Among the lipophilic extracts of seven traditional edible mushrooms, the acetone extract of *Sarcodon aspratus* markedly inhibited the growth of HL60 human leukemia cells and induced apoptosis after 24 h incubation. The major active component was identified as ergosterol peroxide by NMR and ESI-MS analysis. Ergosterol peroxide completely inhibited growth and induced apoptosis of HL60 cells at a concentration of 25 μM.

**Key words:** *Sarcodon aspratus* (Berk.) S. Ito; ergosterol peroxide; apoptosis; HL60 human leukemia cells

Various mushrooms, which are low in calories and rich in flavor and texture, are traditionally eaten in Japan. A well-known physiological effect of mushrooms is the anti-tumor action of their polysaccharides. β-D-Glucans in mushrooms have been reported to suppress Sarcoma 180 and Ehrlich solid cancer tumor formation *in vivo*. It is generally accepted that mushroom polysaccharides exert anti-tumor activity by potentiating the immune response via stimulation of T-cell, B-cell, NK-cell and macrophage function. Lentinian, from *Lentinus edodes*, and shizophyllan, from *Schizophyllum commune*, in addition to other polysaccharides, have been studied as non-toxic immunocceuticals against cancer, and clinical effects have been reported. Mushrooms also contain terpenoids and steroids, including abundant amounts of provitamin D, ergosterol. Although there are fewer reports in the literature on the physiological effects of mushroom terpenoids and steroids than of polysaccharides, anti-tumor, anti-inflammatory and immunosuppressive effects have also been described for these compounds. Thus, mushrooms are drawing attention as beneficial foods for human health, although some species are not widely distributed due to technical difficulties in mass production.

In this study we examined the effect of lipophilic extracts of 7 mushrooms, which are not widely distributed but traditionally eaten in some local areas of Japan. We assessed the anti-tumor activity of mushroom extracts by their effect on the growth of HL60 human promyelocytic leukemia cells (JCRB0085). Fresh fruiting bodies of *Hericium erinaceum* (Fr.) Pers., *Lentinus lepides* (Fr.:Fr.):Fr., *Leucopaxillus giganteus* (Sow.:Fr.) Sing., *Lyophillum decestes* (Fr.:Fr.) Sing., *Pleurocybella porrigens* (Pers.:Fr.) Sing., and *Pleurotus cornucopiae* (Paulet) Rolland var. *citrinopileatus* (Sing.) Ohira were extracted with ethanol. *Sarcodon aspratus* (Berk) S. Ito, is usually sold as a dried foodstuff, thus the dried fruiting bodies were extracted with acetone. Extracts were filtered through filter paper, concentrated by evaporation, and dissolved in ethanol before they were added to cell culture medium (RPMI 1640 medium [Invitrogen, Carlsbad, CA] supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Aurora, OH)) for bioassay. HL60 cells (1 × 10^5 cells/ml) were incubated with 0.1 mg/ml of the mushroom extracts for 24 h before viable cell numbers were determined in order to evaluate inhibitory effects on HL60 cell growth. Extracts from *H. erinaceum*, *L. giganteus*, and *P. porrigens* decreased the number of viable cells by 20–40% of control cells, but *L. lepides*, *L. decestes*, and *P. cornucopiae* failed to inhibit cell growth at a concentration of 0.1 mg/ml (Fig. 1a). The *S. aspratus* extract was the most potent in inhibiting growth of HL60 cells: the number of viable cells was reduced to 20% (Fig. 1a), and apoptotic cell bodies (separation of the cell into discrete membrane-bound fragmentation) and nuclear fragmentation were induced in HL60 cells (Fig. 1b). Only the *S. aspratus* extract induced the nucleosomal DNA fragmentation typical of apoptosis after 24 h incubation (Fig. 1c).
Next, we isolated the active component of the \textit{S. aspratus} extract. Dried fruiting bodies of \textit{S. aspratus} (20.7 g, moisture content 3.7%) were extracted with 400 ml of acetone 3 times. The acetone extract (1.3 g) was applied to an OASIS HLB column (20 ml cartridge (1 g), Waters, Milford, MA) and eluted with MeOH/H\textsubscript{2}O (0:100, 20:80, 40:60, 60:40, 80:20, 100:0). Each of the fractions was added to the HL60 culture medium at a concentration of 0.1 mg/ml, and the cells were incubated for 24 h. Apoptotic cells induced by the \textit{S. aspratus} fractions were detected using a microscope. Only the fraction eluted with 80% methanol (yield, 642 mg) inhibited growth and induced apoptosis of HL60 cells. The active fraction was then applied to a silica gel column (21 × 300 mm, Silica Gel 60 (70–230 mesh, Merck, Darmstadt, Germany)) and eluted with n-hexane/ethyl acetate 10:1 (Eh-1), n-hexane/ethyl acetate 3:1 (Eh-2 and 3), and methanol (Eh-4). The yields of the fractions were 211.0 mg (Eh-1), 168.4 mg (Eh-2), 14.0 mg (Eh-3), and 65.1 mg (Eh-4) respectively. Apoptosis-inducing activity was observed in two of these fractions, Eh-3 and Eh-4. Eh-3 contained fewer components and showed higher apoptosis-inducing activity than Eh-4. The major component of Eh-3, component A, was purified by preparative TLC (silica gel 60 F254; developing solvent, 5% methanol/dichloromethane; RF value, 0.33). The yield of component A (10.7 mg) was approximately 0.8% of the acetone extract.

The electrospray ionization (ESI) mass spectrum of component A was recorded on a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (ApexII 70e, Bruker Daltonics, MA) The most intense peak was detected at \(m/z\) 451.3. The addition of potassium ions shifted the peak to \(m/z\) 467.3, indicating that the peak at \(m/z\) 451.3 was of a sodium additive ion ([M + Na]\(^+\)). The molecular weight of component A was 428.3. The elemental composition of [M + Na]\(^+\) was analyzed by FT-ICR mass spectrometry. The exact mass value of [M + Na]\(^+\) was 451.3184, in good agreement with the calculated value of 451.3183 for \(12\text{C}_{28}\text{H}_{44}\text{O}_{16}\text{Na}\) with 1 \(×\) 10\(^{-4}\) u error. The molecular formula of component A was determined to be \(C_{28}H_{44}O_{13}\). Measurement of \(^1H\) NMR spectra, including two-dimensional NMR experiments, DQF-COSY, HOSQ, and HMBC, were performed on an Avance 800 spectrometer (Bruker Biospin, Karlsruhe, Germany) in CDCl\(_3\) containing 0.03% TMS (Sigma-Aldrich, St. Louis, MO) at 800.30 MHz at 298 K. \(^13C\) NMR spectra, including DEPT, were recorded on a DRX 600 spectrometer (Bruker Biospin) in CDCl\(_3\) at 150.92 MHz at 289 K. The \(^1H\) and \(^13C\) NMR spectra of component A resembled those of ergosterol, except for the presence of two carbon peaks around 80 ppm and the absence of two \(sp^2\) carbon peaks around 140 ppm. The results suggest that component A is an oxidized ergosterol. The NMR spectra of this compound were in good agreement with previously reported data on ergosterol peroxide.\(^4\) The active component was thus identified as \(5\alpha,8\alpha\)-epidioxy-22E-ergosta-6,22-diene-3β-ol (ergosterol peroxide) (Fig. 2a). Using \(^1H\) and \(^13C\) NMR spectral data, ergosterol was also identified as the major component of fraction Eh-2, though this compound did not induce apoptosis in HL60 cells. Ergosterol peroxide isolated from \textit{S. aspratus} inhibited the growth of HL60 cells at a dose of >10 \(\mu\)M (Fig. 2b). Twenty-five \(\mu\)M (10.7 \(\mu\)g/ml) ergosterol peroxide isolated from \textit{S. aspratus} reduced the number of
viable cells to 5% of that of control cells, and induced apoptotic cell bodies and nuclear fragmentation in most of the HL60 cells after 24 h incubation (Fig. 2b and c). Nucleosomal DNA fragmentation was also observed in HL60 cells treated with 25 μM ergosterol peroxide for 24 h (Fig. 2d). These results indicate that ergosterol peroxide inhibited the growth of HL60 cells by induction of apoptosis.

Polysaccharides isolated from *S. aspratus*, as well as from *H. erinaceum* and *L. decastes*, have been shown to exert *in vivo* anti-tumor activities against Sarcoma 180, but only a few reports on lipophilic constituents, such as steroids and terpenoids, of *S. aspratus* exist. We report that the acetone extract of dried fruiting bodies from *S. aspratus* induced apoptosis in HL60 leukemia cells, which is an important mechanism of cancer suppression. We have identified ergosterol peroxide as the major active component of fraction Eh-3, which showed the highest apoptosis-inducing activity. Although ergosterol peroxide, a component widely found in edible mushrooms, is known to inhibit cancer cell growth, the mechanism of inhibition is not known. This is the first report indicating that ergosterol peroxide inhibits the growth of HL60 cells by inducing apoptosis. We are currently investigating the effects of this compound on normal cells and other cancer cell lines to confirm the potency of *S. aspratus* fruiting bodies containing ergosterol peroxide in cancer prevention.

**Acknowledgments**

We thank Ms. Ikuko Maeda and Dr. Takashi Murata of the Instrumental Analysis Center for Food Chemistry of the National Food Research Institute of Japan for technical help on NMR and FT-ICR MS measurements respectively. We thank Dr. Keishi Hata of the Akita Research Institute of Food and Brewing for valuable discussions.

**References**


