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LIGNICOLOUS FUNGI AS POTENTIAL NATURAL SOURCES OF ANTIOXIDANTS

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Abstract - As a result of an interest in natural derived metabolites around the world, higher fungi (Basidiomycotina) have taken on great importance in biochemical investigations. A large number of structurally divergent compounds - both cellular components and secondary metabolites - have been extracted and found to possess significant biological activity, such as an immunomodulative effect on the human body. Effects of fungal biomolecules as potential natural antioxidants have not been examined so far. Biochemical analysis have included *in vitro* testing of the influence of different extracts (water, methanol, chloroform) of selected fungal sporocarps on Fe^{2+}/ascorbate-induced lipid peroxidation (LP) in a lecithin liposome system by TBA assay, as well as various other procedures. Qualitative analysis by TLC revealed a distinction both between different extracts of the same fungal species and between similar extracts of different species. The results obtained on antioxidative activities (LP inhibition and "scavenging" activity) indicate that MeOH extracts manifested a degree of activity higher than that of $CHCl_3$ extracts with respect to antioxidative activity, the extracts can be ranged in the following declining order: *Ganoderma lucidum*, *Ganoderma applanatum*, *Meripilus giganteus*, and *Flammulina velutipes*. The obtained results suggest that the analyzed fungi are of potential interest as sources of strong natural antioxidants in the food and cosmetics industries, whereas synthetic ones have proved to be carcinogenic.

Key words: Antioxidants, Basidiomycotina, lignicolous fungi, secondary metabolites, scavenging activity

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INTRODUCTION

The stress and pressure of modern society take a toll on the body's immune system, which is especially important for ill people, whose weakened immunity is more susceptible to infection and disease. The need to maintain or rebuild a healthy defense system has led researchers in the last decades around the world to search for natural health-supporting properties of plants and microorganisms that have not yet been examined thoroughly enough (Lorenzen and Anke, 1998; Wasser and Weis, 1999; Eisenhut and Fritz, 1991; Wainwright, 1992; Weis *et al.* 1999). Traditional Chinese and Japanese healers (Mizuno, 1996, 1999; Shiao, 1992; Kavagishi, 1995; Cheung, 1997) have respected the world of mushrooms for centuries and used lignicolous macrofungi in particular to cure people (subdiv. Basidiomycotina, cl. Homobasidiomycetes, subcl. Aphyllophoromycetideae: reishi - *Ganoderma lucidum*, shiitake - *Lentinus edodes*, *Ganoderma applanatum*, *Coriolus versicolor*, *Meripilus giganteus*, *Schizophyllum commune*, and subcl. Agaricomycetideae: *Lentinus edodes*, *Flammulina velutipes*, *Pleurotus ostreatus*). Western culture for a long time acknowledged only the nutritional and culinary values of some mushroom species (Breene, 1990). However, investigators have recently become aware of their medicinal qualities and significant biological activities: antimicrobial, antifungal, antiviral, and cytotoxic (antitumor), cardiovascular, antiparasitic, anti-inflammatory, hepatoprotective, and antidiabetic (Molitoris, 1994; Chang, 1999; Gunde-Cimerman, 1995, 1999). The pharmacological effect of fungi is based on their powerful immune-modulating action and immunopotential capability, which support and enhance the overall immune function due to the presence of ß-glucans, lectins, polysaccharides, polysacchari-

de-peptide complexes, triterpenoids, and nucleosides. It has been recorded that fungi can be used as "biological response modifiers" (BRM) or immunomodulators that cause no harm to the body and help it adapt to various environmental and psychological stresses. Researchers have found that mushrooms can directly stimulate both basic (lymphocytes, neutrophils, etc.) and secondary (immunoglobulins: IgE, IgA, IgG) immune responses of the immune system. This stimulus can increase production of immune defenders such as cytokinins, and macrophages, which play vital roles in recognizing and removing foreign antigens, as well as in releasing chemical mediators (tumor necrotic factor, reactive oxygen and nitrogen intermediates, interleukins, etc.). There are a great many medicines in the world based on bioactive compounds isolated from extracts of mushroom sporocarps or mycelium (M i z u n o, 1999), and specific mixtures of these extracts have been used to enhance immunity and stabilize convalescence (L i u, 1999; M a t z u z a w a, 1997). It has been found that secondary biomolecules that potentiate the immune system are very divergent in structure and play no essential role in growth and reproduction of organisms. However, they probably have a function either as constantly formed new products in biochemical evolution of a species ensuring its survival or as agents of an indirect mechanism in its differentiation (E n g l e r *et al.* 1998). The presence of these compounds in plants and microorganisms (fungi) is genetically determined, but also varies as a function of ecological factors and the growth stage of those organisms (M i m i c a - D u k i ć, 1997). The chemical composition of fungi is different in sporocarps, mycelium, and fermentation broth (L o r e n z e n and A n k e, 1998). Inasmuch as secondary metabolites of fungi as antioxidants have still not been examined and lignicolous fungi are widely present on the mountain Fruška Gora, the aim of the present study was to screen secondary metabolites from sporocarps of the following autochthonous species of Basidiomycotina fungi: *Ganoderma lucidum*, *Ganoderma applanatum*, *Meripilus giganteus*, and *Flammulina velutipes*. The species *G. lucidum* (Curt. ex Fr.) Karst. and *G. applanatum* (Pers. ex Wallr.) Pat. (family Ganodermataceae) are known to contain ß-glucans, acid heteroglucans, chitin xyloglucans, and highly oxidized but also highoxidated triterpenoids of the lanostan type (ganoderic acid, ganodeneric acid, ganolucidic acid and lucidenic acid) as active substances (W a s s e r and W e i s, 1999). The species *M. giganteus* (Pers. ex Fr.) Karst. (family Polyporaceae *s. lato*) is one that is not used as a source of active biomolecules, as opposed to the species *F. velutipes* (Curt. ex Fr.) Karst. (family Tricholomataceae), which is well-known as a medically important fungus (W a s s e r and W e i s, 1999; Z h a n g, 1999) and contains ß-glucan proteins: EA_6, EA_6-PII, and the glycoprotein proflamin in the submerged mycelial biomass. Antioxidative effects of sporocarp extracts have not been examined so far. Inasmuch as antioxidative activities have significant therapeutic effects, these fungal species could be used in therapy of a variety of disease states and in healthy nutrition as a source of naturally derived antioxidants. They are easily noticed, collected, and recognized in the field, and their secondary metabolites can be easily identified and extracted (E n g l e r *et al*. 1998).

MATERIALS AND METHODS

1) *Sporocarp sampling*: All wild growing sporocarps were collected from two sites (Iriški Venac I, II) on the mountain Fruška Gora except those of *F. velutipes*, which was found on the Ribarsko Ostrvo Lake (L III) near the Danube River. The samples were brush-cleaned of attached soil and humus, air-dried to constant mass, and pulverized in an electric mill.

2) *Extraction*: A weighed portion (10 g) of dried and pulverized fungal sporocarps was poured into Erlenmeyer flasks with 200-250 ml cold water or the same quantity of a pure solvent in order to get aqueous (H_2O), methanol (MeOH), and chloroform ($CHCl_3$) extracts. The extract was filtered and the solvent in the obtained supernatant was evaporated under reduced pressure. The obtained residues were weighed and redissolved in MeOH and $CHCl_3$ at a concentration of 10% or some other concentration (1.25%, 2.5%, 5%).

3) *Phytochemical screening analysis*: An infusion was prepared of 10 g of dried and pulverized fungal material, which was poured into boiling distilled water and left for 15 min. The solution was filtered (with absorbent cotton) and the filtrate was used in subsequent analysis. Screening of components in fungal material was done in accordance with scientifically known reactions.

4) *Antioxidative activity examination of fungal extracts*: This examination was performed by investigating LP in liposomes, $OH^{\bullet}$ radical production in the Fenton reaction, and $DPPH^{\bullet}$ radicals by the scavenging method.

A. *Determination of LP in liposomes*: The extent of LP

was determined by measuring the pink pigment (Halliwell and Gutteridge, 1986) produced in the reaction of 2-thiobarbituric acid (TBA) and malondialdehyde (MDA) as oxidation products in the peroxidation of membrane lipids. This was done by means of TBA-assay. A commercial preparation of liposomes (PRO-LIPO S, Lucas-Meyer) with pH=5-7 was used as a model-system of biological membranes. Liposomes 225-250 nm in diameter were obtained by dissolving the commercial preparation in dematerialized water (1:10) in an ultrasonic bath (Chatterjee and Agarwal, 1988). In its final volume, the reaction mixture contained, 60 µl of liposome suspension, 20 µl of 0.01M $FeSO_4$, 20 µl 0.01M ascorbic acid, and 20 µl of tested compounds dissolved in 2.88 ml of 0.05M KH_2PO_4-K_2HPO_4 buffer (pH=7.4) to start the peroxidation. The samples were incubated at 37°C for 1 h. Liposome LP was measured using the reaction with TBA. A measured volume (1.5 ml) of TBA-reagent (10.4 ml of 10% $HClO_4$, 3 g of TBA, and 120 g of 20% TCA dissolved in 800 ml of dH_2O) and 0.2 ml 0.1M EDTA were added and the tubes were heated on a boiling water bath for 20 min. After cooling, the reaction mixtures were centrifuged at 4000 rpm for 10 min. Absorbance was measured at 532 nm. The fungal extracts were tested in four concentrations (10%, 5%, 2.5%, and 1.25%) against 0.5M BHT (a synthetic antioxidant). All reactions were carried out in triplicate. The percentage of LP inhibition was calculated using the following equation: $I(\%)=(A_0-A_1)/A_0 \times 100$, where A_0 is absorbance of the control reaction (= full reaction, without the test compound) and A_1 is absorbance in the presence of the inhibitor.

B. *Determination of OH• radical content*: The content of OH• radicals was determined from the following reaction of 2-deoxyribose degradation. These radicals take an H atom from 2-deoxyribose, and the products formed react with the TBA reagent. The TBA reaction product was determined spectrophotometrically at 532 nm (Chessman *et al.* 1988). The reaction mixture contained the following: 0.125 ml of 2-deoxyribose, 0.125 ml of $FeSO_4$ (127 mg $FeSO_4x7H_2O$ in 50 ml of phosphate buffer, pH=7.4), and 10 µl of the tested compound. All samples analyzed were topped off with phosphate buffer to a final volume of 3 ml and incubated for 1 h at 37°C. The reaction was stopped by adding TBA-reagent according to the procedure described in the previous paragraph. The intensity of scavenging activity of OH• radicals was determined by the same equation: $I(\%) = (A_0-A_1)/A_0 \times 100$.

C. *Measurement of radical scavenging activities*: The antioxidant activity of fungal extracts was determined by the DPPH• radical scavenging method (Solar-Rivas *et al.* 2000). The 1, 1-diphenyl-2-pivcrilhydrasil (DPPH•) radical is a stable free radical that has a dark violet color in methanol extract. It has an absorption maximum at 515 nm (λ_{max} = 515 nm), and the peak of the DPPH• radical shrinks in the presence of a hydrogen donor, i.e., a free radical-scavenging antioxidant. This is followed by a change of absorbance and disappearance of the solution's violet color. The reaction mixture contained 1 ml of 90 µM DPPH•-solution and 5, 10, 20, 30, 40, 50, 60, or 70 µl of fungal extract. All reactions were carried out in triplicate. The test tubes were filled with pure MeOH to 4 ml. After incubation for 60 minutes at room temperature, absorbance was measured spectrophotometrically at 515 nm. The percentage of scavenging activity was calculated by the same equation as for LP inhibition: %RSC = 100-Au x 100/Ak, where RSC - is the radical scavenging capacity, Au - absorbance of sample, and Ak - absorbance of the control reaction. From these RSC values, IC_{50} values (the concentration at which 50% of the radical form of DPPH• is neutralized) were calculated by applying regression analysis.

RESULTS

1) *Phytochemical "screening" of fungal extracts*: The obtained results of chemical screening of some classes of secondary biomolecules showed that compounds from the classes of tannins and saponins were present in almost every fungal extract, while flavonoids and alkaloids were present in some of them (flavonoids in *G. applanatum* and alkaloids in the species *G. lucidum* and *M. giganteus*).

2) *Thin-layer chromatographic qualitative analysis* revealed the presence of phenolic compounds in the analyzed fungal extracts. Figures 1 and 2 show developed TL-chromatograms photographed under a UV-lamp at 366 nm (Fig. 1) and 254 nm (Fig. 2), where put in the fungal samples were the following order: 1. *F. velutipes* (MeOH, 10%), 2. *G. applanatum* (MeOH, 1%), 3. *G. lucidum* (MeOH, 10%), 4. *G. lucidum* (MeOH, 0.1%), 5. *M. giganteus* (MeOH, 10 %), and 6. *M. giganteus* ($CHCl_3$, 10%). Considering the presence of an intensive blue fluorescent mark (R_f = 0.43) in all extracts and two other fluorescent marks of lower intensity (R_f = 0.29; R_f = 0.34) in all except sample 2 and the fact that they do not

appear at UV-254 nm (Wagner *et al.* 1984), we presumed that phenols from the classes of tannins, phenolic acids, phenyl propanoids, or cumarins are dominant. However, in all extracts the most intensive mark (R_f = 0.43) did not disappear at UV-254 nm, which indicates

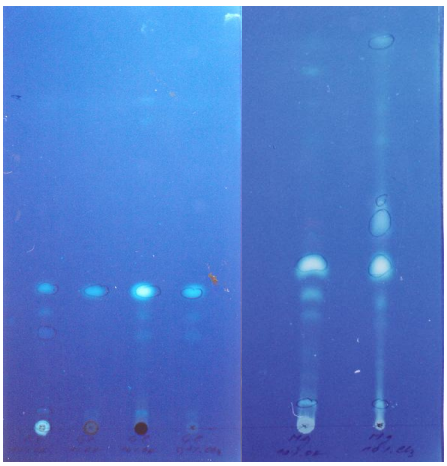

Fig. 1. TLC chromatogram of MeOH and $CHCl_3$ fungal extracts at 366 nm.

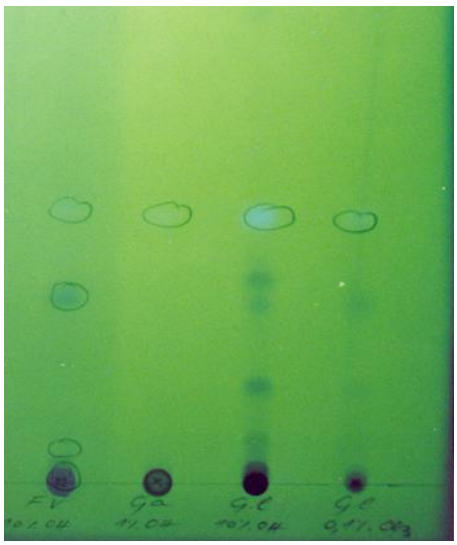

Fig. 2. TLC chromatogram of MeOH and $CHCl_3$ fungal extracts at 254 nm.

the presence of some other compounds. Sample 1 had an intensive blue fluorescent mark at the very beginning of chromatogram (R_f = 0), while in samples 2 and 3 two blue-fluorescent marks near the eluent front (R_f = 0.99; R_f = 0.93). From the TLC of MeOH and $CHCl_3$ extracts of samples 5 and 6, it can be assumed that they are rich in phenolic compounds. The main difference lies in the presence of less polar compounds (probably aglycons) in $CHCl_3$ extract (R_f = 0.51; R_f = 0.59; R_f = 0.99) and the greater amount of polar glycosides in MeOH extract (marks at the start). Methanol extracts of *G. lucidum* had the most registered compounds on TLC, methanol extracts of *G. applanatum* have the fewest.

3) *Results of investigations on antioxidant effects of fungal extracts, on LP inhibition in liposomes*: Preliminary antioxidant screening of lignicolous fungi for LP in liposomes showed that aqueous extracts have no inhibitory effect on LP (most stimulated LP in liposomes), in contrast to methanol (MeOH) and chloroform ($CHCl_3$) extracts. This result can be attributed to the fact that polysaccharides and lectins are dominant in aqueous extracts, and they are not known to have antioxidant potency. All of the examined MeOH extracts manifested a higher degree of LP inhibition than in $CHCl_3$ ones, the highest value being recorded in MeOH extract of *G. applanatum* (61.5%) and $CHCl_3$ extract of *G. lucidum* (38.38%) (Fig. 3). Most of the extracts exhibited inhibition higher than that of the synthetic antioxidant BHT.

Methanol extracts exerted inhibitory action on LP ranging from 14.49% (*G. lucidum*) to 39.67% (*G. applanatum*) for 10% MeOH extracts; from 37.99% (*F. velutipes*) to 44.18% (*M. giganteus*) for 5% MeOH extracts; from 41.77% (*M. giganteus*) to 57.72% (*G. applanatum*) for 2.5% MeOH extracts; and from 12.22% (*F. velutipes*) to 61.25% (*G. applanatum*) for 1.25% extracts (Fig. 3). The most effective was 1.25% MeOH extract of *G. applanatum.* The lowest antioxidant effect in all samples was recorded with 10% extracts and the highest with 1.25% extracts (except in the case of *F. velutipes*, where the highest effect was obtained in a concentration of 2.5%), while all of them exerted stronger inhibitory action than that of the synthetic antioxidant BHT (37.04% inhibition). These results suggested the possibility of using the given extracts in the food and cosmetics industries as natural derived antioxidants instead of synthetic ones, which are known to have a carcinogenic effect.

Chloroform extracts exerted maximum antioxidant action in concentrations of 1.25% for *G. lucidum* and 5% for *M. giganteus*. However, it is still lower than the antioxidant activity of 0.5M BHT (Fig. 3). These results indicate that chloroform extracts are a poorer source of antioxidants than methanol ones. We assumed that terpenoid compounds and fungal sterols are responsible for this activity (according to literature data).

4) *Effect of fungal extracts on production of $OH^{\bullet}$ radi-*

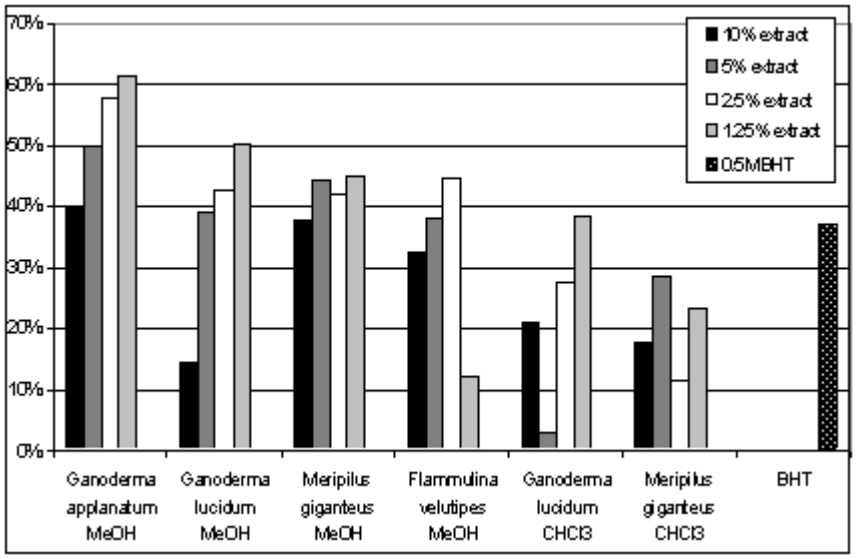

Fig. 3. Inhibitory effect of MeOH and $CHCl_3$ extracts on Fe^{2+}/ascorbate-induced LP (%).

cals: The inhibitory effect of the analyzed extracts on production of $OH^{\bullet}$ radicals generated in the system Fe^{2+}/H_2O_2 was such that none of them exerted antiradical action on $OH^{\bullet}$ radicals, but MDA production did increase. The highest prooxidative effect was recorded with 0.1% extract of *G. lucidum*, the lowest with 5% extract of *M. giganteus*. Because LP is mostly provoked by the hydroxyl radical and all of the fungal extracts caused inhibition of LP, the recorded results appear to be contradictory. Macroelement analyses of extracts showed very high iron content (K a r a m a n, 2002) in *M. giganteus* - 2504.36 µg/g d.m., *G. lucidum* - 2290.92 µg/g d.m., and *F. velutipes* - 2165.28 µg/g d.m., values roughly 20 times higher than in other lignicolous species. We assume that dominance of the Fenton reaction over the antiradical reaction of fungal sporocarps was stimulated by the high presence of Fe^{2+} ions in extracts.

5) *Scavenger activity was determined by the DPPH test* for 0.1% MeOH and $CHCl_3$ extracts. With 0.1% MeOH, the antioxidant effect was similar in the analyzed extracts. The most effective were 0.1% MeOH extracts of *G. applanatum* and *G. lucidum* (which neutralized 91.93% and 88.89% of $DPPH^{\bullet}$ radicals in a concentration of 17.5 µg/ml). Less effective was the extract of *F. velutipes* (which neutralized 76.32% $DPPH^{\bullet}$ radicals in a concentration of 1500 µg/ml). By applying the equation (S h i and N i k i, 1998) $n = \Delta A/(\varepsilon \, l \, C_x)$ - where ΔA is the result of substitution of absorbance of $DPPH^{\bullet}$ radicals at 515 nm from the first and the last reaction, ε is the molar coefficient of extinction of $DPPH^{\bullet}$ radicals ($1{,}25 \times 10^{-4}$ M^{-1} cm^{-1}), l is cell length (civets), and C_x is the pure concentration of $DPPH^{\bullet}$ radicals (90 µM) - we counted the hypothetical n number of electrons (H atoms) given to $DPPH^{\bullet}$ radicals in the process of neutralization. Species with the highest antiradical effect (*Ganoderma*) gave 15×10^{-2} electrons (H atoms). These relations can be easily discerned from the IC50 value, e.g., the concentration at which 50% of the radical form of DPPH is neutralized. The value of IC50 was calculated using the exponentional function of the neutralization level of $DPPH^{\bullet}$ radicals and the samples concentration. For 0.1% MeOH extracts, the lowest IC50 values were obtained for *G. lucidum* (7.5 µg/ml) and *G. applanatum* (10.25 µg/ml), while the highest value was recorded for the species *F. velutipes* (300 µg/ml). For activity of 0.1% $CHCl_3$ extracts in scavenging of on $DPPH^{\bullet}$ radicals, the reverse inhibition efficiency was recorded, but just for *G. applanatum*. The strongest antiradical effect was manifested by the species *G. lucidum* (which neutralized 52.94% at a concentration of 15 µg/ml) and *M. giganteus* (where 35.62% of $DPPH^{\bullet}$ radicals passed over to the neutral protonated form). According to IC50 values, the latter species gave $1{,}5 \times 10^{-2}$ H atoms, which is seven times lower than for *G. lucidum* under the same conditions.

DISCUSSION

The aim of the present study was to examine the antioxidative activity of some autochthonous lignicolous

fungal species. Compounds from the classes of tannins and saponins were present in most of the analyzed fungal extracts, while alkaloids were recorded only in *M. giganteus* and flavonoids only in *G. applanatum*. Aqueous fungal extracts had no inhibitory effect on LP, but rather mostly stimulated LP in liposomes, which was expected in view of the fact that such extracts contain compounds from the groups of polysaccharides, lectins, and the others, known to have no antioxidative effects. On the other hand, MeOH and most of $CHCl_3$ extracts had an inhibitory effect on LP. All of the examined MeOH extracts manifested the higher LP inhibition levels than in $CHCl_3$ extracts and had an inhibitory effect higher than that of the synthetic antioxidant BHT (37.04% inhibition). The most effective were 1.25% MeOH extract of *G. applanatum*, and 1.25% $CHCl_3$ extract of *G. lucidum*.

Methanol extracts of the examined species of fungi had no effect on production of $OH^{\bullet}$ radicals generated in the system Fe^{2+}/H_2O_2. Inasmuch as the fungal extracts inhibited LP (which is for the most part stimulated by hydroxyl radicals), we assume that dominance of the Fenton reaction occurred because a high concentration of Fe^{2+} ions existed (as was demonstrated by earlier analyses of macroelements according to K a r a m a n, 2002).

In regard to the abilities of methanol fungal extracts to neutralize $DPPH^{\bullet}$ radicals, the highest antiradical effect was obtained with 0.1% extracts of *G. lucidum* and *G. applanatum*, which showed the lowest IC50 value at a concentration of 7.5 µg/ml, while the same value was recorded at 300 µg/ml for *F. velutipes*. Significant antiradical effects were recorded for $CHCl_3$ extracts of *G. lucidum* (16.25 µg/ml), *G. applanatum* (19 µg/ml), and *M. giganteus* (62.5 µg/ml).

The obtained results suggest significant antioxidant potency of the analyzed lignicolous fungi and their possible use as natural sources of antioxidants. This applies especially to species of the genus *Ganoderma*. Special attention should be paid to the species *M. giganteus*, whose medical importance has not been investigated up to now, but which in our screening proved to contain a great amount of phenolic compounds, a class of substances known to have considerable scavenger activity. Moreover, the significant antioxidant activity of *F. velutipes* (which is poor in phenolic compounds) indicates the presence of other classes of secondary biomolecules as potential natural antioxidants. Identification of these compounds and the complete chemical analysis of fungal extracts will be the goal of our further investigations.

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ЛИГНИКОЛНЕ ГЉИВЕ КАО ПОТЕНЦИЈАЛНИ ПРИРОДНИ РЕСУРС АНТИОКСИДАНАТА

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С обзиром на све израженији интерес у свету за биоактивним супстанцама природног порекла, нарочито су интензивирана биохемијска испитивања виших гљива (Basidiomycotina). Велики број нових једињења дивергентних структура је екстрахован из спорокарпа, мицелије и екстрацелуларног медијума, за које је утврдјено да чине ћелијске компоненте или секундарне метаболите и испољавајју значајне биолошке активности, као што је имуномодулативни ефекат на људски организам. О биомолекулима гљива као потенцијалним природним антиоксидантима нема доступних литературних података. Биохемијске анализе обухватиле су *in vitro* испитивања утицаја различитих екстраката (водени, метанолни, хлороформски) спорокарпа гљива на Fe^{2+}/аскорбат-индуковану липидну пероксидацију у систему липозома (LP) на Fe^{2+}/аскорбат-индуковану LP ТВА-тестом, и на продукцију веома токсичних $OH^{\bullet}$ радикала, праћењем деградације 2-дезоксирибозе, индуковане Фентоновом реакцијом (Fe^{2+}/H_2O_2), као и испитивањем "скевинџер" активности екстраката гљива DPPH тестом. TLC квалитативна анализа показала је да се екстракти различитих врста гљива као и различити екстракти истих гљива разликују у квалитативном саставу. Резултати антиоксидативних активности (инхибиција LP и "скевиндцер" активност) могу да истакну метанолне екстракте који су испољили јачи степен активности од хлороформских екстраката. Према добијеним антиоксидативним активностима екстракти гљива могли би се рангирати према опадајућим вредностима у следећем низу:

Ganoderma lucidum, Ganoderma applanatum, Meripilus giganteus и *Flammulina velutipes.* Добијени резултати указују на чињеницу да би анализиране гљиве могле наћи значајно место као јаки природни извори антиоксиданата у прехрамбеној и козметичкој индустрији, пошто је за синтетске антиоксиданте доказано да су канцерогени.