Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 120, 287—295, 2011

UDC 635.89:615.28
DOI: 10.2298/ZMSPN1120289K

Maja S. Kozarski[1], *Anita S. Klaus*[2], *Miomir P. Nikšić*[2]

[1] Department for Chemistry and Biochemistry, [2]Department for Industrial Microbiology, Institute for Food Technology and Biochemistry, University of Belgrade-Faculty of Agriculture, Nemanjina 6, 11081 Belgrade, Serbia
e-mail: maja@agrif.bg.ac.rs

EXTRACT FROM WILD STRAIN OF MUSHROOM *GANODERMA LUCIDUM* AS NATURAL ANTIOXIDANT

ABSTRACT: Recently, much attention has been paid to revealing natural biomaterials for clinical purposes since use of synthetic antioxidants is restricted due to their carcinogenicity. Among various natural antioxidants, polysaccharides, in general, have strong antioxidant activities and can be explored as novel potential antioxidants. The aim of this work was to examine the antioxidant properties of hot water extracted polysaccharides from *Ganoderma lucidum* in the form of mature fruit bodies, collected from the Bojčinska forest near Belgrade, the Republic of Serbia. Antioxidant properties were assayed *in vitro*, by the conjugated diene method, reducing power, scavenging abilities against 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ability on ferrous ions. At concentrations of 1 mg/ml, the scavenging ability of *G. lucidum* polysaccharide extract on DPPH radicals was 74.7 %. At 1 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were 11.5, 77.1 and 79.4 %, respectively. The antioxidant activity of the polysaccharide extract increased as the concentration increased to 78.0% at 20 mg/ml. Antioxidant activities of ascorbic acid and α-tocopherol were 63.8 % and 65.4% at 20 mg/ml. Polysaccharide extract from *G. lucidum* showed steady increase in the reducing activity as concentrations increased to 2.9 at 20 mg/ml. Ascorbic acid, used as a positive control, had a reducing power of 3.9 at 5 mg/ml. Chelating effects of the polysaccharide extract on ferrous ion increased with the increased concentrations. At 0.1-20 mg/ml, the chelating ability of *G. lucidum* polysaccharide extract was between 10.3-87.8%. The chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid did not prove to be good chelating agent for ferrous ions in this assay since its chelating ability was 10.3% at 20 mg/ml.

KEY WORDS: antioxidant properties, *Ganoderma lucidum*, polysaccharide extract

INTRODUCTION

Free radical species produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging (C r o s s , 1987; B e c m a n and A m e s , 1998). Almost all organisms are well protected against free radical damage by enzymes,

such as superoxide dismutase and catalase, or compounds, such as ascorbic acid, tocopherols and glutathione (N i k i et al., 1994). In order to reduce the damage of free radicals, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and *tert*-butylhydroquinone (TBHQ) are used. However, according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing, such as BHT and BHA, have already been documented. For example, higher levels of BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis (D e O l i v e i r a et al., 2009; P r a s a d et al., 2009). Recently, much attention has been paid to screening natural biomaterials.

Among various natural antioxidants, polysaccharides, in general, have strong antioxidant activities and can be explored as novel potential antioxidants (J i a n g et al., 2005; N g et al., 2006). Also, polysaccharides are potentially useful for pharmaceutical purposes due to a variety of their biological activities, such as immunological, anti-radiation, anti-blood coagulation, anticancer, anti-HIV and hypoglycemic activities (Y a n g et al., 2005; Y o o n et al., 2003).

Therefore, the aim of the present work is to evaluate the antioxidant properties of hot water extracted polysaccharides from wild strain of the mushroom *Ganoderma lucidum* in the form of mature fruit bodies, collected from the Bojčinska forest near Belgrade, the Republic of Serbia. In recent years, wild edible mushrooms have become increasingly important in our diet for their nutritional and pharmacological characteristics. Their nutritional values and taste components have been studied. However, there is little information available about antioxidant properties of wild edible mushrooms.

Antioxidant properties were assayed *in vitro* in terms of antioxidant activity, by the conjugated diene method, reducing power, scavenging abilities on 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ability on ferrous ions.

MATERIALS AND METHODS

Sample preparation

Fresh wild-growing fruiting bodies of mushroom *Ganoderma lucidum* were collected from the Bojčinska forest near Belgrade, the Republic of Serbia. After collecting, the fruiting bodies were brush-cleaned, air-dried to constant mass at 40 ºC and ground into fine particles which were stored in the dark prior to the analysis.

Extraction of water soluble polysaccharide fraction

Up to 10% of dried powdered tissue was suspended in Milli-Q water. Polysaccharide extraction was done by autoclaving at 121ºC for 45 minutes.

The extract was cooled down and centrifuged at 9000 x g for 20 minutes. Supernatant was concentrated to 10% of the starting volume. Two volumes of 96% ethanol were added and left at 4 ºC overnight. After centrifugation, the pellets were washed with 70% ethanol, and dried in vacuum at 42 ºC. Purification was done by dialysis using ZelluTrans/Roth® 6.0 regenerated cellulose tubular membrane (MWCO: 8.000-10.000) against Milli-Q for 24 h at room temperature in order to remove residual small molecules as polyphenols, peptides and polysaccharides < 8-10 kDa. After centrifugation high MW polysaccharides were ethanol precipitated and vacuum dried for later use.

DPPH (2.2-diphenyl-1-picryhydrazyl) radical scavenging activity

The assay was done according to the modified method of Bilos (1958). In the first series, each polysaccharide powder (0.1–10 mg/ml, 2ml) in Milli-Q water was mixed with 1ml of freshly prepared DMSO solution containing 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 20 ^{o}C for 40 min. 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 by the equation $[1-(A_i-A_j)/Ac]$ 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. The EC_{50} value (milligrams of extract per milliliter) was the effective concentration at which DPPH radicals were scavenged by 50%, and it was obtained by interpolation from linear regression analysis.

Antioxidant activity

The antioxidant activity was determined by the conjugated diene method with slight modifications (Lingnert et al., 1979). Each polysaccharide powder (0.1 to 20 mg/ml, 100µL) in Milli-Q water was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.5). Then, 6.5 mM Tween 20 was added to provide a stable emulsion which was shaken in the dark at 37 °C to accelerate the oxidation. After incubation for 15 h in 0.2 ml of antioxidant mixture, 6 ml of absolute methanol was added. The absorbance of the supernatant mixture was measured at 234 nm against a blank using a UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. The proportional antioxidant activity was calculated from the equation $[(A_0 - A_1)/ A_0]$ x 100, where A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence

of the sample. Ascorbic acid and α-tocopherol were used as positive controls. A value of 100% indicated the strongest inhibitory ability.

Ferric-reducing antioxidant power assay

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 Milli-Q water was mixed with 2.5 ml of 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 ^{o}C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 2000*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of Milli-Q water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using a UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without the 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 the method of D i n i s et al. (1994). Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in Milli-Q water was mixed with 3.7 ml of Milli-Q water 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 min. at room temperature, the absorbance of the mixture was determined at 562 nm against the blank. Blank was the solution with all reagents but without the extract. Lower absorbance indicated higher chelating ability. The EC_{50} value (mg extract/ml) was the effective concentration at which ferrous ions were chelated by 50%. Citric acid and ethylenediaminetetraacetic acid (EDTA) were used for comparison.

RESULTS AND DISCUSSION

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples (S u et al., 2008). At concentrations of 1 mg/ml, the scavenging ability of *G. lucidum* polysaccharide extract on DPPH radicals was 74.7 % (Figure 1). At 1 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were 11.5, 77.1 and 79.4 %, respectively (Figure 1). The radical scavenging ability of the extract and positive controls at 1 mg/ml decreased in the following order: α-tocopherol ≈ ascorbic acid > *G. lucidum* > BHT. At 10 mg/ml, the radical scavenging ability decreased in the following order: ascorbic acid > α-tocopherol ≈ *G. lucidum* > BHT.

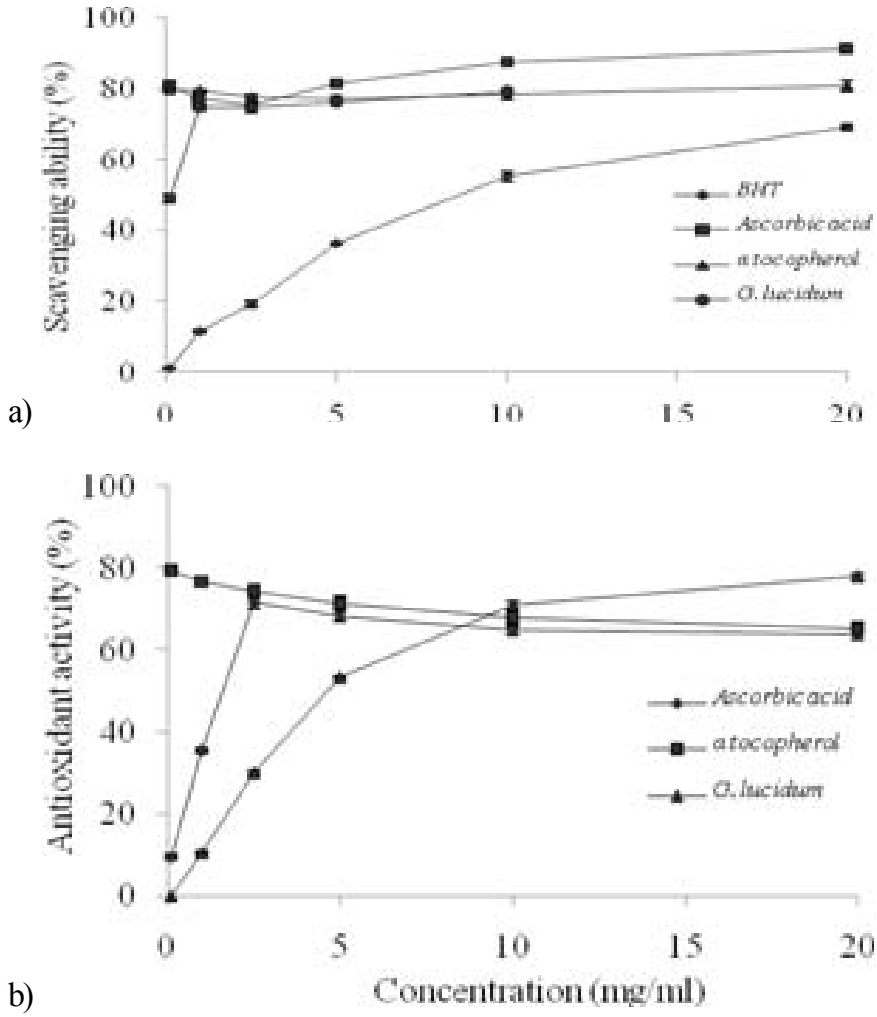

Fig. 1 – Scavenging ability on 1.1-diphenyl-2-picrylhydrazyl radicals (a); antioxidant activity evaluated in the linoleic acid model system (b) of hot water polysaccharide extract from *G. lucidum*. Each value is expressed as mean ± SEM (n = 3).

Using the conjugated diene method, the antioxidant activity of the polysaccharide extract from *G. lucidum* increased as the concentration increased to 78.0% at 20 mg/ml (Figure 1). Antioxidant activities of ascorbic acid and α-tocopherol were 63.8 % and 65.4% at 20 mg/ml, respectively.

Polysaccharide extract from *G. lucidum* showed steadily increasing reducing activity as concentrations increased to 2.9 at 20 mg/ml (Figure 2). Ascorbic acid, used as a positive control, had a reducing power of 3.5 at 20 mg/ml. High reducing power of *G. lucidum* polysaccharide extracts at 20 mg/ml suggested high potential in hydrogen-donating ability (A d e s u n g et al., 2007).

Chelating effects of the polysaccharide extract on ferrous ion increased with the increased concentrations (Figure 2). At 0.1-20 mg/ml, the chelating

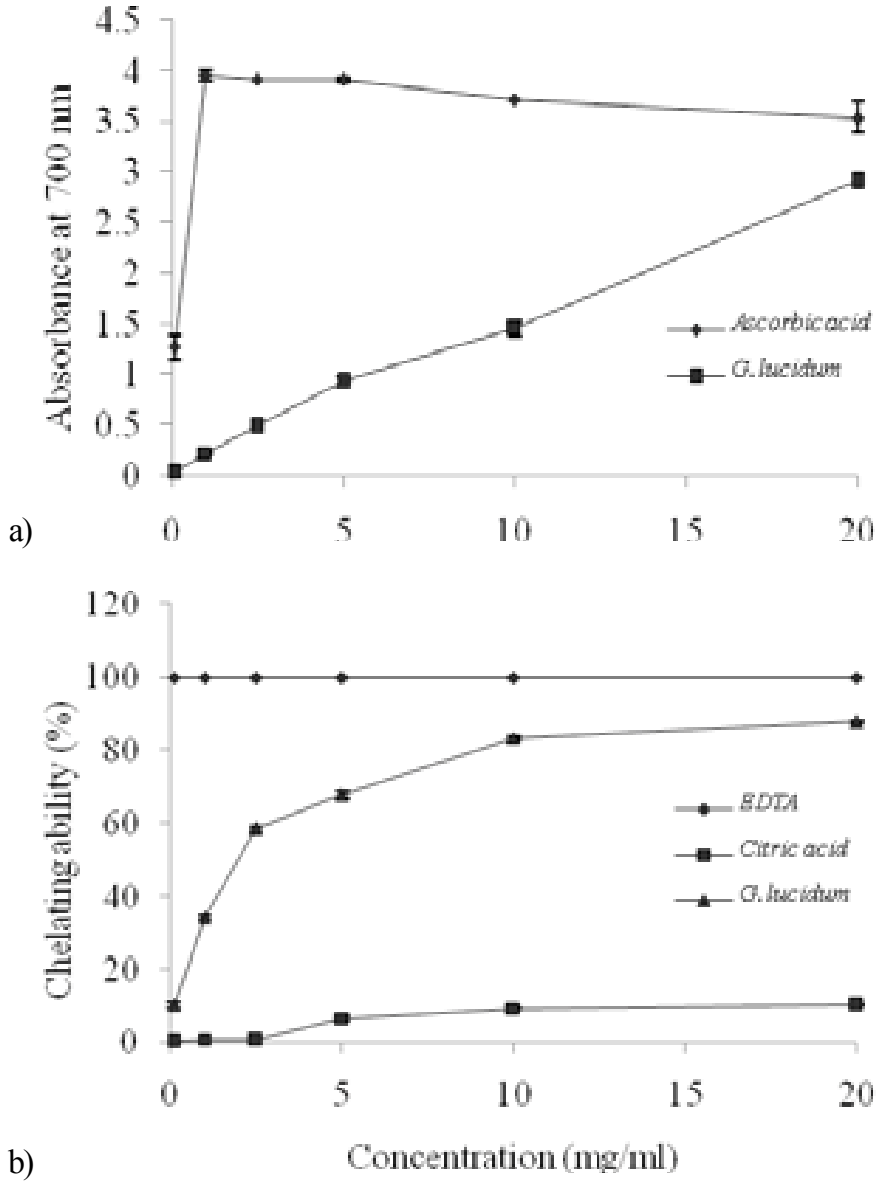

Fig. 2 – Reducing power (a), and chelating ability on ferrous ions (b) of hot water polysaccharide extract from *G. lucidum*. Each value is expressed as mean ± SEM (n = 3).

ability of *G. lucidum* polysaccharide extract was between 10.3-87.8%. The chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid was not a good chelating agent for ferrous ions in this assay, and its chelating ability was 10.3% at 20 mg/ml.

EC_{50} values in antioxidant properties

The effectiveness of antioxidant properties was expressed as EC_{50} (mg/ml) value which represented the effective concentration of mushroom extract required to show 50% antioxidant property (Table 1). Lower EC_{50} value corresponded to higher antioxidant activity of the mushroom extract.

Tab. 1 – EC_{50} values of polysaccharide extract from *G. lucidum* in antioxidant properties. Each value is expressed as mean ± SEM (n = 3).

	EC_{50}[a] (mg extract/ml)
Scavenging ability on DPPH radicals	0.16 ± 0.03
Antioxidant activity	8.89 ± 0.12
Reducing power	2.69 ± 0.17
Chelating ability on ferrous ions	3.48 ± 0.10

[a] EC_{50} value: The effective concentration at which 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%; the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; and ferrous ions were chelated by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.

With regard to scavenging ability on DPPH radicals, the polysaccharides from wild strain of *G. lucidum* showed very good scavenging ability which could be observed in their low EC_{50} values (0.16 ± 0.03 mg/ml). Ascorbic acid and α-tocopherol were both confirmed as excellent scavengers of DPPH radicals (EC_{50} < 0.1 mg/ml). BHT also proved to be a good DPPH radical scavenger (EC_{50} = 11.86 mg/ml). An EC_{50} value of the antioxidant activity was 8.89 ± 0.12 mg/ml. α-tocopherol showed excellent antioxidant activity (EC_{50} < 0.1 mg/ml), whereas ascorbic acid also had a good activity as shown by its low EC_{50} value (1.64 mg/ml).

For reducing power EC_{50} value was 2.69 ± 0.17 mg/ml. Ascorbic acid showed excellent reducing activity (EC_{50} < 0.1 mg/ml). EC_{50} value of the chelating ability of *G. lucidum* extract was 3.48 ± 0.10 mg/ml. The chelator EDTA showed higher activity (EC_{50} < 0.1 mg/ml). An EC_{50} value of the citric acid was < 20 mg/ml.

CONCLUSION

The results of the present study suggest that polysaccharide extract from wild strain of the *Ganoderma lucidum* mushroom acts as natural antioxidant. The extract exhibited good antioxidant activities, and some even showed higher potency than the standard synthetic antioxidants in some instances; for example, *G. lucidum* polysaccharide extract had higher activity in the DPPH assay when compared to BHT. Polysaccharide extract may be a good source for the development of antioxidant food additives. Further investigations are necessary to verify these activities *in vivo*.

ACNOWLEDGMENTS

This study was financed by the Ministry of Science and Technological Development of the Republic of Serbia.

REFERENCES

Adesegun, S. A., Fajana, A., Orabueze, C. I., Coker, H. A. B. (2009): *Evaluation of antioxidant properties of Phaulopsis fascisepala C.B.Cl. (Acanthaceae)*. Evidence-based Complementary and Alternative Medicine, 6: 227-231.

Becman, K. B., Ames, B. N. (1998): *The free radical theory of aging matures*. Physiol. Rev.,78: 547–581.

Bilos, M. S. (1958): *Antioxidant determinations by the use of a stable free radical*. Nature 181: 1199–1200.

Cross, C. E. (1987): Oxygen radicals and human diseases. Ann. Intern. Med., 107: 526–545.

De Oliveira, O. C., Valentim, I. B., Silva, C. A., Bechara, E. J. H., De Barros, M. P., Mano, C. M., Goulart, M. O. F. (2009): *Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues*. Food Chemistry, 115: 469–475.

Dinis, T. C. P., Madeira, V. M. C., Almeida, L. M. (1994): *Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers*. Archives of Biochemistry and Biophysics, 315: 161–169.

Jiang, Y., Wang, H., Lu, L., Tian, G. Y. (2005): *Chemistry of polysaccharide Lzps-1 from Ganoderma lucidum spore and anti-tumor activity of its total polysaccharides* (in Chinese). Acta. Pharmaceutica Sin. 40: 347–350.

Lingnert H., Vallentin, K., Eriksson, C. E. (1979): *Measurement of antioxidative effect in model system*. Journal of Food Processing and Preservation, 3: 87–103.

Ng, T. B., Pi, Z. F., Yue, H., Zhao, L., Fu, M., Li, L., Hou, J., Shi, L. S., Chen, R. R., Jiang, Y., Liu, F. (2006): *A polysaccharopeptide complex and a condensed tannin with antioxidant activity from dried rose (Rosa rugosa) flowers*. J. Pharm. Pharmacol., 58: 529–534.

Niki, E, Shimaski, H., Mino, M. (1994): *Antioxidantism-free radical and biological defense*. Gakkai Syuppan Center, Tokyo, 3-16.

Oyaizu, M. (1986): *Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine*. Japanese Journal of Nutrition 44: 307–315.

Prasad, K. N., Yang, B., Yang, S., Chen, Y., Zhao, M., Ashraf, M., Jiang, Y. (2009): *Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (Litchi sinensis Sonn.) seeds*. Food Chemistry, 116: 1–7.

Su, M. S., Shyu, Y. T., Chien, P. J. (2008): *Antioxidant activities of citrus herbal product extracts*. Food Chemistry, 111: 892–896.

Yang, J. H., Du, Y. M., Huang, R. H., Sun, L. P., Liu, H., Gao, X. H., John, F. K. (2005): *Chemical modification and antitumour activity of Chinese lacquer polysaccharide from lac tree Rhus vemicifera.* Carbohydrate polymers, 59: 101–107.

Yoon, S. J., Yu, M. A., Pyun, Y. R., Hwang, J. K., Chu, D. C., Juneja, L. R., Paulo, A. S. M. (2003): *The nontoxic mushroom Auricularia auricular contains a polysaccharies with anticoagulant activity mediated by antithrombin*. Thrombosis Research, 112: 151–158.

ЕКСТРАКТ ШУМСКОГ СОЈА ГЉИВЕ *GANODERMA LUCIDUM* КАО ПРИРОДНИ АНТИОКСИДАНТ

Маја С. Козарски[1], Анита С. Клаус[2], Миомир П. Никшић[2]

[1] Катедра за хемију и биохемију, [2] Катедра за Технолошку микробиологију, Институт за прехрамбену технологију и биохемију, Универзитет у Београду – Пољопривредни факултет, Немањина 6, 11081 Београд, Србија
e-mail: maja@agrif.bg.ac.rs

Резиме

У данашње време све веће пажња се поклања проналажењу природних биолошких материјала који имају антиоксидативна својстава. Нежељени ефекти, оштећење јетре и карциногенеза, од стране синтетичких антиоксиданата који се користе као адитиви исхрани су увелико потврђени. Међу различитим природним антиоксидантима полисахариди привлаче све већу пажњу као једињења са израженом антиоксидативном активношћу. Циљ рада је био да се испитају антиоксидативна својства врелог воденог екстракта полисахарида гљиве *Ganoderma lucidum* добијеног из зрелих плодоносних тела, прикупљених у Бојчинској шуми у близини Београда, Република Србија. Антиоксидативна својства су испитивана помоћу антиоксидативне методе у модел систему линолеинске киселине, мерењем снаге редукције, способности хватања 1,1-дифенил-2-пикрилхидразил (DPPH) радикала и способности хелирања јона гвожђа. При концентрацији од 1 mg/ml способност хватања DPPH радикала полисахаридног екстракта шумског соја *Ganoderma lucidum* достигла је ниво од 74.7%, док су измерене вредности, при истој концентрацији, за позитивне контроле BHT, аскорбинску киселину и α-токоферол износиле 11.5, 77.1 и 79.4%. Антиоксидативна активност измерена у модел систему линолеинске киселине зависила је од концентрације и повећавала се са повећањем концентрације. Измерена је вредност од 78.0% при концентрацији од 20 mg/ml. Антиоксидативне активности аскорбинске киселине и α-токоферола при концентрацији од 20 mg/ml биле су 63.8 и 65.4%. Редукциона способност полисахаридног екстракта достигла је ниво од 2.9 при 20 mg/ml и 3.9 при 5 mg/ml код аскорбинске киселине која је коришћена као позитивна контрола. Способност хелирања јона гвожђа се повећавала са повећањем концентрације. При концентрацији од 0.1-20 mg/ml измерена способност хелирања била је између 10.3-87.8%. Хелатни ефекат EDTA био је 100% у опсегу од 0.1-20 mg/ml. Лимунска киселина се није показала као добар хелирајући агенс (10.3% при 20 mg/ml).