SE EFFECT ON BIOLOGICAL ACTIVITY OF FLAMMULINA VELUTIPES

I. MILOVANOVIĆ¹, T. STANOJKOVIC², M. STAĐIĆ¹*, J. VUKOJEVIĆ¹ and A. KNEŽEVIĆ¹
¹University of Belgrade, Faculty of Biology, Takovska 43, 11000 Belgrade, Serbia
²Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia
*Corresponding author: stajicm@bio.bg.ac.rs

ABSTRACT

The goals of the study were evaluation of antioxidant, antifungal and anticancer potential of Flammulina velutipes mycelium ethanol extract and examination of Se effect on those activities. Both Se-amended and non-amended mycelium extracts exhibited significant antioxidant and antifungal potential. Se-enriched extract was more effective against Candida krusei and C. albicans and better DPPH• scavengers than non enriched one. Carriers of the antioxidant activity were phenol compounds. Contrary to antioxidant and antifungal potential, tested extracts were many times weaker cytotoxic agents against HeLa and LS174 cell lines than cis-DDP. Thus, Se-enriched mycelium could be supplement with antioxidant and antifungal capacity.

- Keywords: anticancer activity, antifungal activity, antioxidant activity, Flammulina velutipes, Se-enriched mycelium -
INTRODUCTION

A need for new antimicrobial agents exist for a long time due to the emergence of microorganisms resistance as a result of the uncontrolled usage of commercial antibiotics and antimycotics. Preference is given to natural compounds due to their health-beneficiary and environmentally-friendly effect. Besides diseases caused by microorganisms, nowadays, cancer, diabetes, atherosclerosis, as well as neurodegenerative disorders occur frequently. One of the significant triggers for the mentioned diseases and disorders is oxidative stress (LIMÓN-PACHECO and GONSE-BATT, 2009). Since the capacity of cellular antioxidant defence is insufficient in some cases and synthetic antioxidants could have toxic and mutagenic effects, and as awareness about healthy lifestyle is permanently raised, search for natural antioxidants presents current trend. Nowadays, natural remedies are also favoured for cancer treatment, because of the many side effects of the chemotherapy, such as the same effect on cancer and healthy cells, occurrence of mutations which could pass into future generations, as well as more aggressive metastasis of returned disease.

Based on traditional experience and scientific data, numerous mushroom species represent important resources of antimicrobial, antioxidant, and anticancer agents. Although special attention is given to the species of the genera Ganoderma, Lentinus, Trametes, and Pleurotus, other mushrooms also produce active compounds and their biological activities should be tested (ZHANG et al., 2007; CHEN et al., 2008; SHI et al., 2012). One of them is Flammulina velutipes (Curt. Fr.) Karst. known as Golden needle mushroom, Enoki, Enokidake and Enokitake. This species is a famous edible and medicinal mushroom due to synthesis of numerous bioactive molecules, such as polysaccharides, proteins, sterols etc. Significant bioactivities should be tested (BAO et al., 2010; WANG et al., 2012). The inoculum preparation involved the following steps: (i) inoculation of 100.0 mL of synthetic medium (glucose, 10.0 g/L; NH4NO3, 2.0 g/L; KH2PO4, 1.0 g/L; NaH2PO4 x H2O, 0.4 g/L; MgSO4 x 7H2O, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with 25 mycelial discs (Ø 0.5 cm, from 7-day-old culture from malt agar); (ii) incubation at room temperature (22 ± 2ºC), on a rotary shaker (100 rpm), for 7 days; (iii) washing of obtained biomass (3 times) by sterile distilled water (dH2O); (iv) biomass homogenization with 100.0 mL of sterile dH2O in a laboratory blender.

Homogenized inoculum (30.0 mL) was used for inoculation of 400.0 mL modified synthetic medium (with glucose in the amount of 65.0 g/L and peptone in the concentration of 2.0 g/L, previously determined as the optimal carbon and nitrogen sources and concentrations for biomass production) enriched with sodium selenite (Na2SeO3) in the initial Se concentration of 1.3 mg/L. The medium without Se was used as the control. Submerged cultivation was carried out at room temperature on rotary shaker for 21 days. The obtained biomass was filtered, washed 3 times with dH2O at magnetic stirrer and temperature of 30ºC with the aim of removing the remaining Se from cell wall, and dried at 50ºC to constant weight.

Preparation of the fungal extracts

Dry Se-amended and non-amended mycelia (3.0 g) were extracted by stirring with 90.0 mL of 96% ethanol at the 30ºC for 72 h. The
obtained extracts were filtered through Whatman No. 4 filter paper, concentrated under reduced pressure in a rotary evaporator (BUCHI R-114, Switzerland) at 40°C to dryness, and redissolved in 96% ethanol (for the testing of antioxidant activity) or in 5% dimethylsulphoxide (DMSO) (for the analysis of antifungal and anticancer activity).

Antioxidant activity

DPPH assay

Antioxidant activity was defined by measuring bleaching of the purple-coloured methanol solution of stable 1,1-diphenyl-2-picryl-hydrazil radical (DPPH•) (BLOIS, 1958). 1800.0 µL of a methanol solution of DPPH• and 200.0 µL of extract of defined concentration (series of double dilutions from 32.0 mg/mL to 0.5 mg/mL) was analyzed. Each well contained the reaction reagents except the extract. Scavenging effects was calculated by equation:

\[ \text{DPPH scavenging effect (\%) = } \left[ \frac{A_0 - A_{\text{sample}}}{A_0} \right] \times 100, \]

\( A_0 \) - the absorbance of the negative control; \( A_{\text{sample}} \) - the absorbance of reaction mixture.

The EC50 value (mg extract/mL) is the effective concentration at which the DPPH• were scavenged by 50% and was obtained by interpolation from linear regression analysis. Commercial antioxidant, butylated hydroxyanisole (BHA) was used as a positive control.

Determination of total phenolic content

Total soluble phenolic compounds in the ethanolic extracts of Se-amended and non-amended mycelium were estimated with Folin-Ciocalteu reagent according to the method of SINGLETON and ROSSI (1965), using gallic acid as a standard. 1000.0 µL of 10% Folin-Ciocalteu reagent and 200.0 µL of the extract were reacted in the dark for 6 min before addition of 800.0 µL of 7.5% Na2CO3. The reaction mixture was vortexed vigorously and incubated on a rotary shaker (100 rpm) in the dark and at the room temperature for 2 h. The absorbance was measured at 760 nm by spectrophotometer against blank (mixture without extract). The total concentration of phenolic compounds in tested extracts determined as µg of gallic acid equivalents (GAE) per mg of dry extract, using an equation that was obtained from standard gallic acid graph as:

\[ \text{Absorbance } = 0.012 x \text{ total phenols } + \]
\[ + 0.041 \quad (R^2 = 0.999) \]

Determination of total flavonoid content

Total flavonoid content was determined using the methods of PARK et al. (1997). 1000.0 µL of the mycelium extract was diluted with 4300.0 µL mixture containing 4100.0 µL of 80% ethanol, 100.0 µL of 10% aluminium nitrate and 100.0 µL of 1 M aqueous potassium acetate. The mixture was incubated at room temperature for 40 min, and absorbance was measured spectrophotometrically at 415 nm. The amount of total flavonoids was expressed as µg of quercetine equivalents (QE) per mg of dry extract, using an equation that was obtained from standard quercetin hydrate graph as:

\[ \text{Absorbance } = 0.014 x \text{ total flavonoid } - 0.072 \quad \]
\[ \quad (R^2 = 0.989) \]

Antifungal activity

The tested micromycetes (Table 1) are maintained on Malt agar at 4°C in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

Tested micromycetes were cultivated on Sabouraud dextrose agar (SDA) at temperature of 25 ± 2°C for 21 days. Spore suspensions were prepared by washing of agar surface with sterile 0.9% saline containing 0.1% Tween 80 (v/v). Turbidity was determined spectrophotometrically at 530 nm and spore number was adjusted to 106 CFU/mL (NCCLS, 1998).

DMSO extracts of Se-amended and non-amended mycelia were sterilized by filtration through Whatman No. 4 filter paper and 0.2 µm membrane filter. Antifungal potential of the tested extracts was studied by microdilution method using 96-well microtiter plate (SARKER et al., 2007). Series of double extract dilutions (from 32.0 mg/mL to 0.5 mg/mL) was analyzed. Each well contained SDA, spore suspension, resazurine, and crude ethanol extract of defined concentration. The mixture without extract was used as the negative control, while positive control contained commercial antifungal, ketoconazole, instead extract. Tested ketoconazole concentrations ranged from 0.0313 mg/mL to 0.0019 mg/mL (series of double dilutions). Effect of 5% DMSO on the spore germination was also analysed by its addition in the mixture instead SDA. Microtiter plates were incubated at 25 ± 2°C for 72 h. The lowest extract concentration without visible mycelium growth was defined as minimal inhibitory concentration (MIC). Minimal fungicidal concentration (MFC) was determined as the lowest extract concentration with no mycelial growth after reinoculation of 2 µL of the mixture on SDA. The experiments were repeated three times.

Cytotoxic activity

Cell lines

Human cervix adenocarcinoma HeLa and human colon carcinoma LS174 cell lines were ob-
tained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100.0 g/L heat-inactivated (56°C) fetal bovine serum (FBS), 3 mM L-glutamine, 100.0 mg/mL streptomycin, 100.0 IU/mL penicillin, and 25 mM 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and adjusted to pH 7.2 with bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air/5% CO2 (v/v) at 37°C.

Treatment of cell lines

Stock solutions (100.0 mg/mL) of extracts, made in 50.0 g/L DMSO, were dissolved in enriched RPMI 1640 medium to the required working concentrations. Neoplastic HeLa cells (2000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates. 24 h later, after cell adherence, five different doubly diluted concentrations of the extracts were added to the wells. The final concentrations applied to target cells were 200.0, 100.0, 50.0, 25.0 and 12.5 µg/mL, except in the control wells where only the nutrient medium was added to the cells. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of extracts on cancer cell survival was determined by microculture tetrazolium test (MTT test), according to MOSMANN (1983) with modification by OHNO and ABE (1991). 20.0 µL of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffering saline] of concentration of 5.0 mg/mL was added to each well. Samples were incubated for 4 h at 37°C in a humidified atmosphere of 95% air/5% CO2 (v/v). Then, 100.0 µL of 10% sodium dodecyl sulfate was added to extract to dissolve the insoluble product formazan resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the light absorbance (A), which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm 24 h later. The inhibition rate was calculated according to the formula:

\[
\text{Cell growth inhibition rate} (\%) = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100
\]

It was implied that the A of the blank was always subtracted from the A of the corresponding sample with target cells. IC50 was defined as the concentration of the extracts inhibiting cell survival by 50%, compared with a vehicle-treated control. Cis-diamminedichloroplatinum (cis-DDP) was used as a positive control. All experiments were done in triplicate.

Statistical analysis

The results were expressed as the mean ± standard error of data obtained from three parallel measurements. One-way analysis of variance (ANOVA) followed by LSD post-hoc determinations were performed using STATISTIKA software, version 5.0 (StatSoft, Inc) to test any significant differences. P-values less than 0.01 were considered statistically significant.

Table 1 - Antifungal activity (MIC and MFC) of ethanol extracts of Se-non amended and Se-amended mycelium of Flammulina velutipes and commercial antimycotic.

<table>
<thead>
<tr>
<th>Tested organisms</th>
<th>Flammulina velutipes</th>
<th>Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/mL)</td>
<td>MFC (mg/mL)</td>
</tr>
<tr>
<td></td>
<td>Se non-amended mycelium</td>
<td>Se-amended mycelium</td>
</tr>
<tr>
<td>Acremonium strictum W. Gams</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Aspergillus flavus Link</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Aspergillus fumigatus Fresen.</td>
<td>32.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Aspergillus niger Teigh.</td>
<td>32.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Aspergillus terreus Thom</td>
<td>16.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Candida albicans (C.P Robin) Berkhout</td>
<td>8.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Candida kruisi (Castell.) Berkhou</td>
<td>8.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Candida parapsilosis (Ashford) Langeron &amp; Talice</td>
<td>16.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fusarium verticillioides (Sacc.) Nirenberg</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Microsporum gypseum (E.Bodin) Guiart &amp; Grigoraki</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penicillium funiculosum Thom</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Trichoderma viride Pers.</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes (C.P Robin) Sabour.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
RESULTS

Antioxidant activity

Ethanol extracts of both Se-amended and non-amended *F. velutipes* mycelium showed good antioxidant potential that was dependent on the concentration, at higher concentrations extracts were more effective in DPPH• scavenging (Fig. 1). However, significant difference between the extracts, especially at higher concentrations, was noted (P<0.01). Se-enriched mycelium extract had higher scavenging effect than the control, and it exhibited a progressive increase of the activity at concentrations up to 8.0 mg/mL (from 4% to even 15% at concentration of 32.0 mg/mL). These results were confirmed by EC50 values, which were 30.5 ± 0.3 mg/mL in Se-enriched mycelium extract and 43.8 ± 0.6 mg/mL in non-amended one. Commercial antioxidant BHA was more efficient comparing with *F. velutipes* mycelium extracts, with EC50 value of 13.4 µg/mL.

Total phenol compounds were detected in both tested extracts. Their content in the non-amended extract was 9.5 ± 1.8 µg/mg, while the higher concentration of 14.5 ± 1.1 µg/mg was noted in Se-amended extract. However, flavonoids were not detected in any of the extracts. Direct correlation between phenol content and DPPH• scavenging effect existed, and linear relationship with R² = 0.989 in non-amended mycelium extract and R² = 0.957 in Se-amended one were noted.

Antifungal activity

The antifungal potential of ethanol extracts of Se-amended and non-amended mycelium was tested against 14 micromycetes including saprobes as well as plant, animal and human pathogens. In the most cases, MICs of Se-enriched mycelium extract were lower, except for *Candida albicans* where control extract had twice more effect, and for *Acremonium strictum* and *Trichoderma viride* where MICs of Se-amended and non-amended extracts were the same (Table 1). The most sensitive species, with MIC of Se-enriched mycelium extract of 2.0 mg/mL, were *C. krusi* and *C. parapsilosis*. The most resistant species were *Aspergillus fumigatus* and *A. niger*, which growth was inhibited only with the maximum extract concentration (32.0 mg/mL for non-amended and 16.0 mg/mL for Se-amended one). Tested extract concentrations (from 0.5 mg/mL to 32.0 mg/mL) have no inhibitory effect on *Cladosporium* sp. and both causal agents of dermatomycosis, *Microsporum gypseum* and *Trichophyton mentagrophytes*. The maximum tested concentration of extract (32.0 mg/mL) did not show fungicidal effect for any tested fungal species (Table 1).

Sensitivity of the tested species to commercial antymycotic, ketoconazole, was more higher. Thus, the lowest tested concentration of 0.0019 mg/mL was MIC for *Cladosporium* sp., *M. gypseum*, and *T. mentagrophytes*. The concentration of 0.0039 mg/mL was MIC for *Penicillium funiculosum* and *T. viride* and MFC for *Cladosporium* sp., *M. gypseum*, and *T. mentagrophytes*. Mycelium growth of *A. strictum*, *A. flavus*, *A. fumigatus*, *A. albicans*, *C. krusi*, and *C. parapsilosis* was inhibited at ketoconazole concentration of 0.0078 mg/mL, which was also MFC for *A. flavus*, *P. funiculosum* and *T. viride*. Concentration of 0.0156 mg/mL was MIC for *A. niger*, *A. terreus*, and *Fusarium verticillioides* and MFC for most of the tested species, while the highest tested concentra-
tion (0.0313 mg/mL) was MFC for *A. niger* and *A. terreus* (Table 1). 5% DMSO, used as a negative control, had no inhibitory effect on the tested micromycetes.

**Cytotoxic activity**

Comparing with cis-DDP, which was used as the positive control, the tested extracts showed low cytotoxic activity against both HeLa and LS174 cell line (Table 2). IC$_{50}$ of non-amended mycelium extract against HeLa cells was 360- and 130-fold, respectively, higher than values obtained for cis-DDP. Se-amended mycelium extract was weaker cytotoxic agent than control one, especially for HeLa cell line.

**DISCUSSION**

Enrichment of cultivation medium with Se in the initial concentration of 1.3 mg/L and ability of *F. velutipes* HAI 966 mycelium to absorb and incorporate it in significant amount (10.0 µg/g) (MILOVANOVIC *et al.*, 2013) led to enhancement of antioxidant and antifungal capacity, while cytotoxic activity was reduced and was not in correlation with antioxidant potential.

DPPH• scavenging activity of *F. velutipes* ethanol extracts was reported by BAO *et al.* (2010), who demonstrated higher efficiency of mycelium extract than fruiting body one in almost 4-fold. According to SALTARELLI *et al.* (2009), the activity bases on redox properties of phenols, which enable them to act as reducing agents and hydrogen donors. Direct dependence of antioxidant capacity on phenol content was confirmed by CHEN *et al.* (2008) and BAO *et al.* (2010). Lower phenol content in *F. velutipes* HAI 966 mycelium extracts, compared with that in reported data, was responsible for negligible antioxidant potential that was significant only at the highest concentration. Lack of flavonoid in the ethanol extracts was in accordance with results of KARAMAN *et al.* (2009) who also have not found those compounds in mycelium extracts of *F. velutipes*. However, other compounds from those extracts could also be carriers of antioxidant activity (SHI *et al.*, 2012; WANG *et al.*, 2012). Thus, SHI *et al.* (2012) demonstrated significant scavenging effects of polysaccharides, even 90% at concentration of 2.5 mg/mL, while WANG *et al.* (2012) have noted high efficiency of sesquiterpenoids.

Enhanced antioxidant potential of Se-amended *F. velutipes* HAI 966 mycelium extract is in accordance with results of TURLO *et al.* (2010) who noted higher level of antioxidant activity in *Lentinus edodes* after cultivation in Se-enriched medium. The better effect could be explained by the fact that selenomolecules react with free radicals (•OH, •H) and successfully neutralize them (SHEN *et al.*, 2010).

WANG *et al.* (2012) tested antifungal potential of sesquiterpenoids isolated from *F. velutipes* mycelium against numerous species and reported weak activity against *A. fumigatus* and absence of any inhibitory effect on *C. albicans*. Similar, negligible activity of ethanol extract of *F. velutipes* HAI 966 mycelium against *A. fumigatus* was also noted but it was enhanced with Se addition. However, tested *Candida* spp. were very sensitive to the extracts, especially in the Se presence, which was contrary to the results of WANG *et al.* (2012). Results of this study showed that Se acts as an antifungal agent which is in accordance with results of SHAHVERDI *et al.* (2010) who clearly demonstrated antifungal activity of biogenic Se against selected clinical micromycetes. Mycelium enrichment with this element presents a way for getting biogenic Se nanoparticles that could be a potent ingredient for the preparation of antifungal formulations (SHAHERVERDI *et al.*, 2010).

Contrary to Se stimulatory effect on antioxidant activity, this trace element caused regression of cytotoxic activity. Numerous biologically active compounds, such as diverse types of sesquiterpenes, polysaccharides, glycoproteins, ribosome inactivating proteins, and sterols, isolated from mycelium of *F. velutipes*, take important place as cytotoxic agents against various cancer cell lines (LEUNG *et al.*, 1997; NG and WANG, 2004; WANG *et al.*, 2012; YANG *et al.*, 2012; YI *et al.*, 2013). LEUNG *et al.* (1997) showed that soluble homopolysaccharide, composed of glucose and isolated from *F. velutipes* fruiting bodies, were very efficient in Sarcoma-180 cells regression in vivo. The same effect was noted by YANG *et al.* (2012) for alkaline-soluble heteropolysaccharides with a glucan as backbone chain and triple helix structure, which also have strong anti-proliferation activity against lung cancer cells (A549) and human gastric cancer cells (BGC-823) (inhibitory rate was 32.3% and 95%, respectively). The cytotoxic effect of polysaccharides was based on proliferation of B-cell, T-cell or both cells and not on tumor cell kill. Activation of immune system and in such a way production of interferon-gamma with antiproliferate effect on tumor cells was also lied in the base of antitumor activity of *F. velutipes* glycoprotein FVE (CHANG *et al.*, 2010). However, ribosome inactivating proteins (flammulin, velutin, flammmin, and velin) stopped cancer cell proliferation by ribosome inactivation and translation inhibition (NG and WANG, 2004). Efficiency of sesquiterpenoids against human liver carcinoma cell line (HepG2), breast cancer cell line (MCF-7), human gastric cancer cell line (SGC7901) and A549 was modest (IC$_{50}$ was in range between 20.0 and 100.0 µM (WANG *et al.*, 2012), while inhibition rate of human glioma cell line (U251) by sterols, at concentration of 20 µg/mL, was even 57% (YI *et al.*, 2013). However, the sterol was not so efficient against HeLa cell line (IC$_{50}$ > 40.0 µg/mL), but more efficient comparing with tested Se-amended and non-amended mycelium extract.

Although tested extracts showed lower biolog-
ically activities than commercial antymycotic, antioxi-
dant and cytostatic, preference should be given to natural products. Therefore, *F. velutipes* is not only food but also could be considered as a possible base for natural safe remedies for human therapy.

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