***Interprof. J. Health Sci.* 2022, *20(1-2): 01-08*.**
ISSN (online) 2672-9423 ISSN (print) 2672-9628

DETERMINATION OF ADENOSINE AND CORDYCEPIN IN CULTURE AND NATURAL CORDYCEPS

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Received 18 November 2020; Revised 29 April 2022; Accepted 6 January 2022

Abstract: *Cordyceps sinensis* or *Ophiocordyceps sinensis* is parasite fungus in the insects. It contains polysaccharides, nucleoside derivatives, some amino acids and sterol which showed some pharmacological activities. Therefore, it causes the interests to culture in the laboratory especially *Cordyceps militaris.* This study aimed to determine the major contents (cordycepin and adenosine) in culture and natural *Cordyceps*. The culture *Cordyceps* were grown in potato dextrose agar (PDA), potato dextrose broth (PDB) medium and later on the culture materials. The contents of adenosine and cordycepin were analyzed with reversed phase high performance liquid chromatography (RP-HPLC). The results showed that both adenosine (0.20-0.21 mg/g) and cordycepin (0.90-1.76 mg/g) were found in the fruiting body of culture *Cordyceps* but only adenosine (1.66-2.95 mg/g) was the major nucleoside found in the natural *Cordyceps*. This method can be adopted to analyze adenosine and cordycepin in *Cordyceps* dietary products.

Keywords: adenosine, cordycepin, *Cordyceps sinensis*, *Ophiocordyceps sinensis*

INTRODUCTION

Cordyceps sinensis is a fungus that grows on insects in the family Ophiocordycipitaceae. *C. sinensis* is currently known as *Ophiocordyceps sinensis*. *O. sinensis* is mainly found in the meadows above 3,500 meters in the Himalayan regions of Nepal, Bhutan, India and Tibet. It parasitizes larvae of moths or insects to produce a fruiting body. *O. sinensis* is classified as a medicinal mushroom and it has been used in traditional medicine in China and Tibet to treat fatigue and to promote health well-being. The primary and secondary metabolites in the fruiting body are nucleoside derivatives; for example, cordycepin (3′-deoxyadenosine), adenosine and polysaccharides such as rhamnose, galactomannan, mannitol (Surapong N and Sipolkrai P, 2018). Cordycepin (3′-deoxy adenosine) is an adenosine analog and originally isolated from culture of *Cordyceps*. It exhibits pharmacological activities including antifungal, antibacterial, anti-oxidant, anti-inflammatory, anticancer, and decrease blood glucose and cholesterol (Tuli HS and et al., 2015). *Cordyceps* which more than 80 species have been found in Thailand and it was claimed for eternal youth, immortality drug and increase male sexual performance.

Cordyceps militaris is a parasite fungus in the insects and it is a species in the family Clavicipitaceae. The fungus forms 20-50 mm high, club-shaped and orange fruiting bodies. It considers as a medicinal mushroom because its metabolites contain some bioactive nucleosides (Das SK and et al., 2010). It exhibits anti-oxidant, anti-tumor, antiproliferative and anti-inflammatory. Therefore, it causes interests as dietary supplement for revitalization

of various systems of the body. An effort is try to culture *C. militaris* in various culture media to obtain high bioactive compounds.

Because of the high values of *Cordyceps*, an attempt is to culture it in the laboratory. The culture and natural *Cordyceps* showed varieties of bioactive compounds. *C. sinensis* is reported for high bioactive compounds while *C. militaris* is the popular one that is cultured in Thailand. In this study the culture of *C. militaris* was grown in the media and collected to study the chemical components. The natural *Cordyceps* was bought from China and determined the main compositions. Cordycepin and other nucleoside analogs are analyzed with TLC, HPLC and LC-MS. TLC and HPTLC were performed on a silica gel 60 F254 to determine eight kinds of nucleosides, nucleobases and ergosterol as the markers for the quality control of *Cordyceps* (Nga LI, 2012). Quality assessment of *Cordyceps* can be evaluated using HPLC with UV or PDA detectors (Yu L and et al., 2006). In addition, HPLC-MS/MS can be useful for the authentication of *Cordyceps* (Hu H and et al., 2015). The objective of this study was to determine adenosine and cordycepin contents in culture and natural *Cordyceps*.

MATERIALS AND METHODS

Materials

Adenosine and cordycepin were bought from CATO Research Chemicals Inc. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Himedia. Methanol (HPLC grade) was bought from Burdick Jackson, Korea. Yeast extract was bought from Scharlau. D(+)-glucose anhydrous and sodium sulfate were bought from Carlo Erba. RO water and ultrapure water were prepared on a Puris water purifier system. Natural *Cordyceps* were obtained from Myanmar and China.

Cordyceps cultures

Fungal fiber of *Cordyceps* on the culture materials was obtained from mushroom farm at Samutprakarn province, Thailand. It was incubated at 20°C under light for 12 days and obtained orange color. Spread the fungal filament on the PDA medium and incubated for 15 days. Then transferred the fungal fiber to the PDB medium and continue incubating for 12 days. After that transferred the fungal medium to the culture materials, grew for 45 days and collected the mushroom body and culture materials (Samples 1, 2) for analysis. The other samples (Samples 3, 4) were prepared at the same procedure except that it was grown in PDA medium for 20 days and PDB medium for 9 days before transferring to grow in culture materials for 63 days.

PDA and PDB medium were freshly prepared from PDA, and PDB mixed with water and sterilized at 121°C under 15 pound/inch^2 pressure. In addition, culture materials were prepared from Jasmine brown rice, stock soup and sterilized at 121°C under 15 pound/inch^2 pressure. Stock soup composed of peptone, yeast extract, glucose, sodium sulfate, vitamins eggs and potato stock.

Sample preparation

Cordyceps fruiting body was collected and ground to powder. The powder was accurately weighed 100 mg (n=2) and extracted with water using an ultrasonic bath for 2 hours (Xie J-W and et al., 2010). The extract was centrifuged at 4,000 rpm for 10 minutes and the supernatant was filtered and freeze dry overnight. The dry sample was reconstituted in 70% methanol and filtered through syringe filter (0.45 μm) before HPLC analysis

Standard preparation

Standard adenosine and cordycepin were prepared at the concentration of 1,000 μg/mL as the stock solution. Then adenosine standard solution was diluted to 0.31 – 10 μg/mL while cordycepin solution was diluted to 2.5 – 80 μg/mL. They were analyzed using HPLC and their peak areas were recorded.

HPLC analysis

The major components of *Cordyceps* were analyzed with HPLC [Agilent 1260] and acquired data with OpenLab EZChrom software. The analysis was performed on ACE C18 column (4.6 x 250 mm, 5 μm) and was controlled at 25°C. Mobile phase composed of methanol and water in a gradient system, 2% to 22% methanol over 30 minutes, washed the column at 100% methanol for 5 minutes and regenerated to 2 % methanol in 5 minutes (Yu L and et al., 2006). Flow rate was 0.7 mL/minute and the total run time was 40 minutes. The chromatogram was observed at the wavelength of 260 nm.

ESI-MS identification for some nucleosides

The other nucleosides were identified using LC ESI-MS [Ultimate 3000, Dionex /Amazon SL, Bruker. The analytical condition was the same as HPLC analysis except that Zorbax C18 extended column (4.6 x 250 mm, 5 μm) was used. Electrospray mass spectrometer was equipped with quadrupole ion trap. Capillary voltage was set at 4,500 V, nebulizer gas was set at 2 bars and dry gas temperature was 220 °C with a flow rate of 7 mL/min. MS evaluation was performed in both positive and negative modes alternately and scanned at the mass range of m/z 70-1,000 amu. Mass tune was at 250 amu. Chromelon, Trap control and Hystar were used for controlling the system and process mass spectra.

RESULTS AND DISCUSSION

The procedure growing *Cordyceps* in the laboratory was showed in Figure 1. The samples 1-4 were collected, dried and ground to powder as shown in Figure 2. The fruiting body of culture and natural *Cordyceps* were look like worm. The main components of culture *Cordyceps* were adenosine and cordycepin while that of natural *Cordyceps* was only adenosine. The cordycepin content in this study (1.66-2.95 mg/g), which was cultured in jasmine rice, was relatively high in comparison with those cultured (3.41 mg/g) in rice medium (Huang L and et al., 2009). In addition, adenosine and cordycepin were found in the culture materials. In the HPLC condition, standard adenosine and cordycepin were observed at the retention time of 22.4 and 24.4 minutes, respectively (Figure 3). HPLC chromatograms of sample 1-4 showed adenosine and cordycepin eluted at the same retention time as those of standards (Figure 4). This result was similar to the article reported by Yu L and et al., 2006 that there were no cordycepin found in natural *C. sinensis*. However, cordycepin was found in the range of 0.90-1.76 mg/g which was slightly higher than the reported natural *C. sinensis* (0.20-0.38 mg/g). For culture *Cordyceps*, cordycepin was found in the range of 1.66-2.95 mg/g in the fruiting body. The cordycepin content was lower than those found in culture *C. sinensis* from China, but higher than the content (0.98 mg/g) which was reported by Huang L and et al., 2009. This HPLC method was optimized for quantitative analysis of adenosine and cordycepin in *Cordyceps* raw materials. Moreover, it can be applied to determine these main nucleosides in dietary products of *Cordyceps*.

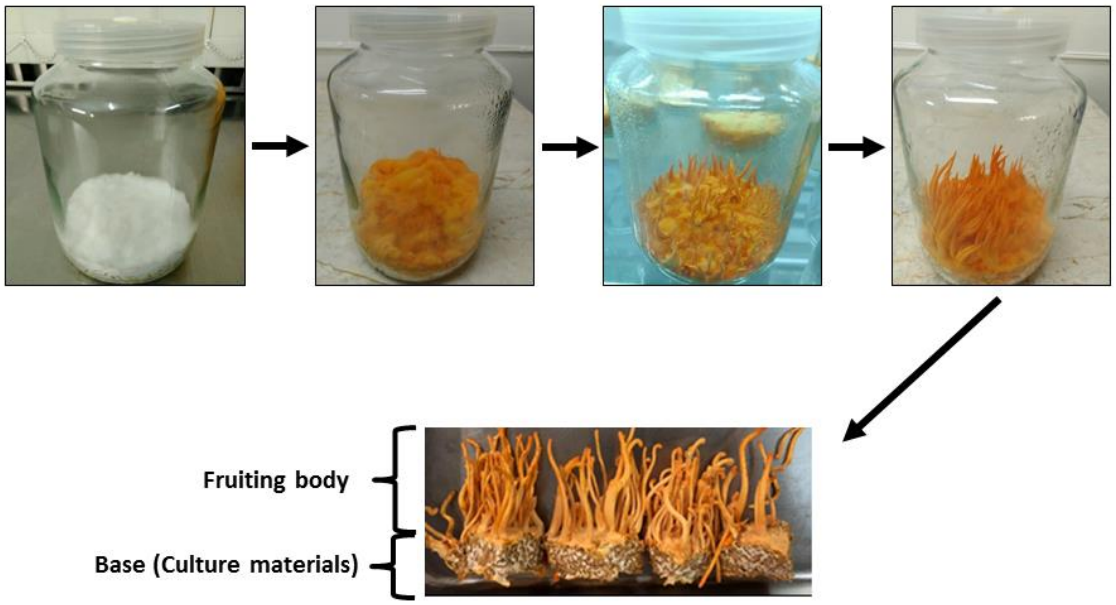


Figure 1. Culture process of *Cordyceps militaris*

Figure 2. *Cordyceps* samples**:** 1-4 culture *Cordyceps* and 5-6 natural *Cordyceps*

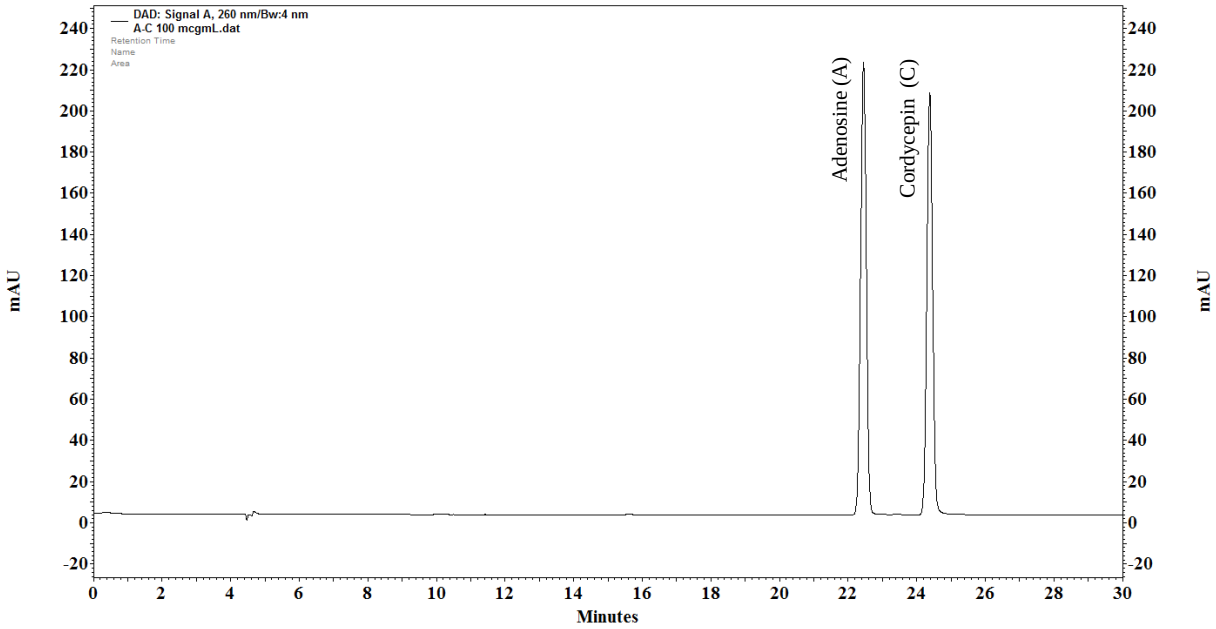


Figure 3. HPLC chromatogram of standard adenosine and cordycepin at the concentration of 100 μg/mL

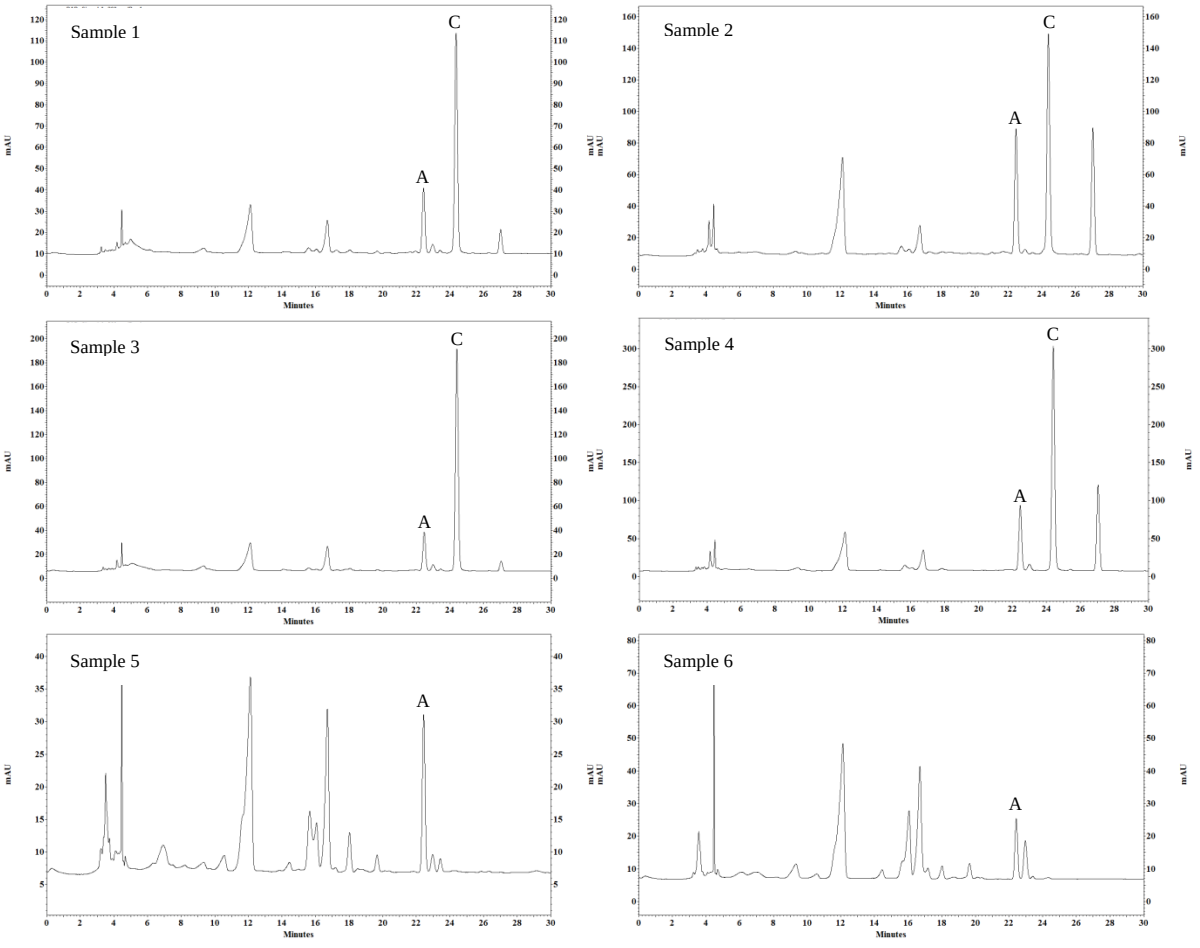


Figure 4. HPLC chromatogram of culture (1-4) and natural (5-6) *Cordyceps* samples

Table 1. Contents of adenosine and cordycepin in culture and natural *Cordyceps*

Samples	Adenosine (mg/g)	Cordycepin (mg/g)
1 (Culture materials)	0.11 ± 0.00	2.47 ± 0.06
2 (Fruiting body)	0.21 ± 0.00	1.66 ± 0.06
3 (Culture materials)	0.11 ± 0.00	4.75 ± 0.09
4 (Fruiting body)	0.20 ± 0.00	2.95 ± 0.02
5 (Fruiting body	0.90 ± 0.07	-
6 (Fruiting body)	1.76 ± 0.19	-

Extracted ion chromatogram from LC ESI-MS showed major adenosine in positive mode and cordycepin clearly in negative mode for culture *Cordyceps* both in fruiting body and culture medium. The chromatogram pattern was the same elution order for both ACE C18 and Zorbax C18 columns except that the peaks were eluted slightly faster and much sharper. Figure 5 showed representatives of extracted ion chromatogram (EIC) of sample 2 and 5 for culture and natural *Cordyceps*, respectively. Other nucleosides which were identified were uridine and guanosine in both culture and natural *Cordyceps*. The EIC confirmed that there was no cordycepin in both natural *Cordyceps*. There was an unidentified compound (m/z 311) which eluted after cordycepin and found only in culture *Cordyceps*.

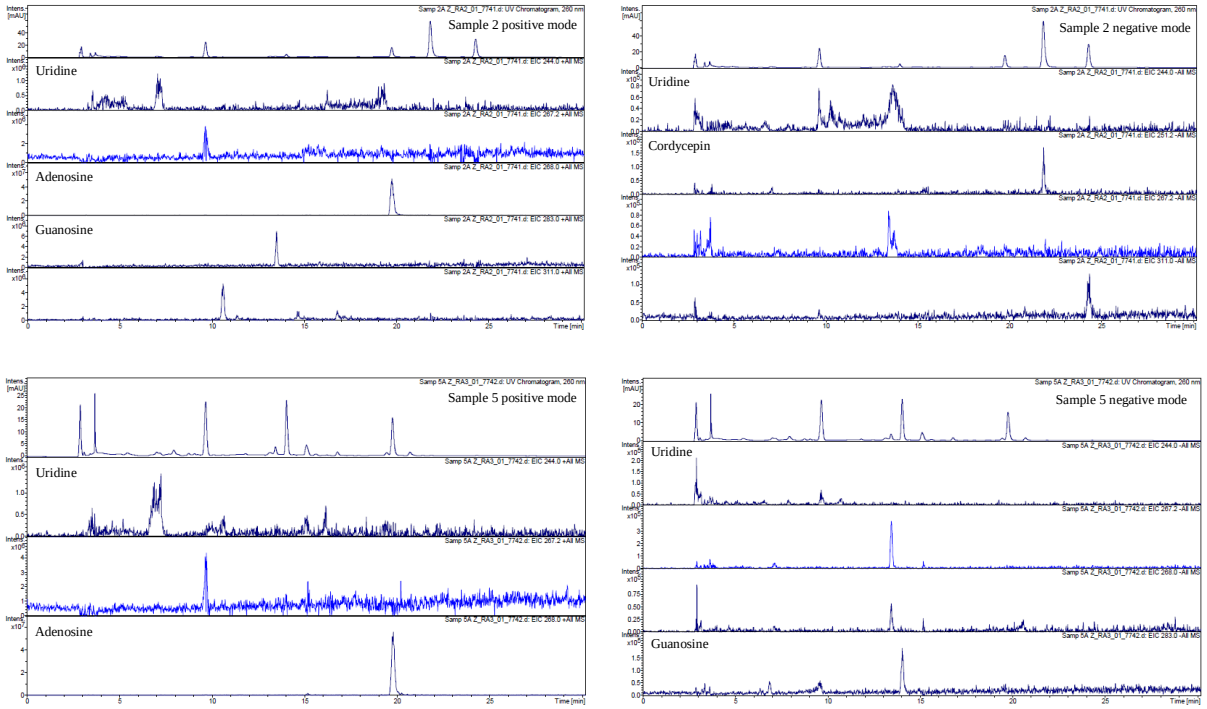
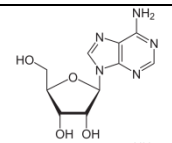
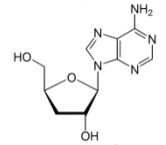
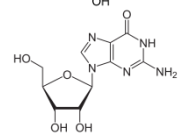
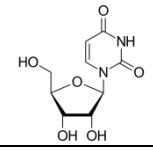


Figure 5. Extracted ion chromatogram (EIC) of culture (sample 2) and natural *Cordyceps* (sample 5)

The molecular ions of identified nucleosides in culture and natural *Cordyceps* were presented in Table 2. The molecular ions of these nucleosides were corresponded to their molecular weights. On the left of Figure 5 showed EIC in positive mode and EIC in negative mode was showed on the right. Adenosine was clearly seen in positive mode while cordycepin was seen in negative mode. Guanosine was observed in both positive and negative modes. Uridine was rarely seen in HPLC chromatogram but its molecular ions were observed. These four identified nucleosides were similar to those reported by Chutvirasakul and et al.,

2016. Inosine was the other nucleoside which was difficult to identify in the low sensitive MS because its molecular weight was 268.

Table 2. Chemical structure, molecular weight and molecular ions of identified nucleosides

Compounds	Chemical structure	MW	$[M+H^+]^+$	$[M-H^+]^+$
Adenosine		267	267.7	265.8
Cordycepin		252	251.7	249.8
Guanosine		283	283.7	281.7
Uridine		244	244.7	242.8

CONCLUSION

This study reported that the contents of adenosine and cordycepin were found in culture *Cordyceps* while only adenosine was mainly observed in natural *Cordyceps*. Some nucleosides such as uridine and guanosine were identified also by ESI MS. The modified HPLC method was suitable for quantitative analysis of nucleoside analogs in *Cordyceps*. It can be applied to analyze *Cordyceps* in dietary products.

ACKNOWLEDGMENTS

The authors acknowledged Prof. Dr. Krisana Kraisintu, Ms. Chitradee Luprasong and Thai Nakorn Patana Co., Ltd. for providing natural *Cordyceps* samples. The authors would like to thank College of Pharmacy, Rangsit University for instrument and facility supports.

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