¹H-NMR-Based Metabolic Profiling of *Cordyceps militaris* to Correlate the Development Process and Anti-Cancer Effect^S

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The study of metabolomics in natural products using the diverse analytical instruments including GC-MS, LC-MS, and NMR is useful for the exploration of physiological and biological effects and the investigation of drug discovery and health functional foods. *Cordyceps militaris* has been very attractive to natural medicine as a traditional Chinese medicine, due to its various bioactive properties including anti-cancer and anti-oxidant effects. In this study, we analyzed the metabolite profile in 50% ethanol extracts of *C. militaris* fruit bodies from three development periods (growth period, matured period, and aging period) using ¹H-NMR, and identified 44 metabolites, which are classified as 16 amino acids, 10 organic acids, 5 carbohydrates, 3 nucleotide derivatives, and 10 other compounds. Among the three development periods of the *C. militaris* fruit body, the aging period showed significantly higher levels of metabolites including cordycepin, mannitol (cordycepic acid), and β-glucan. Interestingly, these bioactive metabolites are positively correlated with anti-tumor growth effect; the extract of the aging period showed significant inhibition of HepG2 hepatic cancer cell proliferation. These results showed that the aging period during the development of *C. militaris* fruit bodies was more highly enriched with bioactive metabolites that are associated with cancer cell growth inhibition.

Keywords: *Cordyceps militaris*, metabolomics, cordycepin, β-glucan, nuclear magnetic resonance, anti-cancer effect

Introduction

Medicinal mushrooms have long been used in Asian culture and recently received considerable attention through discovery of useful natural products with biological activities [1]. *Cordyceps militaris* is one of the entomopathogenic fungal species in the family Cordycipitaceae that belongs to the phylum Ascomycota [2, 3]. It has been widely used for centuries in folk tonics due to its medicinal value and its

various pharmacological properties have been reported [4]. *C. militaris* contains various biologically active compounds including amino acids, nucleosides, polysaccharides, and phenolic compounds [5]. Recently, it has been massively cultivated in liquid media for collecting mycelia and in solid media for fruit body production due to its biological activities such as anti-inflammatory [6], anti-oxidant [7], immune modulating [8, 9], and anti-cancer effects [10–12].

The effective biological activities of *C. militaris* have been

mainly attributed to cordycepin, adenosine, β -glucan, ergosterol, and mannitol (cordycepic acid) [5, 13]. Cordycepin is one of the representative bioactive compounds in *C. militaris* and its biological and pharmaceutical properties have been reported. Its anti-cancer effect induced apoptosis and cell cycle arrest through modulating Wnt [14], mTOR, and Erk1/2 signaling pathways [15, 16]. The structure of cordycepin, also termed as 3'-deoxyadenosine, is a nucleoside analogue and differs from its precursor adenosine by the absence of the hydroxyl group in the 3' position of its ribose [17]. The biosynthesis of cordycepin from adenosine has been recently uncovered with the processes of hydroxyl phosphorylation, dephosphorylation, and reduction from adenosine [18, 19]. Adenosine is an endogenous nucleoside that plays a pivotal role in cytoprotection for numerous instances such as hypoxia, ischemia, and seizure [20]. The most common forms of β -glucan in medicinal mushrooms including *C. militaris* are those comprising D-glucose units with β -1,3 glycosidic linkages. Polysaccharides including β -glucan are associated with anti-inflammatory, antioxidant, anti-cancer, anti-metastatic, immune modulating, hypoglycemic, and hypolipidemic effects [6].

Metabolomics is focused on the quantitative and qualitative high-throughput chemical profiling analysis of biological resources, which is merged and based on multivariate statistics data from nuclear magnetic resonance spectroscopy (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and transform infrared spectroscopy (FTIR) [21, 22]. For identifying metabolic profiling of

Cordyceps, Park *et al.* used ¹H-NMR analysis to characterize the metabolites of cultivated *C. bassiana* mycelia and fruiting bodies [23], and Hyun *et al.* [24] used GC-MS spectrometry to investigate the metabolic profiles of *C. bassiana* according to development stage. In addition, in our previous study, we analyzed the GC-MS spectrometry-based metabolic profiling with *C. militaris* fruit bodies, and identified significant change in the cordycepin content during the development periods of *C. militaris* [25].

The development periods of *C. militaris* fruit bodies can be classified into three periods: growth period, matured period, and aging period based on the maturity of ascospores and perithecia (Fig. 1). The growth period is characterized by the initiation of perithecia formation or the formation of irregular immature perithecia on the developed fruit body. In the matured period, perithecia are matured and filled with matured ascospores, and the filiform ascospores start to release from the perithecia. The aging period can be defined with fewer ascospores releasing from the perithecia and deformed fruit bodies with secondary branch formation.

In the present study, we performed metabolic profiling of *C. militaris* with three different development periods using ¹H-NMR spectroscopy. Especially, we focused on two representative bioactive compounds, such as cordycepin and β -glucan, and analyzed their contents in each sample from the development periods of *C. militaris* fruit bodies. Based on the metabolic profiling, we investigated the correlation between the three development periods of *C. militaris* fruit bodies and their anti-cancer effect, which suggests the possibility that metabolomic profiling can be

Fig. 1. Characteristics of the development periods of *C. militaris* fruit body.

(A) Growth period displays the developing perithecia on the fruit body at 6 weeks' incubation. (B) In the matured period at 8 weeks' incubation, the majority of ascospores inside the perithecia were matured on the fruit bodies. (C) In the aging period, after culturing for 10 weeks, fewer ascospores were observed inside the perithecia on the fruit bodies since most of the ascospores were released. Scale bar = 2 cm

applied as a biomarker in the development of natural medicine and functional food.

Materials and Methods

Solvents and Chemicals

We obtained cordycepin, dimethyl sulfoxide (DMSO), methanol- d_4 , phosphate buffer (KH_2PO_4), 3-(trimethylsilyl)-propionic-2,2,3,3- d_4 acid sodium salt (TSP), deuterium oxide (D_2O) 99.8%, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich Inc. (USA). NMR tube was purchased from Optima Inc. (Itabashi, Japan), and methanol and HPLC-grade water were obtained from Honeywell Burdick and Jackson Inc. (USA). RPMI-1640 medium and Fetal Bovine Serum (FBS) were obtained from GIBCO Inc. (USA).

Fruit Body Production of *C. militaris* and Sample Preparation for ^1H -NMR Spectroscopy

C. militaris strain was obtained from Korean Agricultural Culture Collection (Accession No. KACC 43316). This *C. militaris* strain was used for the fruit body production using a solid medium of brown rice in a 1,000-ml polypropylene bottle and incubating it at 25°C under 1,000 lux of continuous white fluorescent light and 90% humidity [26]. To conduct ^1H -NMR metabolic profiling based on the fruit body development, we sampled fruit bodies with brown rice medium in 100 bottles according to the development periods (e.g., growth period, matured period, and aging period in Fig. 1). We observed the developing perithecia on the fruit body at incubating for 6 weeks and sampled them for growth period. With incubating for 8 weeks, the majority of ascospores inside the perithecia were matured and these fruit bodies were sampled for matured period. At 10 weeks' incubation, we observed fewer ascospores inside the perithecia on the fruit bodies since most of ascospores were released. Sampling of aging period was made with these fruit bodies which are deformed with the secondary branch formation (Fig. 1). In sample preparation for ^1H -NMR spectroscopy, each sample was immediately freeze-dried for 24 h and 1 kg of dried sample was extracted with 50% aqueous ethanol using microwave digestion at 65°C (Transform 800, AR0800-MW-1800, Aurora Instruments Ltd., Vancouver, B.C., Canada). The 50% ethanol extract was freeze-dried and pulverized for ^1H -NMR spectroscopy. Each powdered sample was stored at -70°C using a deep-freezer (Daihan, Korea) before analysis for ^1H NMR spectroscopy, HPLC, β -glucan assay, and MTT assays.

^1H -NMR Metabolomic Profiling

To extract intracellular metabolites for ^1H -NMR spectroscopy, 100 mg of each powdered sample of *C. militaris* was dissolved in 750 μl methanol- d_4 and 750 μl phosphate buffer (90 mM, pH 7.0–7.4) in D_2O with 0.01% TSP as an internal standard using ultrasonication for 50 min (Lab Companion, Korea). The supernatants were collected by centrifuging at 12,000 $\times g$ for 20 min at room

temperature (Labogene, Korea) and filtered using Amicon Ultra 0.5 ml centrifugal filters (Millipore, Germany). Seven hundred microliters of each filtered sample was loaded into 5 mm NMR tubes ($n = 3$). ^1H -NMR spectra were obtained at 300 K on a 600.13 MHz Bruker Advance spectrometer (Germany) using the standard parameter set (ZGPR) pulse sequence with pre-saturation for water suppression. In total, 128 transients were gathered into 32 K data points with a relaxation delay of 2 s with an acquisition time per scan of 1.70 s and a spectra width of 10.0 ppm. The NMR spectra were analyzed using Chenomx NMR Suite software version 8.2 (Canada) and the peaks associated with cordycepin were assigned based on the previously reported ^1H -NMR data [27].

Data Preprocessing and Multivariate Statistical Analysis of ^1H -NMR Data

^1H -NMR data were processed and the peak assignment was conducted using MestReNOVA 6 version 6.0.4 (Mestrelab Research SL, Spain) and Chenomx NMR Suite software (version 8.2, Chenomx Inc.). Multivariate statistical analyses were performed by one-way ANOVA followed by a Tukey's significant difference test using PASW Statistics 22 software (IBM, USA). Significance was determined with a p -value threshold ($p < 0.05$). Metabolite levels were normalized using log2 function. Mean centering and UV scaling were applied for all principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) using SIMCA software (version 15.0, Umetrics, Sweden).

Quantitative Analysis of Cordycepin and β -Glucan

To quantify cordycepin in each of the developmental periods, 10 mg of cordycepin standard was dissolved in DMSO to prepare stock solution. It was serially diluted with DMSO and the concentration of the diluted solutions was determined using AZURA HPLC with UV system at 260 nm (KNAUER, Germany). For HPLC analysis, 10 mg of 50% ethanol extract of *C. militaris* was suspended in 0.5 ml methanol and 0.5 ml HPLC-grade water and sonicated for 50 min at 40 kHz, 300 W at 40°C (Lab Companion). The supernatant was filtered by 0.2 μl PTFE syringe filter (Whatman, UK). The quantity of cordycepin in *C. militaris* was determined by AZURA HPLC fitted with a Phenomenex Gemini C18 Column (110 Å, 250 $\times$ 4.60 mm, 5 μm). The mobile phase A of HPLC-grade water and B of HPLC-grade methanol (85:15 v/v) were used with an isocratic flow rate of 0.8 ml/min. The UV detection was performed at 260 nm. The quantification of β -glucan was proceeded with according to the manufacturer's protocol using the Mushroom and Yeast β -glucan Assay Procedure K-YBGL 09/2009 Kit (Megazyme Int., Ireland).

MTT Cell Proliferation Assay

HepG2 is a hepatocellular carcinoma cell line and was purchased from American Type Culture Collection (ATCC, USA). HepG2 cells were cultured in DMEM medium supplemented with 10% FBS. The cells were incubated at 37°C in 5% CO_2 (SCI-165D,

Fig. 2. Regions of the 600 MHz ¹H-NMR spectra identification of *C. militaris* fruit body.

C. militaris metabolites list was described in Table 1. The chemical shift of 44 metabolites was indicated on the NMR spectrum.

Water-Jacket System, Astec Co. Ltd., Japan). Before the treatment of *C. militaris* extracts, HepG2 cell was seeded in 96-well microtiter plates (1×10^4 cells/well) for starvation condition in DMEM/1% FBS. *C. militaris* extracts were treated at the concentrations of 0, 5, 10, 20, 50, and 100 μ g/ml for 24 h. After incubation, 50 μ l of MTT solution was added to each well (3 mg/ml in phosphate-buffered saline, PBS) and the plate was incubated for 2 h at 37°C. After incubation, absorbance was measured at a wavelength of 570 nm using an ELISA reader (Molecular Devices LLC., USA).

Results and Discussion

¹H-NMR Metabolic Profiling and Multivariate Data Analysis of Development Periods of *C. militaris*

Fig. 2 and Table 1 showed that 44 metabolites were identified in 50% ethanol extracts from different development periods (*e.g.*, growth period, matured period, and aging period) using ¹H-NMR spectra, which were annotated based on chemical shifts (δ), peak multiplicity, and *J* value (Hz). The relative concentrations of detected metabolites were obtained by integrating isolated NMR signals relative to the known concentration of the internal standard. The metabolites detected in the process of differentiating the development periods of *C. militaris* fruit bodies include 18 amino acids and derivatives, 14 organic acids, 7 saccharides and alcohols, 3 nucleosides, 1 vitamin derivative, and 1 alkaloid (Table 1). To compare each of the three development periods of *C. militaris*, we conducted ANOVA statistical analyses with Tukey's post-hoc test ($p < 0.05$) based on the

relative concentrations of the metabolites of each period (Table 1). The levels of leucine, lysine, putrescine, acetoacetate, tyrosine, glycerol, xylose, and guanidoacetate were significantly higher ($p < 0.05$) in the growth period, while the levels of valine, lactate, glutamate, malonate, trimethylamine *N*-oxide (TMAO), choline, and phenylacetate were dominant in the matured period. In the aging period, the levels of ethanol, threonine, proline, pyruvate, succinate, 2-oxoglutarate, glutamine, methylamine, aspartate, asparagine, glucose, betaine, glycine, xylitol, mannitol (cordycepic acid), trehalose, trigonelline, cordycepin, maleate, adenosine, fumarate, phenylalanine, and xanthine were higher compared to other periods. Based on both PCA and PLS-DA, to compare the three development periods and characterize the relevant metabolites to distinguish each period of *C. militaris*, the samples of each period from 50% ethanol extracts of *C. militaris* were clearly separated in both PCA (Fig. 3A) and PLS-DA (Fig. 3B) score plots. From the PLS-DA loading plot (Fig. S1), the intense signals of glycerol, lysine, leucine, guanidoacetate, putrescine, acetoacetate, tyrosine, and xylose indicated the presence of high relative concentrations of these metabolites in the growth period. The matured period was characterized by intense signals of citrate, phenylacetate, malonate, glutamate, lactate, valine, choline, and TMAO. In the case of the aging period, arginine, aspartate, 4-hydroxy phenylacetate, ethanol, asparagine, alanine, mannitol, succinate, xylitol, 2-oxoglutarate, fumarate, trehalose, threonine, phenylalanine, pyruvate, formate,

Table 1. Metabolite assignments and chemical shifts of *C. militaris*.

No.	Metabolite compound	Chemical shift (ppm)
1	Leucine	0.95 (t, $J = 6.5$ Hz), 1.69 (m), 3.76 (m)
2	Valine	0.98 (d, $J = 6.87$ Hz), 1.05 (d, $J = 6.0$ Hz)
3	Ethanol	1.22 (t, $J = 7.07$ Hz), 3.62 (dd, $J_1 = 9.88$ Hz, $J_2 = 3.80$ Hz)
4	Lactate	1.3 (d, $J = 6.91$ Hz)
5	Threonine	1.34 (d, $J = 6.58$ Hz), 4.22 (m)
6	Lysine	1.42 (m), 1.74 (m), 3.00 (t, $J = 7.5$ Hz)
7	Alanine	1.46 (d, $J = 8.31$ Hz)
8	Putrescine	1.78 (m)
9	Arginine	1.94 (m), 3.24 (t, $J = 6.93$ Hz), 3.76 (t, $J = 6.11$ Hz)
10	Glutamate	2.02 (m), 2.14 (m), 2.38 (m)
11	Proline	2.02 (m), 2.06 (m), 4.06 (dd, $J_1 = 8.63$ Hz, $J_2 = 6.42$ Hz)
12	Methionine	2.14 (s), 2.62 (t, $J = 7.58$ Hz)
13	Acetoacetate	2.3 (s), 3.42 (s)
14	Pyruvate	2.34 (s)
15	Succinate	2.38 (s)
16	2-Oxoglutarate	2.43 (t, $J = 6.95$ Hz), 2.98 (t, $J = 6.84$ Hz)
17	Glutamine	2.46 (m)
18	Citrate	2.5 (d, $J = 15.92$ Hz), 2.7 (d, $J = 15.59$ Hz)
19	Methylamine	2.58 (s)
20	Aspartate	2.64 (m), 2.84 (m)
21	Asparagine	2.94 (m), 3.98 (m)
22	Tyrosine	3.02 (dd, $J_1 = 4.76$ Hz, $J_2 = 12.48$ Hz), 7.18 (d, $J = 8.41$ Hz)
23	Malonate	3.1 (s)
24	Glucose	3.17 (d, $J = 5.57$ Hz), 3.19 (d, $J = 6.50$ Hz), 3.38 (m), 3.46 (m), 3.82 (m), 5.2 (d, $J = 4.07$ Hz)
25	TMAO	3.22 (s)
26	Choline	3.22 (s), 4.02 (m)
27	Betaine	3.26 (s), 3.9 (s)
28	Glycine	3.54 (s)
29	Glycerol	3.54 (m), 3.62 (m)
30	Phenylacetate	3.54 (s), 7.64 (m), 7.38 (m)
31	Xylitol	3.62 (m), 3.72 (m)
32	Xylose	3.66 (m), 3.94 (m), 5.18 (d, $J = 3.66$ Hz)
33	Mannitol	3.66 (dd, $J_1 = 11.76$ Hz, $J_2 = 6.2$ Hz), 3.84 (dd, $J_1 = 11.87$ Hz, $J_2 = 2.8$ Hz)
34	Trehalose	3.85 (m), 5.18 (d, $J = 3.8$ Hz)
35	Guanidoacetate	3.78 (s)
36	Trigonelline	4.46 (s), 8.1 (m), 8.86 (m), 9.14 (s)
37	Cordycepin	6.06 (d, $J = 2.50$ Hz), 8.27 (s), 8.41 (s)
38	Maleate	6.02 (s)
39	Adenosine	6.06 (d, $J = 2.50$ Hz), 8.27 (s), 8.41 (s)
40	Fumarate	6.5 (s)
41	4-Hydroxyphenylacetate	6.86 (d, $J = 8.6$ Hz), 7.18 (d, $J = 8.65$ Hz)
42	Phenylalanine	7.32 (m), 7.36 (m), 7.42 (m)
43	Xanthine	7.94 (s)
44	Formate	8.44 (s)

Fig. 3. PCA and PLS-DA plots of multivariate statistical analyses to differentiate the three development periods of *C. militaris* fruit body.

(A) PCA score plot based on two principal components (PC1 64.6% and PC2 24.1%). (B) PLS-DA score plot based on two PLS components: PLS1 component 1 64.6% and PLS2 component 2 24.1% ($R^2Y = 0.995$, $Q^2Y = 0.990$, R^2Y intercept = 0.279, Q^2Y intercept = -0.314).

glucose, cordycepin, betaine, glycine, glutamine, adenosine, maleate, proline, trigonelline, methylamine, methionine, and xanthine were found to be higher than in the other development periods.

Quantification of Cordycepin and β -Glucan in *C. militaris* Extracts

We have previously reported that cordycepin was enriched in the senescence process of *C. militaris* fruit body using GC-MS spectrometry by comparing growth and aging periods, and we suggested that the aging period could be optimal for medical use [25]. Cordycepin has various biological activities, including anti-oxidant, anti-tumor, anti-fungal, anti-bacterial, anti-viral, anti-leukemia, and immunoenhancement effects, and it is the most important bioactive compound of *C. militaris* [17]. In ¹H-NMR spectroscopy to compare the three development periods (e.g., growth, mature, and aging periods), our results showed that cordycepin is predominant in the aging period, which is consistent with the results of HPLC quantification of the three development periods (Table 2). The total amount of cordycepin in the *C. militaris* was 13.356 mg/g at growth period and 29.014 mg/g at mature period. In contrast, the amount of cordycepin at aging period was 39.674 mg/g, which is much higher than those of the growth and mature periods. The cordycepin amount in the aging period was approximately three times higher than that of the growth period.

In addition to cordycepin, we conducted β -glucan assay because it is one of the most potent mushroom-derived substances and is also known to exhibit favorable biological

Table 2. Cordycepin content in *C. militaris* extraction.

Samples	Cordycepin content, mg/g
Growth period	13.356 $\pm$ 0.541
Mature period	29.014 $\pm$ 3.214
Aging period	39.674 $\pm$ 4.057

Values (means $\pm$ standard deviation, SD; $n = 3$).

properties including anti-cancer and immunoenhancement effects [28]. The results of the total glucan and β -glucan contents are presented in Table 3. The total glucan content in the growth, mature, and aging periods of *C. militaris* was 66.91 $\pm$ 5.06%, 62.70 $\pm$ 4.25%, and 70.53 $\pm$ 3.81% (w/v), respectively, while the corresponding β -glucan content was 43.41 $\pm$ 2.44%, 39.84 $\pm$ 2.17%, and 47.86 $\pm$ 2.42% (w/v), respectively. The β -glucan content increased significantly in the aging period and it is approximately 104.7% higher than that in the growth period. Cho *et al.* [29] analyzed the β -glucan content of mature fruiting bodies of *Ganoderma spp.*, *C. militaris*, and *Phellinus linteus*, and found that the β -glucan content of *C. militaris* was 25% (w/w) higher than that of *Ganoderma spp.* and *P. linteus*. These results indicated that fruit bodies from the aging period of *C. militaris* contain

Table 3. β -glucan content in *C. militaris* extraction.

Samples	Glucan content (dry weight basis) % (w/w)	
	Total-glucan	β -glucan
Growth period	66.91 $\pm$ 5.0	43.41 $\pm$ 2.44
Mature period	62.70 $\pm$ 4.2	39.84 $\pm$ 2.17
Aging period	70.53 $\pm$ 3.81	47.86 $\pm$ 2.42

Values (means $\pm$ standard deviation, SD; $n = 3$).

much more β -glucan than other medicinal mushrooms such as *Ganoderma spp* and *P. linteus*.

Metabolic Changes and Cancer Cell Growth Inhibitory Activity of Development Periods of *C. militaris* Fruiting Bodies

To understand the biochemical pathway of *C. militaris* focusing on cordycepin and β -glucan synthesis, we mapped 44 detected metabolites on its relevant pathway in Fig. 4. As with cordycepin, the glucose level was higher in the aging period, which can be used as a carbon source and a precursor for cordycepin and adenosine. In addition, alanine and aspartate were reported to be related with cordycepin content [30], which is also consistent with our results of the enrichment of alanine and aspartate in the aging period. In addition to cordycepin, a total of 21 metabolites, including adenosine, asparagine xanthine, glucose, ethanol, xylitol, mannitol, trehalose, threonine, proline, glutamine, betaine, glycine, phenylalanine, pyruvate, succinate, 2-oxoglutarate, methylamine, maleate, fumarate, and trigonelline were enriched in the aging period. Especially, saccharides and alcohol metabolites (*e.g.*, glucose, trehalose, ethanol, xylitol, and mannitol) accumulated in the aging period rather than in the growth and mature periods, which may be associated with the accumulation of β -glucan in the aging period [31, 32].

Similar to our research, Park *et al.*, performed ¹H-NMR spectroscopy to identify the metabolic profiling of the development period of *C. bassiana* [21]. In this study, the mature period of *C. bassiana* fruit body shows predominant metabolites including phenylalanine, tyrosine, tryptophan, valine, leucine, isoleucine, alanine, lysine, proline, glutamine, arginine, glycine, threonine, 2-hydroxyisovaleric acid, lactic acid, acetic acid, adenosine, uridine, and glucose, and more effective anti-oxidant activity compared other periods. *C. militaris* extracts show significant anti-tumor and anti-metastatic activities [33–35], and cordycepin and β-glucan are correlated with inducing apoptosis of liver cancer cells and protecting from hepatic damage [16, 36, 37]. To correlate the different metabolic enrichments in the three development periods of *C. militaris* fruit body and the growth inhibitory effects on human liver cancer HepG2 cells, we conducted an MTT assay. HepG2 cells were treated with the ethanol extracts obtained from three development periods of *C. militaris* at various concentrations (0, 5, 10, 20, 50, and 100 µg/ml) for 24 h. In Fig. 5, the extract from the aging period shows dramatically inhibited growth of HepG2 cells in a dose-dependent manner ($p < 0.05$). The cell viability decreased significantly to 68.99%, 48.77%,

Fig. 4. Schematic diagram of the metabolic pathway and relative levels of the compounds in *C. militaris* of different three development periods.

This diagram was modified from pathways in (KEGG) database. ANOVA was performed to assess the statistical significance of differences between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns; no significance). Results are presented as means $\pm$ standard deviation (SD). Red squares indicate the metabolites which were significantly different in their metabolites in aging period.

46.11%, 46.67%, and 41.08% for the concentrations of 5, 10, 20, 50, and 100 µg/ml, respectively. The enrichment of bioactive materials including cordycepin and β-glucan in ethanol extract of the aging period is closely related with the inhibitory activity on cancer cell proliferation [35, 38–40]. Therefore, we confirmed that the aging period of *C. militaris* could be optimally utilized in the medical and healthcare industry.

In the present study, we analyzed metabolic profiling and showed the anti-cancer effect of *C. militaris*, which is a successfully cultivated species and has high content of

Fig. 5. Growth inhibition of human hepatic carcinoma HepG2 cells by ethanol extraction of *C. militaris*. HepG2 cells were treated with various concentration (0, 5, 10, 20, 50, and 100 µg/ml) of the *C. militaris* extraction according to each of the development periods.

Values are expressed as means $\pm$ standard error (SE) of three experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control.

cordycepin, cordycepic acid, and other important bioactive compounds. The metabolite profile of *C. militaris* in the three development periods (e.g., growth, mature, and aging periods) were investigated using ¹H-NMR spectroscopy. We have identified 44 metabolites from 50% ethanol extracts of *C. militaris*, which were composed of 18 amino acids and their derivatives, 14 organic acids, 7 saccharides and alcohols, 3 nucleosides, 1 vitamin derivative, and 1 alkaloid. The metabolites were clearly separated according to each of the development periods in PCA and PLS-DA analysis. The aging period of *C. militaris* showed the higher levels of cordycepin, β -glucan, mannitol, xylitol, alanine, aspartate, and fumarate, and the ethanol extract of the aging period demonstrated the most efficacy in the anti-cancer activity on liver cells. Therefore, we suggest that the aging period of *C. militaris* is the optimal harvest time of fruit body in the cultivation for use in food and medical applications in the future.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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