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Original Article

Evaluation of Antioxidant Potential, Anticholinesterase Activity and Element Content of *Chlorophyllum rhacodes* Species

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ABSTRACT

In this study, antioxidant potential, anticholinesterase activity and elemental contents of *Chlorophyllum rhacodes* were evaluated. In order to determine the antioxidant capacity, total antioxidant level (TAS), total oxidant level (TOS), oxidative stress index (OSI), DPPH radical scavenging activity and FRAP ferric reducing power analyses were applied. According to the obtained results, TAS value was determined as 3.125±0.060 mmol/L, TOS value as 6.821±0.062 µmol/L, OSI value as 0.218±0.003, DPPH activity as 55.430±1.438 mg TE/g and FRAP value as 72.540±1.461 mg TE/g. These data revealed that *C. rhacodes* showed a significant level of antioxidant activity. In the evaluation of anticholinesterase activity, inhibitory effects against AChE and BChE enzymes were determined and IC_{50} values were determined as 94.330±1.536 µg/mL and 128.397±1.556 µg/mL, respectively. These results show that C. rhacodes shows limited inhibitory effect on these enzymes but may have a certain level of anticholinesterase potential. According to the results of elemental analysis, Cd (14.78±1.53), Cr (33.17±1.42), Mn (7.35±0.52), Cu (155.77±2.34), Fe (469.30±15.75), Pb (6.98±0.54), Ni (3.66±0.31) and Zn (76.18±2.25) mg/kg levels were detected in mushroom samples. In particular, it was observed that Cd and Cu levels were above the upper limits specified in the literature. This situation shows that *C. rhacodes* tends to accumulate some heavy metals and may be highly sensitive to environmental pollution. In conclusion, *C. rhacodes* species exhibits a biologically remarkable profile with certain antioxidant and anticholinesterase activities and can also be considered as a potential indicator species in the biological monitoring of environmental metal exposure.

KEYWORDS: *Chlorophyllum rhacodes*, antioxidant activity, anticholinesterase, heavy metal, elemental analysis, functional mushroom.

1. Introduction

In addition to the critical role they play in the sustainability of the nutrient cycle in nature, mushrooms are natural resources that attract attention in terms of medical and pharmaceutical sciences with their rich biochemical content [1]. Thanks to their high species diversity and ability to adapt to environmental conditions, mushrooms have the capacity to produce a large number of secondary metabolites [2]. These compounds include a wide spectrum, primarily polysaccharides, phenolic acids, flavonoids, triterpenes, alkaloids and sterol derivatives. Scientific studies show that these bioactive molecules can exhibit antimicrobial, anti-inflammatory, anticarcinogenic, antiviral and immunomodulatory activities in addition to their strong antioxidant effects [3-7]. In particular, reducing cellular damage caused by oxidative stress, regulating inflammatory responses and their regulatory effects on the immune system bring mushrooms to the forefront among natural therapeutic agents. In addition, it is reported that mushroom-derived compounds have the potential for protective and supportive treatment for many disease groups, from neurological diseases to

cardiovascular disorders, from metabolic syndrome to liver failure [8,9]. The evaluation of mushrooms, which have been used in traditional folk medicine for centuries, in modern pharmaceutical formulations has increased the interest in natural product research and has provided significant momentum in new generation drug development processes [10,11]. Today, the evaluation of mushrooms not only as food but also as the main component of functional food, nutraceutical and pharmaceutical products requires a more comprehensive understanding of the medical value of these organisms in line with interdisciplinary approaches. In this study, the antioxidant, anticholinesterase activity and element contents of *Chlorophyllum rhacodes* (Vittad.) Vellinga were determined.

C. rhacodes is an edible macromycete species that is commonly found in habitats such as open forest areas, rich meadows and pine plantations. It has been reported in regions with different climate and geographical characteristics such as Türkiye, India, Czechia and Ukraine. Due to morphological similarity, there is a risk of confusion with some toxic species, especially *Chlorophyllum molybdites*, so care should be taken during identification [12,13]. When evaluated from an ecological point of view, C. rhacodes is a non-mycorrhizal species; however, it has the ability to colonize the rhizosphere region. It usually produces fruit around ant nests and in organic-rich soils, especially in association with tree roots or insect remains. This species is quite adaptable in terms of habitat diversity and is widely collected in some regions [14]. Chemical analyses show that C. rhacodes tends to accumulate various elements. The presence of elements such as silver (Ag), copper (Cu), rubidium (Rb), selenium (Se), zinc (Zn), arsenic (As), cadmium (Cd) and thallium (Tl) has been detected. However, since this species is usually consumed in low amounts, the risk to human health is limited. In addition, studies on the accumulation of radioactive cesium (137Cs) and heavy metals have reported that this species can be used for environmental monitoring and bioindicator purposes [15-17]. When evaluated pharmacologically, C. rhacodes extracts have been shown to have antioxidant and antimicrobial potential. It has been reported that it has natural antioxidant properties, especially due to the presence of phenolic and flavonoid components. Acetone extracts have shown significant antimicrobial effects against some Gram-positive and Gram-negative bacteria, especially *Staphylococcus aureus* [18]. Although it is classified as edible, poisoning cases have been reported as a result of incorrect identifications made by the public. Therefore, it is important to obtain expert opinion and be careful when identifying the species in nature. In conclusion, *C. rhacodes* is an important species for both its potential pharmaceutical benefits and environmental monitoring; however, its safe use depends on correct identification [13,19].

2. Materials and Methods

The *C. rhacodes* samples used in this study were obtained from the Ilgaz Mountain region located within the borders of Kastamonu province of Turkey. The collected samples were dried under controlled conditions in a laboratory environment and brought to a form suitable for analysis. Within the scope of the extraction process, 10 grams of dry mushroom material was weighed and extracted with 250 mL of ethanol solution at 50 °C for approximately 6 hours using a Soxhlet apparatus. The obtained crude extract was concentrated with a Buchi R100 model rotary evaporator operating at 40 °C in order to remove organic solvent. The final product extracts were stored at 4 °C until experimental analyses were performed.

2.1. Antioxidant activity tests

Total antioxidant capacity (TAS) and total oxidant level (TOS) measurements of extracts obtained with ethanol from aboveground parts of *C. rhacodes* were carried out using commercial analysis kits from Rel Assay Diagnostics. The measurements were carried out in accordance with the protocol and instructions provided by the manufacturer. Trolox was used as the reference standard in TAS analyses, while TOS measurements were calibrated according to the hydrogen peroxide standard. The obtained TAS values were expressed in mmol TE/L , and TOS values were expressed in μmol H_2O_2/L [20,21]. Oxidative stress index (OSI) was calculated by converting both parameters to the same unit and then dividing TOS by TAS, and the result was reported in percentage (%). [22].

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of mushroom extracts was determined spectrophotometrically with slight modifications. Stock solutions of the extracts were prepared in DMSO at a concentration of 1 mg/mL. From each stock, 1 mL was mixed with 160 μL of freshly prepared DPPH solution (0.267 mM in methanol). The mixtures were incubated for 30 minutes in the dark at room temperature, and the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Methanol was used as a blank, and DPPH solution without sample was used as the control. Trolox standards (0–500 μM) were prepared under the same conditions to generate a calibration curve, and the antioxidant capacity of the extracts was expressed as mg Trolox equivalent per g extract (mg TE/g extract). All experiments were conducted in triplicate, and results were reported as mean ± standard deviation (SD) [23].

The antioxidant capacity of mushroom extracts was determined using the FRAP (Ferric Reducing Antioxidant Power) method with minor modifications. For the assay, 100 μL of each extract was mixed with 2 mL of freshly prepared FRAP reagent. The FRAP reagent consisted of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ mixed at a ratio of 10:1:1. The reaction mixture was incubated at 37 °C for 4 minutes, and absorbance was recorded at 593 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). The reagent mixture without sample was used as the blank. Trolox solutions (0–500 μM) were prepared as standards to generate a calibration curve. Antioxidant capacities were expressed as mg Trolox equivalent per g extract (mg TE/g). All measurements were performed in triplicate, and values were reported as mean ± standard deviation (SD) [23].

2.2. Anticholinesterase activity tests

The anticholinesterase activity of the ethanolic extracts of *C. rhacodes* was evaluated using the colorimetric method of Ellman et al. [24] with minor modifications. Galantamine was used as the positive control. Stock solutions of the extracts were prepared and tested at concentrations ranging from 200 to 3.125 μg/mL. In a 96-well microplate, 130 μL of phosphate buffer (0.1 M,

pH 8.0), 10 µL of sample solution, and 20 µL of enzyme solution (AChE or BChE) were added sequentially to each well. The mixture was incubated for 10 min at 25 °C in the dark. Subsequently, 20 µL of DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] solution and 20 µL of substrate (acetylthiocholine iodide for AChE or butyrylthiocholine iodide for BChE) were added to initiate the reaction. Absorbance was recorded at 412 nm using a UV-Vis microplate reader (BioTek Synergy HTX, USA). Appropriate blanks without enzyme were included to correct for background absorbance. All assays were performed in triplicate, and IC_{50} values (µg/mL) were determined from dose–response curves. Results were expressed as mean ± standard deviation (SD).

2.3. Elemental contents

Elemental content in the fruit bodies of the mushroom was analyzed using atomic absorption spectrophotometer (Agilent 240FS AA, USA). Before analysis, samples were dried in an oven at 80°C to remove moisture. Then, 0.5 grams of mushroom sample was subjected to mineralization process with a mixture containing 9mL of nitric acid (HNO_3, 65%) and 1mL of hydrogen peroxide (H_2O_2, 30%). This process was carried out using a microwave-assisted digestion system (Milestone Ethos Easy) to ensure complete dissolution of the organic matrix [25].

3. Results and Discussion

3.1. Antioxidant activity

Mushrooms are natural sources that exhibit significant antioxidant activity thanks to their rich phenolic compounds, flavonoids, ascorbic acid, tocopherols and especially polysaccharide structures [26]. These bioactive components play an effective role in reducing oxidative stress caused by free radicals and help prevent cellular damage. Considering that oxidative stress is associated with many chronic diseases such as cancer, cardiovascular diseases, diabetes and neurodegenerative disorders, the potential of mushrooms in this direction is increasingly gaining importance [27,28]. In vitro studies have revealed that many edible and medicinal mushroom species show high activity in different antioxidant tests. These results show that mushrooms should be evaluated not only in terms of nutritional value but also as a functional food and natural antioxidant source [29,30]. In this study, the antioxidant potential of *C. rhacodes* was evaluated. The obtained data are presented in Table 1.

Table 1. TAS, TOS, OSI, DPPH and FRAP values of *Chlorophyllum rhacodes*

Parameters	*Chlorophyllum rhacodes*
TAS (mmol/L)	3.125±0.060
TOS (µmol/L)	6.821±0.062
OSI (TOS/(TASx10))	0.218±0.003
DPPH (mg TE/g)	55.430±1.438
FRAP (mg TE/g)	72.540±1.461

In this study, the antioxidant capacity of *C. rhacodes* was confirmed by both DPPH and FRAP assays. The DPPH radical scavenging activity was determined as 55.430 ± 1.438 mg TE/g. Although a previous study reported 70.46% inhibition for C. rhacodes [31], direct comparison with our results is not possible due to methodological and unit differences. Inhibition percentages represent relative values that depend on assay conditions, whereas Trolox equivalents are absolute values referenced to a standard antioxidant. Nevertheless, both datasets support the notion that C. rhacodes exhibits measurable radical scavenging activity, even though the magnitude of this activity may vary depending on the method employed. The FRAP value (72.540 ± 1.461 mg TE/g) further indicates a substantial ferric reducing power, underscoring the species' capacity to contribute to antioxidant defense. Antioxidant activities of different mushroom species have been reported in the literature using Rel Assay kits. TAS values of *Otidea onotica, Phellinus hartigii, Tricholoma terreum, Lactarius deliciosus, Hericium erinaceus* and *Cantharellus cibarius* were reported as 8.866, 4.98, 2.302, 7.468, 5.426 and 5.511 mmol TE/L, respectively. In the same studies, TOS values were reported as 14.724, 9.27, 12.483, 13.161, 6.621 and 7.289 µmol H_2O_2/L, respectively; OSI values were reported as 0.166, 0.19, 0.542, 0.176, 0.122 and 0.132 [32-37]. These data reveal that there are significant variations in antioxidant and oxidant parameters among mushroom species and underline the biochemical diversity among species. TAS value is an important biochemical indicator reflecting the total of antioxidant compounds synthesized in natural products [38]. The TAS value of *C. rhacodes* used in our study was determined to be higher than Tricholoma terreum; but lower than *O. onotica, P. hartigii, L. deliciosus, H. erinaceus* and *C. cibarius*. This situation shows that C. rhacodes has a significant antioxidant capacity, but it contains a limited number or lower concentration of antioxidant compounds compared to some species. Especially being above species with low TAS values such as *T. terreum* shows that it has the potential to compete with some species. TOS value expresses the total of oxidant compounds synthesized in natural products [38]. According to the data obtained in our study, the TOS value of *C. rhacodes* was determined to be higher than Hericium erinaceus; but lower than *T. terreum, O. onotica, P. hartigii, L. deliciosus* and *C. cibarius*. This finding shows that there is a certain oxidant compound load in the structure of *C. rhacodes*; however, this level is moderate compared to many species. The OSI value is a critical parameter reflecting the extent to which oxidant compounds are suppressed by antioxidant systems [38]. The OSI value of *C. rhacodes* was determined to be lower than *T. terreum*; but higher than *O. onotica, P. hartigii, L. deliciosus, H. erinaceus* and *C. cibarius*. This situation reveals that *C. rhacodes* can partially provide the antioxidant-oxidant balance; however, it can maintain this balance less successfully compared to some species. Since a low OSI value is a desirable situation in terms of keeping oxidative stress under control, it can be said that *C. rhacodes* can form an effective antioxidant defense system under certain conditions. The observed differences in OSI values may be related to differences in the phytochemical composition of the species. Environmental factors such as growth substrate, climatic conditions, and developmental stage are also known to influence the accumulation of these compounds, which may further explain the observed results.

In conclusion, this study shows that *C. rhacodes* can be considered as a potential natural antioxidant source in biological systems. The findings obtained reveal that this

species should be taken into consideration, especially in terms of functional food ingredient or pharmaceutical product development studies. However, advanced cellular and in vivo studies are required to more clearly reveal the effects of in vitro results on biological systems.

3.2. Anticholinesterase activity

Recent studies have shown that mushrooms attract attention not only with their nutritional value and antioxidant capacity, but also with their neuroprotective potential [39]. In particular, their ability to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes suggests that mushrooms may contain natural compounds with anticholinesterase activity. Inhibitors of these enzymes are used in the symptomatic treatment of various neurodegenerative diseases, especially Alzheimer's disease [40,41]. The anticholinesterase effects of mushroom extracts are generally due to secondary metabolites such as phenolic compounds, terpenoids, alkaloids, and sterol derivatives [42]. In this context, mushrooms are evaluated as potential natural cholinesterase inhibitors and are among the promising biological sources in pharmaceutical research. In this study, the anticholinesterase activity of *C. rhacodes* was investigated and the results are presented in Table 2.

Table 2. Anti-AChE and anti-BChE values of *Chlorophyllum rhacodes*

Sample	AChE µg/mL	BChE µg/mL
Chlorophyllum rhacodes	94.330±1.536	128.397±1.556
Galantamine	7.402±0.120	16.217±0.151

In this study, the inhibitory effects of *C. rhacodes* species on cholinesterase enzymes were evaluated and a certain level of inhibition was observed on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Cholinesterase enzymes are responsible for the degradation of neurotransmitters such as acetylcholine in the synaptic cleft, and inhibitors of these enzymes are widely investigated to support cholinergic function in neurodegenerative diseases [43]. The obtained IC_{50} values show that *C. rhacodes* has a low level of inhibitory power against these enzymes and cannot be considered among the strong inhibitors. When compared to galantamine used as a positive control, it is clearly seen that the inhibitory potential of *C. rhacodes* is more limited. However, the fact that some natural products do not show high inhibitory effect alone does not mean that their biological activities are insignificant. Mushroom extracts generally contain a large number of bioactive compounds, and the synergistic effects of these compounds may produce functional results independent of individual IC_{50} values. Therefore, despite the observed weak inhibitory activity, the anticholinesterase potential of this species should not be completely disregarded.

There is no evidence in the literature regarding the cholinesterase inhibition of *C. rhacodes*, and the findings obtained in this study may contribute to the current lack of knowledge in this area. The results obtained suggest that this species should not be evaluated as a direct therapeutic agent, but as a source of potential bioactive compounds. In addition, it should be considered that this inhibitory activity is affected by factors such as the extraction method used, solvent type, test system and analysis parameters. Therefore, it is evaluated that fractionation and compound isolation studies to be carried out under different conditions can reveal the inhibitory capacity of the species more clearly.

3.3. Element contents

Fungi are organisms that have the ability to absorb various elements from their environment. They can be rich in beneficial minerals such as potassium, magnesium, iron, zinc and selenium. However, they are also sensitive to the accumulation of heavy metals (e.g. lead, cadmium) in the environment. Thanks to these features, they can be evaluated as both a food source and a bioindicator of environmental pollution [44-46]. In this study, the elemental contents of *C. rhacodes* were examined and the results obtained are presented in Table 3.

Table 3. Element contents of *Chlorophyllum rhacodes*

Elements	*Chlorophyllum rhacodes* (mg/kg)
Cd	14.78±1.53
Cr	33.17±1.42
Mn	7.35±0.52
Cu	155.77±2.34
Fe	469.30±15.75
Pb	6.98±0.54
Ni	3.66±0.31
Zn	76.18±2.25

In the literature, the Cd content in the fruit bodies of *C. rhacodes* samples collected from the Czech Republic was reported as 0.49, Cr content as 0.08, Mn content as 84.6, Cu content as 85.6, Fe content as 33.7, Pb content as 0.22, Ni content as 0.65 and Zn content as 127 mg/kg [47]. In our study, it was observed that *C. rhacodes* samples collected from Türkiye had higher Cd, Cr, Cu, Fe, Pb and Ni contents and lower Mn and Zn contents. It is thought that these differences may occur depending on many environmental factors such as the geological structure of the habitat where the mushrooms were collected, soil properties, industrial pollution level, growth period of the mushroom and environmental metal exposure. The presence of high levels of toxic elements, especially Cd and Pb, is noteworthy in terms of potential health risks [48], making it necessary to regularly monitor this species when it comes to its consumption in the food chain. The increase in essential elements, such as Cu and Fe, can be considered a positive feature in terms of the potential nutritional value of the mushroom; however, balance is important since the intake of these elements above the limits can also cause toxic effects. In addition, it has been reported that Fe content is in the range of 14.6–835, Zn content is in the range of 28.69–158.00, Pb content is in the range of 0.68–23.05, Ni content is in the range of 0.67–5.14, Mn content is in the range of 5.25–103, Cr content is in the range of 9.63–73.01, Cd content is in the range of 0.16–8.96 and Cu content is in the range of 1.90–109.95 mg/kg in different wild mushroom species [49,50]. According to these value ranges of *C. rhacodes* used in our study, it was observed that Fe, Zn, Pb, Ni, Mn and Cr contents remained within these ranges, while Cd and Cu

contents exceeded the upper limits specified in the literature. This situation indicates that the mushroom may tend to selectively accumulate certain heavy metals. Especially the fact that toxic elements such as Cd are above the determined maximum limits reflects the bioaccumulation potential of this species and reveals its potential to be evaluated as a bioindicator species in terms of monitoring the biological reflections of environmental pollution. At the same time, these results emphasize the importance of regional metal analyses in terms of consumability and food safety of mushrooms.

4. Conclusions

In this study, the biological properties of the *C. rhacodes* species regarding antioxidant, anticholinesterase and element contents were evaluated in detail. The results obtained revealed that this species has remarkable properties in terms of various biological activities. According to the antioxidant test results, it was determined that C. rhacodes showed significant activity especially in free radical scavenging and ferric reduction methods such as DPPH and FRAP. In addition, when the TAS, TOS and OSI values were evaluated, it was seen that this species can maintain the antioxidant-oxidant balance to a certain extent. These properties show that the mushroom has potential as a functional food ingredient and natural antioxidant source. In terms of anticholinesterase activity, it was determined that the inhibitory effect of C. rhacodes on AChE and BChE enzymes was limited compared to the reference substance galantamine. However, the results obtained show that this species can exhibit anticholinesterase effects, albeit at a weak level, and this effect can be evaluated from a pharmaceutical perspective by supporting it with possible synergistic compounds. When evaluated in terms of elemental contents, it was observed that some essential (Fe, Cu) and toxic (Cd, Pb) elements were significantly high in samples collected from Turkey. This situation reveals the sensitivity of the mushroom to environmental metal exposure and that it may be prone to bioaccumulation of certain metals. Especially the fact that Cd and Cu contents are above the upper limits reported in the literature strengthens the potential of this species to be evaluated as a bioindicator of environmental pollutants. In addition, since the high levels of toxic elements may pose potential risks in terms of consumption, periodic monitoring of these species is recommended within the scope of food safety. In conclusion, *C. rhacodes* stands out as a mushroom species that should be carefully examined in terms of both pharmaceutical research and environmental biosurveillance with its antioxidant activity, secondary metabolite-derived biological effects and environmental metal accumulation properties. These findings provide important contributions to the biological profile of the species and should be supported by advanced biochemical, toxicological and molecular studies.

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