan organisms that usually appear in rainy seasons, growing on various substrates (Lenz et al., 2007; Sevindik, 2018; Malakar et al., 2019). It is reported that macrofungi are composed of a high amount of protein, low carbohydrate, raw fiber, minerals, vitamins and secondary metabolites with antioxidant, antimicrobial, antitumoral, cholesterol lowering and immune system boosting effects (Sevindik, 2019; Mushtaq et al., 2020; Pereira et al., 2020; Rybczyńska-Tkaczyk et al., 2021).

Macrofungi are generally identified according to their morphological characteristics. Recent advances in molecular techniques help researchers identify fungal species in a more accurate and precise way, particularly for relatively close species. Identification based on direct sequencing of the internal transcribed spacer (ITS) region of the rRNA encoding gene is a precise tool for identification and constructing phylogenetic relationships among different organisms or species. (Yang et al., 2018). There are numerous studies in the literature which report that macrofungi samples are identified using rDNA-ITS markers and the phylogenetic relations among them are constructed by using data belonging to reference strains deposited in the NCBI GenBank database (Raja et al., 2017).

Reactive oxygen species (ROS) are oxygen bearing free radicals produced during natural energy metabolism reactions (Sevindik et al., 2017). Accumulation of ROS in cells may lead to serious chronic diseases, including diabetes, Parkinson's, Alzheimer's, cardiovascular disorders, and cancer (Sevindik and Akata, 2019). Antioxidant supplementation may be necessary when the natural defence systems of the organisms against ROS are not effective enough. It is known that fungal extracts have antioxidant properties and many studies have been conducted for the determination of the antioxidant potential of the macrofungi extracts (Vamanu and Nita, 2013; Kozarski et al., 2015; Akata et al., 2019; Akyüz et al., 2019; Roman et al., 2020).

Moreover, extracts of macrofungi samples prepared with different solvents and techniques showed various levels of inhibition against pathogen microorganisms (Gücin and Dülger, 1997; Barneche et al., 2016; Fakoya and Adeyemi, 2021; Canpolat et al., 2021; Soliman and El-Sayed, 2021). Antibiotic resistance developed by pathogen microorganisms is a global concern and studies on searching for novel antimicrobial agents revealed that fungal species with various bioactive compounds may constitute serious candidates (Lindequist et al., 2005; Alves et al., 2013; Akgül et al., 2016).

In this study, it was aimed to determine the antioxidant and antimicrobial activities and the content of total phenolic compounds of *B. adusta* collected in 2019 from Niğde, Türkiye.

Material and Methods

Material

Macrofungi sample was collected in May 2019 from Dündarlı district Niğde, Türkiye. Environmental conditions, altitude and coordinates of the location were noted. Macrofungi sample was identified according to the morphological traits using the current literature (Breitenbach and Kränzlin, 1984; Hansen and Knudsen, 1992; Cannon and Kirk, 2007) by Prof. Dr. Ilgaz AKATA (Ankara University). The identified sample was then freeze dried and kept at laboratory conditions until analyses.

Molecular Identification

Molecular identification based on the Internal Transcribed Spacer (ITS) region of the rDNA, which is generally used for discriminating fungal species, was also performed for supporting morphological identification (Schoch et al., 2012; Vu et al., 2019).

Macherey-Nagel™ Nucleospin Plant II DNA extraction kit was used to extract DNA from macrofungi samples with slight modifications in supplier instructions. ITS regions were amplified by Polymerase Chain Reaction

(PCR) with ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al., 1990). 1xPCR mixture includes: 2 µL of each primer (10 pm/µL), 3 µL DNA of the sample, 25 µL DreamTaq master mix and enough sterile distilled water (18 µL) to reach a final volume of 50 µL. The temperature program of the PCR thermal cycler was shown in Table 1.

The thermal cycler was set to 4°C until the PCR product was taken away. After agarose gel electrophoresis PCR product was sent to BM Labosis (Ankara, Türkiye) for sequence analysis. Macrofungi sample was identified by comparing data obtained from sequence analysis with the NCBI GenBank database via the Basic Local Alignment Search Tool (BLAST).

Preparation of Extracts

10 g of dried fungal sample was weighed, ground into a powder, mixed with 100 mL solvent (ethanol or methanol) and incubated at 30 °C for 48 hours at 150 rpm in a shaking incubator. Obtained extracts after incubation were filtered through Whatman No:1 filter paper and solvent was removed by a rotary evaporator under vacuum until dryness. The residue was resuspended with solvent to obtain a 200 mg/mL extract. The crude extract was stored at 4 °C for further use (Lin et al., 2016).

Test Microorganisms and Growth Conditions

The antimicrobial activity of the fungal extracts were tested against five pathogen microorganisms, namely: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DSMZ 50071, *Bacillus subtilis* DSMZ 1971, *Salmonella typhimurium* SL1344 ve *Staphylococcus aureus* ATCC 25923. The strains were routinely cultured in Luria-Bertani (LB) agar medium at 37 °C. A single colony from each corresponding organism was inoculated into LB broth medium and allowed to grow for 24 h at 37 °C. The turbidity of the microbial cultures used for the antimicrobial assay was in accordance with the 0.5 McFarland standard.

Disc diffusion method was performed for evaluating the antimicrobial activities of fungal extracts (Balouiri et al., 2016). 100 µL of microbial cultures in LB broth was plated on LB agar medium with sterile drigalski spatula. 15, 30, 60 µL aliquots of fungal extract were applied on sterile paper discs with a diameter of 6 mm. Discs were then placed on agar medium and incubated at 37°C for 24 hours. Extraction solvents (ethanol and methanol) and empty discs were used as negative control while discs with gentamycin (10µg/disc) and vancomycin (30µg/disc) were used as positive control. After incubation, diameters of inhibition zones around the discs were measured and expressed in mm.

Table 1. Temperature program set for the PCR.

Step	Temperature (°C)	Time (sec)	Cycle
Initial Denaturation	94	120	1
Denaturation	95	20	35
Annealing	55	20	35
Elongation	72	30	35
Final Elongation	72	300	1

Table 2. Inhibition zones against test microorganisms.

Test Microorganisms	Inhibition Zone Diameter (mm)							VA	CN
	<i>Bjerkandera adusta</i>								
	Ethanol Extract (µL)			Methanol Extract (µL)					
	15	30	60	15	30	60			
<i>E. coli</i> ATCC 25922	12±0.6	17±0.6	24±1.5	9.3±0.6	12±0.6	15±0.6	12	20	
<i>P. aeruginosa</i> DSMZ 50071	14±1.2	19±0.6	28±1	10±0	15±0.6	18±1.2	nd	20	
<i>B. subtilis</i> DSMZ 1971	13±0.6	17±1.5	23±1.2	9.3±0.6	13±0.6	16±0.6	8	21	
<i>S. typhimurium</i> SL1344	11±1	15±0.6	21±1.5	8.7±1.2	13±0.6	15±0.6	8	21	
<i>S. aureus</i> ATCC 25923	12±0.6	15±0.6	18±0.6	10±1.5	14±1.2	19±2.1	17	22	

VA: Vancomycin (30µg/disc), CN: Gentamycin (10µg/disc), nd: not detect

Total Phenolic Contents

Total phenolic compound analysis was conducted using Folin–Ciocalteu’s reagent and 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 methanolic extract of the sample, shaken and kept in dark for 5 min. Then, 1mL of (7.5%) Na₂CO₃ solution was added to the mixture and incubated at room temperature for 90 minutes. The absorbance of the blue color formed in the test tubes were measured at 765nm by a spectrophotometer. Total phenolic compounds were expressed as µg GAE/mL (Singleton et al., 1999).

Antioxidant Activity Assay

Antioxidant potential of ethanolic fungal extract was evaluated by widely used DPPH method. DPPH (2,2 diphenyl-1-picrylhydrazyl) is a dark powder material containing stable free radicals showing maximum absorbance at 517 nm (Blois, 1958; Shimada et al., 1992). 0,1 mM DPPH solution and a 5 graded serial dilution of fungal extract was prepared with ethanol. 100 µL extract was added to 2.9 mL DPPH solution, shaken vigorously and incubated for 15 min at room temperature in dark. After incubation the absorbance of the sample was measured by a spectrophotometer at 517 nm. 0.1 mM DPPH solution was used as blind. The DPPH scavenging activity of the fungal extract 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.

$$\% SA = (ADPPH - ASample) / ADPPH \times 100$$

Results and Discussion

Identification of the Macrofungi Sample

Macrofungi sample that constituted the material of this study was identified both morphologically according to the current literature (Breitenbach and Kränzlin, 1984; Hansen and Knudsen, 1992; Cannon and Kirk, 2007) and molecularly by comparing the data obtained from sequence analysis with the NCBI GenBank database. The sequence data deposited in the GenBank database belonging to fungal species are mostly based on the data of ITS fragments of rDNA as a standard marker. (Badotti et al., 2017; Giusti et al., 2021). According to the BLAST results the macrofungi sample showed a similarity rate of 99.92% and was identified as *Bjerkandera adusta* (Willd.) P. Karst (1880).

Antimicrobial Properties

In this study antimicrobial activity of 15, 30 and 60 µL aliquots of ethanol and methanol extracts with a concentration of 200 mg/mL of *B. adusta* against *E. coli* ATCC 25922, *P. aeruginosa* DSMZ 50071, *S. aureus* ATCC 25923, *B. subtilis* DSMZ 1971, *S. typhimurium* SL1344 were investigated. The results are shown in Table 2.

According to the results, the highest antimicrobial activity was found in 60 µL ethanol extract of *B. adusta* against *P. aeruginosa* with an inhibition zone diameter of 28±1 mm while the lowest antimicrobial activity was found in 15 µL methanol extract against *S. typhimurium* with an inhibition zone diameter of 8.7±1.2 mm.

Although Chikwem et al. (2020) has indicated in their study that the methanolic extract of *B. adusta* is superior compared to aqueous and ethanolic extracts against the bacterial strains tested, the results in this study show that the ethanolic extract of *B. adusta* has higher inhibition against test microorganisms. In another study investigating the antimicrobial activity of *B. adusta* ethanolic extract against pathogen microorganisms including *E. coli*, *S. aureus* and *P. aeruginosa* indicated that ethanolic extract of *B. adusta* has strong antimicrobial activity with inhibition zone diameters of 12±0.3, 15±0.1 and 10±0.52 mm, respectively (Soliman and El-Sayed, 2021). In our study, ethanolic extract of *B. adusta* showed inhibition against the mentioned three microorganisms with an inhibition zone diameter of 24±1.5, 18±0.6 and 28±1 mm, respectively.

The difference between the results in this study and in the literature may be due to the geographic differences in the sampling locations, altitude and growth phase of the macrofungi sample. The results also show that the antimicrobial activity of both ethanol and methanol extracts of *B. adusta* is in accordance with the amount of extract applied to the discs meaning that antimicrobial activity increases proportionally with the dosage of *B. adusta* extracts.

Total Phenolic Content and Antioxidant Activity

Many studies reported that macrofungi have high antioxidant potential (Kozarski et al., 2015; Sevindik et al., 2018; Akata et al., 2019; Szwajkowska-Michalek et al., 2022). Most of the studies that investigate total phenolics and antioxidative activities of fungal extracts reveal that there is a positive correlation between these two properties thus confirming that fungi have the potential as natural antioxidants due to the ability of phenolics to inhibit lipid oxidation (Rathee et al., 2012).

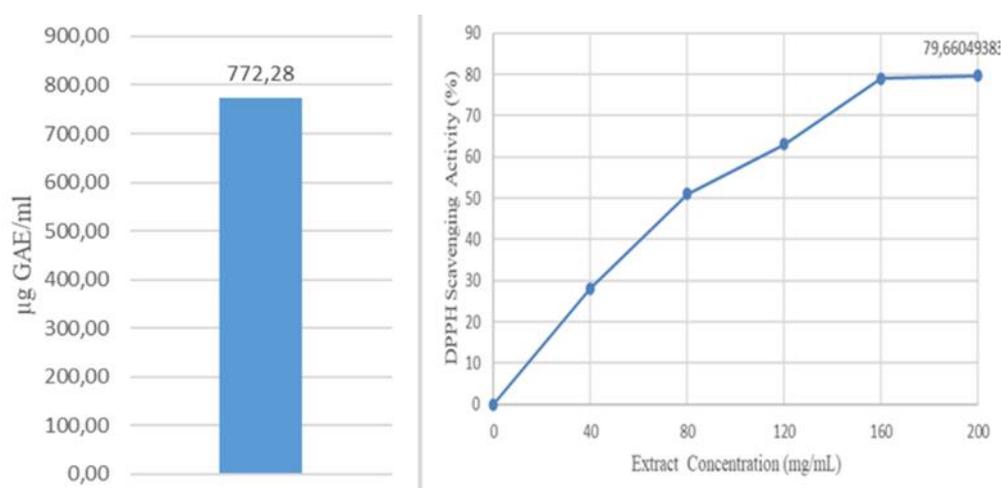


Figure 1. Total phenolic compounds content ($\mu\text{g GAE/ml}$) of *B. adusta* methanolic extract and DPPH scavenging activity (%) of *B. adusta* ethanolic extract, respectively

White rot fungi have their own characteristic enzyme systems that are responsible for the degradation of lignin, and laccase and peroxidase enzymes can be given as examples of extracellular oxidative enzymes produced during their secondary metabolism (Watanabe et al., 2000).

For the determination of antioxidative activity the DPPH assay is one of the most preferred spectrophotometric method. In this study antioxidant potential of *B. adusta* extract was also evaluated by using DPPH free radical scavenging method. According to Figure-1 DPPH scavenging activity of the *B. adusta* ethanolic extract increases in accordance with the increasing concentration and the highest DPPH scavenging activity was found in 200 mg/mL concentration with 79.66%. Phenolic compounds acting as hydrogen donors, singlet oxygen scavengers and reducing agents draw attention with their potential to be beneficial to human health with antioxidant, antitumor and antimutagen properties (Emsen and Güven, 2020). Figure-1 also indicates that *B. adusta* methanol extract constitutes of 772.28 $\mu\text{g GAE/ml}$ phenolic compounds.

The investigation findings showed that ethanolic and methanolic extracts of *B. adusta* may have antioxidant and antibacterial properties. The bioactive elements found in mushroom structures should be further studied, and focus should be given to the potential applications of bioactive materials derived from mushrooms in functional foods. It is recommended that more extensive research be done on mushrooms collected in the wild and that their potential uses be expanded.

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