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Ophiocordyceps pulvinata Induces Caspase-Dependent Apoptosis through Mitochondrial Impairment in Human Leukemia Cells

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A B S T R A C T

Cordyceps is a genus of fungi from the *Cordycipitaceae* family that grows using host insects as the nutritional source. This genus comprises approximately 500 species worldwide. In the present study, culturing 16 species from 3 genuses of Chu-Soh, namely, *Cordyceps*, *Ophiocordyceps*, and *Isaria*, were successfully artificially cultured We then specifically investigated the antitumor effects of *Ophiocordyceps pulvinata* on human cancer cells. Secondary metabolites of *O. pulvinata* (OP-FD) showed a significant anti-proliferative effect on breast cancer and leukemia cells at low concentrations but not on gastric and pancreatic cancer cells at the same concentrations. Virtually no effect was observed in normal human dermal fibroblasts (NHDF) cells. As OP-FD did not lose antitumor activity on heating, further fractionation with organic solvents was conducted to investigate the antitumor effects. The hexane (OP-He) and chloroform (OP-CH) fractions showed a potent cytotoxic effect on human breast cancer and leukemia cells at lower concentrations than OP-FD. Furthermore, OP-He-induced cell death was characterized by mitochondrial impairment, loss of mitochondrial membrane potential ($\Delta\Psi_m$), caspase-9 and-3 activation, sub-G_1 phase DNA, chromatin condensation, and DNA fragmentation. These results indicate that the cytotoxicity of *O. pulvinata* to leukemia cells is due to apoptosis via the caspase-dependent mitochondrial pathway.

Introduction

In Japan, more than 40,000 women are diagnosed with breast cancer and more than 10,000 women die of breast cancer each year. Compared to its incidence 30 years ago, the incidence of breast cancer has increased nearly 4-fold and mortality from breast cancer has increased nearly 3-fold. Breast cancer accounts for approximately half of all

metastatic bone tumors. Furthermore, patients with early-stage breast cancer are at an increased risk of developing leukemia after receiving chemotherapy and radiotherapy (Saito *et al.,* 2009; Sagara *et al*., 2016).

Cordyceps, known as the Kampo formulation of "Chu-So," refers to fungi (mushrooms) that parasitize living insects and form host-specific fruiting bodies after the insects die (Paterson *et al*., 2008). In taxonomy, the genus *Cordyceps* is part of the *Clavicipitaceae* family from the order *Hypocreales* of the division Ascomycota. *Cordyceps* fungi parasitize various different insects depending on the species, and more than 360 species are found within Japan (Takano *et al*., 1996; Yahagi *et al*., 1999). *Cordyceps* has been acknowledged as an important pharmaceutical resource in recent years. However, it has long been used as a natural medicine and medicinal resource (Bok *et al*., 1999, Cheng *et al*., 2013; Nakamura *et al*., 1999; Oh *et al*., 2008; Shao *et al*., 2016; Yu *et al*., 2016). The quantities required to utilize *Cordyceps* as a medicinal resource are difficult to obtain because certain species are rare, while others lack complete information on drug efficacy and active ingredients. Despite the empirically well-known medicinal effects of *Cordyceps*, research on its physiological activities has been hampered due to its extremely small supply available from the natural environment (Kodama *et al*., 2000; Lee *et al*., 2013; Lee *et al*., 2016; Ma *et al*., 2015).

With artificial culturing methods involving insects (Sato *et al*., 2002), the effects of insect components on safety in medicinal and food applications cannot be ruled out, and medicinal effects may vary between different lots. Thus, we established a culture system without the use of insects. We previously screened 16 artificially cultivated *Cordyceps* preparations to study the growth inhibitory effects on human cancer cells. Each *Cordyceps* preparation showed a distinct pattern of cell specificity (Yahagi *et al*., 1999; Yahagi *et al*., 2004; Ogawa *et al*., 2014).

Ophiocordyceps pulvinata, the *Cordyceps* species in the present study, was originally discovered by Mr. Yoshitaka Kaitsu in Iidate Village in Fukushima Prefecture in 1986. *O. pulvinata* is an ascomycete in the genus *Cordyceps* that parasitizes ants from the family *Formicidae* in the order *Hymenoptera*. *O. pulvinata* generates a *Torrubiella-type* fruiting part between the head and chest nodule in a scarf-like manner; however, no details on the physiological activities of components from this mushroom are known. In the present study, we assessed the mechanism of breast cancer and leukemia cell death activity (*in vitro*) of secondary metabolites from artificially cultured *O. pulvinata*. The aim of our work was to decrease the side effects of chemotherapy and the risk of developing leukemia due to radiation therapy in patients with breast cancer.

Materials and Methods

Materials

Cordyceps species Fungal Mycelial Cultibation

Parasitic mushroom, Chu-Soh, *Ophiocordyceps pulvinata* sp.nov were harvested from infected insects at Iidate Village (Fukushima Prefecture, Japan) between 2000-2007. The ascospores isolated from perithecium of *O. pulvinata* were inoculated into an autoclaved culture medium composed of 0.3% yeast extract, 0.5% glucose and 0.016% inosine in a 200 ml-flask, and left in the dark for 14 month at 18 ºC (Yahagi *et al*., 1999). Conidispores were growing from developing colonies of the new

hyphae in the artificial medium 6 month after inoculaton. The hyphae of *Cordyceps* species were carefully removed from the medium by filtration, and the filtrate was centrifuged at 10,000 rpm for 60 minutes. The supernatant was lyophilized to give dark-brownish powder (OP-FD). OP-FD were extracted with methanol. The MeOH extract was suspended in water, and then partitioned with Hexan (OP-He), $CHCl_3$ (OP-CH), EtOAc (OP-Et) and BuOH (OP-Bu).

Chemicals

Cell Counting Kit-8 including 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophhenyl)-2H-tetrazolium,monosodium salt (WST-8) was from Dojindo Co., Kumamoto, Japan. RPMI 1640 medium was from Nissui Pharmaceutical Co., Tokyo, Japan. Fetal bovine serum (FBS) was from Life Technologies Co., Carlsbad, CA, USA. Penicillin- streptomycin was from Roche Diagnostics K. K., Tokyo, Japan. Trypan blue solution was from Nacalai Tesque, Inc., Kyoto, Japan. Aphidicolin were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Cells

Human promonocytic leukemia U937 cells, acute T cell leukemia Jurkat cells and breast cancer MCF-7 cells were obtained from Institute of Development, Ageing and Cancer, Tohoku University. Human bile duct cancer TYBDC-1 cells and human endometrial adenocarcinoma (HEC-1) cells were obtained from the JCRB Cell Bank. Human breast cancer MDA-MB-231(MDA) cells and prosate cancer LNCap cells were purchased from DS Pharma Biomedical Co. (Tokyo, Japan). Normal human dermal fibroblasts (NHDF) cells were purchased from Takara bio Co. (Tokyo, Japan). U937, Jurkat and LNCap were maintained in RPMI 1640 medium. MCF-7 and HEC-1 were grown E-MEM. MDA cells were grown L-15 medium, and TYBDC-1 cells were maintained DMEM and Ham's F12 medium. All cell cultures were supplemented with heat-inactivated fetal bovine serum 10% (v/v), penicillin (100 IU/ml), and streptomycin (100 □ g/ml) at 37 °C in an atmosphere of 95% air/ 5% CO_2.

Cell viability and cytotoxic assay

Cells (2×10^4, in 90 □ l solution) were cultured in 96-well flat-bottom plate and treated with various concentrations of each samples for 48 h at 37 °C in an atmosphere of 95% air and 5% CO_2. Cytotoxic activity and cell viability and cell growth were evaluated by trypan blue (0.5% (w/v)) exclusion and by the WST-8 assay (10 □ l), respectively. The reduction in proportion of living cells was assayed by measurement of absorbance at 450 nm (reference, 600 nm) using the GloMax Multi Detection System.

Nuclear Staining with Hoechst 33258

U937 cells (1×10^6 cells/dish) were plated in 6-cm dish and then treated with or without OP-He. After 48 h incubation, the harvested cells were washed with PBS, and fixed with 1% glutaraldehyde for 30 min. After washing with PBS, the cells were stained with Hoechst 33258 for 10 min. The cells were washed with PBS and their nuclear morphology was observed by fluorescent microscopy (Eclipse E600, Nikon, Tokyo, Japan).

DNA Fragmentation Analysis

U937 cells (1×10^6 cells/dish) were plated in 6-cm dish and then treated with or without OP-He and OP-Bu. After the treatments, the cells were washed with ice-cold PBS and resuspended in lysis buffer (50 mM Tris-HCl, pH8.0, 10 mM EDTA, and 0.5% SDS) with 0.2 mg/ml RNase A for 30 min at 50 °C.

Proteinase K was added and cells were incubated overnight. The DNA was separated using a 2% agarose gel and visualized under UV illumination after staining with ethidium bromide.

Flow Cytometry Analysis of Apoptotic Cells

Cell cycle analysis to detect the sub-G1 phase cells was performed using a cell cycle phase determination kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. U937 cells ($1x10^6$ cells/dish) were plated in 6-cm dish, and treated with or without OP-He. After treatment with OP-He for 48 h, the cells were centrifuged and washed twice with assay buffer. And then, the cells were fixed with fixative and suspended with staining solution containing propidium iodide (PI) and RNase A. The sub-G1 peak was measured and analyzed in the FL2 channel of a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with 488 nm excitation laser. The cells ($2x10^4$ cells) were counted for each sample.

Analysis of FITC-labeled annexin V binding and propidium iodide incorporation

Cell apoptosis was detected using Annexin V-FITC Apoptosis detection kit I (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. U937 cells ($1x10^6$ cells/dish) were plated in 6-cm dish, and treated with or without OP-He. After treatment with OP-He for 12, 24 and 48 h, the cells were centrifuged and washed twice with cold PBS. The cells were re-suspended in binding buffer and then incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark. Samples werethen analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) The cells ($2x10^4$ cells) were counted for each sample.

Statistical analysis

The results of experiments are presented as mean ± standard error (SE). Differences in means were evaluated by two-tailed Student's *t*-test with P values <0.05 considered to be statistically significant.

Results and Discussion

Culture of *Ophiocordyceps pulvinata* and preparation of secondary metabolites

C. militaris and other species were initially cultured using the experimental conditions reported by Kobayashi (Kobayashi, 1941). However, reproducibility was poor because all of the fruiting bodies that developed were synnemata, and no ascocarps formed. In addition, the methods reported by Harada *et al.* and Chen *et al.* employ parasitic insects (Kepler *et al*., 2012; Chen *et al*., 2002; Sato *et al*., 2002) and are associated with safety concerns for use in the medical industry.

Therefore, we successfully developed a stable Chu-Soh culture system that has a high level of safety. It also permits the efficient formation of sexual fruiting bodies on the culture medium that are comparable with those that develop in the wild, and allow the production of different lots with the same efficacy.

Using this artificial liquid medium, we were able to culture samples in large quantities (Yahagi *et al*., 2004). Figure 1B shows the fruiting bodies developed from *O. pulvinata* on agar. The metabolite-containing media obtained from this culture system were filtered through a membrane filter and freeze-dried for use in subsequent experiments.

The freeze-dried secondary metabolite filtrates were approximately 0.03–0.05% w/v for *O. pulvinata*.

Anti-proliferative activity of *O. pulvinata* secondary metabolites on human cancer cells

The anti-proliferative activity of *O. pulvinata* secondary metabolites (OP-FD) was tested with human leukemia cells (U937 and Jurkat), human breast cancer cells (MCF7 and MDA-MB-231), human prostate cancer cells (LNCap), human bile duct cancer cells (TYBDC-1), and. human endometrial adenocarcinoma cells (HEC-1). As shown in Figure 2, OP-FD potently inhibited cell proliferation in a concentration-dependent manner in leukemia cells (U937 and Jurkat) and breast cancer cells (MCF7 and MDA-MB-231). Activity levels observed with other cancer cells were noticeably lower. The IC_{50} value of OP-FD on cell proliferation was 350 μg/mL in the leukemia cells and 640 μg/mL in the breast cancer cells. The IC_{50} value in normal human skin fibroblasts was ≥2500 μg/mL.

To identify active components, OP-FD was subjected to heating and then administered to leukemia cells. The antitumor activity of OP-FD was not affected by heating. Next, a methanol extract of OP-FD was subjected to solvent partitioning using hexane, chloroform, ethyl acetate, and 1-butanol. The soluble fraction of each solvent was administered to the leukemia and breast cancer cells. As shown in Figure 3, OP- He and OP-CH showed potent and concentration-dependent antitumor activity on all cell types. For U937 cells, the IC_{50} values of OP-He and OP-CH were ≤4 μg/mL and 4 μg/mL, respectively, which were substantially lower than those of OP-FD.

Confirmation of apoptosis induction by OP-He

To determine whether the inhibition of cell proliferation by OP-He was due to apoptosis, morphological changes to the nucleus and DNA fragmentation were investigated. Nuclear morphology was observed in cells stained with Hoechst 33258 using a fluorescence microscope. DNA fragmentation was detected using agarose gel electrophoresis of cells treated with RNase and proteinase K. OP-He caused chromatin aggregation (Fig.4A) and DNA fragmentation (Fig.4B) that was characteristic of apoptosis in leukemia cells. OP-He induced a time-dependent increase in the binding of annexin V to phosphatidylserine (PS) that migrated toward the outer layer of the cell membrane (Fig.4C). The presence of PS is another biochemical characteristic of apoptosis. Furthermore, a FACS analysis of the sub-G_1-phase population of propidium iodide-stained cells revealed a marked increase in the sub-G_1 population of OP-He-treated cells (Fig.4D). These results suggest that OP-He inhibits the proliferation of leukemia cells by inducing apoptosis.

Analysis of apoptotic mechanisms induced by OP-He

To determine the mechanism underlying the apoptosis-inducing effect of OP-He on U937 cells, caspase activation was assessed. Treatment with OP-He activated caspase-9 and -3 (Fig.5A). Mitochondria are closely involved in apoptosis induced by various stimuli. Therefore, we measured the mitochondrial membrane potential (MMP) using JC-1. Cells treated with OP-He showed a time-dependent decrease in MMP (Fig.5B).

C. militaris is an extensively studied species that contains an abundance of physiologicall active substances such as cordycepin, β-glucans, and ergosterol (Chen *et al*., 2013; Jeong *et al*., 2012; Jeong *et al*., 2013; Kobori *et al*., 2007; Nallathamby *et al*., 2015; Park *et al*., 2009; Yoshikawa *et al*., 2004). Cordycepin is an adenosine derivative that

potentially inhibits cancer cell proliferation through stimulation of the adenosine A 3 receptor (Tian *et al*., 2015; Yoshikawa *et al*., 2011). Cordycepin also activates AMP-activated protein kinase (AMPK). AMPK plays an important role in intracellular signaling and can suppress cancer cell proliferation (Wu *et al*., 2014; Zhang *et al*., 2014). Moreover, it has been reported that different *Cordyceps* species have varying activities, as exemplified by FTY720, an immunosuppressive compound found in *Isaria cicadae* Miquel that parasitizes *Meimuna opalifera* (Bhatti *et al*., 2013). We conducted antitumor screening in human cancer cells and found that low concentrations of secondary metabolites of *O. pulvinata* (OP-FD) effectively and specifically inhibited the proliferation of leukemia and breast cancer cells but not of normal (NHDF) cells. These findings indicate that susceptibility to OP-FD widely varies among different cancer cells. Acute and chronic toxicity experiments in mice showed no major differences in body weight gain or organ abnormalities between the control and test groups. Based on these findings, unknown components in the metabolic fluid of *O. pulvinata* may serve as new lead compounds for the development of anticancer drugs with minimal side effects. To characterize the active substances in OP-FD, heated OP-FD was administered to leukemia cells. The heated OP-FD preparation was as effective as the unheated preparation in terms of antitumor activity. Next, the methanol extract of OP-FD was subjected to solvent partitioning with hexane, chloroform, ethyl acetate, and 1-butanol and then tested for antitumor activity. The hexane and chloroform fractions had a strong antitumor effect. These results suggest that the active substance is a highly hydrophobic, low-molecular weight compound.

Fig.1 Artificial culture and natural products of *Ophiocordyceps pulvinata*.
(A) Specimens of *Ophiocordyceps pulvinata* in natural field.
Arrow indicates stromata caused to wrap around the neck of ant.
(B) Fruiting bodies produced by *Ophiocordyceps pulvinata* in culture medium.

Fig.2 Inhibitory effect of secondary metabolites of *Ophiocordyceps pulvinata* (OP-FD) on the Growth of human tumor cells Cells were treated with OP-FD [2500-80□ g/ml] for 48h. Then cell viability was determined by WST-8 assay and trypan blue dye exclusion assay. (A) U937(●), Jurkat(□), LNCap(▲) and HEC-1(♦). (B) MDA-MB-231(●), MCF7(□), TYBDC-1(▲) and NHDF(♦).

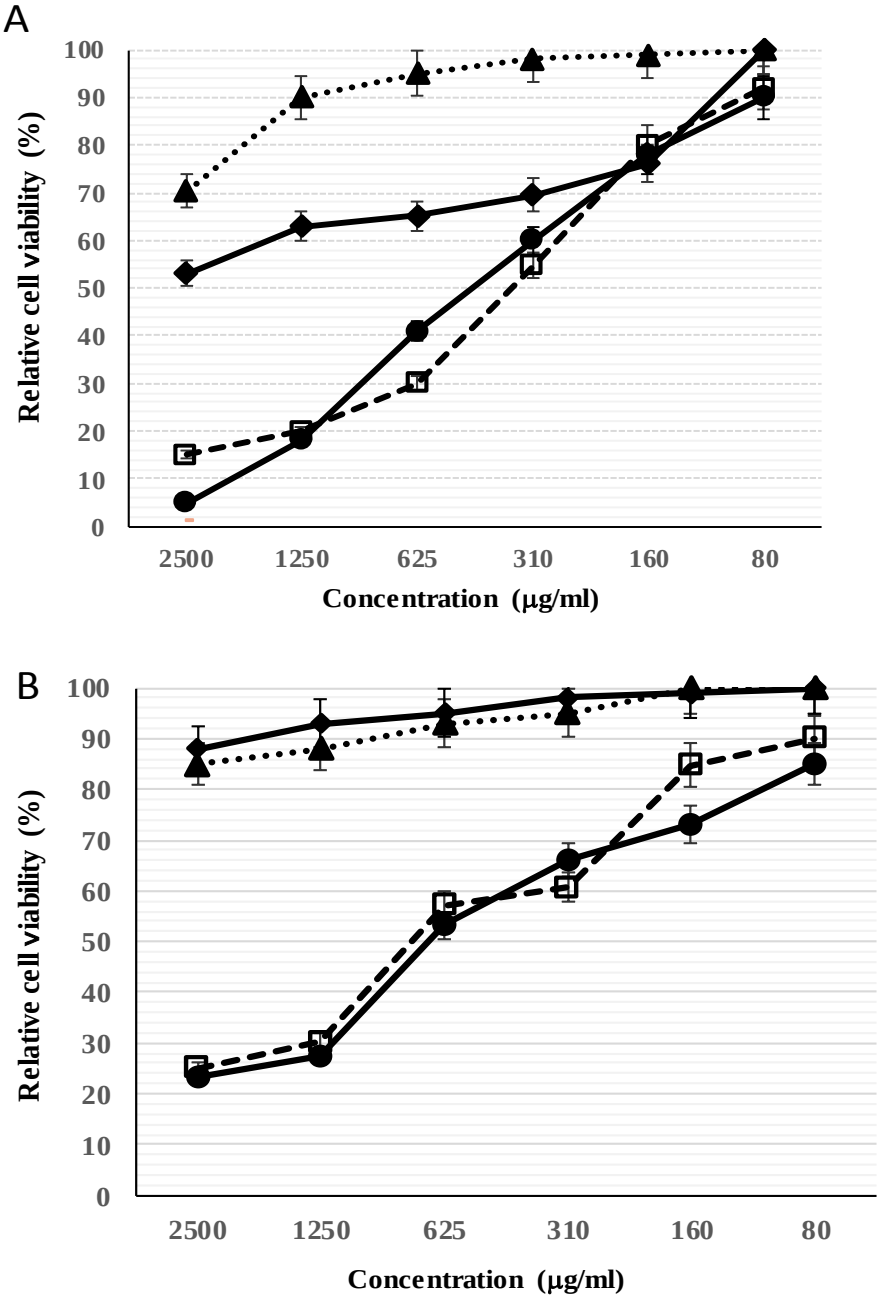
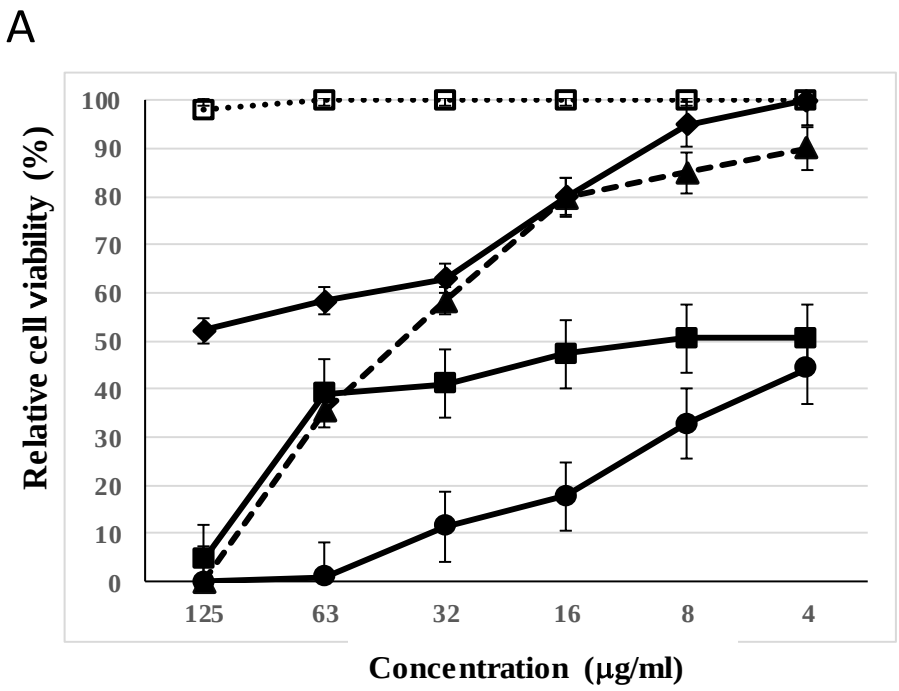


Fig.3 Anti-cancer effect of organic solvent extracted fraction of OP-FD on the Growth of human tumor cells U937 cells (A) and MDA-MB-231 cells (B) were treated with OP-He(●), OP-CH(■), OP-Et(▲), OP-Bu(♦) and OP-H2O(□) [125-4□ g/ml] for 48h. Then cell viability was determined by WST-8 assay and trypan blue dye exclusion assay



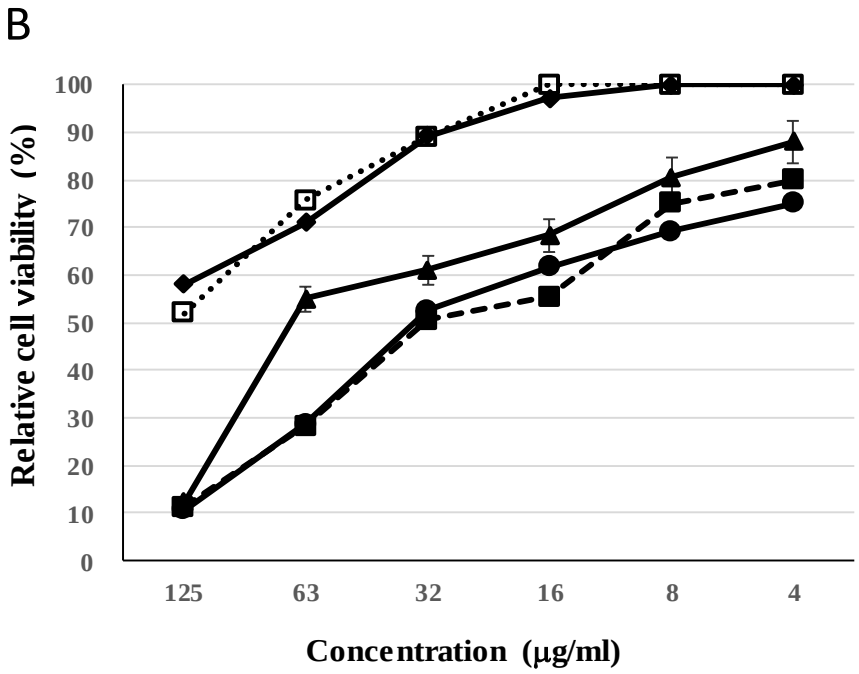


Fig.4 Effect of organic solvent extracted fraction of OP-FD on apotosis induction.

(A) Induction of chromatin condensation by OP-He. U937 cells were treated with OP-He for 48h and stained with Hoechst 33258. The nuclear morphology was observed fluorescent microscopy (magnification x400) (B) Induction of DNA fragmentation by OP-He. U937 cells were treated with OP-He at various concentrations for 48h. DNA fragmentation was analyzed by agarose gel electrophoresis. (C) Analysis of FITC-labeled annexin V binding and propidium iodide incorporation. U937 cells were treated with 16μg/ml OP-He for 48h, and analyzed by flow cytometry after staining with FITC-annexin V and PI. The data shown are representative of three independent experiments with similar results. (D) Increase of the sub-G1 phase cells by OP-He. U937 cells were treated with 16μg/ml OP-He for 48h, and analyzed by flow cytometry after staining with PI. The data shown are representative of three independent experiments with similar results.

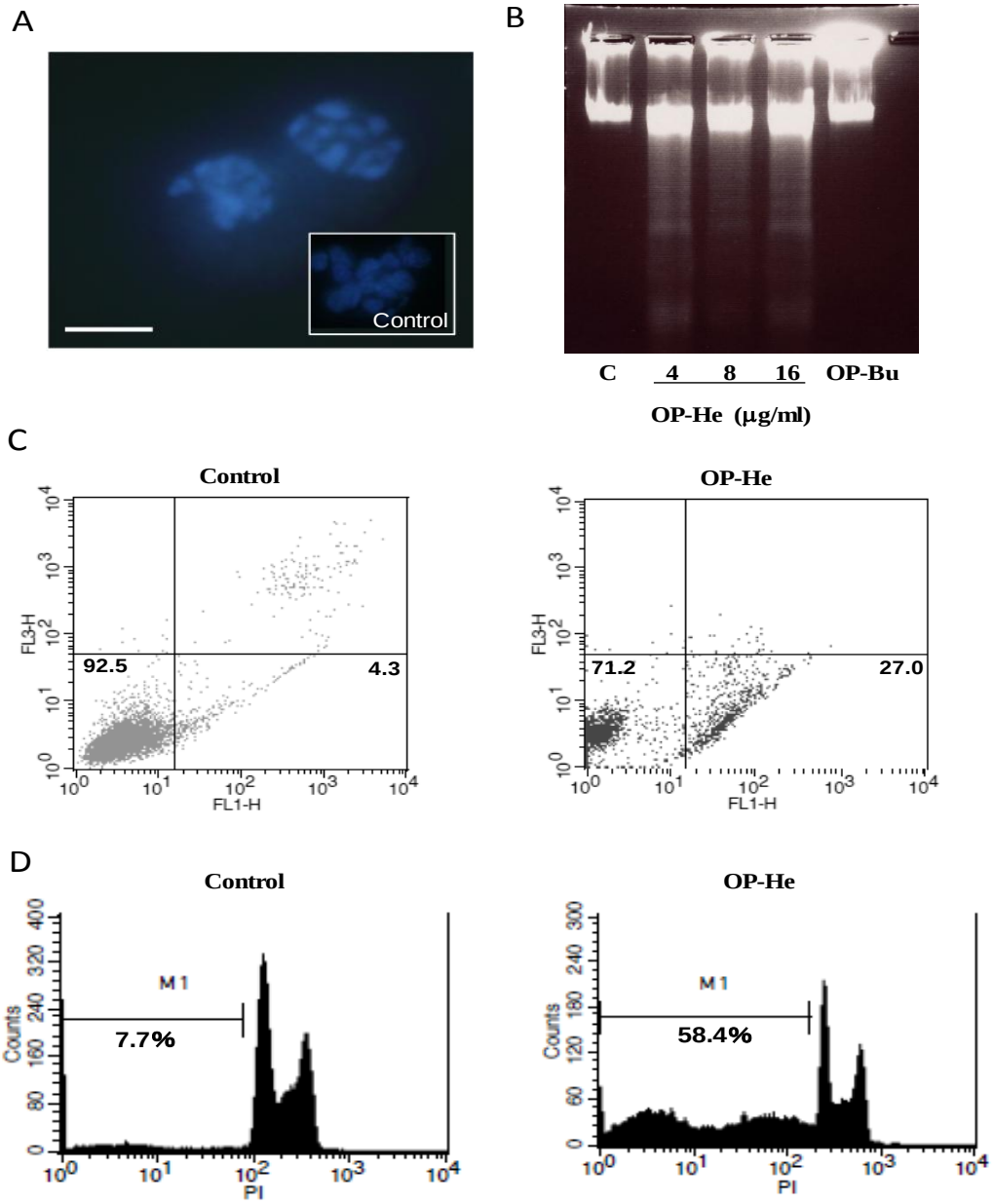
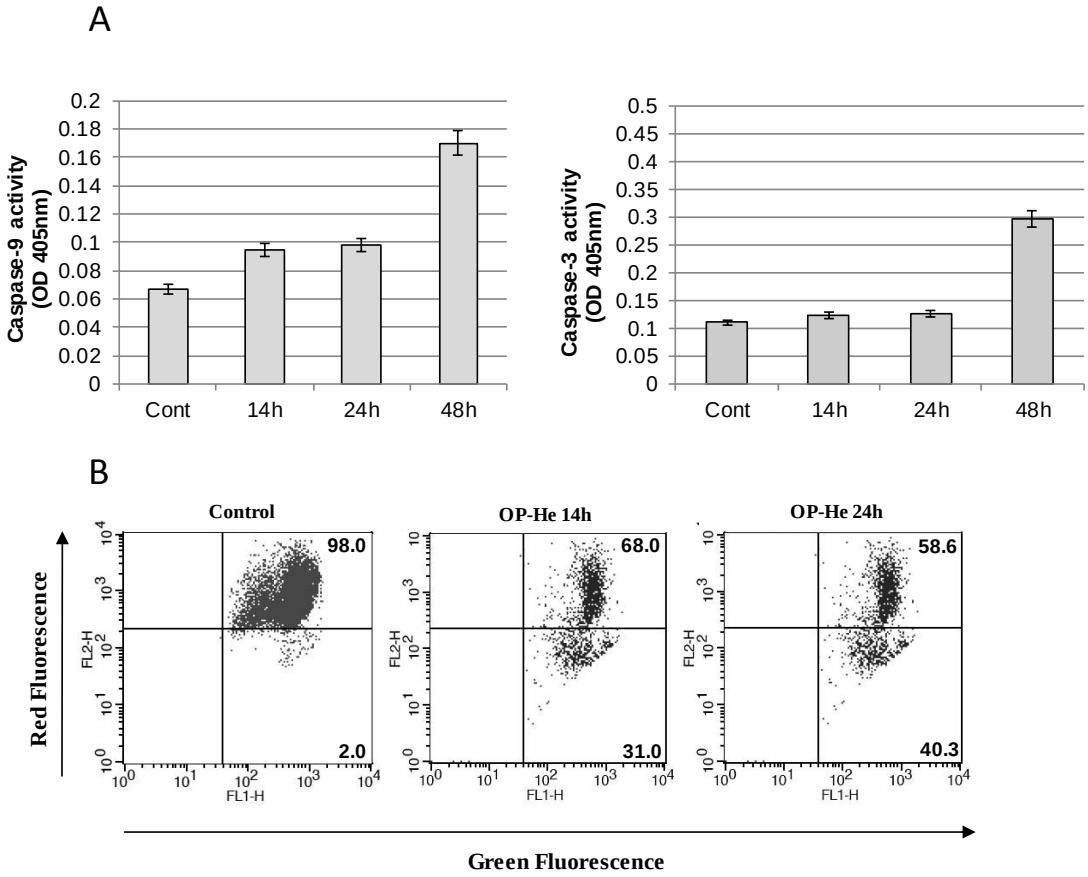


Fig.5 OP-He induced apoptosis through a caspase-mediated pathway in U937 cells. (A) Involvement of the caspase cascade in OP-He-induced apoptosis. U937 cells were treated with 16µg/ml OP-He for the times indicated. Cells were lysed and treated with substrate of caspase-3,-9 and the activity were measured. Error bars: SE from three different cell preparations assayed individually. (B) Effect of OP-He on the loss of ΔΨm. U937 cells were treated with 16 g/ml OP-He for the times indicated and analyzed by flow cytometry after staining with JC-1.



In vivo mechanisms induce cell death in unnecessary or harmful cells. Apoptosis plays an important role in not only the elimination of unnecessary cells during development and regeneration processes but also the elimination of cancer and virus-infected cells. When the cellular switch for apoptosis is turned on, proteases called caspases in the cell are activated. Activated caspases break down multiple protein molecules essential for cell survival and thereby induce cell death (Galluzzi *et al*., 2016). An apoptotic cell is broken into several small fragments (apoptotic bodies) with characteristic morphological features such as chromatin condensation. However, the cell membrane does not rupture, and cell contents never leave the cell. A major feature of apoptosis is the cleavage of chromosomal DNA into nucleosome units. A caspase-activated DNA-degrading enzyme has been reported to be involved in this DNA cleavage process (Hamacher-Brady *et al*., 2015; Larsen *et al*., 2016). Dead cells are processed by phagocytes such as macrophages (Mitra *et al*. 2015). Macrophages recognize a lipid called PS found only on the surface of apoptotic cells. Furthermore, autoimmune diseases can inhibit the ability of macrophages to process apoptotic cells (Cummings *et al*., 2016). In

the present study, chromatin condensation, nuclear fragmentation, and increased annexin V binding were observed in leukemia cells treated with OP-He, indicating the induction of apoptosis. Three major apoptosis signaling pathways are known: death receptor-mediated pathway, mitochondrial pathway, and endoplasmic reticulum stress pathway. Treatment with OP-He reduced the MMP and activated caspase-9 and -3. This result suggests that OP-He induces apoptosis through the caspase-dependent mitochondrial pathway. Because OP-He showed antitumor effects on breast cancer and leukemia cells, it is likely to reduce the side effects in chemotherapy-treated patients with breast cancer and the risk of developing leukemia in patients receiving radiation therapy.

Cordyceps fungi, including *O. pulvinata*, contain various substances. Thus, these fungi may produce not only antitumor effects but also immunostimulatory effects via additive and synergistic interactions. Determining the physiologically active components in these species is expected to produce new lead compounds for anticancer drugs, apart from cordycepin and ergosterol peroxide.

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