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Analysis of Anti-adipogenic Constituents of *Cordyceps militaris* Using High Performance Liquid Chromatography-Diode Array Detection in Different Samples: Comparison with Anti-adipogenic Activity

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Abstract – We previously isolated cordycepin, guanosine and tryptophan from *Cordyceps militaris* as anti-adipogenic constituents. For the quality control of *C. militaris* for anti-adipogenic activity, simultaneous analytical method using high-performance liquid chromatography (HPLC)-diode array detection (DAD) was developed and validated. Quantitation of these compounds in various *Cordyceps* samples from different sources and various extraction methods were conducted using developed method. Our study shows that natural *Cordyceps* and host insect possess higher content than cultured ones and fruiting bodies, respectively. The content of cordycepin showed great difference in different *C. militaris* samples whereas trytophan content was similar in tested samples. Addition of water to extraction solvent greatly increased the yield of guanosine and tryptophan. High temperature and longer extraction time increased yield of guanosine, whereas the content of trytophan was decreased in high temperature during extraction with water. Extraction using ultrasonic apparatus slightly increased extraction efficiency. Cordycepin, however, has little variation in different extraction method tested. Strong anti-adipogenic activity was observed in the samples that contain all the three constituents. Taken together, quantitation of these compounds using developed analytical method might provide basic requirement for the anti-adipogenic activity of *C. militaris*.

Keywords – *Cordyceps militaris*, Quantitation, HPLC-DAD, Cordycepin, Tryptophan, Anti-adipogenic activity

Introduction

Cordyceps, also known as 'winter worm summer grass', is a well known traditional medicine in Asian countries. It has been known as a rich source of biologically active components and used for treatment of several diseases such as cancer, fatigue, hyposexualities, hyperglycemia and hyperlipidemia (Yun *et al*., 2006; Lee *et al*., 2006; Koh*et al*., 2003; Kim *et al*., 2010). Previous phytochemical studies of *Cordyceps* have reported nucleotides and sterols as major constituents (Paterson, 2008; Yu *et al*., 2007). Recently, favorable role of *Cordyceps militaris* in the regulation of obesity has been reported (Shimada *et al*., 2008) and we identified cordycepin, guanosine and tryptophan from *C. militaris*as anti-adipogenic constituents (Liu *et al*., 2011).Although there is no doubt about the biological effects of *C. militaris*, its usage has been limited because of short supply and high price. Therefore, much effort has focused on development of cultivation method to achieve a large-scale production of *Cordyceps*. For example, fermentation technology with various culture conditions and discovering the alternative species has been reported.

Several analytical methods have been developed for the quantification of nucleotides, sterol and fatty acid using various chromatography methods (Yang *et al*., 2009; Li *et al*., 2006; Yang *et al*., 2009). The amount of constituents of *Cordyceps* has been affected by species, habitat, sample preparation methods and so on. However, there are few reports evaluating its content and pharmacological activities. To ensure the pharmacological activity in different samples of *C. militaris*, a fast and accurate analytical method for the quantification of the bioactive components is needed.

In the present study, anti-adipogenic activity of six different *Cordyceps* samples was evaluated employing 3T3-L1, a mouse preadipocyte cell line as an *in vitro* assay system. We also developed analytical method using HPLC-DAD for the quantitative analysis of active constituents and quantified their contents in different productions and different extraction samples.

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Experimental

General experimental procedures – Analysis was performed using a Waters HPLC system equipped with Waters 600 Q-pumps, a 996 photodiode array detector, and Waters Empower software using Kinetex ODS-column (2.6 μm, 4.6 × 100 mm) for quantitation.

Production of *Cordyceps* – Dried *Cordyceps* samples were provided from Rural Development Administration in November 2009. *Cordyceps* samples were produced as previously reported (Hong *et al*., 2010; Choi *et al*., 1999; Sung *et al*., 2002). Briefly, hyphal body suspension of *C. militaris* was injected to living silkworm. After incubation, the silkworm host (CM-1) and fruity bodies (CM-2) were harvested separately. In other preparation, hyphal body suspension of *C. militaris* was injected to silkworm pupae, andthen, pupae (CM-3) and fruity bodies (CM-4) were harvested separately after incubation. Hyphal body suspension of *C. militaris* was cultured in rice bran medium and silkworm pupae medium. After incubation, fruity bodies from rice bran medium (CM-5) and silkworm pupae medium (CM-6) were harvested, respectively. Voucher specimen was deposited in the herbarium of College of Pharmacy at Chungbuk National University (CBNU200811-CM).

Preparation of standard solution – Stock standard solution of cordycepin, guanosine and tryptophan was prepared in methanol at a concentration of 1 mg/ml, respectively. Cordycepin, guanosine and tryptophan were prepared as we previous reported (Liu *et al*., 2011). The purity of each compounds were > 98%, as determined by HPLC analysis. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

Sample preparation for HPLC – The powdered CM-1, CM-2, CM-3, CM-4, CM-5 and CM-6 (each 100.0 mg) were weighed accurately and extracted with 100% ethanol for 24 h at room temperature. This extract was filtered and evaporated in vacuum, and then suspended to 10 ml with ethanol in a volumetric flask. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 mm) before HPLC analysis. For the evaluation of anti-adipogenic activity, the evaporated extract was dissolved in DMSO and diluted with water. For evaluation of solvent effects, powdered CM-5 (100.0 mg) was weighed accurately and extracted with the mixture of ethanol-water (100, 75, 50 and 0% ethanol in water) for 24 h at room temperature. Effects of time and temperature were evaluated by extracting powdered CM-5 (100.0 mg) with ethanol or water for different time periods such as 0.5, 1, 2, 4 and 8 h at room temperature or 70 °C. For the optimization of extraction method, powdered CM-5 (100.0 mg) was extracted using ultrasonic apparatus for different time periods as indicated. Each sample solution was filtered through 0.45 μm membrane filter before HPLC analysis.

Validation of the HPLC methods – The linearity of calibration curves was calculated according to the International Conference on Harmonization (ICH) guidelines. Five concentrations of each compound (0.01, 0.025, 0.05, 0.1 and 0.25 mg/ml) were prepared and analyzed in triplicate. The limit of detection (LOD) and quantification (LOQ) were determined based on the method recommended by ICH (LOD = 3.3 δ/*S*, δ = standard deviation of the response, *S* = slope of the calibration curve). The precision test was carried out by the intra-day and inter-day variability for each compound. The intra-day variability was assayed at five concentrations on the same day and inter-day variability was assayed at five concentrations on three sequential days (1, 3, 5 days). The accuracy of the method set up in this study was determined by the method of standard addition. The dilute sample solutionwas spiked with the mixture standard samples of different concentration, respectively. The resultant samples were analyzed by using the proposed method.

Assessment of anti-adipogenic activity – Anti-adipogenic activity was assessed employing 3T3-L1 mouse preadipocytes as an assay system *in vitro*. Adipocyte differentiation was induced using hormone mixtures (0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μg/ml insulin and 1 μM dexamethasone) and fat accumulation was quantitated using Oil Red O staining, as we previously reported (Shin *et al*., 2010). Relative fat accumulation (%) was calculated as 100 × [(absorbance of sample-treated – absorbance of undifferentiated control) / (absorbance of differentiated control – absorbance of undifferentiated control)].

Statistical analysis – The evaluation of statistical significance was determined by the one-way ANOVA test with a value of $p < 0.05$ considered to be statistically significant.

Results and Discussions

Anti-adipogenic activity of different *C. militaris* samples – We previously reported anti-adipogenic activity of *C. militaris* in 3T3-L1 cells (Liu *et al*., 2011). In the present study, six different *Cordyceps* samples such as CM-1, CM-2, CM-3, CM-4, CM-5 and CM-6 were prepared as described in Experimental section. All the *Cordyceps* samples showed significant but differential

inhibitory activity on fat accumulation in 3T3-L1 cells (Fig. 1). Fruity bodies of *Cordyceps* from rice bran medium (CM-5) and those from silkworm pupae medium (CM-6) showed potent inhibitory activity on fat accumulation. *Cordyceps* grown in pupae (CM-3 and CM-4) exerted stronger inhibitory activity than those grown in silkworm (CM-1 and CM-2). Related to parts used, host insects of *Cordyceps* (CM-1 and CM-3) showed stronger inhibition than fruity bodies (CM-2 and CM-4), respectively.

Optimization of chromatographic conditions – For simultaneous determination of three active components of *C. militaris*, cordycepin, guanosine and tryptophan, the chromatographic condition was first investigated. Various mixtures of water, methanol and acetonitrile in combination with acetic acid were tested as a mobile phase. In our chromatographic condition, mixture of water and methanol showed favorable separation and addition of acetic acid did not improve the peak resolution. The wavelength for detection was set at 220 nm, where the three compounds showed the maximum absorption as measured by DAD. As a result, the optimal mobile phase consisting of 10% methanol in water was subsequently employed for the analysis of CM-5, which led to good resolution and satisfactory peak shape at 220 nm. Under this chromatographic condition, cordycepin, guanosine and tryptophan have retention times of 11.7, 16.5 and 8.5 min, respectively (Fig. 2).

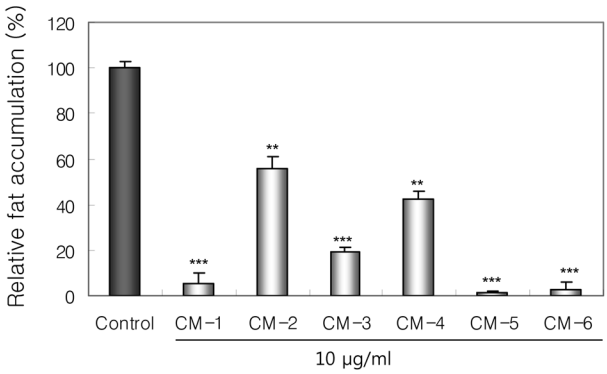

Fig. 1. Anti-adipogenic activity of different *C. militaris* samples. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{**}p < 0.01$, $^{***}p < 0.001$compared with control.

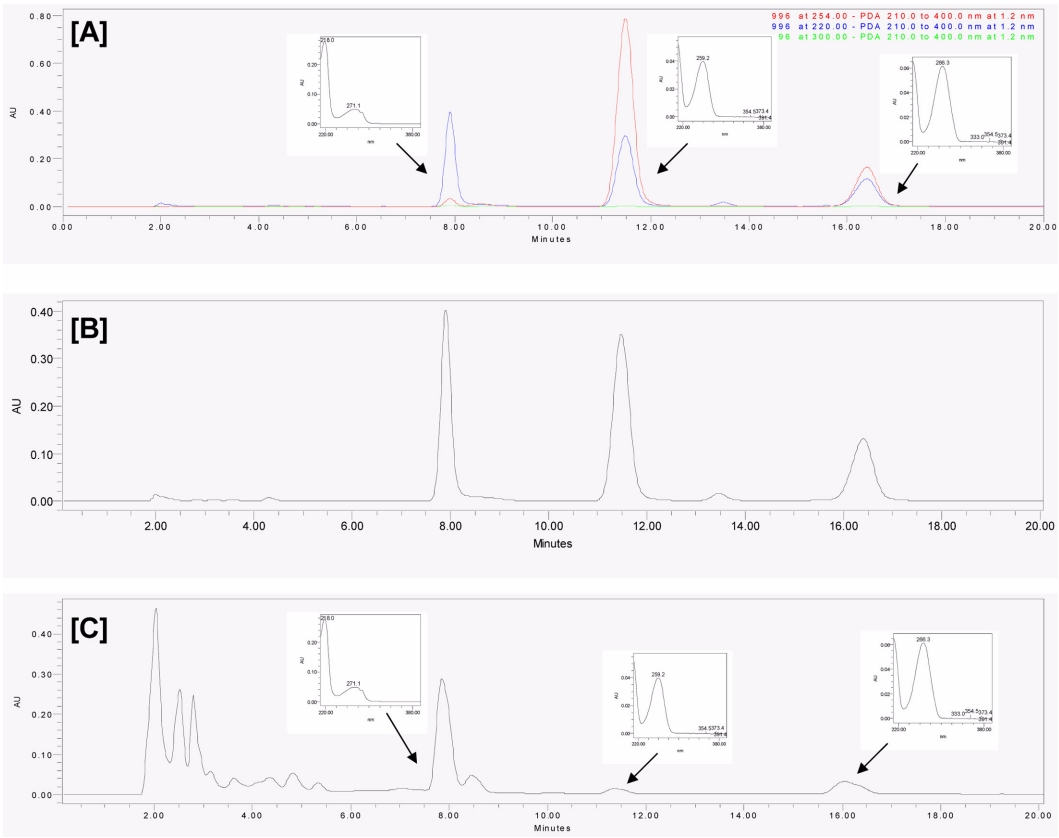

Fig. 2. HPLC chromatogram of established method. (A) HPLC chromatogram of cordycepin, guanosine and tryptophan at 220, 254 and 300 nm; (B) HPLC chromatogram of compounds at 220 nm; (C) HPLC chromatogram of total extract of CM-5 at 220 nm. HPLC analysis was performed using Kinetex ODS-column (2.6 μm, 4.6 × 100 mm) with mobile phase consisting of 10% methanol in waterat a flow rate of 1 ml/min.

Table 1. Linear regression data, LOD and LOQ of compounds

Compounds	Linear regression data			LOD (μg)	LOQ (μg)
	Equation	Ranges (μg/ml)	R^2		
Cordycepin	y = 53.62x – 0.77	1 – 250	0.9989	0.04	0.12
Guanosine	y = 25.29x + 0.01	1 – 250	0.9999	0.05	0.15
Tryptophan	y = 58.21x – 1.28	1 – 250	0.9983	0.05	0.14

Table 2. Inter- and intra-day variability and accuracy of compounds

Compound	Precision				Accuracy		
	Inter-day		Intra-day				
	Spiked amount (μg)	RSD[a] (%)	Spiked amount (μg)	RSD[a] (%)	Spiked amount (μg)	Accuracy (%)	RSD[a] (%)
Cordycepin	5.20	0.10	2.60	0.57	1.57	115.1	0.26
	2.60	0.55	1.04	1.54	1.83	113.8	0.25
	1.04	1.07	0.26	1.10	2.09	112.4	0.19
Guanosine	8.00	0.12	4.00	0.52	2.42	104.9	0.38
	4.00	0.62	1.60	1.26	2.82	107.4	0.38
	1.60	1.22	0.40	1.24	3.21	106.0	0.13
Tryptophan	2.80	1.88	1.40	0.74	0.80	94.4	0.47
	1.40	1.50	0.56	1.83	0.95	102.6	0.31
	0.56	1.31	0.14	1.70	1.10	106.8	0.03

[a] RSD (%) = (SD of amount detected / mean of amount detected) × 100 (n = 3).

Validation of developed analytical method – Specificity was determined by the calculation of peak purity facilitated by DAD. The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. Calibration curves were linear in relatively wide range of concentrations and all showed good linear regressions with high correlation coefficient values ($r^2 > 0.9998$) between peak area (y) and amount of each compound (x). LOD and LOQ for three compounds were less than 0.05 μg and 0.15 μg, respectively (Table 1). The RSD of overall intra- and inter-day variability showed good precision of this method. The developed method had good accuracy with recovery of 94.4 - 115.1% with RSD values less than 10.0% (Table 2).

Content of constituents and anti-adipogenic activity on different *Cordyceps* – The established method has been applied to the determination of the three active components in *Cordyceps* samples. As shown in Fig. 3, the content of constituents has great variation in different cultivation conditions of *Cordyceps*. Especially, the content of cordycepin showed great difference between samples whereas the content of tryptophan was similar in tested samples. The guanosine was contained in small amount

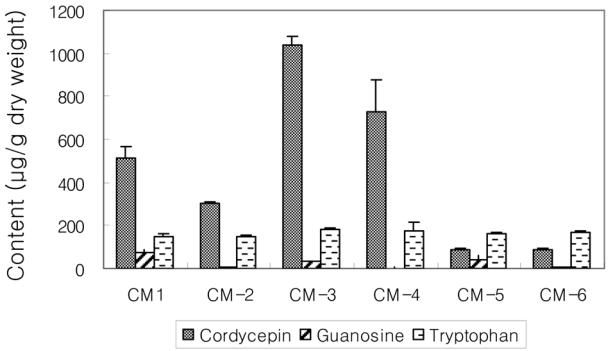

Fig. 3. Analysis of contents of three constituents in different *C. militaris* samples per dry weight. Anti-adipogenic activity of different *C. militaris* samples (B). Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

compared to cordycepin and tryptophan, and even hard to detect in some samples. Our analytical data of *Cordyceps* samples also suggested the variance of content in different parts of *Cordyceps*. In our samples, the content of host insect, CM-1 and CM-3 was higher than that offruiting bodies, CM-2 and CM-4, respectively. In addition, *Cordyceps* grown in pupae (CM-3 and CM-4) has higher content than grown in silkworm (CM-1 and CM-2). Cultured *Cordyceps* (CM-5 and CM-6) possessed low

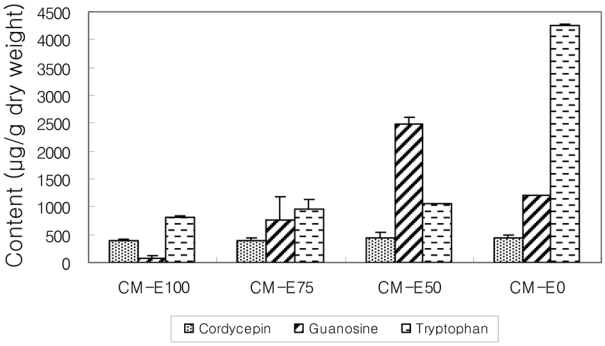

Fig. 4. Effect of extraction solvent on the yield of cordycepin, guanosine and tryptophan from CM-5.CM-5 was extracted for 24 h with the mixture of ethanol and water (100, 75, 50 and 0% ethanol in water were abbreviated as E100, E75, E50 and E0, respectively).

amount of constituents compared to natural *Cordyceps*.

As we identified cordycepin, guanosine and tryptophan as active constituents of *C. militaris*, we tried to compare the content of these constituents and anti-adipogenic activity. Unfortunately, the total content of three constituents did not directly correlate with anti-adipogenic activity. Although the CM-3 and CM-4 have high content of cordycepin than CM-1 and CM-5, the anti-adipogenic activities of CM-1 and CM-5 were more potent than those of CM-3 and CM-4 (Fig. 1). Interestingly, CM-1, CM-3 and CM-5 which contain three constituents showed better activity than CM-2 and CM-4 which contain two of them. However, CM-6 showed different pattern to our supposition. Therefore, we supposed that other constituents as well as three active constituents might be important in the anti-adipogenic activity of *C. militaris*, which needs to be clarified with further study.

Effect of sample preparation method on extraction efficiency – To optimize a sample preparation method for anti-adipogenic constituents, we analyzed the content of constituents in CM-5, which showed the most potent activity, prepared using different extraction methods. First, the effect of extraction solvent was tested. Because the polar fraction of *C. militaris* showed anti-adipogenic activity (Liu *et al*., 2011), samples were extracted with the mixture of ethanol and water (100, 75, 50 and 0% ethanol in water) and analyzed. As shown in Fig. 4, the total yield was greatly increased by addition of water. The yield of guanosine and tryptophan was also increased by addition of water, however, the yield of cordycepin showed little variation among four extraction solvent.

Next the effects of extraction method on extraction efficiency were evaluated. CM-5 was extracted with ethanol or water using ultrasonic apparatus or by shaking for different times. The yield at different extraction temperature such as room temperature and 70 ºC was also

Table 3. Effect of sample preparation method on the yield of cordycepin, guanosine and tryptophan from CM-5[a)]

Time	Compound	Ethanol			Water		
		RT	70ºC	Ultrasonic	RT	70ºC	Ultrasonic
30 min	Cordycepin	376.9	402.1	340.8	417.0	402.4	408.5
	Guanosine	48.1	96.8	74.3	1126.6	1079.0	1049.6
	Tryptophan	627.5	667.2	587.5	1699.4	1052.2	2291.2
1 h	Cordycepin	377.6	416.8	389.3	401.1	416.4	393.6
	Guanosine	47.8	128.6	104.0	1164.9	1310.5	1028.7
	Tryptophan	611.9	772.6	653.8	2019.3	973.2	2367.2
2 h	Cordycepin	399.2	434.5	400.6	409.4	416.0	423.7
	Guanosine	66.3	119.2	106.5	1276.4	1742.6	1297.9
	Tryptophan	623.7	671.2	800.0	2278.6	1002.0	2339.4
4 h	Cordycepin	407.9	420.6	398.3	421.0	419.4	403.4
	Guanosine	79.3	127.6	146.0	1353.9	1261.6	1352.7
	Tryptophan	586.0	746.0	806.0	2400.4	1009.4	2723.2
8 h	Cordycepin	429.8	446.7	436.4	444.3	413.5	426.2
	Guanosine	70.2	226.0	242.9	1245.5	1096.7	1287.4
	Tryptophan	671.7	715.4	779.7	2588.3	1155.2	2878.7
12 h	Cordycepin	436.5	426.1	428.2	430.3	420.1	422.0
	Guanosine	144.3	209.0	213.2	1367.1	1237.2	1025.5
	Tryptophan	690.3	760.6	754.9	2509.8	1296.2	2464.5

[a)] Yields are expressed as μg/g dry weight of CM-5.

tested (Table 3). Consistent with Fig. 3, guanosine and tryptophan showed higher yield in water extraction in all extraction conditions, whereas cordycepin showed similar yield.

The effect of temperature during extraction showed differential effect in ethanol and water extraction. The yield of guanosine showed almost twice increase when extracted at 70 ºC with EtOH whereas little difference in water extraction. Interestingly, however, the yield of tryptophan showed decrease in water extraction at 70 ºC whereas little difference in ethanol extraction. Cordycepin showed similar yield independent of temperature in both ethanol and water extraction.

The effect of extraction method was also investigated by comparing the extraction yield using ultrasonic apparatus and shaking. Extraction using ultrasonic apparatus increased the yield of guanosine in ethanol extraction but little improvement in other conditions. The extraction time also affected the extraction yield. Extraction with ethanol needed longer extraction time for maximum yield. Especially, the yield of guanosine increased as time increased. Extraction of sample with ethanolat room temperature and 70 ºC needed 12 h and 8 h, respectively, for maximum yield of guanosine. The yield of cordycepin and tryptophan in ethanol extraction was also slightly increased in longer extraction time. Extraction of sample with water reached maximum yield in 2 h extraction.

Conclusion

For the quality control of anti-adipogenic activity of *C. militaris*, analytical method was developed and validated, and the content of these compounds in different sample was quantitated. The content of three active constituents showed variance in different *C. militaris* samples and was also greatly affected by the extraction methods. Therefore, quantitation of these compounds using developed analytical method might provide basic equirement for the anti-adipogenic activity of *C. militaris*.

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