

**Antimicrobial and demelanizing activity of *Ganoderma lucidum*
extract, *p*-hydroxybenzoic and cinnamic acids and their synthetic
acetylated glucuronide methyl esters**

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ABSTRACT

Mushroom extracts or isolated compounds may be useful in the search of new potent antimicrobial agents. Herein, it is described the synthesis of protected (acetylated) glucuronide derivatives of *p*-hydroxybenzoic and cinnamic acids, two compounds identified in the medicinal mushroom *Ganoderma lucidum*. Their antimicrobial and demelanizing activities were evaluated and compared to the parent acids and *G. lucidum* extract. *p*-Hydroxybenzoic and cinnamic acids, as also their protected glucuronide derivatives revealed high antimicrobial (antibacterial and antifungal) activity, even better than the one showed by commercial standards. Despite the variation in the order of parent acids and the protected glucuronide derivatives, their antimicrobial activity was always higher than the one revealed by the extract. Nevertheless, the extract was the only one with demelanizing activity against *A. niger*. The acetylated glucuronide derivatives could be deprotected to obtain glucuronide metabolites, which circulate in the human organism as products of the metabolism of the parent compounds.

Keywords: *Ganoderma lucidum*; *p*-hydroxybenzoic acid; cinnamic acid; Acetylated glucuronides; Chemical synthesis; Antimicrobial activity.

1. Introduction

Nature has been a source of medicinal agents for thousands of years. During the last three decades the problem of antibiotic resistance has emerged. Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and nowadays, the need to discover new and more potent of these agents as accessories or alternatives to antibiotic therapy is stronger. Currently, natural compounds are on the focus of some biotechnological companies that are looking for new antimicrobial drugs (Butler, 2004; Lam, 2007). Mushrooms are rich sources of bioactive compounds with an enormous variety of chemical structures. In this respect, mushrooms isolated compounds could be useful in the search of new potent antimicrobial agents (Alves et al., 2012).

There are available in literature some studies reporting antimicrobial activity of different extracts of *Ganoderma lucidum* (Curtis) P. Karst from India (Sheena et al., 2003; Quereshi et al., 2010) and China (Gao et al., 2005). This species is one of the most famous traditional medicinal mushrooms, being used as functional food and in preventive medicines, mostly in the form of extracts with an annual global market value of over \$1.5 billion (Sullivan et al., 2006; Pala and Wani, 2011).

Otherwise, the antimicrobial activity of some phenolic compounds has been described (Lou et al., 2012; Orhan et al., 2010; Alves et al., 2013). *p*-Hydroxybenzoic acid was the most abundant phenolic acid found in wild *G. lucidum* from Portugal, as well as cinnamic acid (0.58 and 0.28 mg/100 g dry weight, respectively; Heleno et al., 2012). Furthermore, these compounds are present in several other mushrooms species (Barros et al., 2009).

Dietary phenolic compounds are widely considered to contribute to health benefits in humans. However, little is known about their bioactive forms *in vivo* and the

mechanisms by which they may contribute toward disease prevention. Moreover, many studies on the biological effects of phenolic compounds have ignored the question of their achievable concentrations in the circulation after ingestion as well as the possibility of metabolism (Rechner et al., 2002). There is accumulating evidence suggesting that phenolic compounds are rapidly metabolized in the human organism. Glucuronidation appears as one of the most prevalent metabolic pathways for phenolic compounds in humans (Rechner et al., 2002). Despite the large data concerning the antimicrobial effects of phenolic acids (Lou et al., 2012; Orhan et al., 2010; Alves et al., 2013), studies dealing with the antimicrobial properties of their metabolites or derivatives are scarce due to the fact that most of these compounds are not commercially available.

The present work aims at contributing to the knowledge of the mechanisms involved in the antimicrobial properties of phenolic compounds, namely phenolic acids and precursors, usually present in mushrooms. With that goal, the antimicrobial activity of *G. lucidum* extract, *p*-hydroxybenzoic and cinnamic acids and their acetylated glucuronide derivatives (protected glucuronides), prepared by chemical synthesis, was evaluated and compared.

2. Materials and methods

2.1. Wild mushroom

Samples of *Ganoderma lucidum* (Curtis) P. Karst. were collected in Bragança (Northeast Portugal) in July 2011. After taxonomic identification of the sporocarps (Phillips, 1981; Hall et al., 2003; Oria de Rueda, 2007), specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança under the number BRESA-gl01-2011. Fruiting bodies were further separated from spores using a

scalpel, lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), and reduced to a fine dried powder (20 mesh).

2.2. Preparation of the extract

The lyophilized *G. lucidum* sample (~10 g) was extracted with methanol (250 mL) at -20 °C for 6 h. The extract was sonicated for 15 min, centrifuged at 4000g for 10 min and filtered through Whatman No.4 paper. The residue was then re-extracted with three additional 150 mL portions of methanol. The combined extracts were evaporated (rotary evaporator Büchi R-210; Flawil, Switzerland) at 40 °C to dryness.

2.3. Compounds identified in *Ganoderma lucidum*

p-Hydroxybenzoic and cinnamic acids are two of the compounds identified in *G. lucidum* (Heleno et al., 2012). For the antimicrobial assays, these compounds were purchased from Sigma (St. Louis, MO, USA).

2.4. Synthesis of acetylated glucuronide derivatives (protected forms of *p*-hydroxybenzoic and cinnamic acids glucuronides)

2.4.1. 2,3,4-tri-*O*-acetyl-1-*p*-hydroxybenzoyl-*D*-glucuronic acid methyl ester (HAGP).

p-Hydroxybenzoic acid (0.100 g, 0.724 mmol), acetobromo- α -*D*-glucuronic acid methyl ester (0.574 g, 1.44 mmol) and potassium carbonate (0.100 g, 0.724 mmol) were dissolved in 10 mL of DMSO under argon and the mixture was stirred for 24h. The reaction mixture was diluted with 50 mL of ethyl acetate and then washed with water (7×10 mL). The organic layer was dried over MgSO₄ and the solvent was evaporated. The product obtained was purified by a column chromatography using silica gel 60A (60-200 micron) and a mixture of ether/petroleum ether (60/40, v/v) as eluent. The

product was isolated as a white solid (0.123 g, 38%). m.p.=125.9-126.2 °C. ¹H NMR (300 MHz, CDCl₃): δ= 2.00 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 3.73 (s, 3H), 4.30 (d, *J* = 9.6 Hz, 1H), 5.29 (t, *J* = 9.2 Hz, 1H), 5.34 (dd, *J* = 9.2 and 7.6 Hz, 1H), 5.42 (t, *J* = 9.2 Hz, 1H), 5.94 (d, *J* = 7.6 Hz, 1H), 6.80 (d, *J* = 8.8 Hz, 2H), 7.81 (d, *J* = 8.8 Hz, 2H) (*). ¹³C NMR (75.4 MHz, CDCl₃): 20.47 (OAc), 20.53 (OAc), 20.58 (OAc), 53.19 (OMe), 69.11 (CH), 69.94 (CH), 71.58 (CH), 72.77 (CH), 91.63 (CH), 115.41 (2×CH), 119.95 (C), 132.58 (2×CH), 161.28 (C), 163.99 (C=O), 167.53 (C=O), 169.45 (C=O), 169.56 (C=O), 169.91 (C=O). HRMS (ESI-TOF) calcd. for C₂₀H₂₂O₁₂ (M⁺+Na) 477.1004, found 477.0995.

(*). The proton of the OH group was not detected in the proton nmr spectrum.

2.4.2. 2,3,4-tri-*O*-acetyl-1-cinnamoyl-*D*-glucuronic acid methyl ester (CAGP).

Cinnamic acid (0.100 g, 0.675 mmol), acetobromo- α -*D*-glucuronic acid methyl ester (0.268 g, 0.675 mmol) and potassium carbonate (0.140 g, 1.01 mmol) were dissolved in 10 mL of DMSO under argon and the mixture was stirred for 24h. The reaction mixture was diluted with 50 mL of ethyl acetate and then washed with water (7×10 mL). The organic layer was dried over MgSO₄ and the solvent was evaporated. The product obtained was purified by a column chromatography using silica gel 60A (60-200 micron) and a mixture of ether/petroleum ether (50/50, v/v) as eluent. The product was isolated as a white solid (0.100 g, 32%). m.p= 169.8-170.2°C. ¹H NMR (300 MHz, CDCl₃): δ= 2.04 (s, 3H), 2.060 (s, 3H), 2.063 (s, 3H), 3.75 (s, 3H), 4.25 (d, *J* = 9.6 Hz, 1H), 5.27 (dd, *J* = 9.2 and 7.6 Hz, 1H), 5.31 (t, *J* = 9.2 Hz, 1H), 5.38 (t, *J* = 9.2 Hz, 1H), 5.92 (d, *J* = 8.0 Hz, 1H), 6.42 (d, *J* = 16.0 Hz, 1H), 7.41-7.43 (m, 3H), 7.53-7.56 (m, 2H), 7.78 (d, *J* = 16.0 Hz, 1H). ¹³C NMR (75.4 MHz, CDCl₃): 20.48 (OAc), 20.55 (OAc), 20.58 (OAc), 53.02 (OMe), 69.05 (CH), 70.12 (CH), 71.82 (CH), 73.06 (CH), 91.57

(CH), 116.08 (CH), 128.43 (2×CH), 129.00 (2×CH), 130.99 (CH), 133.82 (C), 147.66 (CH), 164.51 (C=O), 166.80 (C=O), 169.25 (C=O), 169.42 (C=O), 169.90 (C=O). HRMS (ESI-TOF) calcd. for C₂₂H₂₄O₁₁ (M⁺+Na) 487.1211, found 487.1212.

2.5. Antimicrobial activity

2.5.1. Antibacterial activity. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973), and *Micrococcus flavus* (ATCC 10240), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311), and *Enterobacter cloacae* (human isolate), were used. The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The antibacterial assay was carried out by a microdilution method (CSLI, 2006; Tsukatani et al., 2012) in order to determine the antibacterial activity of extract/compounds tested against the human pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10⁵ CFU/mL. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates by microdilution test. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10⁵ CFU/mL. Mushroom extract/compounds were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and added in Tryptic Soy broth (TSB) medium (100 µL) with bacterial inoculum (1.0×10⁴ CFU per well) to achieve the wanted concentrations (0.005-3 mg/mL for extract and 0.003-0.25 mg/mL for compounds). The lowest

concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MICs obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on reduction of a INT color and compared with positive control for each bacterial strains (CSLI, 2006; Tsukatani et al., 2012). The MBCs were determined by serial sub-cultivation of 2 μ L into microtitre plates containing 100 μ L of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. Streptomycin (Sigma P 7794) and Ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline). Five percent DMSO was used as a negative control.

2.5.2. *Antifungal activity.* *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate), were used. The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar and the cultures stored at 4°C and sub-cultured once a month. In order to investigate the antifungal activity of mushroom extract/compounds, a modified microdilution technique was used (Hanel et al., 1988; Espinel-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore

suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. Extract and compounds were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and added in broth Malt medium with inoculum (0.005-3 mg/mL for extract and 0.003-0.25 mg/mL for compounds). The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μL of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 μL of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, and commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1 - 3000 $\mu\text{g/mL}$).

2.6. Demelanizing activity using micromycetes

All microfungi tested for antifungal activity of *G. lucidum* extract were used to evaluate extract/compounds demelanizing activity. The micromycetes were maintained on malt agar and the cultures were stored at 4°C; 96-well microtiter plates were used. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to an approximate concentration of 1.0×10^5 in a final volume of 100 μL /well. Dilutions of the inocula were cultured on malt agar to verify the absence of contamination and to

check the validity of the inoculum. Determination of minimum demelanizing concentrations (MDC) was performed by a serial dilution technique. The extract/compounds were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and added in broth Malt medium with inoculum (0.005-3 mg/mL for extract and 0.003-0.25 mg/ml for compounds). The microplates were incubated at Rotary shaker (160 rpm) for 72 h at 28° C. A sample of mycelium was taken from the periphery of a colony grown on Malt extract medium enriched with different concentrations of tested extract. The samples were dried and fixed with lactophenol and observed under a light microscope (Mikroskop DMLS Typ 020 518 500. Leica, Wetzlar. Neubauer Zählkammer. Eppendorf, Hamburg, Germany) to examine structural abnormalities (Heleno et al., 2013). The lowest concentration that provoked demelanization of fungal hyphae and conidia was determined as MDC. Samples from the control plate without added extracts were also stained and observed. Solution of 5% DMSO was used as a negative control.

2.7. Statistical analysis

All the assays were carried out in triplicate and the results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1. Synthesis of acetylated glucuronide derivatives

p-Hydroxybenzoic acid (HA) was reacted with acetobromo- α -D-glucuronic acid methyl ester (2 equiv.) affording the corresponding 2,3,4-tri-*O*-acetyl-1-*p*-hydroxybenzoyl-D-glucuronic acid methyl ester (HAGP, **Figure 1**) in 38% yield after purification.

Cinnamic acid (CA) was also reacted with acetobromo- α -D-glucuronic acid methyl ester (1 equiv.) to give the corresponding 2,3,4-tri-*O*-acetyl-1-cinnamoyl-D-glucuronic acid methyl ester (CAGP, **Figure 1**) in 32% yield after purification. The need of more equivalents of the starting glucuronic acid methyl ester in the case of HA is related with the improvement in the reaction yield; with 2 equivalents of the starting reagent we were able to double the yield of the reaction from 16% (with 1 equivalent of the starting reagent) to 38%, but we also detected traces of the di-acetylated compound.

As far as we know, this is the first report on the synthesis of protected glucuronide derivatives of *p*-hydroxybenzoic and cinnamic acids, despite the existent report on the synthesis of a protected glucuronide derivative of ethyl-4-hydroxybenzoate ([Zhang et al., 2012](#)). Despite some studies describing the synthesis of glucuronide derivatives of flavonoids ([Needs and Kroon, 2006](#); [Kajjout and Rolando, 2011](#)), we could only find one paper reporting the synthesis of ferulic acid protected glucuronide and its acyl glucuronide, with 25% yield ([Piazzon et al., 2012](#)).

The yields obtained in the present study are quite good considering that glucuronidation reactions occur in animal metabolism, which involves the participation of specific enzymes allowing the natural occurrence of these compounds in the organism.

3.2. Antibacterial activity

The protected glucuronides prepared (HAGP and CAGP) were submitted to antimicrobial activity evaluation in order to compare the results with the parent acids and the mushroom extract in which they were identified. The results of antibacterial activity were presented in **Table 1**. *G. lucidum* methanolic extract was active against all the tested bacteria with minimal inhibitory concentrations of 0.0125-0.75 mg/mL and bactericidal concentrations of 0.035-1.5 mg/mL. *S. aureus* and *B. cereus* were the most

susceptible bacteria to *G. lucidum* extract, while *P. aeruginosa* was the most resistant. The extract showed higher activity against *S. aureus* and *B.cereus* than the antibiotics ampicillin and streptomycin. These results were better than the ones reported by [Quereshi et al. \(2010\)](#) and [Sheena et al. \(2003\)](#) that described the antibacterial activity of *G. lucidum* methanolic extract against *S. aureus* and *B. cereus* moderate and poor, respectively. The results against *P. aeruginosa* and *E. cloacae* were similar to the ones showed by ampicillin.

HA was active against all the tested bacteria with MICs of 0.003-0.03 mg/mL and MBCs of 0.007-0.06 mg/mL. The majority of the tested bacteria were susceptible to HA; *E. coli* and *L. monocytogenes* were the most resistant one. HAGP also revealed a good antibacterial activity against all the tested bacteria with MICs of 0.007-0.03 mg/mL and MBCs of 0.015-0.06 mg/mL. *S. typhimurium* was the most susceptible to HAGP, while *M. flavus*, *L. monocytogenes* and *E. coli* were the most resistant bacteria to this glucuronide derivative. The antibacterial activity of HAGP decreased in comparison with the activity of its parent compound, unless for *L. monocytogenes* and *E. coli*, in which the activity was maintained. HAGP and the parent compound HA showed better activity than the extract and, even, than the standards.

CA also revealed antibacterial activity against all the tested bacteria with MICs of 0.0007-0.015 mg/mL and MBCs of 0.0015-0.06 mg/mL. It revealed an excellent activity against *P. aeruginosa*, much better than the extract, HA, HAGP and even better than the two standards tested. Curiously, *P. aeruginosa* was the most resistant bacteria to *G. lucidum* extract, but it was the most susceptible to cinnamic acid. The most resistant bacteria to the latter compound were *M. flavus*, *L. monocytogenes* and *E. coli*. CAGP was also active against all the tested bacteria with MICs of 0.007-0.03 mg/mL and MBCs of 0.01-0.06 mg/mL. *B. cereus*, *S. typhimurium* and *E. cloacae* were the

most susceptible bacteria against CAGP, while *S. aureus*, *M. flavus*, *L. monocytogenes* and *E. coli* were the most resistant. Once more, the antibacterial activity of CAGP decreased in relation to the parent compound (cinnamic acid), but was better than the extract and the two standards tested.

It should be noticed that [Alves et al. \(2013\)](#) could not find antibacterial activity of HA and CA at 1 mg/mL against some of the herein tested bacteria: *E. coli*, *S. aureus* and *L. monocytogenes*, probably due to the different method used to screen the antimicrobial activity.

3.3. Antifungal activity

The antifungal activity of *G. lucidum* extract, prepared protected glucuronides and their parent acids was presented in **Table 2**.

The extract showed antifungal activity against all the tested fungi with MICs of 0.005-1.5 mg/mL and MFCs of 0.1-4.5 mg/mL. *T. viride* was the most susceptible fungi to the extract while *A. fumigatus*, *A. niger* and *P. verucosum* var. *cyclopium* were the most resistant. In the case of *T. viride*, the extract activity was better than the one of the standards, bifonazole and ketoconazole.

HA was active against all the fungi, showing MICs of 0.003-0.12 mg/mL and MFCs of 0.015-0.25 mg/mL. *A. versicolor* and *T. viride* were the most susceptible fungi to this phenolic acid, while *A. fumigatus* was the most resistant. HA showed higher activity than the extract and the two standards, for all the tested fungi.

HAGP also showed activity against all the fungi with MICs of 0.007-0.12 mg/mL and MFCs of 0.015-0.25 mg/mL. This compound gave a strong activity against *A. ochraceus*, *P. funiculosum* and *P. ochrochloron*, being *A. fumigatus* the most resistant

fungi. HAGP showed higher activity than the standards, the extract and even than the parent HA.

CA also has activity against all the tested fungi with MICs of 0.007-0.03 mg/mL and MFCs of 0.015-0.06 mg/mL. *A. fumigatus* was the most susceptible fungi, while *A. niger* and *P. ochrochloron* were the most resistant. This acid also gave better results than the extract and the standards, and in some cases, better than HA and HAGP.

CAGP showed antifungal activity against all the fungi with MICs of 0.007-0.06 mg/mL and MFCs of 0.015-0.25 mg/mL. *A. ochraceus* and *P. funiculosum* were the most susceptible fungi, while *A. fumigatus* and *P. verrucosum* var. *cyclopium* were the most resistant. Compared with its parent CA, the protected glucuronide maintained the activity, with the exception of *A. niger* and *P. ochrochloron*, in which the activity increased. Its antifungal activity was higher than the one revealed by the extract and the standards.

Other compounds present in *G. lucidum* have also been reported as antifungal, such is the case of ganodermin. Ganodermin is an antifungal protein isolated from *G. lucidum* with activity against phytopathogenic fungi such as *Botrytis cinerea* (IC₅₀=15.2 μM), *Fusarium oxysporum* (IC₅₀=12.4 μM) and *Physalospora paricola* (IC₅₀=18.1 μM) (Wang and Ng, 2006). Nevertheless, as far as we know this is the first report on antifungal activity of protected glucuronide derivatives of HA and CA.

3.4. Demelanizing activity

In order to investigate the demelanizing activity of *G. lucidum* extract and compounds as an important factor in fungal virulence, eight microfungi were used. Demelanizing activity was obtained only for *G. lucidum* extract toward *A. niger*.

The results were expressed as minimum demelanizing concentrations (MDC), which were defined as sublethal and subinhibitory concentration necessary to provoke demelanization in fungus during 72 h. The subinhibitory concentration was achieved at 0.75 mg/mL, while sublethal concentration was observed at 0.1 mg/mL of *G. lucidum* extract (**Figure 2a-f**). The colored conidiophores of some *Aspergillus* and *Penicillium* species contains pigments belonging to the group of melanins: a green colored chromoprotein and a black insoluble pigment (Eisenman and Casadevall, 2012). Melanin production by fungi contributes to the virulence of pathogens of humans as well as those of food crops (Rosa et al., 2010). It was shown that this pigment has an important role in the protection of the fungus against immune effector cells; it is able to scavenge reactive oxygen species generated by alveolar macrophages and neutrophils of the host (Brakhage and Liebmann, 2005). Morphological changes in melanization of *A. niger* are obvious from **Figure 2** and showed depigmentation; samples were treated with *G. lucidum* extracts at MDC (0.75 and 0.1 mg/mL). Observing morphological changes of conidiphores it was determined that demelanized cultures of tested fungi interestingly possessed unusually small number of heads (**Figure 2a, 2b**) in comparison to those in untreated culture (**Figure 2c**). The reduction of head numbers and demelanization of *A. niger* spores is also recorded under light microscope (**Figure 2d and 2e**) in compurgation with untreated control (**Figure 2f**). Thus, we may presume that the extracts of *G. lucidum* might directly be involved in the inhibition or modification of the mechanism of demelanization. The results for demelanizing activity are important, since MDC is sublethal to fungus being needed smaller doses of extract, in comparison to inhibitory and fungicidal doses.

Overall, HA, CA and their protected glucuronide derivatives (HAGP and CAGP) revealed high antimicrobial (antibacterial and antifungal) activity, even better than the one showed by commercial standards. Despite the variation in the order of parent acids and the protected glucuronide derivatives, their antimicrobial activity was always higher than the one revealed by the extract. Nevertheless, the extract was the only one with demelanizing activity against *A. niger*, which is certainly related to other compounds besides the ones mentioned in the present study. It should be highlighted that HA and CA are also present in other mushroom species and even in other matrices, which increases the general impact of the results reported herein. The synthesized acetylated glucuronide derivatives could be deprotected to obtain glucuronide metabolites, which circulate in the human organism as products of the metabolism of the parent compounds.

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Figure 1. Glucuronidation of *p*-hydroxybenzoic and cinnamic acids. HA- *p*-hydroxybenzoic acid; HAGP- *p*-hydroxybenzoic acid glucuronide protected form; CA- cinnamic acid; CAGP- cinnamic acid glucuronide protected form.

Figure 2. (a) Demelanized mycelium of *A.niger* treated with *Ganoderma lucidum* extract at 1 mg/mL; (b) Mycelium of *A.niger* treated with *G. lucidum* extract at 0.75 mg/mL; (c) Normal mycelium of *A. niger* without treatment; (d) Culture of *A. niger* with few amount of heads, treated with *G. lucidum* extract at 0.1 mg/mL; (e) Culture of *A. niger* with smaller amount of heads treated with *G. lucidum* extract at 0.75 mg/mL recorded under light microscope; (f) Typical culture of *A. niger* with numerous heads, recorded under light microscope (d-f).

Table 1. Antibacterial activity (MIC and MBC, mg/mL) of *Ganoderma lucidum* extract, *p*-hydroxybenzoic and cinnamic acids, and their synthesized acetylated glucuronide derivatives.

Bacteria	Extract	HA	HAGP	CA	CAGP	Streptomycin	Ampicillin
	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC	MIC MBC
<i>Staphylococcus aureus</i>	0.025 0.035	0.003 0.007	0.015 0.03	0.0015 0.003	0.03 0.06	0.04 0.09	0.25 0.37
<i>Bacillus cereus</i>	0.0125 0.035	0.003 0.007	0.015 0.02	0.0015 0.003	0.007 0.015	0.09 0.17	0.25 0.37
<i>Micrococcus flavus</i>	0.5 0.75	0.015 0.03	0.03 0.06	0.015 0.03	0.03 0.06	0.17 0.34	0.25 0.37
<i>Listeria monocytogenes</i>	0.3 0.75	0.03 0.06	0.03 0.06	0.007 0.06	0.03 0.06	0.17 0.34	0.37 0.49
<i>Pseudomonas aeruginosa</i>	0.75 1.5	0.003 0.007	0.015 0.03	0.0007 0.0015	0.007 0.03	0.17 0.34	0.74 1.24
<i>Salmonella typhimurium</i>	0.35 0.75	0.003 0.007	0.007 0.015	0.0015 0.003	0.007 0.015	0.17 0.34	0.37 0.49
<i>Escherichia coli</i>	0.35 0.75	0.03 0.06	0.03 0.06	0.007 0.06	0.03 0.06	0.17 0.34	0.25 0.49
<i>Enterobacter cloacae</i>	0.35 0.75	0.006 0.007	0.015 0.03	0.0015 0.003	0.007 0.01	0.26 0.52	0.37 0.74

HA- *p*-hydroxybenzoic acid; HAGP- *p*-hydroxybenzoic acid glucuronide protected form;

CA- cinnamic acid; CAGP- cinnamic acid glucuronide protected form.

Table 2. Antifungal activity (MIC and MFC, mg/mL) of *Ganoderma lucidum* extract, *p*-hydroxybenzoic and cinnamic acids, and their acetylated glucuronide derivatives.

Fungi	Extract	HA	HAGP	CA	CAGP	Bifonazole	Ketoconazole
	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC	MIC MFC
<i>Aspergillus fumigatus</i>	1.5 3.0	0.12 0.25	0.12 0.25	0.007 0.015	0.03 0.25	0.15 0.20	0.20 0.50
<i>Aspergillus versicolor</i>	0.1 4.5	0.003 0.03	0.06 0.25	0.007 0.06	0.015 0.03	0.10 0.20	0.20 0.50
<i>Aspergillus ochraceus</i>	0.75 1.5	0.015 0.07	0.007 0.015	0.007 0.03	0.007 0.015	0.15 0.20	1.50 2.0
<i>Aspergillus niger</i>	1.5 3.0	0.03 0.07	0.015 0.03	0.03 0.06	0.015 0.03	0.15 0.20	0.20 0.50
<i>Trichoderma viride</i>	0.005 0.1	0.007 0.015	0.007 0.03	0.015 0.03	0.015 0.06	0.15 0.20	1.0 1.0
<i>Penicillium funiculosum</i>	0.09 1.5	0.03 0.07	0.007 0.015	0.015 0.06	0.007 0.015	0.20 0.25	0.20 0.50
<i>Penicillium ochrochloron</i>	0.35 0.7	0.06 0.07	0.007 0.015	0.03 0.06	0.015 0.03	0.20 0.25	2.5 3.5
<i>Penicillium verrucosum</i>	1.5 3.0	0.06 0.07	0.03 0.06	0.007 0.03	0.06 0.12	0.10 0.20	0.20 0.30

HA- *p*-hydroxybenzoic acid; HAGP- *p*-hydroxybenzoic acid glucuronide protected form;

CA- cinnamic acid; CAGP- cinnamic acid glucuronide protected form.

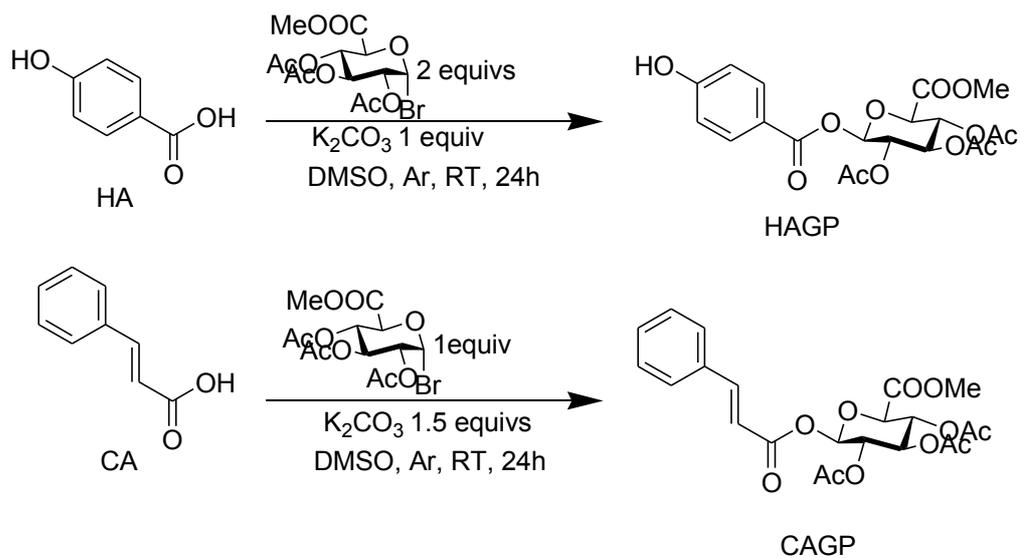


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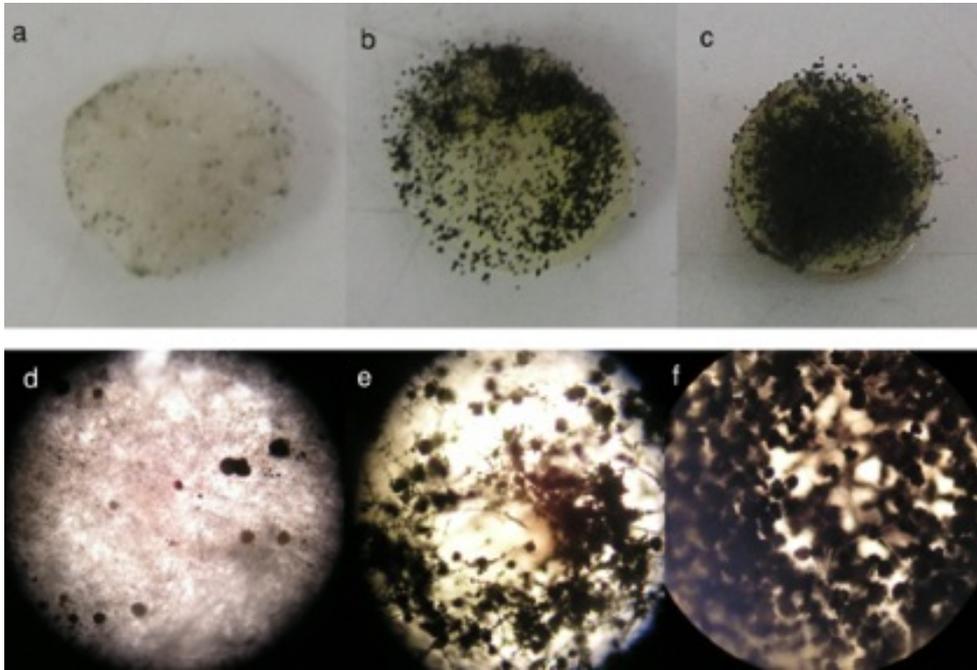


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