

Investigation of antioxidant activities of *Ganoderma* mushroom

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Ganoderma lucidum is a highly popular medicinal mushroom that has been used for centuries to treat various diseases. *Ganoderma lucidum* contains biologically active components and these components give *Ganoderma lucidum* its antimicrobial and antioxidant properties. In this study, we aimed to investigate the antioxidant activities of *Ganoderma lucidum*. *Ganoderma lucidum* DMSO extracts for both mycelium and fruiting body were prepared. DPPH radical scavenging activity and ferric-reducing antioxidant power (FRAP) assays were applied in order to understand the antioxidant features of *G. lucidum*. The chemical composition of the DMSO extracts of *G. lucidum* was analyzed using gas chromatography. The chemical compounds and antioxidant activities were evaluated using computational methods. The ethanol extracts of the fruiting body of *G. lucidum* (GL) exhibited DPPH radical scavenging activity with an IC₅₀ value of 15.01 ± 0.06 mg/ml. However, the mycelium part of *Ganoderma* (GE) showed the poorest antiradical-scavenging capacity (IC₅₀ = 30.53 ± 0.03) and the least press diminishing capacity (41.17 ± 0.019) within the water-dissolvable *Ganoderma lucidum* may show antioxidant activities, and structural characteristics of its bioactive ingredients may play a crucial role in the mechanism of antioxidant action.

Keywords: antioxidant, computational chemistry, DPPH, *Ganoderma lucidum*

INTRODUCTION

Ganoderma lucidum (GL) is a fungus with a woody structure and a shiny appearance that goes by several names, including "Reishi," "Ling Zhi," and "Mannentake". *Ganoderma* is a traditional medicinal mushroom that has been used for millennia to improve longevity, healing, and health in east countries such as China, Japan, and Korea [1-3]. Depending on the climate conditions such as humidity, heat, lighting, etc., the color, bitterness and shape of Reishi mushroom differ from each other even if they have the same strain [4]. *Ganoderma* species belong to the group *Basidiomycota*, class *Homobasidiomycetes*, order *Aphyllphorales*, family *Polyporaceae* [5]. Latest pharmacologic studies with *Ganoderma* extracts have revealed that they have antibacterial properties and they also inhibit lipid peroxidation/oxidative DNA damage. Treatment of *S. aureus* infections has limitations since it creates resistance to antibacterial compounds and reduces the effectiveness of the chemicals used, as well as making the microbe more tolerant and harmful. Various *Ganoderma* extracts have been proven to inhibit the growth of both Gram-positive and Gram-negative bacteria [1]. Many animal model and molecular based studies

have conducted to proven the various pharmacological effects of *G. lucidum* in traditional Chinese medicine [6]. According to ancient Chinese medicine, while GL has functions such as increasing body resistance against microbial infections and prolonging life, recent studies with *Ganoderma* extracts have revealed various therapeutic effects [5] such as anticancer, anti-inflammatory, antitumor, antioxidant, immunomodulatory, antidiabetic, immunodeficiency, antiviral, anti-hypertensive, antibacterial, antifungal, antiatherosclerotic, antiaging, antiandrogenic, antihepatotoxic, able to scavenge free radicals, neuroprotective, increasing sleep, suppressing cholesterol synthesis, hypoglycemia, preventing lipid peroxidation and oxidative DNA damage, hepatoprotective, preserving gut health, preventing obesity, migraines, high blood pressure, loss of appetite, gastritis, hemorrhoids, menstrual pain, constipation and cardiovascular issues [7]. These mushrooms are widely utilized in Chinese medicine, where they are believed to boost energy, enhance the immune system, and extend lifespan [8]. Additionally, triterpenoids, the main components of *G. lucidum*, are reported to play an important role in the pharmacological effects mentioned. During the past three decades, more than 150 triterpenoid compounds have been isolated from the genus

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Ganoderma sp. Among the triterpenoids, ganoderic acid A (GA), ganoderic acid B (GB), ganoderic acid G (GG), ganoderic acid E (GE), ganoderenic acid D (GED), and ganoderic acid D (GD) are the main components. The effect of *G. lucidum* on cancer is based on glucan and triterpenes that it contains. Beta glucans are thought to activate the immune system, and triterpenes are thought to have a cytotoxic effect against various cancer cells [9].

Ganoderma lucidum polysaccharides are known as antitumor agents, but although *GL* extracts contain biologically active compounds like glycosides, carbohydrates, phenolic compounds and triterpenoids, their antibacterial activity is partly due to the inhibitory properties of polysaccharides. When it comes to antibacterial agents, extracts can inhibit both Gram-negative and Gram-positive bacteria, so some studies have focused on the activity of isolated polysaccharides, as well as research on extracts derived from mycelium and fruiting body. In general, extracts in organic solvents such as dichloromethane, hexane, methanol and ethyl acetate, as well as aqueous extracts of *G. lucidum* act against *Enterobacter aerogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [10].

Staphylococci are invasive Gram-positive bacteria that cause a variety of diseases in humans and animals. These include bacteriuria, enterocolitis, osteomyelitis, pneumonia, acne, carditis, meningitis, arthritis and septicemia. Antibiotic-resistant *Staphylococci* have evolved as an unavoidable genetic response to the selective pressure of antimicrobial therapy. The ability of microbial species to adapt rapidly to changes in their environment was demonstrated by the development of resistance when an antibiotic enters clinical use [11]. When an antibacterial compound is applied, limitations arise in the treatment of *S. aureus* infections, as it creates resistance and reduces the effect of the compounds used, and it also makes the microorganism more tolerant and more dangerous [12, 13]. Therefore, it is important to investigate new potential treatments.

In this study, it was aimed to identify the chemical composition and investigate the antioxidant features of *Ganoderma* mushroom. Computational chemistry methods were applied to evaluate the antioxidant activity of *G. lucidum* for the first time.

EXPERIMENTAL

Materials and methods

Ganoderma lucidum powder was used for preparation of mushroom extracts (Gano Excel Co.).

300 mg of powder were mixed with 10 mL of DMSO and placed on a shaker for 24 h at room temperature. Then the solution was filtered by 0.22 μ m syringe filter. DMSO extraction method was used for both fruiting body (*GL*) and mycelium (*GE*) of *G. lucidum* separately [14].

DPPH radical scavenging activity assay

The antioxidant capacity of two *Ganoderma* extracts in three solvent types: aqueous (a mixture of 82 parts of distilled water with 10 parts of methanol and 8 parts of acetic acid); ethanol; and hexane, was determined by the stable DPPH radical scavenging activity described by the method of Villano *et al.* (2007) [15].

DPPH \cdot radical, a stable free radical, reacts with an antioxidant and is reduced to DPPH-H which is detected by the decrement in absorbance in spectrophotometry. The degree of discoloration reveals the antioxidant chemicals in extracts' scavenging activity in terms of their capacity to donate hydrogen. To prepare the reaction mixture, 1 ml of DPPH \cdot solution was added to each of 4 ml-sample solutions from three solvent types. The absorbance of the resulting solution was measured by spectrophotometry at 517 nm, following incubation at 20°C for 30 min. The IC₅₀ values of the extracts in the DPPH assay were calculated, and low IC₅₀ values referred to higher antioxidant capacity. The results were compared to ascorbic acid (AA) used as a reference.

Ferric-reducing antioxidant power (FRAP) assay

With a few minor adjustments, the Oyaizu 1986 assay was used to test the ferric reduction ability of the samples and the reference [16]. The test was based on the reducing-power of the transformation of ferrous (Fe²⁺) to ferric (Fe³⁺) ions, which produces a blue complex and increases absorbance at 700 nm. Samples of varying concentrations (0.1-1.5 mg) were combined with phosphate buffer (2.5 ml) in 1 ml of methanol. The mixture was incubated at 50°C for 20 min. To the mixture aliquots of trichloroacetic acid (2.5 ml) were added and the mixture was centrifuged at 3000 rpm for 10 min. A freshly made ferric chloride solution (0.5 ml) and distilled water (2.5 ml) were combined with the solution's upper layer (2.5 ml). The absorbance was measured at 700 nm, ascorbic acid was used as reference standard. Increased absorbance of the reaction mixture indicates increase in reducing power [17].

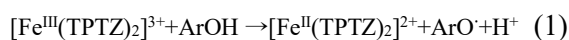
GC-MS analysis

The *G. lucidum* and *G. excellium* were purchased from Gano Excel company. One capsule (480 mg) from each was opened and transferred to a 15 mL-tube separately. Then, 10 mL of ethanol was added to each, and sonicated for 30 min. The dried samples were treated for 90 min at 30 °C with 150 µl of hydroxylammonium chloride (25.0 mg/mL) in pyridine. The samples were derivatized with the addition of 270 µl MSTFA + 30 µl TFA and incubation at 90 °C for 90 min after cooling to room temperature. A vial of GC-MS was used to transfer 50 ml of the derivatized samples for analysis. GC-MS analysis of the constituent sugars was performed on a Shimadzu GC-2010 plus gas chromatograph (Shimadzu Scientific Instruments, Columbia, MA, USA), equipped with an Rtx®-5MS column (30 m × 0.25 mm ID, 0.10 µm film thickness) (Restek, USA). Helium was used as carrier gas. The oven temperature program was set as follows: keeping at 50 °C for 2 min, ramping at 4 °C min⁻¹ up to 250 °C and keeping at 250 °C for 8 min. The temperatures of the transfer line and ion source were set at 270 and 250°C, respectively. Mass spectra were scanned from 40–400 m/z at a rate of 0.5 scans s⁻¹ and the electron impact ionization energy was 70 eV. Data handling was supported by the software GC-MS solution, ver. 2.51 (Shimadzu). The compounds of extracts were identified using the National Institute Standard and Technology (NIST) library. The relative percentages of the compounds were calculated based on the GC peak areas.

Theoretical calculations

Geometry optimizations and ferric reducing mechanism (Eq. 1) modeling of candidate antioxidant molecules myristic acid, arabitol, mannitol, inositol, and stearic acid were performed by HF/6-31+Gdp method in hexane, ethanol and mixture. IEF-PCM method was used for the solvent optimizations. All computational calculations were implemented in GaussView 5 [18] molecular visualization and Gaussian 09 software [19].

The chemical mechanism of the ferric reducing reaction is given in Eq. 1 [20]. In the mechanism modeling of ferric reducing, the energy changes during the capture of e⁻ in the Fe^{III+} → Fe^{II+} conversion of the candidate antioxidant molecules determined above, were investigated.



Statistical analysis

The results were expressed as mean ± SD of at least three independent determinations. Multi-group

comparisons were analyzed using one-way ANOVA followed by a post hoc Tukey test. At p<0.05, differences were accepted statistically significant.

RESULTS AND DISCUSSION

DPPH radical scavenging activity assay

The antioxidant properties of *Ganoderma* extracts (*GE* and *GL*, respectively) with three different solvents; aqueous, ethanol (EtOH), and hexane, were evaluated using two methods: DPPH· radical scavenging activity and ferric reducing antioxidant power (FRAP). Summary of the results is presented in Table 1.

Table 1. Antioxidant activity of *GL* and *GE* mushrooms extracted in three solvent types, with DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. Values are the mean ± standard deviation (n = 3). Differences between samples in the three extracted solvents in each assay are statistically significant (P <0.01). NA= no activity.

Samples	Solvent	DPPH activity Ic ₅₀ values (Mg/MI)	FRAP G /Mmol Aa equivalent
<i>Gl</i>	Aqueous	67.4 ± 0.01	8.4 ± 0.02
	Ethanol	15.01 ± 0.06	4.46 ± 0.03
	Hexane	30.95 ± 0.05	7.50 ± 0.01
<i>Ge</i>	Aqueous	72.2 ± 0.01	41.17 ± 0.01
	Ethanol	30.53 ± 0.03	26.78 ± 0.03
	Hexane	Na	37.5 ± 0.01
Ascorbic acid	-	9.12 ± 0.01 µg/MI	-

According to the results of the DPPH radical scavenging assay, *GL* exhibited radical scavenging activity in all solvent types, which was highest in the ethanolic extract. The EtOH extracts of *GL* exhibited DPPH· radical scavenging activity with an IC₅₀ value of 15.01 ± 0.06 mg/ml. *GE* showed moderate DPPH· radical scavenging activity compared to *GL*, while no activity was observed by the *GE* hexanic extract. Ascorbic acid, known for its significant antioxidant activity, was used as the reference. (IC₅₀ = 9.12 ± 0.01 µg/ml).

Ferric reducing antioxidant power (FRAP) assay

Considering the results obtained using the FRAP assay, the EtOH extract of *GL* possessed the highest reducing power relative to that of ascorbic acid (4.46 ± 0.03 g /mmol AA) used as the reference. When compared to the *GL* extracts, the *GE* extracts displayed much lower antioxidant activity based on their ascorbic acid equivalences (41.17 ± 0.01, 26.78 ± 0.03, 37.5 ± 0.01 (g/mmol AA) for aqueous, ethanolic and hexanic extracts, respectively. The antioxidant ability of *GL* and *GE* could be attributed to the presence of a variety of biomolecules in *GL*

and *GE* and their specific ability to prevent oxidation. Data from GC-MS analysis indicated that sugars (disaccharides and monosaccharides) and sugar alcohols are the most abundant biomolecules (more than 50%), followed by fatty acids (dicarboxylic fatty acids, saturated fatty acids, mono and polyunsaturated fatty acids) in both samples (Table 2).

Table 2. GC-MS results of *Ganoderma* (*GE* and *GL*).

RT	Compound	<i>GE</i> %	<i>GL</i> %
8.158	Succinic acid	0.90	2.39
13.533	Malic acid	1.88	8.70
14.300	Erythritol	-	2.19
20.492	Xylitol	9.20	10.08
21.792	Azelaic acid	-	2.07
21.433	Ribose	2.27	-
22.942	Myristic acid	1.15	-
25.758	Sorbitol	3.28	6.74
26.150	Fructose	5.14	3.19
26.333	Arabitol	5.89	1.92
27.750	Glucose	8.97	10.65
28.042	Mannitol	10.19	1.94
28.100	Galactose	8.55	8.18
29.500	Inositol	6.01	3.85
30.325	Palmitic acid	3.36	4.15
32.000	Linoleic acid	1.23	7.71
32.333	Oleic acid	11.44	10.92
36.275	Stearic acid	7.51	6.05
39.392	Eicosanoic acid	1.69	2.05
41.158	Sucrose	4.91	-
42.992	Turanose	2.39	4.15
44.967	Palatinose	4.05	3.05
	Total	100.00	100.00

The antioxidant activity of the studied samples might be related to the important bioactive molecules such as reducing sugars, organic acids like malic acid and succinic acid, also unsaturated fatty acids, which predominated over saturated fatty acids in both samples [21, 22]. However, sugars and sugar alcohols also exhibit anti-radical capacity, which is mostly dependent on the number of aliphatic hydroxyl groups in sugar alcohols of monosaccharides [23]. Sugars and sugar alcohols are soluble in water and ethanol due to the presence of multiple hydroxyl groups in their chemical structure. Neither sugars nor sugar alcohols are soluble in

hexane due to the polarity mismatch between the solute and solvent particles. The hydroxyl groups are less reactive compared to the functional groups found in potent antioxidants like vitamin C, E and phenolic compounds which can effectively donate hydrogen atoms to neutralize free radicals [23].

Compared to *GL*, *GE* exhibited poorer antiradical-scavenging ability ($IC_{50} = 72.2 \pm 0.01$) and lower ferric reducing ability (41.17 ± 0.019) in the aqueous solvent. These results could be explained by pointing out the fact that while higher amounts of sugars were found in *GE*, higher amounts of organic acids were recorded in *GL* (about 11%) in which malic acid predominates over succinic acid. Unsaturated fatty acids predominated over saturated fatty acids in both samples, and their amount is higher in *GL* compared to *GE*. The antioxidant effect of unsaturated fatty acids can be explained by their chemical structure and biological activity. Unsaturated fatty acids contain one or more double bounds through which they interact with free radicals. The hydrogen atoms adjacent to the double bonds are particularly active and bring about the hydrogen donating ability to unsaturated fatty acids, and therefore lead to the neutralization of free radicals and prevent oxidative damage [22]. Biomolecule content found in the studied samples was comparable to the one reported by Mau *et al.* (2001), for cultivated *G. lucidum* from Serbia [24]. However, *GL* and *GE* from different origins, measured by the same *in vitro* assays, previously showed higher antioxidant properties than that we obtained here [25]. The outcomes of two antioxidant assays indicate that both samples revealed antioxidant properties. Nevertheless, the ethanolic extracts outperformed aqueous and hexane extract in terms of antioxidant efficacy in both samples suggesting that the type of solvent for extraction and their specific interactions with free radicals affect the impact of individual molecules on the antioxidant capacity of samples. It is expected that hydrophilic ingredients soluble in polar solvents, including the sugars possessed by the samples, are mostly soluble in ethanol and water yet organic acids and fatty acids are mostly soluble in nonpolar solvents like hexane [26], which suggests the difference in antioxidant activity from the different solvents. Therefore, the structural characteristics of bioactive ingredients, as well as the possible interactions between them and their solvents play a crucial role in the mechanism of antioxidant action.

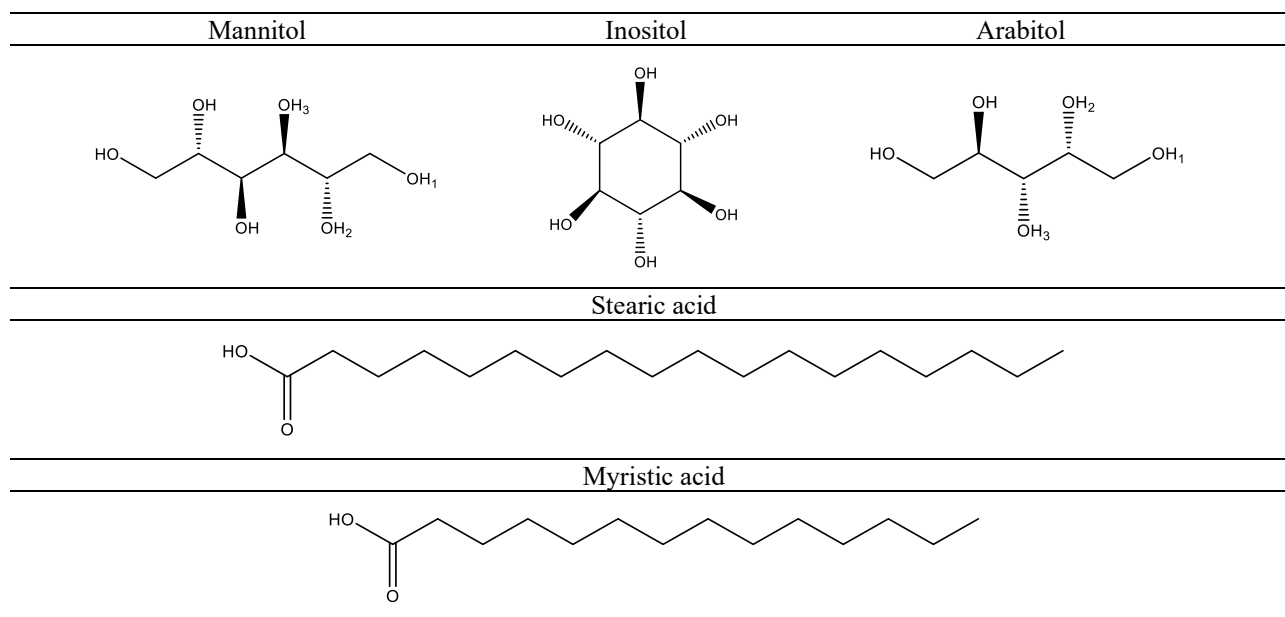
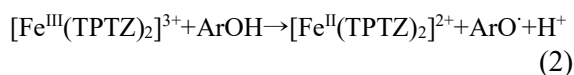


Fig. 1. 2D structures of candidate antioxidant molecules.

GC/MS analysis

The components obtained after derivatization of the capsules containing *Ganoderma lucidum* (*GE* and *GL*) are given in Table 2. Various candidate antioxidant molecules were detected using GC-MS analysis (Fig. 1). When Table 1 is examined, from both DPPH and FRAP methods, chosen as antioxidant activity determination methods in this study, is found that the antioxidant activity of *GL* extract is higher than that of *GE*. The antioxidant activity of *GE* is demonstrated with a more significant difference, especially in the examination made with the FRAP method. When the GC-MS analyses of these extracts given in Table 1 are evaluated in terms of the percentage of active ingredients, it is thought that the antioxidant activity of *GE* and *GL* extracts may be caused by arabitol, mannitol, inositol, and stearic acid molecules (Fig. 1). Myristic acid was detected in *GE* extracts. At this point, the molecular modeling of the FRAP method was made using quantum chemical methods, and it was examined which of the molecules listed above caused this activity. The chemical mechanism of the FRAP method is given in Eq. 2 below [20]. In the mechanism modeling of FRAP, the energy changes during the capture of e^- in the $Fe^{III+} \rightarrow Fe^{II+}$ conversion of the candidate antioxidant molecules determined above were investigated.

Theoretical calculations



For the mannitol compound, the calculated reaction energies of the ferric-reducing mechanism

were evaluated using the HF/6-31G+dp method in all investigated solvents. The antioxidant activity of each of the OH bonds of the mannitol compound, which has 3 different inductive effects, in the analyzed solvent, is higher than the antioxidant activities of stearic acid, inositol, arabitol and myristic acid molecules. It was determined that H^+ transfer, which plays a critical role in the ferric reducing mechanism, is easier. On the other hand, calculations performed to determine which $-OH$ bond confers the highest antioxidant activity revealed that the $-OH$ groups located at the mannitol-OH3 position ($1206.06 \text{ kJ}\cdot\text{mol}^{-1}$) in the mannitol molecule and at the arabitol-OH2 position ($1210.85 \text{ kJ}\cdot\text{mol}^{-1}$) in the arabitol molecule exhibit the lowest bond dissociation energies, indicating their greater contribution to antioxidant activity. It contributes more because it breaks more easily than $-OH$ bonds. On the other hand, in accordance with the literature, it was observed that the increase in the polarity of the solvent supported the antioxidant activity as it facilitated the charge separations [27]. The dipole moment order of the selected solvents in this study is hexane (1.9 D) < ethanol (16.2 D) and aqueous solvent (68.0 D). The basidiomycetous fungus *Ganoderma lucidum* (family *Ganodermataceae*) has been utilized in traditional Eastern medicine for generations. This medicinal mushroom is thought to help people live longer by preserving their vitality [28]. The polysaccharides from *Ganoderma lucidum* are used as a biological macromolecule material in medicine, food and

cosmetics [29]. According to the GC-MS results, it was determined that both capsules were rich in polysaccharides, consistent with the literature. *Ganoderma lucidum*'s considerable antioxidant effect, which reduces oxidative damage, is thought to be responsible for at least some of its health advantages [30]. The extracts of *G. lucidum* have shown *in vitro* antioxidant activities which may be due to the presence of flavonoids and phenolics. The solubility of bioactive components varies depending on the extracting solvents chosen. Generally, some active phytochemicals are insoluble in water, but soluble in alcohol, according to some studies [27]. Since the biological properties of the molecules detected in various herbal extracts can be defined by computational chemistry methods [31], it may be possible to use the molecules identified in *Ganoderma* mushroom as drug candidates for treatment purposes in various diseases in the future.

CONCLUSIONS

In this study, in order to comprehend the antioxidant properties of *G. lucidum*, DPPH radical scavenging activity and ferric-reducing antioxidant power (FRAP) assays were used. Using gas chromatography, the chemical make-up of the DMSO extracts of *G. lucidum* was examined. Applying computational approaches, the chemical components and antioxidant activities were assessed. With an IC₅₀ value of 15.01–0.06 mg/ml, the ethanol extracts of *G. lucidum* demonstrated DPPH radical scavenging action. The antioxidant activity of *GL* (fruiting body) extract was found to be greater than that of *GE* (mycelium) extract. Arabitol, mannitol, inositol, and stearic acid which may be the source of the antioxidant action in *GE* and *GL* extracts, were identified using GC/MS. In conclusion, the antioxidant properties of *Ganoderma lucidum* were identified.

Declaration of competing interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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