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Identification of a novel component leading to anti-tumor activity besides the major ingredient cordycepin in *Cordyceps militaris* extract

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ABSTRACT

In accordance with our previous study that was carried out to identify novel anti-tumor ingredients, chromatographic separation in combination with an anti-tumor activity assay was used for analysis of *Cordyceps militaris* extract in this study. Various modes of chromatography including reversed-phase, cation-exchange and anion-exchange were used to separate components of *Cordyceps militaris*, which showed various chemical properties. Anti-tumor activity of each fraction was assessed by a Hoechst staining-based apoptosis assay using malignant melanoma MeWo cells. By these repeated approaches through chromatographic segregation and cell biological assay, we finally succeeded in identifying the target substance from a certain fraction that included neutral hydrophilic components using a pre-column and post-column chlorine adduct ionization LC–APCI–MS method. The target substance was a mono-carbohydrate, xylitol, that induced apoptotic cell death in MeWo cells but not in normal human OUMS-24 fibroblasts. This is the first study showing that *Cordyceps militaris* extract contains a large amount of xylitol. Thus, our results will contribute greatly to uncovering the mysterious multifunctional herbal drug *Cordyceps militaris* as an anti-tumor agent.

1. Introduction

Melanoma, which arises from melanocytes and is the most serious skin cancer frequently shows metastatic malignancy. Surgery followed by chemotherapy is only effective in the early non-metastatic stage. Standardized therapy for melanoma in the advanced metastatic stage in a clinical setting has not been established. Clinicians use only a palliative approach (palliative medicine) in most cases. Recently, new approaches in a clinical setting have led to improvements in serious outcomes. The new approaches include immunotherapy based on immune checkpoint inhibition using antibodies to programmed cell death 1 receptor (PD-1) and its ligand (PD-L1) [1]. PD-1 is expressed on the surface of T-cells, and PD-L1 is expressed on the surface of melanoma cells. However, even an innovative approach cannot be used in all cases of melanomas but is limited to PD-L1-expressing melanomas, and further advances are therefore required. Long-term use of current anti-tumor drugs is unfavorable due to many unexpected and sometimes severe side-effects. Thus, new highly potential and low-risk anti-tumor drugs are needed.

The use of herbal medicines in cancer therapy is now being reconsidered due to their effectiveness with few severe side-effects. The herbal medicinal mushroom *Cordyceps militaris* has been reported to show potential anti-tumor properties including cancer-specific apoptosis and anti-angiogenic capacity. Chou et al. [2] demonstrated that extracts of *Cordyceps militaris* exhibit a potent cytotoxic effect against many cancer cell lines, especially human leukemia cells. A water extract of *Cordyceps militaris* has been shown to induce apoptosis of human lung carcinoma A549 cells by inhibition of telomerase activity [3]. A methanolic extract of *Cordyceps militaris* inhibited the proliferation of various human tumor cell lines including MCF-7 cells (breast cancer), NCI-H460 cells (non-small lung cancer), HCT-15 cells (colon cancer) and HeLa cells (cervical cancer) [4]. Apoptosis of human chronic

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myeloid leukemia K562 cells was observed in an *in vitro* assay with *Cordyceps militaris* extract [5]. Cordycepin is thought to be one of the active anti-tumor ingredients of *Cordyceps militaris*. Thus, various methods for extracting cordycepin effectively from *Cordyceps militaris* have been investigated [4,6–8]. However, it was recently shown that *Cordyceps militaris* extract has a more potent apoptotic effect than the effect of cordycepin alone, implying that presence of another component(s) besides cordycepin in the extract for induction of cancer-specific apoptosis [9].

The structure of cordycepin is very similar to that of the cellular nucleoside adenosine, and cordycepin therefore acts like a nucleoside analogue to disrupt cancer processes, especially synthesis of DNA and RNA for cell proliferation, eventually leading to apoptosis [7,10–13]. We also found that cordycepin has a significant anti-melanoma function [14]. We therefore considered cordycepin to be a central anti-tumor component in *Cordyceps militaris*, but we are now aware of the existence of other factors for cancer suppression in *Cordyceps militaris* [14]. Previously, we were not able to investigate and identify such novel components in *Cordyceps militaris*. It is difficult to obtain a large amount of natural *Cordyceps militaris* due to its rare appearance in nature. Detailed analysis and identification of ingredients in natural materials require an adequate yield and purity of the natural materials. To overcome this problem, we previously reported an innovative and inexpensive method for isolation and culture of *Cordyceps militaris* to produce a high-quality extract with a high yield of active ingredients under sterilized conditions [14]. The aim of this study was to identify another important anti-tumor substance(s) besides cordycepin using our *Cordyceps militaris* extract.

1.1. Strategy for separation and identification of an active ingredient

Various modes of chromatography including reversed-phase, cation-exchange and anion-exchange were used to separate the components of *Cordyceps militaris* extract, which have diverse chemical properties. The anti-tumor activity of each fraction was investigated by an apoptosis assay using MeWo cells. Fractions exhibiting a high level of anti-tumor activity in the apoptosis assay were further separated and analyzed. Fig. 1 shows a flow scheme of the procedure for separation and specification of active ingredients in *Cordyceps militaris* extract. Table 1 shows the chemical property of each fraction determined in the procedure.

1.2. Identification of an active ingredient

Through our studies, the idea arose that a kind of sugar and its related compounds such as sugar alcohols, monosaccharides and disaccharides might be candidates of anti-tumor ingredients in *Cordyceps militaris* extract. However, we had difficulties in both separation and detection of sugar alcohols, monosaccharides and oligosaccharides. Sugar alcohols, monosaccharides and oligosaccharides are highly hydrophilic and generally lack intrinsic fluorescent or chromophoric moieties. Their structures are similar, and most of them are epimers of each other. Various chromatographic methods including GC [15–18], GC–MS [19,20], HPLC [20–32], and LC–MS [20,33–38] for separation and determination of sugars have been reported. Gas chromatography coupled to a flame ionization detector (GC-FID) [15–18] or a mass spectrometer (GC–MS) [19,20] has been extensively used for separation and determination of sugars. Although the GC-FID and GC–MS methods provide good separation of monosaccharides with increased sensitivity, the combined methods are very time-consuming because of the multiple processes involved, and there is a problem of the appearance of very complex separation patterns by products from stereochemical isomeric reaction of monosaccharides. In recent years, LC methods using reversed-phase columns have been developed and have provided improvements for rapidity and user convenience. However, native sugars cannot be retained on widely used reversed-phase columns such as an ODS column owing to their high hydrophilicity, and reversed-phase liquid chromatography has therefore not been used for native sugar separation with high resolution. Consequently, many types of hydrophilic solid phases have been used for the LC method of sugar analysis. In order to overcome the above-described problem, we focused on two types of columns responding to amino propyl group-bonded stationary phase [31,32] and amide group-bonded stationary phase [33,34] as columns for separation of saccharides. We found that these two different columns were required to properly separate the targeted saccharides. Although both of these columns are in hydrophilic interaction mode in the stationary phase, we fortuitously found that the separation behaviors of sugar alcohols and saccharides were different. By using an improved method, we finally succeeded in identifying various sugar alcohols, monosaccharides and oligosaccharides from different retention times of the sugars obtained by using both columns. Various detection systems including an electrochemical detector (ECD) [24], pulsed-amperometric detector (PAD) [28], refractive index detector (RID) [26,30], evaporative light scattering detector (ELSD) [25,32] and charged aerosol detector (CAD) [29] have also been reported for direct sugar analysis. However, these detectors were not able to identify our targeted sugar and its related compounds because of their low selectivity. The emergence of mass spectrometry has increased the sensitivity of sample detection by selection of appropriate molecular and fragment ions to avoid interference from co-extracted sample materials. Liquid chromatography-electrospray ionization mass spectrometry (LC–ESI-MS) has been widely applied to all kinds of analytical research to obtain qualitative and quantitative information on analytes. However, the poor ionization efficiency of sugar alcohols makes the step of derivatization, which is indispensable for LC–ESI-MS analysis, incomplete, resulting in insufficient analytic identification [36]. To overcome this problem, we used an LC/MS method using an APCI ion source with chlorine adduct ionization that does not require the step of derivatization of sugars [37,38].

In this study, a pre-column and post-column chlorine adduct ionization LC–APCI–MS method was therefore used for identification and determination of sugar alcohols and saccharides.

2. Materials and methods

2.1. Sample

Cordyceps militaris extract was kindly supplied by CAITAC Corp. (Okayama, Japan).

2.2. Chemicals

Xylitol, D(+)-glucose, lactose monohydrate, acetic acid (special grade), phosphoric acid (special grade), methanol and ultrapure water (LC–MS grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D(−)-Ribose, *meso*-Erithritol, D(+)-Xylose, D(−)-arabinose, L(−)-sorbose, (+)-mannose, D(+)-galactose, *myo*-inositol, D(−)-mannitol, dulcit (galactitol), maltose monohydrate and acetonitrile (LC/MS grade) were from Kanto Chemical Co., Inc. (Tokyo, Japan). Cordycepin, D(−)-lyxose, ribitol, D(+)-arabitol, D-psicose, D(+)-talose, D(−)-fructose, D(+)-allose, L-gulose, D-sorbitol, D(+)-trehalose and heptafluorobutyric acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). D-Threitol, D-ribulose, D-xylulose and D-tagatose were purchased from Sigma Ardrich (St. Louis, MO, USA). Sucrose was purchased from Showa Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2.1. Preparation of sugar-related compound standard solutions

Stock standard solutions (each 1000 μg/mL) were prepared by dissolving each reference substance in ultrapure water. Working standard solutions for generating calibration curves were further obtained individually by appropriate dilution of the stock standard solutions with

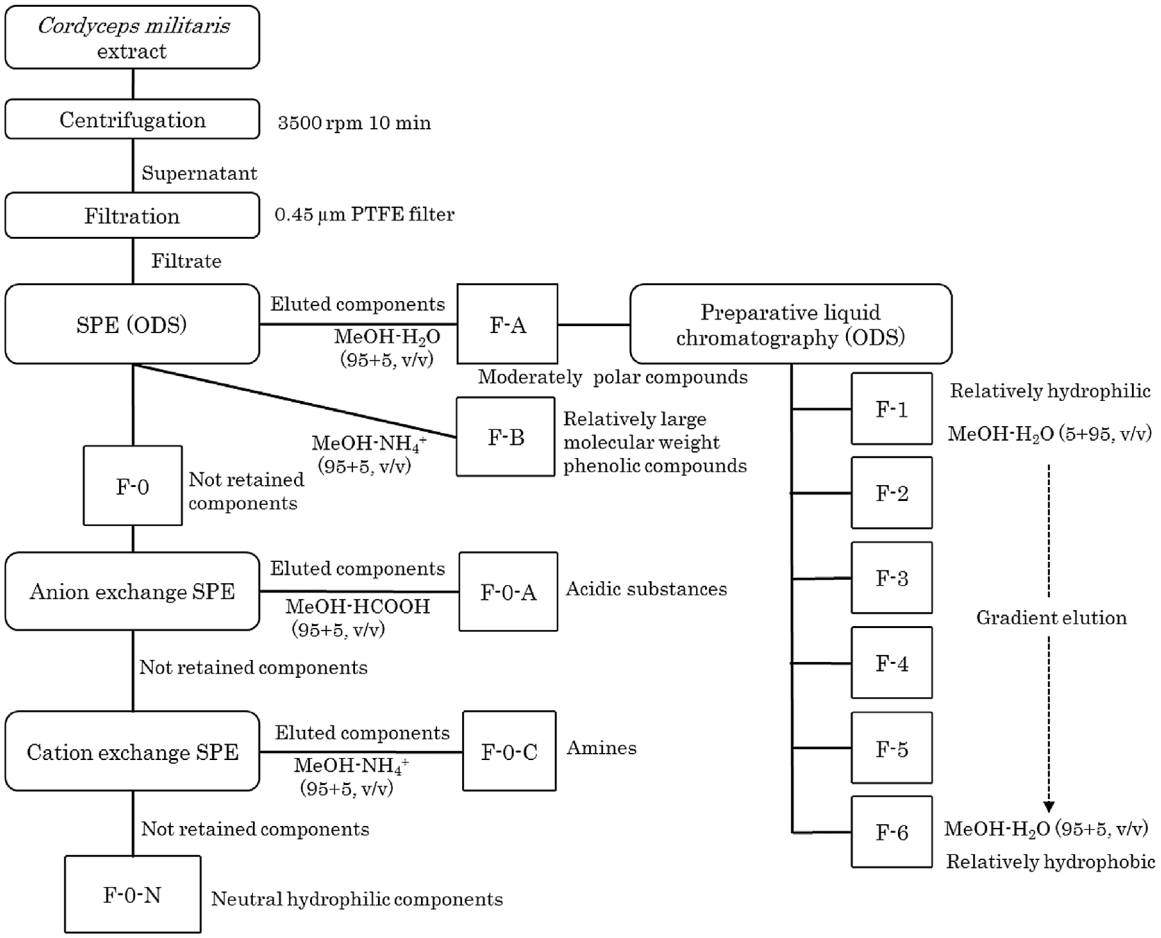


Fig. 1. Flow scheme of identification of antitumor components in *Cordyceps militaris* extract by using solid-phase extraction and preparative liquid chromatography.

Table 1
Chemical property of the substance group included in each fraction of *Cordyceps militaris* extract.

Fraction	Range	Chemical property of estimated component
F-0	–	Highly polar compounds that could not be retained on ODS SPE Cationic, anionic and neutral hydrophilic compounds Pale yellow color
F-1	0.33–6 min	Relatively polar compounds that could be retained on ODS SPE Pale yellow color
F-2	6–10 min	Relatively polar compounds that could be retained on ODS SPE Almost colorless
F-3	10–17.5 min	Moderately polar compounds Pale yellow color
F-4	17.5–25 min	Moderately polar compounds Including cordycepin and many component Orange color
F-5	25–32.5 min	Relatively hydrophobic compounds Pale orange color
F-6	32.5–40 min	Relatively hydrophobic compounds Pale yellow color
F-B	–	Compounds that could be strongly retained on ODS SPE and eluted with methanol-ammonium aqueous solution Brown color

acetonitrile-ultrapure water (95 + 5, v/v). All of the solutions were stored at 4 °C until use.

2.2.2. Preparation of adenine, adenosine and cordycepin standard solutions

Stock standard solutions (each 1000 μg/mL) were prepared by dissolving each standard substance in methanol. Working standard solutions for generating calibration curves were further obtained by mixing and appropriate dilution of the stock standard solutions with acetonitrile-ultrapure water (5 + 95, v/v). All of the solutions were stored at 4 °C until use.

2.3. Cell lines

The human malignant melanoma cell line (MeWo) was purchased from American Type Culture Collection (ATCC, Manassas, VA). MeWo cells and OUMS-24 cells (normal human fibroblasts; kindly provided by Dr. Masayoshi Namba) were cultured in D/F medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS.

2.4. Solid-phase extraction column

An octadecyl silyl (ODS-) silica gel solid-phase extraction (SPE) column, Inertsep C18 (1 g/6cc), was purchased from GL Sciences Inc. (Tokyo, Japan). A reversed-phase and weak cation-exchange mixed mode polymer SPE column, Oasis WAX Plus Short Cartridge, and a reversed-phase and weak cation-exchange mixed mode polymer SPE column, Oasis WCX Plus Short Cartridge, were purchased from Waters Corp. (Milford, MA, USA).

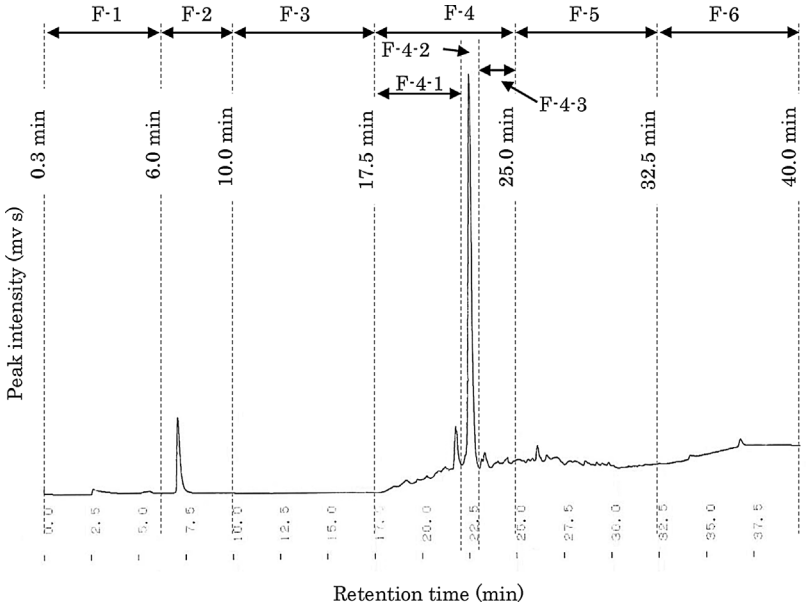


Fig. 2. Preparative chromatogram of fraction F-A separated from *Cordyceps militaris* extract.

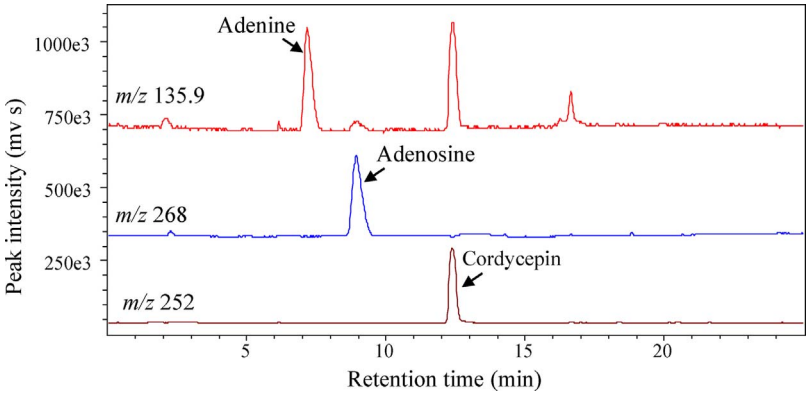


Fig. 3. Overlaid extracted ion chromatograms of adenine, adenosine and cordycepin in the *Cordyceps militaris* extract obtained from LC–ESI-MS analysis using an ODS column.

Table 2
Concentrations of components detected in *Cordyceps militaris* extract and each fraction.

Component	Concentration (mg/mL)			
	Cordyceps militaris extract	F-4 (F-4-2)	F-0	F-0-N
Adenine	0.0038	N.A.*	N.A.	N.A.
Adenosine	0.025	N.A.	N.A.	N.A.
Cordisepin	0.31	0.12	N.A.	N.A.
Xylitol	23	N.A.	9.2	4.6
Fructose	0.48	N.A.	0.19	0.096
Mannitol	1.3	N.A.	0.52	0.26
Glucose	4.5	N.A.	1.8	0.9
Trehalose	0.33	N.A.	0.13	0.066

* Not analyzed.
Concentration of each fraction was calculated as the final concentration in cell culture of growth and apoptosis assay using MeWo cells.

2.5. Apparatus

2.5.1. Preparative liquid chromatograph

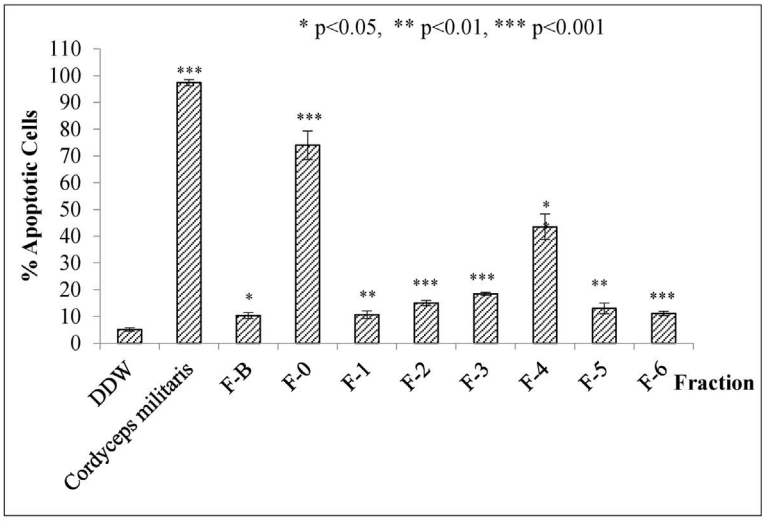
Chromatographic separation of the fractions that included moderately polar compounds was carried out on a preparative liquid chromatography system consisting of an LC-10ADVP pump, an LC-20AT pump, an SIL-20AP auto sampler, a CTO-20A column oven, an SPD-20A UV/VIS detector, a DGU-20A3 degasser, an SCL-10AVP system controller, an FRC-10A fraction collector and a C-R8A Chromatopac data system (Shimadzu Corporation, Kyoto, Japan). The column used in this study was a reversed-phase preparative column (L-column2 ODS, 5 μm, 10 mm × 250 mm, Chemicals Evaluation and Research Institute, Japan (CERI)).

2.5.2. Liquid chromatograph mass spectrometer (LC–MS)

Analysis of the known components and highly polar compounds such as sugar alcohols, monosaccharides and disaccharides contained in the *Cordyceps militaris* extract was carried out on a liquid chromatograph mass spectrometer (LC–MS) system. The LC–MS apparatus consisted of two LC-10ADvp pumps, an SIL-HTA auto sampler, a CTO-10Avp column oven, a DGU-14AM degasser, an LCMS-2010A mass spectrometer and an LCMS-solution data system (Shimadzu Corporation, Kyoto, Japan).

2.5.3. Sample preparation and SPE separation

A scheme of the separation process is shown in Fig. 1. Ten mL of the *Cordyceps militaris* extract was centrifuged at 3500 rpm for 10 min, and the insoluble small particle-suspended substance remaining in the obtained supernatant liquid was removed by filtration using a polytetrafluoroethylene (PTFE) membrane disposable syringe filter (GL Sciences Inc.,Tokyo, Japan, 0.45 μm, 25 mm). A 5-mL volume of the filtrate was applied to an ODS SPE column pre-conditioned with 10 mL of methanol and 10 mL of ultrapure water. After sample loading, the ODS SPE column was washed with 10 mL of ultrapure water. Both of the effluents were collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to 5 mL at 45 °C or below using a rotary evaporator (F-0). The component retained in the ODS SPE column was then



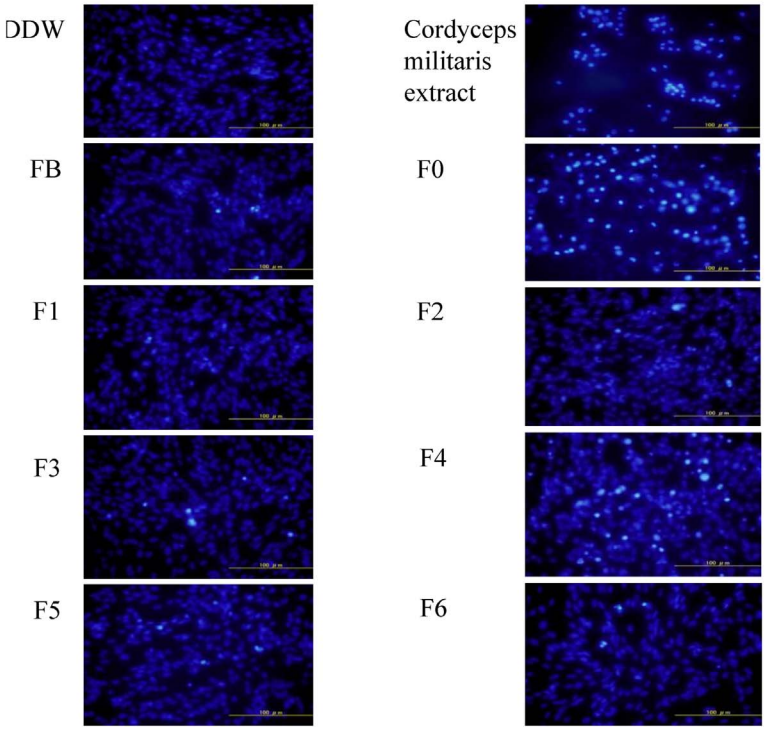


Fig. 4. MeWo cells were treated with each fraction of *Cordyceps millitaris* extract separated by column chromatography and compared to crude *Cordyceps millitaris* extract. The prepared fractions were recovered to be the same volume as that of the original extract and were added to the cell culture to a final concentration of 10% (v/v) and then kept for 48 h. The upper panel shows the quantified results of apoptotic assessments. Among the fractions, F-0 fraction showed the best ability for inducing apoptosis in MeWo cells. The lower panel shows representative pictures of the appearance of cells with fraction-induced apoptosis, which were stained by Hoechst 33342.

eluted with 10 mL of methanol-water (95 + 5, v/v). The effluents were collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to dryness at 45 °C or below using a rotary evaporator. The above operation was repeatedly carried out for a total of 120 mL of the *Cordyceps militaris* extract. The residue was dissolved in 20 mL of ultrapure water (F-A). The component that remained in the ODS SPE column was then eluted with 10 mL of methanol-ammonia aqueous solution (95 + 5, v/v). The effluents were collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to dryness at 45 °C or below using a rotary evaporator. The residue was dissolved in 20 mL of ultrapure water (F-B).

2.5.4. Preparative liquid chromatography

The sample fraction F-A was further divided into six fractions using a fraction preparative liquid chromatograph with an ODS column (Figs. 1 and 2). The mobile phases were ultrapure water (mobile phase A) and methanol (mobile phase B). The gradient conditions were set to a linear gradient of A:B (95:5, v/v) (5 min hold at this composition) for 20 min, (5:95, v/v) (10 min hold at this composition). The flow rate was 2 mL/min and the injection volume was 200 μL. The components were detected at 200 nm. Fraction collecting conditions and chemical properties of the substance group included in each fraction are shown in Table 1. The above operation was repeated automatically and a total of 10 mL of fraction F-A was treated. Each effluent was collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to dryness at a temperature below 40 °C using a rotary evaporator. The residue was dissolved in 10 mL of ultrapure water.

2.5.5. Ion-exchange chromatographic separation of hydrophilic substances

The anion-exchange SPE columns were pre-conditioned with the same volume (10 mL) of methanol-formic acid (98 + 2, v/v), methanol and ultrapure water in that order. The cation-exchange SPE columns were pre-conditioned with the same volume (10 mL) of methanol-aqueous ammonia solution (95 + 5, v/v), methanol and ultrapure water in that order. As shown in Fig. 1, 10 mL of fraction F-0 was loaded to an anion-exchange SPE column, OASIS Plus WAX, connected to a cation-exchange SPE column, OASIS Plus WCX, and the effluent including neutral hydrophilic components was collected. The combined

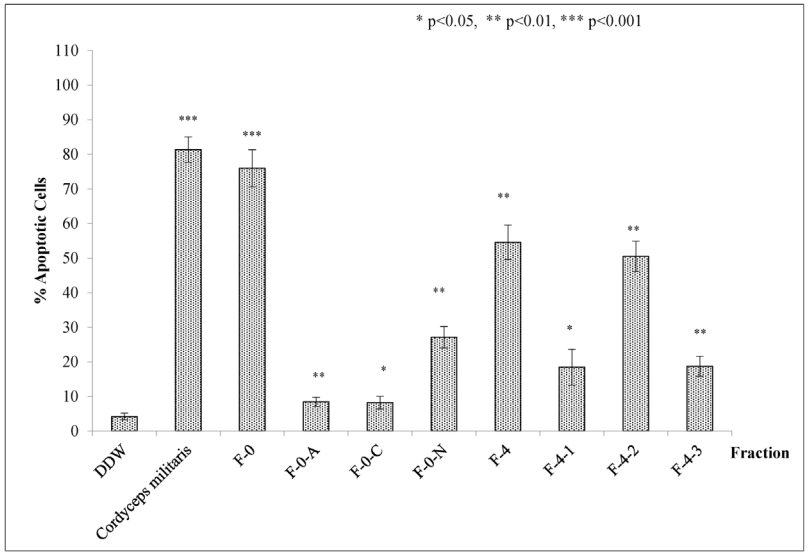


Fig. 5. MeWo cells were treated with each fraction prepared by further separation of the active fractions, F-0 and F-4. Fractions F-0-A, F-0-C and F-0-N were from fraction F-0, and fractions F-4-1, F-4-2 and F-4-3 were from fraction F-4. They were recovered to be the same volume as that of fraction F-0 or F-4, and they were also matched to the volume of the original extract. All of the prepared samples were added to the cell culture to a final concentration of 10% (v/v) and then kept for 48 h. The method of assessments was the same as that in Fig. 3. Among the fractions derived from fraction F-0, fraction F-0-N showed the best ability for inducing apoptosis in MeWo cells.

Table 3
Retention times of sugar-related compounds obtained from an amino propyl column and an amide column.

Analyte	Retention time (min)		Monitor ion (*m/z*)	M	Carbon number	Remarks
	Amino column	Amide column				
Erythritol	6.86	4.81	157, 159, 121	122	4	Sugar alcohol
Threitol	6.88	4.88	157, 159, 121	122	4	Sugar alcohol
D-Ribulose	5.73	3.94	185, 187	150	5	Ketose
D-Xylulose	6.36	4.18	185, 187	150	5	Ketose
D-Ribose	–	4.45	185, 187	150	5	Aldose
D-Lyxose	–	5.99	185, 187	150	5	Aldose
D-Xylose	–	6.70	185, 187	150	5	Aldose
D-Arabinose	–	7.33	185, 187	150	5	Aldose
Ribitol	9.50	7.80	187, 189, 151	152	5	Sugar alcohol
Xylitol	9.51	8.38	187, 189, 151	152	5	Sugar alcohol
Arabitol	9.96	8.64	187, 189, 151	152	5	Sugar alcohol
D-Psicose	8.39	6.19	215, 217	180	6	Ketose
D-Talose	–	7.33	215, 217	180	6	Aldose
D-Tagatose	10.03	8.98	215, 217	180	6	Ketose
D-Fructose	10.73	10.49	215, 217	180	6	Ketose
D-Allose	–	10.93	215, 217	180	6	Aldose
D-Sorbose	11.04	10.96	215, 217	180	6	Ketose
D-Mannose	–	12.71	215, 217	180	6	Aldose
L-Gulose	–	11.94	215, 217	180	6	Aldose
D-Glucose	13.19	14.05	215, 217	180	6	Aldose
D-Galactose	–	14.15	215, 217	180	6	Aldose
myo-Inositol	17.51	20.94	215, 217	180	6	Cyclitol
Sorbitol	12.51	13.11	217, 219, 181	182	6	Sugar alcohol
Mannitol	13.15	13.65	217, 219, 181	182	6	Sugar alcohol
Galactitol	13.22	13.99	217, 219, 181	182	6	Sugar alcohol
Sucrose	17.46	19.90	377, 379, 341	342	12	Disaccharide
Lactose	18.38	22.18	377, 379, 341	342	12	Disaccharide
Maltose	18.44	21.58	377, 379, 341	342	12	Disaccharide
Trehalose	19.70	22.65	377, 379, 341	342	12	Disaccharide

column was washed with 10 mL of ultrapure water. These effluents obtained from a total of 40 mL of fraction F-0 were collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to 10 mL at a temperature below 45 °C using a rotary evaporator (F-0-N). The neutral hydrophilic components included in fraction F-0-N were analyzed using LC–MS.

Anionic components of fraction F-0 such as organic acid were then eluted with 10 mL of methanol-formic acid (98 + 2, v/v) from the anion-exchange SPE column separated from the cation-exchange SPE column. A total of 40 mL of fraction F-0 was treated. The effluent was collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to dryness at a temperature below 45 °C using a rotary evaporator. The residue was dissolved in 10 mL of ultrapure water (F-0-A).

Cationic components of F-0 such as amines were then eluted with 10 mL of methanol-aqueous ammonia solution (95 + 5, v/v) from the cation-exchange SPE column. A total of 40 mL of fraction F-0 was treated. The effluent was collected in a 100-mL eggplant-shaped flask and the solvent was evaporated to dryness at a temperature below 45 °C using a rotary evaporator. The residue was dissolved in 10 mL of ultrapure water (F-0-C).

2.5.6. LC/MS analysis of adenine, adenosine and cordycepin

Adenine, adenosine and cordycepin are known components of *Cordyceps militaris* extract. Therefore, we determined these components in fraction F-A by LC–MS. These analytes were separated by an ODS column (L-column2 ODS, 3 μm, 2.1 mm × 250 mm, CERI) maintained at 40 °C. The mobile phases were 2.5 mmol/L heptafluorobutyric acid solution (mobile phase A) and acetonitrile (mobile phase B). The gradient conditions were set to a linear gradient of A:B (95:5, v/v) (5 min hold at this composition) for 20 min, (30:70, v/v). The flow rate was 0.2 mL/min and the injection volume was 5 μL. For detection of the analytes, the effluent was introduced into a mass spectrometer in the electrospray ionization positive mode (ESI+), and adenine, adenosine and cordycepin were detected at *m/z* 135.9, *m/z* 268.0 and *m/z* 252, respectively, by selected ion monitoring (Fig. 3). Finally, these contents in the extract were measured and the contents are shown in Table 2.

2.5.7. LC/MS analysis of sugar alcohols, monosaccharides and disaccharides

Fraction F-0-N was diluted to 500 or 2000 times with acetonitrile and subjected to LC–MS. For identification of the components included in the neutral hydrophilic fraction F-0-N, typical sugar alcohols, monosaccharides and disaccharides were analyzed as reference substances by two different chromatographic conditions. In the first

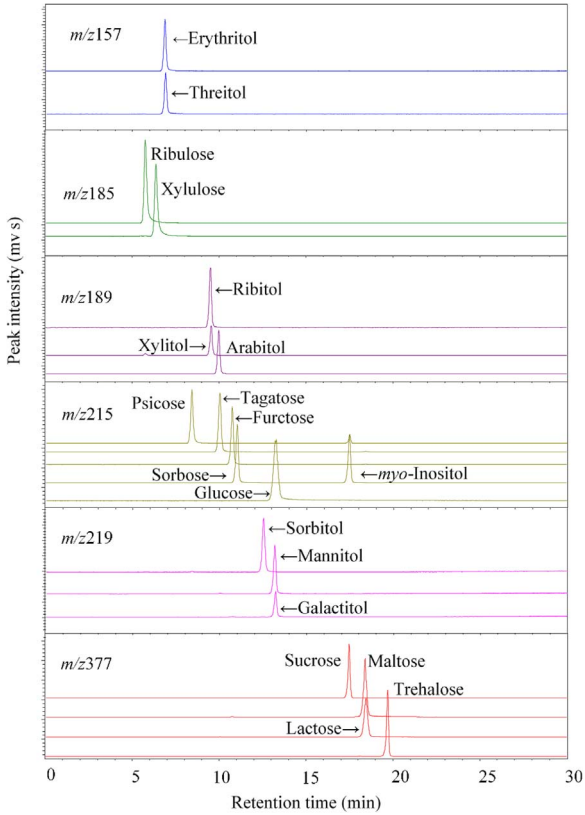


Fig. 6. Overlaid extracted ion chromatograms of sugar-related compound standard solutions (20 μg/mL) obtained from LC-APCI-MS analysis using an amino-propyl column.

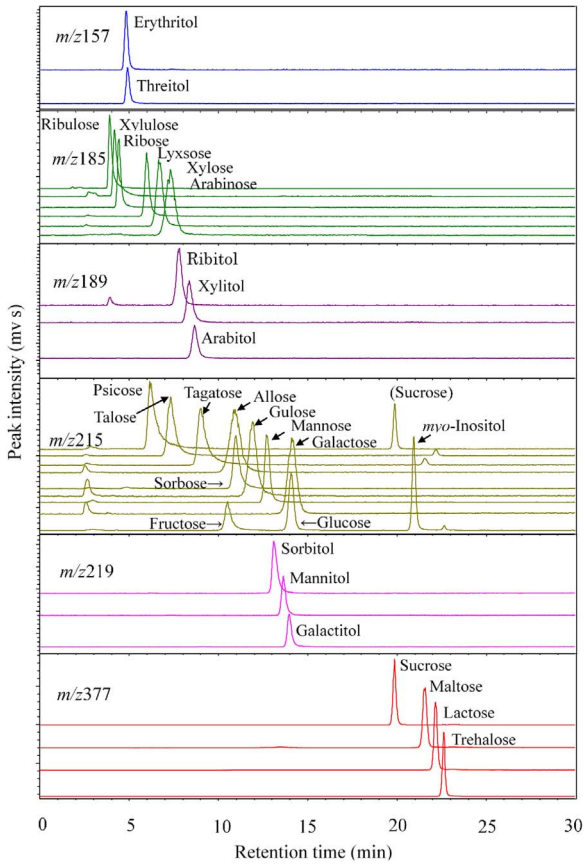


Fig. 7. Overlaid extracted ion chromatograms of sugar-related compound standard solutions (20 μg/mL) obtained from LC-APCI-MS analysis using an amide column.

method, an amino propyl group-bonded silica gel column (TSKgel NH2-100, 3 μm, 2.0 mm × 150 mm, Tosoh Corporation (Tokyo, Japan)) was used as a conventional normal phase column. The mobile phases were ultrapure water (mobile phase A) and acetonitrile-dichloromethane (95 + 5, v/v) (mobile phase B). The gradient conditions were set to a linear gradient of A:B (10:90, v/v) for 30 min, (50:50, v/v) (5 min hold at this composition). The flow rate was 0.2 mL/min and the injection volume was 10 μL.

In the second method, an ethylene-bridged hybrid particle column with a trifunctionally bonded amide group (XBridge BEH Amide, 3.5 μm, 2.1 mm × 100 mm, Waters Corp. (Milford, MA, USA)) was used for hydrophilic interaction chromatography (HILIC). The mobile phases were 0.1% ammonium aqueous solution (mobile phase A) and 0.1% ammonium acetonitrile solution (mobile phase B). The gradient conditions were set to a linear gradient of A:B (5:95, v/v) for 30 min, (40:60, v/v). The flow rate was 0.2 mL/min and the injection volume was 5 μL. Acetonitrile-dichloromethane (90 + 10, v/v) was mixed with the effluent as a post column reagent via a low dead volume tee at the flow rate of 0.1 mL/min. For the detection of analytes, the effluent was introduced into a mass spectrometer in the atmospheric pressure chemical ionization negative mode (APCI-), and sugar alcohols, monosaccharides and disaccharides were detected as their corresponding chlorine adduct ions ($[M + Cl]^-$) by selected ion monitoring. The monitor ions of the analytes are shown in Table 3. Identification of the components was performed with the retentions time obtained by the two chromatographic conditions and monitor ions.

2.6. Evaluation of apoptosis

Apoptotic cells were detected as described previously [14]. Briefly, the cell culture stained with Hoechst33342 solution (Invitrogen) at the final concentration of 4 μg/mL was incubated for 30 min, and apoptotic cells (shrinking nuclei stained with Hoechst at higher intensity) were observed and evaluated by a fluorescence microscope. Apoptotic cells were counted and are presented as a percentage.

3. Results and discussion

Through our studies on *Cordyceps militaris*, we have had the idea that another unidentified factor(s) besides cordycepin in the herbal extract contributes to the *Cordyceps militaris*-derived anti-tumor effects. Therefore, we tried to identify a soluble candidate(s) from the extract. At first, the insoluble solid debris in the *Cordyceps militaris* extract was eliminated by centrifugation and subsequent filtration, and then the cleared supernatant of the extract was subjected to SPE separation (Fig. 1). Fraction F-A containing moderately polar compounds that was retained on the ODS SPE column was separated into six fractions by reversed-phase preparative liquid chromatography (Figs. 1 and 2). In this first stage of screening, fractions F-0 and F-4 showed good ability for inducing apoptosis in MeWo cells (Fig. 4). The main peak detected in fraction F-4 was identified as cordycepin by LC/MS analysis (see Materials and Methods). The results of quantitative analysis are shown in Table 2, i.e., 0.12 mg/mL of cordycepin was included in the separated F-4 fraction. The other fractions, F-B, F-1, F-2, F-3, F-5 and F-6, showed relatively poor ability for inducing apoptosis in MeWo cells. Therefore, we focused on F-0 and F-4 fractions. At first, fraction F-4 was further separated into 3 fractions, F-4-1, F-4-2 and F-4-3 (Fig. 2), and then another apoptosis assay was performed. As a result, fraction F-4-2 that included mainly cordycepin showed significantly higher activity than the activities of the other fractions (Fig. 5), indicating that cordycepin in this fraction plays a major role in apoptotic cell death. To seek a potential anti-tumor component besides cordycepin, we next investigated the cordysepin-absent F-0 fraction, which had much higher potential for anti-tumor activity. It was thought that highly polar compounds such as fatty acids, amines and sugars were included in fraction F-0. Therefore, fraction F-0 was further separated into 3

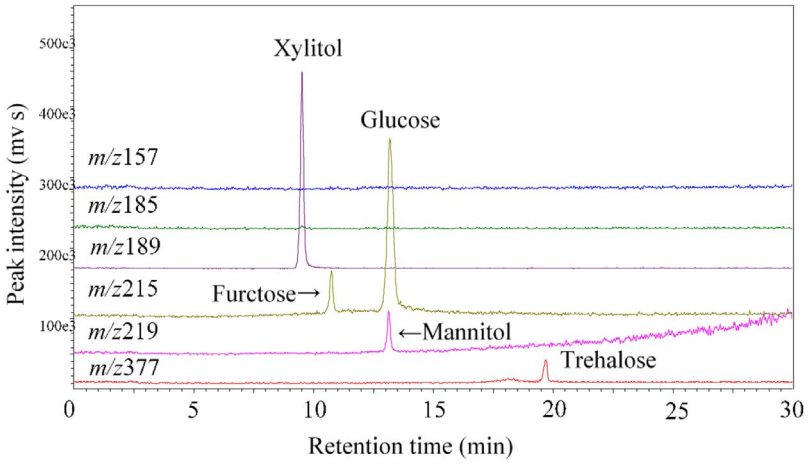


Fig. 8. Overlaid extracted ion chromatograms of fraction F-0-N (final concentration of 0.2%(v/v)) of *Cordyceps militaris* extract obtained from LC-APCI-MS analysis using an amino-propyl column.

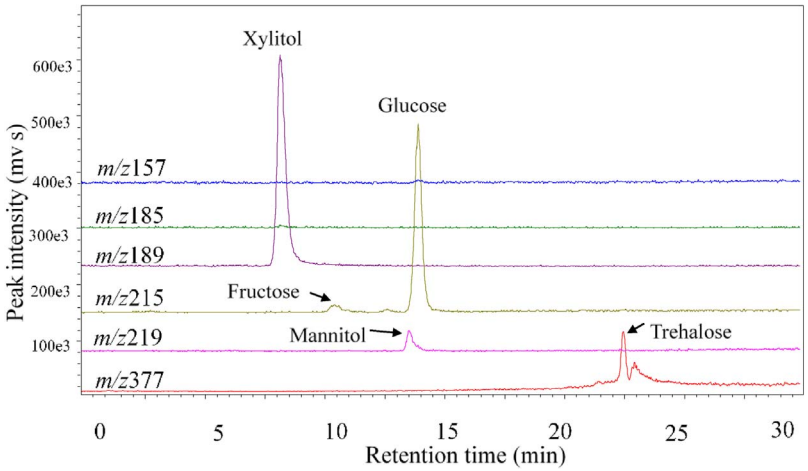


Fig. 9. Overlaid extracted ion chromatograms of fraction F-0-N (final concentration of 0.05%(v/v)) of *Cordyceps militaris* extract obtained from LC-APCI-MS analysis using an amide column.

fractions, F-0-A, F-0-N and F-0-C, by using anion-exchange SPE and the cation-exchange SPE (Fig. 1), and then an apoptosis assay was performed again.

The components retained on the anion-exchange SPE column were considered to be anionic compounds such as organic or inorganic acids (F-0-A). On the other hand, the components retained on the cation-exchange SPE column were considered to be cationic compounds such as amines (F-0-C). The components not retained on either SPE column were considered to be neutral hydrophilic compounds such as sugar alcohols, monosaccharides and oligosaccharides (F-0-N). Among these sub-fractions, fraction F-0-N showed the best ability for inducing apoptosis in MeWo cells (Fig. 5). Thus, the target fraction could be narrowed down to F-0-N by chromatographic separation and apoptosis assay.

To identify the specific sugar compound being dissolved in the F-0-N fraction, which probably shows central anti-tumor activity in that fraction, we established a method to separate and determine sugars according to different retention times on specific chromatography (see Materials and Methods). Table 3 shows the retention times of reference substances of sugar-related compounds obtained from an amino-propyl column and an amide column. The overlaid extracted ion chromatograms of sugar-related compounds obtained from the amino-propyl column and amide column are shown in Figs. 6 and 7, respectively. Symmetrical and sharp peaks were observed in sugar alcohols, ketoses and disaccharides by using the amino-propyl column. The amino-propyl column gave a small broad tailing peak shape to aldoses such as ribose, lyxsose, xylose, arabinose, talose, mannose, gulose and galactose except for glucose under this condition. These phenomena were caused by the formation of glycosylamines (Schiff bases) from reaction between the amino groups on the stationary phase and an aldehyde group in aldose. Although the shape was relatively broad, the amide column gave peaks to all analytes. The separation of analytes was incomplete, but retention times were different, except for erythritol/threitol, allose/sorbose and galactose/glucose. These methods were then applied to fraction F-0-N to analyze the sugar-related compounds contained in the fraction, and five peaks were detected (Figs. 8 and 9). These compounds were identified as xylitol, fructose, mannitol, glucose and trehalose by using the values of retention time of reference compounds. Thus, we succeeded in identifying the sugars contained in fraction F-0-N and in determining their contents in the fraction. The concentrations of the sugar-related compounds detected in *Cordyceps militaris* extract and in each fraction are shown in Table 2. In addition, the expected ingredients adenine, adenosine and cordycepin were also detected in *Cordyceps militaris* extract at relatively low concentrations (Fig. 3 and Table 2). Surprisingly, the *Cordyceps militaris* extract contained a remarkably high level (23 mg/mL) of xylitol. This has not been reported so far.

Generally, it is difficult to identify sugar alcohols by the LC/MS method due to the very poor ionization efficiency of sugar alcohols. In this study, we attempted to overcome this problem and we finally succeeded in establishing a modified LC/MS method using an APCI ion source with chlorine adduct ionization that does not require the step of derivatization of sugars. That enabled us to identify the sugars included in *Cordyceps militaris* extract for the first time. Thus, the presence of xylitol even at a high concentration might have been hidden so far in *Cordyceps militaris* extract. We expected in this study that sugars or sugar alcohols would be identified as active anti-tumor ingredients by the newly developed analytical method and procedure.

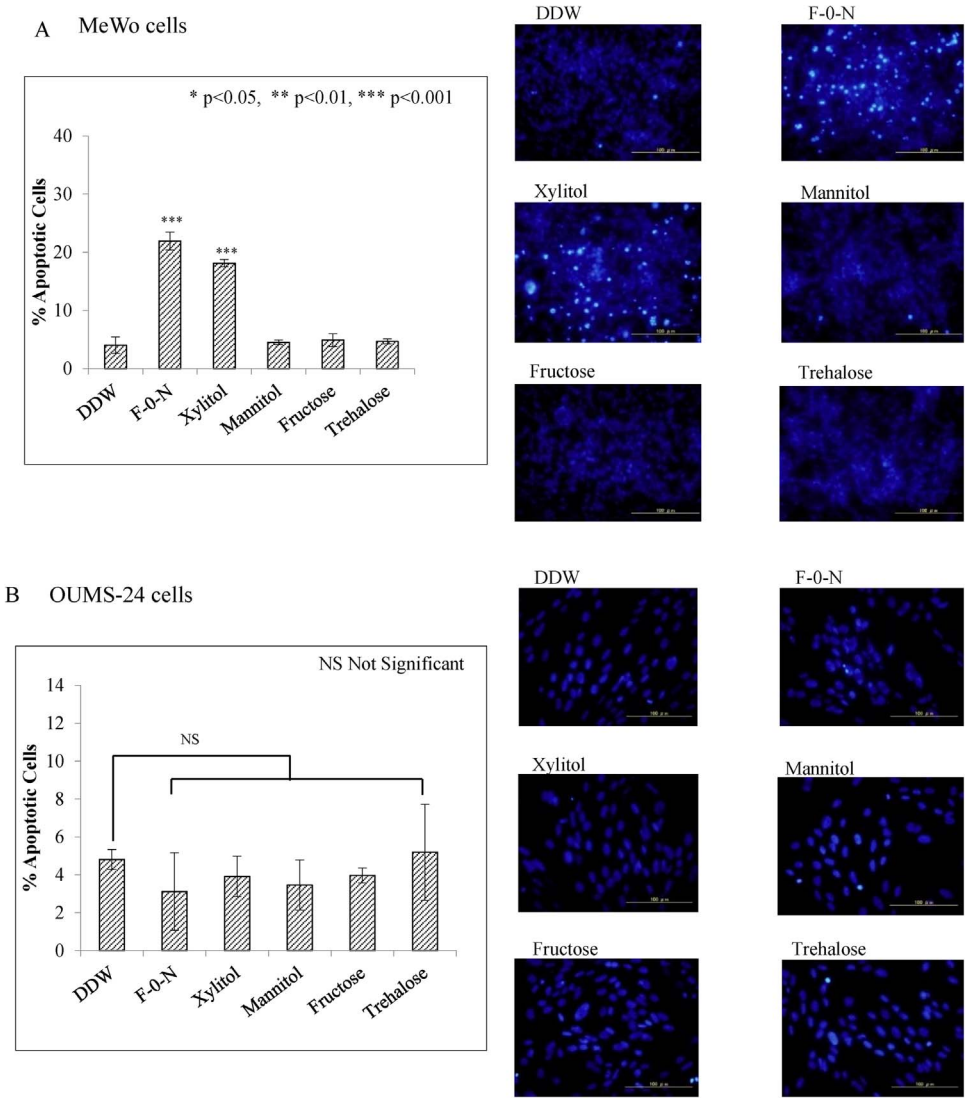


Fig. 10. MeWo cells (A) and normal human OUMS-24 fibroblasts (B) were treated with each sugar-related compound (xylitol at 2.34 mg/mL, mannitol at 0.05 mg/mL, fructose at 0.13 mg/mL and trehalose at 0.03 mg/mL) and fraction F-0-N. Since fraction F-0-N was used at the final concentration of 10% (v/v) in cell culture, the concentrations of sugar-related compounds used were also adjusted to be equivalent to the concentration included in the 10% of fraction F-0-N. After 48-h treatment of the cells with each compound, apoptotic assessments were carried out. Quantification of apoptotic cells showed that xylitol has the best ability for inducing apoptosis in MeWo cells, but no differences were found in OUMS-24 cells.

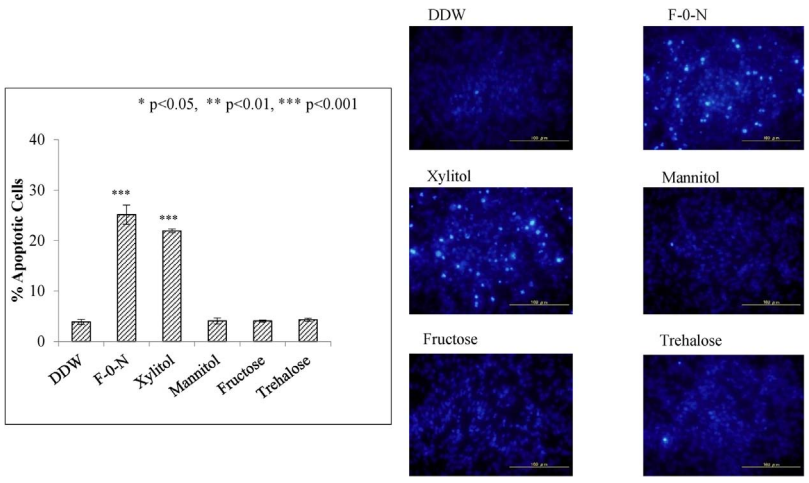
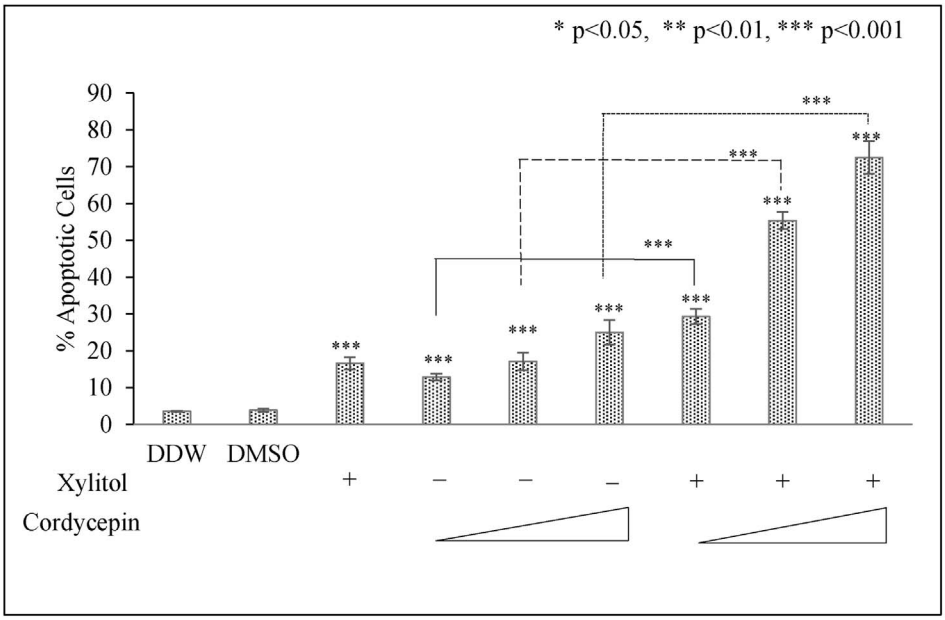


Fig. 11. MeWo cells were treated again with each sugar-related compound (xylitol, mannitol, fructose and trehalose) and fraction F-0-N. Final concentrations of the sugar-related compounds used were all 2.34 mg/mL. After 48-h treatment of the cells with each compound, apoptotic assessments were carried out.

In order to examine selective anti-tumor activity in each sugar we identified, MeWo cells and normal human OUMS-24 fibroblasts were treated with each sugar-related compound (xylitol, mannitol, fructose and trehalose) and fraction F-0-N (Fig. 10). Since fraction F-0-N was used at the final concentration of 10% (v/v) in the cell culture, the concentrations of sugar-related compounds used were also adjusted to be equivalent to the concentration included in the 10% of fraction F-0-N. After treatment of the cells with each material for 48 h, apoptotic assessments were carried out. The quantification of apoptotic cells showed that xylitol has the best ability for inducing apoptosis in MeWo cells, but no appreciable differences were found in OUMS-24 cells. Furthermore, MeWo cells were treated with an equal concentration



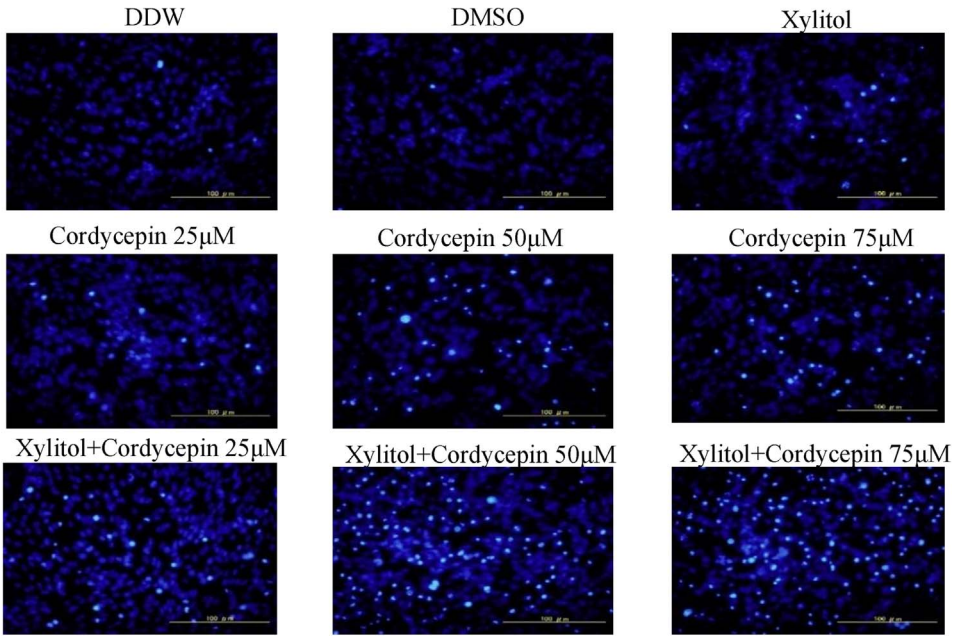


Fig. 12. MeWo cells were treated with increasing doses of cordycepin (25 μM, 50 μM, and 75 μM) in the presence or absence of xylitol (1.17 mg/mL). After 48-h treatment of the cells, apoptotic assessments were carried out.

(2.34 mg/mL) of each sugar-related compound (xylitol, mannitol, fructose and trehalose). After treatment of the cells with each compound for 48 h, xylitol showed the best ability for inducing apoptosis in MeWo cells (Fig. 11). It has been reported that xylitol has a strong ability to induce apoptosis in human lung cancer A549 cells [39]. Our results obtained for melanoma are in agreement with the results of that study. The former study showed that xylitol-mediated cell death occurred in parallel with activation of autophagy, which is important in cellular homeostasis. Accelerated mal-autophagy may play a role in apoptotic death in cancer cells. In addition, Wu et al. reported that xylitol was able to induce glutathione (GSH) depletion when combined with selenite, a new synthetic Se compound, resulting in apoptotic cell death in human hepatoma SMMC-7221 cells [40]. GSH is critical to protect cells from oxidative stresses. Thus, it seems that multiple mechanisms are involved in xylitol-mediated apoptotic cell death. These results taken together with our results of analysis and evaluation of apoptosis indicate that xylitol is a central anti-tumor ingredient in addition to cordycepin in *Cordyceps militaris* extract.

To evaluate the significance of xylitol in *Cordyceps militaris* extract for anti-tumor activity, we lastly examined whether xylitol has the ability to enhance cordycepin-triggered apoptosis. MeWo cells were treated with cordycepin in the presence or absence of xylitol. Single treatment with cordycepin induced apoptosis in MeWo cells, and the ratio of apoptotic cells increased up to about 30% in a dose-dependent manner. When we combined cordycepin with a constant concentration of xylitol, the cordycepin-mediated apoptosis was significantly enhanced compared to that in the case of single treatments with cordycepin at increasing concentrations (25, 50 and 75 μM) (Fig. 12). These results suggest that xylitol plays a critical role in enhancing the anti-tumor effect of cordycepin and that the inclusion of xylitol we succeeded in identifying will lead to a better understanding of the anti-tumor role of the mysterious *Cordyceps militaris* extract.

4. Conclusion

Xylitol was identified as an anti-tumor component of *Cordyceps*

militaris extract. It effectively induced apoptosis in MeWo cells. Xylitol is widely used as a functional food additive and sweetener. Xylitol is expected to have a beneficial effect on other cancers as well. Although cordycepin is the primary compound for the anti-tumor activity, xylitol, which is abundantly contained in *Cordyceps militaris* extract, might also contribute to the anti-tumor activity synergistically with cordycepin. Further experiments are needed to elucidate the mechanism by which xylitol induces apoptosis.

Acknowledgements

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