Effect of Time and Temperature on the Extraction of
Cordyceps militaris in Pilot Scale

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Abstract. Cordyceps militaris is a valuable medicinal mushroom that has been interested in research by scientists around the world, which has high value in promoting health, immunoregulatory, antioxidant, anti-inflammatory and cancer prevention. Following our previous research, the extraction process in pilot scales was built on the results of the project Nguyen Tat Thanh University “Research and propose the process of extracting extracts from Cordyceps militaris in Kon Tum province”, which was deployed and accepted in 2020. After evaluating the quality characteristics of raw powder materials, the condition of concentration and preservation of Cordyceps militaris extract was conducted. To achieve the required moisture (<20%), it was necessary to concentrate Cordyceps militaris extract at three standards: 60°C, 70°C and 80°C in 150 minutes, 120 minutes and 105 minutes, respectively. Considering the three standards, it was found that the cordycepin content at 80°C was statistically significantly lower than the remaining two temperature landmarks. Concentrate the extract at 70°C for 120 minutes to obtain a concentrated extract with cordycepin content of 2938.97 ± 94.66 (µg / g DW) with the moisture content of 8.78 ± 1.95 (%).

1. Introduction
Due to the increasing market demand and the confirmed use of Cordyceps, Cordyceps militaris is being cultivated widely in many localities in the country [1]. Recognizing the outstanding values that Cordyceps militaris brings, currently in the world there are many types of research on extracting active ingredients from this precious ingredient. However, the comparative between research in the laboratory and development of a pilot scale extraction technology process is still limited. At the same time, the problem of storage conditions of Cordyceps militaris extract has not been given adequate attention.

In 2014, the study of Hsiu Ju Wang et al. on optimizing the condition for extracting cordycepin from Cordyceps militaris species was based on the experimental design assisted by ultrasound. Four factors: extraction time (min), ethanol concentration (%), extraction temperature (°C) and extraction frequency (kHz) were studied. The results showed that the highest cordycepin yield of 7.04 mg/g (86.98% ± 0.23%) was obtained with an extraction time of 60 min, ethanol concentration of 50%, extraction temperature of 65 °C and extraction frequency of 56 kHz [2].

The optimization of cordycepin extraction conditions from Cordyceps militaris was focused on the study of Lin et al. in 2018 [3]. Cordyceps militaris water extract from surface liquid culture has anti-oral cancer activity. Research in Korea in 2005 evaluated the effect of Cordyceps militaris extract has
a strong antioxidant effect, counteracts durable DPPH free radicals, and has local anti-inflammatory activity of edema in the ear due to oil in rats [9]. *Cordyceps militaris* have high value in promoting health, anti-inflammatory, antioxidant activity and growth of tumors, helping to improve resistance, regulating immunity. In recent years, medicinal properties of this mushroom are scientifically researched and has been published in many specialized journals.

Many scientific works in the world have proven Cordyceps’ ability to prevent and treat cancer. Research by Yoo HS et al. demonstrated that *Cordyceps militaris* methanol extract inhibited the growth of human umbilical vein endothelial cells HUVEC and fibroid tumor HT1080 cells, with concentrations of 100 and 200 mg/L. *Cordyceps militaris* methanol extract inhibited tubular formation of endothelial cells in vitro and in vivo, by inhibiting the growth of B16-F10 malignant tumor cells in mice compared with the control group [10]. Cordycepin, extracted from *Cordyceps sinensis* mycelium, has anti-tumor effects on mouse melanoma cells, human lung carcinoma cells and oral cancer cells, human gallbladder cancer cells. [3]. Cordycepin from *Cordyceps sinensis* is a potential active ingredient in neuroblastoma therapy with activation of caspase-3, PARP, and LC-3 A / B leading to cyclic apoptosis. (apoptosis) and autophagy on the human neuroma cell lines SK-N-SH and BE (2) - M17 [11]. Cordycepin captures the cell cycle at the G0 / G1 phase, inhibits proliferation and induces apoptosis of human lung cancer cells H1975 through the phosphorylation of EGFR, AKT and ERK ½ signaling pathways [4]. In addition, *C. sinensis*’s selenium-rich extract is a potential adjunct in the treatment of cervical cancer [11] or inhibits breast cancer cell metastasis through decreased cytokine expression in the model. mice in vivo [12].

Li Ma et al. studied the cordycepin effects on diabetic mice by alloxan. The results showed that cordycepin in *Cordyceps militaris* did not increase insulin concentration in blood but decreased blood glucose through the effect of increasing glycogen concentration in liver. At the same time, cordycepin has a protective effect on the kidneys and reduces spleen injury due to urination [5]. The therapeutic effect of diabetes from the active substances extracted of *Cordyceps sinensis* is more clearly confirmed with specific analysis of changes in body weight, a significant decrease in blood glucose levels by promoting glucose metabolism and a strong inhibition of serum cholesterol and triglyceride concentrations, and a decrease in blood urea nitrogen, creatinine, uric acid, plasma lipid index, oral glucose tolerance tests, insulin tolerance and concentration plasma insulin levels in a mouse model of diabetes in vivo [3][6].

In 2017, the Center for Science and Technology Progress Application in Kon Tum province (now Research, Application and Service of Science and Technology) implemented the project “Applying scientific and technological advances to building propagation models, production of the fungus *Cordyceps militaris* in Kon Tum province” and has obtained positive results. The project has mastered the technology and successfully built a breeding and production model of *Cordyceps militaris* (*Cordyceps militaris*). The quality of the *Cordyceps militaris* mushroom produced at the Center has a relatively high content of pharmaceutical ingredients, cordycepin content of 3.52 mg / g of fresh cordyceps, adenosine content of 10.40 mg / g of fresh cordyceps.

In 2018, Doan Thi Phuong Thuy and her colleagues studied the factors affecting the extraction process. The optimal condition was selected based on the amount of cordycepin collected, quantified by high performance thin layer chromatography method. It was shown that, by using ethanol 50% as solvent, adenosine and cordycepin could be effectively extracted at 65°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 [7].

Research on the construction of extraction process for adenosine and cordycepin from cultured *Cordyceps militaris* (*Cordyceps militaris*) by Le Thi Huyen Trang et al (2017). Factors affecting extraction efficiency were investigated including: extraction method, solvent type, medicinal / solvent ratio, extraction time, number of extraction times, extraction temperature. Evaluation criteria: extraction efficiency of adenosin and cordycepin from the remedy. Content of adenosin and cordycepin in medicinal herbs was quantified by HPLC in the extract. The results of investigating and selecting the optimal parameters of the *Cordyceps militaris* extraction process: using ultrasonic
extraction, with extraction conditions including: the extraction solvent is water, with the rate of medicinal herbs / solvent is 1:20, extraction at 50 °C, for 45 minutes, the number of extraction times is 2. The process for high extraction efficiency reached 96.85% with cordycepin and 85.99% with adenosin. The obtained investigation procedure is highly stable and repeatable and can be used in standardized extracts from *Cordyceps militaris* cultured to produce medicinal and functional food products [8].

Following our previous research, the extraction process in pilot scales was built on the results of the project Nguyen Tat Thanh University “Research and propose the process of extracting extracts from *Cordyceps militaris* in Kon Tum province”, which was deployed and accepted in 2020. *Cordyceps militaris* in Kon Tum province were extracted with hot extraction method with reflux conduction, 60% ethanol, extracts 1 times and the mixing speed was 300 rpm. Applying RSM CCD optimization software, the optimal condition for extracting cordycepin active ingredient from *Cordyceps* is 70 °C for 28 minutes with a solvent / material ratio of 40 (ml / g). After extraction time under optimal conditions, the amount of cordycepin obtained is 1014.35 ± 9.37 (µg / g dry matter). However, this study is an empirical result in the laboratory, the application of this extraction procedure on a large scale requires adjustment of specifications and appropriate equipment selection.

2. Materials and methods

2.1. Materials and chemicals

*Cordyceps militaris* grown on cultivation base of brown rice were collected from Research, application, and service center for science and technology Kon Tum province (Kon Tum, Vietnam). Prior to extraction, host materials were removed completely. Fresh fruiting bodies or mycelia was freeze-dried and ground into powder (diameter at approximate 5 mm). The dried powder was added into extraction solvent, which was then put into a shaking water bath at different conditions. The heated mixture solutions were filtered using No. 1 Whatman filter paper, and then through a 0.22 µm syringe filter (Merck, Germany). The chemical substances and solvents used in this analysis were bought from Sigma Chemical Corporation (St. Louis, MO, USA).

2.2. Scale pilot with extracting capacity of 10 g/time

*Cordyceps militaris* raw materials will be grinded by a dedicated blender to form a fine powder with the size of less than 0.5mm. Each grinding involves around 30g of raw material. Next, the extraction process was carried out with a 1000ml double neck flask on a water bath fitted with a condenser system. The capacity of each extraction is 10g of raw material. Afforded extracts will be filtered and replenished with sufficient volume with extraction solvent. The cordycepin content in the extract was then recorded.

2.3. Study the effect of temperature and time of extract concentration

Followed by the process of concentration on Heidolph vacuum evaporator system with a volume of 50ml / time in a 100ml rotary evaporator. The pressure was allowed to elevate from 30-60 mmHg and rotation number was 100 rpm. *Cordyceps militaris* extract was examined for the cordycepin concentration at different temperature and time conditions: temperatures: 60°C, 70°C and 80°C, extract times: 90 minutes, 105 minutes, 120 minutes, 135 minutes and 150 minutes. The product after concentration will be assessed for moisture index and cordycepin content (µg / g DW).

2.4. Determination of Cordycepin contents

In mobile phase solution, known amounts of cordycepin were dissolved to give different concentrations for calibration. Samples were extracted in specific condition, and then filtered through a 0.22 µm filter membrane. All HPLC analysis work was carried out on a 1260 Infinity II LC system.
(Agilent, Germany), which consists of a reverse phase column Gemini 5µm C18 Column 250 x 4.6 mm and a guard column Phenomenex C18 (catridge 30 x 4 mm). The mobile step was a blend of pH 6.3 of methanol and potassium dihydrogen phosphate (15:85). Elution was done at a solvent flow rate of 1.0 ml / min, and the volume of injection was 10 µl. A variable-wavelength UV detector (L–4250) at 260 nm was used for detection. All samples were run in triplicates. For drawing calibration curves the standard cordycepin solvent was injected five times in a row.

![Figure 1. Standard curve of cordycepin](image)

2.5. Statistical analysis
The results were shown as the means for three replicates. One way Variance Analysis (ANOVA) was used to compare the mean values. p values below 0.05 were considered as significant difference. Differences among the samples were measured using Tukey's SPSS test. (version 23, IBM, USA).

3. Results and discussion

3.1. Characteristics of raw material Cordyceps militaris
*Cordyceps militaris* fruity powder features: orange - yellow color, characteristic aroma, and fatty taste. *Cordyceps militaris* raw material powder has a moisture content of 9.67 ± 0.25%, suitable for storage and extraction. The pre-extracted *Cordyceps militaris* material has been prepared carefully, but its inorganic content is low, with a total ash of 4.03 ± 0.51% and an acid insolubility of 0.36 ± 0.05%.

3.2. Scale pilot with extracting capacity of 10 g/time
Extracting *Cordyceps militaris* powder at a capacity of 10 g / time by water-bath extraction with a 500ml flask with a volume of 500 ml to obtain an extract with cordycepin content of 1033.53 ± 26.95 (µg / g DW ). The extracts in this phase will be used for further experiments.

| Table 1. Extract of Cordyceps militaris extract at pilot scale |
|---|---|---|
| Num. | Sample mass (g) | Cordycepin (µg/g DW) |
|---|---|---|
| 1 | 10.0356 | 1064.99 |
| 2 | 10.0025 | 1042.48 |
| 3 | 10.2640 | 1001.24 |
| 4 | 10.2530 | 1025.41 |
| Average ± SD | 10.1388 ± 0.139 | 1033.53 ± 26.95 |
3.3. Study the effect of temperature and time of extract concentration

*Cordyceps militaris* extracts were investigated for concentration under different conditions and evaluated based on moisture index and cordycepin content. Research results are presented in detail in Figures 1 and 2.

![Figure 1](image_url)

**Figure 1.** The effect of temperature and time of extraction of the extract on moisture.

When the temperature of 60°C is selected for concentration, *Cordyceps militaris* extract takes longer to reach the moisture content of the concentrate (below 20%). Specifically, when the time increases from 90 to 135 minutes, concentrated extracts achieve moisture in the range from 66.15% to 97.43%. Continue concentration to 150 minutes at a temperature of 60°C to obtain a concentrated concentrate with a moisture content of 11.33 ± 2.6 (%).

Similarly, the graph showing the humidity at 70°C and 80°C also tends to decrease with increasing time, but the time to reach the required high is shorter than at 60 °C. At 80°C, it only takes 105 minutes to achieve a humidity of 6.82 ± 1.73 (%).

Based on the above research results, to achieve the required high humidity, it is necessary to concentrate the *Cordyceps militaris* extract at the landmarks of 60°C, 70°C and 80°C with 150 minutes, 120 minutes, and 105 minutes respectively.
Figure 2. Effect of temperature and time of extract concentration on cordycepin content (µg / g DW).

The concentration of cordycepin obtained under the condensed conditions had a statistically significant difference (p < 0.05). At the temperature of 60°C, the cordycepin content changed over time and reached the highest concentration of 2823.64 ± 75.6 (µg / g DW). In the temperature range of 70°C and 80°C, the cordycepin content tends to increase steadily over time and inversely proportional to the humidity.

Considering the three standard highs of 60°C at 150 minutes, 70°C high at 120 minutes and 80°C high at 105 minutes, it was found that the low cordycepin content was statistically significant at the 80°C mark, with the remaining two temperature landmarks. The concentration of cordycepin at the mark of 60°C and 70°C was not significantly different, that is, choose one of the two conditions to conduct the concentration to have the same quality. However, there is a big difference between the concentration time at the two temperature points, the choice of the temperature of 60°C will have disadvantages such as the concentration time is more than 1.25 times longer; costs incurred for fuel and waste of machinery materials, ... In addition, due to limited time, the study only evaluated two indicators of moisture and cordycepin content in extracts, the selection of Concentration temperature of 60°C with a duration of 150 minutes may appear more degenerate ingredients affecting the quality and activity of extracts. For the above reasons, the research team chose concentration conditions to conduct the next experiments of 70°C with a time of 120 minutes.
4. Conclusion

*Cordyceps militaris* fruity powder features: orange - yellow color, characteristic aroma, and fatty taste. *Cordyceps militaris* raw material has a moisture content of 9.67 ± 0.25%, suitable for storage and extraction. The pre-extracted *Cordyceps militaris* material has been carefully prepared, but its inorganic content is low, with a total ash of 4.03 ± 0.51% and an acid insolubility of 0.36 ± 0.05%.

Extraction at pilot scale with capacity of 10g / time produces an extract with cordycepin content of 1033.53 ± 26.95 (µg / g DW). To achieve the required high humidity, it is necessary to concentrate *Cordyceps militaris* extract at 60°C, 70°C and 80°C at 150 minutes, 120 minutes and 105 minutes, respectively. Considering the three standard highs of 60°C at 150 minutes, 70°C high at 120 minutes and 80°C high at 105 minutes, it was found that the low cordycepin content was statistically significant at the 80°C mark. with the remaining two temperature landmarks. The cordycepin content at the mark of 60°C and 70°C was not significantly different, so one of the two above concentration conditions could be selected. Concentrate the extract at 70°C for 120 minutes to obtain a concentrated extract with cordycepin content of 2938.97 ± 94.66 (µg / g DW) with moisture content of 8.78 ± 1.95 (%).

5. References

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