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Composition and Antitumor Activity of the Mycelia and Fruiting Bodies of *Cordyceps militaris*

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Abstract Cordyceps militaris contains many kinds of bioactive components with the various physiological activities, but has less natural sources of fruiting bodies. Compared with the fruiting bodies from the same origin, the fermented mycelia contained significantly higher amount of protein, crude fat and polysaccharides. The mycelia had more amount of the total amino acids, with proline (3.84 ± 0.12 g $100g^{-1}$), leucine (2.96 ± 0.10 g $100g^{-1}$), and aspartic acid (2.68 ± 0.06 g $100g^{-1}$) as the top three aboundant amino acids, while glutamic acid (3.56 ± 0.07 g $100g^{-1}$), aspartic acid (2.52 ± 0.13 g $100g^{-1}$), and valine (2.35 ± 0.11 g $100g^{-1}$) in fruiting bodies. The tested minerals in the mycelia were much more than those in fruiting bodies, especially Selenium which was nondetectable in fruiting bodies. However, the mycelia had lower level of cordycepin and mannitol. The mycelia also exhibited higher antitumor activity against six tested human cancer cell lines, with the range of IC_{50} (μg ml^{-1}) from 25.03 ± 1.37 to 39.81 ± 0.54. In the animal test for liver cancer cell Hep G2, even high-dose treatment (250 mg kg-1 BW/day) of the extract of the mycelia inhibited the growth of tumor by only 27.6%, significantly lower than the positive control, CTX (30 mg kg^{-1} BW/day), but had no influence on body weight. The results confirm the potential of cultivated mycelia to be a good substitute of natural products.

Keywords: *component analysis, amino acids, nucleosides, minerals, anti-tumor test*

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1. Introduction

Cordyceps militaris, an entomopathogenic ascomycete fungus, known as "winter worm summer grass" in Chinese because of its appearance during different seasons, is an important traditional Chinese medicinal mushroom [1]. It contains many kinds of bioactive components, including nucleosides, amino acids, vitamins, trace minerals and polysaccharides [2,3,4]. Due to the various physiological activities, C.militaris has been used extensively for multiple medicinal purposes in China, Japan, Korea and other oriental countries for many years.

Consequently, the demand for natural fruiting bodies of C.militaris is continuously increasing because of its medicinal uses, while the wild resources are decreasing rapidly due to non-sustainable collection. In addition, the production in artificial culture has been proved to be extremely difficult and only feasible at laboratory scale so far. So many attempts have been made to obtain useful and potent substances from a submerged mycelial culture, which gives rise to potential advantages of higher mycelial production in a compact space and shorter time with less chance of contamination. Moreover, the artificial mycelial biomass shows a close chemical composition [5,6] and has a similar pharmacological efficacy to natural products of Cordyceps sp. [7,8]. So, the cultivated mycelia of Cordyceps fungi have become a main substitute of the natural species for commercial health food or drug formulation. The anti-tumor activities of natural Cordyceps and cultivated fungal mycelia have been detected in several previous studies [9,10].

In this study, we analyzed the composition of C.militaris mycelia from submerged culture, compared with that of fruiting bodies to clarify the chemical differences among them. In addition, the growth inhibitory effect of the extracts from mycelia and fruiting bodies on human cancer cell lines was also demonstrated.

2. Materials and Methods

2.1. Strain

Cordyceps militaris (Strain number C-8) is isolated from fresh fruiting body of natural *Cordyceps* sp., collected in October 2008, Qinghai province, China. Based on its microscopic morphology and ITS sequences analysis (Accession Number EU825999 in GenBank), the fungus C-8 was identified as *C.militaris* [11]. A reference living culture was kept at Institution of Agro-product Processing, Jiangsu Academy of Agricultural Sciences, China. The stock culture was maintained on a potato dextrose agar (PDA) slant and subcultured every month.

Slants were incubated at 20°C for 7 days and subsequently stored at 4°C.

2.2. Seed Culture

The inoculum was prepared in Erlenmeyer flask of 250 ml with 100 ml of potato glucose medium complemented with 0.5 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4$, and 0.1 g L^{-1} vitamin B_1. The flask was inoculated with cells from the stock slants and incubated at 20 °C in a shake incubator at 120 rpm for 5 days in the dark.

2.3. Submerged Cultivation

The strain was cultivated in a 10L airlift fermentation system (Zhenjiang Green Bio-engineering Co., Ltd., Zhenjiang, China) with working volume of 7 L and inocula 10%. The media contained 25 g L^{-1} molasses, 10 g L^{-1} corn steep powder, 0.25 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $CaCl_2$, 0.5 g L^{-1} KH_2PO_4 and 0.5 g L^{-1} K_2HPO_4 [12]. The fermentation was carried out at 20°C, pressure 0.05 Mpa, aeration 0.75 vvm and agitation rate 80 rpm. Culture broth was centrifuged at 4000 rpm for 15 min, and the mycelia sediment obtained was washed twice with distilled water. Dry mycelia were obtained by lyophilization until a constant mass.

2.4. Solid Cultivation for Fruiting Body

For solid cultivation, 10 ml inoculum was inoculated on the sterilized medium containing 30 g rice, 1 g silkworm chrysalis powder and 45 ml nutrition liquid (containing 2% dextrose, 1% peptone, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.01% Vitamin B_1) and incubated at 20°C for 20 days in the dark. After then, light culturing was taken until orange fruiting bodies formed. The fruiting bodies were harvested and freeze-dried.

The mycelia and fruiting bodies were finely ground into powder using liquid nitrogen, and then stored at 4°C prior to further analysis.

2.5. Analytical Methods

2.5.1. Proximate Composition

Protein concentrations were determined using the method of Lowry et al. [13] with bovine serum albumin as the standard by a UV-1600PC spectrophotometer (Mapada, Shanghai, China). Crude fat were extracted by SZF-06A Soxhlet extraction equipment (Shanghai Xinjia Electron Co., Ltd, China). Polysaccharide was analyzed according to the method of Xiao et al. [14].

2.5.2. Measurement of Amino acids

Amino acids were determined using an automatic amino acid analyzer (Hitachi L-8900, Japan). Hydrolysis of the samples was performed in the presence of 6 mol/L HCl at 110°C for 24 h under a nitrogen atmosphere. The hydrolysate was evaporated and the residue was redissolved in 1mL 0.02 mol L^{-1} HCl. The sample was filtered through a 0.45 μm filtermembrane prior to analysis.

2.5.3. Measurement of Minerals and Trace elements

Total calcium (Ca), copper (Cu), Cobalt (Co), iron (Fe), Magnesium (Mg), Zinc (Zn), and Selenium (Se) contents were determined according to the methods of Chinese national standard GB/T 5009 drafted and published by the ministry of health. Ca, Cu, Co, Fe, Mg and Zn were detected using atomic absorption spectrophotometer (AA320N, Shanghai, China), and Se was analyzed using atomic fluorescence spectrometer (SK-2003Z, Beijing, China).

2.5.4. Measurement of Nucleoside and Mannitol

The sample (0.5 g) was suspended in 20 ml distilled water and sonicated for 50 min at 20 kHz, 100 W (XL2020, Heat Systems Inc., USA) at 60°C. The supernatant obtained by centrifugation was used for the determination.

Adenosine and cordycepin concentrations were determined by HPLC equipped with a UV detector (Agilent1100, USA) and ZORBAX SB C-18 column (4.6 mm × 250mm, 5 μm) [15]. The mobile phase was consisted of methanol and water (15/85, v/v). The flow rate was 1.0 ml min^{-1} and the column temperature was 30°C. The chromatogram was monitored by UV absorbance at 260nm.

Mannitol was determined by HPLC with a refractive index detector system [16] and Cosmosil NH_2 column (4.6 mm × 250mm, 5 μm). The mobile phase is 0.05 g L^{-1} EDTA calcium disodium solution with the flow rate of 0.5 ml min^{-1} and the column temperature of 85°C.

2.6. Cell growth inhibition assay

The anti-tumor activity *in vitro* was tested on six different human cancer cell lines, colonic carcinoma cell HCT-116, non-small lung cancer cell A549, stomach cancer cell MGC-803, liver cancer cell HepG2, leucocythemia cell HL-60 and galactophore cancer cell MCF-7, which were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, GrandIsland, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) at 37°C in a 5% CO_2 incubator.

Mycelia and fruiting bodies were extracted with methanol in a ratio of 1:50 (w/v) for 2 h under continuous stirring. The resulting extracts were evaporated at 50 °C and 0.08 MPa vacuum conditions. The MTT assay was used to measure the inhibition ability of the extract against cancer cell proliferation [17]. The cell cultures were exposed to various concentrations of the extracts and incubated for 24h. The culture medium was removed and 20μl of 5mg ml^{-1} MTT was added. Four hours later, the supernatant was discarded and 100μl DMSO was added to each well. The mixture was shaken and measured at 570 nm using an Automated Microplate Reader (Bio-Tek ELX 800, USA). Cell inhibition ratio (I%) was calculated by the following equation:

$$I\% = (1- A_{treated} / A_{control}) \times 100$$

Where $A_{treated}$ and $A_{control}$ are the average absorbance of three parallel experiments from treated and control groups, respectively. The IC_{50} was defined as the concentration that caused 50% inhibition of cell proliferation and was calculated by SAS statistical software.

2.7. Animal Test

The *in vivo* anti-tumor activity of the extract of mycelium and fruiting bodies was further tested in ICR mice. The mice (18-22g, all male) were obtained from the Animal Center of China Pharmaceutical University, Nanjing, China. Upon arrival, the animals were kept in the animal room for 3 days to adapt to the environment. On the 4^{th} day, each mouse was injected with 2×10^6 cancer cells (from the cell culture described above) subcutaneously to induce tumors. The recipient mice were randomly divided into four groups, eight mice each, a control group which was treated with double distilled water, a positive control group treated with a proven cancer drug, Cytoxan (CTX, cyclophosphamide, Sigma, Cat.C0768), and two groups treated by the extract with different concentrations. Both CTX and the extract were pre-dissolved in distilled water and then filter-sterilized, and administered to the mice once every day by intraperitoneal (i.p.) injection. Body weight and tumor size of mice were measured daily. After 27 days of treatment, the mice were sacrificed and dissected to remove the tumors for weighing.

The animal test was conducted in accordance with the principles for laboratory animal use and care as found by National Academy of Sciences and published by National Institutes of Health.

2.8. Statistical Analysis

All tests were carried out in triplicate. Data are presented as means ± SD. Analysis of variance (ANOVA) was done by the SAS 8.0 program.

Table 1. Protein, crude fat and polysaccharide in the mycelia and fruiting bodies of *C.militaris* (g 100g^{-1})

	Mycelia	Fruiting bodies
Protein	30.23±0.06^a	25.37±0.14^b
Crude fat	4.58±0.10^a	3.35±0.13^b
Polysaccharide	5.36±0.07^a	4.32±0.13^b

Means (n=3) with different letters in the same row are significantly different (P<0.05).

3. Results

3.1. Crude Fat, Protein and Polysaccharide

Protein, crude fat and polysaccharide in mycelia were 30.23, 4.58 and 5.36 g 100g^{-1} respectively, significantly higher than those in fruiting bodies (Table 1). These results disagrees with a report by Hsu et al. [6] that the fermented mycelia had lower protein and fat contents (14.8 and 6.63%, respectively) than did the fruiting body (30.4 and 9.09%).

Protein levels in natural and fermented *Cordyceps* mycelia have been reported to be approximately 5.6-31.6 g 100g^{-1} [18,19]. Fat contents reported previously were above 5 g 100g^{-1} [6,20,21]. And *Cordyceps* contains high amount of polysaccharide with a wide range [22]. Compared to other reports, *C.militaris* C-8 had higher protein content and lower fat content, which may due to different fungal geographical origins.

3.2. Amino Acid Analysis

Amino acids content and composition in fermentation mycelia and fruiting bodies were presented in Table 2. Six amino acids in the fermented mycelia, alanine, isoleucine, leucine, lysine, proline and histidine, were significantly higher than those in the fruiting bodies, as well as the total level of amino acids, among which isoleucine, leucine and lysine are essential amino acids. While glutamic acid and tyrosine in the fermented mycelium were significantly lower than those in the fruiting bodies. The top three aboundant amino acids and their levels in the fermented mycelia are proline (3.84 ± 0.12 g 100g^{-1}), leucine (2.96 ± 0.10 g 100g^{-1}), and aspartic acid (2.68 ± 0.06 g 100g^{-1}), and those in fruiting bodies are glutamic acid (3.56 ± 0.07 g 100g^{-1}), aspartic acid (2.52 ± 0.13 g 100g^{-1}), and valine (2.35 ± 0.11 g 100g^{-1}), respectively.

Hsu et al. [6] also found that glutamic acid and aspartic acid were the two main amino acids in all samples tested, but the level of total amino acids in the mycelium was only half that in the natural *Cordyceps*.

Table 2. Amino acid composition of the mycelia and fruiting bodies of *C.militaris* (g 100g^{-1})

Amino acid	Mycelia	Fruiting bodies
Aspartic acid	2.68±0.06	2.52±0.13
Threonine*	1.38±0.03	1.38±0.08
Serine	1.13±0.05	1.20±0.03
Glutamic acid	2.86±0.07^b	3.56±0.07^a
Glycine	1.54±0.09	1.30±0.07
Alanine	2.09±0.03^a	1.60±0.06^b
Valine*	2.46±0.08	2.35±0.11
Methionine*	0.88±0.06	0.82±0.09
Isoleucine*	1.44±0.07^a	0.89±0.07^b
Leucine*	2.96±0.10^a	1.71±0.09^b
Tyrosine*	1.79±0.05^b	2.06±0.07^a
Phenylalanine*	2.25±0.11	2.14±0.09
Lysine*	1.70±0.08^a	1.26±0.03^b
Proline	3.84±0.12^a	1.34±0.08^b
Histidine	0.48±0.03^a	0.30±0.05^b
Argnine	1.45±0.09	1.36±0.08
Total	30.93±0.18^a	25.79±0.17^b

Means (n=3) with different letters in the same row are significantly different (P<0.05).
*Essential amino acids.

Table 3. Mineral composition of the mycelia and fruiting bodies of *C.militaris* (μg g^{-1})

Mineral composition	Mycelia	Fruiting bodies
Cu	24.3±1.18^a	15.9±0.71^b
Fe	936.7±1.54^a	171.4±1.35^b
Mg	266.4±1.35^a	216.2±1.97^b
Zn	88.0±2.04^b	110.2±2.54^a
Ca	3395.4±3.45^a	141.9±1.27^b
Co	2.07±0.81^a	0.651±0.94^b
Se	0.40±0.24	-

-, Not detectable
Means (n=3) with different letters in the same row are significantly different (P<0.05)

3.3. Mineral Analysis

The mineral contents in the fermented mycelium and fruiting bodies were significantly different (Table 3). Except Zn, the contents of other six trace minerals in the fermented mycelia were significantly higher than those in fruiting bodies. Fe and Co contents in the fermented mycelia were 5.46 and 3.18 times higher than those in the fruiting bodies, respectively, and even Ca 23.93 times higher. In addition, Se, a trace mineral with important function in the metabolic process of human body, was

0.40μg g^{-1} in the fermented mycelia, while not found in the fruiting bodies.

Table 4. Significant markers of the mycelia and fruiting bodies of *C.militaris*(mg g^{-1})

	Mycelia	Fruiting bodies
Cordycepin	0.16±0.38 [b]	1.72±0.71 [a]
Adenosine	6.37±0.91 [a]	0.96±0.86 [b]
Mannitol	86.12±2.57 [b]	120.81±1.64 [a]

Means (n=3) with different letters in the same row are significantly different (P<0.05)

3.4. Nucleoside and Mannitol Analysis

Cordycepin, adenosine and mannitol was used usually as marker for the quality control of *Cordyceps* spp. Table 4 shows the analysis of cordycepin, adenosine and mannitol in the samples, obviously indicating that the content of adenosine in the fermented mycelium was much higher than that in the fruiting bodies, while by contrary with cordycepin and mannitol.

Trace amounts of cordycepin, 0.16 mg g^{-1}, were detected in *C.militaris* fermented mycelium in this study. Adenosine, inosine or cordycepin have been used as indexing ingredients for quality control, which differentiated *Cordyceps* from different species and the counterfeit [2,3,6]. However, actually, the existence of cordycepin in natural *Cordyceps* is controversial in the past decades. There was a great variation of nucleoside content in different sources of *Cordyceps*. Recently, cordycepin has been detected in natural *Cordyceps* with very low contents in some publication [23]. Many studies have reported that oxygen supply could evidently influence the formation and accumulation of bioactive metabolites in the submerged cultivation of medicinal fungus [24]. Xie et al. [25] also reported that with the increasing of medium capacity dry mycelia weight decreased but cordycepin production enhanced, and obtained the highest cordycepin yield of 1.8 mg g^{-1}.

Apparently, the results of chemical composition in our study had a great variation with those in other reports. Such variation may occurs in different fungal species origin, or even within the same species, the composition is affected by a variety of culturing conditions, such as cultivation substrate, with respect to carbon and nitrogen sources and their ratio, oxygen, humidity and temperature, and so on, consequently bringing about the different transformation mechanisms of corresponding substances in the different culture process. So it's difficult to differentiate *Cordyceps* from counterfeits or evaluate the quality of *Cordyceps* on the basis of chemical composition.

Table 5. IC_{50} (μg ml^{-1}) of samples for different human tumor cell lines

Cell line	HCT-116	A549	MGC-803	Hep G2	HL-60	MCF-7
Mycelia	39.81±0.54[a]	25.73±1.09[a]	27.62±1.24[a]	25.03±1.37[a]	36.95±0.72[a]	27.73±2.67[a]
Fruiting bodies	46.41±0.79[b]	53.47±0.93[b]	56.34±1.65[b]	135.33±1.56[b]	72.03±2.03[b]	36.21±1.44[b]

Means with different letters within a column are significantly different (P <0.05).

3.5. Cell Growth Inhibition Assay

Table 5 summarized the inhibitory effect of methanol extract of fermented mycelia and fruiting bodies on six cancer cell lines, HCT-116, A549, MGC-803, HepG2, HL-60 and MCF-7. The IC_{50} (μg ml^{-1}) of samples were given. Obviously, fermented mycelia had significantly stronger anti-tumor activity than fruiting bodies. HepG2 was the most susceptible to the mycelia, while MCF-7 to fruiting bodies. The anti-tumor activity of the mycelia for the cancer cells was Hep G2 > A549 > MGC-803 > MCF-7 > HL-60 > HCT-116, different from that of the fruiting bodies, MCF-7 > HCT-116 > A549 > MGC-803 > MGC-803 > Hep G2 (Table 5).

Lin and Chiang [26] evaluated the anti-tumor activity of the fermentation broth of *Cordyceps militaris* cultured in the medium of *Radix astragali*, and found that the fermentation broth inhibited the growth of four tumor cells including human gastric cancer AGS cells, human breast cancer MCF-7 cells, human hepatocellular carcinoma HepG2 cells and murine colorectal adenocarcinoma CT26 cells with IC_{50} 465μg ml^{-1}, 37μg ml^{-1}, 25μg ml^{-1}, and 20μg ml^{-1}, respectively.

3.6. Animal Test

The dosages of the methanol extract of the mycelia were 100 mg kg^{-1} BW/day and 250 mg kg^{-1} BW/day for the study of anti-tumor activity for HepG2 *in vivo*. CTX with 30mg kg^{-1} was the positive control group. CTX (Cyclophosphamide) is an alkylating agent, the most commonly used anticancer drug. But high dose of CTX could cause a lethal cardiotoxicity leading to fatal complications such as congestive heart failure, arrhythmias, cardiac tamponade and myocardial depression [27].

Table 6. Effect of samples on the transplant tumor model of Heps in mice

Group	Dosage (mg kg^{-1})	Body Weight (g)		Tumor Weitht (g)
		Before treatment	After treatment	
Control	0	18.40±0.70	27.20±2.82 [a]	1.52±0.31 [a]
CTX	30	18.20±0.42	18.90±1.60 [b]	0.36±0.21 [b]
C-8	250	18.30±0.48	26.20±1.40 [a]	1.10±0.33 [b]
C-8	100	18.40±0.70	25.90±1.91 [a]	1.53±0.27 [a]

Means with different letters within a row are significantly different (p <0.05)

As shown in Table 6, CTX treatment had great effect on both body weight and tumor growth, with the tumor weight decreasing by 76.3% and the loss of body weight by 30.5%. High-dose treatment (250 mg kg^{-1} BW/day) of the extract of the mycelia had significant effect on tumor growth with the reduction of tumor weight 27.6%. All of the mice in the mycelia extraction groups did not show any loss in body weight, suggesting that mycelia extraction did not affect the normal metabolism of the mice.

Although many studies reported that cordycepin is definitely the key compound responsible for the anti-tumor activity of *Cordyceps* products [28,29,30], only trace amount of cordycepin was found in the mycelia of *C. militaris* in our study, revealing that there are other compounds also contributing to the anti-tumor activity, maybe synergistically with cordycepin [31]. Actually, adenosine [32], polysaccharides [33] and Se, which exhibited higher contents in the mycelia, are also often associated with the pharmacological activity [34,35].

4. Conclusions

In this study, the composition and anti-tumor activity of the mycelia and fruiting bodies of *C. militaris* were investigated. Many bioactive compounds have been found in the fruiting body and mycelia of *Cordyceps* spp., including proteins, polysaccharides, amino acids, nucleosides, mannitol and trace minerals. Many of these bioactive components have multifunctional pharmacological effect *in vivo*, so many scientists have great interest in this special herbal material. But little scientific information is available to evaluate different *Cordyceps* forms from the same fungal origin by bioactive ingredients and composition systematically and comprehensively. The information obtained in this work was helpful and useful to the development of *C. militaries* cultivation process for efficient production of functional foods or drugs. In addition, further more studies on the mechanism of antitumor activity are needed.

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Statement of Competing Interests

The authors have no competing interests.

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