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# *Ganoderma lucidum* as a cosmeceutical: antiradical potential and inhibitory effect on hyperpigmentation and skin extracellular matrix degradation enzymes

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**Abstract:** Three different polysaccharide extracts of a wild European source of the mushroom *Ganoderma lucidum* were screened for their free radical-blocking potential, which could strengthen the skin's barrier function, and provide a skin-lightening effect via potential inhibition of tyrosinase. The anti-collagenase and anti-elastase activities, which can help to restore skin elasticity and tensile strength, were also evaluated for the three extracts. Carbohydrates were the most abundant components of the extracts, followed by smaller quantities of proteins, phenols and flavonoids. The glucan fraction represented between 48% and 61% of carbohydrate content in all three extracts. None of the analyzed extracts showed overt toxicity to spontaneously-transformed immortal human keratinocytes (HaCaT) at concentrations up to 2 mg/mL, and displayed superior scavenging ability on 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The hot water crude polysaccharide extract (HWCP) and partially purified fraction (HWPP) were found to be effective inhibitors of lipid peroxidation (LPx), with an almost two-fold increased inhibition of LPx compared with ascorbic acid ($EC_{50}$=1.65±0.08 mg/mL), a common additive in cosmeceutical formulations and used at mg levels. Among the investigated extracts, HWCP showed the strongest inhibition potential on tyrosinase and skin extracellular matrix (ECM) degradation enzymes. These diverse functionalities indicate that *G. lucidum* may represent a promising source of natural cosmeceutical ingredients.

**Keywords:** *Ganoderma lucidum*; cosmeceutical; maturate skin; oxidative stress; polysaccharide extracts

**Abbreviations and acronyms:** 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS); bovine serum albumin (BSA); catechin equivalents (CEs); dimethyl sulfoxide (DMSO); heavy water ($D_2O$); 1,1-diphenyl-2-picrylhydrazyl (DPPH); 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (DSS); effective concentrations of each extract that are required to show 50% antioxidant properties ($EC_{50}$); extracellular matrix (ECM); ethylenediaminetetraacetic acid (EDTA); epigallocatechin gallate (EGCG); N-(3-[2-furylacryloyl)-Leu-Gly-Pro-Ala (FALGPA); ferric-reducing antioxidant power (FRAP); Fourier-transform infrared spectroscopy (FTIR); gallic acid equivalents (GAE); spontaneously transformed immortal human keratinocytes (HaCaT); hydrogen atom transfer (HAT); hot alkali polysaccharide extract (HWAP); hot water crude polysaccharide extract (HWCP); hot water polysaccharide extract purified by dialysis (HWPP); 50% inhibitory concentration ($IC_{50}$); dihydroxyphenylalanine (L-DOPA); lipid peroxidation (LPx); N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MAAPVN); metalloproteinases (MMPs); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); nuclear magnetic resonance (NMR); reactive oxygen species (ROS); single electron transfer (SET); sirtuin 1 (SIRT1); transforming growth factor β (TGF-β); thin layer chromatography (TLC).

## INTRODUCTION

Oxidative stress induced by reactive oxygen species (ROS) plays an important role in the process of human skin aging [1,2]. The skin is an interface between the body and the environment and is constantly exposed to atmospheric oxygen, ultraviolet (UV) irradiation, pollutants and xenobiotics. Oxidative damage caused by these exogenous sources can impair skin structure and function, leading to the phenotypic features of extrinsic aging [1]. In addition, excessive consumption of alcohol, improper diet, physical inactivity and mechanical stress can contribute to oxidative damage of skin [3-5].

The endogenous defense systems against ROS are often insufficient to combat oxidative processes in mature skin. ROS that are not neutralized can target biomolecules and lead to cellular dysfunction or death and accelerated aging [6]. Furthermore, ROS can induce the overexpression of MMPs, which increase collagen fragmentation and can downregulate TGF-β and promote collagen synthesis [7]. ROS can also increase elastase activity and trigger wrinkle formation by cleaving elastin and further degradation of collagen fibrils in the ECM [5,7]; it may also be an essential causative factor for hyperpigmentation or even carcinogenic processes in the skin [8].

*Ganoderma lucidum* (Curtis) P. Karst. (lingzhi) is a medicinal mushroom with a wide variety of bioactive components, possessing nutraceutical and pharmacological benefits [9]. Popularly known as the "mushroom of immortality", *G. lucidum* has been widely used in China for medicinal purposes for more than 2000 years [10]. Historically, lingzhi was grown in the wild and consumed only by wealthy individuals. Nowadays, large quantities are cultivated for production at an industrial level and are widely consumed [10]. *G. lucidum* is used in cancer treatment as an alternative to or alongside modern medicine to enhance the immune response, alleviate the side effects of chemotherapy and protect cellular DNA by increasing cellular antioxidant capacity [11]. Moreover, it has been used in the prevention of hypertension and hypercholesterolemia, treatment of diabetes, maintenance of gut health and stimulation of probiotics [9,10]. The use of bioactive extracts from mushrooms in the design of cosmeceutical formulations for topical application is receiving increasing attention [12-14]. Modern trends in the cosmetics industry prioritize ingredients or extracts from natural sources with nontoxic effects and the ability to delay the aging process [12,14].

The objective of the present study was to investigate the potential therapeutic effect on mature skin of polysaccharide extracts from the fruiting bodies of a wild European source of *G. lucidum*. These effects are expected to influence oxidative stress via the potential antiradical activity of the extracts and inhibitory effect on tyrosinase and enzymes responsible for ECM degradation.

## MATERIALS AND METHODS

### Reagents and chemicals

DPPH, ABTS, MTT, porcine pancreatic elastase type I [EC 3.4.21.36], (N-methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone, MAAPVN, collagenase from *Clostridium histolyticum* [EC 3.4.24.3], tyrosinase from mushroom [EC 1.14.18.1], FALGPA, EGCG, L-DOPA, potassium persulfate, ferrous sulfate, potassium ferricyanide, ferric chloride, trifluoroacetic acid (TFA), polyoxyethylene sorbitan monolaurate (Tween 20), silica gel H plates, ferrous chloride, ferrozine, 2-propanol, acetone, lactic acid, DMSO, $D_2O$ (deuteration degree min 99.96%), RPMI-1640, HEPES, streptomycin, penicillin, L-glutamine, Fast Blue BB diazonium dye, Coomassie Brilliant Blue G-250, standards including BSA (98% electrophoresis purity), gallic acid (97.5-102.5% by titration), (+)-catechin, kojic acid (≥99%), ascorbic acid (≥99%), EDTA (≥99%), and DSS were purchased from Merck KGaA (Darmstadt, Germany). D-Glucuronic acid (D-GlcA), D-Galacturonic acid (D-GalA), D-Glucose (D-Glc), D-Galactose (D-Gal), D-Mannose (D-Man), L-Fucose (L-Fuc), D-Xylose (D-Xyl), D-Arabinose (D-Ara), L-Rhamnose (L-Rha), and linoleic acid (analytical grade) were from Fluka (Buchs, Switzerland). Absolute methanol (Methanol Optigrade) was provided by LGC Promochem (Germany). ZelluTrans/Roth1 dialysis membranes were from Carl Roth GmbH & Co. KG (Germany). HaCaT cells were obtained from CLS-Cell Lines Service GmbH (Eppelheim, Germany). An analytical mushroom β-glucan kit was obtained from Megazyme Int. (Wicklow, Ireland).

### Mushroom material

Fresh wild-growing fruiting bodies of mushroom *G. lucidum* were collected from the forest/park Košutnjak near Belgrade, Republic of Serbia. After collection, carpophores were identified according to the methods of classical herbarium taxonomy to confirm the correct species [15]. The representative voucher specimens were deposited at the herbarium of the Department for Industrial Microbiology at the Faculty of Agriculture-University of Belgrade (under reference number GLK-12) together with their mycelial cultures, and stored at 4°C in the culture collection for mushrooms until further use. Samples were brush-cleaned, air-dried at 40°C to a constant mass and ground into fine powder prior to extraction and analysis.

### Preparation of extracts

Crude polysaccharide extracts were prepared by hot water extraction of 100 g of powdered sample [16]. After centrifugation, the resulting pellets were washed with ethanol 70% (v/v), dried under a vacuum and stored in the cold as hot water-extracted crude polysaccharides, HWCP. A portion of the HWCP sample was dialyzed against Milli-Q water (MQ) for 24 h at room temperature to remove residual small molecules as polyphenols, peptides and polysaccharides <8-10 kDa. After centrifugation, high molecular weight polysaccharides were ethanol precipitated and vacuum dried. This material was partly purified and designated HWPP. Alkali-soluble polysaccharides were extracted from the original hot water filter cake by autoclaving in 2 L of 1 M NaOH solution [16]. This extract was designated HWAP.

### Chemical composition of extracts

To explain the differences observed, we measured: (i) the total polysaccharide content of the extract using the phenol-sulfuric acid method [17], with results expressed as g of Glc equivalents per 100 g of dry weight (DW) of the extract; (ii) the protein content by the Bradford method [18], with results expressed as g of bovine serum albumin (BSA) equivalents per 100 g of DW of the extract; (iii) the total phenolic content using a Fast Blue BB (FBBB) method [19], with the content of total phenols expressed as gallic acid equivalents (GAE) per 100 g of DW of the extract; (iv) the flavonoid content [19], with results expressed as g of catechin equivalents (CEs) per 100 g of extract; (v) the content of total α- and β-glucans, after prior partial hydrolysis, using the Mushroom and Yeast β-glucan Assay Procedure K-YBGL 07/11.

### FT-IR spectroscopy

FT-IR spectra were obtained using a Fourier transform infrared spectrophotometer (Thermo-Nicolet Model 6700, Thermo Scientific, USA), equipped with a Smart Orbit (Diamond) ATR accessory and OMNIC 7.3 software, in the 4000-400 $cm^{-1}$ range at a resolution of 4 $cm^{-1}$ in transmission mode.

### Analysis of monosaccharide composition

TLC and NMR spectroscopy were used to determine the monosaccharide composition. Each polysaccharide extract was hydrolyzed separately with 2 M TFA at 121°C. After hydrolysis, the final residue was dissolved in 2 mL of MQ and used for further analysis. TLC was run on silica gel H plates [16]. Authentic sugars were: D-GlcA, D-GalA, D-Glc, D-Gal, D-Man, L-Fuc, D-Xyl, D-Ara, and L-Rha. NMR spectra were measured on a Bruker AVANCE III 500 spectrometer (500.26 MHz for $^1H$ nuclei), using a 5 mm broad-band probe head at 25°C in $D_2O$ with DSS as the internal standard [20]. Signals were assigned according to the web-based version of the computer program CASPER (Widmalm Research Group, Stockholm, Sweden) [21].

### Evaluation of radical-blocking capacities

Five methods were used to evaluate radical-blocking capacities: DPPH free radical ($DPPH^{\bullet}$) scavenging ability [20], inhibition of LPx in a linoleic acid model system [20], FRAP assay [20], ferrous-ion chelating ability [20], and $ABTS^+$ radical ($ABTS^{+\bullet}$) scavenging ability [22]. Results were expressed as $EC_{50}$ (mg/mL) values, representing the effective concentrations of each extract required to show 50% antioxidant activities. Extracts were analyzed at the concentration range of 0.04-5 mg/mL. Ascorbic acid and EDTA were used as positive controls.

### Enzyme inhibition activities

#### Assay of tyrosinase activity

Tyrosinase activity [23] was determined in a 96-well plate using an absorbance microplate reader (ELx808, BioTek Instruments, Inc., USA) controlled by Gen5™ Software to measure absorbance at 475 nm. An aliquot of the extract (40 µL) in 0.067 M of phosphate buffer (pH 6.8) containing 5% DMSO was incubated with 80 µL of phosphate buffer (pH 6.8) and 40 µL of tyrosinase (46 units/mL) at 23°C for 10 min. Next, 40 µL of 2.5 mM L-DOPA in 0.067 M of phosphate buffer (pH 6.8) was added. Extracts were analyzed at the concentration range of 0.04-5 mg/mL, using kojic acid as a reference. The anti-tyrosinase activities of the extracts were expressed as $IC_{50}$ values, which were calculated using linear regression analyses as the concentration of extract required for 50% inhibition *in vitro*.

#### Assay of elastase activity

An extract of appropriate concentration (50 µL) in 0.2 M tris-HCl buffer (pH 7.6) containing 5% DMSO was incubated with 100 µL of tris-HCl buffer (pH 7.6) and 25 µL of elastase (0.3 units/mL) at room temperature for 20 min, after which 25 µL of the substrate MAAPVN (10 mM) was added and the plates incubated for a further 40 min at 25°C [24]. Absorbance was read at 405 nm. Extracts were analyzed at a concentration range of 0.04-5 mg/mL. (N-methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone was used as a reference. Anti-elastase activities were expressed as $IC_{50}$ values.

#### Assay of collagenase activity

Extracts of appropriate concentration (0.04-5 mg/mL) were dissolved in 50 mM of Tricine buffer (pH 7.5 with 0.4 M NaCl, 10 mM $CaCl_2$) containing 5% DMSO [25]. Next, 20 µL of collagenase (0.16 units/mL) was allowed to react with 40 µL of each extract and 70 µL of Tricine buffer in the dark at 37°C for 20 min [25]. After preincubation, 40 µL of 0.8 mM FALGPA were added to each well and the microplate was further incubated in the dark at 37°C for 30 min. Absorbance was measured at 340 nm. EGCG was used as a positive control, and anti-collagenase activities were expressed as $IC_{50}$ values.

### Cytotoxicity analysis

Stock solutions of polysaccharide extracts were prepared in DMSO [19]. HaCaT cells were cultured as a monolayer in nutrient medium at 37°C in 5% $CO_2$ and a humidified air atmosphere. HaCaT (5,000 c/w) cells were seeded into 96-well microtiter plates and incubated for 20 h before different concentrations of investigated extracts were added to the wells in the range 0.0625-2 mg/mL. Nutrient medium alone was added to the cells in the control wells. Corresponding concentrations of extracts in nutrient medium but without cells were used as blanks. Cell survival was determined by MTT testing [26], 24 h after the investigated extracts were added. The data were expressed as the concentration of sample required to decrease cell viability by 50% ($IC_{50}$) compared with the controls.

### Statistical analysis

All experiments were carried out in triplicate and expressed as the mean±standard deviation (SD). Statistical analyses were performed with the Statistica 8.0 software package (StatSoft Inc., Tulsa, OK), using one-way analysis of variance (ANOVA) for all collected data. Differences between the means for each treatment were determined using Duncan's multiple range tests ($P<0.05$). The correlation coefficients ($r$) between antioxidant activities and polysaccharide fractions were determined.

## RESULTS

### Extraction yields

Extraction yields of HWCP, HWPP, and HWAP from *G. lucidum* were 1.8±0.4, 0.4±0.1 and 6.2±0.7 g/100 g of mushroom DW, respectively. HWPP was obtained from HWCP by dialysis using a dialyzing membrane that allowed molecules <8-10 kDa to pass through. Thus, the removal of low molecular mass compounds led to a significant reduction of about 80% of the yield of the crude hot water extract. Hot alkaline extraction enables intensive degradation of the cell wall and transforms water-insoluble compounds into water-soluble components [16], resulting in a significantly higher yield of extract (about 70%) compared with hot water extraction.

## Chemical composition of *G. lucidum* polysaccharide extracts

The chemical compositions of *G. lucidum* extracts are presented in Table 1. Carbohydrates were the most abundant compounds, followed by proteins, phenols and flavonoids. HWPP yielded the highest content of total carbohydrates (82.7±2.8 g/100 g) with molecular masses greater than 8-10 kDa after dialysis. HWAP had a significantly lower carbohydrate content (27.9±1.3 g/100 g DW) compared with HWCP and HWPP. The glucan fraction comprised between 46% and 61% of the carbohydrate content in all three extracts. Given the powdery nature of the material, the glucan fraction could be readily extracted and isolated; it consisted almost exclusively of β-glucans.

Phenols were found in considerable quantities in all extracts. The highest content was detected in HWCP (Table 1). The phenol content in HWPP was statistically different and approximately 43% lower than that in HWCP. Dialysis and ethanol reprecipitation were insufficient for the removal of small polyphenols from HWPP, indicating possible formation of polysaccharide-polyphenol conjugates. The flavonoid content of the tested samples was ranked in the order of HWCP>HWAP>HWPP, being about 75%, 83% and 69% of the total phenolics, respectively.

Proteins were present in all extracts (Table 1). Deproteinization of the polysaccharide fractions was not achieved with thermal treatment (autoclaving at 120°C for 2 h). Dialysis was also insufficient for the removal of all proteins in HWPP, resulting in a 38% reduction in the total protein content of HWCP.

## FT-IR spectroscopy

The FT-IR spectra of polysaccharide extracts HWCP, HWPP, and HWAP isolated from *G. lucidum* showed typical patterns of polysaccharides with similar structural characteristics (Fig. 1). The FT-IR spectrum of HWCP showed a wide intense absorption band at 3000 $cm^{-1}$ to 3500 $cm^{-1}$, characteristic for -OH stretching vibration related to inter- and intramolecular hydrogen bonds with a peak at 2950-2850 $cm^{-1}$, which corresponded to $CH_2$ stretching [27,28]. The peaks at 1200-1000 $cm^{-1}$ were related to C-O absorption and ring vibrations, and included a prominent sharp peak at 1079 $cm^{-1}$, characteristic for stretching of C-O and C-C bonds in beta-linked glucose residues; a peak at 1055 $cm^{-1}$ corresponded to C-O-C stretching [20,27,28]. The weak absorptions at 890 $cm^{-1}$ and 850 $cm^{-1}$ were specific for β- and α-glycosidic linkages, respectively [20,27,28]. The presence of residual proteins can be attributed to bands at 1642 $cm^{-1}$, 1455 $cm^{-1}$ and 1403

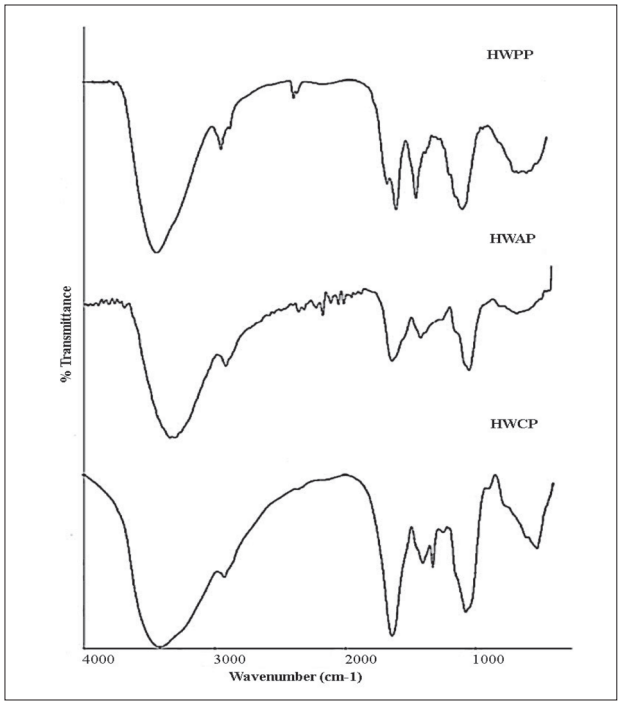


**Fig. 1.** FT-IR spectra of polysaccharide extracts isolated from *G. lucidum*.

**Table 1.** Chemical composition of polysaccharide extracts derived from the fruiting body of a wild European source of *G. lucidum*.

| Extract | Total polysaccharides | Glucan content | | | Total proteins | Total phenolics | Total flavonoids |
|---|---|---|---|---|---|---|---|
| | | Total | α | β | | | |
| HWCP | 40.7±2.2[b] | 23.4±1.1[b] | 1.8±0.3[b] | 21.6±0.8[b] | 3.9±0.3[a] | 4.5±0·2a | 3.4±0.2[a] |
| HWPP | 82.7±2·8a | 39.4±0.7[a] | 3.2±0.6[a] | 36.2±1.2[a] | 2.4±0.1[b] | 2.6±0.1[c] | 1.8±0.2[c] |
| HWAP | 27.9±1·3c | 13.9±1.0[c] | 1.7±0.5[b] | 12.2±0.7[c] | 1.0±0.1[c] | 3.0±0.1[b] | 2.5±0.1[b] |

All values are expressed per dry weight of extract (g/100 g). Data are the mean±SD (n=3). Within the same column, means followed by different letters are significantly different at $P<0.05$.

**Table 2.** Quantitative ratio of the monosaccharide components after acid hydrolysis in samples HWCP, HWPP and HWAP according to their $^1$H-NMR spectra.

| Extract | Monosaccharide α/β | | | | % Aromatic compounds | Aliphatic compounds*** |
|---|---|---|---|---|---|---|
| | Glc | Gal | Man | Fuc and/or Xyl ** | | |
| HWCP | 1*/1.6 | - | 0.17/- | - | 35 | + |
| HWPP | 1/1.5 | 0.13/0.13 | 0.22/0.1 | -/0.13 | 5 | + |
| HWAP | 1/1.6 | 0.1/0.25 | 0.23/0.22 | 0.16/0.25** | - | ++ |

* H(1)-integral of α-Glc is calibrated to 1. ** Signals overlapped; assignation uncertain. *** Many overlapping signals; the presence notified.

cm$^{-1}$ [20]. The characteristic N-H vibration at 3400 cm$^{-1}$ was overlapped by stretching of the O-H vibration at 3000-3500 cm$^{-1}$ [20]. The frequency at 1642 cm$^{-1}$ also corresponded to the bending vibration O-H of adsorbed water molecules. Additionally, the peak at 1642 cm$^{-1}$ was due to overlap with absorptions of C=C and C=O stretching vibrations, indicative of aromatics [20]. Vibrations between 1410 cm$^{-1}$ and 1310 cm$^{-1}$ were indicative of O-H groups of phenolic compounds, as was a band at 1403 cm$^{-1}$, which was related to aliphatic groups originating from the aromatic pigments [20].

The FT-IR spectrum of the HWPP sample showed the same characteristic absorption bands as the HWCP sample, except that absorption at 1642 cm$^{-1}$ related to the presence of the protein was somewhat reduced when compared with the FT-IR spectrum of HWCP, indicating that a certain amount of protein molecules had been removed by dialysis. However, the presence of bands for amide I at 1642 cm$^{-1}$ and amide II at 1568 cm$^{-1}$ were indicative of the presence of some residual protein in this polysaccharide extract. Absorptions that were related to the frequency of phenolic compounds were also altered to some extent.

The FT-IR spectrum of HWAP retained similar structural features, with absorptions related to the polysaccharide structure as well as to the content of proteins and phenolic compounds, although the intensity of some absorptions was altered. The peak at 1080 cm$^{-1}$ characteristic for β-glycosidic bonds was reduced, probably due to alkaline degradation of polysaccharide molecules during extraction [29]. In addition, a characteristic weak peak at 890 cm$^{-1}$ corresponding to β-glycosidic linkages had disappeared, indicating that hot alkaline treatment of polysaccharide caused degradation, especially at the basic chain [16].

The absence of a characteristic absorption band related to ester-carbonyl stretching absorption at 1740 cm$^{-1}$ in the FT-IR spectra [20] of all three samples indicated that the investigated polysaccharide extracts did not contain uronic acid in their structure, which was also confirmed by TLC.

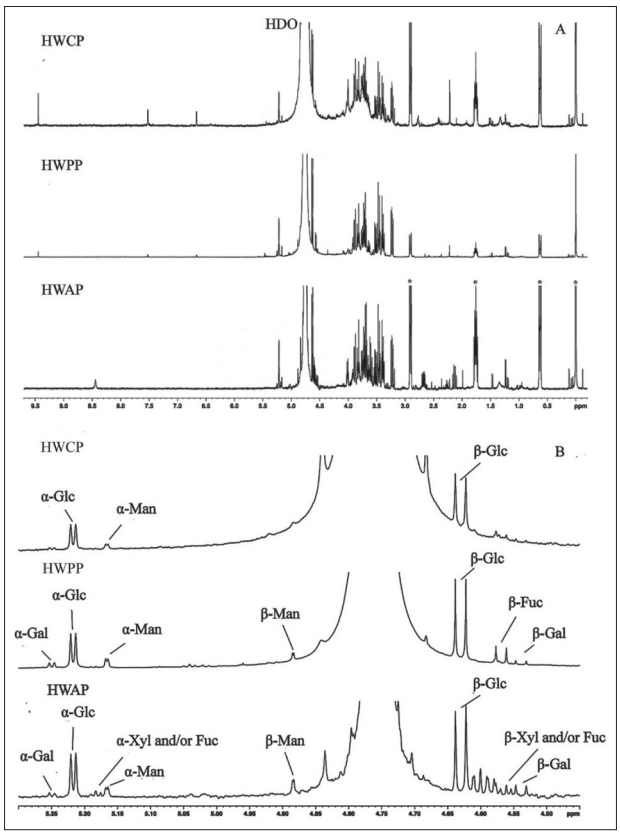


**Fig. 2. A** – The full $^1$H-NMR spectra of HWCP, HWPP and HWAP upon acid hydrolysis (the signals labeled with asterisk originate from 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt-DSS). **B** – The $^1$H-NMR spectra-anomeric regions.

## Monosaccharide composition

All three polysaccharide extracts from *G. lucidum* showed D-Glc as the predominant component after total acid hydrolysis, with smaller amounts of D-Gal and

**Table 3.** $EC_{50}$ and $IC_{50}$values of polysaccharide extracts from *G. lucidum* in radical-blocking properties and enzyme inhibition.

| **Properties** | **$EC_{50}$ (mg extract/mL)±SD** | | |
|---|---|---|---|
| | **HWCP** | **HWPP** | **HWAP** |
| DPPH• scavenging ability | 0.06±0.001[a] | 0.09±0.009[c] | 0.08±0.000[b] |
| $ABTS^{+\bullet}$ scavenging ability | 0.07±0.00[a] | 0.11±0.01[b] | 0.10±0.01[b] |
| Inhibition of LPx | 0.89±0.07[a] | 0.91±0.06[a] | 4.84±0.32[b] |
| FRAP | 0.49±0.13[a] | 0.61±0.11[a] | 1.30±0.17[b] |
| Chelating ability | 1.40±0.12[a] | 3.61±0.23[b] | 1.23±0.29[a] |
| **Enzyme inhibition** | **$IC_{50}$ (mg extract/mL)±SD** | | |
| Anti-tyrosinase activity | 0.37±0.05[a] | 0.85±0.07[c] | 0.62±0.07[b] |
| Anti-elastase activity | 1.01±0.09[a] | 1.55±0.16[b] | 2.11±0.31[c] |
| Anti-collagenase activity | 0.49±0.06[a] | 0.76±0.26[b] | 1.13±0.32[c] |

$EC_{50}$ value: the effective concentrations of each mushroom extract providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay; $IC_{50}$ value: concentration of extract required for 50% inhibition *in vitro*. Each value is expressed as the mean±SD (n=3). Means with different letters within a row are significantly different ($P<0.05$).

traces of D-Man and D-Fuc and/or D-Xyl, on the basis of TLC data, by comparison of their chromatographic mobility with monosaccharide standards. No uronic acid was found in the thin-layer chromatogram when a solvent for the separation of acidic components was used (chromatogram not shown).

The $^1$H-NMR spectra of HWCP, HWPP and HWAP extracts after acid hydrolysis were relatively similar (Fig. 2A). The monosaccharide components were identified and quantified based on their H-1 signals (Table 2, Fig. 2B). According to these data, D-Glc was the main constituent, with -anomer dominating in all three samples. Among the other monosaccharides, weak signals of D-Man, D-Fuc, and/or D-Xyl and D-Gal were also present.

## *G. lucidum* polysaccharide extracts as anti-aging agents

### Anti-radical potential

The results regarding the anti-radical activity of *G. lucidum* polysaccharide extracts are presented in Table 3. The higher the anti-radical capacity, the lower the value of $EC_{50}$. The mean values of $EC_{50}$ indicated that all extracts of *G. lucidum* were potent antioxidants. $EC_{50}$ values for the scavenging activities on DPPH• of the tested samples were in the order of: HWCP<HWAP<HWPP, and for the prevention on the generation of $ABTS^{+\bullet}$ they were: HWCP<HWPP≈HWAP. Furthermore, the extracts showed comparable values of $EC_{50}$ with ascorbic acid (0.08±0.001 mg/mL) in scavenging activities of DPPH• and prevention of $ABTS^{+\bullet}$ generation (0.06±0.000 mg/mL). HWCP and HWPP were potent inhibitors of LPx, exerting an almost two-fold higher potential for LPx inhibition compared to ascorbic acid (1.65±0.08 mg/mL), a common additive in cosmeceutical formulations and used at mg levels. There was no significant difference in $EC_{50}$ values for the reducing power of HWCP and HWPP. Ascorbic acid showed a lower $EC_{50}$ value in the FRAP assay (0.056±0.007 mg/mL) and higher reducing power than the analyzed extracts. Likewise, no significant difference at $P<0.05$ was found between the $EC_{50}$ values of HWCP and HWAP with respect to chelating ability. For comparison, the chelator EDTA, widely used in the cosmetics industry, showed a higher activity ($EC_{50}$<0.04 mg/mL).

### Correlation between the anti-radical potential and components of polysaccharide extracts

Regression analysis revealed a highly significant correlation between the $EC_{50}$ of scavenging activities on DPPH• and prevention of $ABTS^{+\bullet}$ generation and total phenols and flavonoids, (Table 4). A decrease in $EC_{50}$ value correlated with higher phenol and flavonoid contents. A significant correlation was also observed for total proteins. In contrast, regression analyses revealed a significant role for the carbohydrate components of the extracts in the inhibition of LPx in the linoleic acid model system and FRAP assay. A highly significant correlation was observed for total protein in both assays. A highly significant correlation was also observed between the chelating ability and the flavonoid content (Table 4).

**Table 4.** Correlation coefficient (*r*) between total polysaccharide, glucan, protein, phenol and flavonoid content vs. $EC_{50}$ values in antioxidant activities of HWCP, HWPP and HWAP.

| | correlation coefficient, *r** |
|---|---|
| **$EC_{50}$ in DPPH$^{\cdot}$ scavenging ability** | |
| polysaccharides | +0.391 |
| glucans | +0.355 |
| proteins | -0.635 |
| phenols | -0.938 |
| flavonoids | -0.957 |
| **$EC_{50}$ in $ABTS^{+\cdot}$ scavenging ability** | |
| polysaccharides | +0.341 |
| glucans | +0.309 |
| proteins | -0.657 |
| phenols | -0.892 |
| flavonoids | -0.836 |
| **$EC_{50}$ in inhibition of LPx** | |
| polysaccharides | -0.799 |
| glucans | -0.818 |
| proteins | -0.847 |
| phenols | -0.314 |
| flavonoids | -0.069 |
| **$EC_{50}$ in FRAP** | |
| polysaccharides | -0.678 |
| glucans | -0.675 |
| proteins | -0.835 |
| phenols | -0.380 |
| flavonoids | -0.136 |
| **$EC_{50}$ in chelating ability** | |
| polysaccharides | +0.929 |
| glucans | +0.928 |
| proteins | +0.073 |
| phenols | -0.567 |
| flavonoids | -0.720 |

*r** – all values are statistically significant ($P<0.05$); for absolute values of *r*, 0-0.19 is regarded as a 'very weak', 0.2-0.39 as a 'weak', 0.40-0.59 as a 'moderate', 0.6-0.79 as a 'strong', and 0.8-1 as a 'very strong' correlation.

## Enzyme inhibition activities

The inhibitory activities for tyrosinase, elastase and collagenase exhibited by HWCP, HWPP, and HWAP are presented in Table 3, and they show that all extracts had the ability to inhibit these enzymes. HWCP displayed the strongest inhibitory activity among the investigated extracts and exhibited a moderate potential when compared with standard inhibitors. The anti-tyrosinase $IC_{50}$ value of kojic acid, which is currently used in topical dermatological products, was 0.079±0.004 mg/mL, while an $IC_{50}$ value of 0.23±0.05 mg/mL was obtained for (N-methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone, a common irreversible inhibitor of human leukocyte and neutrophil elastase that was found to be an effective inhibitor of porcine elastase in the present study. The $IC_{50}$ value of EGCG, which has anti-collagenase activity, was 0.068±0.03 mg/mL. This compound is an established ingredient in dermocosmetics to prevent skin aging and maintain skin balance.

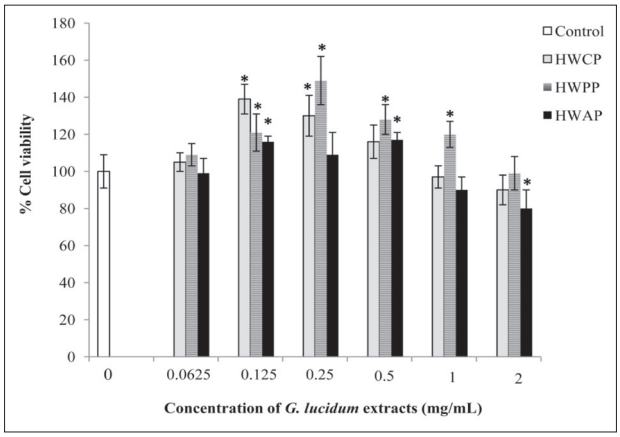


**Fig. 3.** Viability of HaCaT cells after treatment with polysaccharide extracts. Data are expressed as the mean±SD (n = 3). Significantly different from the control, *$P<0.05$ after 24 h.

## Cytotoxicity analysis

*G. lucidum* extracts were subjected to an *in vitro* cytotoxicity assay in HaCaT cells (Fig. 3). After incubation for 24 h, HWCP, HWPP and HWAP were found to be nontoxic to cells at concentrations up to 2 mg/mL. High levels of cell viability (more than 80%) were observed, meaning that $IC_{50}$ values could not be determined for the extracts. Furthermore, HWCP and HWPP induced significant proliferation of up to 50% in HaCaT cells at the concentration range 0.125-0.250 mg/mL ($P<0.05$), as compared with the control.

## DISCUSSION

A major barrier to the acceptance of natural products in the pharmaceutical and cosmetic industries is their complexity. However, this complexity can bring significant advantages. For example, certain components of natural products can reduce the cytotoxicity of the whole product, and interactions between different biologically active components can be responsible for

their *in vivo* effects [30]. Moreover, synthetic skincare products containing active ingredients may cause adverse reactions such as allergic contact dermatitis, irritant contact dermatitis, phototoxic, photoallergic reactions, or even infections [31]. The demand for multifunctional products is also driving innovation in the cosmetic industry, as price-conscious consumers are opting for products that can provide sufficient vitality and skin protection.

The results of the present research highlight the potential capacity of polysaccharide fractions derived from the fruiting bodies of a wild European source of *G. lucidum*: for free radical blocking, which may help to strengthen the skin's barrier; for a skin-lightening effect as inhibitors of tyrosinase, and for the anti-collagenase and anti-elastase activities to help restore skin elasticity and tensile strength.

Moreover, no obvious toxicity was observed in HaCaT keratinocytes, which represent the first line of defense of the body against the outside environment [32]. This is the first report of the preventive effect of *G. lucidum* originating from Serbia in the potential development of cosmeceutical formulations and the slowing of skin aging.

The presence of different bioactive molecules from the classes of polysaccharides, proteins and phenolic and flavonoid compounds in the HWCP, HWPP and HWAP fractions may correlate with their observed bioactivities.

Carbohydrates were the most abundant compounds in the extracts. A significant and strong correlation was observed between polysaccharides and the prevention of LPx in a linoleic acid model system. The ROS-scavenging mechanism of polysaccharides may be similar to that of phenol compounds, i.e. via H atom transfer (HAT) reactions [33]. However, HAT reactions are more likely to occur in neutral polysaccharides, which were also observed in the investigated fractions, while electron transfer (ET) typically occurs in acidic polysaccharides [33]. Additionally, chain-breaking/free radical scavengers can act via secondary or preventive mechanisms, which may include inhibition of lipid hydroperoxide breakdown to unwanted volatile products, regeneration of primary antioxidants, singlet oxygen quenching and deactivation of metals [6].

All three polysaccharide extracts were shown to be good sources of β-glucans. β-glucans, despite their considerable molecular weight, are known to enter the *stratum corneum* and epidermis, penetrating deep into the dermis [34]. β-glucans do not directly enter the cell, but penetrate skin through the intercellular space. It has been suggested that β-glucans form a thin film above the *stratum corneum* and epidermis to promote moisturization [34]. Within the dermis, they can stimulate collagen synthesis through direct interaction with fibroblasts and through indirect cytokine-mediated interaction with macrophages [34]. Collagen synthesis is one possible mechanism by which the elasticity of the skin is enhanced.

Polyphenols were present in all investigated extracts, even after purification procedures to remove molecules with molecular masses lower than 8-10 kDa. Natural polyphenolic compounds are the most abundant antioxidants, and they exert their antioxidant effect by quenching free radical species and/or promoting the endogenous antioxidant capacity [6]. The ABTS and DPPH assays are typically classified as SET reactions, but these two indicator radicals may in fact be neutralized either by direct reduction via electron transfers or by radical quenching via the HAT mechanism [35]. Furthermore, polyphenolics can modulate the activity of nuclear factor kappa B (NF-κB) or sirtuin 1 (SIRT1), thus exerting protective effects [6,36]. SIRT1 acts as a "rescue gene", capable of repairing damage caused by the action of free radicals and of preventing the premature death of cells [6]. SIRT1 in the epidermis regulates cell migration, redox response, inflammation, epidermis re-epithelialization, and granulation formation, it and plays a crucial role in wound repair [36]. It is an important regulator of the keratinocyte differentiation pathway and a potential regulator of skin aging [37].

Flavonoids, subclasses of phenols, were also detected in all three extracts. A strong and significant correlation was observed between ferrous-ion-chelating ability and flavonoid content. Metal ions are required for tyrosinase and collagenase activity [38]. In contrast, however, metal ions contain unpaired electrons that react quickly with peroxides and form alkoxyl radicals in the body [39]. Natural flavonoids can form stable metal complexes through their multiple OH groups

and carbonyl moiety, whenever present [39]. The use of natural chelators such as flavonoids is preferred to the use synthetic counterparts, due to reduced toxicity [40]. Likewise, natural flavonoids have a photoprotection potential because of their ability to absorb UV light and to act as direct and indirect antioxidants as well as anti-inflammatory and immunomodulatory agents, thus providing an exciting platform for the development of photoprotection [41].

It has been confirmed that mushroom extracts are a promising source of natural phenolic and flavonoid compounds [42-45]. Hot water extracts of medicinal mushrooms can be a source of bioactive flavonoids that could not be isolated at lower temperatures. Thus, daidzein was identified in *Trametes versicolor* hot water extract [45]. Also, amentoflavone and catechin were isolated in two-fold greater concentrations, as compared with the ethanol extract of the same mushroom [45].

Dialysis and thermal treatment (autoclaving at 120°C) were insufficient to remove all proteins from the extracts. Proteins can provide protection against harmful ROS, as many amino acids possess a strong antioxidant potential due to the presence of polar R groups [46]. Bioactive peptides might be effective inhibitors of enzymes that are involved in the turnover of ECM proteins and melanin production [47].

All three extracts showed no toxicity to keratinocytes *in vitro*. Our previous report confirmed a lack of toxicity up to 10 mg/mL of the commercial water extract obtained from an industrial strain of *G. lucidum* (Fujian, China) [48]. Furthermore, this extract stimulated the *in vitro* proliferation of HTR-8/SVneo cells, which are essential for normal placentation, establishment of pregnancy, and maintenance of fetal growth in humans [49].

Finally, the results of the present study strongly support the existing scientific data on the use of ingredients from natural sources as anti-aging agents and their application in the cosmeceutical industry. The polysaccharide extracts of *G. lucidum* may represent promising alternative raw materials for use in cosmetic products. However, these findings require further verification in clinical trials to examine the stability, skin permeation, and efficacy of the final cosmeceutical product.

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**Author contributions:** MK participated in all the experiments, wrote the draft and performed the literature search; AK participated in all the experiments and reviewed the manuscript; DJ performed the chemical analysis, FTIR spectroscopy and reviewed the manuscript; NT performed the NMR analysis and reviewed the manuscript; WAAQIWM performed the literature search and critically reviewed the manuscript; MN supervised all phases of the research and reviewed the manuscript. All authors read and approved the final manuscript.

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