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ORIGINAL ARTICLE

Chemical composition, antiproliferative and antioxidant activity of differently processed *Ganoderma lucidum* ethanol extracts

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Abstract The content of phenolic compounds (TPC) and glucans, as well as the effectiveness of antiproliferative and antioxidant activity of differently processed *Ganoderma lucidum* ethanol extracts were determined and compared. The content of glucans (total, α- and β-) strongly depended on the extraction time and particle size, but only interaction of these parameters influenced the TPC. Gallic acid, quercetin, *trans*-cinnamic acid, kaempferol, hesperetin and naringenin were detected in extracts by HPLC–DAD. The most abundant phenols were hesperetin (1.875–3.222 μg/g) and naringenin (1.235–2.856 μg/g). The ethanol extracts exhibited noteworthy antioxidant activity, but the significant amount of phenolic compounds was strongly linked to polysaccharides, and hence reduced their antioxidant capacity. The results of the antiproliferative activity in vitro showed that the analyzed extracts were the most effective against HeLa cells. Significant correlations were observed between the antiproliferative effect and the TPC/glucan content of extracts.

Keywords *Ganoderma lucidum* · Ethanol extracts · Phenolic compounds · Glucans · Antioxidant capacity · Antiproliferative activity

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Introduction

The increasing demand for the natural drug and food supplements is expected to further increase the growth of mushrooms that have been recognized as one of the most potential raw materials in the production of functional food and drinks. Mushrooms have been widely used for centuries in traditional Chinese medicine as panacea and cure in treatment of many diseases. Recent studies confirmed that they represent an unlimited source of pharmaceutical substances with potent and unique properties. Non-edible mushrooms with coarse texture and therapeutic effects represent medicinal mushrooms (Wasser 2005). During the past decades, *Ganoderma lucidum* is recognizable as one of the most important medicinal mushroom, which contains highly bioactive secondary metabolite with an enormous variety of chemical structures. These bioactive chemical molecules extracted from *G. lucidum* fruit body, mycelium and spores include phenols, terpenoids, steroids, nucleotides, as well as their derivatives—glycoproteins and polysaccharides (Galor et al. 2011; Yuen and Gohel 2005). Polysaccharides and triterpenoids are considered as important marker components with beneficial properties in the treatment of various diseases, but recent studies also recognize the importance of phenolic compounds (Ferreira et al. 2009; Yuen and Gohel 2005). Many compounds of crude extract have a synergistic effect, resulting to an increase in biological activity of the extract (Liu et al. 2002).

Medicinal mushrooms synthesize various antibacterial, antifungal and antiviral substances, which can be purified and applied in clinical practice. They are also shown antitumor effects in animals and humans, which led to introducing them into the phase I, II, and III clinical trials (Wasser 2010). Thus, recent research studies showed that

Ganoderma lucidum extracts possess significant anticancer potential (Cao and Lin 2006; Chen et al. 2010). The substances responsible for chemopreventive and/or tumoricidal activity of Ganoderma genus are polysaccharides and triterpenes, but their capacity highly depends on the extraction and purification methods (Wasser 2010; Yuen and Gohel 2005).

In general, a wide range of extraction and isolation procedures have been set up for the extraction of relevant bioactive compounds from Ganoderma fruit body, especially polysaccharides (Ferreira et al. 2015). It is well established that *Ganoderma lucidum* contains the diverse structure of bioactive polysaccharides, mostly in the form of glucans with different types of glycosidic linkages, such as (1–3), (1–6)-β-glucans and (1–3)-α-glucans (Wasser 2002). It was previously reported that polysaccharides with cytotoxic activity were mainly in the form of β-glucans (Silva 2003).

In addition to anticancer effects of *G. lucidum,* as the most investigated, recent studies have focused also on the evaluation of its antioxidant effects. There are many reports regarding antioxidant properties of *Ganoderma lucidum,* not only methanol (Heleno et al. 2012; Karaman et al. 2010; Kim et al. 2008) and ethanol extracts (Ćilardžić et al. 2014; Sheikh et al. 2014), but also aqueous extracts (Heleno et al. 2012; Kozarski et al. 2011, 2012). The type of solvent has a significant influence on the dynamic of extraction process and determinate the chemical composition of extract. Thus, the extraction parameters can be optimized in order to obtain an extract with desired bioactive effects. Some previous studies showed that methanol extracts of *G. lucidum* have higher antioxidant potential compared with water extracts (Rawat et al. 2013). However, the methanol is highly toxic and is not a suitable solvent for the extracts with application in food industry (or methanol must be removed before use in food production).

Despite the fact that knowledge about chemical composition of *Ganoderma lucidum* is very important, the data about optimal extraction conditions and compounds which mostly contribute to the antioxidant activity of extracts is limited. Although *G. lucidum* represents poor source of phenolic compounds, some investigations reported that these compounds provide the greatest contribution to the antioxidant activity of its extracts (Ćilardžić et al. 2014; Saltarelli et al. 2009). The major flavonoids with effective antioxidant activity in the ethanol extract from mycelia of *G. lucidum* are quercetin, myricetin, and morin (Saltarelli et al. 2015).

The aim of the present work was to investigate the influences of particle size and extraction time on the content of phenolic compounds and glucans of *Ganoderma lucidum* ethanol extracts. Also, to examine the influence of chemical composition on effectiveness of the antiproliferative and antioxidant activity of *G. lucidum* ethanol extracts.

Materials and methods

Standards and reagents

Gallic acid (GA), Folin–Ciocalteu's phenol reagent, hydrochloric acid, sodium acetate trihydrate, glacial acetic acid and sodium carbonate were purchased from Merck (Darmstadt, Germany). 2,4,6-Trypyridyl-*s*-triazine-s-triazine (TPTZ), ferric chloride hexahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylicacid (Trolox), sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium chloride, 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulfate and phenolic standards rutin, naringenin, chlorogenic acid, *trans*-cinnamic acid, quercetin, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid, resveratrol and kaempferol were purchased from Sigma-Aldrich (Steinheim, Germany), while hesperetin and catehin were purchased from Fluka (Steinheim, Germany). Benzoic acid was obtained from Lachnema (Nerastovice, Czech Republic) and gallic acid from Alfa Aesar (Lancaster, Great Britain). The purities of standards were up to 99%. The solvents used were of analytical grade dimethylsulfoxide J.T. Baker (NJ, USA), glacial acetic acid POCH (Gliwice, Poland) and acetonitril HPLC grade J.T. Baker (NJ, USA). An analytical mushroom β-glucan kit was obtained from Megazyme Int. (Wicklow, Ireland).

Sample preparations

Ganoderma lucidum (W. Curt.: Fr.) P. Karst. fruiting bodies were obtained from Jingsu Alphay Bio-Tech, Co. Ltd. (PR Chine). Fruit bodies were separated from spores using brushes and then air-dried at 40 °C to constant mass. The fruit bodies were prepared for extraction by cutting into pieces (about 1 cm) and by grounding into fine particles. The average particle size of ground fungi was measured on sieve shaker Analysette 3 Pro (Fritsch, Idar – Oberstein, Germany). The average milled particle size was 0.13 mm. Six samples of ethanol extracts (E1–E6) were produced in the experiment. The production parameters are presented in Table 1. Fragmented mushroom sample (40 g) was extracted using 60% ethanol (1000 ml), at 25 °C, stirred using a rotary shaker (120 rpm). Liquid phase (extracts) was separated by filtration through 70 g/m^3 filter paper. After filtration, the solvent was partially removed by vacuum evaporation (Rotavapor R-200/205, Buchi,

Table 1 The parameters of extract production

Parameters	E1	E2	E3	E4	E5	E6
Extraction time (days)	15	15	30	30	1	1
Particles size (mm)	10	0.13	10	0.13	10	0.13

Labortechnik AG, Meiersegg strasse, Flawil, Switzerland) and its complete removal was performed by lyophilisation (Modulyo D-230, Thermo Savant, Holbrook, NY, USA).

Determination of total phenolics and antioxidant capacity

Determination of total phenolic content (TPC) in the samples of *G. lucidum* extract was conducted by the Folin-Ciocalteu method described by Singleton and Rossi (1965). For the determination of antioxidative characteristics of samples, the DPPH, Ferric ion Reducing Antioxidant Power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) methods were used. DPPH-reducing activity was evaluated following modified procedure described by Kaneda et al. (1995). FRAP assay was performed according to the procedure by Benzie and Strain (1996). The TEAC assay was conducted according to the procedure described by Re et al. (1999).

HPLC analyses of phenolic compounds

Phenolic compounds were determined by the HPLC method using an Agilent 1100 Series liquid chromatograph (Palo Alto, CA, USA) equipped with UV/DAD detector. Chromatographic separation was performed on Poroshell 120 EC-C18 (4.6 × 100 mm 2.7 μm) column. The solvent system had a constant flow rate of 1.0 ml/min. The mobile phase was distilled water with 0.1% glacial acetic acid (solvent A) and acetonitrile with 0.1% glacial acetic acid (solvent B). The gradient was: 0–3.25 min, 8–10%B; 3.25–8 min, 10–12%B; 8–15, 12–25%B; 15–15.8 min, 25–30%B; 15.8–25 min, 30–90%B; 25–25.4 min, 90–100%B; 25.4–30 min, 100%B. Injection volume was 5 μl and temperature was kept constant at 25 °C. Detection wavelengths were chosen according to absorption maximum of analyzed phenolic compounds and included 225 nm (vanillic acid, benzoic acid), 280 nm (gallic acid, 4-hydroxybenzoic acid, catehin, syringic acid, *trans*-cinnamic acid, hesperetin, naringenin), 305 nm (coumaric acid, resveratrol), 330 nm (chlorogenic acid, caffeic acid) and 360 nm (rutin, quercetin, kaempferol). Quantification was performed by external standard method. The standard stock solutions (1, 2.5, 5, 10, 15, 25 mg/l) were made with dimethylsulfoxide (DMSO). All standard calibration curves showed high degrees of linearity ($r^2 > 0.99$). Samples were filtered through 0.45 μm filter prior to direct injection in HPLC.

Measurement of glucan content

The contents of total and α-glucans were determined in the polysaccharide extracts using the Mushroom and Yeast β-glucan Assay Procedure (Megazyme Int.) according to the method describe by authors (Kozarski et al. 2012). The β-glucan content was calculated by subtracting the α-glucan from the total glucan content. All values of glucan contents were expressed as g/100 g of a dry weight (DW) of the extracts. Results were calculated with the Megazym program Mega-CalcTM.

Cell lines and culture conditions

Human cervical carcinoma (HeLa), human alveolar basal adenocarcinoma (A549) and human colon carcinoma (LS174) cell lines were maintained as monolayer cultures in the Roswell Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co, Saint Louis, Mo, USA). The endothelial cell line EA.hy 926 (permanent human cell line derived by fusing human umbilical vein endothelial cells—HUVEC, with human lung adenocarcinoma epithelial cells—A549), was maintained in the nutrient medium, Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Steinheim, Germany). Nutrient mediums were prepared in sterile ionized water, supplemented with penicillin (192 U/ml), streptomycin (200 μg/ml), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM), 10% of heat-inactivated fetal calf serum (FCS) (pH 7.2) and D-glucose (4.5 g/l). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

MTT assay

Antiproliferative activity of the investigated extracts was determined using 3-(4,5-dymethylthiazol-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, Steinheim, Germany) assay, according to the procedure described previously (Rakić et al. 2012; Supino 1995).

Precisely, cells were seeded into 96-well cell culture plates (Thermo Scientific Nunc™, Sigma-Aldrich, Steinheim, Germany), at a cell density of 4000 cells/well (HeLa and Ea.hy 926), 6000 cells/well (A549), and 10,000 cells/well (LS174) in 100 μl of culture medium. After 24 h of growth, cells were exposed to the serial dilutions of the tested extracts. The investigated compounds were dissolved in DMSO at a concentration of 50 mg/ml as stock solution, and prior the use diluted with nutrient medium to the desired final concentrations (in range up to 500 μg/ml).

Each concentration was tested in triplicates. After incubation periods of 24 or 48 h, 20 µl of MTT solution (5 mg/mL in phosphate buffer solution, pH 7.2) were added to each well. Samples were incubated for 4 h at 37 °C, with 5% CO_2 in a humidified atmosphere. Formazan crystals were dissolved in 100 µl of 10% sodium dodecyl sulfate (SDS). Absorbances were recorded after 24 h, on an enzyme-linked immune sorbent assay (ELISA) reader (Thermo Labsystems Multiskan EX 200–240 V, Termo Fisher Scientific Oy, Vanta, Finland), at the wavelength of 570 nm. IC_{50} values (µM), were determined from the cell survival diagrams. The percentages of surviving cells relative to untreated controls were determined. The IC_{50} value, defined as the concentration of the compound causing 50% cell growth inhibition, was estimated from the dose–response curves.

Statistical analyses

All measurements were done in triplicate and data were expressed as mean value ± standard deviation (SD). The experimental data were subjected to the analysis of variance (ANOVA). Analysis was conducted in a factorial arrangement where time extraction and concentration of added *G. lucidum* were analyzed factors. Tukey's test was used to determine difference ($p \leq 0.01$) between the mean values. The correlation between TPC, glucans (total, α- and β-), antioxidant activity and antiproliferative effect was also determined. All statistical analyses were performed using statistical program Statistica 12 (Statsoft Inc., Tulsa, Oklahoma, USA).

Results and discussion

Phenolic compounds analyses

The total phenolic content (TPC) of samples and their composition are shown in Table 2. Investigated factors (particle size and extraction time) had no significant influence on TPC independently. However, the interaction of these two factors had highly significant effect on TPC ($F = 20.45$; $p = 0.00$), which indicates that particle size and extraction time affect only in conjunction.

Considering tested samples, the TPC were ranged from 8.6 ± 1.0 (E4) to 13.9 ± 0.3 (E6) g/100 g gallic acid equivalents (GAE) (Table 2). The transfer of soluble compounds from the interior of the small fungi particles to the alcohol-water solution was intensive, and extraction period was shorter. After the complete extraction of phenolic compounds, longer extraction period allowed them to degrade or react with other substances in solution. Hence, the content of total phenolic compounds was decreased with the extended extraction period. In the case of chopped *G. lucidum* samples, transfer of the phenolic compounds from the inside of the particles was slower, but their concentration was increased by longer extraction period.

In the previous study, TPC of hot water extracts of *G. lucidum* and *G. appalantum* was found to be 3.3 and 4.7 g/100 g, respectively (Kozarski et al. 2012). In the research of TPC in the purified phenolic and polysaccharide extracts of *Ganoderma lucidum* fruit body and spore, the reported values were 2.86, 5.5, 1.5 and 4.3 g/100 g GAE, respectively (Heleno et al. 2012). TPC of ethanol extract of wild *G. lucidum* collected from Madhya Pradesh (Central India) was 7.14 ± 0.09 g/100 g GAE (Sheikh et al. 2014). TPC of ethanol extracts was significantly higher than reported in mentioned studies. The obtained results showed that the extraction procedure had a significant effect on the TPC, wherein the highest concentration of phenolic compounds were achieved in extracts produced from ground mushroom with extraction time of 24 h.

The ethanol extracts of *G. lucidum* were analyzed by HPLC–DAD and the results are presented in Table 2. Detected phenolic compounds can be classified into phenolic acids (gallic acid and *trans*-cinnamic acid) and flavonoids (quercetin, kaempferol, hesperetin and naringenin).

Table 2 Total phenolic content (TPC) and composition of phenolic compounds in ethanol extracts of *Ganoderma lucidum*

Compounds	Samples (µg/g)					
	E1	E2	E3	E4	E5	E6
TPC (g/100 g GAE)	9.4 ± 0.2^{a}	$11.8 \pm 0.3^{a,b}$	13.7 ± 0.4^{b}	8.6 ± 1.0^{a}	8.8 ± 1.2^{a}	13.9 ± 0.3^{b}
Gallic acid	0.142 ± 0.003^{a}	0.915 ± 0.001^{b}	nd.	0.892 ± 0.002^{c}	nd.	1.016 ± 0.004^{d}
Trans-cinnamic acid	0.054 ± 0.002^{a}	0.084 ± 0.001^{b}	0.046 ± 0.000^{c}	0.061 ± 0.000^{d}	0.060 ± 0.003^{d}	0.104 ± 0.002^{e}
Quercetin	nd.	0.798 ± 0.001^{a}	nd.	0.844 ± 0.001^{b}	0.124 ± 0.001^{c}	0.968 ± 0.003^{d}
Kaempferol	0.598 ± 0.002^{a}	0.871 ± 0.004^{b}	0.584 ± 0.001^{c}	0.860 ± 0.000^{d}	0.657 ± 0.002^{e}	0.918 ± 0.001^{f}
Hesperetin	1.875 ± 0.001^{a}	2.569 ± 0.003^{b}	1.949 ± 0.000^{c}	3.218 ± 0.000^{d}	2.171 ± 0.001^{e}	3.222 ± 0.004^{d}
Naringenin	2.543 ± 0.002^{a}	1.578 ± 0.001^{b}	2.562 ± 0.002^{c}	1.235 ± 0.002^{d}	2.856 ± 0.002^{e}	2.812 ± 0.003^{f}

nd. not detected

Different letters in same row denote a significant difference according to Tuckey's test, at $p < 0.01$

The composition and content of individual phenolic compounds were significantly different between the extracts from minced and chopped mushrooms. The minced mushroom particles released higher content of phenolic compounds than the cut particles, which was expected in accordance with the literature data (Luthria 2008). However, an exception was naringenin, whose concentration was higher in samples from chopped mushroom. The reason for that is not entirely clear, but one of possible explanation is the following: the highest concentration of all determined individual phenolic compounds was after 24 h of extraction, wherein at that moment the naringenin concentration in the samples E5 and E6 (produced from milled and chopped mushroom, respectively) was very similar. During the prolonged extraction, concentration of analyzed individual phenols was decreased due to oxidation and/or reactions with other compounds in solution.

Although, the phenolic compounds represent one of the most important groups with bioactive effect in *Ganoderma lucidum*, only few studies were conducted to determine the content and composition of its phenolic compounds. Kim et al. (2008) studied the content of phenolic compounds in *Ganoderma lucidum* cultivated in Korea and reported total phenol content 162 μg/g dw. Detected following phenolic compounds were gallic acid, pyrogallol, 5-sulfosalicylic acid, protocatechuic acid, catechin, benzoic acid, myricetin, quercetin, kaempferol, hesperetin, formononetin and biochanin. Furthermore, the phenolic compounds were evaluated in phenol extracts, made from fruiting body, spores and mycelium produced in different media (Heleno et al. 2012). Total phenol content in phenol extracts was ranged from 2.5 to 12.3 μg/g, and detected phenolic compounds in extracts were the *p*-hydroxybenzoic, *p*-coumaric and cinnamic acid. The flavonoids in the ethanolic extracts from mycelia of *Ganoderma lucidum* strains Gl-4 and Gl-5 were analyzed, and quercetin, myricetin, and morin were identified (Saltarelli et al. 2015). Gallic acid (1103 μg/g) was the only detected phenolic compound in methanol extract of wild growing *Ganoderma lucidum* collected from Fruška gora, Serbia. However, its content was significantly higher than described by the other studies (Karaman et al. 2010).

Our research showed greater TPC concentration in analyzed ethanol extracts comparing to previously reported results (Heleno et al. 2012; Sheikh et al. 2014). It is well known that gallic acid is compound with strong antioxidant activity (Rice-Evans et al. 1996), and its highest concentration was found in sample E6. The most abundant phenols in all samples were hesperetin and naringenin, while *trans*-cinnamic acid was present in very low concentration.

Antioxidant activity

Many different in vitro assays are used for the determination of antioxidant capacity, since there is not defined standard method. Three methods, the DPPH, Ferric ion reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC), were selected to analyze the samples.

The all produced extracts showed significant antioxidant capacity (Table 3). In contrast to the TPC, antioxidant activity was significantly depended on the extraction time and particle size, as well as their mutual interaction.

The results of antioxidant assays clearly revealed that the largest amount of soluble compounds with antioxidant capacity was extracted after 1 day from the milled particles. Thus, the extraction process was finished after 1 day and extended extraction may adversely influence the quality of extracts due to degradation of soluble bioactive compounds. Unlike the milled particles, dynamic of extraction of chopped mushroom was different, where the extraction process was finished after 15 days. Based on the results of antioxidant capacity, extraction time is inversely proportional to the size of the particles. However, the increase in the antioxidant capacity of extracts due to the reduction of particle size or extension of the extraction time is limited; for each particle size, optimum extraction time should be determined.

The ethanol extracts had significantly higher antioxidant activity (4.32–6.65 FRAP units and 2.52–6.00 mmol Trolox Equivalent) compared with special brandy produced with same amount of *G. lucidum* (40 g/L) (0.432 FRAP units and 1.043 mmol Trolox Equivalent) (Pecić et al. 2016). The main reason for such a big difference is the fact that the evaporation of solvent and concentration of bioactive compounds significantly affects the increase of the antioxidant capacity.

Table 3 Antioxidant capacity of *Ganoderma lucidum* ethanol extracts evaluated with DPPH, FRAP and TEAC assays

Samples	DPPH (mM TE)	FRAP (FRAP unit)	TEAC (mM TE)
E1	$2.08 \pm 0.16^{a,b}$	4.85 ± 0.10^{a}	3.64 ± 0.06^{a}
E2	$2.33 \pm 0.14^{b,c}$	5.40 ± 0.26^{b}	4.40 ± 0.08^{b}
E3	1.40 ± 0.05^{a}	4.32 ± 0.08^{c}	2.52 ± 0.08^{c}
E4	$2.05 \pm 0.06^{a,b}$	6.46 ± 0.10^{d}	3.78 ± 0.07^{a}
E5	$1.81 \pm 0.12^{a,b}$	$4.48 \pm 0.06^{a,c}$	3.29 ± 0.09^{d}
E6	3.07 ± 0.20^{c}	6.65 ± 0.13^{d}	6.00 ± 0.19^{e}

Different letters in same column denote a significant difference according to Tuckey's test, at $p < 0.01$

mM TE mM Trolox Equivalent

Table 4 α-, β- and total glucans content in *G. lucidum* extracts

	Glucan content (g/100 g)		
	Total	α	β
E1	18.55 ± 0.04[a]	2.91 ± 0.06[a]	15.64 ± 0.01[a]
E2	15.81 ± 0.04[b]	2.43 ± 0.04[a]	13.38 ± 0.01[b]
E3	16.53 ± 0.07[c]	2.93 ± 0.03[b]	13.62 ± 0.02[c]
E4	13.42 ± 0.05[d]	1.79 ± 0.04[c]	11.63 ± 0.03[d]
E5	14.72 ± 0.03[e]	1.68 ± 0.02[d]	13.03 ± 0.05[e]
E6	9.44 ± 0.03[f]	0.54 ± 0.01[e]	8.90 ± 0.04[f]

Different letters in same column denote a significant difference according to Tuckey's test, at $p < 0.01$

Glucan content

The content of glucans (total, α- and β-) was measured using Megazyme β-glucan assay kit. The results are presented in Table 4. The results of ANOVA showed that total glucan contents of ethanol extracts was strongly depended on the extraction parameters and varied from 9.44 ± 0.03 g/100 g (E6) to 18.55 ± 0.04 g/100 g (E1). Total glucan content of *Ganoderma lucidum* hot water polysaccharide and spore extracts were 47.1 ± 0.6 g/100 g and 20.8 ± 0.5 g/100 g, respectively (Kozarski et al. 2011, 2012).

The concentration of α-glucans is not negligible and valued from 5.72 to 17.72%. The percentage of α-glucans in ethanol extracts was similar with the content in hot water extracts, but enormously smaller than in spore extract of *G. lucidum* (Kozarski et al. 2011).

β-glucan content was determined by subtracting α-glucans from total glucans. The content of β-glucans in ethanol extracts was from 8.90 ± 0.04 to 15.64 ± 0.01 g/100 g. The reported amount is much lower than in hot water *Ganoderma lucidum* extract 41.4 ± 1.4 g/100 g (Kozarski et al. 2012). In the other study, β-glucans content in *Ganoderma lucidum* was determined by the enzymatic HPLC method, and the obtained value was lower (7.9 ± 0.2 g/100 g) compared to our ethanol extracts (Su et al. 2016).

The polysaccharides is mainly water-soluble compounds, hence hot water extraction is the most commonly used procedure for their separation (Ferreira et al. 2015; Wasser 2002). In our research, 60% v/v ethanol was used as a solvent for extracts production, and this is the main reason why is the content of glucans greater in our extracts compared to purified phenolic extracts (obtained by extraction with 96% v/v ethanol).

Obtained data indicate that particle size had the important effect on the content of α-, β- and the total glucans in the ethanol extracts. Specifically, increasing the particle size also increases the amount of glucans in the ethanol extracts.

Cytotoxicity in cancer cell lines

The antiproliferative activity of Ganoderma extracts (E1-E6) was evaluated, using colorimetric MTT assay. The study was performed in several human neoplastic cell lines (HeLa, A549, LS174) and transformed endothelial cell line EA.hy 926. The results are summarized in Table 5 in terms of IC_{50} values with their standard deviations for the 24 and 48 h incubation periods.

The obtained cytotoxicity results revealed that extracts E1-E6 mostly exhibited antiproliferative activity against all cell lines used in range of concentrations up to 500 μg/ml. It is shown that the analyzed extracts were most effective against HeLa—human cervical carcinoma, while the least sensitive was LS174—colon carcinoma cell line. The extract E4 expressing the highest antiproliferative activity against HeLa, Ea.hy 26 and LS174 cell, contained the

Table 5 Results of the MTT assay presented as IC_{50} values obtained after 24 and 48 h treatments

Cell line complex	IC50 ± SD (μg/ml)							
	HeLa		A549		EA.hy 926		LS174	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
E1	357.6 ± 30.9[a]	209.5 ± 0.6[a]	>500[a]	459.9 ± 1.8[a]	484.6 ± 6.2[a]	282.8 ± 9.4[a]	394.5 ± 8.3[a]	390.8 ± 9.1[a]
E2	364.8 ± 1.5[a]	244.3 ± 3.7[b]	375.1 ± 14.1[b]	290.9 ± 15.5[b]	385.3 ± 25.7[b]	242.5 ± 5.2[b]	>500[b]	342.3 ± 1.7[b]
E3	>500[b]	391.2 ± 14.8[c]	>500[a]	>500[c]	450.7 ± 31.7[a,c]	370.1 ± 4.1[c]	>500[b]	>500[c]
E4	223.1 ± 6.9[c]	119.3 ± 1.0[d]	>500[a]	344.8 ± 7.9[d]	408.4 ± 13.5[b,d]	242.8 ± 1.0[b]	438.3 ± 10.6[c]	285.3 ± 1.2[d]
E5	334.7 ± 5.8[d]	184.7 ± 0.4[e]	>500[a]	363.5 ± 4.6[e]	476.2 ± 10.5[a,c]	450.8 ± 4.2[d]	>500[b]	412.1 ± 5.4[e]
E6	394.3 ± 4.5[e]	335.5 ± 11.5[f]	400.1 ± 5.5[c]	197.7 ± 5.4[f]	442.5 ± 43.3[c,d]	336.7 ± 19.2[c]	>500[b]	>500[c]

The sign > (in front of the maximum value of the concentration) indicates that IC_{50} value is not reached in the examined range of concentrations
Different letters in same column denote a significant difference according to Tuckey's test, at $p < 0.01$

smallest amount of TPC (8.6/100 g), but its content of particular phenolic compounds detected by HPLC was very high. Considering our results, it can be concluded that those phenolic compounds also influenced the antiproliferative effect.

Recent study regarding Ganoderma extracts from Serbia exhibited cytotoxic effect on breast and cervical cell lines, confirming the results we have just reported (Stojković et al. 2014). Ethanol extracts obtained from basidiocarps of *Ganoderma lucidum* cultivated on alternative and commercial substrate manifested a more powerful cytotoxic effect against HeLa (38.11–109.04 μg/ml) and A549 (26.48–167.08 μg/ml) cell lines compared to our extracts (Ćilardžić et al. 2014). However, it should take into account that the mentioned cell lines was cultured with the indicated concentrations of ethanol extracts for a longer period (72 h). Comparison of the results leads to the suggestion that there was significant difference regarding cytotoxicity of the investigated extracts, and that also cell line and the period of treatment caused significant antiproliferative differences in vitro.

The cytotoxic effect of *Ganoderma lucidum* is usually related with the content of polysaccharides (Liu et al. 2015), but there are also recent studies that have confirmed the cytotoxic effect of ganoderic acids, which are one of the most important bioactive compounds in ethanol extracts (Li et al. 2010).

Correlation between TPC and β-glucans and antioxidant and antiproliferative activity of *Ganoderma lucidum* extracts

The correlations between total phenolic content, antioxidant activity, glucans content (total, α- and β-) and antiproliferative activity against cell lines (HeLa, A549, LS174) for all analyzed samples (E1-E6) were calculated (Table 6). Statistically significant positive correlation was observed between antiproliferative activity against HeLa cell line after 48 h treatment and TPC, and antiproliferative activity against A549 cell line after 48 h treatment and total glucans. Hence, it can be concluded that phenolic compounds also affect cytotoxic activity against HeLa cell line. Also, the strong correlation was found between antiproliferative activities on cell lines HeLa 24 h, HeLa 48 h and LS147 48 h.

The correlation between the TPC and antioxidant capacity was not significant, but other bioactive compounds and their interactions had important influence on the antioxidant activity. The most active sample E6 showed the highest content of phenolic compounds and the smallest content of total glucans. According to the results, the content of β-glucans was in negative correlation with TPC ($r = -0.362$) and antioxidant capacity

Table 6 Correlation between TPC, antioxidant capacity, glucans (total, α-, β-) and antiproliferative effect

r	DPPH	FRAP	TEAC	Total glucans	α-Glucans	β-Glucans	HeLa 24	HeLa 48	A549 24	A549 48	Ea.hy 926 24	Ea.hy 926 48	LS174 24	LS174 48
TPC	0.281	0.092	0.327	−0.333	−0.150	−0.396	0.797	**0.942**	−0.537	−0.209	−0.176	0.776	0.582	0.748
DPPH		0.786	**0.996**	−0.705	−0.742	−0.674	−0.198	0.336	−0.734	**−0.885**	−0.257	−0.307	0.072	0.064
FRAP			0.798	−0.772	−0.714	−0.778	−0510	−0.205	−0.457	−0.777	−0.517	−0.527	−0.062	−0.256
TEAC				−0.749	−0.770	−0.723	−0.174	0.706	−0.706	**−0.912**	−0.300	−0.285	0.148	0.088
total glucans					**0.959**	**0.994**	0.176	0.119	0.434	**0.835**	0.264	−0.097	−0.454	−0.222
α-Glucans						**0.921**	0.285	0.036	0.385	**0.857**	0.103	−0.191	−0.365	−0.173
β-Glucans							0.131	−0.176	0.444	0.808	0.321	−0.058	−0.476	−0.235
HeLa 24								**0.943**	−0.133	0.313	0.267	0.398	0.448	**0.848**
HeLa 48									−0.286	0.072	0.105	0.294	0.524	**0.879**
A549 24										0.765	0.610	0.344	−0.472	0.065
A549 48											0.468	0.168	−0.387	0.056
Ea.hy 926 24												0.658	−0.291	0.492
Ea.hy 926 48													0.479	0.628
LS174 24														0.459

r correlation coefficient

Bolded numbers indicate statistically significant correlation ($p < 0.05$)

($r_{DPPH} = -0.674$, $r_{FRAP} = -0.778$, $r_{TEAC} = -0.723$). These results indicate that a significant amount of phenolic compounds in analyzed samples were linked to polysaccharides, and therefore reduced their activity. This type of influence of polysaccharides on antioxidant capacity in polysaccharide extracts was previously confirmed by Heleno et al. (2012). According to the results, the antioxidant assays were not equally sensitive on the component with the antioxidant capacity. The results of DPPH and TAEC were in significant correlation.

Conclusion

The results showed that, extraction parameters (extraction time and particle size) strongly influenced the transfer process of chemical compounds from *Ganoderma lucidum* particles to the ethanol–water solution, thus determined the quality and quantity of analyzed compounds. The glucans content was affected by particle size and extraction time, as well as their interaction, while the phenolic content was influenced only by the interaction of these factors. The profiles of phenolic compounds were different in the ethanol extracts made with the different particle size. Gallic acid and quercetine were detected in the all extracts made from grounded particles, but in the case of the extracts from chopped mushroom gallic acid was only found in sample E1, and quercetin in sample E5. The type and quantity of bioactive compounds affected the antiproliferative and antioxidative capacity of analyzed ethanol extracts. The correlation between the TPC and antioxidant capacity was not significant, and these results suggested that a significant amount of phenolic compounds were strongly linked to polysaccharides, and hence their capacity was reduced.

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