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Quantitative analysis of cordycepin in *Cordyceps militaris* under different extraction methods

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Abstract *Cordyceps militaris* (CM) is one of the most important medicinal mushrooms known to possess various biological activities. Cordycepin (CP) is a bioactive compound present in the fruiting bodies of CM and is known to have anti-tumor, anti-metastatic immunomodulatory and anti-inflammatory activities. In this study, we aim to analyze CP quantitatively under various CM extraction conditions. CP was measured using high-performance liquid chromatography, quantified using a reversed phase column using a gradient elution system of water and acetonitrile, and detected with a UV absorbance wavelength of 260 nm. The CP content of CM was the highest in 100% ethanol extract of the fruiting bodies and 60% ethanol extract of the mycelium. This study provides an efficient analysis method to determine the optimal extraction conditions for CP that can be used as a basis for developing functional foods and pharmaceutical products derived from CM.

Keywords Cordycepin · *Cordyceps militaris* · Extraction method · High-performance liquid chromatography · Quantitative analysis

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Introduction

Medicinal fungi have long been valued in traditional Chinese medicine as they are considered to be an abundant source of natural products. *Cordyceps* species are the most important medicinal mushrooms known to possess various biological activities [1]. One of the most utilized of these species is *Cordyceps militaris* (CM), an entomopathogenic fungus belonging to the Ascomycetes class. It is widely distributed in North and South America, Europe and Asia, ranging from sub-tropical to temperate regions of the world [2,3]. Previous reports have demonstrated that CM extract exhibits many pharmacological activities, which include inhibiting the proliferation of human glomerular mesangial cells, improving insulin secretion and insulin resistance, and inhibiting the growth of U937 leukemia cells [4-6]. Other studies have shown its anti-angiogenic, anti-fibrotic, anti-inflammatory, anti-oxidant, and anti-viral effects [7-11]. Another *Cordyceps* species is *C. sinensis*, a parasitic fungus on the larvae of Lepidoptera. *C. sinensis* is highly valued as a health food because of its various biological activities. It has long been used as a very effective and nutritious traditional medicine in Chinese society. In our previous study, we reported a wild form of *Cordyceps* fungi, *Paecilomyces japonica*, which grows on silkworm larvae. Its fruiting bodies have been shown to possess anti-tumor and immunostimulating activities [12].

Various classes of compounds have already been isolated from CM, including active substances such as cordycepin (CP), adenosine, polysaccharides, ergosterol, mannitol, amino acids, and fatty acids [13,14]. CP was first reported as a metabolite isolated from CM culture broth and is present in its fruiting bodies and also considered to be its major active compound in CM [15]. It is a nucleoside analog (3′-deoxyadenosine) of adenosine where 3'-OH is replaced by 3'-H [16]. The nucleoside adenosine is used to make ribosomes in the nucleolus. Nucleolus is a highly organized structure within a cell which produces ribosomes as its primary

function. It can easily be interrupted by nucleic acids or agents that interfere with protein synthesis. CP is a unique substance that can be incorporated into ribosomal RNA production by the nucleolus action therefore preventing normal processing and interfering with the synthesis of molecules [17]. It also acts as an effective agent in preventing cancer metastasis [18].

CP can also be found in *C. sinensis* and *C. kyushuensis* [19,20]. The importance of CP is attributed to its many biological activities, including anti-tumor, immunomodulatory and anti-inflammatory activities [21-23]. It is also considered an "antibiotic of nucleic acids" that might prevent healthy cells from developing into cancer cells due to the fact that it is a nucleoside analog. A recent study suggested that CP may play a role in inhibiting airway remodeling in asthma and could be used as a novel approach in treating allergy-induced asthma [24,25]. Furthermore, CP was shown to be a potential therapeutic agent in treating neuronal disorders, such as Alzheimer's disease and diabetic nephropathy [26,27].

Research on novel constituents of the medicinal fungus CM with regards to their efficacy, proper standardization, and content analysis are required to promote their availability in the medicinal field. Previous studies have already reported the determination of CP using high-performance liquid chromatography (HPLC) [28,29]. HPLC is the commonly used analytical technique for monitoring chemical products.

In this study, we conducted a quantitative HPLC analysis of the CP obtained from CM by applying different extraction conditions to assess which method gives the greatest yield of CP. This study aimed to determine the best extraction method to help revolutionize fungus-based pharmaceutical products.

Materials and Methods

Materials

The CM sample was obtained from NI & Pharm Inc., Republic of Korea.

Instruments and reagents

Chromatographic analysis was performed using an HPLC system equipped with a pump (Waters 1525 Binary HPLC Pump, Milford, MA, USA), an auto-sampler, and a UV detector (Waters 2489 Binary HPLC Detector). CP was obtained from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea. HPLC-grade solvents of water, acetonitrile, and methanol (MeOH) were purchased from J. T. Baker (Philipsburg, NJ, USA). Ethanol (EtOH) was purchased from Samcheon Chemical (Pyeongtaek, Korea).

Sample extraction and preparation

CM samples were prepared by weighing 2 g each of fruiting bodies and mycelium. The extraction solvents used were distilled water (DW), and EtOH with different ratios of DW (20% EtOH, 40% EtOH, and 60% EtOH) and pure EtOH. The extractions were conducted at 70 °C for 5 h using the ultrasonic extraction method. To compare the extractions more accurately, a commercial CM was also subjected to extraction in the same manner as described above. For quantitative evaluation of the CM extract, it was dissolved in MeOH and filtered using a syringe filter (0.45 μm). Each sample was dissolved in MeOH at 20 mg/mL.

HPLC conditions

Quantitative analysis of CP was performed using a reversed-phase HPLC system. Chromatographic separation was performed using a YMC Pack Pro C18 column (25 cm×4.6 mm, 5 μm). The analysis was conducted with a gradient elution system using a mobile phase composed of 0.5% acetic acid in water (A) and acetonitrile (B). The elution system was as follows: 100% A at 0 min, 0% A at 15 min, 0% A at 20 min, 100% A at 21 min, and 100% A at 25 min. The temperature of the column was maintained at 30 °C, and the flow rate was set at 1.1 mL/min. The injection volume was 10 μL, and the detector wavelength was set at 260 nm.

Calibration curve

A standard stock solution of CP was prepared by dissolving the compound in MeOH (1 mg/mL). The working solutions used to construct the calibration curve were prepared by serially diluting the stock solutions to the desired concentrations. The contents of the analytes were determined from the corresponding calibration curves. The calibration function of CP was calculated using peak area (Y) and concentration (X, mg/10 mL), and mean values ± standard deviation (n =4) are presented.

Results and Discussion

Quantitative analyses of CP in the mycelium and fruiting bodies of CM under five extraction conditions were performed using HPLC with a reversed-phase column. The HPLC method produced good separation, and the use of UV absorbance at 260 nm was effective for detection. The calibration curve was created by plotting the peak areas of the prepared concentrations and was analyzed using linear regression. The correlation coefficient r^2 of the standard was 0.9991. The calibration curve data of the standard are shown in Table 1. The structure of CP is shown in Fig. 1. The retention time was 7.7 min (Table 1), which is considered excellent. The HPLC chromatograms of CP and the extracts are shown in Fig. 2. Table 2 shows the extraction yield and CP content from the fruiting bodies and mycelia according to the five extraction methods.

Extracting the fruiting bodies with 20% EtOH gave the highest extraction yield, while EtOH gave the lowest yield. Extracting the mycelium with DW gave the highest extraction yield, while 60%

Table 1 Calibration curve of CP

Compound	t_R	Calibration equation[a]	Coefficient of determination, r^2 [b]
CP	7.717	Y = 11766X- 22368	0.9991

[a] Y = peak area, X = concentration of CP (mg/mL)
[b] r^2 = correlation coefficient based on five data points in the calibration curve

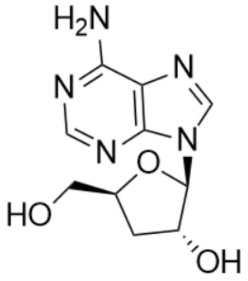

Fig. 1 Chemical structure of CP from CM

EtOH gave the lowest yield. However, as shown in Table 3, the CP content from the fruiting bodies was the highest in the EtOH extract. From the mycelium, the CP content was the highest in the 60% EtOH extract. To assess our results, fruiting bodies obtained from a commercial source were extracted and quantitatively analyzed. The results revealed that although the DW extract gave the highest extraction yield, the CP content was the highest in the EtOH extract (Table 2). It can be observed that the extraction yield is higher when EtOH/DW was used as solvent. The effect of EtOH concentration influences the extraction yield of CM. Initially, the yield increases at 20% EtOH but gradually decreases when the EtOH concentration is increased. However, as the EtOH increases, the CP content is also increased with 100% EtOH having the highest content of CP content.

CP in CM has been quantified successfully using our optimized HPLC-UV method. Different extraction conditions were utilized to identify the optimal conditions in maximizing the yield. The results showed that 20% EtOH gave the highest extraction yield from CM fruiting bodies, while DW gave the highest extraction yield from the mycelium. CP was the best extracted in DW using an ultrasonic extraction method.

Ni et al. reported that extracting fruiting bodies under reflux using DW as the extraction solvent produced the best extraction yield [30]. Additionally, Wang et al. reported that extraction of fruiting bodies using ultrasonication and 50% EtOH as the extraction solvent was the most efficient in extraction CP [31]. In our study, highest CP was obtained by extracting the fruiting bodies with 100% EtOH, meanwhile CP was highest when extracted with 60% EtOH in the mycelium. Large solvent volume dissolves target compounds more effectively, and results in an enhancement of extraction yield. This explains why CP content was higher when EtOH to DW ratio was increased. Our results supported previous findings that using EtOH and DW as solvent is the best when extracting CP [32]. Optimized extraction of CM can be achieved by considering the best extraction solvent and extraction method. Lee reported a yield of 3.28 μg/mL from CM grown in medium for 8 weeks that was ultrasonically extracted with 80% EtOH for 40 min. In terms of HPLC analysis, we used a different gradient elution system compared to previously reported studies. Here, we utilized 0.5% acetic acid in water and

Table 2 Extraction yield of CM under different extraction conditions

Sample	Extraction solvent	Yield (%)
Fruiting body	DW	13.0
	20% EtOH	13.5
	40% EtOH	9.6
	60% EtOH	11.5
	EtOH	7.7
Mycelium	DW	7.2
	20% EtOH	5.2
	40% EtOH	5.3
	60% EtOH	4.4
	EtOH	4.7
Commercial fruiting body	DW	15.2
	20% EtOH	15.7
	40% EtOH	12.3
	60% EtOH	11.0
	EtOH	8.2

Table 3 Content of CP in CM extracts under different extraction conditions

Sample	Extraction solvent	Content (mg/g ext.)
Fruiting body	DW	7.792±0.107
	20% EtOH	9.484±0.906
	40% EtOH	10.253±0.106
	60% EtOH	11.519±0.249
	EtOH	15.206±0.120
Mycelium	DW	2.717±0.025
	20% EtOH	3.093±0.134
	40% EtOH	4.150±0.149
	60% EtOH	4.208±0.066
	EtOH	3.961±0.161
Commercial fruiting body	DW	4.988±0.015
	20% EtOH	3.776±0.008
	40% EtOH	6.128±0.071
	60% EtOH	7.448±0.136
	EtOH	8.656±0.022

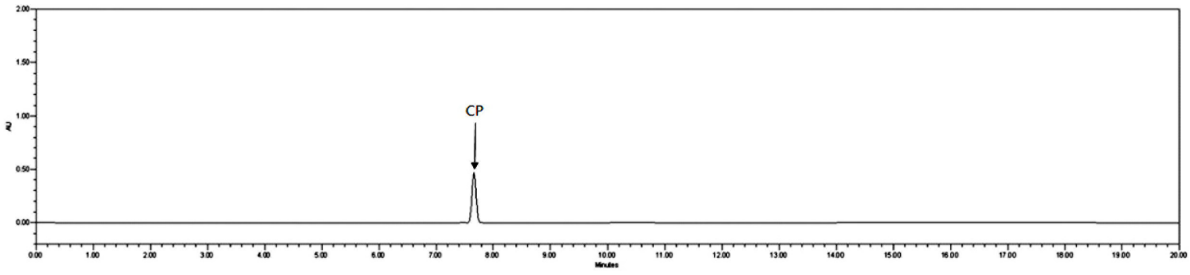

Fig. 2 HPLC chromatogram of CP

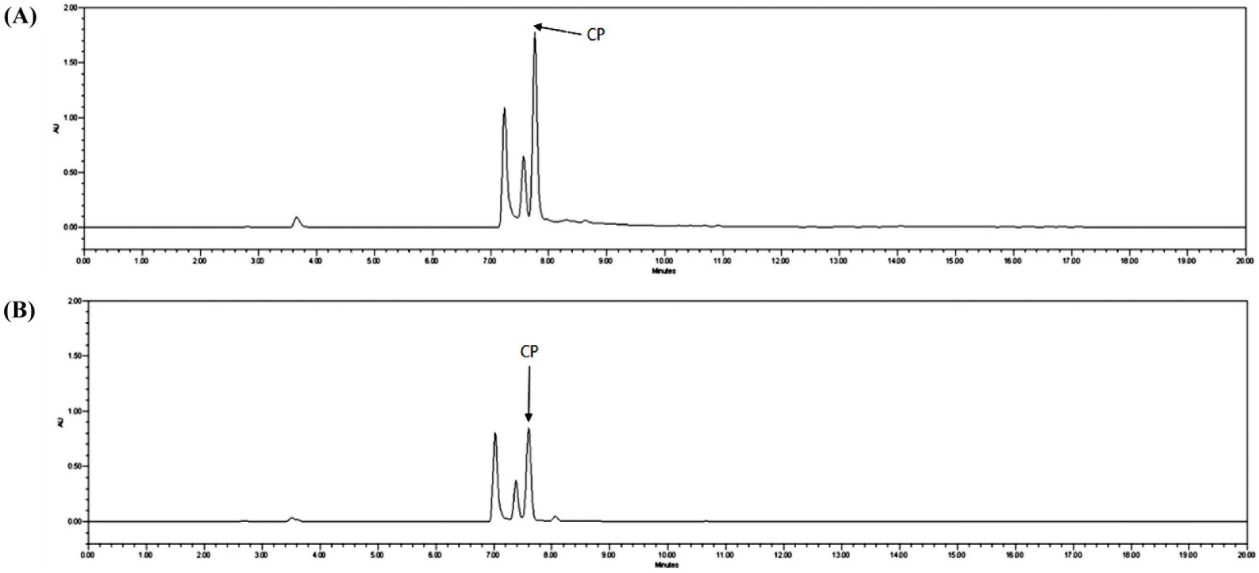

Fig. 3 HPLC chromatograms of EtOH (A) and DW (B) extracts of fruiting bodies

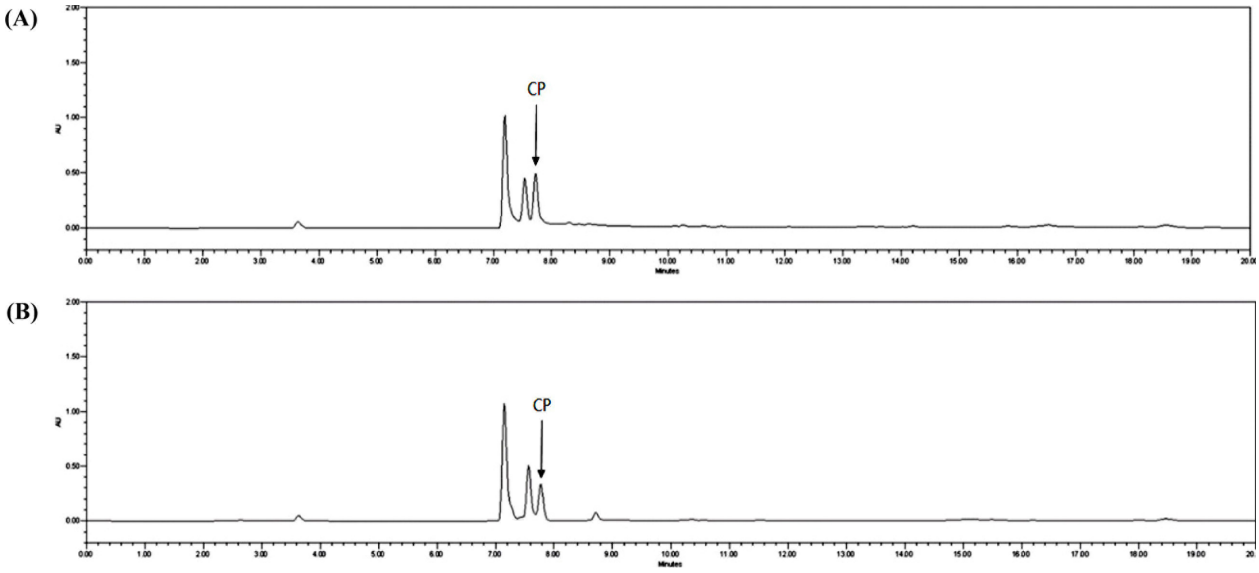

Fig. 4 HPLC chromatograms of EtOH (A) and DW (B) extracts of mycelium

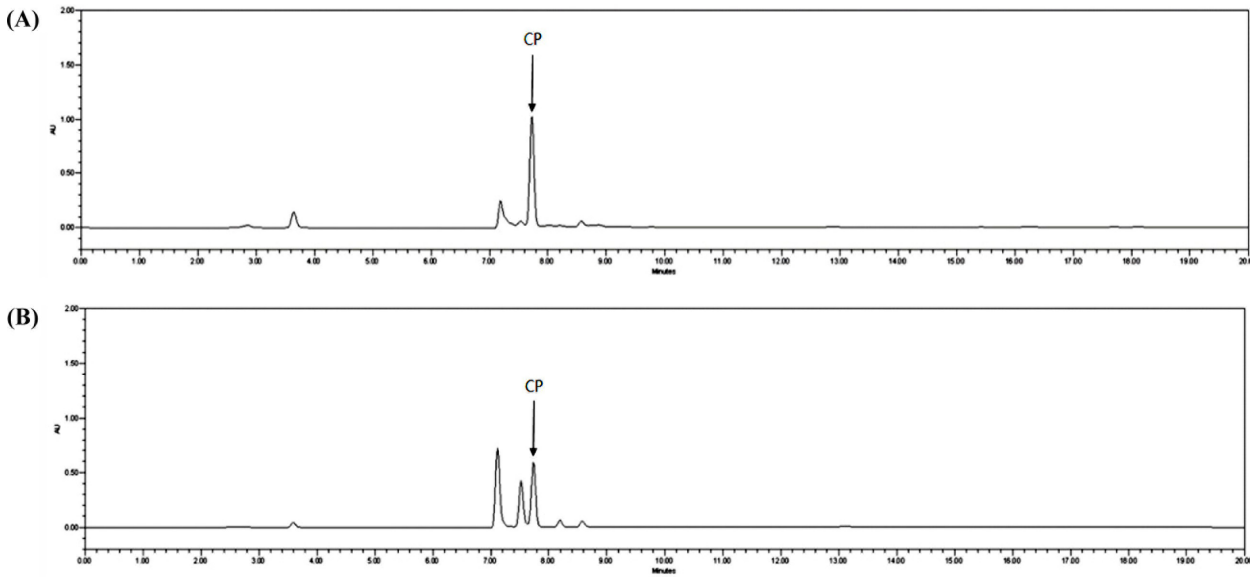

Fig. 5 HPLC chromatograms of EtOH (A) and DW (B) extracts of commercial fruiting bodies

acetonitrile while Ni et al. and Wang et al. reported the use of MeOH and DW. CP was eluted faster using our method with clear resolution of the peak of interest. Additionally, Our experimental results showed better extraction efficiency than that of Lee [33]. The difference in these results could be attributed to the difference in extraction times.

The results of our study could help establish a method in obtaining a higher quantity of CP than is currently attainable from CM extract. This study provides an efficient analysis method to determine the optimal extraction conditions for CP that can be used as a basis for developing functional foods and pharmaceutical products derived from CM. This would be useful in providing information regarding the best extraction method for CM extracts and in revolutionizing fungus-based pharmaceutical products.

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