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Phytochemical Analysis and Antiradical Properties of *Sarcodon imbricatus* (L.:Fr) Karsten

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The chemical composition of *Sarcodon imbricatus* (L.:Fr) Karsten (Hydnaceae) was evaluated to assess it as source of nutrients and nutraceuticals. The analyzed mushroom contains ergosterol and ergosterol peroxide. *S. imbricatus* methanolic extract showed a moderate antiradical activity (measured by DPPH radical scavenging activity). The combination of bioactive compounds and rich nutritional composition (high contents in protein, low content in fat, and its content of unsaturated fatty acids) makes the mushroom a good food.

Keywords: *Sarcodon imbricatus*, mushroom, ergosterol, ergosterol peroxide, fatty acid composition, proteins, sugars, DPPH.

Mushrooms have been recognized as a source of food for thousands of years. They can be valued as nutritional foods as they are a good source of proteins (10-40%), carbohydrates (3-21%), and fiber (3-35%) [1]. Mushrooms are low in calories and high in vitamins and minerals. Lipid contents range between 1.75 and 15.5% [2].

In addition to their nutritional value, many mushrooms have gained importance for their biological activity. Antiinflammatory, antioxidant, antimicrobial, immunostimulant and antitumor properties have been shown by wild and cultivated mushrooms. [3]

Recently we have been interested in *Sarcodon glaucopus* [4] and *S. cyrneus* [5]. These two bitter species were particularly interesting for the presence of cyathane diterpenes, which show peculiar biological properties [4a, 5]. These compounds have been indicated as those responsible for the bitter taste of these species.

S. imbricatus (L.:Fr) Karsten (Hydnaceae) is an edible species. Two previous studies evaluated the chemical composition of this mushroom [6], but no analysis of secondary metabolites was reported. As a result, we decided to investigate *S. imbricatus* to determine the possible presence of cyathane diterpenes, as well as to ascertain the most abundant metabolites of this mushroom. Furthermore, we studied the fatty acid profile, total soluble sugars, total proteins and total polyphenols. The antioxidant property of *S. imbricatus* methanolic extract was also determined using the DPPH radical scavenging activity.

Dry *S. imbricatus* was extracted with *n*-hexane and MeOH at room temperature for 24 h. The *n*-hexane extract was esterified using an ethereal solution of

diazomethane and studied by GC-MS to determine the fatty acid composition. Methyl palmitate, methyl oleate and methyl linoleate, the latter accounting for 42% of the total amount, were the most abundant compounds.

The methanolic extract was dissolved in EtOAc and washed with water. ^{1}H NMR spectral analysis of the crude ethyl acetate phase showed the absence of cyathane diterpenes in the extract. Purification by column chromatography gave only two products that were identified by NMR spectroscopy as ergosterol (**1**) [7] and ergosterol peroxide (**2**) [8].

Ergosterol peroxide (**2**) is widely distributed in mushrooms and shows a plethora of biological activities, such as antileukemic and anticancer [8], apoptotic-inducing [9], and anti-inflammatory [10].

Total soluble sugars, as g sucrose/100 g dry weight, were determined colorimetrically by the anthrone method directly on the dry fruiting bodies [11]. The total soluble sugar quantity, expressed as g of sucrose equivalents/100 g dry weight, was 1.55%.

Total proteins, expressed as g bovine serum albumin equivalents/100 g dry weight, were determined colorimetrically by a slightly modified Lowry method [12] on the lyophilized fruiting bodies; the amount found was quite high (17%).

A well-established method for determining total polyphenols relies on color development due to the reduction of the Folin-Ciocalteu reagent by the reductant (polyphenol). This particular method utilizes a reference standard, such as gallic acid, with absorbance readings taken with a spectrophotometer. [13] Total polyphenols (0.5%) are expressed as g gallic acid /100 g dry weight.

The antioxidant activity of the methanolic extract was evaluated using the DPPH radical scavenging activity assay [14]. The IC_{50} (concentration in mg/mL required for 50% inhibition of DPPH radical) was 0.43±0.11. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as reference compound (IC_{50}=0.014±0.001). This result shows that the radical scavenging activity of this extract is fairly good.

In conclusion, we have studied the chemical composition of *S. imbricatus* and evaluated its radical scavenging activity. The results showed the absence of cyathane diterpenes, and this could explain the absence of bitterness of this species, and the presence of ergosterol and ergosterol peroxide (0.8 and 0.2% of dry weight, respectively); these are important biologically active compounds. The mushroom showed a high content of proteins and a low content of fat, with the precious contribution of unsaturated fatty acids. These data confirm that *S. imbricatus* can be considered a good food.

Experimental

General: UV spectra were obtained on a UV/VIS V530 JASCO instrument. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, on a Bruker Avance-DRX 400 spectrometer at 400 and 100.62 MHz, respectively, using TMS as the internal standard. GC-MS analysis was performed on a HP-6890 instrument equipped with a mass selective detector HP-5973. Column chromatography was performed using Merck silica gel 60 (70–120 mesh). Thin layer chromatography was performed on Merck silica gel 60 F254.

Material collection: *S. imbricatus* was collected in October 2005 near Perugia (Italy) and identified by Prof. Rita Pagiotti. A voucher specimen (RP #63) is deposited at the Dipartimento di Biologia Applicata, Sezione Biologia Vegetale e Geobotanica, Università degli Studi di Perugia. Fresh mushrooms were frozen and lyophylized immediately after collection.

Extraction**:** Lyophilized fruiting bodies (30 g) were extracted at r.t. for 24 h with *n*-hexane (1 L). After vacuum filtration, the solvent was evaporated giving 550 mg of *n*-hexane extract. The residue was extracted with MeOH (1 L) at r.t. for 24 h. After vacuum filtration the methanolic extract was evaporated giving 3 g of residue.

Esterification of the* n*-hexane extract: The *n*-hexane extract was dissolved in 200 mL of Et_2O and methylated using an ethereal solution of diazomethane. At the end of the reaction the solvent was removed and the residue purified by column chromatography. Elution with dichloromethane-EtOAc (19:1) gave two fractions: the less polar was composed of a mixture of fatty acid methyl esters (475 mg), and the most polar (90 mg), analyzed by NMR and MS spectroscopy, was identified as ergosterol (**1**) [7].

Gas chromatography of fatty acid methyl esters: GC analysis of fatty acids methyl esters was performed

on a Dani 1000 (Norwalk, CT, USA) instrument equipped with a split-splitless injector and a flame ionization detector (FID). A fused silica WCOT capillary column CP-Select CB for FAME (50 m x 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy) was used. The injector and detector temperature was 250°C; the oven temperature was 130°C, and then increased to 225°C at 3°C/min; the final temperature was held for 10 min. The carrier gas (He) flow rate was 1 mL/min and the split ratio was 1:70.

For the acquisition and integration of chromatograms Clarity (DataApex Ltd., Prague, Czech Republic) software was used.

To confirm the identity of the fatty acid methyl esters a GC-MS QP-2010 (Shimadzu, Japan) was used with the same capillary column and the same chromatographic conditions as those described above for GC-FID analysis of fatty acid methyl esters.

Isolation of ergosterol (**1**) ***and ergosterol peroxide*** (**2**)***:*** The methanolic extract (3 g) was suspended in 200 mL of EtOAc and washed with H_2O (3x 25 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated, leading to a brown syrup (500 mg). This extract was fractioned by SiO_2 gel column chromatography using dichlomethane-EtOAc 9:1. Two compounds were obtained: ergosterol (**1**) (150 mg) and ergosterol peroxide (**2**) (60 mg). The structures of these two compounds were determined by comparison with reported literature data [7,9].

Determination of total soluble sugars: Dry mushrooms (0.2 g) were extracted at r.t. for 24 h with 20 mL of 80% EtOH. After this time, the suspension was filtered and diluted to 100 mL with 80% EtOH. Of this ethanolic solution, 1 mL was mixed with 10 mL of anthrone solution [prepared with thiourea (5 g), anthrone (0.25 g) and 473 mL of 12.68 *M* sulphuric acid]. The mixture was heated in a boiling water bath and left for 1 h in the dark. The absorbance was measured at 620 nm against a blank containing all the reagents, except the carbohydrate solution, which had been substituted with distilled water. The average of five measurements was used to calculate the sugar content.

Determination of total proteins: Dry mushrooms (1 g) were extracted with 50 mL of 1N NaOH solution at r.t. for 24 h. The suspension was then filtered and 0.1 mL of the filtrate diluted to 2 mL with 1N NaOH. To the solution, were added 0.5 mL of a 2% solution of $CuSO_4 \cdot 5\ H_2O$, 0.5 mL of a 1% solution of sodium and potassium tartrate, and 49 mL of a 2% solution of Na_2CO_3. To this solution 2 mL of a 1:1 mixture of 1N NaOH and Folin reagent were added. The mixture was kept for 20 min. in the dark before the absorbance was measured at 740 nm against a blank containing all the reagents, except the protein solution, which had been substituted with distilled water. The average of five measurements was used to calculate the sugar content.

Determination of total polyphenols: Dry mushrooms (1 g) were extracted with 100 mL of MeOH at r.t. for 24 h. The suspension was then filtered and diluted to 100 mL with MeOH. To 1 mL of this solution, 10 mL of Folin reagent and 8 mL of a 7.5% Na_2CO_3 solution and 1 mL of H_2O were added. The mixture was kept for 20 min. in the dark before the absorbance was measured at 765 nm against a blank containing all the reagents, except the polyphenol solution, which had been substituted with distilled water. The average of five measurements was used to calculate the polyphenol content.

Determination of antiradical activity: Fresh DPPH stock solution at a concentration of 9 mg/100 mL MeOH was prepared. Methanol was used instead of the sample for blank measurements. An aliquot of 1 mL of different concentrations of either standard antioxidant or *Sarcodon imbricatus* methanolic solution was added to 1 mL of DPPH solution. The mixture was vortexed for a few seconds and left to stand in the dark for 20 min at room temperature. Trolox was used as a control.

The activity was measured of the total methanolic extract prepared by extraction of 1 g of dry mushroom with 100 mL of MeOH at r.t. for 24 h. After this time the suspension was filtered and the filtrate evaporated under reduced pressure. The DPPH test was performed, as previously described, on the residues obtained after evaporation. All determinations were performed in triplicate.

References

[1] Manzi P, Gambelli L, Marconi S, Vivanti V, Pizzoferrato L. (**1999**) Nutrients in edible mushrooms: an inter-species comparative study. *Food Chemistry*, ***65***, 477-482.

[2] Bernas E, Jaworska G, Lisiewska Z. (**2006**) Edible mushrooms as a source of valuable nutritive constituents. *Acta Scientiarum Polonorum, Technlogia Alimentaria*, ***5***, 5-20.

[3] Poucheret P, Fons F, Rapior S. (**2006**) Biological and pharmacological activity of higher fungi: 20-year retrospective analysis. *Cryptogamie, Mycologie*, ***27***, 311-333.

[4] (a) Curini M, Maltese F, Marcotullio MC, Menghini L, Pagiotti R, Rosati O, Altinier G, Tubaro A. (**2005**) Glaucopines A and B, New cyathane diterpenes from the fruiting bodies of *Sarcodon glaucopus*. *Planta Medica*, ***71***, 194-196. (b) Marcotullio MC, Pagiotti R, Campagna V, Maltese F, Fardella G, Altinier G, Tubaro A. Glaucopine C, a new diterpene from the fruiting bodies of *Sarcodon glaucopus*. (**2006**) *Natural Product Research*, ***20***, 917-921.

[5] (a) Marcotullio MC, Pagiotti R, Maltese F, Obara Y, Hoshino T, Nakahata N, Curini M. Neurite Outgrowth activity of cyathane diterpenes from *Sarcodon cyrneus*, cyrneines A and B. (**2006**) *Planta Medica*, ***72***, 819-823; (b) Marcotullio MC, Pagiotti R, Maltese F, Oball-Mond Mwankie GN, Hoshino T, Obara Y, Nakahata N. (**2007**) Cyathane diterpenes from *Sarcodon cyrneus* and evaluation of their activities of neuritegenesis and nerve growth factor production. *Bioorganic Medicinal Chemistry*, ***15***, 2878-2882; (c) Obara Y, Hoshino T, Marcotullio MC, Pagiotti R, Nakahata N. (**2007**) A novel cyathane diterpene, cyrneine A, induces neurite outgrowth in a Rac1-dependent mechanism in PC12 cells. *Life Sciences*, ***80***, 1669-1677.

[6] (a) Barros L, Baptista P, Correia DM, Casal S, Oliveira B, Ferreira ICFR. (**2007**) Fatty acid and sugar compositions, and nutritional value of five wild edible mushrooms from northeast Portugal. *Food Chemistry*, ***105***, 140-145; (b) Barros L, Ferreira M.-J, Queiros B, Ferreira LCFR, Baptista P. (**2007**) Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chemistry*, ***103***, 413-419.

[7] Zhou W-X, Nes WD. (**2000**) Stereochemistry of hydrogen introduction at C-25 in ergosterol synthesized by mevalonate-independent pathway. *Tetrahedron Letters*, 2791-2795.

[8] Kobori M, Yoshida M, Ohnishi-Kameyama M, Takei T, Shinmoto H. (**2006**) 5α,8α-Epidioxy-22*E*-ergosta-6,9(11),22-trien-3β-ol from edible mushroom suppresses growth of HL60 leukemia and HT29 colon adenocarcinoma cells. *Biological & Pharmaceutical Bullettin*, ***29***, 755-759.

[9] Takei T, Yoshida M, Ohnishi-Kameyama M, Kobori M. (**2005**) Ergosterol peroxide, an apoptosis-inducing component isolated from *Sarcodon aspratus* (Berk.) S. Ito. *Bioscience Biotechnology and Biochemistry*, ***69***, 212-215.

[10] Kobori M, Yoshida M, Ohnishi-Kameyama M, Shinmoto H. (**2007**) Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. *British Journal of Pharmacology*, ***150***, 209-219.

[11] Sunderwirter SG, Olson CG, Johnson G. (**1964**) Paper chromatographic-anthrone determination of sugars. *Journal of Chromatography*, ***16***, 176-180.

[12] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (**1951**) Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry*, ***193***, 265-275.

[13] Singleton VL, Rossi JA Jr. (**1965**) Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, ***16***, 144-158.

[14] Peterson DM, Hahn MJ, Emmons CL. (**2002**) Oat avenanthramides exhibit antioxidant activities *in vitro*. *Food Chemistry*, ***9***, 473-478.