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Chemical Properties and Physiological Activities of Stromata of *Cordyceps militaris*

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Abstract The chemical properties and physiological activities of the freeze-dried stromata of *Cordyceps militaris* were examined. A proximate analysis exhibited that the stromata consisted mainly of crude carbohydrate (74.3%), crude protein (11.5%), and moisture content (8.7%), with a low content of crude ash (4.2%) and fat (1.3%). The carbohydrate was mostly composed of glucose (88.6%). A large quantity of essential fatty acids, including linolenic acid (31.9%) and linoleic acid (12.3%), and unsaturated fatty acid of oleic acid (33.8%) was also observed. An analysis of the component amino acid showed a relatively high ratio of the essential amino acids, lysine (101.2 mg/g), methionine (62.7 mg/g), and acidic amino acids of glutamic acid (57.5 mg/g) and aspartic acid (43.9 mg/g), whereas a low of tyrosine content (4.7 mg/g). An examination of the cordycepin content indicated that the stromata and mycelium-embedded media (silkworm pupae) contained 0.2% and 0.5%, respectively. From the examination of the physiological activities, based on methanol extract (M), ethylacetate extract (EA), and hot-water extract (HW) fractions of the stromata, the hot-water extract (HW) fraction showed the most potent intestinal immune modulating activity, anticoagulant activity, and anticomplementary activity, whereas the ethylacetate extract (EA) fraction exhibited a radical scavenging activity. Therefore, the results from the present study indicate that the stromata of *C. militaris* contain various healthy chemical ingredients, and that especially boiled water extract of whole body would apprear to provide beneficial physiological activities.

Key words: *Cordyceps militaris*, cordycepin, physiological activity

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The genus *Cordyceps* (Ascomycotina; Pyrenomycetes; Clavicipitales; Clavicipitaceae), known as a group of entomopathogenic fungi, forms stromata on its insect hosts. This genus is composed of approximately 750 species, among which 75 species have been recorded to produce toxins in insects [3]. As much members of the *Cordyceps* genus have the potential to be biocontrol agents against insect pests. In particular, since the occurrence of *Cordyceps militaris* is widespread across the world, this fungus has attracted much attention from researchers. *C. militaris* affects the physiological system, inhibiting human glomerular mesangial cell proliferation [38], and Yang *et al.* [35] investigated hypolipidermic effect of exo-polymers from submerged mycelial culture. Cunningham *et al.* [8] also reported that cordycepin (3'-deoxyadenosine), the nucleoside derivative and isomer of quinic acid, separated from *C. militaris* inhibits the synthesis of DNA and RNA, and protein kinase. In addition, cordycepin exhibits an anticancer activity against fibrosarcoma, such as bladder cancer, colon cancer, and lung cancer [14].

Although several physiologically-active substances have been examined in *C. militaris*, they have rarely been developed for commercial use due to the lack of sufficient stromata. Recently, Korean mushroom cultivators developed artificial media for the mass production of stromata, thereby providing an opportunity for further pharmacological examination. In addition, it is also necessary to examine the kinds and yields of physiologically-active substances from artificially-cultured stromata prior to their commercial use. Accordingly, the present study was undertakan to characterize the chemical compositions, the amounts, and physiological activity of artificially-cultured stromata of *C. militaris*.

MATERIALS AND METHODS

Microorganism and Materials

The *Cordyceps militaris* (KCCM 60303) was obtained from KCCM and initially inoculated in PDB (potato dextrose broth) for preculture using the method as described by Song *et al.* [31], followed by silkworm pupae media. After cultivation, the stromata of *C. militaris* were freeze-dried for use in the experiment. The RPMI-1640 medium and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY, U.S.A.). The heat inactivated horse serum and fetal bovine serum were obtained from Cell Culture Laboratories (Cleveland, OH, U.S.A.), and the penicillin, streptomycin, and amphotericin B from Flow Laboratories (Irvine, Scotland). The Alamar Blue™ was obtained from Alamar Bio-Sciences Inc. (Sacramento, CA, U.S.A.). The acetylthiocholine iodide (ASCh) and 5,5'-dithiobis-(2-nitro)-benzoic acid (DTNB) were purchased from Sigma Co. (St. Louis, MO, U.S.A.). The activated partial thromboplastin time (aPTT) reagent was obtained from Dade Behring Inc. (Deerfield, IL, U.S.A.). The 12-*O*-tetradecanoylphobol-13-acetate (TPA) was obtained from Research Biochemicals International (Natick, MA, U.S.A).

Cells and Culture Conditions

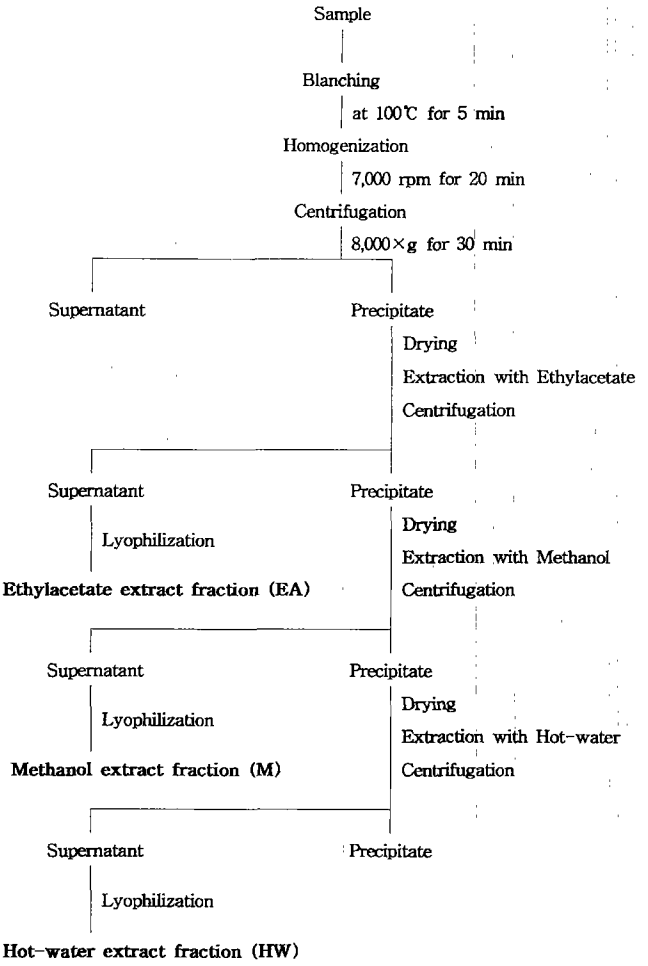
PC12 cells originated from rat pheochromocytoma and human promyelocytic leukemia HL-60 cells obtained from the Health Science Research Resources Bank were cultured in an RPMI-1640 medium supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, and 1% antibiotic-antimycotic (Gibco-BRL, NY, U.S.A.). The cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2, and subcultured by mechanically removing them from the substratum with squirts of fresh medium. The cells were grown to subconfluence in 100-mm culture dishes.

Preparation of Various Fractions by Systemic Extraction

The freeze-dried stromata were blanched at 100°C for 5 min and homogenized using with an Ultra-turrax T-50 (Janke Kunkel IKA-Labortechniker, Germany; 7,000 rpm, 20 min). After centrifugation (8,000 ×g, 30 min), the supernatant was removed and the precipitate initially fractionated with ethylacetate (EA), which is a solvent with a low polarity, followed by fractionation with methanol (M) and hot-water (HW), in an increasing order of polarity (Scheme 1).

Chemical Analysis

The contents of moisture, crude protein, crude carbohydrate, crude fat, crude ash, and crude fiber of stromata were determined according to AOAC [1] methods, and to determine the crude protein, a 6.25 conversion factor was used.



Scheme 1

The sugar component of the polysaccharides was analyzed with alditol acetates after the hydrolysis of the polysaccharides with 2 M TFA for 1.5 h at 121°C [16], and then analyzed using a GLC with an SP-2380 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco, U.S.A.) according to the method of Zhao *et al.* [37]. The temperature programme was: 60°C for 1 min, 60→215°C (30°C/min), 215°C (18.8 min), 215→250°C (8°C/min), and 250°C (5.7 min). The molar ratios were calculated from the peak areas and response factors using a flame-ionization detector (FID).

To determine the amino acid content, 5 g of the sample was mixed in 5 ml of distilled water. Then, 500 mg of sulfosalicyclic acid was added to the mixture, after which the mixture was stored at 4°C for 1 h, and centrifuged at 1,300 ×g for 15 min. The supernatant was filtered through a 0.45 μm filter paper, and pre-treated by the method described by Lindroth and Mopper [22]. The determination of the amino acid content using high performance liquid chromatography (HPLC) was performed using a modified version of the method developed by Hodgin *et al.* [12]. The amino acid content was analyzed using an ODS-

μ-Bondapak C_{18} column (3.9 mm×30 cm) and an HPLC (Shimadzu, Japan) equipped with a fluorescence detector (Shimadzu, FLD-6A, Japan). All quantitative analyses were performed by relating the peak areas of individual amino acids to those of external standard amino acids (Wako, Japan).

The preparation of the sample for the fatty acid analysis was performed according to the AOAC method outlined in Section 28.065 [2] using a CarboWax capillary column (30 m×0.32 mm). An HP 5890 gas chromatography equipped with a flame ionization detector was also used. A chromatogram was constructed using a linear temperature program, which was ramped from 140°C to 180°C for 8 min followed by 3 min of an initial holding period at 140°C. There was also a final holding incubation for 3 min at 180°C. The temperature of the injector and detector was 220°C and 250°C, respectively, and the identification of the peak, retention time, peak area, and area ratio was measured and compared with a standard calibration solution.

In order to determine the cordycepin content [17], 1 g of the stromata or mycelium-embedded media (silkworm pupae) was homogenized in 50 ml of 5% ice perchloric acid for 10 min, stirred for 2 h, then the total volume of the mixture was adjusted to 100 ml with 5% perchloric acid. After the mixture was filtered, 0.4 ml of a 3 mmol/l KOH solution was added to 5 ml of the filtrate and cooled for 20- 30 min. The filtrate was then injected into an HPLC (Shimadzu, Japan) and the cordycepin content analyzed. The column was a Phenomenex Luna 5 C18(2) (15 cm×4.6 mm) and the elution was performed using 30 mmol/l 2-diethylaminoethanol (pH 7.4) at a flow-rate of 1 ml/min. The column effluent was monitored at 260 nm. All values expressed are means of triplicate determinations.

Physiological Activity Assay

Acetylcholinesterase (AChE) inhibition assay. The AChE assay was performed using the colorimetric methood of Ellman *et al.* [9] with acetylthiocholine iodide (ASCh) as the substrate. For the enzyme source, PC12 cell cultures were homogenized in a Glass-Col homogenizer with 5 volumes of a homogenation buffer (10 mM Tris-HCl, pH 7.2, containing 1 M NaCl, 50 mM $MgCl_2$, and 1% Triton X-100), and centrifuged at 10,000 ×g for 30 min. The resulting supernatant was used as the enzyme source. The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate format. Each freeze-dried extract (10 μl) dissolved in 5% DMSO was mixed with the enzyme solution (10 μl) and incubated at 37°C for 15 min. The absorbance was read at 405 nm immediately after adding an Ellman reaction mixture [70 μl; 0.5 mM acetylthiocholine, 1 mM 5,5'-dithiobis-(2-nitro)-benzoic acid] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. The reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. A blank reaction was measured by substituting saline for the enzyme. The percentage of the enzyme activity inhibited was calculated and compared with that of the control activity (100%). The degree of enzyme inhibition was calculated as follows: Inhibition (%)=100 (1- inhibited reaction/ uninhibited reaction).

Anticoagulant assay. The activated partial thromboplastin time (aPTT) was measured at 37°C by an automatic blood coagulator (Clot-1A; Hospitex Dianostics, Milan, Italy), as described by Fox *et al.* [10]. Briefly, a sample of 100 μl of human citrated platelet-poor plasma (1:10 v/v, 3.8% sodium citrate) was warmed at 37°C, and then 100 μl of a prewarmed activator reagent was added. The mixture was incubated at 37°C for 18 s. Prewarmed 20 mM calcium chloride (100 μl) was then added and the aPTT was recorded for clot formation time.

Inhibition assay of TPA-induced O_2^- generation. An inhibitory test of TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells was performed according to the method of Murakami *et al.* [25]. In order to determine the inhibitory effect of the O_2^- generation, a test compound dissolved in 5 μl of DMSO was added to a DMSO-induced differentiated HL-60 cell suspension in PBS (1 ml), and incubated at 37°C for 15 min. The cells were washed with PBS twice to remove any extracellular test compound and to avoid the O_2^- scavenging effect. TPA (10 nM) and a cytochrome *c* solution (1 μg/ml) were added to the reaction mixture, which was then incubated for another 15 min. The reaction was terminated by placing it on ice. After centrifugation at 250 ×g, the visible absorption at 550 nm was measured. The inhibitory effects were expressed based on the decreasing ratio of the absorbance of the testing compounds to a control experiment.

Radical scavenging assay. The radical scavenging activity of the stromata extracts was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method [4]. Two milliliters of 0.5% TFA in the sample (final concentration 0.08 mg/ml) passed through nylon membrane filters (0.45 μm, Gelman Sci., Michigan, U.S.A.) was added to a methanol solution (1.0 ml) containing a 0.2 mM DPPH radical. The mixture was shaken vigorously, and after 10 min, the amount of residual DPPH was determined by the absorbance at 517 nm (A_{517}) using a spectrophotometer (Shimadzu UV-160A, Kyoto, Japan). The percent inhibition of the DPPH radical was 100- [(A_{517} of reaction mixture- A_{517} of sample/ A_{517} of control, which contained no sample)×100]. All tests and analyses were run in triplicate.

Anticomplementary assay. The activity was measured according to the method of Yamada *et al.* [34]. Normal human serum (NHS) was obtained from a healthy adult. Various concentrations of the sample in water (50 μl) were mixed with 50 μl of NHS and 50 μl of GVB^{++} (gelatin veronal-buffered saline, pH 7.4) containing 500 μg Mg^{++} and 150 μg Ca^{++}. The mixtures were pre-incubated at

37°C for 30 min, and 350 μl of GVB^{++} was added. IgM-hemolysin-sensitized sheep erythrocytes (250 μl) at 1×10^8 cells/ml were added to the mixtures diluted serially (10-160 folds), and then incubated at 37°C for 1 h. After the addition of phosphate-buffered saline (PBS, pH 7.2) and centrifugation, the absorbance of the supernatants was measured at 412 nm. The NHS was incubated with water and GVB^{++} as the control. The anticomplementary activity was expressed as the percent inhibition of the total complementary hemolysis (TCH_{50}) for the control [18]. The degree of inhibition of TCH_{50} was calculated according to (TCH_{50} of control - TCH_{50} of sample)/TCH_{50} of the control 100.

Intestinal immune system modulating assay. The activity was measured according to the procedure of Hong *et al.* [13]. 180 μl of a Peyer's patch cell suspension (2×10^6 cells/ml in RPMI 1640-FBS) prepared from the small intestine of C3H/HeJ mice (5-7 weeks old, Daehan Biolink Co., Korea) was cultured with 20 μl of a test sample in a 96-well flat bottom microtiter plate for 5 days at 37°C in a humidified atmosphere of 5% CO_2-95% air. The resulting culture supernatant (50 μl) was incubated with a bone marrow cell suspension (2.5×10^5 cells/ml) from C3H/HeJ mice for 6 days. After 20 μl of Alamar Blue™ solution was added to each well, the cells were continuously cultured for 5-24 h [27]. The fluorescence intensity was measured to evaluate cell numbers using a Spectrafluor Plus (Tecan, Austria) at an excitation wavelength of 544 nm and emission wavelength of 590 nm during cultivation. The intestinal immune system modulating activity was expressed as the stimulation of the cell growth of the bone marrow cells compared with that of the control, in which Peyer's patch cells were incubated with distilled water instead of the test sample.

RESULTS AND DISCUSSION

Proximate and Chemical Analysis

Table 1 shows the composition of the stromata used in the present study. As illustrated, the stromata exhibited a substantial content of crude carbohydrate (74.3%), especially crude fiber (31.3%), and crude protein (11.5%). However, the stromata were composed of a low content of crude ash (4.2%) and fat (1.3%). Kweon *et al.* [20] have reported that various edible mushrooms were composed of high content of fiber, proteins, microelements, and low fat content. The composition of the carbohydrate in the stromata included a large quantity of glucose (88.6%) and small amounts of mannose (9.4%) and galactose (1.9%) (Table 1), whereas, Song *et al.* [32] found that the anticomplementary active exopolymer produced from *C. militaris* by a submerged mycelial culture was composed of glucose (78.6%), galactose (19.1%), and arabinose (2.2%). This result indicates a compositional difference between the stromata and the exopolymer produced by the mycelial culture.

Table 1. Proximate and chemical properties of stromata of *Cordyceps militaris*.

Component	Content (%)[e]
Moisture	8.7±0.01
Crude protein[a]	11.5±0.12
Crude fat	1.3±0.00
Crude carbohydrate	74.3±1.01
Crude fiber	31.3±0.29
Crude ash	4.2±0.01
Component sugar[b]	**Content (mol %)**
Arabinose	trace
Xylose	trace
Rhamnose	trace
Mannose	9.4±0.02
Galactose	1.9±0.00
Glucose	88.6±1.34
Cordycepin[c]	**Content (%)**
Stromata	0.2±0.00
Mycelium-embeded media[d]	0.5±0.01

[a]To determine crude protein, a 6.25 conversion factor was used.
[b]The sugar component of polysaccharide was determined by GC as alditol acetate derivatives and analyzed by GLC using an SP-2380 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco, U.S.A.) equipped with an FID.
[c]To determine cordycepin content, the HPLC column was a Phenomenex Luna 5 μ C18(2) (15 cm×4.6 mm), and the column effluent was monitored at 260 nm.
[d]Mycelium-embeded media: silkworm pupae as host.
[e]All values expressed are the mean of triplicate determinations.

The examination of the cordycepin content indicated that the stromata contained 0.2%, whereas the mycelium-embedded pupae contained 0.5% (Table 1). Therefore, this result suggested that the whole-body take-up in the pupae provided more cordycepin. When Melling *et al.* [24] used a glucose, yeast extract, casamino acid, and salts medium, cordycepin concentrations of about 500 mg/l were obtained from the culture medium. In addition, since no cordycepin was produced from shaken cultures, although the mould grew well, the growth characteristics of *C. militaris* were further studied in an attempt to improve the yield of cordycepin.

The stromata contained sixteen kinds of amino acids (Table 2): lysine 101.2 mg/g; methionine 62.7 mg/g; glutamic acid 57.5 mg/g; aspartic acid 43.9 mg/g; glycine 38.1 mg/g; threonine 26.2 mg/g; alanine 26.0 mg/g; valine 23.1 mg/g; arginine 22.3 mg/g; serine 21.2 mg/g; leucine 20. 6 mg/g; phenylalanine 15.8 mg/g; isoleucine 15.1 mg/g; histidine 10.5 mg/g; cysteine 9.5 mg/g; and tyrosine 4.7 mg/g. Essential amino acids such as methionine and lysine were present in a large quantity.

The stromata contained a high ratio of essential fatty acids, such as linoleic acid (12.3%) and linolenic acid

Table 2. Amino acid composition of stromata of *Cordyceps militaris*.

Component amino acid[a]	Content (mg/g)[b]
Alanine	26.0±0.27
Arginine	22.3±0.20
Aspartic acid	43.9±0.38
Cysteine	9.5±0.05
Glutamic acid	57.5±0.36
Glycine	38.1±0.29
Histidine	10.5±0.07
Isoleucine	15.1±0.07
Leucine	20.6±0.12
Lysine	101.2±1.20
Methionine	62.7±0.52
Phenylalanine	15.8±0.08
Serine	21.2±0.20
Threonine	26.2±0.26
Tyrosine	4.7±0.00
Valine	23.1±0.22

[a]The amino acid was analyzed on an ODS-μ-Bondapak C_{18} column (3.9 mm×30 cm), using HPLC (Shimadzu, Japan) equipped with a fluorescence detector (Shimadzu, FLD-6A, Japan).
[b]All analyses were made in triplicate.

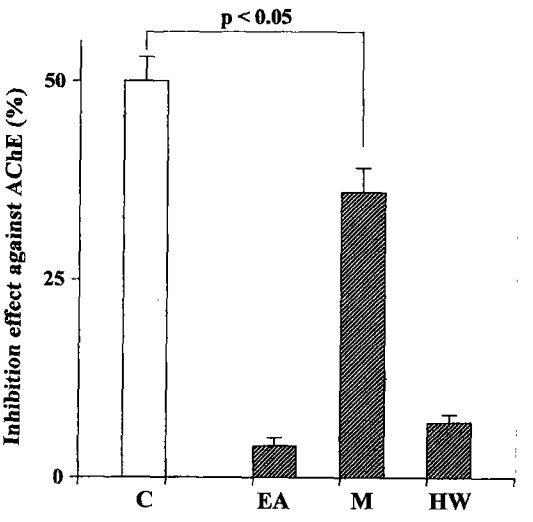


Fig. 1. Inhibitory effect on AChE activities of stromata of *Cordyceps militaris* fractionated by solvent extraction.
The percentage of enzyme activity values for each fraction was calculated as compared to the control activity (100%). The final sample concentration was 100 μg/ml. Values represent the mean (n=4) S.E. $p<0.05$; Significance between the positive control and samples. □, Positive control (Tacrine, 100 μg/ml); ▨, Sample. C, Positive control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

(31.9%), as well as oleic acid (43.3%) (Table 3). Since these unsaturated fatty acids are known to be important for various physiological activities, the stromata of *C. militaris* possessing a high ratio of unsaturated fatty acids would appear to provide beneficial physiological activities.

Physiological Activity

Acetylcholinesterase (AChE) inhibition activity. Alzheimer's disease (AD) is primarily caused by a cholinergic dysfunction. The degree of cognitive impairment in AD has been reported to correlate well with a central cholinergic deficit [7]. This provides a rationale for the most recent approach to AD drug therapy. Thus, it is suggested that the elevation of the acetylcholine (ACh) level may be helpful to improve the symptoms of cognitive deficit in AD [6]. Several investigators have tried to supplement the acetylcholine (ACh) level at synaptic sites by the administration of ACh precursors, cholinergic agonists, or AChE inhibitors [23], such as tacrine (tetrahydroaminoacridine) [29] and galantamine [5], which prevent ACh hydrolysis.

In order to identify a natural active constituent that has a potent inhibitory effect on AChE, the solvent-extracts of the stromata were examined. Among the extracts, the methanol extract (M) exhibited the highest inhibitory effect (36%). This effect, however, was low when compared to the 50% in the positive control, indicating that the stromata of *C. militaris* are not effective for the inhibition of AChE (Fig. 1).

Anticoagulant activity. Blood coagulation is a major cause of mortality and disability, leading to cerebral hemorrhage, myocardial infarction, peripheral ischemia, arteriosclerosis, and pulmonary embolism [11]. During the past several years, extensive investigations on the therapeutic agents to develop effective anticoagulants have been conducted.

We have examined the anticoagulant activity in three solvent-extracts of the stromata (Fig. 2). It was observed that a hot-water extract (HW) of the stromata contributed anticoagulant activity (coagulating time, 73 sec) significantly. This result indicates that the anticoagulant active ingredients appear to be the macromolecules extracted with hot-water.

Sulfated polysaccharides from seaweeds, a complex group of macromolecules, have been known to possess antithrombotic activity. So far, the studies for the potential antithrombotics from natural sources have focused mostly on mammalian tissues and seaweeds [28]. Recently, pharmacological function to certain aliments in edible

Table 3. Fatty acid composition of stromata of *Cordyceps militaris*.

Component fatty acid[a]	Content (%)[b]
Palmitic acid ($C_{16:0}$)	14.70.05
Palmitoleic acid ($C_{16:1}$)	0.740.00
Stearic acid ($C_{18:0}$)	6.40.01
Oleic acid ($C_{18:1}$)	33.80.42
Linoleic acid ($C_{18:2}$)	12.30.03
Linolenic acid ($C_{18:3}$)	31.90.48

[a]The fatty acid was analyzed on a CarboWax capillary column (30 m× 0.32 mm) using an HP 5890 gas chromatography (Hewlett Packard) equipped with an FID.
[b]All analyses were made in triplicate.

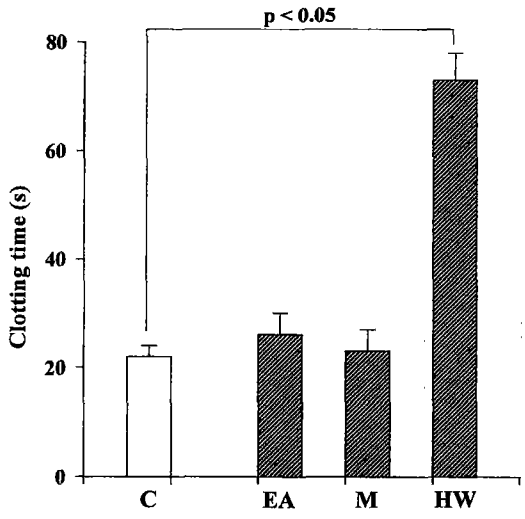


Fig. 2. Anticoagulant activities of stromata of *Cordyceps militaris* fractionated by solvent extraction.
The clotting time was measured using the aPTT method. The final sample concentration was 100 μg/ml. Values represent the mean (n=4) S.E. $p<0.05$; Significance between the control and samples. □, Control (saline); ▨, Sample. C, Control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

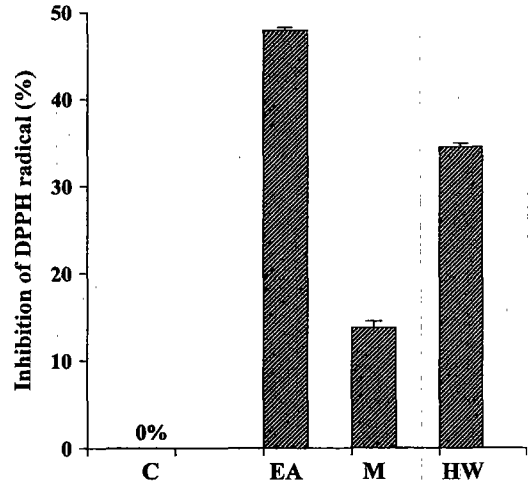


Fig. 4. Scavenging activity of stromata of *Cordyceps militaris* fractionated by solvent extraction on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.
The percentage of inhibitory effect values for each fraction was calculated as compared to the control radical generation (100%). The final sample concentration was 100 μg/ml. Values represent the mean (n=4) S.E. □, Control (saline); ▨, Sample. C, Control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

plants and fungi has received increasing attention. Therefore, it is necessary to identify the active components obtained from stromata of *C. militaris*.

Inhibition activity of TPA-induced O_2^- generation. 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters are reported to trigger superoxide (O_2^-) generation in epithelial cells and leukocytes [30]. Superoxide (O_2^-) is one of the precursors of several types of reactive oxygen species (ROS), resulting in oxidative stress-related diseases, including cancer. Therefore, the present study focused on searching for O_2^- generation inhibitors as effective and promising candidates for the prevention of these diseases. Unfortunately, no stromata extract revealed any inhibitory activity during the examination of phytochemicals with an inhibitory effect on O_2^- generation in leukocytes *in vitro* (Fig. 3).

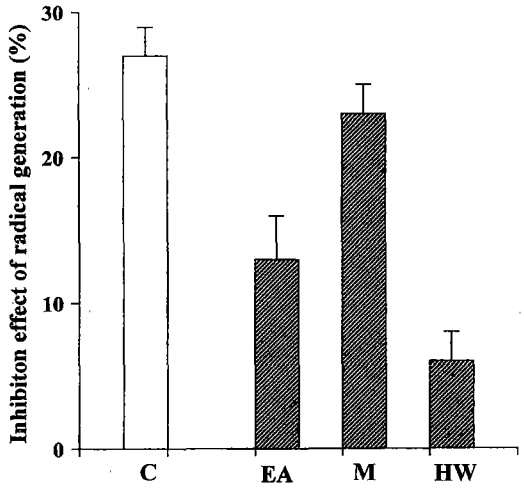


Fig. 3. Inhibitory effect on TPA-induced O_2^- generation of stromata of *Cordyceps militaris* fractionated by solvent extraction.
The percentage of inhibitory effect values for each fraction was calculated as compared to the control O_2^- generation (100%). The final sample was 100 μg/ml. Values represent the mean (n=4) S.E. □, Positive control (genistine, 100 μg/ml); ▨, Sample. C, Positive control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

Radical scavenging activity. The scavenging activities of the solvent extracts on the DPPH radical are shown in Fig. 4. The ethylacetate extract (EA) showed the most potent scavenging activity (47.9%), followed by the hot-water extract (HW, 37.5%). However, the methanol extract (M) did not show any free radical scavenging activity. This result suggested that ethylacetate extracts (EA) appear to have a relatively strong activity.

The plants and fungi offer a large range of phenolic compounds, both polar and nonpolar, among which tochophenols are best known as efficient naturally occurring liposoluble antioxidants. Their antioxidant activity has been attributed to the presence of polar phenolic compounds and essential oils [21]. The inhibitory substances of O_2^- generation from *C. militaris* stromata will be further identified.

Anticomplementary activity. The complement system plays an important role in host resistance to the actions of the primary humoral mediation of Ag-Ab reactions. Although the complement function has been viewed as a predominantly nonspecific resistance mechanism, there is

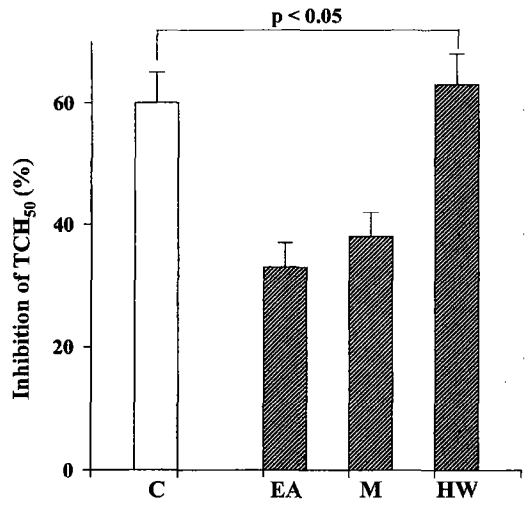


Fig. 5. Anticomplementary activities of stromata of *Cordyceps militaris* fractionated by solvent extraction.
The percentage of anticomplementary effect values for each fraction was expressed as the percentage inhibition of the total complement hemolysis (TCH_{50}) of the control. The final sample concentration was 100 μg/ml. Values represent the mean (n=4) S.E. $p<0.05$; Significance between the positive control and samples. □, Positive control (LPS, 100 μg/ml); ▨, Sample. C, Positive control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

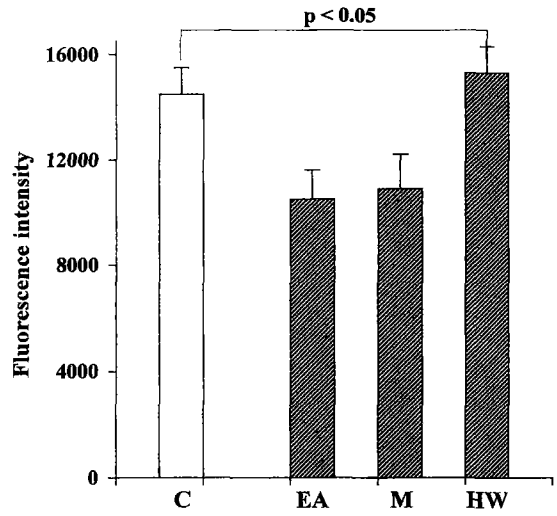


Fig. 6. Intestinal immune system modulating activities of stromata of *Cordyceps militaris* fractionated by solvent extraction.
The intestinal immune system modulating effect values for each fraction was expressed as the stimulation of the cell growth of the bone marrow cells. The final sample concentration was 100 μg/ml. Values represent the mean (n=4) S.E. $p<0.05$; Significance between the positive control and samples. □, Positive control (LPS, 100 μg/ml); ▨, Sample. C, Positive control; EA, Ethylacetate extract fraction; M, Methanol extract fraction; HW, Hot-water extract fraction.

accumulating evidence that complement is involved in the induction and regulation of specific immune responses. In particular, complement activation appears to be intrinsically associated with the activation of macrophages and lymphocytes [26], the localization and retention of antigens in germinal centers, the generation of B cell memory [19], cellular co-operation, and the regulation of cyclical antibody production [33].

The solvent extracts of the stromata were examined for anticomplementary activity using the complement fixation test, and the activity was exhibited as $ITCH_{50}$ (Fig. 5). Among the stromata extracts, the hot-water extract (HW) showed the highest activity (63%), followed by the methanol extract (M, 38%) and the ethylacetate extract (EA, 33%). This result shows that the stromata possessed a high anticomplementary activity.

Various polysaccharide and polysaccharide-containing materials such as endotoxic lipopolysaccharide are known to activate the complement system. In particular, a considerable number of medicinal herbs and edible plants have been found to possess anticomplementary activity; for example, arabinogalactan from *Angelica acutiloba* [18]. The fact that the hot-water extract fraction (HA) has the anticomplementary activity, shows the possibility that stromata of *C. militaris* may contain a kind of regulator of the complement system.

Intestinal immune system modulating activity. Gut-associated lymphoreticular tissues (GALT) play an important role in host defense, including the IgA response of the intestinal immune system [15]. Peyer's patches are important lymphoid organs of the intestine, and known to induce IgA production. Therefore, the intestinal immune system including Peyer's patches not only contributes to the defense system of the mucosa, but also regulates systemic inflammation, resulting in the suppression of allergic reactions and autoimmune diseases.

To examine their modulating activities of the intestinal immune system, the stromata were crushed, fractionated with ethylacetate, followed by fractionation with methanol and water, in an increasing order of polarity. Among these extracts, only the hot-water extract (HW) showed a potent intestinal immune system modulating activity (Fig. 6). None of the other extracts showed any activity even at a concentration of 100 μg/ml.

Because natural fungi including stromata of *C. militaris* are generally administered orally, there is a possibility that these natural sources express their clinical effects through the intestinal immune system. Yu *et al.* [36] have found that one of the traditional herbal medicines, rhizomes of *Atractylodes lancea*, showed modulating activity for the intestinal immune system *in vitro* through activation of T cells in Peyer's patches to stimulate secretion of hematopoietic growth factors. In addition, three polysaccharides, (one arabinogalactan and two pectic polysaccharides) should play an important role in the potent intestinal immune modulating action. Therefore, further studies on these active substances for intestinal immune system modulation will provide us with important information.

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