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(RESEARCH ARTICLE)

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Cordycepin in the fruiting body of *Cordyceps militaris* cultured from 5 different materials in Vietnam: Analysis and comparison

Chung Duong Dinh *

Faculty of Pharmacy, Nguyen Tat Thanh University, 298-300A Nguyen Tat Thanh Street, Ward 13, District 4, Ho Chi Minh City, 700000, Vietnam.

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Abstract

Cordyceps militaris is frequently employed in pharmacology and nutrition for its well-documented health benefits. The advancement of *Cordyceps militaris* farming technology has facilitated the establishment of a reliable supply source capable of effectively meeting the expanding market demand. This study aimed to analyze and compare cordycepin concentrations in the fruiting bodies of *Cordyceps militaris* cultivated from five raw material sources in Vietnam. The results revealed significant variations in cordycepin concentrations among the samples, with average concentrations (measured in mg/g) as follows: Sample 1 (0.8), Sample 2 (1.2), Sample 3 (0.6), Sample 4 (1.0), and Sample 5 (0.9). Notably, *Cordyceps militaris* from Sample 2 exhibited the highest cordycepin concentration, while Sample 3 had the lowest. Differences in cordycepin content between *Cordyceps militaris* samples from different raw material sources may be attributed to variations in manufacturing technology. The results of this study provided the necessary information to evaluate the quality of *Cordyceps militaris* products on the market and propose quality standards for raw materials in the food and pharmaceutical industries.

Keywords: *Cordyceps militaris*; Content cordycepin; Fruiting body; Materials in Vietnam

1. Introduction

Cordyceps mushrooms, belonging to the Ascomycota group, are parasitic fungi with over 680 species, primarily distributed in North America, South America, Europe, and Asia (1). The Ascomyceta genus Cordyceps comprises over 500 species, with *Cordyceps sinensis* and *Cordyceps militaris* being the most extensively researched (2, 3). While *Cordyceps sinensis* grows naturally, cultivating it in vitro is challenging and impossible. Many species of Cordyceps are industrially cultivated for medicinal and nutraceutical products or for refining pharmacologically active compounds, with *Cordyceps militaris* being the most common worldwide (4). *Cordyceps militaris* shares chemical composition and pharmacological properties with *Cordyceps sinensis* (5, 6). *Cordyceps militaris* strains have been shown to produce the highest levels of cordycepin (7), (8), (9), (10), (11).

Cordyceps militaris holds significant economic and medical value (12), (13). Studies have demonstrated various biological activities of *Cordyceps militaris*, including antioxidant capabilities to eliminate free radicals, anti-inflammatory properties to protect blood vessels, immune-modulating effects, antiviral properties, and inhibition of cancer cell proliferation (14).

It is imperative to know what chemicals are in *Cordyceps militaris*, especially cordycepin, adenosine, hydroxyethyl adenosine, and polysaccharides (15), (16). Additionally, chemical analyses have revealed approximately 17 different amino acids in Cordyceps (17), along with D-mannitol, lipids, and various trace elements (Na, K, Ca, Mg, Al, Mn, Cu, Zn, Bo, and Fe, with phosphorus being the most abundant) (18). It contains non-saturated fatty acids, with linoleic acid

* Corresponding author: Chung Duong Dinh

comprising 61.3% of the total fatty acid content [22]. Cordyceps biomass also contains significant amounts of vitamins (100g of Cordyceps contains 0.12g of vitamin B12; 19mg of vitamin A; 116.03mg of vitamin C, as well as riboflavin, vitamin E, vitamin K...), and about 25-30% protein, 8% fat, and mannitol (19). Among its chemical constituents, cordycepin has been reported as a highly promising bioactive compound (20-22).

Cordycepin was first isolated from *Cordyceps militaris* mushrooms in 1950 (23). It can enhance the production of cytokines such as IL-12, IFN-γ, IL-4, and IL-10, playing a crucial role in stimulating the immune system against tumours (24). By stopping the production and release of inflammatory cytokines like IL-1β, IL-6, and IL-18 (25), cordycepin protects experimental mice against kidney problems caused by diabetes. It also prevents hyperglycemia in alloxan-induced diabetic mice (26).

Cordycepin induces apoptosis in human leukaemia cells by generating ROS (reactive oxygen species), disrupting mitochondrial function, activating caspases, and cleaving polypolymerase (27). Cordycepin can be an effective antiviral agent against dengue virus in Vero cells at a concentration of 26.94 μM (27). It has been discovered as a significant new inhibitor of Epstein-Barr virus at concentrations of 125 μM (28). Cordycepin has the potential to combat COVID-19 (29), acting as an inhibitor of the polypolymerase and early protein synthesis processes of the virus and destabilizing the RNA of SARS-CoV-2 (30). Cordycepin has been found to lower the expression of NRP1/CD304 in cells, which may prevent SARS-CoV-2 from invading cancer cells via NRP1 (31) and reversing platelet aggregation in humans (32). It exhibits antioxidant activity (33-35) and anti-inflammatory effects through the NF-κB and Mitogen-Activated Protein Kinase pathways (36). Cordycepin has been found to inhibit MAPKs such as ERK 1/2, JNK, and p38 kinase in BV2 microglia cells to suppress the proliferation of cisplatin-resistant lung cancer cells A549 (37). It also protects against inflammation-induced injury in various diseases, including acute lung injury (34) and rheumatoid arthritis (38). Cordycepin has been revealed to inhibit smooth muscle cell proliferation, reducing vascular constriction (39). Furthermore, it inhibits tyrosinase, showing potential for cosmetics (40). Therefore, *Cordyceps militaris* as an herbal medicine may hold significant value.

Traditionally, cordycepin is primarily derived from wild Cordyceps specimens (38). However, the quantity and efficiency of extraction from wild Cordyceps specimens cannot meet demand due to their low biological conversion rates. Due to the scarcity and value of this natural resource, it cannot meet market demand (41).

Therefore, the development of cultivated Cordyceps as an inevitable trend has seen many successful publications controlling technological factors, cultivation conditions, and techniques such as submerged fermentation (42, 43) or solid substrate fermentation on nutrient bases such as various grains or nutrition sources from silkworm pupae (44-47). Although manufacturers have produced products, there is significant variation in quality factors. However, the level of cordycepin varies depending on the cultivation environment used (48). Therefore, *Cordyceps militaris* is necessary to produce high-quality specimens. Hence, when considering the quality of products originating from Cordyceps in the market, whether natural or artificial, the active ingredients adenosine and cordycepin are used as markers and analyzed to assess product quality. Liquid chromatography methods have been studied, and chromatographic conditions have been proposed to quantify these active components, such as HPLC-Rp (49-52). Vietnam views the development of cultivation and the development of a production chain, supplying products related to Cordyceps to the market as a strategy for agricultural, food, and pharmaceutical development.

Therefore, to seek suitable, safe, high-quality, stable raw materials for health support/pharmaceutical product development directions, this study was conducted to evaluate the quality of post-cultivation products, providing reference materials to propose quality standards for active ingredients (cordycepin) in raw materials for the food and pharmaceutical industries.

2. Material and methods

2.1. Materials

In this study, the materials used include mycelium and fruiting bodies of the *Cordyceps militaris* strain, as illustrated in Figure 1. Sample sources from manufacturers in Vietnam include sample NC1 from Ecordy (lot number: 8938532905055), sample NC2 from L'angfarm (lot number: 8936003720534), samples NC3 to NC5 from Tam An (lot number: 8938528054019), sample NC4 from CordyHappy (lot number: 8936149850133), and samples from Viefarm (lot number: 8936145270119).

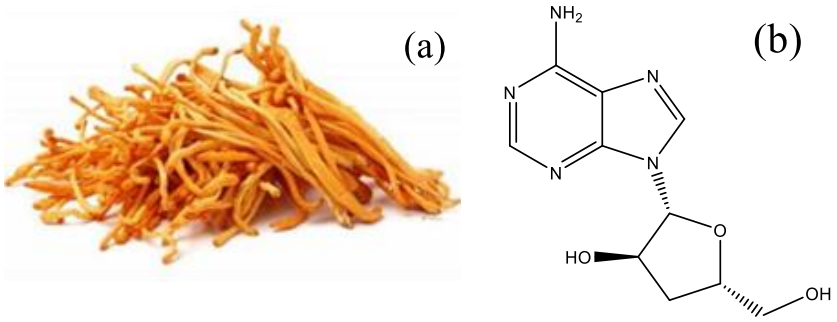


Figure 1 Cordyceps fruiting bodies (a); Cordycepin (b)

2.2. Reagents

Acetonitrile, Ethanol, Methanol (HPLC-grade provided by Merck, Germany), and Cordycepin (≥98%) are supplied by Sigma-Aldrich (Sigma, Singapore). Ultrapure water for chromatography is obtained from a Pall ultrapure water filtration system (USA) and other required chemicals meeting analytical grade standards (Merck, Germany).

2.3. Sample preparation

The sample was prepared using a previously published method with some adjustments (53). Specifically, approximately 100 mg of the test sample (previously ground, sieved through a 180-mesh sieve, and moisture content determined) was transferred into a 50 mL Falcon tube containing about 20 mL of 10% methanol. The mixture was sonicated for 25 minutes (power 200 W, frequency 37 kHz, at 60 oC). Subsequently, the sample was cooled, and the volume was adjusted to 25 mL with the same solvent. Next, the sample was centrifuged for 15 minutes at a speed of 6000 rpm, followed by filtration through a 0.45 µm nylon membrane, and the filtrate was collected for chromatographic injection. The cordycepin content was calculated using the following formula:

$$X = \frac{C_m \times D \times 10^{-3}}{m_{C\hat{a}n}(1 - \mathrm{H})} (\mathrm{mg/g})$$

Where: X: Cordycepin content in the sample (mg/g) Cm: Concentration of the sample measured from the standard curve (µg/ml) D: Dilution factor (25 ml) m_scale: Actual weighing mass (g) H: Moisture content of the sample (%)

2.4. Analytical Method

2.4.1. Moisture assay

The initial moisture content was determined using the loss-on-drying method [8]. Sample NC1: 10.13 ± 0.09; Sample NC2: 10.93 ± 0.12; Sample NC3: 5.27 ± 0.09; Sample NC4: 5.00 ± 0.06; and Sample NC5: 10.13 ± 0.09. The results indicate that the moisture content of the study samples, ranging from 5.0% to 10.9%, falls within the permissible limit (≤ 13%).

2.4.2. Cordycepin assay

Cordycepin in the study samples was analyzed against a standard cordycepin reference solution with a 20 µg/mL concentration. The analysis was performed on an Agilent 1260 chromatographic system equipped with a DAD detector, using a Luna C8 column (150 mm x 4.6 mm; 5µm) for chromatographic separation. The injection volume was 10 µl. Column temperature was maintained at 25 C, and the signal was monitored at a wavelength of 260 nm. The mobile phase consisted of a mixture of methanol and ultrapure water in a ratio of 10:90 v/v, with isocratic elution at a flow rate of 1.0 ml/min (50),(51),(49),(52). The analytical procedure was validated according to the guidelines of AOAC 2016."

2.5. Statistical analysis

The analysis results are presented as mean values with standard deviations.

3. Results

3.1. The analytical procedure validation

The chromatographic procedure was repeated six times with a standard cordycepin sample at a determined concentration of approximately 20 µg/mL. Chromatographic parameters were recorded, including retention time, peak area, and theoretical plate number. The results showed that the relative standard deviation (RSD) values for all chromatographic parameters were ≤ 2.0%, indicating the stability and high compatibility of the system. Therefore, the cordycepin quantification process is suitable and can be applied in research.

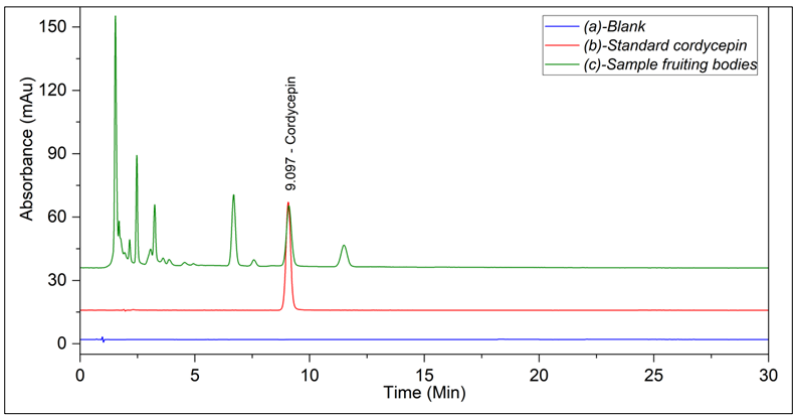


Figure 2 Chromatogram of the test sample under Luna C8 150 x 4.6 mm, 5 µm separation conditions with isocratic elution using methanol-water (10:90; v/v)

Table 2 Results of System Suitability

No.	tR	k'	mAU*S	Sym.	Rs	N
Mean	9.10	5.09	505.16	0.90	6.04	6689
RSD (%)	0.10	0.12	0.29	0.51	1.52	

Note: to – Retention time; k' – Capacity coefficient; Sym.-Symmetry factor; Rs – Resolution and N- Theoretical plates

The results of the linear regression survey have indicated that cordycepin exhibits linear correlation within the concentration range of 2.83 µg/ml to 175.07 µg/ml. with a correlation coefficient R > 0.999. falling within the AOAC's permissible range [29]. The limit of quantification was determined through statistical values from the linear regression and reached 0.64 µg/ml. Figure 3 illustrates the linear regression plot of cordycepin. demonstrating the linear relationship between concentration and peak area.

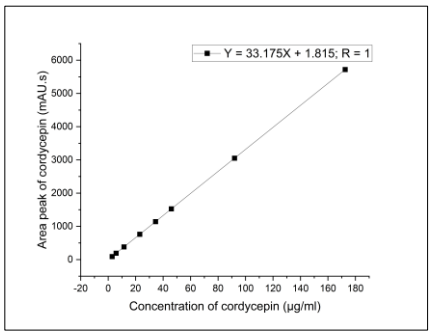


Figure 3 Linear regression plot of cordycepin

The accuracy was assessed through recovery efficiency and determined by adding standard cordycepin to the *Cordyceps militaris* fruiting body samples at three different levels compared to the amount of cordycepin determined in the samples. Subsequently. sample processing. chromatographic analysis. and results were presented in Table 3. The actual

recovery efficiency of cordycepin was determined to be in the range of 94.11% to 104.85%. According to AOAC guidelines for food matrix samples. with content above 0.1%. the recovery rate should be between 95-105%. According to the European Council regulations. with a content above 0.1%. the recovery efficiency should be between 80-110%.

Table 3 Recovery evaluation results

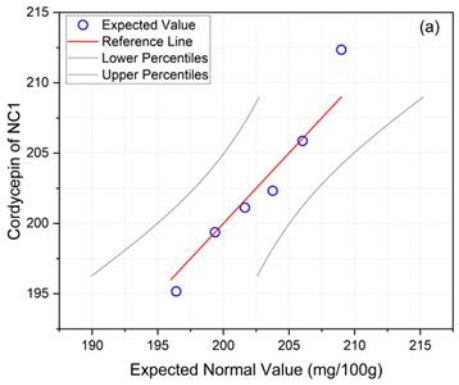
No.	Add (µg/ml)	Initial (µg/ml)	Mean ± SD (%)
Level 1	25.00	4.76	97.11 ± 3.15
Level 2	50.00	4.76	104.85 ± 1.55
Level 3	75.00	4.76	97.61 ± 1.87

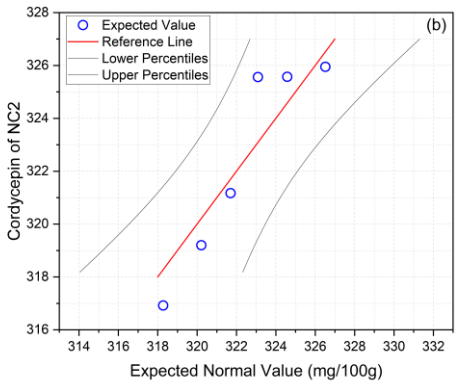
3.2. Quantification of cordycepin

The study was conducted following the analysis procedure on five samples of *Cordyceps militaris* fruiting bodies available in circulation in Vietnam. with each sample being tested six times to calculate the average value. The cordycepin content in the test samples varied significantly from 2.02 mg/g to 3.58 mg/g. The results are presented in Table 4. The cordycepin content was lower than reported by Hur in 2008. where *Cordyceps militaris* purchased in Kyong-dong. Seoul. South Korea. had a cordycepin content of 9.0 mg/g (0.9%) (54). The 2008 study by Hur indicated that the stroma contained 9.7 mg/g of cordycepin (54). Kang et al. 2014 reported a cordycepin content of 9.17 mg/g (44). Yu. Zhao et al. reported in 2006 that cordycepin content in cultivated *Cordyceps militaris* ranged from 3.57 mg/g to 7.23 mg/g (55).

Table 4 Cordycepin content in the study samples

N.o	NC1 (mg/100g)	NC2 (mg/100g)	NC3 (mg/100g)	NC4 (mg/100g)	NC5 (mg/100g)
1	195.17	319.20	358.23	265.76	221.25
2	199.37	325.95	359.89	263.19	224.60
3	202.32	325.57	356.81	262.09	223.12
4	205.87	316.92	362.23	255.89	218.88
5	212.35	325.56	349.76	253.99	227.84
6	201.12	321.17	361.81	260.09	225.55
Mean	202.70	322.40	358.12	260.17	223.54
SD	5.89	3.86	4.59	4.48	3.19
RSD (%)	2.9%	1.2%	1.3%	1.7%	1.4%





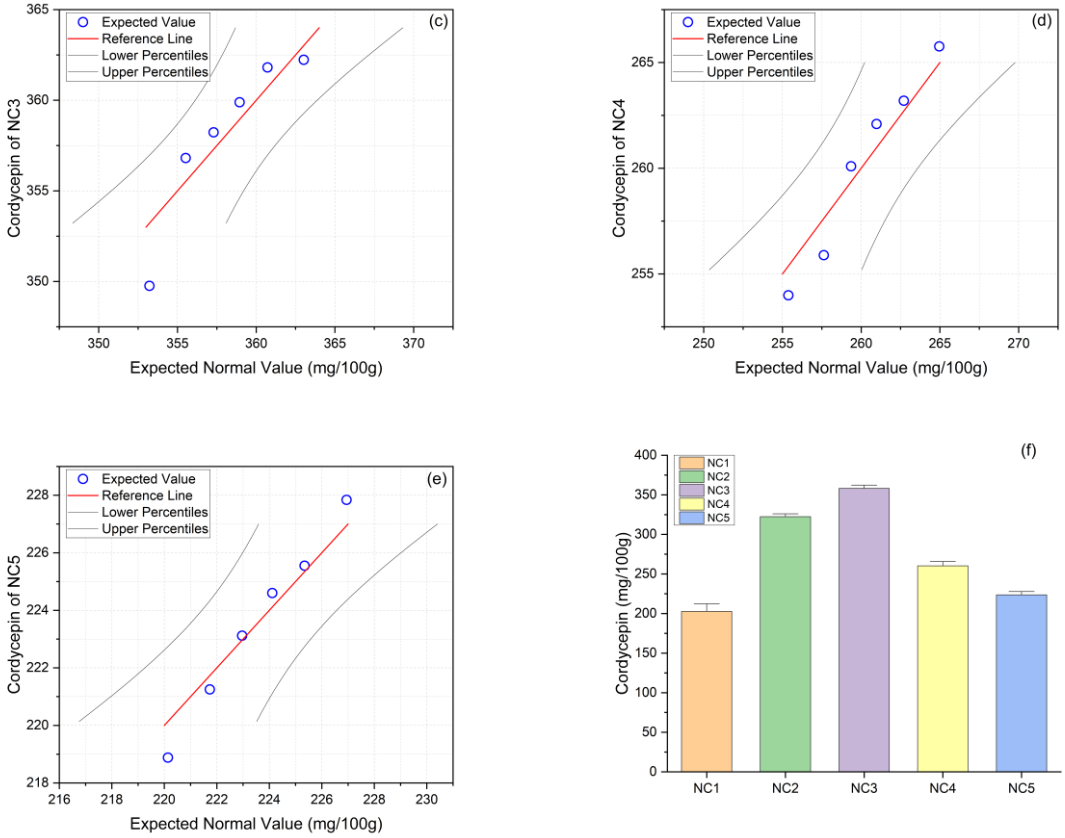


Figure 4 Cordycepin content in the study samples 1-5 (n = 6)

4. Discussion

Cordyceps militaris has become popular and garnered global scientific attention due to its numerous nutritional and medicinal properties (12). (13). In Vietnam. *Cordyceps militaris* mushrooms are primarily commercially used as dried fruiting bodies. Studies have shown that cereal grains and some substrates are better for cultivating *Cordyceps militaris* than insect-based substrates (56). Cereal grains support good development (47). and when combined with silkworm pupae. they create an ideal environment for the growth of *Cordyceps militaris* and secondary metabolites (57). Adding silkworm pupae to cultivation has shown the highest infection rate of *Cordyceps militaris* and the highest yield. The cordycepin content in dried fruiting bodies reaches 5.25 mg/g (58). The stroma formation rate in the silkworm substrate is lower than that in the rice bran substrate. with the highest cordycepin content recorded from strain KSP8 at over 8 mg/g (7). However. research groups focusing on small-scale laboratory production of *Cordyceps militaris* aim to investigate factors influencing cordycepin synthesis.

In contrast. large-scale industrial production considers cost efficiency. substrate efficacy. cultivation time. and scalability. Studies have shown that optimizing nutrient sources for *Cordyceps militaris* cultivation can result in higher quality and cordycepin content. reaching 8.621 mg/g (59) and 9.17 mg/g (44). The influence of light on the growth of *Cordyceps militaris* has been studied. with different LED (60-63) wavelengths tested. yielding the best cordycepin content of 6.1 mg/g. Supplementing glucose. maintaining a pH between 4 and 8. and maintaining room temperature from 15-25°C are essential for increasing yield and quality. Additionally. harvesting after 65 days will yield the highest cordycepin content (64).

The most suitable plant growth regulators. triacontanol and diethyl aminoethyl hexanoate. have been studied to enhance cordycepin content to 6.13 mg/g (65). In a study by Liang et al. 2014. cordycepin activity reached 25.07 mg/g (66).

Furthermore. such variations are attributed to various factors. one of the most crucial being the strain used. As previously discussed. the genetic structure of hypoxanthine 5.45 g/L; và L-alanine significantly impacts the formation

of ascomata and. most importantly. the ability to synthesize cordycepin for each strain. Kang et al. successfully developed a strain of *Cordyceps militaris* (KSP8) in 2017 capable of producing cordycepin levels up to 6.63 mg/g (7).

Finally. because *Cordyceps militaris* is harvested fresh. preserving it for long-term use while maintaining its colour. flavour. and medicinal properties requires selecting an appropriate drying method. as each drying method will affect the quality of the product differently. Research has shown that infrared drying of *Cordyceps militaris* at 50°C retains volatile compounds better than conventional hot air drying at the same temperature. resulting in cordycepin levels below 1.1 mg/g (67). In contrast. Wu. Zhang et al. in 2019 demonstrated that combining infrared drying with freeze-drying at 60°C yielded three times higher cordycepin content (<3.3 mg/g) (68). Cordycepin obtained from *Cordyceps militaris* material processed through freeze-drying is 18.9% higher than material processed through conventional hot air drying. yielding 1.95 mg/g (69).

This study indicates that industrially produced dried fruiting body samples have lower cordycepin content than small-scale experimental studies. However. the results of this research provide scientific data for reference. supporting manufacturers of food/pharmaceutical products from *Cordyceps militaris* materials in selecting raw materials. Our findings suggest actions such as requiring *Cordyceps militaris* and related products to clearly label cordycepin content on the label. along with information on active ingredient content in the main quality target section. This will contribute to providing quality and safe products for consumers.

5. Conclusion

According to the study. the initial evaluation of cordycepin content in the fruiting bodies of cultivated *Cordyceps militaris* mushrooms and dried fruiting body products circulating in the Vietnamese market has been conducted. The findings also serve as a basis for proposing quality standards for *Cordyceps militaris* fruiting bodies as raw materials for the food or pharmaceutical industries.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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