Screening of extraction conditions by Plackett–Burman design for extraction of *Cordyceps militaris* Cordycipitaceae

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Abstract. *Cordyceps* is the product of a fungal genus that develops on the larvae of insects. Cordycepin, namely 3’-deoxyadenosine, the most significant adenosine counterpart of other *Cordyceps*, has demonstrated numerous and excellent pharmacological activities such as antioxidant, anti-cancer, and antimicrobial. In this research, Plackett – Burman method was used to test six extraction parameters, including time (30-120 minutes), the concentration of solvent (40%-70%), temperature (30°C-85°C), number of extraction (1-3 times), solvent to material ratio (40-200 ml/g) and pH (4-7) for the extraction of *Cordyceps militaris* in Kon Tum Province, Vietnam. Based on Plackett–Burman design, the results illustrated that the time (coefficient = -3.87), temperature (coefficient = -5.32) and solvent ratio to the substance (coefficient = 4.37) were the most important variables influencing Cordycepin extraction. This study identified significant main factors, including time, temperature, and solvent, that could be considered for the next stage of the optimization technique using response surface methodology.

1. Introduction
The value in medicinal plants for their phytochemical components, biological activities and antioxidant function has been studied since long ago [1-4]. *Cordyceps* is entomopathogenic fungi, which belongs to the fungus *Ascomycota* group. More than 350 stime, tpecies of *Cordyceps* have been identified worldwide dependent on the fungus and/or insect host. Previous study has shown that *Cordyceps* have incredible effects such as anti-cancer, reducing stress and antioxidant, anti-fibrotic liver and lung, regulating blood sugar levels, preventing osteoporosis, regulating immune system and strengthening fitness [5]. Generally, *Cordyceps* have specific characteristics such as forms of insect in winter and plant in summer. Their spores may get into a specific live insect. Then, the hyphae develops within the host and destroys the host by feeding. They will spend the winter within the host, finally forming fruiting bodies in the summer on the surface of the corpse of the egg insect [6].

Because of the abundance and extraordinary curative properties of *C. sansensis*, such natural alternatives such as *C. liangshanensis*, *C. militaris*, and *C. cicadicola* are sold in market [7]. A genus specific to the *Cordyceps, C. militaris*, displays the broadest variety of hosts, expanding to 2 orders, 13 families[8]. This fungus presents a broad variety of pharmacological functions, including antioxidant,
anti–stress, antifatigue, and anticancer. The fruit *C. Militaris* contains a number of active components such as polysaccharides, cordycepin, amino acids, fatty acids, and other chemical compounds [9]. Cordycepin is the principal active component of *C. militaris*. In addition, cordycepin has the most biological and pharmacological properties, such as antioxidant, antineoplastic, and anti-inflammatory activity, apoptosis of tumor cells and reduced tumor cell proliferation [10].

However, small cordycepin profitability poses an obstacle to marketing. Since *C. militaris* can be effectively produced in artificial environments, including submerged culture and solid environments, which is the leading candidate for the development of cordycepin. Therefore, there is significant research initiative to increase the yield of cordycepin, which has centered on conditions of production, strain development, biosynthesis process and optimization of cordycepin extraction methods.

So far, in order to screen factors effected extraction condition, the method of alternating each variable is usually used, ie sequentially investigating each element while fixing the remaining elements. The disadvantage is that it is a time and material consuming method and cause difficulty in evaluate the influence of the factors together. Moreover, if the initial fixed value were not suitable, it would lead to falsifical results. Applying the Plackett–Burman method is overcome the above disadvantages. Plackett–Burman design is a relatively new technique used to monitor "key points" in dynamic systems, with utility focused on its capacity to identify potentially important variables within a specific interaction group. While Plackett–Burman design only gives the results of the main factors affecting the extraction process, the specific optimization value is not determined. This is why the next stage of Plackett–Burman design uses a response surface methodology to find the optimization condition for extraction.

In this study, Plackett–Burman design was used to screen six parameters for the extraction of cordycepin from *Cordyceps militaris* Cordycipitaceae in Kom Tum Province, Vietnam, including time (30-120 minutes), concentration of solvent (40%-70%), temperature (30°C-85°C), number of extraction (1-3 times), solvent to material ratio (40-200 ml/g) and pH (4-7).

2. Materials and methods

2.1. Materials
*Cordyceps militaris* grown on brown rice were collected from Research, Application and Service Center for Science and Technology Kon Tum Province (Kon Tum, Vietnam). Only the fruiting bodies were harvested and used to extraction. First, fresh fruiting bodies or mycelia was freezedried and grinded into powder (diameter at approximate 0.5 mm). The provided dried powder was added into extraction solvent and the prepared solutions were put into a laboratory shaking water bath at different condition (Table 1). Second, the heated mixture solutions were filtered using No. 1 Whatman filter paper. Finally, the extracted was determinated cordycepin contents by HPLC method. The chemical substances and solvents used in this analysis including cordycepin, ethanol, acid acetic, amoniac, and so on, which were bought from Sigma Chemical Corporation (St. Louis, MO, USA).

| No. | Variables with designate | Lower (−1) | Higher (+1) |
|-----|--------------------------|------------|-------------|
| 1   | Time (minutes)           | 30         | 120         |
| 2   | Concentration of solvent (%) | 40       | 70          |
| 3   | Temperature (°C)         | 30         | 85          |
| 4   | Number of extraction     | 1          | 3           |
| 5   | Ratio solvent to material| 40         | 200         |
| 6   | pH                       | 4          | 7           |
2.2. Determination of Cordycepin contents
The standard sample contained 20µg/ml cordycepin and was dissolved to give different concentrations for calibration. The extracted samples were centrifuged in 15,000 revolutions per minute, and then filtered through a 0.22 µm filter membrane (Merck, Germany). 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. The mobile step was a blend of methanol and acid orthophosphoric 0.01% (5:95). 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.

2.3. Extraction condition screening using the Plackett – Burman design
Plackett–Burman design was used to test 6 parameters (n=6) for extraction of Cordyceps militaris Cordycipitaceae including time, temperature, pH, concentration of solvent, number of extraction and ratio solvent to material ratio (Tables 1 and 2). Plackett-Burman design includes two-factorial (−1 and +1) design that determines significant variables for the production. Various number or “n” factors can be screened in an ‘n + 1’ run design. The key benefit of saturated designs is the minimal number of measurements required for effect for a specific factor. [11].

2.4. Statistical analysis
The results were shown as the means for three replicates. The statistically designed method is to classify the relevant variables and their corresponding coefficients, such that the rates of variables will produce a desirable output. The results are evaluated using the Student's t – test for statistical significance. If p value < 0.05, it was considered to be significant. The project design, the study and the tests were collected using the software application Design Expert version 11.0.4.0 (Stat – Ease Inc., Minneapolis, United States).

3. Results and Discussion
Precious experiments had utilized several methods of obtaining Cordycepin from C. militaris for scale as hydrothermal reflux, shaking [12], ultrasonic–assisted extraction [13] and microwave–assisted extraction. The previous result showed that hydrothermal reflux had extraction rate marginally lower (95.02%) than ultrasonic extraction (96.12%) [14]. Moreover, the hydrothermal reflux method is much less costly and simpler [15][16]. Thus, our research aimed to extract cordycepin from C. militaris in Kontum provide in the large–scale industrial. Hydrothermal reflux in large-scale industrial active compounds processing is considerably easier to apply [17][18].

Cordycepin is a polar molecule, therefore, to effectively extract cordycepin, the presence of polar solvent in the extract is needed. In most studies, cordycepin was extracted using polar solvent like methanol 100%, methanol, ethanol, and water [13]. Methanol is a polar solvent and extensively utilized in the industry, but it is toxic and hard to completely remove from extract [10][19][21]. In the large–scale industrial, the extracts are usually evaporated to facilitate in transportation, storage and mixing with other ingredients. Water is a useful solvent with low cost and non–toxicity. However, it is needed high temperature, pressure, time to evaporate [22][23][24]. In study of Zhang et al. in 2011, using solvent water and ethanol mixture (water:ethanol=1:1) is better than 100% ethanol [19]. Therefore, water and ethanol mixture were used in our research to extract cordycepin from C. militaris, which could decrease temperature, conserve time for evaporation and maintain essential active component like cordycepin.

The experimental analysis can be seen in Table 2, C. militaris has achieved strong cordycepin yields in run 9 and 10. Based on the design matrix used for screening critical variables, at run 10 the maximum cordycepin was 86.7982 mg/100g and the lowest in run 12 (49.0085 mg/100g) was recorded.
Table 2: PB design matrix of six variables

| Run | Factor 1: Time (minutes) | Factor 2: Concentration of solvent (%) | Factor 3: Temperature (°C) | Factor 4: Number of times extraction | Factor 5: Ratio solvent to material | Factor 6: pH | Cordycepin mg/100g |
|-----|--------------------------|---------------------------------------|---------------------------|------------------------------------|-------------------------------|-------------|------------------|
| 1   | +                        | +                                     | –                         | –                                  | –                             | –           | 50.6962          |
| 2   | +                        | –                                     | +                         | +                                  | –                             | +           | 64.7856          |
| 3   | –                        | +                                     | +                         | –                                  | +                             | –           | 63.4898          |
| 4   | –                        | –                                     | –                         | +                                  | –                             | +           | 67.8528          |
| 5   | +                        | +                                     | –                         | –                                  | –                             | +           | 68.9435          |
| 6   | +                        | –                                     | –                         | +                                  | –                             | –           | 65.8416          |
| 7   | –                        | +                                     | +                         | +                                  | –                             | –           | 67.4721          |
| 8   | –                        | –                                     | –                         | –                                  | –                             | –           | 64.9559          |
| 9   | +                        | +                                     | –                         | +                                  | +                             | –           | 72.6667          |
| 10  | –                        | –                                     | +                         | –                                  | +                             | –           | 86.7982          |
| 11  | –                        | –                                     | +                         | –                                  | +                             | +           | 67.7981          |
| 12  | +                        | –                                     | +                         | –                                  | +                             | +           | 49.0085          |

The Model p value of 0.0399 and $R^2 = 86.94\%$ indicate the model is significant. There is only a 3.99\% chance that a "Model F–Value" this large could occur due to noise. P-value < 0.05 suggests significant model terms. In this research, time, temperature and solvent-to-material ratio are important modeling concepts in this work. Generally, the large volume of solvents more easily dissolves target materials and results in increased extraction yield [13]. The outcomes showed that cordycepin improved dramatically as the concentration of solvent, number of extraction and solvent to substance ratio increased (Figure 1).

Figure 1. Main effects of the medium conditions on *C. militaris* cordycepin extraction.
The specific effect of each factor on cordycepin extraction efficiency calculated by Design expert 11 software is presented in Table 3. As the absolute value of the influence level increased, the stronger the correlation was with the extracted cordycepin content. When the level of influence is positive, the factor and cordycepin content are positively correlated. The extraction period and temperature in the cordycepin extraction process are the key factors, which restrict the yield of cordycepin [25].

Coefficient of time extraction was $-3.87$ with $p$-value $=0.0489$. This effect may be attributed to the active ingredients not dissolving as the solubility of dissolving–out compounds becomes saturated with a rise in the extraction period. In contrast, the loss of cordycepin decreased with the viscosity of extracts when the extraction time increased [13].

It is not surprising that temperature appeared to be the most contributing variable during cordycepin extraction from *C. militaris* based medium with the highest percent contribution and $p$-value of 0.0162 (Table 3). Extraction temperature may have a significant impact on bioactive product recovery during liquid-solid extraction. In the study of Hsiu Ju Wang et al. (2014), cordycepin extraction yields increase as the extraction temperature rises from 30 to 60°C and decreases when the temperature is over 70°C [13]. Similar to other studies like Hsiu Ju Wang (2014) [13], Zhang Hong (2011) [19] and Wencheng Zhang (2017) [25], the current study indicated that the higher temperature, the lower cordycepin extraction. Growing the extraction medium temperature can increase the solvent's diffusiveness in the cells. However, it can contribute to faster volatilization of the solvent, higher energy consumption and impurity extraction. Moreover, the high temperature could impulse decomposition process of cordycepin, which is a sensitive purine alkaloid.

Table 3. Coefficient of each variable, confidence interval (CI) at 95% confidence level based on ‘t’ statistic and sum of the squares for extraction of cordycepin in 6 variable Plackett–Burman design

| No. | Variables with designate                  | Coefficient | 95% CI       | Sum of squares | p-value |
|-----|------------------------------------------|-------------|--------------|----------------|---------|
| 1   | Time (minutes)                           | $-3.87$     | $-7.71$      | $-0.03$        | 179.61  | 0.0489* |
| 2   | Concentration of solvent (%)             | 2.49        | $-1.36$      | 6.33           | 74.12   | 0.1572  |
| 3   | Temperature (°C)                         | $-5.32$     | $-9.16$      | $-1.48$        | 339.29  | 0.0162* |
| 4   | Number of extraction                     | 2.24        | $-1.60$      | 6.08           | 60.12   | 0.1944  |
| 5   | Ratio solvent to material                | 4.37        | 0.53         | 8.21           | 229.26  | 0.0328* |
| 6   | pH                                       | $-0.90$     | $-4.74$      | 2.94           | 9.70    | 0.5736  |

*Variables showed significant effects on cordycepin extraction.

4. Conclusion
One of the well-known therapeutic entomopathogenic fungi, *Cordyceps militaris* has been commonly used to treat different diseases. The experimental methodological design provides an effective approach for determining the relevant variables. The research identified the main extraction conditions that affect the process of extracting from *Cordyceps militaris* based on the Plackett – Burman design. Six factors were selected for the evaluation: time, concentration of solvent, temperature, number of extraction, solvent to material ratio, and pH. The design survey model of main factors affecting cordycepin extraction has good predictability, statistically significant with $p$-value $=0.0399$ and $R^2 = 86.94\%$. Based on the results of the Plackett - Burman matrix, the three main factors affecting the cordycepin extraction process were time extraction (coefficient $=-3.87$), temperature (coefficient $=-5.32$) and solvent ratio to the substance (coefficient $=4.37$) ($p<0.05$). These significant factors found could be considered for the next stage of optimization technique using response surface methodology.

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