SUPPLEMENTARY MATERIAL

Steroidal composition and cytotoxic activity from fruiting body of *Cortinarius xiphidipus*

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Abstract

From the fruiting body of ectomycorrhizal fungi *Cortinarius xiphidipus*, sterols were identified from the crude extract and the cytotoxic effect of ergosta-4, 6, 8(14), 22-tetraen-3-one was evaluated. Ten sterols including Ergosta-3,5,7,9(11), 22-pentaene, (22E)-ergosta-5,7,9(11),22-tetraen-3b-ol, (3 β ,22E)-ergosta-5,7,22-trien-3-ol, (22E)-ergosta-7,22-dien-3-ol, Neoergosterol, (3 β)-ergosta-5,8-dien-3-ol, (3 β)-ergosta-7-en-3-ol, Stigmasterol, Stigmasterol 22,23-dihydro and (22E)-ergosta-4,6,8(14),22-tetraen-3-one were identified from the crude extract. The cytotoxic activity of the sterol fraction containing ergosta-4, 6, 8(14), 22-tetraen-3-one was assessed on four tumor cell lines (Neuro-2a, Saos-2, MCF7 and LNCaP-C42). The cytotoxic activity against the four tumor cell lines tested, being Neuro-2a and Saos-2 the most sensitive, with a half-maximal inhibitory concentration (IC₅₀) of 20.8 ± 2.2 and 27.8 ± 1.0 μ g/mL, respectively. This is the first report of this antarctic fungi collected in the Magallanes and Chilean Antarctica Region. This work represents a potential source for the development of anticancer drugs.

Keywords: *Cortinarius xiphidipus*; mycorrhizal fungus; fruiting body; ergosterols; cytotoxicity

Experimental Section

Collection of biological material

Fruiting bodies of *Cortinarius xiphidipus* were collected in southern Chile during the autumn season, specifically in the Magallanes National Reserve (53°06' S - 71°01' W), where the average annual temperature is close to 7 °C. *Nothofagus* forests cover most of the study area. *Cortinarius xiphidipus* grows as a mycorrhizal fungus associated with *Nothofagus pumilio* (Lenga). The collected and stored material was transferred to the Natural Products Chemistry Laboratory of the Department of Botany at the University of Concepción for further analyses and deposited in the Herbarium of University of Concepcion section Fungi under number CONC-F1610.

Obtaining of total extract

Fruiting bodies of *C. xiphidipus* (2 Kg) were extracted in 1.5 L of methanol (MeOH). The extract was filtered in N°1 Whatman paper and the solvent was evaporated at reduced pressure in a rotary evaporator at 40 °C (Heildolph Unimax 2010). Dried extract was fractioned in an extract of hexane and ethyl acetate (EtOAc). Both extracts were concentrated until dry and weighted in order to obtain total yield.

Extraction and isolation

The total extract (7.5 g) was fractioned by chromatography in a Merck 60 F₂₅₄ silica gel column. Solvents of growing polarity (n-hexane-EtOAc y MeOH) were used as eluent phase. Fractions were collected (F₁-F₁₁), concentrated and subsequently were monitored by TLC, using aluminum chromatofolium covered by Silica gel 60 F₂₅₄ (Merck, Germany). A mixture of 1:1 hexane-ethyl acetate was used as mobile phase. For the visualization of compounds, a mixture of 1:1 anisaldehyde/sulfuric acid with heat to 105°C until spots were visualized was used. For the purification of the compound, fractions F₅-F₆ and F₇ containing the ergosta-4,6,8(14),22-tetraene-3-one, were concentrated and chromatographed in a 30x1.5 cm silica gel 60 column 0.063-0.200 mm-Merck, using the mixture of mobile phase hexane-petroleum ether (9:1) and five fractions were obtained. The R_f obtained was 0.5.

Identification of steroidal components

Gas chromatography coupled to mass spectrometry (CG-MS)

Composition of the hexanic extract was determined in a gas chromatographer coupled to a mass spectrometer (Agilent 7890), equipped with an Agilent automatic injector and using a capillary column HP-5 (30 m x 0,25 mm ID) with a film of 0.25 mm thickness. For the splitless injection the injector temperature was 250 °C. The oven temperature was programmed from 100 °C (held for 5 min) to 285 °C with increment of 10 °C for min and the final temperature being held for 15 min. The temperature of detector was 300 °C. Identification of the compounds was performed by using the NIST/EPA/NIH MASS 2005 Spectral Library (NIST 05, 2005) database. Structures of the compounds were designed with the ChemDraw[®] program.

Nuclear magnetic resonance spectroscopy (NMR)

Structural elucidation was determined by means of the conventional spectroscopic technique; Nuclear Magnetic Resonance NMR (Bruker Model Ascend™ 400 MHz). An analysis of ¹H-NMR was performed using deuterium- chloroform (CDCl₃) as solvent. Values are expressed in ppm (δ), using TMS as internal standard.

Cytotoxicity assay in tumor cell lines

Cytotoxic activity of the extract was assessed on the cell lines of mouse neuroblastoma (Neuro-2a) (ATCC, American Type Culture Collection, Rockville, MD; CCL-131), human osteosarcoma (Saos-2) (ATCC: HTB-85), human breast cancer (MCF7) (ATCC: HTB-22), and human prostate cancer (LNCaP-C42). Cells Neuro-2a and LNCaP/C42 were maintained in medium RPMI 1640 (GIBCO) with 1 mM sodium piruvate (GIBCO); and cells Saos-2 and MCF7 were maintained in medium Dulbecco's Modified Eagle's Medium DMEM (GIBCO). All culture media were supplemented with 100% fetal bovine serum (FBS), (GIBCO), 2mM glutamine, 100 µg/mL penicillin, 100 U/mL streptomycin, and 2.5 µg/mL amphotericin B (GIBCO). Cultures were maintained in incubator at 37°C with injection of 5% CO₂. For the incubation of the cytotoxic activity, cells were seeded in plates

of 96 wells, at a density of 190.000 cells/mL for neuro 21, 125.000 cells/mL for Saos 2; 150.000 cells/mL for MCF7; and 225.000 cells/mL for LNCaP-C42, in a volume of 200 μ L. After 24 hours of incubation, cells were exposed to 30 μ L of the extract during 24 hours. The extract containing the compound to be evaluated was dissolved in dimethyl sulfoxide (DMSO), and was serially diluted at a ratio of 1:2 in culture medium. Concentration of DMSO in the well did not exceed 0.5%. The concentration evaluated ranged from 0.6 μ g/mL to 118 μ g/mL. After the incubation period with the extract ended, this was eliminated, and 60 μ L of MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-difenil-tetrazolium bromide, and Invitrogen at a concentration of 0.83 mg/mL were added to all cell lines, except MCF7, which was incubated with 1.7 mg/mL of MTT. It was removed after 20 minutes at 37°C, and the formazan salts were dissolved in 100 μ L of DMSO. Optic density was measured in a microplate reader (Synergy H1, Biotek, Vermont, USA), at 570 nm. The obtained dose/response curves were analyzed by means of Prism 4 statistical software (GraphPad, San Diego, California, USA), and the estimated value of IC₅₀ was obtained at 24 hours of exposure, defined as the concentration that reduces viability by 50%.

Statistical analysis

Data analysis was performed using Statistica software (version 6.0, StatSoft, Inc, Tulsa, OK, USA). The data were treated with one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Differences were considered statistically significant when $p < 0.05$.

The IC₅₀ value (the concentration of the sample required to inhibit 50% of the viability) was calculated from the extract concentration and cell viability by using Graphpad 6 (GraphPad Software Inc, San Diego, CA).