# EXTRACTION OF ADENOSINE AND CORDYCEPIN FROM SPENT SOLID MEDIUM OF *CORDYCEPS MILITARIS* CULTURE

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## ABSTRACT

The highly prized medical fungus *Cordyceps militaris,* known in Viet Nam as Dong Trung Ha Thao, is nowadays successfully domesticated and cultivated on artificial solid media. Apart from the fruiting bodies as the main product, the spent solid medium is also considered valuable due to its high content of the bioactive compounds cordycepin and adenosine. In this study, with the aim to obtain concentrates of bioactive compounds from this material within a practical time, several methods of extraction were studied using ethanol as solvent and with and without the support of heat and ultrasound. It was shown that, by using ethanol 50 % as solvent, adenosine and cordycepin could be effectively extracted at 65 $^{o}$C in 6 hours. By vacuum evaporation, the concentration of the active compounds was increased 10 times, while high recovery yield was obtained, facilitating further usage as dietary supplements.

*Keywords*: *Cordyceps millitaris* R6, extraction, cordycepin, adenosine.

## 1. INTRODUCTION

*Cordyceps militaris,* a member of *Ascomycota*, is a parasite of insect's larval stage, forming fruiting bodies expanding outside the insect larvae or pupae [1-3]. *C. militaris* have been known for long time as a valuable folk medicine, especially in East Asia [1-3]. *C. militaris* polysaccharides and other bioactive compound such as adenosine and cordycepin (3′-deoxyadenosine) have been proven to have antitumor activities against cervical and liver cancer cells *in vitro,* antioxidant, antibacterial, antifungal, antihuman tumor cell lines, anti-inflammatory, anti-brotic, and anti-angiogenetic activities [1, 2]. Therefore, in recent years *C. militaris* is extensively cultivated in liquid as well as on solid media [1, 3] and is the most successfully cultivated *Cordyceps* species [3]. In solid media different supplemented grain types and seeds are used, but most is brown rice together with silkworm powder. *Cordyceps militaris* grows rather well on such solid media (for example on Figure 1). The fruiting bodies expand up from the solid medium; meanwhile the mycelia spread on surface and goes deeply into the solid phase. The fruiting bodies of *C. militaris* cultivated on such solid media yielded in rather high amount of adenosine and cordycepin depending on strain, cultivation condition and

supplemented media [3]. The fruiting bodies therefore normally are harvested and frozen dried for long storage and use. The spent medium solid also contains around 10–15 % bioactive compounds compared to fruiting body. The methods of extraction of cordycepin and adenosine from fruiting body of *C. militaris* have been investigated, for example using methanol or ethanol with support of heat, ultrasound and microwave [4-6]. However, to the best of our knowledge, up to date there is no reported extraction method applied for the spent solid medium after harvesting the fruiting bodies, except the column chromatographic extraction reported by He Ni et al. in 2009 [7].

The aim of this study is to investigate the extraction of adenosine and cordycepin from the spent solid medium after harvesting the fruiting bodies of *C. militaris* R6, which cultivated in the Center for Research and Development in Biotechnology, Hanoi University of Science and Technology. An appropriate extraction method, which is simple, economical and upscaleable, will expand more options to develop new value added products from this spent solid.

## 2. MATERIALS AND METHODS

### 2.1. Materials

The solid medium for the cultivation of *Cordyceps militaris* R6 was based on brown rice, silkworm powder, coconut milk and other supplemented ingredients. After 2 months of cultivation at tightly controlled condition of temperature, humidity and air, the fruiting bodies were harvested. The spent solid medium was collected and dried at 42 $^{o}$C until water content was around 6 %, then stored at -20 $^{o}$C until extraction. For the extraction, the solid was ground finely. The mixture of solid material and solvent was homogenized by IKA T10 Basic Ultra Turrax dispersers.

### 2.2. Extraction methods

Cordycepin and adenosine were extracted with ethanol, which was considered the most safe and effective solvent compared to others, such as methanol [5, 6]. Temperature, ratio of solvent/solid, ethanol concentration and extraction duration were varied to obtain the best yield.

First, the extractions were performed for 6 - 24 h with ethanol at high temperature, varied from 45 to 65 $^{o}$C. The solvent and solid ratio was also varied from 5 to 200 based on the dry matter of solid phase. The extraction at elevated temperature of 65 $^{o}$C was also supported by ultrasound with sonication water bath (Elma, S60, Germany). The extraction was similarly performed at room temperature of 30 $^{o}$C, in which the influence of time, ethanol concentration and solvent/solid ratio were also assessed.

*Figure 1. C. militaris* R6 fruiting bodies on solid medium (left) and the spent solid medium after harvesting of the fruiting body (right).

The concentration of adenosine and cordycepin in the extracts liquid was determined by HPLC. The effectiveness of extraction was expressed as amount of extracted adenosine or cordycepin from 1 gram of dry matter of spend solid medium (µg/g of dry matter).

### 2.3. Concentration of the extracts

The obtained extracts containing 50 % ethanol were concentrated under vacuum of 60 - 350 mbar (temperature 40 - 80 $^{o}$C) using a B215 Evaporator (Buchi, Switzerland), or by boiling at atmospheric pressure.

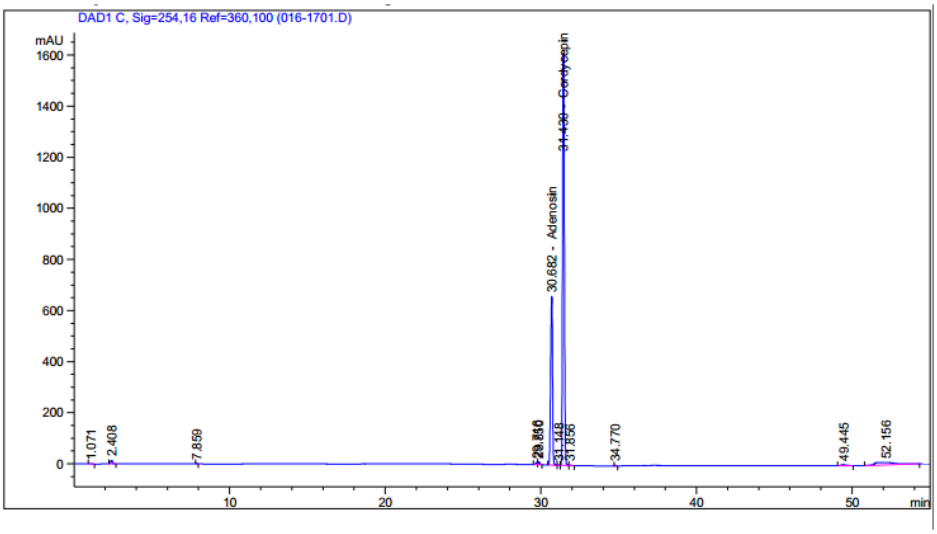


*Figure 2*. HPLC chromatograph of standard of Adenosine and Cordycepin (Sigma) at concentration of 0.1 mg/ml.

### 2.4. Determination of Adenosine and Cordycepin contents

The samples were injected in Vertisep C18 column (250 x 4,6 mm, 5 µm, Vertichrom, Thailand) on a HPLC Agilent 1200 Series (Germany) and eluted by a gradient program of MeOH and HPLC grade water (Merck) as follows: 0-20 min/8 % MeOH, 20-30 min /8-70 % MeOH; 30-50 min/70 % MeOH; 50-60 min/8 % MeOH. The flow rate was 0.6 ml/min. Temperature of separation on column was maintained at 30$^{o}$C. Cordycepin and adenosine from Sigma (Singapore) were used as external standard for quantification. Being detected by DAD at 254 nm, the retention time of adenosine and cordycepin was 30.6 and 31.4 min, respectively (Figure 2).

The moisture content of the spent solid medium was determined by using MA35 equipment (Satorius, Germany) when needed.

## 3. RESULTS AND DISCUSSIONS

### 3.1. Extraction at high temperature without and with support of ultrasound

As mentioned in Material and Methods, the extraction experiment was performed in elevated temperature, which was also used by other authors but for fruiting bodies of *C. militaris* [4, 6, 8]. The extraction effectiveness, presented in amount of adenosine (A) and cordycepin (C)

(μg/g of dry matter of the spent solid) under different conditions varied of temperature, concentration of ethanol in solvent, duration and ratio of solvent to solid medium were shown in Table 1 to Table 4.

*Table 1*. Influence of ethanol concentration.

| Ethanol concentration (%) | A (μg/g) | C (μg/g) |
|---|---|---|
| 40 | 343.6 ± 14.4 | 1998.2 ± 83.9 |
| 50 | 446.2 ± 18.7 | 2060.0 ± 94.8 |
| 60 | 415.0 ± 17.4 | 2070.3 ± 76.6 |
| 80 | 423.9 ± 17.8 | 1895.2 ± 94.8 |

Liquid/solid ratio of 100; temparature of 65 °C, 24 h of extraction.

*Table 2.* Effect of elevated temperature.

| Temperature (°C) | A (μg/g) | C (μg/g) |
|---|---|---|
| 45 | 205.3 ± 8.6 | 2031.2 ± 85.3 |
| 55 | 278.4 ± 11.7 | 1979.7 ± 91.1 |
| 65 | 446.2 ± 18.7 | 2060.0 ± 76.2 |

Liquid/solid ratio of 100; ethanol concentration of 50 %, 24 h of extraction.

Starting with solvent/solid ratio of 100, at 65 $^{o}$C, and extraction for 24 h, the changing of ethanol concentration from 40–80 % did not affect significantly on the extraction yield of A and C which ranged in 350–450 μg/g and c.a. 2000 μg/g dry matter of solid, respectively (Table 1). This result supported for the findings of Wang *et al.* [6]. These authors have used heat combining ultrasonication to extract cordycepin from fruiting bodies of *Cordyceps militaris* and observed that the yield did not change much when increase ethanol concentration from 40 – 80 %. The best temperature for extraction of cordycepin was found around 60 $^{o}$C. The yield at 60 $^{o}$C has significant increasing compared to at 40 $^{o}$C [6]. In this study, we also found similar results but for adenosine, meanwhile there was no difference on extraction yield of C when temperature increased (Table 2). It may be that the cordycepin in spent solid medium is accumulated differently from in fruiting bodies or the amount of cordycepin in this media mostly "maximum". Similarly, the effect of extraction time was not clearly observed when prolonged from 6 h to 24 h (Table 3). This mean it is not necessary to perform the extraction up to 24 h, but 6 h to save time and energy for heating. As expected, the ration solvent/solid resulted in a remarkable influence to the extraction yield (Table 4). The higher ratio increased the yield but the relation was not linear. The ratio of 5 could extract nearly a half of A and C in comparison to ratio of 100 (Table 4).

*Table 3*. Influence of extraction time.

| Extraction time (h) | A (μg/g) | C (μg/g) |
|---|---|---|
| 6 | 437.3 ± 18.4 | 1998.2 ± 83.9 |
| 12 | 433.0 ± 18.2 | 2020.0 ± 92.9 |
| 24 | 446.2 ± 18.7 | 2060.0 ± 76.2 |

Ethanol 50 %, liquid/solid ratio of 100 and temperature of 65 °C.

*Table 4*. Effect of solvent/solid media ratio.

| Ratio solvent/solid | A (μg/g) | C (μg/g) |
|---|---|---|
| 5 | 206.2 ± 8.7 | 1009.4 ± 42.4 |
| 10 | 197.1 ± 8.3 | 1277.2 ± 58.8 |
| 20 | 315.5 ± 13.2 | 1551.2 ± 57.4 |
| 100 | 446.2 ± 18.7 | 2060.0 ± 86.5 |
| 200 | 544.4 ± 22.9 | 1771.6 ± 81.5 |

Temparature of 65 °C, 24 h of extraction, ethanol concentration of 50 %.

*Table 5*. Influence of extraction time using ultrasound.

| Extraction time (min) | A (μg/g) | C (μg/g) |
|---|---|---|
| 40 | 223.0 ± 9.4 | 1650.0 ± 69.3 |
| 60 | 273.8 ± 11.5 | 1663.0 ± 76.5 |

Ethanol 50 %, liquid/solid ratio of 100 and temperature of 65 °C, ultrasonic 50 Hz.

Ultrasound resulted in a supportive effect on the extraction of A and C. The yield of extraction after 1 h treatment was 1663 μg/g (C) (Table 5), which was slightly lower than the yield obtained after 6 h (1998.2 μg/g) without sonication (Table 3), under the same condition of temperature and ratio of solvent/solid. This result was in agreement to the finding of Wang *et al.* [6]. The maximum cordycepin yield was obtained after 60 minutes of treatment [6]. Even though the sonication can increase the effectiveness of extraction of bioactive compounds, this method is not feasiable due to the cost of equipment and maintainance, particularly in large scale. This method is more suitable for treatment of small volume sample for analysis (for example for HPLC).

### 3.2. Extraction at room temperature

In Asia, the extract of functional ingredients from herbal (plant)/animal/fungi is obtained by immersing the material in rice-wine for long time at room temperature. In this study, the extraction was also performed at room temperature (25-35 $^{o}$C) with ratio of solvent/solid varying from 3 to 5 and the ethanol concentration varying from 30 % to 96 %. The incubation time was from 2 to 12 weeks. The extraction yield of A and C from these experiments were presented in Table 6 - 8.

The results show that after 2 weeks the yield of A and C both was almost highest as until 12 weeks (Table 6). Similarly, the varying of ethanol concentration in solvent did not result in significant increasing of yield (Table 7).

Obviously, at room temperature the extraction yield was much lower than at temperature of 65 $^{o}$C. For example, the extraction of C at room temperature after 2 weeks was less than 50 % compared to extraction of C at 65 $^{o}$C for 6 h (410.0 μg/g compared to 1009 μg/g of dry matter) at the same solvent/solid ratio (Table 8 and Table 4).

*Table 6*. Extraction of Adenosine (A) and Cordycepin (C) at different time of extraction at room temperature.

| Time of extraction (weeks) | A (μg/g) | C (μg/g) |
|---|---|---|
| 2 | 180.0 ± 7.2 | 350.0 ± 16.1 |
| 6 | 194.6 ± 9.9 | 367.0 ± 15.4 |
| 9 | 186.0 ± 11.3 | 353.0 ± 17.1 |
| 12 | 183.0 ± 8.5 | 356.0 ± 13.2 |

Ethanol concentration of 50 % and solvent/ solid ratio of 4:1.

*Table 7*. Influence of ethanol on extraction of Adenosine and Cordycepin.

| Ethanol concentration (%) | A (μg/g) | C (μg/g) |
|---|---|---|
| 40 | 175 ± 7.35 | 352.0 ± 14.8 |
| 50 | 180 ± 7.2 | 350.0 ± 16.1 |
| 80 | 188 ± 6.96 | 374.0 ± 13.8 |
| 96 | 200 ± 9.60 | 380.0 ± 18.2 |

Room temperature (30 °C), 2 weeks of extraction, solvent/solid ratio of 4:1

*Table 8*. Effect of solvent/spent solid medium ratio on extract at room temperature.

| Solvent/solid ratio | A (μg/g) | C (μg/g) |
|---|---|---|
| 3 | 184.0 ± 9.2 | 300.0 ± 15.0 |
| 4 | 180.0 ± 7.2 | 350.0 ± 14.0 |
| 5 | 213.0 ± 9.2 | 410.0 ± 17.6 |

Room temperature (30 $^{o}$C), ethanol concentration of 50 %, 2 weeks.

So far, there are different extraction methods that reported but mostly for fruiting body. On the other hand, these methods require the supporting of ultrasonic, microwave, supercritical fluid or column chromatography [4, 7-9]. The extraction method we applied here is very simple and it is feasible for large scale.

### 3.3. Concentration of extract

Higher ratio of solvent/solid resulted in lower concentration of adenosine and cordycepin in extract. To increase the concentration of extract, the solvent was evaporated. The recovery yield was depending on condition of evaporation (Table 9). The higher temperature used (less vacuum), the lower of recovery yields were. High temperature of 100 $^{o}$C (atmostpheric condition) resulted in a lower yield of c.a 40 % for both A and C (Table 9), even though the evaporation was fast. In contrast, the temperature of 40 $^{o}$C did not affect much to A and C, hence the recovery was almost maintained after evaporation. This suggests that to remain the quality of extract, the evaporation should be done at low temperature. Apart from that, the ratio of solvent/spent solid should be kept lower and the ethanol concentration should be higher to save

time for removal of solvent. The higher content of bioactive compounds in concentrated extract, the more options can be applied afterward.

*Table 9*. Recovery yield of Adenosine and Cordycepin in concentration.

| Temperature (°C) | Pressure (mbar) | Duration time (h) | Recovery yield (%) | |
|---|---|---|---|---|
| | | | Adenosine | Cordycepin |
| In initial extract | | | 100 | 100 |
| 100 | 0 | 0.5 | 41 | 39 |
| 80 | 60-350 | 1 | 97 | 65 |
| 60 | 60-350 | 1.3 | 103 | 79 |
| 40 | 60-350 | 2 | 108 | 95 |

Concentration ratio of 10, i.e. from initial volume of 100 ml to 10 ml after concentration.

## 4. CONCLUSIONS

In this study, we applied a simple solvent extraction method with ethanol at elevated temperature to obtain bioactive compounds, including adenosine and cordycepin, from the spent solid culture media of the medical fungus *Cordyceps militaris* R6. The results showed that an appropriate condition for the extraction of the target compounds was: temperature of 65 $^{o}$C, ratio of ethanol 50 % to spent solid media of 100 and time for extraction of 6 h. To preserve both bioactive compounds, the concentration process should be conducted at low temperature (40 $^{o}$C) with the aid of vacuum. This simple extraction method could be easily applied for large-scale production.

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