SUPPLEMENTARY MATERIAL

Supercritical carbon-oxide extracts from cultivated and wild-grown *Ganoderma lucidum* mushroom: Differences in ergosterol and ganoderic acids content, antioxidative and enzyme inhibitory properties

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Abstract

In the present study, we investigated the effect of supercritical carbon-oxide ($scCO_2$) extraction on antioxidant capacity, enzyme inhibitory potential, and levels of ergosterol and ganoderic acid in both cultivated and wild-grown *G. lucidum*. Extraction yields were slightly higher for wild samples (1.29%) than for cultivated ones (1.13%). The levels of ganoderic acid and ergosterol were higher in cultivated in comparison to wild samples. In addition, the total phenolic content in cultivated samples (13.42 mg GAE g^{-1}) was higher than in wild samples (10.38 mg GAE g^{-1}). In general, cultivated samples exhibited stronger antioxidant potential when compared with wild ones. Regarding enzyme inhibitory properties, it was validated that the wild samples (14.01 mg OE g^{-1}) possessed greater lipase activity in comparison to cultivated samples (5.36 mg OE g^{-1}). Based on our findings, cultivated *G. lucidum* might be considered a valuable source of natural bioactive agents in the preparation of health-promoting products.

Keywords: *G. lucidum*; supercritical ($scCO_2$) extraction; ergosterol; ganoderic acids; antioxidant capacity; enzyme inhibition activity.

Experimental

Culture collection

The basidiocarp of *G. lucidum* was collected in the region of Mt. Mali Jastrebac (central Serbia), during the summer of 2021. Its identification was done according to the macroscopic features and the micromorphology of the reproductive structures. A small fragment of the fresh fruiting body was extracted on Malt agar medium (MA) for isolation of pure cultures of *G. lucidum* ICTMF211, which are then maintained in the Culture Collection of the Innovation Centre of the Faculty of Technology and Metallurgy, Belgrade (ICTMF).

Spawn preparation

Wheat grain was used for spawn production. The grains were washed with water and boiled for 30 minutes until they softened. Boiled grains were drained, supplemented with 2% $Ca_3(PO_4)_2$ and 0.5% $CaCO_3$ (Sigma-Aldrich, St. Louis, USA), mixed manually, placed in bottles, and sterilized in an autoclave at 121 °C for 15 min. After cooling, each bottle was inoculated with 25 mycelial disks (Ø 0.5 cm) obtained from 7-day-old culture and incubated at 22 ± 2 °C in the dark for 2 weeks.

Fruiting body growth

Wheat straw (small pieces) and oak dust in a ratio of 2.5:1 were dipped in dH_2O. After 12 h the wheat straw was centrifuged at 1800 rpm for 5 min to remove excess water. A mass of 0.7 kg was inserted into the polypropylene bags and autoclaved at 121 ℃ for 2 h. The final humidity was 80%. Once the substrate reached room temperature inoculation with overgrown spawn was performed. The inoculated bags were incubated at room temperature (22 ± 2 °C) in the dark for 2 weeks. Humidity was maintained by spraying water twice a day, until mushroom formation (about 40 days) (da Silva et al. 2012). Mushrooms were harvested on the 20th day after mushroom formation.

Both mushrooms' samples, collected and harvested, were lyophilized in a gamma 1-16 freeze-drying system (Christ, Osterode am Harz, Germany) and ground in a Retsch ZM 200 mill (Retsch, Haan, Germany; pore size 0.12 mm) to a fine powder.

63 **Supercritical CO_2 extraction**

64 Supercritical CO_2 extraction from *G. lucidum* was carried out in the High Pressure
65 Extraction Adsorption (HPEA) 500 unit (Eurotechnica GmbH) (Fig. S1). The unit can be used for
66 the integrated supercritical fluid extraction (SFE) and impregnation process or for the SFE process
67 only. SFE from cultivated and wild mushroom raw material was performed at 350 bar and 50 °C
68 (Table S1). For each experiment 20 g of mushroom powder was used. The mushroom powder was
69 placed in the 280 mL stainless steel extractor, designed to be operated at the maximum pressure of
70 550 bar and temperature of 120 °C. Liquid CO_2 supplied from a CO_2 cylinder with a siphon tube
71 was cooled in a cryostat to prevent vaporization and pumped into the system by a liquid metering
72 pump (Milton Roy, France) until the operating pressure was obtained. After reaching the operating
73 conditions, a continuous flow of supercritical fluid commenced. The operating pressure was
74 maintained by the back pressure regulator (BPR). The average extraction time was 1.4 h. Extraction
75 yields were calculated after the consumption of approximately 50 gCO_2 g^{-1} mushroom material,
76 whereby the mushroom material was exhausted. The extraction yield (y) was calculated using the
77 following equation:

78 $$y(\%) = \frac{m_e}{m_s} \times 100, \quad (1)$$

79 where m_e is the mass of obtained extract, while m_s is the mass of mushroom material at beginning
80 of the process. All the experiments were performed in duplicates.

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82 **Analytical procedure**

83 ***HPLC analysis***

84 HPLC fingerprint of the extract and quantification of identified compounds was achieved
85 by HPLC (Agilent Technologies 1200). Detection was performed using Diode Array Detector
86 (DAD), and the chromatograms were recorded at λ = 260 nm (for ganoderic acids (GA) A (GLA),
87 B (GLB), and C2 (GLC2)), and 282 nm (for ergosterol). HPLC separation of ergosterol was
88 achieved using Phenomex Syringe Hydro RP C18 (5 μm), 150 × 4.6 mm i.d. column, at 35 ºC, with
89 a flow rate of 1 mL min^{-1} and mobile phase, A [H_2O], B [MeOH], elution, combination of gradient
90 mode: 5-30% A, 0-20 min; isocratic 30% A, 5min; 30-35% A, 25-30 min. The samples were
91 prepared dissolving 29.36 and 30.66 mg of the extract cultivated and wild-grown, respectively
92 (obtained by the procedure previously described) in 1 mL of MeOH, filtered through 0.2 μm PTFE

93 filters prior to HPLC analysis. The injected volume was 5 μL. A standard solution of sterol alcohol
94 ergosterol was prepared at a final concentration of 0.68 mg mL^{-1} in methanol. The volume injected
95 was 5 μL, the same as the investigated extract.
96 HPLC separation of triterpenoids was achieved using a LiChrospher 100 RP 18e (5 μm),
97 250 × 4 mm i.d. column, at 35 ºC, with a flow rate of 1 mL min^{-1} and mobile phases A (H_2O, with
98 0.5% CH_3COOH) and B (MeOH), elution being the combination of gradient and isocratic modes:
99 0-30% B, 0-55 min; 30% **B**, for 5min; 30-90% **B**, 60-100 min; 90% B for 5 min; 90-100% B, 105-
100 120. The samples were prepared dissolving 29.36 and 30.66 mg of the extract cultivated and wild-
101 grown respectively (obtained by the procedure previously described) in 1 mL of MeOH, filtered
102 through 0.2 μm PTFE filters prior to HPLC analysis. The injected volume was 5 μL. Standard
103 solutions for the determination of triterpenoids were prepared at a final concentration of 0.67 mg
104 mL^{-1} (the same concentration for ganoderic acids A, B and C2) in methanol. The volume injected
105 was 5 μL, the same as the investigated extract.
106 The identification was carried out thanks to retention time and spectra matching. Once
107 spectra matching succeeded, results were confirmed by spiking with respective standards to
108 achieve a complete identification by means of the so-called peak purity test. Those peaks which
109 not fulfilling these requirements were not quantified. Quantification was performed by the external
110 standard method, and the results were expressed as mean value ± SD of three measurements.
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112 **Total phenolic content**

113 Referring to our previous paper (Uysal et al. 2017), total phenolic contents were determined
114 on the basis of a standard Folin-Ciocalteu assay. Briefly, 50 µl of an aliquot samples solution in
115 methanol (5 mg mL^{-1}) was mixed with 100 µL of 1:10 Folin-Ciocalteu reagent. The mixture was
116 shaken well and then added 75 µL of sodium carbonate (7.5%). The mixture was incubated (120
117 min) in the dark. Finally, the absorbance of the solution was recorded at 765 nm. Gallic acid was
118 used as standard and the results were expressed as gallic acid equivalent (mg GAE g^{-1}) for total
119 phenolic content.
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121 **Antioxidant properties**

122 In order to evaluate the antioxidant activity (AA) of the extracts, different
123 spectrophotometric experiments as ferrous ion chelating, phosphomolybdenum and radicals

124 scavenging tests (ferric reducing antioxidant power (FRAP), 2,2′-azino-bis(3-
125 ethylbenzothiazoline)-6-sulfonic acid (ABTS), cupric reducing antioxidant capacity (CUPRAC)
126 and 1,1-diphenyl-2-picrylhydrazyl (DPPH)) were performed as previously reported. The findings
127 were given as standard compounds equivalents of EDTA or Trolox (mg EDTAE g^{-1} and mg TE g^{-1}
128). The concentration of the extracts was 0.5-5 mg mL^{-1}. The procedures were conducted
129 accordingly to the corresponding assay methods given in our earlier work (Uysal et al. 2017).
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131 **Enzyme inhibitory activities**

132 The *in vitro* enzyme inhibitory effects of extracts on α-amylase, α-glucosidase,
133 cholinesterases (acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE)), and tyrosinase
134 were evaluated, as previously reported (Uysal et al. 2017). The used concentration of the
135 investigated extracts ranged from 0.5-5 mg mL^{-1}. The enzyme inhibitory actions of extracts were
136 assessed as equivalents of kojic acid (KAE) for tyrosinase, galantamine for acetyl cholinesterase
137 (AChE) and butyryl cholinesterase (BChE), and acarbose for α-amylase and α-glucosidase,
138 respectively.
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140 **Statistical Analysis**

141 The antioxidant and enzyme inhibitory results were reported as mean ± standard deviation
142 of three parallel experiments. The differences between the two essential oils were analyzed using
143 the student t-test ($p < 0.05$). The statistical procedures were achieved by GraphPad Prism 8
144 software.
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150 **Table S1.** Summary of supercritical extraction procedure and yield efficiencies from *G. lucidum* mushrooms

Raw Material	Extraction parameter	Yield (%)	Reference
Spores	35 Mpa; 4h; 25 ºC; CO_2 flow rate 10 Kg h^{-1}	2.98	Fu et al. (2009)
	5 – 60 MPa; 0.5 – 6 h; 32 – 85 ºC; CO_2 flow rate 5 – 80 Kg h^{-1}	37.00	Li et al. (2016a)
	30 Mpa; 2 h; 40 ºC; CO_2 flow rate 25 L h^{-1}	24.16	Chen et al. (2012)
	35 Mpa; 3 h; 48 ºC; unknown CO_2 flow rate	29.50	Li et al. (2016b)
Fruiting body	30 Mpa; 4.5 h; 40 ºC; CO_2 flow rate 3.2 L h^{-1}	2.07	Cor et al. (2014)
	105 Mpa; 4 h; 50 ºC; CO_2 flow rate 12 L h^{-1}	5.66	Zhu et al. (2020)
	10.3 Mpa; 2 h; 40 ºC; CO_2 flow rate 0.24 L h^{-1}	1.20	Hsu et al. (2001)

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Table S2. Ganoderic acid and ergosterol quantification by HPLC analysis of *G. lucidum* mushroom

	Cultivated	**Wild grown**
Ganoderic acid A (mg g^{-1})	2.13 ± 0.11*	0.85 ± 0.09
Ganoderic acid B (mg g^{-1})	1.22 ± 0.07	0.52 ± 0.04
Ganoderic acid C2 (mg g^{-1})	0.12 ± 0.01	0.04 ± 0.01
Ergosterol (mg g^{-1})	5.23 ± 0.12	2.27 ± 0.10

152 * Values expressed represent means ± S.D. of three parallel colorimetric measurements. Different letters indicate significant differences

153 in the extracts ($p < 0.05$)

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168 **Table S3.** Antioxidant activity of *G. lucidum* mushroom

Assays name	Cultivated	Wild grown
Total phenolic content (mg GAE g^{-1})	13.42 ± 0.09^{a*}	10.38 ± 0.28^{b}
Toal flavanoid content (mg RE g^{-1})	1.78 ± 0.24^{a}	1.42 ± 0.17^{b}
DPPH scavenging ability (mg TE g^{-1})	2.61 ± 0.11^{a}	1.89 ± 0.06^{b}
ABTS scavenging ability (mg TE g^{-1})	25.50 ± 0.03^{a}	12.89 ± 0.09^{b}
CUPRAC (mg TE g^{-1})	57.70 ± 1.78^{a}	52.70 ± 0.88^{b}
FRAP (mg TE g^{-1})	12.38 ± 0.12^{a}	11.32 ± 0.33^{b}
Phosphmolybdenum assay (mmol TE g^{-1})	0.89 ± 0.01^{b}	0.94 ± 0.02^{a}
Metal chelating ability (mg EDTAE g^{-1})	19.70 ± 1.25^{a}	17.10 ± 1.06^{b}

169 [*] Values expressed represent means ± S.D. of three parallel colorimetric measurements. GAE: Gallic acid equivalents; RE: Rutin
170 equivalent; TE: Trolox equivalents; EDTAE: EDTA equivalents. Different letters ($^{a-b}$) indicate significant differences in the extracts (p
171 < 0.05)

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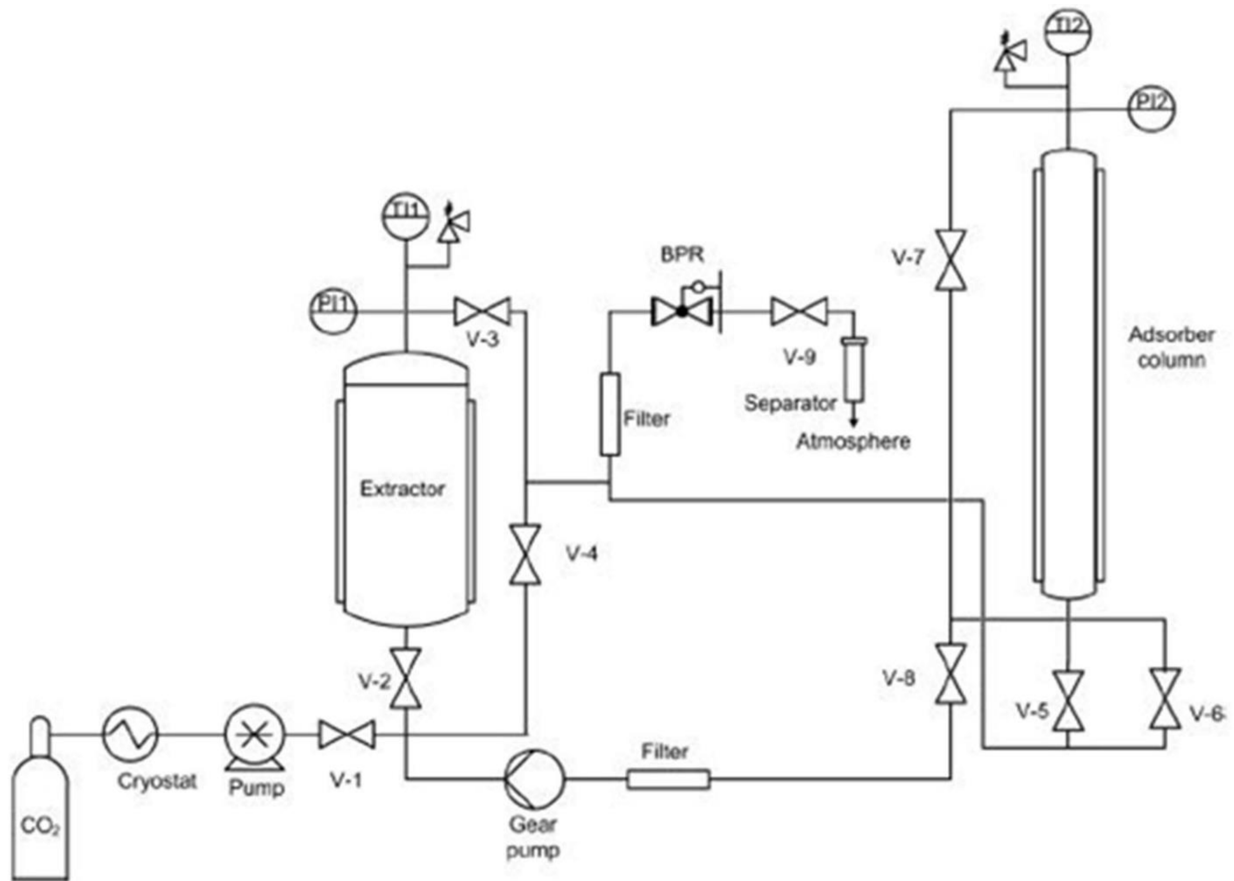
181 **Table S4.** Enzyme inhibitory activity of *G. lucidum* mushroom

Assays name	Cultivated	Wild grown
AChE inhibition (mg GALAE g^{-1})	$1.01 \pm 0.02^{a*}$	0.93 ± 0.01^{b}
Tyrosinase inhibition (mg KAE g^{-1})	17.25 ± 1.68^{a}	18.84 ± 0.63^{a}
Amylase inhibition (mmol ACAE g^{-1})	0.33 ± 0.01^{a}	0.35 ± 0.01^{a}
Glucosidase inhibition (mmol ACAE g^{-1})	0.39 ± 0.01^{a}	0.39 ± 0.01^{a}
Lipase inhibition (mg OE g^{-1})	5.36 ± 0.39^{b}	14.01 ± 2.81^{a}

182 [*]Values expressed are means ± S.D. of three parallel colorimetric measurements. GALAE: Galantamine equivalent; KAE: Kojic acid
183 equivalent; ACAE: Acarbose equivalent; OE: Orlistat equivalent. Different letters ($^{a-b}$) indicate significant differences in the extracts (p
184 < 0.05)



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186 **Fig S1.** Schematic view of HPEA 500 unit

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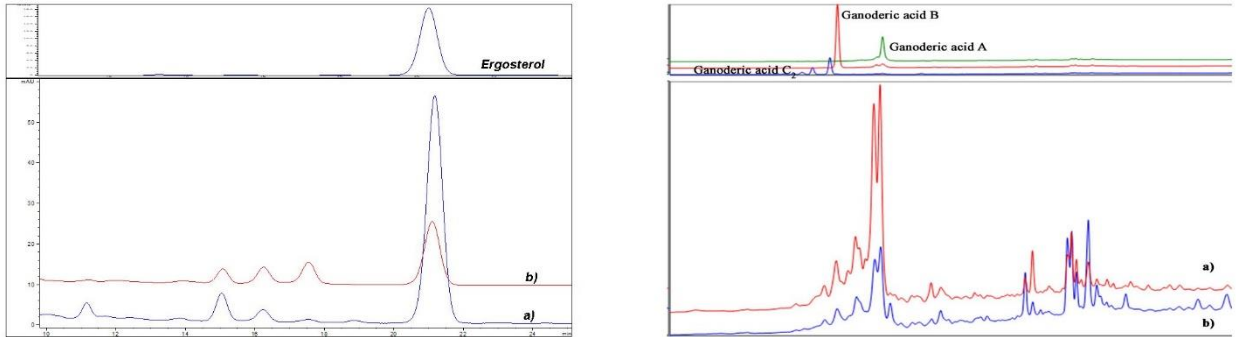

196 **Fig S2.** HPLC chromatogram of cultivated (a) and wild grown (b) *G. lucidum* mushroom $scCO_2$

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