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ANTIOXIDANT PROPERTIES OF HOT WATER EXTRACTS FROM CARPOPHORE AND SPORES OF MUSHROOM *GANODERMA LUCIDUM*

ABSTRACT: *Ganoderma lucidum* (Leyss.:Fr.) Karst is one of the medicinal mushrooms, which possesses enviable antioxidant properties. Objective of this investigation was to evaluate antioxidant activity, reducing power, scavenging abilities on 1.1-diphenyl-2-picryl-hydrazyl (DPPH) radicals and chelating effects on ferrous ions of hot water extracts obtained from carpophore and spores of this mushroom. Hot water extract from carpophore (Gl-I) showed high antioxidant activity of 85.7 ± 0.7%, at 10 mg/ml, while antioxidant activity of hot water extract from spores (Gl-Is) was 9.2 ± 0.3% at 10 mg/ml. Reducing power of Gl-I reached a plateau of 3.4 ± 0.1 at 20 mg/ml, and 0.3 ± 0.0 at 20 mg/ml for Gl-Is. At 10 mg/ml, scavenging ability on DPPH radicals of Gl-I increased to 96.8 ± 2.5%, whereas Gl-Is scavenged DPPH radicals by 69.6 ± 2.5% at 10 mg/ml. Gl-I chelated 81.6 ± 3.6 % of ferrous ions at 20 mg/ml, while the chelating effect of Gl-Is was 73.8 ± 1.7%. The antioxidative activities of hot water extracts from carpophore and spores of the mushroom *G. lucidum* were concentration dependent and increased with an increase in the concentration.

KEY WORDS: *Ganoderma lucidum*, antioxidant activity, scavenging effect, reducing power, chelating effect, polysaccharide, mushroom extract

INTRODUCTION

Free radicals are abundantly present in food components and have an effect on many reactions in food systems. Also, they are produced during normal and pathological cell metabolism as a consequence of exogenous chemical and endogenous metabolic processes. Free radicals, especially oxygen derived ones, are highly reactive and capable of oxidizing biomolecules which results in cell death and tissue damage. Degenerative diseases including brain dysfunction, cancer, cardiovascular disease, cataract, diabetes, and immune-system decline are supposed to be associated with these processes (A m e s et al., 1993). Oxidative damages of DNA, proteins and other macromolecules accumulate with

age and are considered to be decisive factors of endogenous damage (F r a g a et al., 1990). All organisms possess natural defense systems against free radical damage, such as superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and oxygen gas. In addition, they contain anti-oxidants, such as α-tocopherol, ascorbic acid, carotenoids, glutathione and polyphenols (N i k i et al.,1994), that trap free radicals, thus inhibiting the oxidative mechanisms that lead to degenerative diseases. If the mechanism of antioxidant protection becomes unbalanced by aging, a constant supply of natural products with antioxidant activity would be suitable to help the human body to reduce oxidative damage. Naturally occurring antioxidants found in whole grains, fruits, vegetables, teas, herbs may have a role in natural protection.

Mushrooms have also been reported as organisms with antioxidant activity. This is correlated with their phenolic and polysaccharide compounds (M a u et al., 2005; D u b o s t , O u and B e e l m a n , 2007; S o n g and V a n G r i e n s v e n , 2008). They are not only appreciated for their taste and high nutritional value, but also as a significant source of biologically active compounds of medicinal value. *Ganoderma lucidum* (Leyss.:Fr.) Karst, commonly known as lacquered mushroom, is not edible due to its coarse and hard texture and bitter taste, but it is the most often used one in traditional medicine of Far Eastern people. For thousands of years it has been well known for its treatment of various diseases, including cancers. Recent investigations also point to high antioxidative properties of this mushroom.

MATERIALS AND METHODS

Preparation of polysaccharides

Hundred grams of fine mushroom powder was washed with 96% ethanol at room temperature for 24 h under stirring, filtered and dried in vacuum (60 min at 42°C). Dried filtercake was extracted with 2 l Milli-Q water (MQ) by autoclaving (45 min. at 121°C), the extract was chilled and centrifuged for 20 min at 9000 g. Supernatant was concentrated to 10% of its initial volume, and polysaccharides were precipitated by addition of 2 volumes of cold 96% ethanol and left at 4°C overnight. After centrifugation, the pellets were washed with 70% ethanol, dried in vacuum and dialyzed using a ZelluTrans/Roth® 6.0 regenerated cellulose tubular membrane (MWCO: 8.000-10.000) against MQ for 24 h at room temperature to remove residual small molecules as polyphenols, peptides and polysaccharides < 8-10 kD. After centrifugation, the high molecular weight polysaccharides were ethanol precipitated and vacuum dried for later use.

Evaluation of the Antioxidant Properties

This examination was performed by measuring DPPH free radical scavenging activity, reducing power, antioxidative effect and chelating ability on ferrous ions. Each extract was analyzed in three replicates for each antioxidant test, and the results were reported as mean values ± standard deviation.

Antioxidant Activity

The antioxidant activity was determined by the conjugated diene method (L i n g n e r t et al., 1979) with slight modification. Each polysaccharide powder (0.1 to 10 mg/ml, 100 μl) in MQ was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer. Then, 6.5 mM Tween 20 was added to provide a stable emulsion and the mixture was incubated for 15 h in the dark at 37ºC, while shaken to accelerate oxidation. Afterwards, 0.2 ml of the antioxidant mixture was added to 6 ml of absolute methanol. The absorbance of the supernatant mixture was measured at 234 nm against a blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. The antioxidant activity was calculated as follows: antioxidant activity (%) = $[(A_0 – A_1)/ A_0]$ x 100, where A_0 was the absorbance of the control reaction and A1 the absorbance in the presence of the sample. Ascorbic acid and α-tocopherol were used as the positive control. Value of 100% indicated the strongest inhibitory ability.

DPPH Free Radical Scavenging Activity Assay

The assay was done according to the modified method of Bilos (1958). In the first series, each polysaccharide powder (0.1–10 mg/ml, 2 ml) in MQ was mixed with 1 ml of freshly prepared DMSO solution of 0.2 mM DPPH. In the second series, each sample was mixed with 1 ml DMSO solution. The reaction mixture was vortexed vigorously for 1 min and kept in the dark at 20ºC for 40 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against the blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). DPPH free radical scavenging activity was calculated according to the following equation: % scavenging = [1-(Ai-Aj)/Ac] x100, where Ai was the absorbance of 2 ml extract mixed with 1 ml DPPH solution, Aj was the absorbance of 2 ml extract mixed with 1 ml DMSO solution and Ac was the absorbance of blank-2 ml of DMSO mixed with 1 ml of DPPH solution. Ascorbic acid, BHT and α-tocopherol dissolved in DMSO were used as the positive control.

Reducing Power

The reducing power was determined according to O y a i z u (1986). Each polysaccharide powder (0.1 to 20 mg/ml, 2.5 ml) in MQ was mixed with 2.5 ml 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was vortexed and incubated at 50°C for 20 minutes. Then, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 2000 g for 10 min. The upper layer (5 ml) was mixed with 5 ml of MQ and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. Higher absorbance indicated higher reducing power. Ascorbic acid was used as the positive control.

Chelating Ability on Ferrous Ions

Chelating ability was determined according to D i n i s et al. (1994). Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in MQ was mixed with 3.7 ml of MQ and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. Citric acid and EDTA were used for comparison. The control did not contain ferrous chloride or ferrozine, complex formation molecules. Lower absorbance indicated higher chelating power.

Statistical Analysis

All measurements were done in triplicate and data were expressed as mean ± standard deviation. Free statistical regression calculations online (http://easycalculation.com/statistics/regression.php) was used to calculate EC_{50} values by linear regression analyses.

RESULTS AND DISCUSSION

After hot water extraction and alcohol precipitation, the obtained polysaccharides from the carpophores and the spores of the mushroom *G. lucidum* were refined by dialyses and dry extracts were used for the investigation of antioxidant properties.

Antioxidant Activity

Conjugated diene is formed from a moiety with two double bonds separated by a single methylene group which occurs in polyunsaturated fatty acids. Formed conjugated diene can be monitored spectrophotometrically using its characteristic absorption at 234 nm. This method is suitable for the investigation of an early stage of lipid peroxidation (M o o n and S h i b a m o t o , 2009).

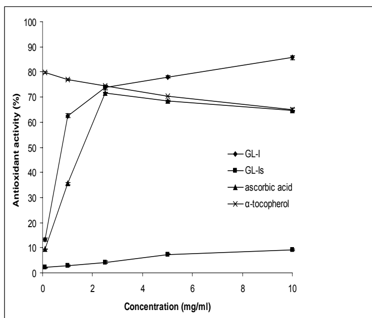

Fig. 1 – Antioxidant activity of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

Gl-I showed high antioxidant activity of 85.7 ± 0.7%, at 10 mg/ml, while antioxidant activity of Gl-Is was 9.2 ± 0.3% at 10 mg/ml (Figure 1.). Gl-I had higher antioxidant activity than Gl-Is at all other concentrations as well. Antioxidant activities of ascorbic acid and α-tocopherol were 64.7 ± 0.1 and 65.0 ± 0.5% at 10 mg/ml. Gl-I was even more effective than ascorbic acid and α-tocopherol at 5 and 10 mg/ml.

DPPH Free Radical Scavenging Activity Assay

The proton radical scavenger reacts with the stable free radical DPPH (deep violet color) and converts it to 1.1-diphenyl-2-picrylhydrazine with discoloration, suggesting that antioxidant activity of *G. lucidum* is due to its proton

donating ability. Medicinal mushrooms are free radical inhibitors or scavengers, acting as primary antioxidants which possess capability of terminating the chain reaction by reacting with free radicals which are major propagators of the autoxidation chain of fat (G o r d o n, 1990).

At 10 mg/ml, scavenging ability on DPPH radicals of Gl-I was very high and increased to 96.8 ± 2.5%, whereas Gl-Is scavenged DPPH radicals by 69.6 ± 2.5% at 10 mg/ml (Figure 2). Obviously, Gl-I appeared more effective than Gl-Is at all concentrations tested. However, ascorbic acid and BHT, the positive controls used in this test, scavenged DPPH radicals by 87.6 ± 0.3 and 55.2 ± 0.2% at 10 mg/ml, whereas α-tocopherol scavenged DPPH radicals by 96.1 ± 0.1% at 5 mg/ml.

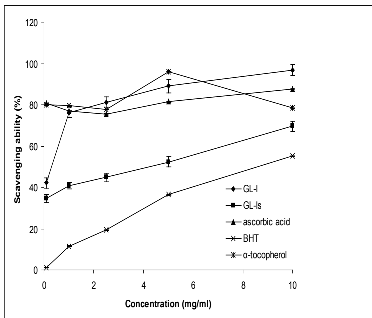

Fig. 2 – Scavenging ability of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

Reducing Power

Test solutions changed color from yellow to different shades of green and blue, depending on the reducing power. Conversion of the Fe^{3+}/ferricyanide complex to the ferrous form was caused by the presence of reducers. It seems that *G. lucidum* possesses hydrogen-donating ability.

Reducing power of Gl-I reached a plateau of 3.4 ± 0.1 at 20 mg/ml and only 0.3 ± 0.0 at 20 mg/ml for Gl-Is (Figure 3.). Compared with Gl-Is, Gl-I was

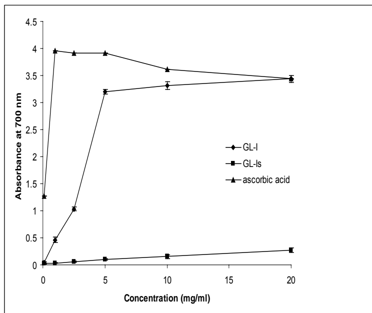

Fig. 3 – Reducing power of Gl-I and Gl-Is from *Ganoderma lucidum*.
Each value is expressed as mean ± standard deviation (n = 3).

more effective (i.e. the difference between Gl-I and Gl-Is was approximately thirty three-fold at 5 mg/ml). Ascorbic acid, used as a positive control, had a reducing power of 3.4 ± 0.0 at 20 mg/ml, the same as Gl-I.

Chelating Ability on Ferrous Ions

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, such as mushroom extracts, the complex formation is disrupted and red color of the complex is decreased. Change of color points to chelating activity of extracts and captures the ferrous ion before ferrozine does it (W o n g a n d C h y e, 2009).

Gl-I chelated 81.6 ± 3.6 % of ferrous ions at 20 mg/ml, while the chelating effect of Gl-Is was 73.8 ± 1.7% (Figure 4). Lower absorbance indicated higher chelating ability. However, the chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid was not a strong chelator in this assay (it chelated only 10.3 ± 0.1% at 20 mg/ml). With regard to ferrous ion chelation, Gl-Is was less active than Gl-I.

The antioxidative properties of hot water extracts from carpophore and spores of the mushroom *G. lucidum* in all applied tests were concentration dependent and increased with increasing concentration.

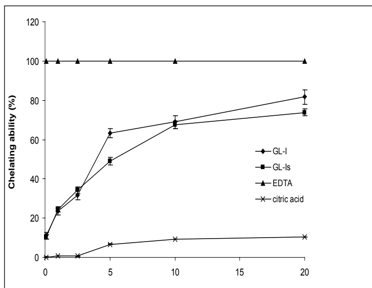

Fig. 4 – Chelating ability of Gl-I and Gl-Is from *Ganoderma lucidum*.
Each value is expressed as mean ± standard deviation (n = 3).

EC_{50} values in antioxidant properties

The results of antioxidant activity, DPPH free radical scavenging activity, reducing power and chelating effect on ferrous ion were normalized and expressed as EC_{50} (mg/ml) values which represented the effective concentration of each mushroom extract required to show 50% of antioxidant properties. Lower EC_{50} value corresponds to higher antioxidant activity of the mushroom extract.

Gl-I showed very good antioxidant activity, which was confirmed by low EC_{50} value (1.2 ± 0.0 mg/ml). On the other hand, EC_{50} for Gl-Is was high (44.8 ± 0.9 mg/ml) which indicated weak antioxidant activity. However, α-tocopherol showed excellent antioxidant activity (EC_{50} < 0.1 mg/ml), while ascorbic acid was also quite active, as it was shown by its low EC_{50} value (1.6 ± 0.0 mg/ml).

The investigated extracts appeared to be very good DPPH radical scavengers, especially Gl-I having the EC_{50} value 0.1 ± 0.1 mg/ml, while EC_{50} value of Gl-Is was 3.6 ± 0.5 mg/ml. Ascorbic acid and α-tocopherol were both excellently scavenging DPPH radicals (EC_{50}<0.1 mg/ml), while BHT was a good DPPH radical scavenger (EC_{50} = 8.5 ± 0.0 mg/ml).

EC_{50} value of the reducing power for Gl-I was very good (0.5 ± 0.1 mg/ml), whereas Gl-Is showed weak reducing power (38.69 ± 6.6 mg/ml). Ascorbic acid showed excellent reducing activity (EC_{50}<0.1 mg/ml).

EC_{50} values of the chelating abilities on ferrous ions were good for both Gl-I (3.86 ± 0.0 mg/ml) and Gl-Is (4.9 ± 0.3 mg/ml). EDTA showed excellent chelating activity (EC_{50} < 0.1 mg/ml), while citric acid was not a good chelator (EC_{50} > 20 mg/ml).

CONCLUSION

Our results suggest that mushroom *Ganoderma lucidum* and its extracts could be very good sources of naturally-derived antioxidants. The applied tests showed that hot water extract obtained from fruiting body of this mushroom had better antioxidant effects than that of the spores, probably due to the fact that spore cell walls were not broken. Use of mushrooms in daily diet might be beneficial for human health in preventing or reducing oxidative damage. Although EDTA is an excellent metal chelator, ascorbic acid, α-tocopherol and BHT also appear to have good antioxidant activity, reducing power and scavenging ability on DPPH radicals. However, these compounds are synthetic antioxidants.Since some of them, such as BHT, may possess mutagenic activity (Namiki, 1990), it would be very useful to replace them with natural antioxidants in food and pharmaceuticals. Mushroom and its extracts could be used for that purpose.

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АНТИОКСИДАТИВНЕ АКТИВНОСТИ ВРЕЛИХ ВОДЕНИХ ЕКСТРАКАТА ИЗ КАРПОФОРА И СПОРА ГЉИВЕ *GANODERMA LUCIDUM*

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Резиме

Ganoderma lucidum (Leyss.: Fr.) Karst је једна од медицински важних гљива, која поседује изузетна антиоксидативна својства. Циљ овог истраживања био је потврда антиоксидативне активности, редукционе способности, могућности хватања 1,1-дифенил-2-пикрилхидразил (DPPH) радикала и способности хелирања јона гвожђа врелих водених екстраката добијених из карпофора и спора ове гљиве. Врели водени екстракт из карпофора (Gl-I) показао је високу антиоксидативну активност 85.7 ± 0.7%, при 10 mg/ml, док је антиоксидативна активност врелог воденог екстракта спора (Gl-Is) била 9.2 ± 0.3% при 10 mg/ml. Редукциона способност екстракта Gl-I достигла је ниво 3.4 ± 0.1 при 20 mg/ml и 0.3 ± 0.0 при 20 mg/ml за екстракт Gl-Is. При концентрацији 10 mg/ml способност хватања DPPH радикала екстракта Gl-I достигла је 96.8 ± 2.5%, док је екстракт Gl-Is везао 69.6 ± 2.5% DPPH радикала при 10 mg/ml. Екстракт Gl-I хелирао је 81.6 ± 3.6 % феро јона при концентрацији 20 mg/ml, а хелирајући ефекат екстракта Gl-Is био је 73.8 ± 1.7%. Антиоксидативне активности врелих водених екстраката из карпофора и спора гљиве *G. lucidum* зависиле су од концентрације и повећавале су се са повећањем концентрације.