Pharmacological Activities of the Mycelial Extract of Cultured *Cordyceps sinensis*

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For defining the possibilities of the commercial mass liquid culture of *Cordyceps sinensis*, the pharmacological activities of mycelia were analyzed. The mycelium of *C. sinensis* consists of carbohydrate (5.1%) and fat (1.3%), and contains a low content of protein (0.7%) and ash (0.5%), and 92.4% moisture. The molecular sugar ratio of carbohydrate was composed mainly of glucose, mannose (1.0 : 0.9), in addition a small amount of galactose and arabinose (0.2 : 0.1). The cellular materials of mycelia were fractionated into ethylacetate (EA), MeOH (M) and hot-water extract fraction (HW). HW fraction showed the most potent intestinal immune system modulating activity, anti-coagulant activity, and anti-complementary activity, and M fraction had the inhibition activity of radical generation as effective as genistine. These results reveal that the mycelium of liquid cultured *C. sinensis* showed pharmacological activities and could be used for commercial purpose.

Key words : Cordyceps sinensis, *mycelium, pharmacological activity.*

The genus *Cordyceps* (Clavicipitaceae), known as a group of entomopathogenic fungi, forms fruiting body in their insect hosts, and 750 species in this genus have been known.[1)] Natural habitats of *C. sinensis* is damp ground covered with fallen leaves in valley. The occurrence of these fungi is probably influenced by many factors such as habitat environment, climate, and availability of hosts, etc.[2)] Some species of *Cordyceps,* such as *C. sinensis, C. militaris, C. martialis, C. ophioglossoides, C. soborifera, C. hawkesii,* and *Beauveria bassiana* have been used for medical purpose.[3)] In particular, *C. sinensis,* which forms fruiting body on moth larva, has been used not only the potential bio-insecticide but also herbal medicine. The pharmacological functions of *C. sinensis* are known as inhibition of human glomerular mesangial cell proliferation,[4)] contribution of hypotensive activity,[5)] and enhancement of Kupffer cell function.[6)]

Even though, the physiologically active substances of natural and cultured stromata have been elucidated from various *Cordyceps* spp., only a few substances are using commercially, because of difficulty for mass production. For overcoming the difficulty, the artificial solid media for mass production of stromata have been developed in Korea. But comparing to the liquid culture, the yield of solid culture is not sufficient. For example, the incubation days by solid culture are longer than those by liquid culture. However, by the liquid culture, only the mycelia are forming. So before the commercial development of mass production by the liquid culture, the physiologically active substances in artificial liquid culture must be investigated.

This paper describes the results of searching for pharmacological activities of artificially cultured mycelia of *Cordyceps sinensis*, and investigating for the possibility of practical use.

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Abbreviations: EA, ethylacetate-soluble fraction; M, methanol-soluble fraction; HW, hot-water soluble fraction.

Materials and Methods

Microorganism and materials. *Cordyceps sinensis*, from Sanming Micrological Institute in China was cultured in a modified potato dextrose broth containing yeast extract (potato 200 g, dextrose 20 g and 0.5 g yeast extract per liter). Potato dextrose broth was purchased from Difco Co. (Detroit, MI, USA). RPMI-1640 medium and Hank's balanced salt solution (HBSS) for the cultivation of PC12 and HL60 cells were obtained from Gibco-BRL Co. (Grand Island, NY, USA). Heat-inactivated horse serum and fetal bovine serum were obtained from Cell Culture Laboratories (Cleveland, OH, USA), and penicillin, streptomycin, and amphotericin B from Flow Laboratories (Irvine, Scotland). Alamar Blue™ was obtained from Alamar Bio-Sciences Inc. (Sacramento, CA, USA). Acetylthiocholine iodide (ASCh) and 5,5'-dithio-bis-(2-nitro)- benzoic acid (DTNB) were purchased from Sigma Co. (St. Louis, MO., USA). Activated partial thromboplastin time (aPTT) reagent was obtained from Dade Behring Inc. (New York, DE, USA), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) from Research Biochemicals International

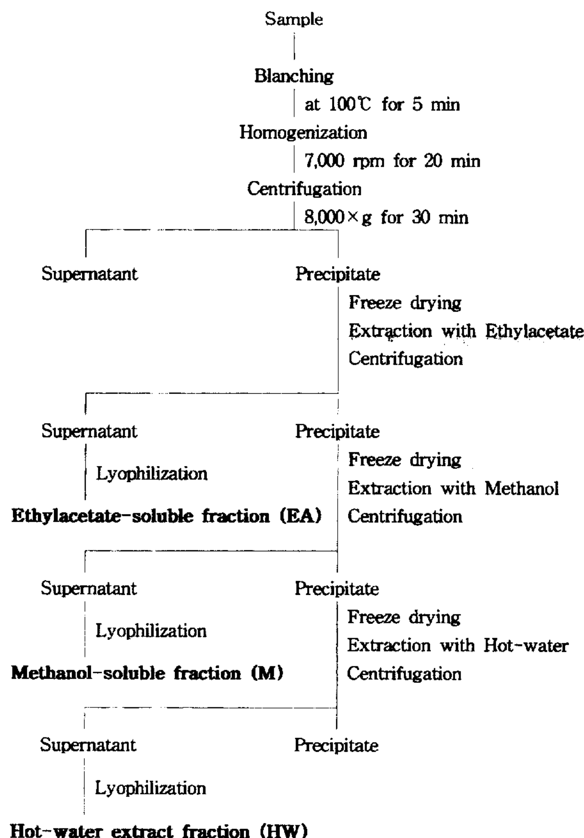

Fig. 1. Flow chart of extraction from cultured *C. sinensis*.

(Natick, MA., USA). All other reagents were analytical grade.

Culture conditions of cells. PC12 cells from rat pheochromocytoma and human promyelocytic leukemia HL-60 cells, obtained from the Health Science Research Resources Bank, were cultured in RPMI-1640 liquid media supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, and 1% antibiotic-antimycotic (Gibco-BRL, New York, USA), at 37°C and 55% humidified atmosphere containing 5% CO_2-95% air. The batch fermentation for cultivating *Cordyceps sinensis* was carried out at 150 rpm, 25°C, pH 5 for 7 days in 5 *l* jar fermenter.

Fractionation. Dried mycelia (50 g) were blanched at 100°C for 5 min and homogenized by Ultra-Turrax T-50 (Janke Kunkel IKA-Labortechniker, Germany, 7000 rpm, 20 min). After centrifugation (8,000 ×g, 30 min), the supernatant was removed, and the precipitant was initially extracted at room temperature with ethylacetate (1 *l*), followed by further extraction with MeOH (1 *l*) and hot-water (1 *l*), in an increasing order of their polarity (Fig. 1). Each extracts was centrifuged to remove insoluble materials, and their supernatants were lyophilized and named as ethylacetate-soluble fraction (EA, yield; 2.8%), MeOH-soluble fraction (M, yield; 5.8%) and hot-water extract fraction (HW, yield; 29.3%).

Chemical analysis. Moisture, crude protein, crude carbohydrate, crude fat, and crude ash contents of the mycelium were determined according to AOAC methods.[7] All values were mean of quadruplicate determinations. Carbohydrate composition of the mycelium was analyzed as alditol acetates after hydrolysis of polysaccharide with 2 M TFA for 1.5 hr at 121°C,[8] and analyzed via GLC using an SP-2380 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco, USA) as the method of Zhao *et al.*[9] The temperature program 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 molecular weights using a flame-ionization detector (FID).

Acethylcholinesterase (AChE) inhibition assay. The AChE assay was performed through the colorimetric method of Ellman *et al.*[10] using acetylthiocholine iodide as a substrate. For the enzyme source, PC12 cell cultures were homogenized with 5 volumes of 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 crude enzyme source. The rates of hydrolysis by AChE were recorded spectrophotometrically at 405 nm using a 96-well microtiter plate by microplate reader (Bio-Rad 3550-UV, Japan). Each fraction (10 μ*l*) in 5% DMSO was mixed with enzyme solution (10 μ*l*), and incubated at 37°C for 15 min. Absorbance was read at 405 nm immediately after adding an Ellman reaction mixture (70 μ*l*; 0.5 mM acetylthiocholine, 1 mM 5,5'-dithiobis-(2-nitro)-benzoic acid) in 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. Blank reaction was measured by substituting saline for the enzyme. The percentage of enzyme activity value for the inhibitors was calculated based on that of the control activity.

Anti-coagulant assay. 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 have been made for development of effective anti-coagulants.[12,13] The activated partial thromboplastin time (aPTT) was measured at 37°C with the use of an automatic blood coagulator (Clot-1A; Hospitex Dianostics, Milan, Italy) as described by Fox *et al.*[14] Briefly, a sample of 100 μ*l* of human citrated platelet-poor plasma (1 : 10 v/v, 3.8% sodium citrate) was warmed at 37°C, adding the same quantity of prewarmed activator reagent. The mixture was incubated at the same condition for 18 s. Prewarmed 20 mM calcium chloride (100 μ*l*) was then added, and aPTT was expressed as the time required for clot formation.

Inhibition assay 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.[15] Superoxide (O_2^-) is one of the precursors to several types of reactive oxygen species (ROS), a causative agent of oxidative stress-related diseases including cancer. Inhibitory test of TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells was performed as described by Marker *et al.* and Murakami *et al.*[16,17] In order to

determine the inhibitory effect of O_2^- generation, 5 μ*l* of DMSO, in which each lyophilized fractions was dissolved, was added to tube containing DMSO-induced differentiated HL-60 cell suspension in PBS (pH 7.2, 1 m*l*), and the tube was incubated at 37°C for 15 min. The cells were washed twice with PBS for removal of extracellular compound. TPA (10 nM) and cytochrome *c* solution (1 μg · ml^{-1}) were added to the tube, and then incubated for additional 15 min. The reaction was terminated by placing the tube on ice. After centrifugation at 250 ×g, the visible absorption at 550 nm was measured. Inhibitory effect was expressed by a decreasing ratio of absorbance of test samples to control.

Anti-complementary assay. The anti-complementary activity was measured according to the described procedure of Yamada *et al.*[18] Normal human serum (NHS) was obtained from a healthy adult. Various concentrations of sample in water (50 μ*l*) were mixed with 50 μ*l* of NHS and 50 μ*l* 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 μ*l* of GVB was added. IgM-hemolysin-sensitized sheep erythrocytes (250 μ*l*) at 1 × 10^8 cells/*ml* was added to the mixtures diluted serially (10 to 160 fold), and then incubated at 37°C for 1 h. After the addition of PBS (pH 7.2) and centrifugation, the absorbance of the supernatant was detected at 412 nm. NHS was incubated with water and GVB as a control. The anti-complementary activity was expressed as the percentage inhibition of the total complementary hemolysis (TCH_{50}) of the control. The degree of inhibition of TCH_{50} was calculated as followings:

$$ITCH_{50}\,(\%) = \frac{(TCH_{50}\text{ of control} - TCH_{50}\text{ of sample})}{TCH_{50}\text{ of control}} \times 100$$

Intestinal immune system modulating assay. The intestinal immune system modulating activity was measured according to the procedure of Hong *et al.*[19] One hundred and eighty μ*l* of Peyer's patch cell suspension (2 × 10^6 cells/*ml* in RPMI 1640-FBS) prepared from a small intestine of C3H/HeJ mice (5-7 weeks old, Daehan Biolink Co.) were cultured with 20 μ*l* of test sample in a 96-well flat bottom microtiter plate for 5 days at 37°C under a 55% humidified atmosphere of 5% CO_2 - 95% air. The culture supernatant (50 μ*l*) was incubated with bone marrow cell suspension (2.5 × 10^5 cells/*ml*) from C3H/HeJ mice for 6 days. After 20 μ*l* of Alamar Blue™ solution was added to each well, and the cells were then continuously cultured for 5-24 h.[20] The fluorescence intensity was measured to count cell numbers using 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 bone marrow cell growth compared with that of the control, in which Peyer's patch cells were incubated with distilled water instead of test sample.

Table 1. Chemical compositon of mycelium of cultured *Cordyceps sinensis*.

Component	Content (%)[c]
Moisture	92.4 ± 0.3
Protein[a]	0.7 ± 0.0
Lipid	1.3 ± 0.1
Carbohydrate	5.1 ± 0.2
Ash	0.5 ± 0.0
Component sugar[b]	**Content (mol. %)**
Arabinose	6.6 ± 0.1
Xylose	trace
Rhamnose	trace
Mannose	41.1 ± 0.5
Galactose	7.5 ± 0.1
Glucose	44.9 ± 0.6

[a]To determine crude protein, the 6.25 conversion factor was used.
[b]Component sugar of polysaccharide was determined 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, USA) equipped with an FID.
[c]All values expressed are mean of triplicate determinations.

Results and Discussion

Chemical composition of the *C. sinensis*. Table 1 showed the chemical composition of *C. sinensis* mycelium. The moisture content of mycelium contained 92.4%, and carbohydrate occupied 5.1%. Molecular ratio of the composed carbohydrate in the mycelium is glucose, mannose, galactose and arabinose (1 : 0.9 : 0.2 : 0.1). According to Yin and Tang[21], stromata of *C. sinensis* consists of the following constituents: water, 10.8%; fat, 8.4%; crude protein, 25.3%; crude fiber, 18.5%; carbohydrate, 28.9%; ash, 4.1%. Even though carbohydrate content in stromata is higher than that in mycelia, the moisture content of mycelia is also higher than that of stromata Although chemical composition of the mycelia of cultured *C. sinensis* was rarely known, the present results showed that carbohydrate content was less than the stromata.

Acethylcholinesterase (AChE) inhibition activity. Alzheimer's disease (AD) is primarily caused by cholinergic dysfunction. The elevation of the acetycholine (ACh) level should be helpful in improving the symptoms of cognitive deficits in AD.[22,23] Several research groups have tried to supplement the ACh level in synaptic sites through the administration of ACh precursors, cholinergic agonist or AChE inhibitors, such as tacrine (tetrahydroaminoacridine)[24] and galantamine[25] which prevents ACh hydrolysis. AChE plays as a key role in cholinergic transmission in the central nervous system (CNS) of mamals.[26] To find out the inhibitor of AChE, the AChE assay was performed with the extract of *C. sinensis* mycelium. Among the three fractions, EA and M fractions have relative

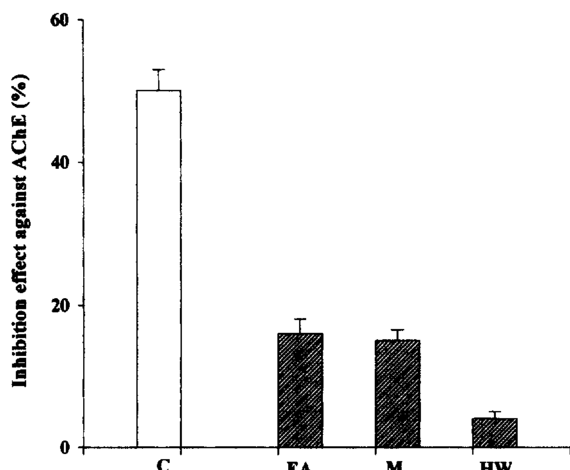

Fig. 2. Inhibitory effect on AChE activities of the solvent extracts from mycelium of cultured *Cordyceps sinensis*. The percentage of enzyme activity values for each fraction was calculated as compared to the control activity (100%). The final concentration of the sample was 100 μg · ml^{-1}. Values represent mean (n = 4) ± S.E. p<0.05; significance between the positive control and samples. □, Positive control (Tacrine, 100 μg·ml^{-1}); ▨, Sample (100 μg·ml^{-1}) C, Positive control; EA, Ethylacetate-soluble fraction; M, Methanol-soluble fraction; HW, Hot-water extract fraction. The significance probability is <0.05.

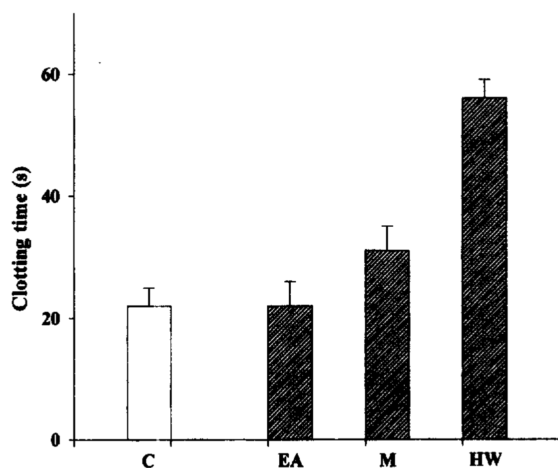

Fig. 3. Anti-coagulant activities of the solvent extracts from mycelium of cultured *Cordyceps sinensis*. The clotting time was measured through aPTT method. The final concentration of sample was 100 μg · ml^{-1}. Values represent mean (n = 4) ± S.E. p<0.05; significance between the control and samples. □, Control (saline); ▨, Sample (100 μg·ml^{-1}) C, Control; EA, Ethylacetate-soluble fraction; M, Methanol-soluble fraction; HW, Hot-water extract fraction. p<0.05.

high inhibitory activity of 16% and 14% respectively, compared to 50% of AChE inhibition activity of the positive control (Tacrine). But HW fraction has low activity (Fig. 2).

Anti-coagulant activity. The anti-coagulant activities in three fractions were analyzed (Fig. 3). HW had relatively higher anti-coagulant activity than other fractions. The control showed 21 s of clotting time. Sulfated polysaccharides from seaweeds, a complex group of macromolecules, have been known to possess the antithrombotic activity.[12,13] Recently, pharmacological function to certain aliments in edible plants and fungi has received an increasing attention. This is the first time that the anti-coagulant activity of *C. sinensis* was found out in mycelia.

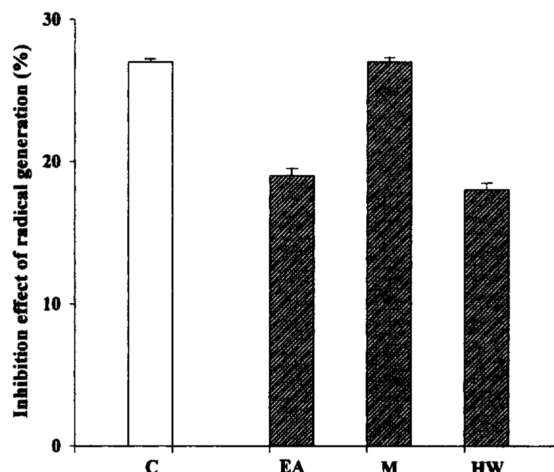

Fig. 4. Inhibitory effect on TPA-induced O_2^- generation of the solvent extracts from mycelium of cultured *Cordyceps sinensis*. The percentage of inhibitory effect values for each fraction was calculated as compared to the control O_2^- generation (100%). The final concentration of sample was 100 μg · ml^{-1}. Values represent mean (n = 4) ± S.E. p<0.05; significance between the positive control and samples. □, Positive control (genistine, 100 μg · ml^{-1}); ▨, Sample (100 μg · ml^{-1}) C, Positive control; EA, Ethylacetate-soluble fraction; M, Methanol-soluble fraction; HW, Hot-water extract fraction. p<0.05.

Inhibition activity of TPA-induced O_2^- generation. Among the fractions examined, M fraction showed the inhibition activity of TPA-induced O_2^- generation, as much as the positive control, genistine (27% inhibition, Fig. 4). The plants and fungi offer a large range of phenolic compounds, among which tochophenols are best known as efficient natural liposoluble antioxidants.[27,28] The inhibitory substances of O_2^- generation from *C. sinensis* mycelium should be analyzed whether phenolic compounds or not.

Anti-complementary activity. The complement system plays an important role in host resistance as actions of the primary humoral mediation of Ag-Ab reactions. Although complement function has been viewed in the context of a predominantly non-specific resistance mechanism, there are lots of evidence that complement is involved in the induction and regulation of specific immune response. Specifically, complement activation appears to be intrinsically associated with the activation of macrophages and lymphocytes,[29] the localization and retention of antigens in germinal centers, the generation of B cell memory,[30] cellular co-operation, and the regulation of cyclical antibody production.[31]

Among the fractions, only the HW fraction showed the highest activity (70%) as shown in Fig. 5. Various polysaccharide and lipopolysaccharide are known to activate the complement system in human serum. A lot of medicinal herbs and

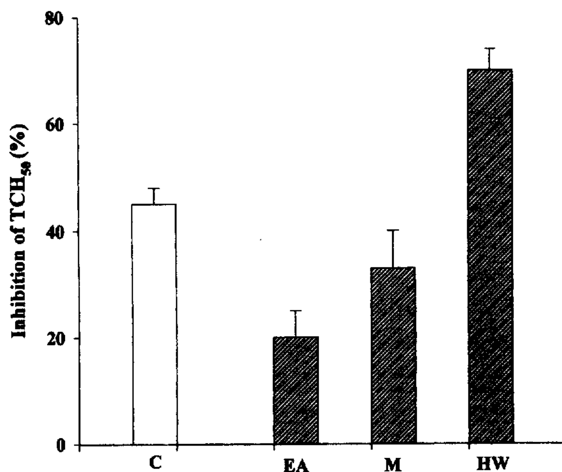

Fig. 5. Anti-complementary activities of the solvent extracts from mycelium of cultured *Cordyceps sinensis*. The percentage of anti-complementary effect values for each fraction was expressed as the percentage inhibition of the total complement hemolysis (TCH_{50}) of the control. The final concentration of sample was 100 μg · ml^{-1}. Values represent mean (n = 4) ± S.E. $p<0.05$; significance between the positive control and samples. □, Positive control (LPS, 100 μg · ml^{-1}); ▨, Sample (100 μg · ml^{-1}) C, Positive control; EA, Ethylacetate-soluble fraction; M, Methanol-soluble fraction; HW, Hot-water extract fraction.

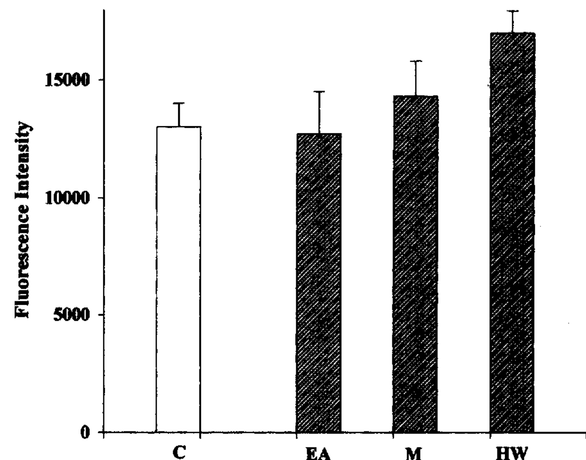

Fig. 6. Intestinal immune system modulating activities of the solvent extracts from mycelium of cultured *Cordyceps sinensis*. The intestinal immune system modulating effect values for each fraction was expressed as stimulation of cell growth of bone marrow cells compared with that of control. The final concentration of sample was 100 μg · ml^{-1}. Values represent mean (n = 4) ± S.E. $p<0.05$; significance between the positive control and samples. □, Positive control (LPS, 100 μg·ml^{-1}); ▨, Sample (100 μg · ml^{-1}) C, Positive control; EA, Ethylacetate-soluble fraction; M, Methanol-soluble fraction; HW, Hot-water extract fraction.

edible plants have been known to possess anti-complementary activity; for example, arabinogalactan from *Angelica acutiloba*,[32] the acidic heteroglycan and neutral glucan from *Coix lachryma-jobi* var. *ma-yuen*,[33] and three acidic heteroglycan from the leaves of *Panax ginseng*.[34] The fact that HW fraction has the anti-complementary activity, shows the possibility that mycelia of *C. sinensis* may contain a kind of water soluble regulator of the complement system.

Intestinal immune system modulating activity. The gut-associated lymphoreticular tissues (GALT) play an important role in host defense system including IgA response of the intestinal immune system.[35] Peyer's patches, important lymphoid organs of the intestine, are known to be inductive sites for 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 disease.[35]

HW fraction showed the most potent intestinal immune system modulating activity, and M fraction had the intermediate activity (Fig. 6). EA fraction showed no activity even at a high concentration of 200 μg · ml^{-1} (data not shown). Because natural fungi, including mycelium of *C. sinensis*, 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 rhizomes of *Atractylodes lancea*, one of traditional herbal medicine, showed intestinal immune system modulating activity *in vitro* through activation of T cells in Peyer's patches to stimulate secretion of hematopoietic growth factors. In addition, arabinogalactan and two pectic polysaccharides were identified to have the potent intestinal immune modulating activity.

In this study, we have found out that the mycelia of *C. sinensis* by liquid culture have the various pharmacological activities. The three fractions are crude, not purified, but have relatively high activities. Considering the control was purified chemicals, the crude fractions have high pharmacological activities. So, the liquid culture of *C. sinensis* could be used as mass production for commercial purpose, and the detailed subfractionation for unveiling the pharmacological activities should be carried out.

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