African Journal of Microbiology Research Vol. 3(12) pp. 957-961 December, 2009
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2009 Academic Journals

Full Length Research Paper

Determination and analysis of cordycepin and adenosine in the products of *Cordyceps* spp.

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Accepted 5 November, 2009

***Cordyceps sinensisis*, a kind of precious natural crude drugs and edible mushrooms, were used as tonic food in East Asia area and enjoyed an extensive praise for its medicinal functions. *Cordyceps militaris*, as a substitute for *C. sinensis*, is a widely distributed species, which can be cultivated in various medium. In this study, the contents of major bioactive components, cordycepin and adenosine in fruiting bodies and mycelia from the nature *C. sinensis* and artificial cultural *C. militaris* were investigated using improved HPLC method. The results showed the mean contents of cordycepin and adenosine in the fruiting bodies of *C. militaris* were 2.654 ± 0.02 and 2.45 ± 0.03 mg/g, those in *C. sinensis* were 0.9801 ± 0.01 and 1.643 ± 0.03 mg/g, while those in the mycelium of *C. militaris* were 0.9040 ± 0.02 and 1.592 ± 0.03 mg/g, respectively. The concentration of cordycepin and adenosine in the fruiting bodies of *C. militaris* were higher than that in natural *C.* sinensis, while the fermented mycelium of *C. militaris* were similar with natural *C.* sinensis.**

Key words: *Cordyceps* spp, high performance liquid chromatography, adenosine, cordycepin, separation condition.

INTRODUCTION

Cordyceps includes several *Cordyceps* species, which are widely used for medicinal purpose or food additives. Among them, *Cordyceps sinensis*, DongChong-XiaCao in Chinese, is a complex of larva corpus of *Hepialus armoricanus*. For several centuries in China, *C. sinensis* was widely used as a kind of tonic food and herbal medicine for preventing or curing various diseases. For example, *C. sinensis* plays an important role in the treatment of respiratory and cerebrovascular diseases, enhancement of body immunomodulatory function and regulation of liver and renal metabolism (Koh et al., 2002; Zhu et al., 1998a, 1998b). Moreover, it also has been used as an antioxidant (Li et al., 2001; Yamaguchi et al., 2000) and antitumor agent (Dai et al., 2000; Zhang et al., 2004; Rao et al., 2007; Russell et al., 2008). *C. sinensis* is only found in the prairie soil at an elevation of 3600 - 5000 m. It is mostly distributed in Tibet, Qinghai, Sichuan, Yunnan and Gansu province in China (Zhou et al., 2009). Due to the limited distribution, high price, over-exploitation and difficulty in artificial culture to obtain mycelium, the resource of *C. sinensis* has been endangered. According to the recent investigation of Chinese Academy of Science, the product of *C. sinensis* had reduced 95% compared to that of 25 years ago, which made it necessary to conduct the substitute research. *Cordyceps militaris* is a widely distributed species, which can be cultivated in various medium. With more extensive distribution and feasibility in artificial culture, *C. militaris* becomes one of the most valuable substitutes of *C. sinensis*. *C. militaris* was approved as New Resource Food by Ministry of Health, People's Republic of China in 2008. Some reports showed that *C. militaris* had similar medical effect with *C. sinensis* in antioxidant (Li et al., 2001) and antitumor (Kima et al., 2008; Lin et al., 2008). Therefore, many products in the market, including various developmental stages of *C. militaris* are all called as Chongcao in China; this gave the public same information with *C. sinensis*.

HPLC is one of the main means in quality monitoring of food and medicine products. It has been widely used in

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variety determination. For the moment, it is acknowledged that the major bioactive components in *cordyceps* are adenosine and cordycepin (Guo et al., 1998; Hsu et al., 2002; Fan et al., 2006). Hitherto, there are various HPLC methods that had been widely used in the determination of adenosine and cordycepin from *C. sinensis* or *C. militaris* (Guo et al., 2006; Li et al., 2006; Huang et al., 2003). In these research reports, the main purpose was to detect the contents of bioactive compounds and establish a standard of quality control for *C. sinensis* and *C. militaris*. However, they have not mentioned that what main components of medium used in the culture of *C. militaris*. The medium have great effect on the accumulation of bioactive components (Gu et al., 2007; Shih et al., 2007). The fruiting bodies of *C. militaris* were cultured in rice medium, silkworm chrysalis medium, and wheat medium, respectively. The purposes of this study were to identify a most valuable substitute from several kinds of fruiting bodies products and fermented mycelia of *C. militaris* and provide scientific foundation for culture of high quality *C. militaris* and quality control of *C. militaris* products on the basis of cordycepin and adenosine quantitative analysis. This study further established foundation for pharmacology experiments of *C. militaris* substituting *C. sinensis*.

MATERIALS AND METHODS

Biological materials

The dried fruiting bodies of *C. sinensis* were purchased from a local health food store. The dried fruiting bodies of *C. militaris*, cultured in rice medium, silkworm chrysalis medium and wheat medium, respectively and the strain of *C. militaris* mycelium were provided by Heilongjiang Xinyisheng Pharmacy Co., Ltd. (China).

Preparation of *Cordyceps militaris mycelia*

Selected medium was used to cultivate *C. militaris* for mycelia, which contained ingredient as follows: 35 g/l sucrose, 5 g/l peptone, 2.5 g/l yeast extract, 0.5 g/l $MgSO_4$, 1 g/l KH_2PO_4 and 0.05 g/l vitamin B_1 (pH 5.2). At first, two agar blocks obtained from a stock culture tube were inoculated into a 250 ml Erlenmeyer flask with 50 ml selected medium and then placed on a shaker at 180 rpm at 28℃ for 4 days. Then 25 ml of above culture was transferred to four 1L Erlenmeyer flasks with 250 ml medium, respectively. The fermentation was performed in these flasks on a 220 rpm rotary shaker at 28℃ for a week. After the fermentation, the mycelium and supernatant was separated from the broth with centrifugation at 6000 rpm for 20 min. Then the fresh mycelium was dried using vacuum freeze drying machine (ALPHA 1-2LD, CHRIST, Germany) for 15 h. The dried mycelium and supernatant were stored in a refrigerator at -20℃.

Extraction of cordycepin and adenosine from different samples

Various samples, under similar method, were prepared to extract cordycepin and adenosine, respectively. Treatment process is shown as following: *C. sinensis* contains two parts, corpse and capillary, which were grinded into powder (diameter at approximate 50 meshes) together in liquid nitrogen. Then, approximate 1.0 g of *C. sinensis* was precisely weighed and added into 10 ml methanol-water (50/50, V/V) in a 50 ml centrifuge tube which was subsequently placed in a ultrasonic machine for extracting cordycepin and adenosine at a power of 75 watt. After the centrifugation, the sample extraction procedure was repeated another twice. Supernatant obtained from the three times centrifugation was mixed and exactly measured of its volume. The sample was filtrated through a 0.45 μm filter prior to HPLC analysis. The extraction of cordycepin and adenosine from fruiting bodies and mycelium of *C. militaris* was also carried out in a similar procedure.

Determination of the bioactive components

All HPLC analysis work was carried out on a Waters 2695 Separation Module (Waters, Milford, MA, USA), which consists of a Waters 2996 Photo-diode Array Detector, an auto injector, and a reverse phase column (Waters Symmetry Shield RP 18/4.6 × 150 mm, 5 μm). Standards of cordycepin and adenosine were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). The standard adenosine and cordycepin solvent was consecutively injected five times to draw calibration curves. The injection volume was 2, 4, 8, 16 and 22 μl, respectively. The determination condition of the samples was set as follows: the mobile phase adopted in the analysis consists of water and methanol were in the ratio 85:15(V/V), 90:10(V/V) and 92:8 (V/V), respectively. The separation was conducted in isocratic elution with a flow rate of 1.0 ml/min. The detection wavelength of photo-diode array was set at 210 - 400 nm and the column temperature was 30℃. The injection volume was 10 μl.

Data analysis

Data collection and analysis was performed using Empower PDA software (Waters Corporation). The results were shown as the means of three replicates.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

In this study, the effect of chromatographic condition on the separation was investigated; especially flow rate and mobile phase. The separation was compared when using different solvent as mobile phase, such as methanol, acetonitrile, ethanol and buffer salt solution. The results shown the best separation was obtained under a specific concentrations of methanol and water. Based on the flow rate of mobile phase, the elution time was determined. It was found that 1.0 ml/min was a proper flow rate. As cordycepin and adenosine are polarity organic matters, with the increase of percentage of methanol in mobile phase, the difference of retention time of cordycepin and adenosine become small which resulted in a poor separation. When using water-mathanol (85:15, V/V) and water-mathanol (90:10, V/V) as mobile phase, the retention time was less than two minutes. Considering both the elution time and retention times, flow rate of 1.0 ml/min and mobile phase of water-methanol (92:8, V/V) was the optimized chromatographic condition used for all following analysis (Figure 1).

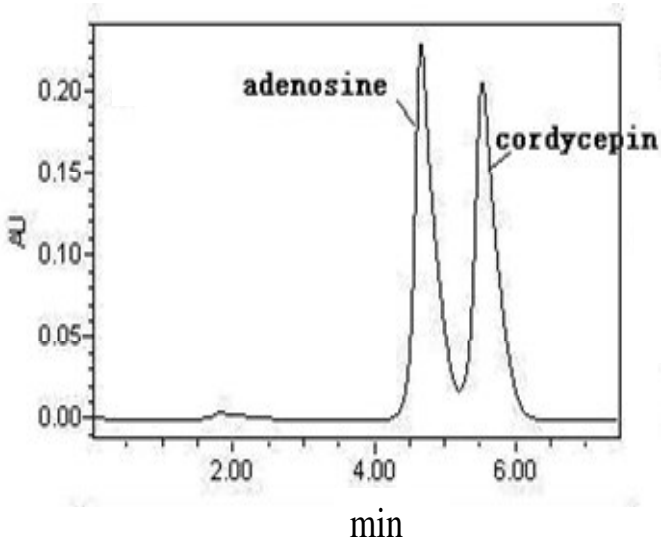

Figure 1a. Retention time of adenosine and cordycepin under different percentage of water and methanol in mobile phase; water-methanol (85:15, V/V), retention time of adenosine and cordycepin 4.668 and 5.553 min, respectively.

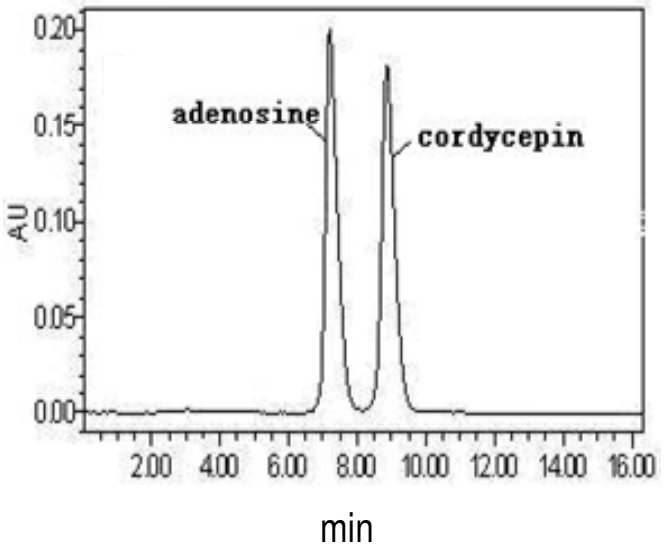

Figure 1b. Retention time of adenosine and cordycepin under different percentage of water and methanol in mobile phase; water-methanol (90:10, V/V), retention time of adenosine and cordycepin 7.204 and 8.864 min, respectively.

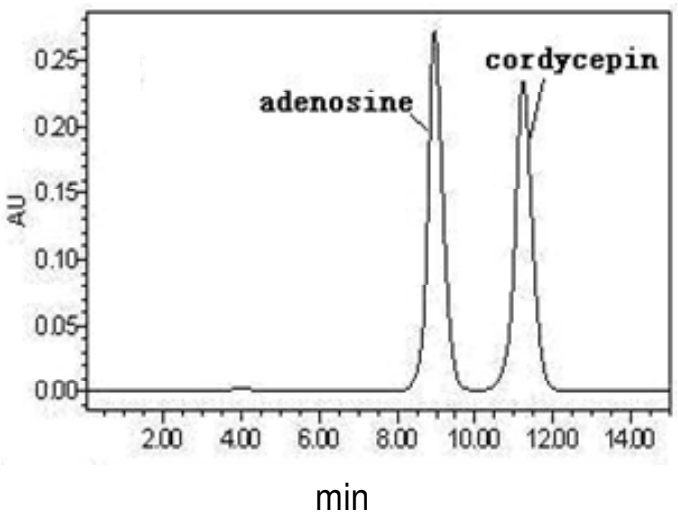

Figure 1c. Retention time of adenosine and cordycepin under different percentage of water and methanol in mobile phase; Water-methanol (92:8, V/V) Retention time of adenosine and cordycepin 8.955 and 11.230 min, respectively.

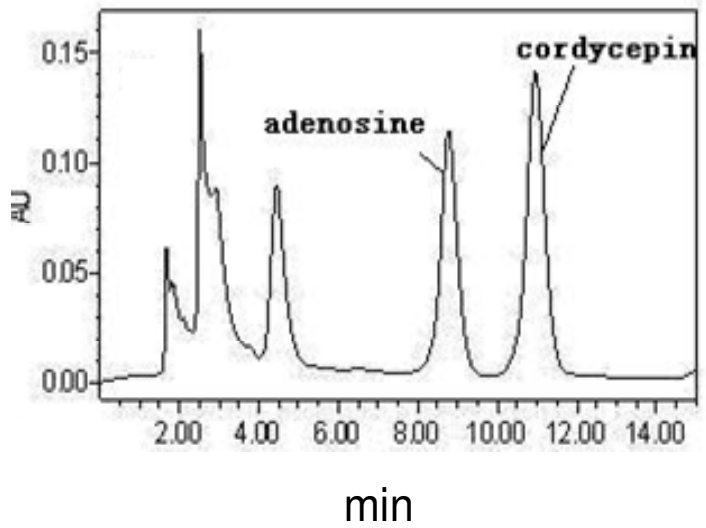

Figure 1d. Retention time of adenosine and cordycepin under different percentage of water and methanol in mobile phase; A *C. militaris* sample separated under mobile phase consisting of water-methanol (92:8, V/V).

HPLC is one of the main means in quality monitoring of food and medicine products. Under an ideal chromatographic conditions including flow rate, mobile phase, detection wavelength and column temperature, the elution time is short and it has a good separation between analyses. The flow rate had great effect on the elution time. Such that when the rate was too low, the separation between the analyses experience a low-efficiency; when efficiency; when too high, the elution time becomes too short and the peak of cordycepin and adenosine could not be separated from interference peaks caused by other matters in the sample usually appearing mainly in the beginning 5 min. At a flow rate of 1.0 ml/min, the elution time was shorter than 15 min and the retention time of analyses was later than 6 min, which was proper condition for the determination of adenosine and cordycepin in *C. sinensis* and *C. militaris.* As for the separation of cordycepin and adenosine, various mobile phases had

been used including phosphate buffer-methanol, water-acetonitrile, methanol- formic acid and gradient elution. However, the separation was not very good and they have close retention times because of their similar structure. On the basis of our experiment, cordycepin and adenosine could be separated when using methanol and water as mobile phase. In this study, we established an optimized chromatographic condition with a mobile phase water-methanol (92:8, V/V), flow rate 1.0 ml/min and detection wavelength 254 nm, which had good separation on adenosine and cordycepin in *cordyceps* products within 15 min.

Analysis of cordycepin and adenosine from different samples

Under an optimum chromatographic conditions, to the standards of adenosine, the retention time (Tr) was 8.960, limit of detection (based on a signal to noise ratio of 3:1) 0.30 and processed channel (UVλ) 254.0, Linear range 2.50 - 120 µg/ml. To the standards of cordycepin, the Tr 11.23, limit of detection (based on a signal to noise ratio of 3:1) 0.25 and processed channel (UVλ) were 254.0, Linear range 2.85 - 130 µg/ml. Based on the chromatographic conditions, we established the standard curves cordycepin and adenosine standards. The regression equations of calibration curves and their coefficients were calculated as follows: for cordycepin, Y = 3.41136E - 7X + 0.5240 (R = 0.9991); for adenosine, Y= 3.33179E - 7X +0.5243 (R = 0.9992). The separation was conducted in isocratic elution with a flow rate of 1.0 ml/min. The detection wavelength of photo-diode array was set at 210 - 400 nm, and the column temperature was 30℃. The injection volume was 10 µl.

The HPLC analysis results showed the contents of cordycepin and adenosine in the fruiting bodies of *C. militaris* culture on the rice medium, silkworm chrysalis medium and wheat medium were 3.412 ± 0.01 and 2.702 ± 0.02, 1.848 ± 0.01 and 2.090 ± 0.03, 2.720 ± 0.03 and 2.539 ± 0.02 mg/g, respectively. The mean contents of cordycepin and adenosine in artificial cultural *C. militaris* fruiting bodies were 2.654 ± 0.02 and 2.45 ± 0.03 mg/g, respectively; those in *C. sinensis* were 0.9801 ± 0.01 and 1.643 ± 0.03 mg/g, while those in the mycelium of *C. militaris* were 0.9040 ± 0.02 and 1.592 ± 0.03 mg/g, respectively. Among the different products of cultured *C. militaris*, the fruiting body cultivated in rice medium had the highest contents of cordycepin and adenosine. The cordycepin contents in each fruiting body product of *C. militaris* were higher than those in dried *C. sinensis*. And the adenosine's contents in three kinds of fruiting bodies were higher than that in dried *C. sinensis* as well, while both of the cordycepin and adenosine's contents in mycelium of *C. militaris* were similar to those of *C. sinensis*.

There are many other papers which report the similar research work, determination of adenosine and cordycepin as well (Li et al., 2006; Guo et al., 2006; Peng et al., 2008). However, they haven't mentioned that what kind of medium used in the culture or the main components of the medium. The medium have great effect on the accumulation of bioactive components (Gu et al., 2007; Shih et al., 2007), which was confirmed in this study as well. As for the fruiting bodies of *C. militaris*, concentrations of adenosine and cordycepin of rice medium were obvious higher than the other two medium.

In previous research report, the main purpose was to detect the contents of bioactive compounds and establish a standard of quality control for *C. sinensis* (Li et al., 2006; Guo et al., 2006). And a lot of work had been done to illustrate the function and structure of major bioactive ingredients in *Cordyceps* (Li et al., 2001, 2006; Gu et al., 2007). However, this study put emphasis on establishing a most valuable substitute of *C. sinensis* and an optimum medium. We must be aware of that as one of the well known Chinese Traditional Medicine; *C. sinensis* is faced with an extinction crisis. According to contemporary exploitation speed, *C. sinensis* will be used up in a near future. In this study, the analysis had been conducted to detect the concentration of cordycepin and adenosine in several *C. militaris* products cultured in different medium with the purpose of finding an ideal substitute of *C. sinensis* and solving its extinction crisis. From the date shown that the fruiting body of *C. militaris* (rice medium) was highest in the concentrations of cordycepin and adenosine compared to other two fruiting bodies kinds of *C. militaris* (cultured in silkworm chrysalis medium and wheat medium, respectively). So the *C. militaris* fruiting body (rice medium) could be chosen to be the proper substitute of *C. sinensis* based on the comparison of different medium in the concentrations of bioactive components. And the rice medium is the best medium in accumulating adenosine and cordycepin. Mass production of this kind fruiting body will bring large amount of substitute of *C. sinensis.* This study found an ideal substitute and provided a feasible scheme for the solving of *C. sinensis* extinction crisis.

ACKNOWLEDGEMENTS

This research is financially supported by the Shanghai Science and Technology Committee, Shanghai Leading Academic Discipline Project (Project Number: B209) and Heilongjiang Xinyisheng Pharmaceutical Co., Ltd., P. R. China.

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