Antiviral activities of *Boletus edulis, Pleurotus ostreatus* and *Lentinus edodes* extracts and polysaccharide fractions against Herpes simplex virus type 1

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Summary

Antiviral properties of water and methanol extracts from *Lentinus edodes*, *Boletus edulis* and *Pleurotus ostreatus* were evaluated against herpes simplex virus type 1 (HSV-1). Pre-treatment of Vero cells with 75 mg·ml^{-1} water extracts before virus addition, inhibited 60% of the virus infection and its addition during the virus adsorption period inhibited it up to 80%. Moreover, the mushrooms water extracts were able to significantly inhibit the in vitro virus replication, showing the concentration of a substance required to reduce plaque number in Vero cells by 50% (IC_{50}) values from 26.69 mg·ml^{-1} to 35.12 mg·ml^{-1}. Methanol extracts exhibited a lower antiviral activity in all cases. In order to identify the type of compounds responsible for the antiviral activity, the low molecular weight (LMW) and high molecular weight (HMW) fractions were evaluated. The activity was found to correlate with the β-glucans present in the polysaccharide fractions, which showed higher antiviral activity than the complete water extracts except for *B. edulis* where other compounds (probably chitin-binding lectins) might have contributed to the observed activity.

Keywords

edible mushroom; antiviral activity; herpes simplex virus type 1; water extract; methanol extract; chitin, β-glucan

Herpes simplex virus type 1 (HSV-1) is a highly prevalent pathogen among children and adults, causing primary infections that are presented clinically as herpes labialis or as primary gingivostomatitis. The virus is also able to establish a latent infection in the nervous system that can be frequently reactivated [1]. Major therapeutic agents for HSV infections are nucleoside analogues such as acyclovir and vidarabine. However, the increased and prolonged use of these compounds, in particular in immunocompromised patients, has led to viral resistance against most of these drugs [2, 3]. The drug-resistant HSV retain their pathogenicity and may be associated with progressive and relapsing disease. Thus, it is necessary to explore and discover novel potential antiherpetic approaches.

Many edible mushrooms were described in the traditional folklore of the Asian culture as medicinal remedies towards a variety of disorders and diseases. Recent studies, with a better scientific background, confirm the presence of specific secondary metabolites, which are bioactive compounds devoid of undesirable side effects. Mushroom extracts were reported to have antibacterial, hematological, antiviral, antitumor, hypotensive and hepatoprotective effects [4, 5]. The fractions showing antiviral properties were extracted from both mycelia and fruiting bodies. The isolated active compounds appeared to play a direct role acting as inhibitors of viral enzymes, synthesis of viral nucleic acids or absorption and uptake of viruses into mammalian cells. The direct antiviral effects were exhibited in particular by small molecules and the indirect antiviral effects were the results of the activity of polysaccharides or other complex molecules [6].

Several triterpenes from *Ganodema lucidum* were reported as active agents against human immunodeficiency virus type (HIV-1) [7]. Ganoder-

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madiol, lucidadiol and applanoxidic acid G, isolated from *G. pfeifferi* and other *Ganodema* strains, showed in vitro antiviral activity against influenza virus type A. Moreover, ganodermadiol was active against HSV-1 [8]. Several neutral or acidic protein-bound polysaccharides isolated from water extracts of *G. lucidum* fruiting bodies showed inhibitory activities against HSV-1 and HSV-2 [9]. In vitro antiviral activity against influenza viruses type A and B was demonstrated for two isolated phenolic compounds obtained from the fruiting body of the basidiomycete *Inonotus hispidus* [10]. Furthermore, water-soluble lignins isolated from *Inonotus obliquus* were also reported to inhibit HIV protease [11]. Anti-HIV and anti-human immunodeficiency virus activities were described for a water-soluble lignin (EP3) presented in the mycelial culture medium of *Lentinus edodes* [12, 13]. Moreover, sulphated lentinan from *L. edodes* completely prevented HIV-induced cytopathic effect [14] and, administrated to HIV positive patients together with didanosine, was a more efficient immunomodulating treatment than the drug by itself [15].

The protein-bound polysaccharides PSK and PSP from *Coriolus versicolor* showed also in vitro antiviral effect on HIV and cytomegalovirus [16]. Velutin, a ribosome-inactivating protein from *Fammulina velutipes*, caused the inhibition of HIV-1 reverse transcriptase [17]. Other saccharide-binding proteins, such as lectins from *Boletus edulis* and *Pleurotus citrinopileatus*, showed high antiviral activities. The *P. citrinopileatus* lectin was a homodimeric 32.4 kDa protein able to inhibit the HIV-1 reverse transcriptase with the concentration of the substance required to reduce the plaque number in Vero cells by 50% (IC_{50}) being $0.3\,\mu\text{mol}\cdot\text{l}^{-1}$ [18, 19].

The present work studies the antiviral activity against herpes virus type 1 of water and methanol extracts, and their polysaccharide fractions, obtained from the fruiting bodies of three edible mushrooms commonly consumed in European countries. The responsible compounds and their potential mechanism of action are also postulated.

MATERIALS AND METHODS

Biological material and samples preparation

Mushroom strains used in this investigation were *Boletus edulis* (Fries/Bull.), *Lentinus edodes* (Berkeley) and *Pleurotus ostreatus* (Imbach). Fresh fruit bodies were purchased at a local supermarket in Spain.

Mushrooms were cut to small pieces, frozen at −20 °C and freeze-dried. Dehydrated material was homogenized in a mill (JR MF10basic; IKA Labortechnik, Staufel, Germany) and sieved (Orto Alresa, Madrid, Spain) until particle size was smaller than 0.3 mm. Mushroom powder was stored at −20 °C for further analysis. Samples were prepared in duplicate.

Methanol extracts were prepared by mixing the mushroom powders (10 mg) with $250\,\mu\text{l}$ of methanol. Suspensions were shaken on a Vortex mixer during 2 min and centrifuged (8 854 ×*g* for 2 min). Supernatants were collected and used in the assays.

Water extracts were obtained by mixing the mushroom powders (10 mg) with $250\,\mu\text{l}$ of Milli-Q water (Millipore, Billerica, Massachusetts, USA) and treating them as described for methanol extracts. Resulting aqueous supernatants were immediately used for the antiviral assays or separated into a high molecular weight (HMW) or low molecular weight (LMW) fractions as described later.

Cells and viruses

Vero cells (African green monkey kidney cell line) were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). They were used as host for HSV-1. The cells were grown using Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer 1 mol·l⁻¹, 1% non-essential amino acids and 1% L-glutamine (all from Life Technologies, Madrid, Spain). Maintenance medium for Vero cells was as described above but with 2% FBS.

Herpes virus simplex type 1 (HSV-1; KOS, a thymidine kinase-positive and acyclovir-sensitive reference HSV-1 strain, ATCC VR 1493) was obtained from ATCC, prepared in aliquots and stored at −80 °C until use. Virus titer was determined by plaque reduction assay in Vero cells and expressed as plaque forming units (PFU) per millilitre. An acyclovir (Sigma, Barcelona, Spain) aqueous solution was used as positive control against HSV-1 replication.

Cytotoxicity assay

The cytotoxic effect of the different extracts on Vero cells was tested using MTT assay, according to a published method [20]. The compund 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) is a yellow water-soluble tetrazolium dye that is reduced by live cells, but not by dead cells, to a purple formazan product

that is insoluble in aqueous solutions. Monolayers of Vero cells in 24-multiwell plates were incubated with MEM containing different concentrations of the mushroom extracts for 48 h at 37 °C. Cells were then washed with phosphate-buffered saline (PBS), 0.5 mg·ml⁻¹ of MTT were added to each well and incubated 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulphate in a mixture of dimethyl formamide and water 1:1, v/v, adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise; Tecan, Crailsheim, Germany) with the extraction solution as a blank. The data were plotted as dose-response curves, from which the concentration required to reduce the number of viable Vero cells by 50% (CC_{50}) after 48 h of incubation with the different extracts were obtained.

Evaluation of virucidal activity

Virus samples containing 10^5 PFU·ml⁻¹ were mixed and incubated at 37 °C for 1 h with MEM containing different extract concentrations or MEM alone (control). Samples were then diluted and used to infect confluent Vero cells for 1 h at 37 °C. After incubation, the virus inocula were removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed with acetone:methanol (50:50) at 4 °C, stained with a 1% solution of crystal violet and the number of the plaques counted. The percentage of inhibition of plaque formation (*IPC*) was calculated as follows:

$$IPF = \frac{(PC - PT)}{PC} \times 100 \qquad (1)$$

where *PC* is mean number of plaques in control and *PT* is mean number of plaques in test.

Influence of various treatment periods on the anti-HSV-1 activity of the extracts

Vero cells and viruses were incubated with the extracts at different stages during the viral infection cycle in order to determine the mode of antiviral action.

1. Cells pre-treatment

Monolayers of Vero cells in 24-multiwell plates were pre-treated with MEM containing different concentrations of the extracts for 3 h at 37 °C. Cells were then washed with PBS and infected with 120 PFU of HSV-1. After incubation for 1 h at 37 °C, the virus inocula were removed, the cells

washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed, stained and the number of the plaques counted. Control consisted of untreated cells infected with HSV-1.

2. Adsorption period

Cells were infected with 120 PFU of HSV-1 in presence of different concentrations of the extracts for 1 h at 37 °C. Then, the virus inocula and the extract were removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) for 48 h at 37 °C. The infected cells were fixed, stained and the number of plaques counted. Control consisted of cells infected without the extract.

3. Intracelullar replication

Cells were infected with 120 PFU of HSV-1. After incubation for 1 h at 37 °C, the virus inocula were removed, the cells washed with PBS and then overlaid with maintenance medium (with 0.5% agarose) containing different concentrations of the extracts or only medium (control). After incubation for 48 h at 37 °C, the infected cells were fixed, stained and the number of plaques counted.

The concentration of a substance required to reduce plaque number in Vero cells by 50% (IC_{50}) as compared to control, was calculated from the dose-response curves generated from the data.

Isolation of LMW and HMW fractions and determination of total phenol and peptides

Mushroom water extracts were submitted to filtration using Microcon filters (Millipore) with a cut-off value of 10 000 Da and a microcentrifuge (8 854 ×g). The filtrate was considered as LMW fraction and the concentrated fraction retained in the filter as HMW fraction after mixing of the fraction with the same volume of water.

The total phenol concentration was determined by the Folin-Ciocalteu method according to the procedure of RAMIREZ-ANGUIANO et al. [21]. The amount of peptides and polypeptides present in LMW aqueous extracts (or proteins in the HMW fractions) was determined using a commercial assay (Sigma) based on the BRADFORD method [22]. Gallic acid and bovine serum albumin BSA were used as standards for phenol and protein quantification, respectively.

Isolation of polysaccharide fractions

In order to isolate only the polysaccharides present in the water extract, mushroom powders (1 g) were mixed with sea sand (4 g) and submitted

to accelerated solvent extraction (ASE) at room temperature during 25 min (5 cycles of 5 min, GIL-RAMÍREZ, A. et al. [23]). Extracted fractions were cooled down to 4 °C and mixed with two volumes of cold ethanol under vigorous stirring allowing polysaccharides precipitation, and maintained at 4 °C overnight.

Total polysaccharides present in the mushrooms were also isolated according to JEURINK et al. [24]. Briefly, the mushroom powders were mixed with distilled water at 120 °C (1 g/100 ml) for 20 min and cooled down to 4 °C. Polysaccharides were precipitated by adding two volumes of cold ethanol, vigorous stirring, and allowing polysaccharide precipitation overnight at 4 °C.

The obtained precipitates (from the water extracts and from the complete mushroom) were collected by centrifugation (10 000 ×*g* for 20 min at 4 °C), re-dissolved in distilled water and the entire precipitation procedure was repeated. The precipitates were dialysed with Spectra/Por 3 molecular-porous membrane tubing (MWCO: 3500; Spectrum Medical Industries, Houston, Texas, USA) against distilled water to remove compounds of low molecular weight during at least 24 h with three or four changes of the distilled water. After the dialysis, the polysaccharides remaining in the dialysis tube were collected and freeze-dried. The freeze-dried polysaccharides were weighed and stored at –20 °C until further use.

Determination of total saccharide content

The polysaccharide extracts were analysed for their total saccharide content with the modified phenol-sulphuric acid method described by FOX and ROBYT [25]. Test solutions (25 μl) or standards of known glucose concentration with 25 μl of 5% (w/v) phenol were added to a microtube. The microtube contents were stirred in a vortex for 30 s, placed on crushed ice, added 125 μl of concentrated H_2SO_4 and heated in a water bath at 80 °C for 30 min. Afterwards, absorbance at 490 nm was determined (Evolution 600; Thermosceintific, Horsham, United Kingdom).

Determination of α- and β- glucans

The β-glucan content of the obtained polysaccharides (PSC) extracts (50 mg) was determined using a β-glucan determination kit specific for mushrooms and yeasts (Megazyme, Barcelona, Spain) following the instructions of the user's manual. Absorbance at 510 nm was measured the spectrophotometer. The procedure calculates the β-glucan content directly from mushroom powder by subtracting the values obtained from a first assay in which the content of total glucans

(α-glucan + β-glucan), D-glucose in oligosaccharides, saccharose and free D-glucose are quantified, to a second assay, in which the content of α-glucans (glycogen and starch), D-glucose in saccharose and free D-glucose are quantified. Therefore, if these assays are carried out using the HMW fractions obtained after dialysis (polysaccharide fraction), mono-, di- and oligosaccharides as well as broken or small polysaccharides are lacking. Thus, the values obtained with the first assay should be due to the total glucans and in the second assay to α-glucans. Therefore, in these types of samples water-insoluble total, α- and β-glucans of a molecular weight higher than 3.5 kDa can be quantified.

Determination of chitin

Chitin determinations were carried out according to VETTER [26] following an adapted method of SMITH and GILKERSON [27]. Briefly, PSC extracts (20 mg) were mixed with 2.5 ml of 6 mol·l⁻¹ HCl in tubes and incubated in oil bath at 106 °C during 24 h. Hydrolysed samples were centrifuged at 1 420 ×*g* for 60 min. Supernatants (50 μl) were brought to a final volume of 200 μl with 6 mol·l⁻¹ HCl and mixed with 400 μl of 2.5% $NaNO_2$, shaken in a vortex and allowed to stand at room temperature for 15 min. Afterwards, 12.5% ammonium sulfamate (200 μl) was added. The mixture was shaken and incubated at room temperature for 5 min. After the liberation of the excess of $NaNO_2$, 0.25% 3-methyl-2-benzothiozolinone hydrazine hydrochloride hydrate (MBTH; 200 μl) was added, shaken once more and incubated at 37 °C for 30 min. After incubation, 0.5% $FeCl_3$ (200 μl) was added and further incubated at 37 °C during 5 min. Samples were cooled down to room temperature and the absorbance at 650 nm was measured by the spectrophotometer.

Statistical analysis

One way analysis of variance (ANOVA) was performed using a Statgraphics Plus 3.1 software (Statistical Graphics, Rockville, Maryland, USA). Fisher's least significant differences procedure (LSD) was used as the mean comparison test.

RESULTS AND DISCUSION

Cytotoxicity of mushrooms extracts

The water and methanol extracts obtained from *L. edodes*, *B. edulis* and *P. ostreatus* were initially evaluated for cytotoxicity on pre-formed monolayers of Vero cells by MTT method. The CC_{50} data obtained (Tab. 1) indicated that metha-

Tab. 1. Antiviral activities of edible mushroom extracts against herpes simplex virus type 1.

Mushroom	Sample	CC_{50} [μg·ml^{-1}]	IC_{50} [μg·ml^{-1}]	SI
L. edodes	Water extract	384.15 ± 12.26[a]	27.04 ± 1.18[a]	14.21
	Methanol extract	791.37 ± 21.26[b]	96.44 ± 8.26[b]	8.2
	Polysaccharide fraction	787.83 ± 15.41[b]	4.70 ± 0.48[c]	167.62
B. edulis	Water extract	490.42 ± 13.78[a]	35.12 ± 2.06[a]	13.96
	Methanol extract	1 142.56 ± 27.45[b]	86.17 ± 9.26[b]	13.25
	Polysaccharide fraction	851.47 ± 17.15[c]	5.96 ± 0.61[d]	142.86
P. ostreatus	Water extract	404.42 ± 15.36[a]	26.69 ± 1.87[a]	15.15
	Methanol extract	1 004.21 ± 31.04[b]	94.24 ± 7.26[b]	10.65
	Polysaccharide fraction	784.24 ± 13.72[c]	4.80 ± 0.39[c]	163.38
L. edodes + B. edulis	Polysaccharide fraction	834.25 ± 12.58[c]	5.90 ± 0.54[d]	141.40
L. edodes + P. ostreatus	Polysaccharide fraction	792.34 ± 14.58[b]	4.53 ± 0.41[c]	174.91
B. edulis + P. ostreatus	Polysaccharide fraction	798.75 ± 20.14[b]	6.02 ± 0.59[d]	132.68
L. edodes + B. edulis + P. ostreatus	Polysaccharide fraction	801.19 ± 18.58[b]	5.74 ± 0.46[d]	139.58
Acyclovir	Water solution	678.36 ± 14.58[d]	0.20 ± 0.03[e]	3 391.8

Values are the mean ± standard deviation of three separate experiments.
a, b, c, d, e – denotes statistically significant differences ($p < 0.05$) between samples extracts from the same mushroom.
CC_{50} – (cytotoxic concentration 50%) concentration required to reduce 50% the number of viable Vero cells after 48 h incubation with the extracts; IC_{50} – (effective concentration 50%) concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of four determinations ± standard deviation; SI – selectivity index, ratio CC_{50}/IC_{50}.
Vero cells were infected with HSV-1 (120 PFU) and incubated at 37 °C for 1h. The unabsorbed viruses were removed and the cells washed with PBS. The infected cells were further incubated in the presence of different extracts concentrations at 37 °C for 48 h. The cells were then fixed, stained and the number of plaques counted.

nol extracts showed a lower cytotoxicity than the water extracts. Among the different mushrooms, *Lentinus edodes* extracts presented the highest toxicity.

Virucidal activity of mushroom extracts

In order to investigate the direct inhibitory effect of the mushroom extracts against HSV-1, a virus suspension was treated at 37 °C for 1 h with different concentrations of the extracts. Pre-incubation of HSV-1 with mushroom extracts resulted in dose-dependent reduction of remaining virus infectivity when compared with the untreated control (Fig. 1). However, the concentrations with 50% virucidal activities against the virus were higher than 3.75 mg·ml^{-1} for water extracts and 18.75 mg·ml^{-1} for methanol extracts. These results indicated that methanol extracts almost lacked extracellular virucidal activity, and water extracts provoked only very low direct effects. *Lentinus edodes* water extracts showed higher virucidal effects than the water extracts from the other mushrooms. When *L. edodes* water extracts were applied at 3.75 mg·ml^{-1}, they were able to provoke 50% inhibition, while *B. edulis* and *P. ostreatus* extracts at similar concentrations only reached 25% and 15% inhibition, respectively.

Influence of various treatment periods on the anti-HSV-1 activity of the extracts

Vero cells were pre-treated for 3 h with different concentrations of the methanol and water extracts. Afterwards, extracts were removed, cells washed and infected with the HSV-1 virus. Results suggested that in cells pre-treated with 75 μg·ml^{-1} and 100 μg·ml^{-1} water extracts, the virus infection was inhibited by approx. 60% and 90%, respectively (Fig. 2). However, 100 μg·ml^{-1} methanol extracts reduced the virus infectivity only by 50% to 40%. The inhibition rates were similar among the three studied mushrooms species. These data suggested that mushroom extracts interfered with the HSV-1 infection process at the initial infection steps perhaps by blocking virus attachment or adsorption to Vero cells.

In order to investigate the influence of extracts on virus adsorption, cells were infected with HSV-1 in presence of different concentrations of the extracts for 1 h at 37 °C. Then, the virus inocula and the extract were removed, the cells washed with PBS and maintained for 48 h at 37 °C. Addition of 50 μg·ml^{-1} and 75 μg·ml^{-1} water extracts reduced the virus infectivity by 60% and 80%, respectively (Fig. 3). In that case, methanol extracts were less effective than the water ones.
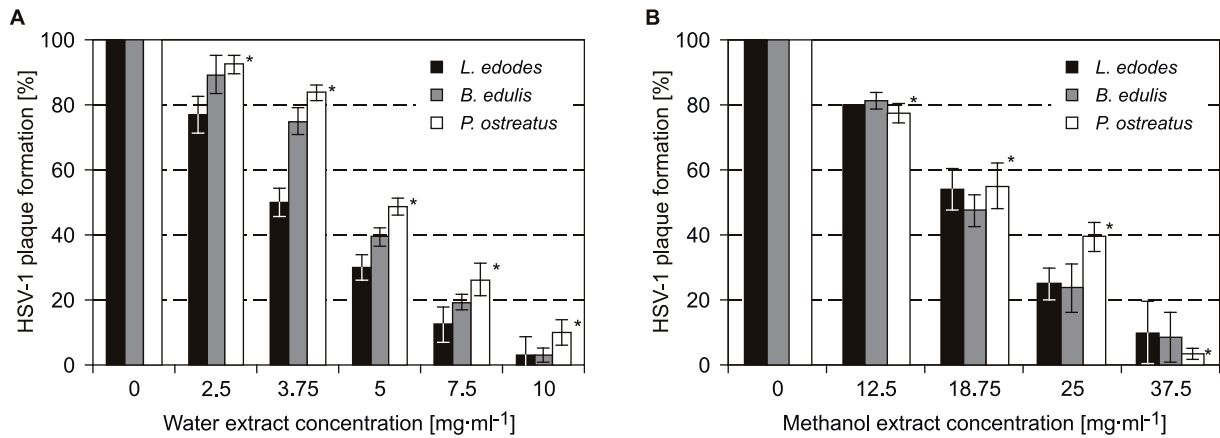
Fig. 1. Virucidal effect of water and methanol extracts obtained from edible mushrooms against HSV-1.

Each bar is the mean of four determinations ± standard deviation. * – denotes statistically significant differences ($p < 0.05$) between control assays (without extract supplementation) and those carried out in the presence of the extracts.
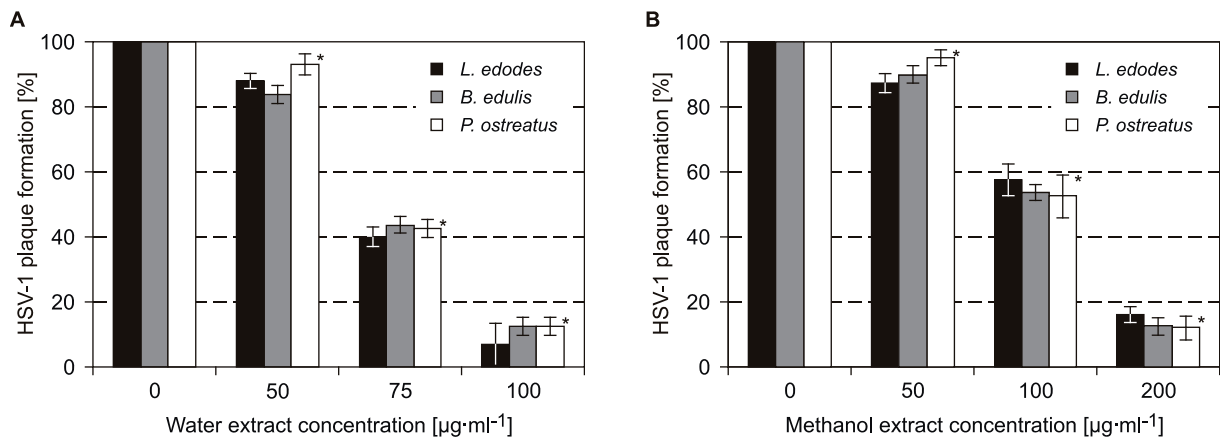

Fig. 2. Effect of cell pre-treatment with water and methanol extracts from edible mushrooms on HSV-1 infectivity.

Each bar is the mean of four determinations ± standard deviation. * – denotes statistically significant differences ($p < 0.05$) between control assays (without extract supplementation) and those carried out in the presence of the extracts.
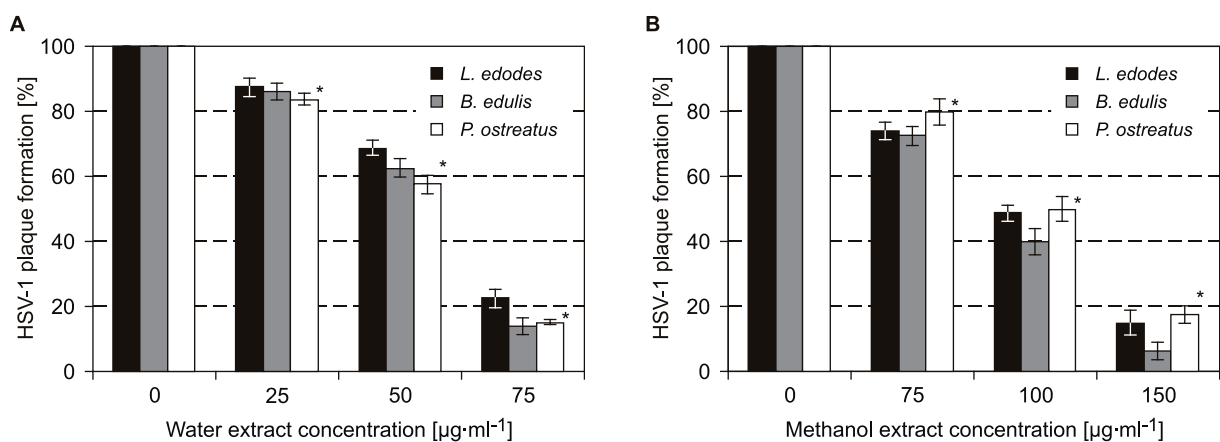

Fig. 3. Effect of water extracts and methanol extracts from edible mushrooms during HSV-1 adsorption period.

Each bar is the mean of four determinations ± standard deviation. * – denotes statistically significant differences ($p < 0.05$) between control assays (without extract supplementation) and those carried out in the presence of the extracts.

Comparing data obtained during the adsorption stage with those obtained in the pre-treatment step, when water extracts were applied at 75 μg·ml^{-1}, at cell pre-treatment the infectivity was reduced by 60%, while the same concentration added during the adsorption period lead to a 80% reduction. These results indicate that mushroom water extracts affect mainly the virus adsorption step.

The antiviral activity of the extracts on the intracellular replication of the virus was evaluated by adding different concentrations of the mushroom extracts to previously HSV-1 infected Vero cells, and incubation for 48 h at 37 °C. Both water and methanol mushroom extracts showed a dose-dependent inhibition of virus replication. The water extracts were more efficient against HSV-1 replication than the methanol extracts, showing IC_{50} values ranging from 26.69 μg·ml^{-1} to 35.12 μg·ml^{-1} (Tab. 1). *P. ostreatus* and *L. edodes* water extracts showed a potent inhibition effect against HSV-1 replication with the lowest IC_{50} values of the studied species. Comparing these data with those obtained during the adsorption period, for the same water extract concentration (25 μg·ml^{-1}), it can be seen that this concentration reduced the virus infectivity by 20% when added during adsorption period, and by 50% when added after virus infection. Thus, mushroom extracts mainly inhibited HSV-1 intracellular replication, although they were also able to disrupt the virus adsorption step. Other mushroom strains, such as *Ganoderma lucidum*, showed higher IC_{50} values (300 μg·ml^{-1}) indicating that the species might be an interesting source of antiviral compounds [28].

The antiviral activities of edible mushrooms have been usually ascribed to the effects of water extracts and frequently related to the presence of water-soluble polysaccharides. ZHANG et al. [29] reported the antiviral activity against HSV-1 of β-glucans isolated from the sclerotium of *Pleurotus tuber-regium* and KIM et al. [30] reported the antiherpetic activity of the acidic protein-bound polysaccharide isolated from water-soluble extracts from *G. Lucidum* fruiting bodies. Also, sulphates of a β-(1→3)-D-glucan from *Lentinus edodes* (lentinan) was reported to possess a considerable anti-HSV-1 activity [14]. However, other type of compounds have also been identified as responsible for the antiviral activities. For instance, two phenolic compounds were isolated from *Inonotus hispidus* fruiting bodies with a remarkable activity against influenza viruses type A and B [10], a peptide with HIV-1 reverse transcriptase inhibitory activity was isolated from *Russula paludosa* [31] and three triterpenes from *Ganoderma pfeifferi* were active against influenza virus type A and HSV-1 [8]. Therefore, in order to better define the antiherpetic compounds in the mushroom strains selected in this study, two different fractions were separated, LMW and HMW fractions. The latter was further treated to isolate the PSC present in water extracts.

Antiviral activity of LMW fractions

Since triterpenes are not extracted with water and the obtained methanol extracts showed lower antiviral activity, the high activity found in water extracts cannot be due to compounds of this type. The LMW fraction contained mainly phenolic compounds and peptides, the contents of phenolics being higher than that of peptides. *L. edodes* was the mushroom with the highest phenolic content (Tab. 2). The antiviral activity of these fractions was measured under the same conditions as previously described for water extracts. Nevertheless, these fractions were not able to significantly reduce the virus infectivity at any of the previously described stages of the HSV-1 viral infection cycle (pre-treatment period, adsorption stage or replication period) suggesting that antiviral compounds of *B. edulis, L. edodes* and *P. ostreatus* water extracts were HMW molecules.

Antiviral activity of HMW fractions

The HMW fraction of the water extracts contained many proteins and a few easily extractable polysaccharides. *P. ostreatus* was the mushroom

Tab. 2. Composition of LMW and HMW fractions of the mushroom water extracts.

Water extracts	LMW compounds		HMW compounds	
	Phenols [%]	Peptides [%]	Proteins [%]	PSC [%]
Lentinus edodes	33.15 ± 0.92 [a]	3.33 ± 0.11 [b]	27.40 ± 1.20 [a]	7.54 ± 4.32 [b]
Boletus edulis	18.96 ± 0.08 [a]	2.21 ± 0.22 [b]	31.45 ± 2.01 [a]	7.57 ± 0.81 [b]
Pleurotus ostreatus	11.36 ± 1.24 [a]	3.00 ± 0.34 [b]	43.46 ± 5.64 [a]	12.91 ± 3.10 [b]

Values are the mean ± standard deviation of two separate experiments.
LMW – low molecular weight, HMW – high molecular weight. a, b – denotes statistically significant differences ($p < 0.05$) between peptides and phenolic content or proteins and polysaccharides.

Tab. 3. Polysaccharides contents in the water extracts and in the polysaccharides extracts isolated from the mushrooms.

PSC in the water extracts	Total [g·kg-1]	β-glucans [g·kg-1]	α-glucans [g·kg-1]	Chitins [g·kg-1]
Lentinus edodes	15.13 ± 1.24 [a]	13.25 ± 1.70 [a]	1.34 ± 0.29 [a]	9.22 ± 0.85 [a]
Boletus edulis	15.68 ± 3.00 [a]	2.79 ± 0.02 [b]	0.20 ± 0.01 [b]	12.46 ± 0.78 [b]
Pleurotus ostreatus	19.88 ± 4.39 [a]	13.89 ± 0.12 [a]	0.49 ± 0.15 [c]	11.55 ± 2.22 [b]
PSC extracts from mushrooms				
Lentinus edodes	189.22 ± 27.50 [a]	186.50 ± 31.57 [a]	12.35 ± 7.45 [a]	44.35 ± 8.56 [a]
Boletus edulis	243.12 ± 17.10 [b]	64.46 ± 0.60 [b]	25.26 ± 1.59 [b]	118.71 ± 10.93 [b]
Pleurotus ostreatus	209.61 ± 58.74 [b]	196.95 ± 57.96 [a]	38.06 ± 12.34 [b]	30.14 ± 17.60 [a]

Contents are expressed in grams per kilogram of mushroom powder dry weight. Values are the mean ± standard deviation of two separate experiments.
PSC – polysaccharides; a, b, c – dtenotes statistically significant differences ($p < 0.05$) between the three mushroom species.

with the highest content of both proteins and polysaccharides (Tab. 2). Since many publications identified fungal polysaccharides, saccharides-binding proteins or proteoglucans as responsible for the antiviral activities in other mushrooms or for other viruses, a more detailed analysis of the polysaccharide fraction (including possible proteoglucans) was carried out. Results indicated that the polysaccharides easily extractable at room temperature were mainly β-glucans followed by chitins in *P. ostreatus* and *L. edodes* mushrooms, while *B. edulis* contained more chitins, determined as glycosaminoglucan hexosamine (Tab. 3). Water extracts from the latter strain were also the extracts that showed lower antiviral activity compared with the other two strains (Tab. 1). However, IC_{50} of 35.1 μg·ml-1 suggested a rather interesting antiviral activity considering that only 2.8 g·kg-1 β-glucan were detected and so other compounds, such as proteins, might be involved in the antiviral activities observed.

Antiviral activity of polysaccharides extracts

In order to further clarify whether the antiviral compounds were polysaccharides or proteins, the mushroom polysaccharides were isolated and their cytotoxicities and antiherpetic activities measured under the same conditions as previously described for water extracts, except for virucidal effects. This activity was not measured in the polysaccharide fractions since water extracts presented a very low virucidal activity. Moreover, if the fungal β-glucans were the antiviral compounds, the real antiviral activity might be underestimated since plain water extraction is not the best method to extract them. Therefore, the antiviral activities of these new fractions were compared with those obtained by the water extraction. The cytotoxicity assays of these polysaccharide fractions (Tab. 1) indicated

a lower toxicity values than the water extracts. Among the different mushrooms, *Boletus edulis* extracts presented the lowest toxicity.

When Vero cells were pretreated with 50 μg·ml-1 of the polysaccharide fractions, virus infection was inhibited by approx. 50% in all cases (Fig. 4A). However, when the water extracts were applied at this concentration, only 10% inhibition was observed. Moreover, if 50 μg·ml-1 of the polysaccharide fractions were applied during the virus adsorption period, the infectivity was reduced by 60–65% (Fig. 4B), while a reduction of 40% was observed when water extracts were used. Since these fractions contained 10- to 15-fold more polysaccharides than the water extracts (Tab. 3), polysaccharides could be identified as the compounds responsible for the antiviral activity of the water extracts of the mushrooms.

The antiviral activity of the polysaccharide fractions on the intracellular replication of the virus was also evaluated, showing IC_{50} values ranging from 4.70 μg·ml-1 to 5.96 μg·ml-1 (Tab. 1). Worth to mention is the fact that these enriched extracts presented a very high selectivity indexes, identifying them as an interesting source of antiviral compounds.

When the different polysaccharides present in the extracts were individually measured (Tab. 3), the β-glucans represented the highest percentage of polysaccharides in *P. ostreatus* and *L. edodes* extracts, while *B. edulis* contained the levels of chitins higher than the levels of other β-glucans. These results pointed to β-glucans as the compounds responsible for the antiviral activity of *P. ostreatus* and *L. edodes* extracts in agreement with previous reports mentioning sulphated β-glucans as responsible for the antiviral activity against other viruses in in vitro and in vivo studies [14, 15, 29]. However, also other compounds
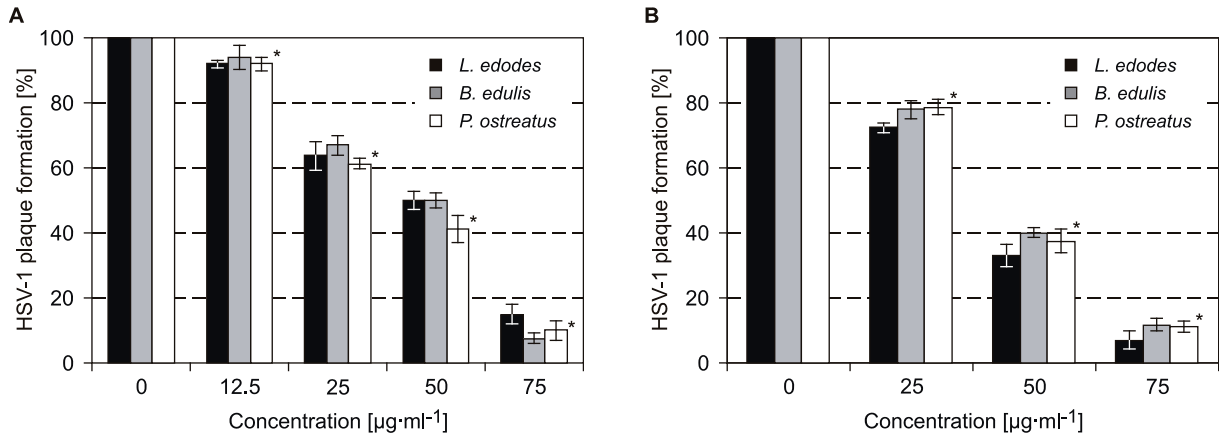
Fig. 4. Effect of cell pre-treatment with polysaccharide-rich fractions from edible mushrooms on HSV-1 infectivity and during HSV-1 adsorption period.

A – Effect on HSV-1 infectivity. B – Effect during HSV-1 adsorption period.
Each bar is the mean of four determinations ± standard deviation. * – denotes statistically significant differences ($p < 0.05$) between control assays (without polysaccharide supplementation) and those carried out in the presence of the polysaccharide fractions.

might be involved in the antiviral activities found in *B. edulis* as, although this mushroom showed an IC_{50} higher than the other two species, values of 5.9 μg·ml^{-1} indicate an interesting antiviral activity. Perhaps in this case, the lectins described in this mushroom might be chitin-binding lectins, due to the high chitin levels found, and might be responsible for the observed activity [18, 32] since fungal chitins lacked antiviral activities by themselves. Nevertheless, all the PSC fractions were able to in-

hibit the virus adsorption step, but they mainly inhibited the HSV-1 infection by interfering with the intracellular virus replication. Similar results were previously described for other mushroom strains, such as *Agaricus brasiliensis*. The antiviral polysaccharides isolated from this mushroom were able to inhibit the initial stage of the virus replication [33].

In order to investigate if the combination of the polysaccharide fractions from the three mushrooms exhibited synergistic effects, the ex-
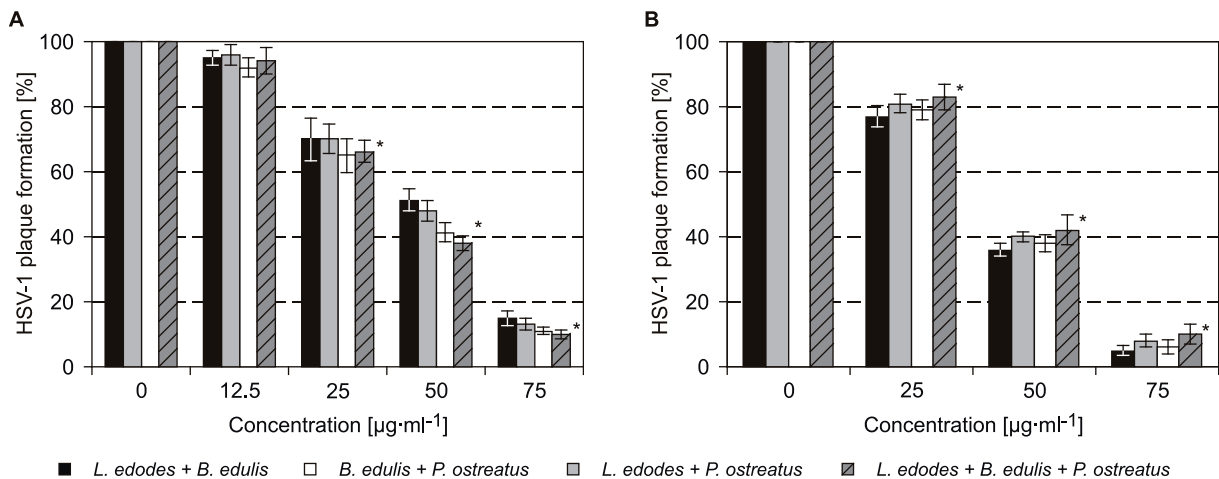

■ *L. edodes + B. edulis*   □ *B. edulis + P. ostreatus*   ▨ *L. edodes + P. ostreatus*   ▨ *L. edodes + B. edulis + P. ostreatus*

Fig. 5. Effect of cell pre-treatment with combination of polysaccharide-rich fractions on HSV-1 infectivity and during HSV-1 adsorption period.

A – Effect of on HSV-1 infectivity. B – Effect during HSV-1 adsorption period.
Polysaccharide-rich fractions were applied in pairs or all three fractions together. Each bar is the mean of four determinations ± standard deviation. * – denotes statistically significant differences ($p < 0.05$) between control assays (without polysaccharide supplementation) and those carried out in the presence of the polysaccharide fractions.

tracts were mixed in a ratio of 1:1 (v/v) in pairs and also pooled all together in a ratio of 1:1:1 (v/v/v). The antiviral activity of these mixtures was measured under the same conditions as previously described for the individual polysaccharide fractions. The polysaccharide combinations showed values of virus infectivity at any of the stages of the HSV-1 viral infection cycle (pre-treatment period, adsorption stage or replication period) that were similar to those determined with each individual polysaccharide fraction (Fig. 5, Tab. 1). No synergy effects were detected within the polysaccharides of the three mushrooms.

CONCLUSION

Concluding, the PSC fractions obtained from *L. edodes, B. edulis* and *P. ostreatus* might be a novel source of compounds able to prevent HSV-1 infections, since 50 μg·ml^{-1} of their polysaccharide extracts applied as a pre-treatment inhibited the virus infectivity by approx. 50% and, applied during the virus adsorption period, the infectivity was reduced by 60-65%. These results could be of interest since the antiviral studies are normally focused on treating the viral infection but not on preventing it. Furthermore, these polysaccharide extracts showed also a high inhibitory activity against the intracellular virus replication, therefore they might be used as a treatment after the virus infection. In order to investigate these interesting possibilities, animal studies should be carried out before clinical trials with mushroom polysaccharides can be planned.

REFERENCES

1. Whitley, R. J.: Herpes simplex viruses. In: Knipe, D. M. – Howley, P. M. – Griffin, D. F. – Lamb, R. A. – Martin, M. A. (Ed.): Fields virology. Vol. 2. 4th ed. Philadelphia : Lippincott Williams & Wilkins, 2001, pp. 2461–2509. ISBN 0-7817-1832-5.
2. Chakrabarti, S. – Pillay, D. – Ratcliffe, D. – Cane, P. A. – Collinghan, K. E. – Milligan, D. W.: Resistance to antiviral drugs in herpes simplex virus infections among allogeneic cell transplant recipients: risk factors and prognostic significance. Journal of Infectious Diseases, *181*, 2000, pp. 2055–2058.
3. Reusser, P.: Herpes virus resistance to antiviral drugs: a review of the mechanisms, clinical importance and therapeutic options. Journal of Hospital Infection, *3*, 1996, pp. 235–248.
4. Mattila, P. – Suonpä, K. – Piironen, V.: Functional properties of edible mushrooms. Nutrition, *16*, 2000, pp. 694–696.
5. Fan, L. – Pan, H. – Soccol, A. T. – Pandey, A. – Soccol, C. R.: Advances in mushroom research in the last decade. Food Technology and Biotechnology, *44*, 2006, pp. 303–311.
6. Brandt, C. R. – Piraino, F.: Mushrooms antivirals. Recent Research and Development. Antimicrobial Agents and Chemotherapy, *4*, 2000, pp. 11–26.
7. El–Mekkawy, S. – Meselby, M. R. – Nakamura, N. – Tekuza, Y. – Hattori, M. – Kakiuchi, N. – Shimotohno, K. – Kawahata, T. – Otake, T.: Anti-HIV-1 and anti HIV-1-protease substances from *Ganoderma lucidum*. Phytochemistry, *49*, 1998, pp. 1651–1657.
8. Mothana, R. A. A. – Awadh, N. A. A. – Jansen, R. – Wegner, U. – Mentel, R. – Lindequist, U.: Antiviral lanostanoid triterpenes from the fungus *Ganoderma pfeifferi* BRES. Fitoterapia, *74*, 2003, pp. 177–180.
9. Eo, S. K. – Kim, Y. S. – Lee, C. K. – Han, S. S.: Antiherpetic activities of various protein bound polysaccharides isolated from *Ganoderma lucidum*. Journal of Ethnopharmacology, *68*, 1999, pp. 175–181.
10. Awadh, A. A. N. – Mothana, R. A. A. – Lesnau, A. – Pilgrim, H. – Lindequist, U.: Antiviral activity of extracts and compounds from *Inonotus hispidus*. Fitotherapia, *74*, 2003, pp. 483–485.
11. Ichimura, T. – Watanabe, O. – Maruyama, S.: Inhibition of HIV-1 protease by water-soluble lignin-like substance from an edible mushroom *Fuscoporia obliqua*. Bioscience, Biotechnology and Biochemistry, *62*, 1998, pp. 575–577.
12. Suzuki, H. – Okubo, A. – Yamazaki, S. – Suzuki, K. – Mitsuya, H. – Toda, S.: Inhibition of the infectivity and cytophatic effect of human immunodeficiency virus by water-soluble lignin in an extract of the culture medium of *Lentinus edodes* mycelia (LEM). Biochemical and Biophysical Research Communications, *160*, 1989, pp. 367–373.
13. Suzuki, H. –Liyama, K. – Yoshida, O. – Yamazaki, S. – Yamamoto, N. – Toda, S.: Structural characterization of the immunoactive and antiviral water-solubilized lignin in an extract of the culture medium of *Lentinus edodes* mycelia (LEM). Agricultural and Biological Chemistry, *54*, 1990, pp. 479–487.
14. Yoshida, O. – Nakashima, H. – Yoshida, T. – Kaneko, Y. – Yakamoto, I. – Matsuzaki, K.: Sulfation of the immunomodulating polysaccharide lentinan: a novel strategy for antivirals to human immunodeficiency virus (HIV). Biochemical Pharmacology, *37*, 1998, pp. 2887–2891.
15. Gordon, M. – Guralnik, M. – Kanekko, Y. – Mimu-

ra, T. – Goodgame, J. – DeMarzo, C. – Pierce, D. – Baker, M. – Lang, W.: A phase II controlled study of a combination of the immune modulator, lentinan, with didanosine (ddI) in HIV patients with CD4 cells of 200–500/mm3. Journal of Medicine, *26*, 1998, pp. 305–330.

16. Colins, R. – Ng, T. B.: Polysaccharopeptide from *Coriolus versicolor* has potential for use against human immunodeficiency virus type 1 infection. Life Science, *60*, 1997, pp. 383–387.

17. Wang, H. X. – Ng, T. B.: Isolation and characterization of velutin, a novel low-molecular-weight ribosome-inactivating protein from winter mushroom (*Fammulina velutipes*) fruiting bodies. Life Science, *68*, 2001, pp. 2151–2158.

18. Zheng, S. – Li, C. – Ng, T. B. – Wang, H. X.: A lectin with mitogenic activity from the edible wild mushroom *Boletus edulis*. Process Biochemistry, *42*, 2007, pp. 1620–1624.

19. Li, Y. R. – Liu, Q. H. – Wang, H. X. – Ng, T. B.: A novel lectin with potent antitumor, mitogenic and HIV-1 reverse transcriptase inhibitory activities from the edible mushroom *Pleurotus citrinopileatus*. Biochimica et Biophysica Acta, *1780*, 2008, pp. 51–57.

20. Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, *65*, 1983, pp. 55–63.

21. Ramirez-Anguiano, C. – Santoyo, S. – Reglero, G. – Soler-Rivas, C.: Radical scavenging activities, endogenous oxidative enzymes and total phenols in edible mushrooms commonly consumed in Europe. Journal of the Science of Food and Agriculture, *87*, 2007, pp. 2272–2278.

22. Bradford, M. M.: A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 1977, pp. 248–254.

23. Gil-Ramírez, A. – Clavijo, C. – Palanisamy, M. – Ruiz-Rodríguez, A. – Navarro-Rubio, M. – Pérez, M. – Marín, F. R. – Reglero, G. – Soler-Rivas, C.: Screening of edible mushrooms and extraction by pressurized water (PWE) of 3-hydroxy-3-methyl-glutaryl CoA reductase inhibitors. Journal of Functional Foods. Available online 15 November 2012. DOI: 10.1016/j.jff.2012.10.013.

24. Jeurink, P. V. – Lull-Noguera, C. – Savelkoul, H. F. J. – Wichers, H. J.: Immunomodulatory capacity of fungal proteins on the cytokine production of human peripheral blood mononuclear cells. International Immunopharmacology, *8*, 2008, pp. 1124–1133.

25. Fox, J. D. – Robyt, J. F.: Miniaturization of three carbohydrate analysis using a microsample plate reader. Analytical Biochemistry, *1*, 1991, pp. 93–96.

26. Vetter, J.: Chitin content of cultivated mushrooms *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*. Food Chemistry, *102*, 2007, pp. 6–9.

27. Smith, R. L. – Gilkerson, E.: Quantitation of glycosaminoglycan hexosamine using 3-methyl-2-benzothiazolone hydrazone hydrochloride. Analytical Biochemistry, *98*, 1979, pp. 478–480.

28. Eo, S. K. – Kim, Y. S. – Lee, C. K. – Han, S. S.: Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. Journal of Ethnopharmacology, *72*, 2000, pp. 475–481.

29. Zhang, M. – Cheung, P. C. K. – Ooi, V. E. C. – Zhang, L.: Evaluation of sulfated fungal β-glucans from the sclerotium of *Pleurotus tuber-regium* as a potential water-soluble anti-viral agent. Carbohydrate Research, *339*, 2004, pp. 2297–2301.

30. Kim, Y. S. – Eo, S. K. – Oh, K. W. – Lee, C. K. – Han, S. S.: Antiherpetic activities of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* alone and in combinations with interferons. Journal of Ethnopharmacology, *72*, 2000, pp. 451–458.

31. Wang, J. – Wang, H. X. – Ng, T. B.: A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*. Peptides, *28*, 2007, pp. 560–565.

32. Yao, Q. – Wu, C. F. – Luo, P. – Xiang, X. C. – Liu, J. J. – Mou, L. – Bao, J. K.: A new chitin-binding lectin from rhizome of *Setcreasea purpurea* with antifungal, antiviral and apoptosis-inducing activities. Process Biochemistry, *45*, 2010, pp. 1477–1485.

33. Faccin, L. C. – Benati, F. – Rincao, V. P. – Mantovani, M. S. – Soares, S. A. – Gonzaga, M. L. – Nozawa, W. – Carvalho-Linhares, R. E.: Antiviral activity of aqueous and ethanol extracts and of an isolated polysaccharide from *Agaricus brasiliensis* against poliovirus type 1. Letters in Applied Microbiology, *45*, 2007, pp. 24–28.