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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 *Aspergillus 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.

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., in press). *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.,

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in press), 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 *G. 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 sonificated for 15 min, centrifuged at 4000*g* 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 G. 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 24 h. 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 $MgSO_4$ and the solvent was evaporated. The product obtained was purified by a column chromatography using silica gel 60A (60–200 μm) 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. ^{1}H NMR (300 MHz, $CDCl_3$): 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)(*). ^{13}C NMR (75.4 MHz, $CDCl_3$): 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 $C_{20}H_{22}O_{12}$ (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 24 h. 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 $MgSO_4$ and the solvent was evaporated. The product obtained was purified by a column chromatography using silica gel 60A (60–200 μm) 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. ^{1}H NMR (300 MHz, $CDCl_3$): 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). ^{13}C NMR (75.4 MHz, $CDCl_3$): 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 $C_{22}H_{24}O_{11}$ (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 (Clinical and Laboratory Standards Institute, 2009; 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 1.0×10^5 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 1.0×10^5 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 (1.0×10^4 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 μL into microtitre plates containing 100 μL 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 and Raether, 1988; Espinel-Ingrof, 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 μg/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