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OPTIMIZATION OF TRITERPENOIDS EXTRACTION FROM VIETNAMESE GANODERMA LUCIDUM USING RESPONSE SURFACE METHODOLOGY AND ANTICANCER EVALUATION OF THE EXTRACT[#]

Ngo Thi Thuy Linh[1], Tran Do Dat[1], Nguyen Tan Tai[2], Nguyen Thi Linh[1], Phan Le Thao My[1], Nguyen Thi Kim Ngan[1], Nguyen Thi Van Anh[1], Vuong Hoai Thanh[2], Nguyen Duc Viet[2], Hoang Minh Nam[2, 3], Mai Thanh Phong[2, 3], Nguyen Huu Hieu[1, 2, 3, *]

[1]*VNU-HCMC Key Laboratory of Chemical Engineering and Petroleum Processing (Key CEPP Lab), 70000, Viet Nam*

[2]*Faculty of Chemical Engineering, Ho Chi Minh City University of Technology, 268 Ly Thuong Kiet Street, Ward 14, District 10, Ho Chi Minh City, Viet Nam*

[3]*Vietnam National University Ho Chi Minh City, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Viet Nam*

[*]Email: *nhhieubk@hcmut.edu.vn*

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Abstract. Triterpenoids are considered to be the major pharmaceutical active compounds found in the macrofungus *Ganoderma lucidum (G. lucidum)*. Extraction conditions of triterpenoids from the Vietnamese red *G. lucidum* were optimized by response surface methodology using ultrasound –assisted extraction (UAE) method. A Box – Behnken experimental design was used to determine the simultaneous influences of three independent variables, namely solvent-to-material ratio, extraction time, and ultrasonic power on the yield of triterpenoids. The highest yield of triterpenoids obtained under optimum conditions including extraction time of 55 min, ultrasonic power of 480 W, solvent-to-material ratio of 27:1 mL/g was 9.5768 ± 0.228 mg/g according to ultraviolet-visible spectroscopy (UV-Vis) analysis. Besides, the hot water extraction method was carried out in order to compare with UAE in terms of the yield of triterpenoids. The results of anticancer evaluation of the extract obtained indicated that with the half-maximal inhibitory concentration value of 67.25 ± 0.82 µg/mL on the human Hep-G2 liver cancer cell lines, triterpenoids extracted from *G. lucidum* could be regarded as a potential agent for medicinal treatment.

Keywords: Ganoderma lucidum, ultrasound – assisted extraction, response surface methodology, triterpenoids.

Classification numbers: 1.3.

1. INTRODUCTION

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The application of herbal plants and medicinal fungi in human's diseases treatment has been known for over millennia. *Ganoderma lucidum (G. lucidum)*, also called Lingzhi, was well - known in China, Japan, and South Korea because of its promising health recognized benefits [1]. As being documented thousands of years ago, the medicinal values of this mushroom have been recorded with many pharmaceutical uses for several types of illnesses such as cancer, hepatopathy, arthritis, hypertension, neurasthenia, and debility [2]. The rapid development of analytical methods and equipment has led to the detection of numerous bioactive compounds from Lingzhi, among them, polysaccharides and triterpenoids were found to be two main phytochemicals of *G. lucidum* [3]. Triterpenoids are the most important active substances of ganoderma spores showing numerous bioactivity properties [4]. From the fruiting bodies of *G. lucidum*, more than 150 triterpenoids have been reported representing five major structural classes [5 - 8].

In general, solid-liquid extraction, which was an ancient technology, has been employed to isolate substances from plants and fungi. This technique consists of selecting, washing, or leaching of targeted compounds from solids by applying appropriate solvents [9]. With the development of extraction techniques, non-conventional technologies have been used as green technologies such as supercritical fluid extraction, vertical extraction, electrical energy extraction, and ultrasonic-assisted extraction (UAE) [10]. Among these methods, UAE has been considered as one of the upcoming techniques due to its high efficiency such as reduction in the amount of solvent, extraction time, and energy, as well as low temperature required [11].

Extraction conditions can alter the extract biochemical profiles and thus, influencing their bioactive potential. By optimizing the operational parameters of extraction process, a maximum extraction efficiency of targeted compounds could be reached [12]. Even though the chemical constituents and bioactivities of the fruiting bodies of *G. lucidum* have received great attention and well supported by various studies. The objective of this study is to evaluate and optimize the UAE conditions of the triterpenoid extraction of Vietnamese red *G. lucidum* using response surface methodology (RSM) with the employment of Box-Behnken design (BBD) in comparison with the hot – water extraction method (HWE) for green chemical process development and economic value enhancement of this Vietnamese fungus. In addition, the anti-cancer activity evaluation was carried out to determine the bioactivity of the extract obtained under optimal extraction conditions against the liver cancer Hep–G2 cell lines.

2. EXPERIMENTAL AND METHODS

2.1. Materials and chemicals

The dried fruiting body of Vietnamese *G. lucidum* was provided by National Institute of Medicinal Materials with the moisture value of materials at 12 %, Viet Nam. Ursolic acid, vanillin, 99.0 % methanol (CH_3OH), 70.0 - 72.0 % perchloric acid ($HClO_4$), acetic acid (CH_3COOH), and ascorbic acid were provided from Xylong, China. 99.5 % ethanol (C_2H_5OH) was purchased from ChemSol, Viet Nam. All chemicals were used as received without further purification. Double-distilled water was used in all experiments.

2.2. Experimental procedure

The Vietnamese *G. lucidum* fruiting body was dried at 50 ℃ to remove moisture and ground in a waring blender before 2.00 g was weighed accurately and mixed with ethanol, which was

selected as a solvent for triterpenoids extraction at 55 ºC. Extraction was performed with the use of ultrasonic equipment (Transicom Engineering, 1200 W, 40 kHz). After filtration, the sample was concentrated by using the vacuum evaporation at 100 mbar and 50 ℃ to partly remove solvent to obtain crude extracts.

2.3. The yield of triterpenoid determination

The yield of triterpenoids determination was performed based on the colorimetric method [13,14]. Tubes contained the extract samples or ursolic acid standards were left to evaporate to dryness in a water bath at 100 ℃. Then 0.4 mL of 5 % vanillin-glacial acetic acid and 1.0 mL of perchloric acid solution were added to each tube, followed by the heating treatment at 60 ℃ for 15 min in a water bath. After being cooled at room temperature for 2 min, each tube was mixed with 5.0 mL of glacial acetic acid before being measured the absorbance at 548 nm for determination of triterpenoids yield. All of determinations were made in triplicate. The yield of triterpenoids (%) was calculated by the following equation:

$$Y\ (\%) = \frac{C \times V \times n}{m} \times 100\ \% \quad (1)$$

where *Y* is the yield of triterpenoids (%); *C* is the concentration of triterpenoids obtained from the calibrated regression equation (mg/mL); *V* is the volume of triterpenoids solution (mL); *n* is the dilution factor; and *m* is the dried sample mass (g).

2.4. Hot water extraction (HWE)

To compare the efficiency of the extraction method, Vietnamese *G. lucidum* powder was extracted by HWE method under conditions as follows: solvent-to-material ratio of 30:1 mL/g, extraction time of 55 min, and extraction temperature of 70 ℃. This extract was then marked as extract M_1.

2.5. Optimization of ultrasonic parameters by RSM and statistical analysis

According to the results of single – factor experiments, the extraction conditions was optimized by using a three – factor – three – level BBD including X_1 (solvent – to – material ratio), X_2 (extraction time), and X_3 (ultrasonic power) test to evaluate their simultaneous effects. The complete quadratic equation used was as follows:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where *Y* is the response variable; $\beta_o, \beta_i, \beta_{ii}$, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction, respectively; X_i and X_j are the independent variables ($i \neq j$). Table 1 presents that each independent variable was coded at three levels: -1 (low value), 0 (intermediate value), and +1 (high value).

Table 1. Independent variables and their levels used for BBD.

Independent variables	Unit of measurement	Coded symbols	Levels		
			-1	0	1
Solvent-to-material ratio	mL/g	X_1	25:1	30:1	35:1

Extraction time	min	X_2	30	60	90
Ultrasonic power	W	X_3	360	480	600

2.6. Effects of simultaneous factors of main factors

By evaluation of the combined effects of main factors on the yield of triterpenoids, the extraction conditions of triterpenoids were optimized using RSM – BBD. Based on the results of analysis of variance (ANOVA), Design-Expert 11.0 software was employed to generate the three-dimensional (3D) surface and contour plots.

2.7. Anti-cancer activity investigation of the extract

The MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been applied for estimating anticancer activities of natural compounds in cell cultures [15]. This assay has been relied on the abilities of mitochondrial dehydrogenase enzymes in living cells, which could lead to the yellow solutions at beginning into a dark blue formazan product [16]. In the study, triterpenoids extracted from *G. lucidum* was tested for anti-cancer activities on liver cancer Hep–G2 cell lines. The percentage of cellular suppression (% I_{MTT}) was calculated according to the equation:

$$\% I_{MTT} = \frac{A_{(+)} - A_{sample}}{A_{(+)} - A_{(-)}} \times 100\ \% \quad (3)$$

where $A_{(+)}$ is absorbance of positive control, $A_{(-)}$ is absorbance of negative control, and A_{sample} is absorbance of sample.

3. RESULTS AND DISCUSSION

3.1. Response surface modeling using Box–Behnken design and statistical analysis

Table 2 presented the extraction yields of triterpenoids under different extraction conditions. The second-order polynomial model was proposed revealing the relationship between the extraction yield of triterpenoids (Y) and the coded variables over their selected ranges was as follows:

$$Y = 9.65 - 0.31X_1 - 0.19X_2 - 0.48X_3 + 0.41X_2X_3 - 0.30{X_1}^2 - 0.54{X_2}^2 - 0.96{X_3}^2 \quad (4)$$

where Y (mg/g) was the yield of triterpenoids, X_1, X_2, and X_3 were solvent-to-material ratio (mL/g), extraction time (min), and ultrasonic power (W).

Table 3 summarized the analysis of variance (ANOVA) for the response surface quadratic model. The high model F-value (F = 22.01) and low *p*-value ($p < 0.0001$) indicated a significant fit of this model. The values of determination coefficient (R^2) and adjusted determination coefficient (Adj – R^2) of the model were 0.9754 and 0.9311, respectively, which suggested that 97.54 % of the variations could be explained by the fitted model and 0.9311 % of the total variations were illustrated by the model. In addition, the low value of coefficient of the variation (C.V. % = 2.30 %) showed a high degree of precision and good reliability of the experimental values.

As can be seen from the Table 3, the linear coefficients (X_1, X_2, and X_3), the two-factor interaction term X_2X_3, and the quadratic terms (X_1^2, X_2^2, and X_3^2) were the main factors

affecting the yield of triterpenoids with very small p – value ($p < 0.05$). The other term coefficients were not significant ($p > 0.05$).

*Table 2***.** Factors and levels for RSM, and BBD with the independent variables.

Run	X_1	X_2	X_3	Y (Yield of triterpenoids, mg/g)
1	–1	–1	0	9.4004
2	1	–1	0	8.6504
3	–1	1	0	9.0008
4	1	1	0	8.2123
5	–1	0	–1	9.1023
6	1	0	–1	8.4205
7	–1	0	1	8.1412
8	1	0	1	7.9121
9	0	–1	–1	9.3125
10	0	1	–1	8.1572
11	0	–1	1	7.3152
12	0	1	1	7.8121
13	0	0	0	9.8098
14	0	0	0	9.4801
15	0	0	0	9.6713

Table 3. ANOVA of regression model for the response and variables.

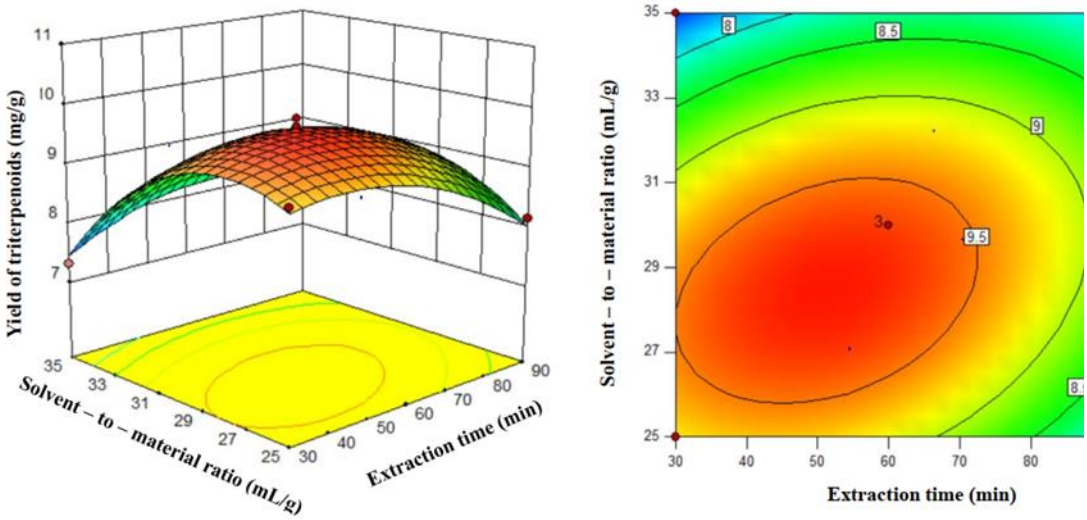
Source	Sum of squares	df	Mean square	F-value	*p*-value
Model	7.9300	9	0.8800	22.01	0.0017[a]
X_1-solvent-to-material ratio	0.7500	1	0.7500	18.71	0.0075[a]
X_2 - extraction time	0.2800	1	0.2800	6.99	0.0458[a]
X_3 - ultrasonic power	1.8200	1	1.8200	45.37	0.0011[a]
X_1X_2	0.0003	1	0.0003	0.009	0.9280[b]
X_1X_3	0.010	1	0.0510	1.26	0.3119[b]
X_2X_3	0.6800	1	0.6800	17.04	0.0091[a]
X_1^2	0.3200	1	0.3200	8.05	0.0363[a]
X_2^2	1.0700	1	1.0700	26.79	0.0035[a]
X_3^2	3.4200	1	3.4200	85.34	0.0002[a]
Residual	0.2000	5	0.0400		
Lack of Fit	0.1500	3	0.0490	1.81	0.3758
Pure error	0.0520	2	0.0260		
Cor total	8.13	14			
R^2	0.9754		SD	0.20	
Adj-R^2	0.9311		C.V.%	2.30	
Pred- R^2	0.69				
Adep. precision	13.487				

[a] $p < 0.05$; [b] $p > 0.05$

3.2. Simultaneous effect of the main factors

3.2.1. Solvent-to-material ratio and extraction time

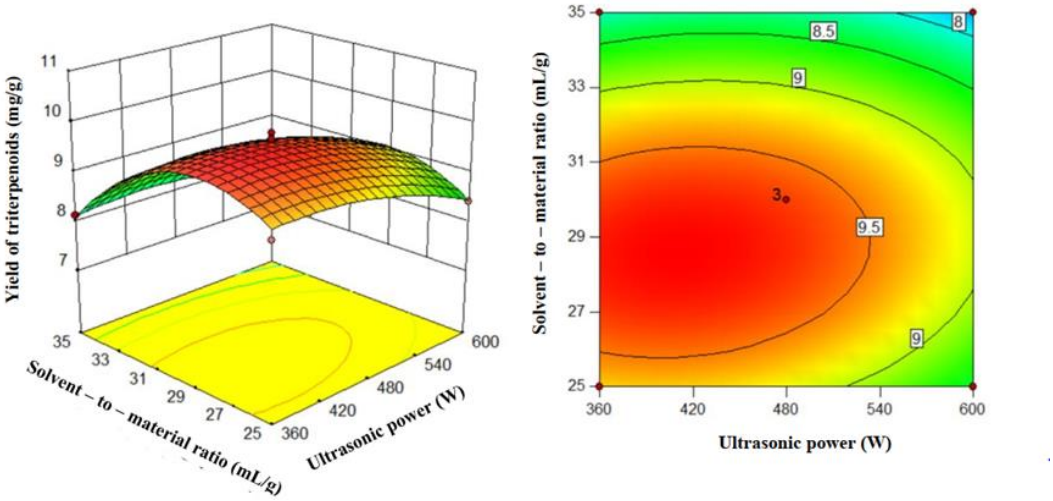
Figure 1 presents the combined influences of solvent-to-material ratio and extraction time on the yield of triterpenoids of the extract. As can be seen from the Figure 1, when the solvent-to-material ration rose from 25:1 to 30:1 mL/g and extraction time increased from 30 to 60 min, the yield of triterpenoids increased prominently and obtained its highest value. However, with the increase in the solvent – to – material ratio and extraction time, the yield of triterpenoids was reported to experience a decrease. The higher yield of triterpenoids could be explained owing to the improvement in extraction efficiency as higher extraction time and appropriate amount of solvent used. Meanwhile, longer extraction time and higher solvent – to – material ratio could result in a decrease in the yield of triterpenoids as the triterpenoids would degrade and solvent molecules would absorb more energy. This result can be found similar to reported research [12, 13].



(a) (b)

Figure 1. Contour plots (a) and response surface plots (b) showing the simultaneous effect of solvent-to-material ratio (X1) and the extraction time (X2) on the yield of triterpenoids.

3.2.2. Solvent-to-material ratio and ultrasonic power



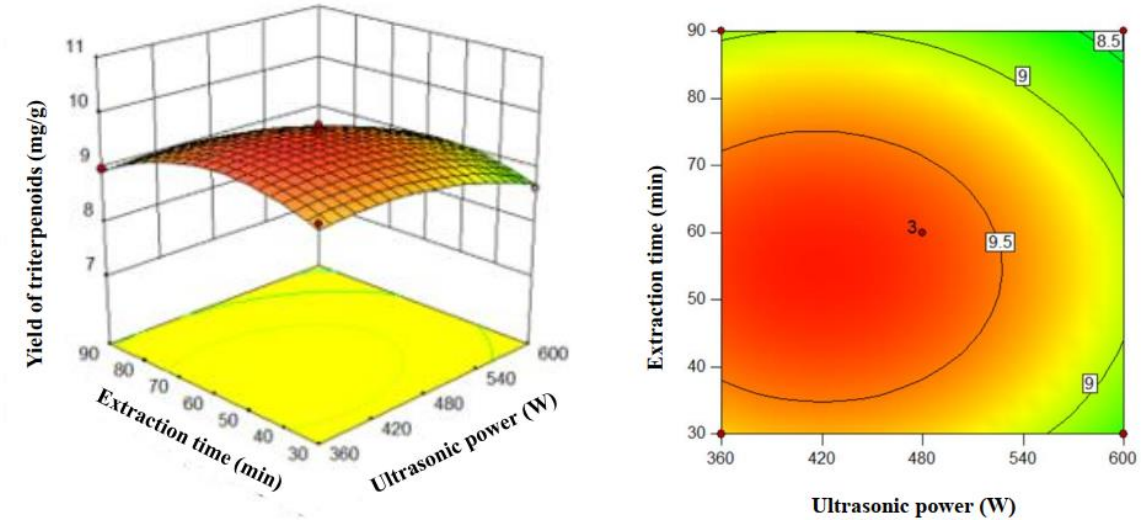
(a) (b)

Figure 2. Contour plots (a) and response surface plots (b) showing the simultaneous effect of solvent-to-material ratio (X1) and the ultrasonic power (X3) on the yield of triterpenoids.

Figure 2 demonstrates the interactive relationship between solvent-to-material ratio and ultrasonic time on the yield of triterpenoids. It can be seen that with an increase in solvent-to-material from 25:1 to 26.568:1 mL/g along with an increase in ultrasonic power from 360 to 420 W, the yield of triterpenoids was enhanced significantly. This phenomenon could be explained according to the enhancement of extraction yield and effective dissolution of substances within the cells when an increase in solvent – to – material ratio and ultrasonic power was observed, whereas a decline in the yield of triterpenoids could be explained because of ultrasound-induced thermal effect and negative influence on the cell wall material break and mass transmission. This result is persistent with reported research [17].

3.2.3. Extraction time and ultrasonic power

The simultaneous effects between extraction time and ultrasonic power on the yield of triterpenoids is illustrated in Figure 3. It can be seen that the yield of triterpenoids rose with the increase in the extraction time from 30 to 54.59 minutes and ultrasonic power between 360 and 600 W. The reason for this is that rising extraction could lead to improvement of extraction efficiency while ultrasonic power could facilitate the release of substances within the cell walls. However, too long extraction time as well as too high ultrasonic power would result in triterpenoids decomposition according to previous studies [14,15].



(a) (b)

Figure 3. Contour plots (a) and response surface plots (b) showing the simultaneous effect of the extraction time (X_2) and ultrasonic power (X_3) on the yield of triterpenoids.

The optimized conditions using RSM for maximizing the content of triterpenoids were found to be solvent-to-material ratio of 27:1 mL/g, extraction time of 55 min, and ultrasonic power of 480 W. Under these conditions, the maximal value for triterpenoid content of 9.5768 mg/g of could be reached, which was very close to the predicted value of 9.6537 mg/g. The model was proved to be validated at these optimal points.

3.3. Comparison between UAE and HWE method

Figure 4 shows the influence of different extraction methods on the yield of triterpenoids. Under optimal extraction conditions including solvent – to – material ratio of 27:1 mL/g, extraction time of 55 min, and ultrasonic power of 480 W, the yield of triterpenoids obtained was of 9.5768 mg/g. In comparison, HWE was conducted generating the yield of triterpenoids of 3.687 mg/g. This result could be considered as suitable with the results reported by Hwang *et al*. when comparing the differences of triterpenoids yield obtained from chaga mushroom (*Inonotus obliquus*) under different extraction methods in 2019 [18]. It is cited that high temperature might lead to destruction of triterpenoids structure while UAE could greatly improve the triterpenoids extraction efficiency as continuous compression and rarefaction are generated with the assistance of solvent used for extraction [17,18].

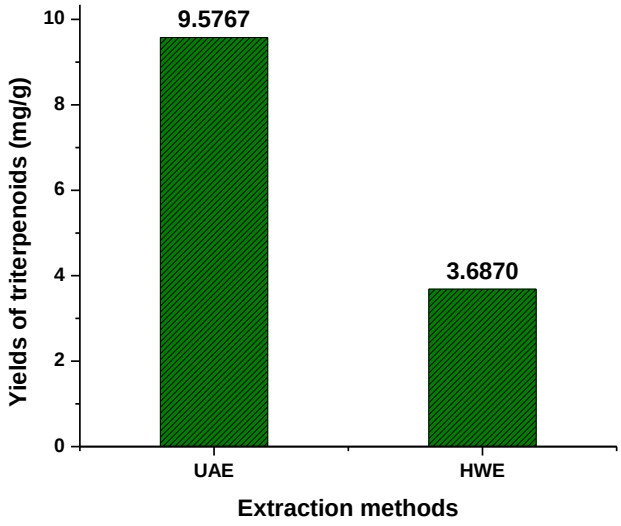


Figure 4. Effect of different extraction methods on the yields of triterpenoids.

3.4. Anti-cancer activity investigation of the extract

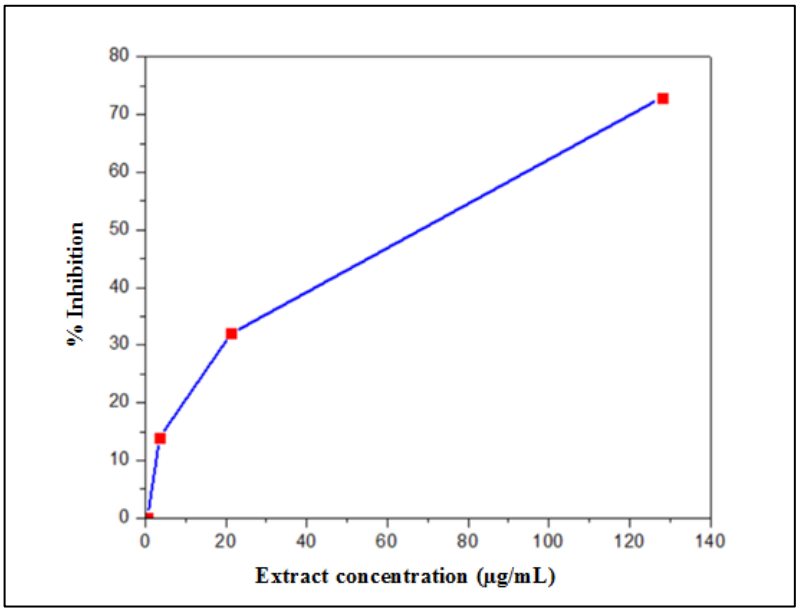


Figure 5. Anti-cancer activity (% inhibition) of the extract at different concentrations against Hep-G2.

The test results of anti-cancer activity the extract from *G. lucidum* on the cell line of Hep-G2 are depicted in the Figure 5. The result showed that the extract could prevent the growth of Hep-G2 cancer cells with the half-maximal inhibitory concentration (IC_{50}) value of 67.25 ± 0.82 µg/mL. The rich content of triterpenoids and polysaccharides of *G. lucidum* could be utilized to account for the anti-cancer activities of the extract [19]. For polysaccharides, its anticancer mechanism might have been known by producing and enhancing varieties of host's immune responses as well as inducing no harm to host's body [20]. Furthermore, triterpenoids have been reported as an anti-cancer agent because of its strong cytotoxic effects with several mechanisms on human cancer cell lines, particularly cell cycle arrest [21].

4. CONCLUSIONS

In this study, triterpenoids were extracted from Vietnamese red *G. lucidum* by using UAE method. The highest yield of triterpenoids obtained under the optimal extraction conditions including solvent – to – material ratio of 27 mL/g, extraction time of 55 min, and ultrasonic power of 480 W was reported to be of 9.5768 ± 0.228 mg/g based on ultraviolet-visible spectroscopy analysis. In addition, UAE was found to be a more efficient method for the extraction of triterpenoids from Vietnamese *G. lucidum* compared with HWE method, which generated the yield of triterpenoids of 3.687 mg/g. The results of bioactivities evaluation indicated that the anti-cancer activity against Hep-G2 cell lines of the extract with the IC_{50} value was of 67.25 ± 0.82 µg/mL. As a result, further studies regarding of purification and isolation of triterpenoids compounds with the aim at industrial purpose could be conducted in the future.

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CRediT authorship contribution statement. NTTL: Conceptualization, Methodology, Investigation, Writing - original draft. TDD: Methodology, Investigation, Writing - original draft. NTT: Methodology,

Investigation, Writing - original draft. NTL: Writing - review & editing. PLTM: Investigation, Writing - review & editing. NTKN: Investigation, Writing - review & editing. NTVA: Writing – review, editing and data analysis. VHT: Writing - review & editing. NDV: Validation, Formal analysis, Data curation, Supervision. HMN: Resources, Visualization, Project administration, Funding acquisition. MTP: Resources, Visualization. NHH: Conceptualization, Resources, Writing - review & editing, Visualization.

Declaration of competing interest. We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere. We have no conflicts of interest to disclose. This paper was written by listed authors who are all aware of its content and approve its submission.

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