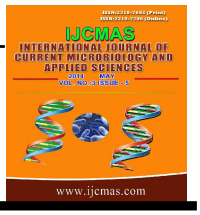
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 607-617**
http://www.ijcmas.com



Original Research Article

Specific antiproliferative activity against human cancer cells with metabolites from several species related to the genus Cordyceps

Yukiko Ogawa[1]*, Nobuo Yahagi[2], Remiko Yahagi[2], Hidemitsu Kobayashi[1]

[1]Divisions of Microbiology, Department of Pharmacy, Faculty of Pharmaceutical Science, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859-3298, Japan
[2]Natural Medical Plant and Microbiological Organism Research, Mamurogawa-machi, Mogami-gun, Yamagata, 999-5604, Japan
Corresponding author

Keywords

Cordyceps species, antitumor activity, cell specificity, medicinal plants, breast cancer cells

A B S T R A C T

The present study was succeeded in culturing 16 species from 3 genuses of Chu-Soh, namely, Cordyceps, Ophiocordyceps, and Isaria, and screened their metabolites for antiproliferative activities against human cancer cells. Culture filtrates (metabolite-containing media) of 13 species from Chu-Soh were lyophilized and resuspended in phosphate buffer to investigate their in vitro antiproliferative activities against 5 human cancer cell lines. Six species, including Cordyceps formicarum, out of 13 species had very little effect when tested at low concentrations. However, 3 species, including Cordyceps militaris, out of 13 species significantly inhibited cell proliferation in almost all the cancer cell lines tested. Culture filtrates from C. roseostromata showed very strong antiproliferative activities against leukemia cells (U937) and breast cancer cells (MCF7), but not in normal human dermal fibroblasts. Cordyceps ophigoglossoides, Ophiocordyceps pulvinata sp. nov., and Isaria sp. nov. (Konaabutake), effectively and specifically inhibited cell proliferation in breast cancer cells (MCF7), leukemia cells (U937), and gastric cancer cells (KatoIII). The antiproliferative metabolites obtained from these 4 species were highly specific to human cancer cell lines. These findings suggest that the unknown compounds in the metabolite-containing media from cultures of these mushrooms are potential lead compounds for developing anticancer drugs with extremely mild side effects.

Introduction

In current cancer therapies, anticancer drugs have the disadvantage of targeting a substantial number of normal as well as cancer cells. This nonspecific cellular toxicity causes severe side effects in patients with cancer. Majority of anticancer drugs used in the clinic were developed using metabolites derived from group A β-hemolytic streptococcus (group A streptococcus) (Shen et al., 1992) or actinomycetes such as Streptomyces caespitosu (Taechowisan et al., 2007,

Homma et al., 2007) as lead compounds. A preferred way of reducing side effects from a view of anticancer drug development is to discover novel lead compounds with high cell specificity. We focused on a unique collection of mushrooms of the genus Cordyceps, which contains several species that have barely been studied before.

Mushrooms of the genus Cordyceps and Isaria (Takano et al., 1996, Yahagi et al., 1999), which contain more than 350 recognized species, are distributed in temperate regions all over the world. In Japan, these species of mushroom are called "Toh-Chu-Kasoh" or "Chu-Soh," and some species have been highly valued for their nutritional fortification since early times. The spores infect and infest various living insects. They proliferate using the bodies of the dead insects as nutrients and grow to form fungal fruiting bodies. There are several technical challenges that have delayed the study of Cordyceps until recent times. First, the fruiting bodies are quite small and hard to find, which makes it difficult to isolate the wild strains. Second, artificial culture has not been established for almost all species. In addition, although artificial culture conditions are established, the fungi proliferate extremely slowly and require 1 or 2 years to reach stationary growth phase, which is also one of reasons that the genus Cordyceps was not a target of study.

Despite the small number of studies on these species, it was anticipated that cordycepin found in C. sinensis, C.militaris, and Isaria japonica is a potential anticancer drug because cordycepin showed strong anticancer activity (Lee et al., 2013, Kodama et al., 2000). Fingolimod (FTY720) (Bhatti et al., 2013), which was found in I. sinclairi, has been recently commercialized as a drug for preventing the recurrence of multiple sclerosis. Some successful results have been achieved in other species of the genus Isaria. However, some biological activities have been reported in C. sinensis, C. militaris, and C sphecocephala (Zhu et al., 1998, Oh et al., 2008, Huang et al., 2000, Nakamura et al., 1999, Cheng et al., 2013), but further studies of the genus Cordyceps have been severely limited. In the present study, we succeeded in the artificial culture of 16 previously unstudied species from the genus Cordyceps and screened their metabolites for antiproliferative activities against human cancer cell lines.

Materials and Methods

Cordyceps species Fungal Mycelial Cultibation

Parasitic mushroom, Chu-Soh, Cordyceps species〔Cordyceps militalis (Vuill.) Fr., Cordyceps martialis Spegazzini, Cordyceps roseostromata Y. Kobayasi et D Shimizu, Cordyceps ophioglossoides (Ehrhart:Fries)〕, Ophiocordyceps species〔Ophiocordyceps purpreostromata Y. Kobayasi, Ophiocordyceps sinensis, Ophiocordyceps sphecocephala (Klotzsch ex Berk), Ophiocordyceps formicarum Y. Kobayasi, Ophiocordyceps pulvinata sp.nov. (the formal name is Torrubiella collanatus sp.)〕and Isaria species〔Isaria japonica Yasuda, Isaria farinosa (Holmsk.)Fr., Isaria sp. (Minomushitake), Isaria sp. nov. (Konaabutake) 〕were harvested from infected insects at Mogami-Gun (Yamagata Prefecture, Japan), Iizaka-mura (Fukushima Prefecture, Japan) and Towada (Aomori Prefecture, Japan) between 2000-2007. Photo of four specimens in natural field were shown in Fig. 1A-1D). The

ascospores isolated from perithecium of Cordyceps, Ophiocordyceps and Isaria species were inoculated into an autoclaved culture medium composed of 0.3% yeast extract, 0.5% glucose and 0.016% inosine in a 200ml-flask, and left in the dark for 14 days 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 (Fig. 1E). 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.

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 calf serum (FCS) 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. Alamar BlueTM and aphidicolin were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan) and Wako Pure Chemical Industries, Osaka, Japan.

Cells

Human promonocytic leukemia U937 cells, breast cancer MCF-7 cells, colon cancer HCT15 cells, pancreatic cancer KLM1 cells and gastric cancer KatoⅢ cells were obtained from the Cell resource Center of the Biomedical Research, Institute of Development, Ageing and Cancer, Tohoku University, Sendai, Japan, respectively. Normal human dermal fibroblasts (NHDF) cells were purchased from Takara bio Co. (Tokyo, Japan). Cells were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal calf serum 10% (v/v), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37 °C in an atmosphere of 95% air/ 5% CO2.

Cell viability and cytotoxic assay

Cells (2×104, in 90 μl solution) were seeded into a 96-well flat-bottom plate and treated with various concentrations (0-2500 μg/ml) of culture filtrates (metabolites-containing media) in Cordyceps, Ophiocordyceps and Isaria species (10 μl) for 48 h at 37 °C in an atmosphere of 95% air and 5% CO2. 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 μl), 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.

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 Chu-Soh Species and Preparation of Secondary Metabolites

C. militaris and other species were initially cultured using the experimental conditions reported by Kobayashi. 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 (Sato et al., 2002, Chen 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 1E shows the fruiting bodies developed from C. roseostromata on agar. Although fruiting bodies of wild-type strains were red in color (Fig. 1D), those produced through artificial culture were frequently devoid of this color. 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 0.03% w/v for I. japonica, and approximately 0.03–0.05% w/v for all other Chu-Soh species.

Antiproliferative Activity of Chu-Soh Secondary Metabolites in Human Cancer Cells

We successfully cultured 16 Chu-Soh species. We then assessed the antiproliferative activity of human leukemia (U937), breast (MCF7), colon (HCT15), pancreatic (KLM1), and gastric cancer cells (Kato III) in the metabolites from 13 of these species (Table 1). Three of the 13 species [C. militaris, I. japonica Yasuda, and I. farinosa (Holmsk.) Fr.] significantly inhibited the proliferation of most of the cancer cell lines tested at low concentrations (160 μg/ml). This low concentration had no effect on normal human dermal fibroblasts (NHDF) (data not shown for the latter 2 species). Six species (C. formicarum, O. sinensis, O.purpreostromata, C. martialis, O. sphecocephala, Isaria sp.) had very little effect when tested at 160 μg/ml.

Figure 2A shows the antiproliferative activity of C. militaris metabolites in each cell line at concentrations ranging from 80–1,250 μg/ml. The antiproliferative effects of C. militaris metabolites were similar in all the cancer cell lines tested. In contrast, cultured filtrates from C. roseostromata at the same concentrations showed strong anti-proliferative activity in leukemia (U937) and breast cancer cells (MCF7), but only weak antiproliferative effects in all other cell lines tested (Fig. 2B).

Metabolites from C. ophigoglossoides, O. pulvinata sp. nov., and Isaria sp. nov. (Konaabutake) effectively and specifically inhibited cell proliferation in breast cancer (MCF7), leukemia (U937) and gastric cancer cells (KATO III) at low concentrations (Table 1). In addition to metabolites from these 3 species, C. roseostromata metabolites also had antiproliferative effects that differed significantly depending on the cancer cell type.

The metabolite-containing media harvested from Chu-Soh species contained many substances including polysaccharides, and therefore it is challenging to accurately quantify the levels of cell-specific active compounds that are present in each Chu-Soh species.

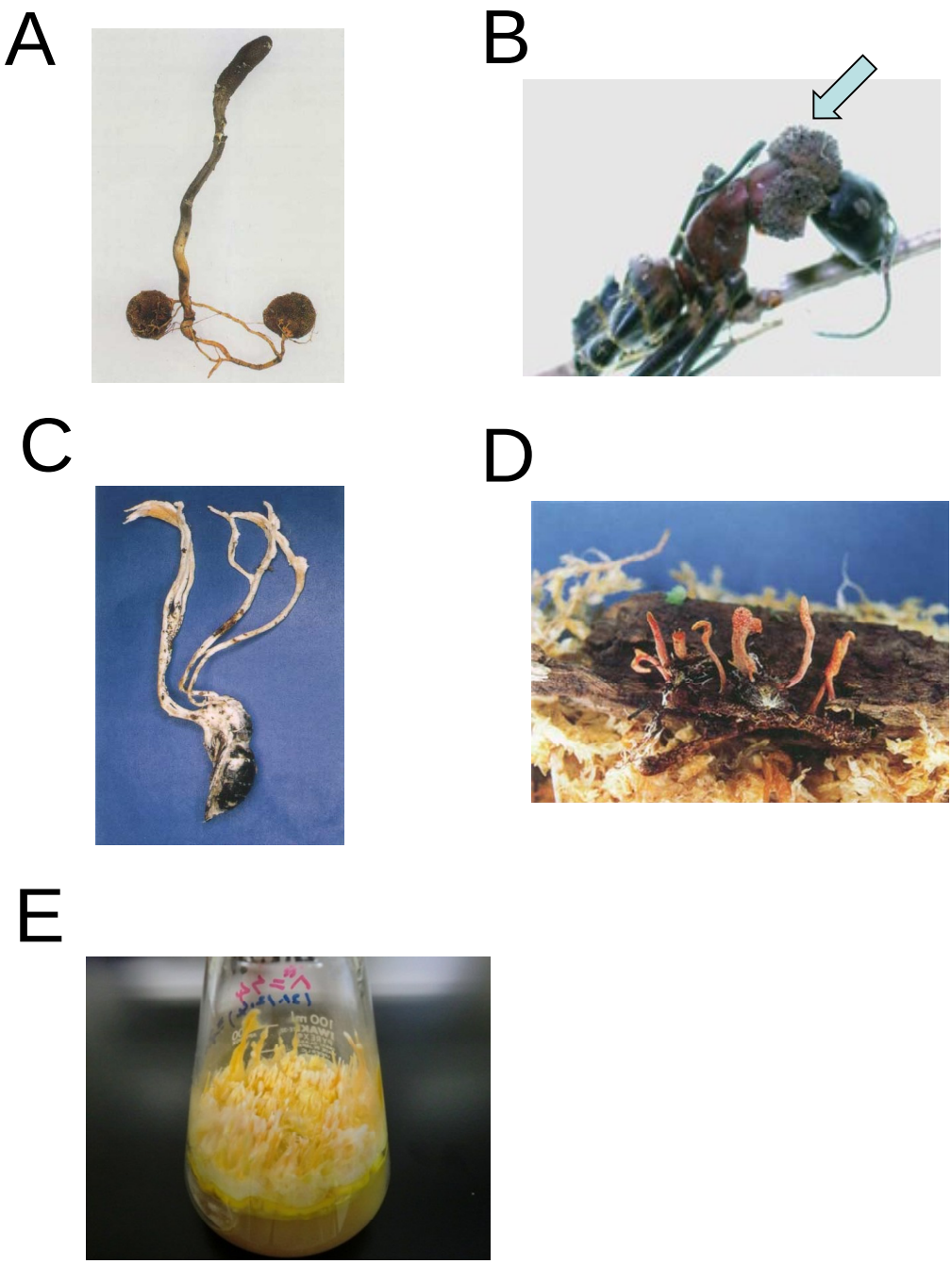


Fig. 1 Artificial culture and natural products of various species *of Cordyceps*, *Ophiocordyceps* and *Isaria*

(A) Specimens of *Cordyceps ophioglossoides* in natural field.
(B) Specimens of *Ophiocordyceps pulvinata* sp.nov in natural field.
Arrow indicates stromata caused to wrap around the neck of ant.
(C) Specimens of *Isaria* sp. nov.(Konaabutake) in natural field.
(D) Specimens of *Cordyceps roseostromata* in natural field.
(E) Fruiting bodies produced by *Cordyceps roseostromata* in culture medium.

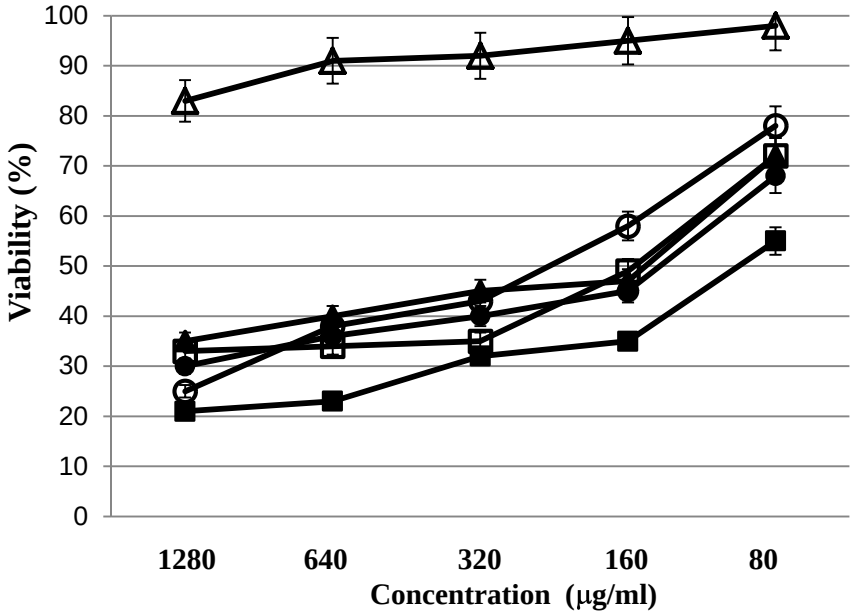
Table.1 Growth Inhibitory Effect of Secondary Metabolites of *Cordyceps, Ophiocordyceps* and *Isaria* species

Cordyceps species	U937	MCF7	HCT15	KLM1	KatoⅢ	NHDF
Cordyceps militaris	++	++	++	+	++	-
Cordyceps roseostromata	+++	++	+	+	+	-
Cordyceps ophioglossoides	-	++	+	-	-	-
Ophiocordyceps pulvinata sp.nov	++	+	-	-	-	-
Isaria sp.nov (Konaabutake)	-	+	+	-	++	-
Cordyceps formicarum	-	-	-	-	-	-

Cytotoxic activity of *Cordyceps*, *Ophiocordyceps and Isaria* species. **A**. Cell viability (%) was quantified by the WST-8 assay. Cancer cells: U937(leukemia cells), MCF7 (breast cancer cells), HCT15(colorectal cancer cells), KLM1(pancreatic cancer cells) and KatoⅢ(gastric cancer cells). Normal cells: normal human dermal fibroblasts (NHDF). 160 μg/ml of *Cordyceps*, *Ophiocordyceps and Isaria* species was administered to each cells. Cell viability; +++ : 30% or less, ++ : 31～50%, + : 51-70% and - : more than 71%**.** Data are the means of triplicate assay mean ± SD.

Figure 2

A



B

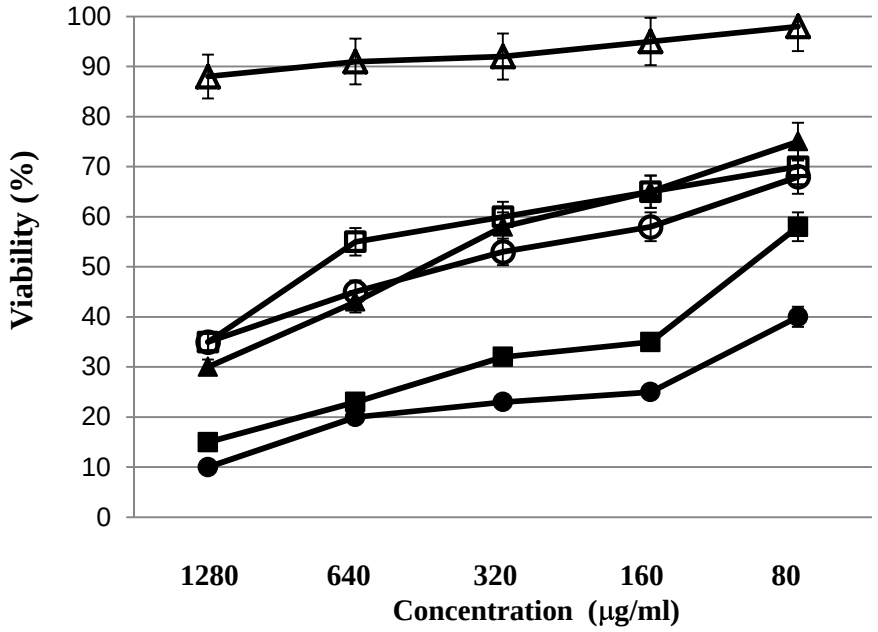


Fig. 2 Inhibitory Effect of Secondary Metabolites of *Cordyceps militalis* (CMFD) and *Cordyceps roseostromata* (CRFD) on the Growth of human tumor cells
Cells were treated with CMFD(A) or CRFD(B)〔1250-80μg/ml〕 for 48h . Then cell viability was determined by WST-8 assay and trypan blue dye exclusion assay.
U937(●), MCF7(■), HCT15(▲), KLM1(○), KatoIII(□) and NHDF(△)

Nevertheless, our observations that the metabolites from each Chu-Soh species exert effects on different cancer cells at concentrations that have no effect on normal cells suggest that different active substances are obtained from the metabolite-containing media from different mushroom species.

Discussion

The quite rare species of mushroom, Chu-Soh and its metabolites are distributed primarily in Asia as an effective folk medicine for preventing and delaying development of diabetes, metabolic diseases affecting cardiovascular system, and cancer. Some species of Chu-Soh are commercially available as a raw material for expensive Chinese herbal medicine or dietary supplement (Paterson *et al*., 2008). However, only a few studies showing the scientific evidence of effectiveness have been reported. Till date, studies on bioactivities of Chu-Soh reported include immunoregulatory activities (Shin *et al*., 2010), the decrease in insulin resistance and the increase in insulin secretion *in vivo* (Choi et al., 2004), and antitumor activities (Park *et al*., 2009) by the hot water extract of *C. militaris.* In addition, antioxidation activities (Yamaguchi *et al*., 2000), anti-inflammatory activities (Rao *et al*., 2007), antihyperlipemia effects (Koh *et al*., 2003), and antitumor activities (Bok *et al*., 1999) by the hot water extract of *C. sinensis* have been reported. Among them, ergosterol peroxide and cordycepin were

paid attention as compounds having antitumor activities. Ergosterol peroxide inhibited the proliferation of HT29 colon cancer cells at the concentration that barely affected the proliferation of fibroblast (Kobori *et al*., 2007). In contrast, there are relatively many reports regarding cordycepin (Jeong *et al*., 2012, Jeong *et al*., 2013, Yoshikawa *et al*., 2004, Chen *et al*., 2013, Yoshikawa *et al*., 2011, Paterson et al., 2008, Shin *et al*., 2009). Cordycepin had inhibitory activities against cancer cell proliferation by stimulating adenosine A_3 receptors because this compound is an adenosine derivative (Yoshikawa *et al*., 2011). Because cordycepin was reported to be present not only in *C. militaris*, but also in *C. sinensis* and *I. japonica* (Jeong *et al*., 2012, Jeong *et al*., 2013, Yoshikawa *et al*., 2004, Chen *et al*., 2013, Yoshikawa *et al*., 2011, Paterson *et al*., 2008, Shin *et al*., 2009), developing new anticancer drugs was anticipated (Kodama *et al*., 2000). However, no further progress has been reported for clinical application. While the metabolite-containing media obtained from artificial cultures of *C. militaris*, *C. sinensis*, and *I. japonica* strongly inhibited cell proliferation in almost all the cancer cell lines tested at a broad range of concentration, the cell specificity was not observed (data not shown). Therefore, when intending to develop anticancer drugs from these species of Chu-Soh, frequent occurrence of toxicity to normal cells, i.e., side effects, is a matter of concern.

In this study, we screened culture metabolites of 13 species from the genus *Cordyceps* for antiproliferative activities against human cancer cell lines. As a result, 6 species, including *C. formicarum*, out of 13 species had very little effect at a broad rage of concentration (data not shown). In contrast, culture filtrates from *C. ophigoglossoides, O. pulvinata sp.nov.*, and *Isaria sp. nov.*（Konaabutake） effectively and specifically inhibited cell proliferation in breast cancer cells (MCF7), leukemia cells (U937), and gastric cancer cells (KatoIII) at a rage of low concentration. Those from *C. roseostromata* showed very strong antiproliferative activities against leukemia cells (U937) and breast cancer cells (MCF7), but significantly weak activities against the other cancer cell lines. These findings indicate that antiproliferating effects of these 4 Chu-Soh species of the genus *Cordyceps* markedly vary in different cancer cell types.

This study is the first report of a new research being conducted in accordance with our plan. Antiproliferative activities of culture filtrates from *C. roseostromata* are rarely observed in normal human skin fibroblasts at a range of concentration where the effects are observed in cancer cells. Moreover, in our preliminary experiments on acute and chronic toxicities in mice, there was not a large difference in weight gain between the control and test groups, and no abnormalities in isolated organs were observed in the test group (data not shown). Taking into consideration data from the above, some ingredients in metabolite-containing media of *C. roseostromata* are expected to be potential novel lead compounds for developing anticancer drugs with extremely mild side effects. Focusing on low-molecular-weight compounds in active ingredients, we have been currently performing isolation and purification by high-performance liquid chromatography after fractionation with various organic solvents and determining the chemical structures primarily by

instrumental analyses including a nuclear magnetic resonance method. We will precisely perform experiments to elucidate the mechanism of anticancer activities of purified fractions by chromatography and toxicity tests in mice, of which details will be reported.

Further, culture filtrates from 3 other species of genus *Cordyceps* (*C. ophigoglossoides*, *O. pulvinata sp.nov.*, and *Isaria sp. nov.* (Konaabutake) also cell-specifically inhibit proliferation of cancer cells at a range of low concentration. Accordingly, we expect that there is a high potential to discover new lead compounds for anticancer drugs from these species, and we will be starting a study on them in the near future.

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