

Research Article

Ultrasound-Assisted Enzymatic Extraction of Adenosine from Vietnamese *Cordyceps militaris* and Bioactivity Analysis of the Extract

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Vietnamese *Cordyceps militaris* (*C. militaris*) has long been recognized as one of the most valuable traditional Chinese medicines. In this study, adenosine was extracted from Vietnamese *C. militaris* by ultrasound-assisted enzymatic extraction method (UAEE) using water as a solvent. Then, the effects of five single factors on adenosine content including pH, enzyme-to-material ratio, ultrasonic power, ultrasonic time, and ultrasonic temperature were determined. After that, three factors consisting of ultrasonic power, ultrasonic time, and ultrasonic temperature were chosen based on their effects on adenosine content. The simultaneous influence of these factors on the adenosine content was investigated by response surface method using central composite design. The adenosine content was evaluated by high-performance liquid chromatography method. Under the optimal conditions, the extract was evaluated for antioxidant and anticancer bioactivities. In addition, different extraction methods including aqueous extraction (AE), ultrasound-assisted extraction (UAE), and enzyme-assisted extraction (EAE) methods were carried out to compare with UAEE. As a result, it can be concluded that UAEE is a promising method for adenosine extraction and further studies regarding isolation and purification need to be conducted.

1. Introduction

For over millennia, fungi were used as crude drugs in Asian countries, especially China. Belonging to Clavicipitaceae Ascomycotina, there are over 400 *Cordyceps* species that have been studied due to their valuable bioactivities [1, 2]. With such species diversity, *Cordyceps* (*C. militaris*) was reported to distribute all over the world, except for Antarctica, from subtropical to tropical regions [3, 4]. *C. militaris* is an entomopathogenic fungus which remains dormant in the soil until host insects get in contact with it [5]. With the discovery of many potentially bioactive

compounds from *C. militaris* such as cordycepin, cordycepic acid (d-mannitol), adenosine, polysaccharides, and proteins, many studies have been carried out to determine the artificial cultivation and medicinal uses for this fungus [6].

Adenosine, whose molecular formula is $C_{10}H_{13}N_5O_4$ and molecular weight is approximately 267.245 g/mol, is a major nucleoside found in *Cordyceps*. Figure 1 shows the chemical structure of adenosine. As an energy transfer and signal transducing in cells, adenosine was recognized for its ability of cytoprotection or prevention of tissue damage with a range of bioactivities such as anti-inflammatory, anti-cancer, and antioxidant properties [7].

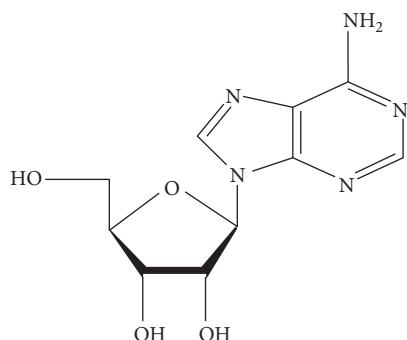


FIGURE 1: Chemical structure of adenosine.

Several traditional and modern adenosine extraction methods have been recorded. The conventional methods such as maceration, infusion, percolation, decoction, and Soxhlet extraction, besides their low cost, bear the disadvantages of low performance, hazardous and flammable organic solvents requirement, and long extraction time [8]. To avoid these undesirable drawbacks, nontraditional methods were applied frequently, including supercritical fluid extraction method, pressurized liquid extraction method, microwave-assisted extraction method, ultrasonic extraction method, and hollow-fiber membrane extraction method [9]. Among these techniques, ultrasound-assisted enzymatic extraction (UAEE) has been a common option due to low solvent volumes requirement, short extraction time, and performance enhancement of the main products [10]. In this study, the adenosine content was studied by using response surface methodology (RSM) with the employment of central composite design (CCD) to investigate the influence of extraction conditions on adenosine content from Vietnamese *C. militaris*. The extract under optimum conditions was tested in terms of anticancer and antioxidant activities.

2. Materials and Methods

2.1. Materials. Dried Vietnamese *C. militaris* was purchased from LINH CHI NONG LAM cultured in Center for Technology Business Incubation-Nong Lam University (CTBI-NLU). Dried Vietnamese *C. militaris* was well ground in a blender after achieving the 14 percent of moisture content and sieved to obtain particle size of 750 μm [11]. Besides, 98% standard adenosine was provided by Biopurify Phytochemicals Ltd, China, 99.5% ethanol was purchased from Chemsol, Vietnam, and methanol for high-performance liquid chromatography (HPLC) grade was purchased from Merck, India. Viscozyme, pectinase, and Alcalase were purchased from Novozymes, Denmark; phosphoric acid, sodium dihydrogen phosphate dehydrate, disodium hydrogen phosphate dodecahydrate, and ascorbic acid were provided by Xylong, China. Double distilled water is used in all experiments. All chemicals were used without further purification.

2.2. Comparison of Different Extraction Methods

2.2.1. Aqueous Extraction. The extraction of Vietnamese *C. militaris* powder was carried out under the extraction condition of ultrasonic temperature of 50°C.

2.2.2. Enzyme-Assisted Extraction. The extraction of Vietnamese *C. militaris* powder was carried out under the extraction conditions including ultrasonic temperature of 30°C and enzyme-to-material ratio of 200:1 $\mu\text{L/g}$.

2.2.3. Ultrasound-Assisted Extraction. The extraction of Vietnamese *C. militaris* powder was carried out under the extraction conditions including ultrasonic temperature of 50°C, ultrasonic time of 120 min, and ultrasonic power of 480 W.

2.3. Selection of Appropriate Enzyme for Adenosine Extraction Procedure. The influences of pectinase, Viscozyme, and Alcalase on adenosine content were compared according to the adenosine content evaluation of extracts so as to select the most appropriate enzyme for adenosine extraction procedure.

2.4. Sample Preparation. The phosphate-citrate buffer was prepared to adjust the pH of the solution. The mixture of 1.00 g of Vietnamese *C. militaris* powder, 25 mL of the phosphate citrate buffer solution, and 200 μL of enzyme was mixed in 100 ml Erlenmeyer flask and sonicated for 120 min in the ultrasonic bath (Transicom Engineering, 1200 W, 40 KHz, Singapore). Then, the enzyme was denatured by putting the mixture into hot water bath at about 80°C–85°C for 2 min, followed by the addition of 25 mL of water. Subsequently, the mixture was kept under shaking conditions for 2 hours using a shaker. Before obtaining the final liquid phase, the mixture was filtered using a 110 mm filtration paper. The extract was collected after the removal of solvent using a vacuum rotary evaporator.

2.5. Effect of Single Factors on the Adenosine Content. In this study, the effects of five single factors, including pH, enzyme-to-material ratio, ultrasonic power, ultrasonic time, and ultrasonic temperature on adenosine content, were investigated. Main factors were appraised according to their influence on the adenosine content.

2.6. Effect of Multifactors on the Adenosine Content. Simultaneous effects of ultrasonic temperature, ultrasonic time, and ultrasonic power on adenosine content were investigated at three levels as shown in Table 1. Statistics was processed using Design-Expert software version 11.0 to build the regression model and graphs. The extraction conditions to obtain the highest adenosine content were determined from the regression model.

2.7. HPLC Method. The adenosine content was evaluated by HPLC method. Calibration curve preparation and sample analysis were implemented by the Agilent (1200 Series, Agilent Technologies Inc., Palo Alto, Calif., USA) system with Pursuit XR_s C18 reversed phase column (250 mm \times 4.6 mm, 5 μm) at CEPP Lab.

TABLE 1: Investigated levels of extraction conditions.

Conditions		Encoded levels		
		-1	0	+1
Ultrasonic time (min)	X_1	100	120	140
Ultrasonic temperature ($^{\circ}\text{C}$)	X_2	45	50	55
Ultrasonic power (W)	X_3	360	480	600

Adenosine concentration in the extract was determined by HPLC method. The extract was diluted three times using methanol as solvent and filtered through $0.45\ \mu\text{m}$ membrane before being pumped into the injection loop.

Calibration samples are prepared with the adenosine concentrations of 5, 10, 20, 40, 80, and 100 mg/L in methanol. A binary gradient solvent system was used; it consisted of solvent A being water and solvent B being methanol. The program was set up based on the elution profile with the following proportions (v/v) of solvent B: 0–30 min, 10–40%; 30–40 min, 10%. The injection volume was $20\ \mu\text{L}$, the column temperature was kept unchanged at 28°C , the flow rate was 1 mL/min, and the detection wavelength was set at 260 nm.

2.8. Bioactivities of the Extract

2.8.1. Antioxidant Activity. The antioxidant activity test was conducted using 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay. First, 0.2 mM of DPPH solution was prepared in distilled water. Then, the extract was diluted in water to obtain six solutions with the concentrations of 0.15, 0.25, 0.35, 0.45, 0.55, and 0.65 mg/mL, respectively. Subsequently, 7.00 mL of DPPH 0.2 mM was mixed with 3.00 mL of sample solution, before being incubated at room temperature in 30 min, and the absorbance was determined at a wavelength of 517 nm. Ascorbic acid was used as a positive control sample. The IC_{50} values of both ascorbic acid and the extract were evaluated from the regression model of sample concentration and radical scavenging activity.

2.8.2. Anticancer Activity. Testing samples were sent to room 601, 18 A of Laboratory of Applied Biochemistry, Institute of Chemistry, Vietnam Academy of Science and Technology located at Hoang Quoc Viet street, Cau Giay district, Ha Noi, to have anticancer tests conducted.

The anticancer test was carried out using MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The cell toxicity tests were first reported to be used at The National Cancer Institute (NCI), USA, for screening and detecting compounds which have the potential to kill or inhibit cancer cells in vitro. Adenosine extracted from Vietnamese *C. militaris* was tested for anticancer activities on two cell lines, Hep-G2 (liver cancer cells) and MCF-7 (breast cancer cells).

Percentage of cellular suppression ($\%I_{\text{MTT}}$) was calculated according to

$$\%I_{\text{MTT}} = \frac{A_0 - A_m}{A_0} \times 100\%, \quad (1)$$

where A_0 is the optical density of the control sample and A_m is the optical density of the extract.

3. Results and Discussion

3.1. Selection of Appropriate Enzyme for Adenosine Extraction Procedure. Figure 2 presents the influence of different kinds of enzyme, namely, pectinase, Viscozyme, and Alcalase, on the adenosine content in the extracts under the extraction conditions including enzyme-to-material ratio of 200 : 1 $\mu\text{L/g}$, ultrasonic time of 60 min, ultrasonic temperature of 55°C , and ultrasonic power of 480 W.

As can be seen from Figure 2, Alcalase gave the highest adenosine content among the others, whose liability is suited with the previous research [12]. This is due to the fact that as Vietnamese *C. militaris* mainly contains proteins, it is stated that the best protease could be Alcalase with reaction temperature about 55°C in order to hydrolyze the protein residue as well as degrade the other chemical compounds [12]. In addition, Alcalase was reported to have an ability to increase the hydrophilic biocomponent content of the extraction process compared to other enzymes [13]. It is reported that enzymatic category is one of essential factors for the pretreatment process based on the nature of extraction materials [14]. Particularly, Vietnamese *C. militaris* is rich in protein contents and hence the application of proteases such as Alcalase could lead to higher adenosine content compared to the results achieved by using other types of enzymes. Therefore, it can be concluded that Alcalase works effectively in this extraction condition.

3.2. Effect of Single Factors on the Adenosine Content

3.2.1. The Effect of pH Value. The effect of pH value on the adenosine content is shown in Figure 3. As can be seen from Figure 3, the adenosine content increased gradually when pH value rose from 6.0 to 6.5. It was reported that the optimal condition for Alcalase was of alkaline pH range [15]. However, when the pH value reached over 6.5, the adenosine decreased significantly. This was caused by the adenosine breakdown due to Alcalase activities. Moreover, the change in pH value would affect the structure and bioactivities of the enzyme [16]. This result is consistent with reported studies [17]. It is cited that the pH value plays an important role in enzymatic extraction [14]. The pH value could strengthen enzyme activities, which has a positive influence on the process of breaking cell walls leading to the enhancement in the extraction efficacy. However, higher pH value may deactivate enzyme and exert a negative effect on the solubility of solvents and target compounds. Thus, a reduction in adenosine content could be observed.

3.2.2. The Effect of Enzyme-to-Material Ratio. Figure 4 illustrates the effect of enzyme-to-material ratio on the adenosine content. The investigated range of enzyme-to-material ratio was from 150 to 350 $\mu\text{L/g}$. It is clear that when the enzyme-to-material ratio increased from 150 to 200 μL , the adenosine content rose noticeably and then went down sharply as the enzyme-to-material ratio increased. In terms of biochemical aspect, it is stated that enzyme could work efficiently with a small amount compared with the material

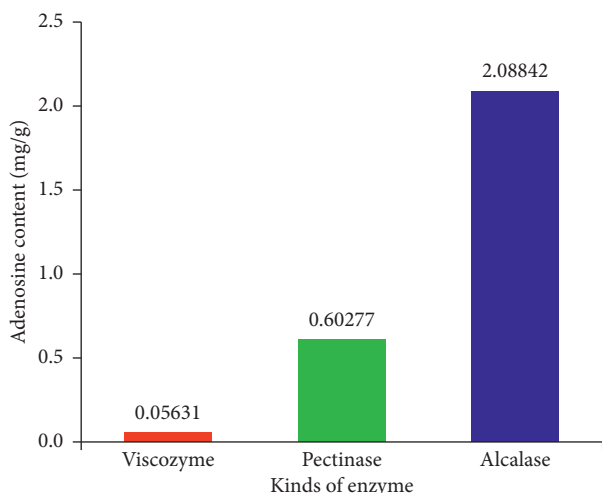


FIGURE 2: Effect of different kinds of enzymes on adenosine content.

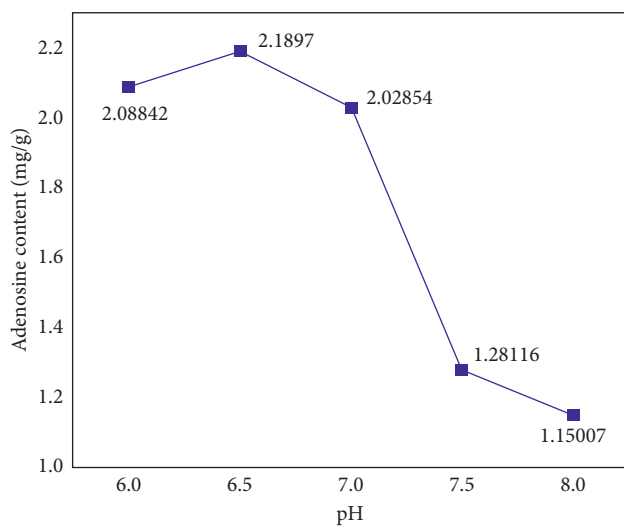


FIGURE 3: Effect of the pH values on adenosine content.

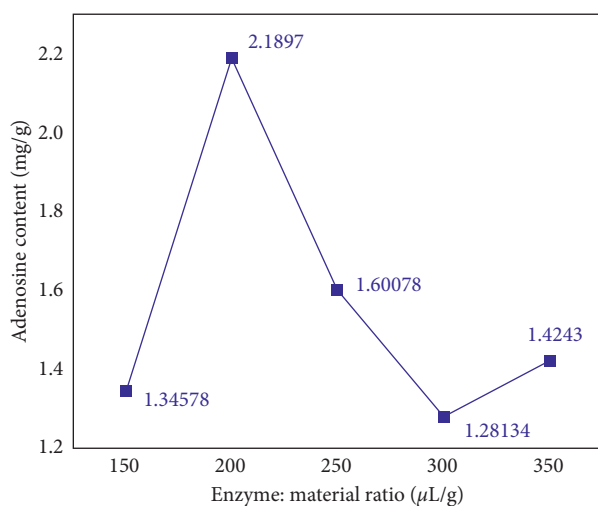


FIGURE 4: Effect of enzyme-to-material ratio on the adenosine content.

mass. Therefore, due to the strong correlation between enzyme-to-material ratio and hydrolysis time, using a proper amount of enzyme and hydrolysis time, good results could be obtained [18]. Normally, each enzyme has a specific limit active ratio. When the concentration reached certain values, the enzyme could no longer assist the extraction process [17]. The results suggest that the overuse and insufficiency of enzyme quantity could induce undesirable results. This might relate to the impacts of other factors like hydrolysis time and temperature on enzyme activities. Although the enzyme causes degradation of cell walls, improving extraction efficacy, it could degrade target compounds when overused or it could not break plant barriers completely when used with inadequate amounts.

3.2.3. The Effect of Ultrasonic Power. Figure 5 presents the effect of ultrasonic power on the adenosine content. In this study, the ultrasonic power ranged from 240 to 720 W. The adenosine content kept enriching in content as the ultrasonic power ran from 240 to 480 W and peaked at its maximum value when the ultrasonic power was 480 W. An explanation for this phenomenon is that when the ultrasonic power was under 480 W, the adenosine content was low because of the inadequate power used to break or expanse the cell. Consequently, the higher ultrasonic power leads to the breakdown of plant tissue and cell walls owing to the influence of gas invasion generated by the ultrasonic waves, the diffusion coefficient, and the increase in solubility of many compounds in the solvent [19, 20]. However, when the ultrasonic power was greater than 480 W, mass destruction and reagglomeration of chemical particles would occur, resulting in the inhibition of particle expansion. Therefore, slight decline in adenosine content could be observed [21]. Additionally, it has been reported that the ultrasonic power could exert an impact on the selectivity of the compounds of interest depending on the ratio of extraction molecules [22]. It might be suggested that choosing appropriate ultrasonic power is key to higher extraction yield because of the proportion of adenosine content in raw materials.

3.2.4. The Effect of Ultrasonic Time. The effect of extraction time on the adenosine content is demonstrated in Figure 6. The adenosine content increased significantly in the first 120 min. After reaching its peak when the extraction time was 120 min, the adenosine content declined. It is reported that prolonged extraction time could affect the adenosine content due to its decomposition [23]. This result was in agreement with the previous result [24]. From this study, it is proposed that utilizing fine powder of materials for extraction without agitation could take longer time. Although fine powder effectively contributes to enhancement of diffusivity, it could cause a difficult interaction between solvent and target compositions due to creating a fixed bed of fine powder at the bottom of extraction equipment.

3.2.5. The Effect of Ultrasonic Temperature. The effect of ultrasonic temperature is shown in Figure 7. From Figure 7,

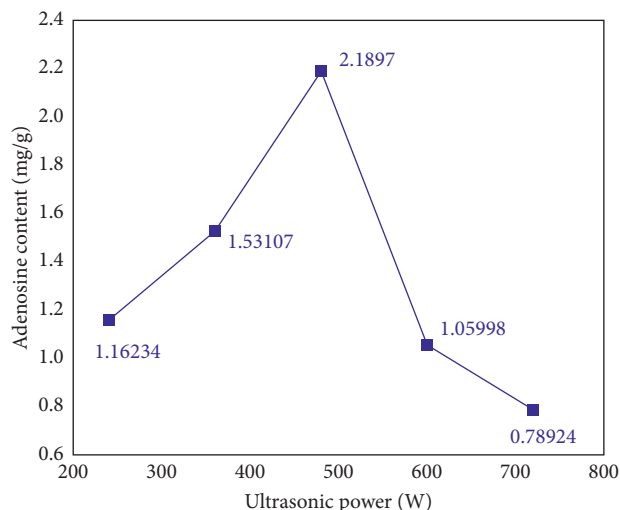


FIGURE 5: Effect of ultrasonic power on the adenosine content.

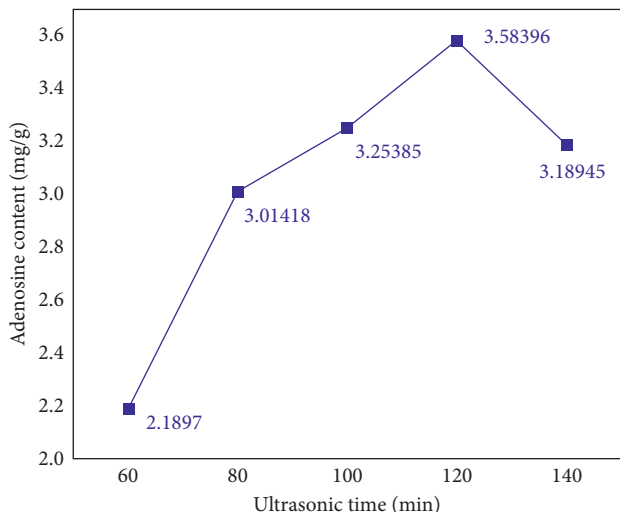


FIGURE 6: Effect of ultrasonic time on adenosine content.

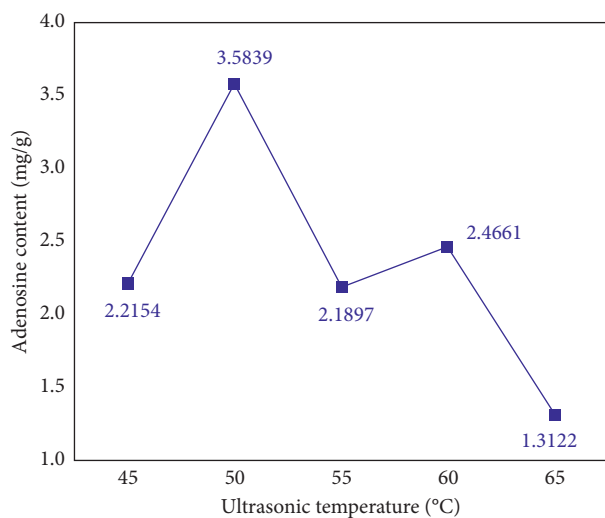


FIGURE 7: Effect of ultrasonic temperature on the adenosine content.

it can be seen that as the ultrasonic temperature was on the upward trend, the adenosine content rose and obtained its highest value at 50°C. This came as a result of the increase in mass transfer, gas cavitation in ultrasound extraction process, and the activation of enzyme caused by increase in ultrasonic temperature. However, the adenosine content decreased when the temperature continued to rise. It is reported that high temperature might induce the impurities of products and thermal degradation [25]. Therefore, after reaching the highest point, an increase of temperature would lead to a decrease in adenosine content because high temperature causes adenosine decomposition and enzyme inactivation. According to preliminary studies, this result could be considered as suitable with reported research [26]. Consequently, it could be proved that temperature has been taken into account because temperature acceleration was attributed to generation of insufficient energy of bubble's implosion to remove cell walls. Thus, selecting optimal point of temperature has been imperative and differs according to each substance of interest.

3.3. Model Building and Statistical Analysis. Experimental design and observed responses were presented in Table 2. Arrangement of experiments based on Box-Behnken model consisted of 15 experiments, including three central experiments. Regression analysis was carried out to determine the optimal conditions for extracting adenosine and survey the relationship between variables. Quadratic polynomial equation of the model is as follows:

$$\begin{aligned}
 Y = & 2.79 + 0.0079 X_1 + 0.0433 X_2 - 0.0582 X_3 \\
 & - 0.0198 X_1 X_2 - 0.0561 X_1 X_3 \\
 & + 0.0744 X_2 X_3 - 0.8294 X_1^2 - 0.8093 X_2^2 - 0.8009 X_3^2,
 \end{aligned}
 \tag{2}$$

where Y was the adenosine content (mg/g), and X_1 , X_2 , and X_3 were ultrasonic time, ultrasonic temperature and ultrasonic power, respectively.

The statistical analysis of each coefficient was checked by F -test and p value, and the analysis of variance analysis (ANOVA) for the RSM was presented in Table 3. The ANOVA of quadratic regression model illustrated that the model was highly significant and had a good fit of the model, showing through the high model F value ($F = 346.97$) but a very low p value ($p < 0.0001$). The lack of fit F value with the value of 11.27 implied the suitability of the model to predict the variations. The determination coefficient ($R^2 = 0.9984$) indicated the goodness-of-fit of the model as only 0.16% of the total variations could not be explained by the model. Moreover, the predicted regression coefficient R_{pre}^2 with the value of 0.9757 was in accordance with the adjusted regression coefficient R_{adj}^2 with the value of 0.9955 ($R_{adj}^2 - R_{pre}^2 < 0.2$), showing a high rate of correlation between experimental and predicted data. In addition, the coefficient of variation (C.V. %) with the low value of 3.04% ($< 5.00\%$) indicated the reproducibility of the model as the dispersion of data points was around the mean. Table 3 also shows that the linear coefficients (X_2 , X_3), the quadratic

TABLE 2: Box-Behnken experimental design and observed responses.

Run	X_1	X_2	X_3	Y (adenosine content, mg/g)	
				Experimental	Predicted
1	-1	-1	0	1.0447	1.0179
2	+1	-1	0	1.1493	1.0849
3	-1	+1	0	1.2023	1.1440
4	+1	+1	0	1.2277	1.1318
5	-1	0	-1	1.2023	1.1259
6	+1	0	-1	1.2813	1.2636
7	-1	0	+1	1.1598	1.0419
8	+1	0	+1	1.0143	0.9588
9	0	-1	-1	1.2703	1.2458
10	0	+1	-1	1.1768	1.1835
11	0	-1	+1	1.0433	0.9026
12	0	+1	+1	1.2475	1.1379
13	0	0	0	2.7777	2.7332
14	0	0	0	2.7947	2.7332
15	0	0	0	2.8116	2.7332

TABLE 3: ANOVA for quadratic model.

Source	Sum of squares	Mean squares	F value	p value
Model	6.43	0.7143	346.97	<0.0001
X_1 : ultrasonic time	0.0005	0.0005	0.2444	0.6420 ^b
X_2 : ultrasonic temperature	0.0150	0.0150	7.30	0.0427 ^a
X_3 : ultrasonic power	0.0271	0.0271	13.17	0.0151 ^a
$X_1 X_2$	0.0016	0.0016	0.7619	0.4227 ^b
$X_1 X_3$	0.0126	0.0126	6.12	0.0562 ^a
$X_2 X_3$	0.0222	0.0222	10.76	0.0219 ^a
X_1^2	2.54	2.54	1233.66	<0.0001 ^a
X_2^2	2.42	2.42	1174.77	<0.0001 ^a
X_3^2	2.37	2.37	1150.31	<0.0001 ^a
Residual	0.0103	0.0021		
Lack of fit	0.0097	0.0032	11.27	0.0826
Pure error	0.0006	0.0003		
Cor. total	6.44			
R^2	0.9984	Std. dev.	0.0454	
Adj- R^2	0.9955	C.V.%	3.04	

^a $p \leq 0.05$; ^b $p > 0.05$.

coefficients (X_1^2 , X_2^2 , and X_3^2), and the products of the coefficients ($X_1 X_3$, $X_2 X_3$) had significant effects on the adenosine content ($p \leq 0.05$). The effects of other coefficients on the adenosine content were not significant ($p > 0.05$).

3.4. Effect of Multifactors on the Adenosine Content

3.4.1. Ultrasonic Time and Ultrasonic Temperature. Figure 8 shows the simultaneous effects of ultrasonic temperature and ultrasonic time on the adenosine content. According to previous study, the selection of probably ultrasonic time and ultrasonic temperature could be considered as important factors in enhancing extraction efficiency [27]. When ultrasonic temperature rose from 45 to 50°C, the adenosine content experienced an increase. However, as ultrasonic temperature reached 55°C, the adenosine content decreased gradually. It is reported that the stability of adenosine under high temperature was different [27]. In the range of ultrasonic time from 100 to 120 min, the adenosine

content increased rapidly but when ultrasonic time increased over 120 min, the adenosine content declined significantly. It is stated that hydroxyl radicals produced by cavitation when increasing ultrasonic time could lead to degradation of adenosine as the increase of contacting surface between raw material and solvent makes the mass transfer processes become easier [28]. Therefore, the adenosine content increased significantly. Nevertheless, the long interval time would result in the decrease in the adenosine content due to adenosine decomposition. Besides, ultrasonic temperature affects to the cavitation, adenosine solubility, diffusion coefficient, and enzyme activation.

3.4.2. Ultrasonic Time and Ultrasonic Power. Figure 9 indicates the simultaneous effects of ultrasonic temperature and ultrasonic power on the adenosine content of the extract. It can be seen that the adenosine content increased when ultrasonic time rose from 100 to 120 min and the ultrasonic power increased from 360 to 480 W. When the ultrasonic

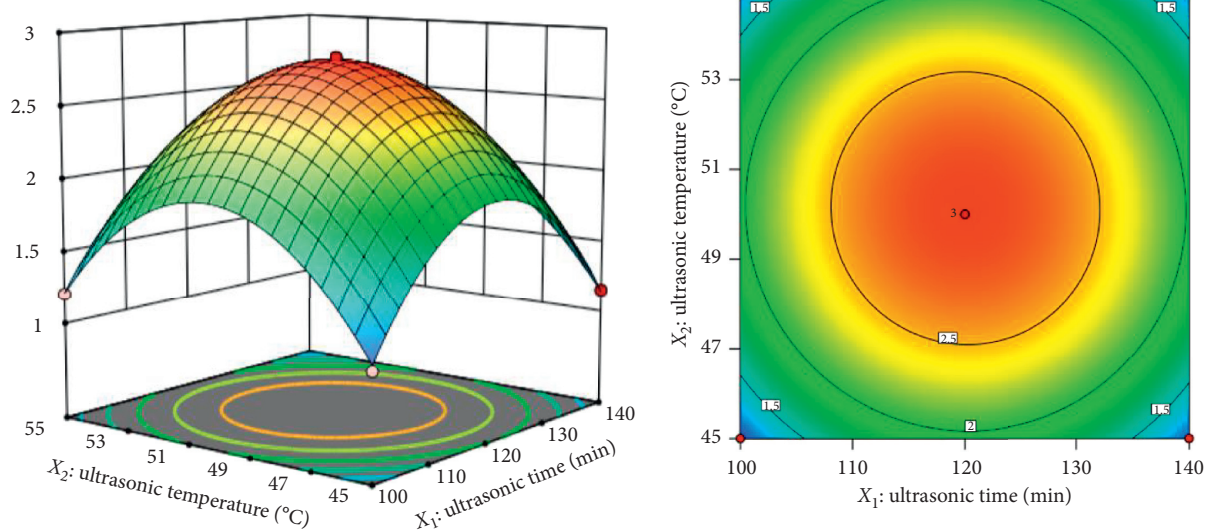


FIGURE 8: Simultaneous effect of ultrasonic time and ultrasonic temperature.

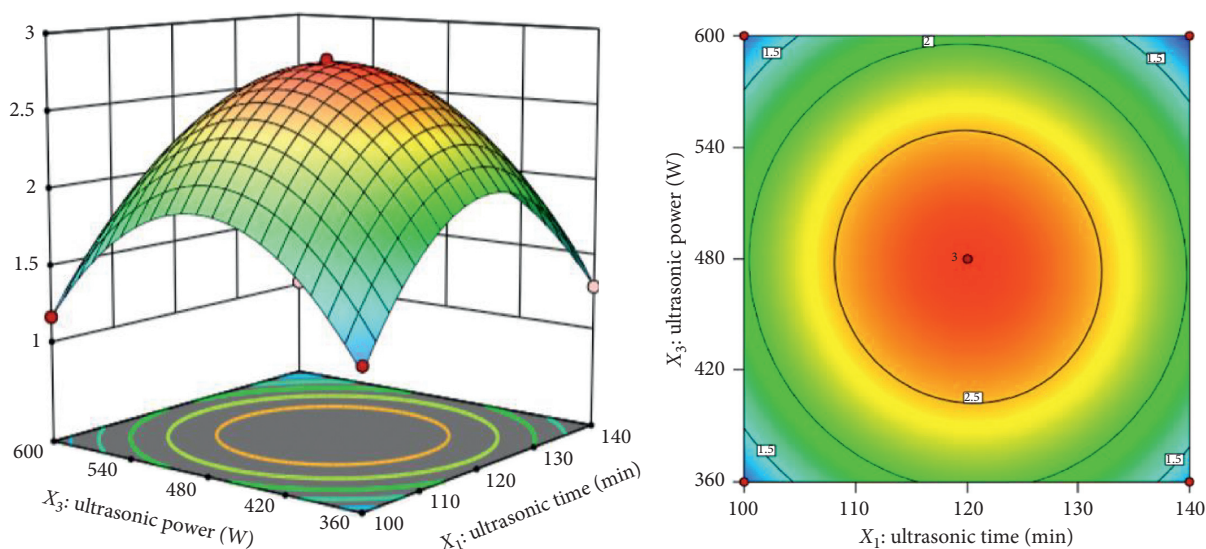


FIGURE 9: Simultaneous effect of ultrasonic time and ultrasonic power.

time and ultrasonic power were higher than 120 min and greater than 480 W, respectively, the adenosine content decreased. These might be caused by the integration of physical and chemical mechanisms related mainly to cavitation [29]. From this result, it is deduced that long extraction time of fine powder materials could create a fixed bed during sedimentation, which reduces mass transfer kinetics. Nevertheless, this problem might be solved by using proper ultrasonic power. If ultrasonic power was larger, adenosine content would fall dramatically due to the molecular degradation and selectivity of target molecules alteration.

3.4.3. Ultrasonic Temperature and Ultrasonic Power. Figure 10 indicates the multi-interaction between ultrasonic temperature and ultrasonic power, which creates effects on

the adenosine content of the extract. It can be seen that the adenosine content increased when temperature rose from 45°C to 50°C and the ultrasonic power increased from 360 to 480 W. When the ultrasonic temperature and ultrasonic power were higher than 50°C and greater than 480 W, respectively, the adenosine content experienced a decrease. This could be owing to the effect of ultrasonic temperature on the cavitation phenomena and enzyme activation as well as the effect of ultrasonic capacity on the structural destruction of adenosine compound.

Using Design-Expert software, the optimal extraction parameters obtained from the model were ultrasonic time with the value of 120.107 min, ultrasonic temperature with the value of 50.108 C, and ultrasonic power with the value of 475.767 W. Under these extraction conditions, the adenosine content obtained was 2.736 mg/g. Experimental tests

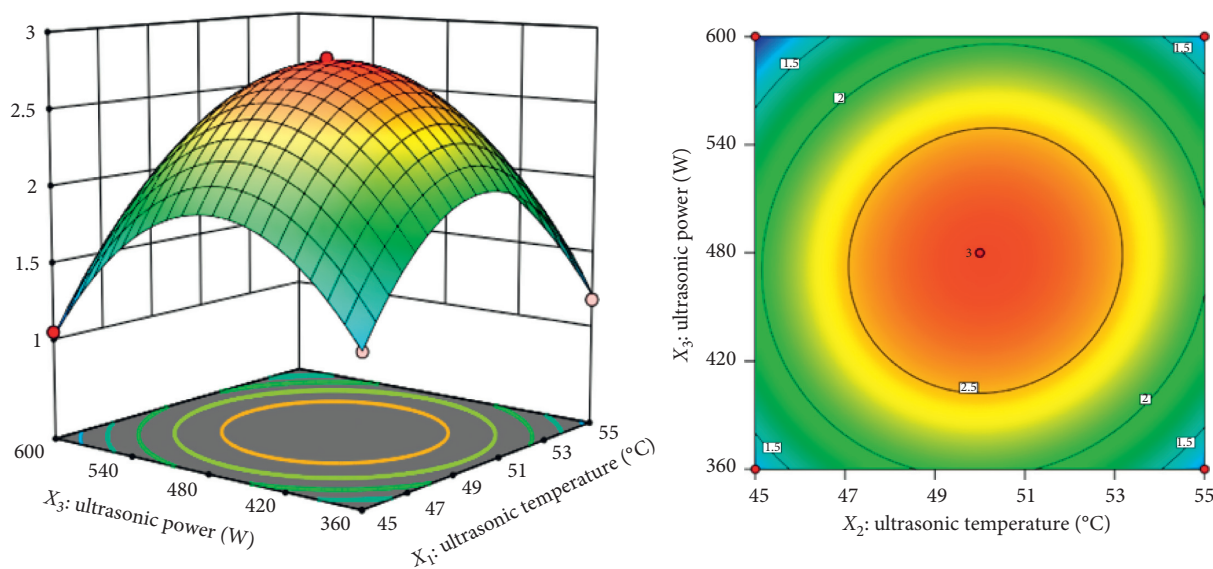


FIGURE 10: Simultaneous effect of ultrasonic temperature and ultrasonic power.

were conducted, however, to suit the experimental conditions, and the extraction parameters were adjusted as the central experiment. Under modified conditions, the adenosine content obtained from the experiment was 2.852 mg/g. The result was 4.06% different from the predicted value of the model. Thereby, the model was consistent with experimental data.

3.5. Comparison of Different Extraction Methods. Figure 11 describes the adenosine content extracted by different extraction methods. As illustrated in Figure 11, it can be seen that the adenosine content obtained from UAE was 1.589 mg/g and from EAE was 2.088 mg/g, while the adenosine content obtained from the AE method was only 0.419 mg/g. In comparison with EAE, AE, and UAE, the application of UAEE greatly facilitates the adenosine content extraction process. Besides, the mean adenosine content in Vietnamese *C. militaris* fruiting bodies was 2.45 ± 0.03 mg/g while in the mycelium it was about 1.592 ± 0.03 mg/g using UAE only with methanol: water 50:50 (*v*:*v*) solvent. [30]. These results showed that the UAEE method is appropriate for adenosine extraction from *C. militaris*.

3.6. Bioactivities of the Extract

3.6.1. Antioxidant Activity. DPPH radical scavenging activity of Vietnamese *C. militaris* extract is presented in Figure 12. From the result, it could be concluded that the antioxidant activity of the extract with IC_{50} value of $357.04 \mu\text{g/mL}$ was 37 times lower than the ascorbic acid activity with IC_{50} value of $9.75 \mu\text{g/mL}$. Therefore, the extract could be reported to have a higher antioxidant activity compared with the *Solanum hainanense* Hance with an IC_{50} value of $1734 \mu\text{g/mL}$ [23]. The antioxidants in Vietnamese *C. militaris* were mainly cordycepin and adenosine according to some preliminary studies [31]. In one study, the

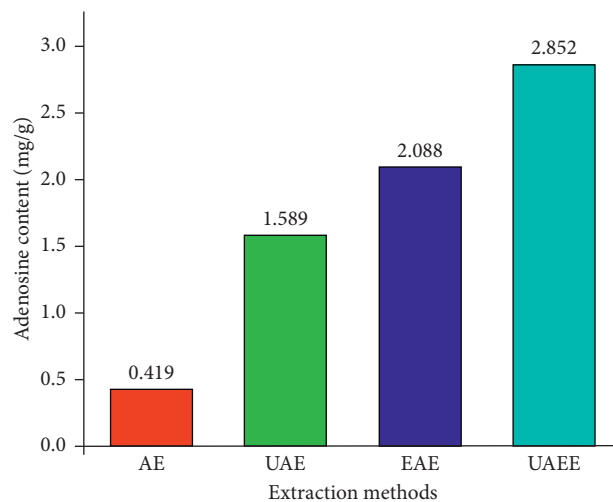


FIGURE 11: Adenosine content extracted by different extraction methods.

cordycepin showed its ability to shield the internal organs of aged rats from oxidative stress by minimizing the intensity of lipid peroxidation and improving the activities of enzymatic and nonenzymatic oxidants [32]. Other research indicates that the extraction conditions, including pH, extraction time, extraction temperature, and enzyme concentration, had profound impacts on the DPPH radical-scavenging activity of Vietnamese *C. militaris* [31].

3.6.2. Anticancer Activity. The test results of anticancer activity of Vietnamese *C. militaris* extract on two lines of Hep-G2 and MCF-7 cancer cells are illustrated in Table 4. Accordingly, Vietnamese *C. militaris* extract was able to resist both strains of Hep-G2 and MCF-7 with IC_{50} values of 101.32 and more than $128.00 \mu\text{g/mL}$, respectively. It can be seen that extract had better anticancer effect on Hep-G2 than

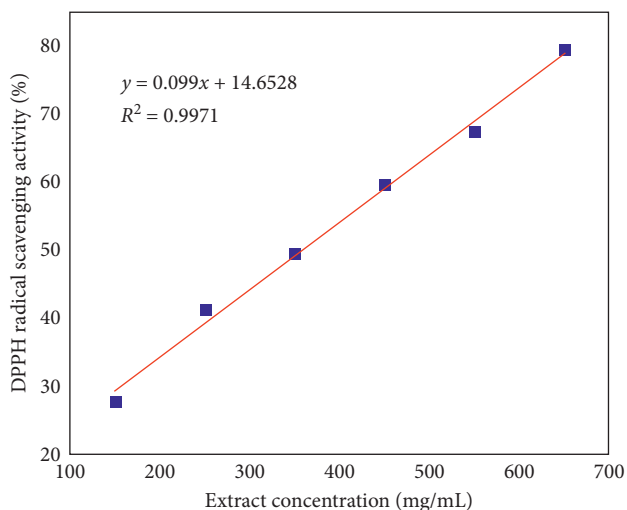


FIGURE 12: DPPH radical scavenging activity of the extract.

TABLE 4: IC₅₀ values of extract.

Samples	IC ₅₀ values (μg/mL)	
	Extract	Ellipticine
Hep-G2	101.32	0.45
MCF-7	>128.00	0.39

MCF-7. The positive control with IC₅₀ values of 0.45 and 0.39 μg/mL on Hep-G2 and MCF-7 cancer cells, respectively, indicated higher cancer resistance than the extract.

It could be proved that the anticancer activity of Vietnamese *C. militaris* extract on Hep-G2 and MCF-7 was lower than that of the extract obtained in this study with an IC₅₀ value of 390 and 400 μg/mL, respectively [33]. At the same time, Vietnamese *C. militaris* extract was more resistant to cancer Hep-G2 cells than other drugs such as *Matricaria recutita* L. with the IC₅₀ value of 300 μg/mL [34] and *Parkia javanica* seeds with the IC₅₀ value of 480 μg/mL [35]. Cancer is the name of many diseases where the growth of abnormal cells is most likely to divide without stopping and spread into surrounding tissues. As mentioned above, cordycepin is one of the most common and crucial bioactive anticancer agents found in *Cordyceps* species [36]. Since it has a similar structure to adenosine, RNA polymerase cannot distinguish between cordycepin and adenosine. As a result, cordycepin is incorporated into a growing RNA molecule leading to prevention of further elongation. This causes the lack of oxygen in the 3' position of its ribose part, thus producing a prematurely terminated RNA molecule [37]. In accordance with several previous studies, cordycepin can induce apoptotic effects on breast, liver, leukemia, lung cancer, and glioblastoma [38].

4. Conclusions

In this study, adenosine is extracted from Vietnamese *C. militaris* by ultrasonic-assisted enzyme method with simple extraction process, using environmentally friendly Alcalase

enzyme. The highest adenosine content was 2.852 mg/g obtained under optimal extraction conditions including 120.107 min in ultrasonic time, 50.108 C in ultrasonic temperature, and 475.767 W in ultrasonic power. In comparison with the other extraction methods, UAEE is stated to get the highest adenosine content, which was 6.8 times higher than that obtained by AE, 1.8 times higher than that obtained by UAE, and nearly 1.3 times higher than that obtained by EAE. The antioxidant activity of Vietnamese *C. militaris* extract using UAEE method was evaluated with the IC₅₀ value of 357.04 μg/mL, leading to the possible application in functional foods or natural antioxidant products. In addition, the anticancer activity test results indicated the cytotoxic activity of the extract on Hep-G2 and MCF-7 cancer cells with an IC₅₀ value of 101.32 and more than 128 μg/mL, respectively. Therefore, Vietnamese *C. militaris* extract can be considered as a natural anticancer agent, although its anticancer concentrations were usually presented in high levels.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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