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64. THÀNH PHẦN FLAVONOID VÀ HOẠT TÍNH KHÁNG OXY HÓA TỪ CÂY CỎ MỰC (*Eclipta prostrata* L.) THU HÁI Ở VƯỜN QUỐC GIA YOK ĐÔN. Đoàn Mạnh Dũng, Trần Thanh Tâm Toàn, Nguyễn Thị Quỳnh Hương, Phan Tứ Quý, Nguyễn Mậu Thành, Nguyễn Nho Dũng, Nguyễn Thị Huyền, Nguyễn Văn Bốn, Đỗ Mai Nguyễn, Nguyễn Thị Thu, Đỗ Xuân Hưng, Nguyễn Anh Dũng, Nguyễn Quang Vinh..... 410

65. KHẢO SÁT ĐỘC TÍNH CẤP ĐƯỜNG UỐNG VÀ TÁC ĐỘNG GIẢM STRESS CỦA CAO CHIẾT RỄ TƠ SÂM NGỌC LINH TRÊN CHUỘT NHẮT. Hà Thị Loan, Trần Nguyễn Lệ Quyên, Cao Huệ Trinh, Nguyễn Trần Phước Huy, Huỳnh Đại Quốc, Vũ Thị Thắm, Phan Diễm Quỳnh, Nguyễn Thành Vũ, Đỗ Thị Hồng Tươi 417

66. PHÂN LOẠI VÀ ĐÁNH GIÁ HOẠT TÍNH SINH HỌC CỦA CHỦNG VI NẤM WQF7 NỘI SINH TRÊN CÂY ĐỈNH TÙNG (*Cephalotaxus mannii* Hook.f.) TẠI HÀ GIANG. Vũ Thị Hạnh Nguyên, Phạm Ngọc Sơn, Quách Ngọc Tùng, Phạm Quỳnh Anh, Lê Phương Chi, Nguyễn Văn Thế, Đỗ Thị Thảo, Phí Quyết Tiến 424

67. PHÂN LOẠI, ĐẶC TÍNH SINH HỌC CỦA CHỦNG *Penicillium crustosum* WQF11 NỘI SINH TRÊN CÂY ĐỈNH TÙNG (*Cephalotaxus mannii* Hook.f.). Phạm Ngọc Sơn, Vũ Thị Hạnh Nguyên, Lê Phương Chi, Phạm Quỳnh Anh, Quách Ngọc Tùng, Lê Thị Thanh Xuân, Nguyễn Thị Thanh Lợi, Phí Quyết Tiến..... 430

68. ĐÁNH GIÁ HOẠT TÍNH KHÁNG KHUẨN VÀ ỨC CHẾ TYROSINASE CỦA CAO CHIẾT THÂN RỄ NGHỆ TRẮNG (*Curcuma aromatica* Salisb.). Võ Thị Ngọc Mỹ, Nguyễn Vũ Hồng Vân, Lê Thị Trúc Huỳnh..... 436

69. XÂY DỰNG QUY TRÌNH ĐỊNH LƯỢNG MIR-9-3P TRONG HUYẾT TƯƠNG BẰNG PHƯƠNG PHÁP STEMLOOP RT-qPCR. Huỳnh Hữu Luân, Dương Thị Chung Thủy, Phan Ngô Hoang, Nguyễn Thị Ngọc Thanh, Nguyễn Thị Huệ 442

70. SỰ BIẾN ĐỔI AMINO ACID CỦA CÁC BIẾN THỂ OMICRON CỦA SARS-CoV-2 TRONG CÁC CA NHIỄM Ở VIỆT NAM. Lê Thị Yến, Huỳnh Phước, Nguyễn Thị Thương Huyền, Thái Kế Quân..... 449

71. SỬ DỤNG CÔNG NGHỆ MASTER MIX SẤY KHÔ CHO NGHIÊN CỨU PHÁT TRIỂN KIT REAL-TIME PCR. Mai Hoàng Khánh Chi, Nguyễn Duy Khánh, Ngô Hồng Gấm 454

72. SO SÁNH HOẠT ĐỘNG CHỐNG OXY HOÁ VÀ KHÁNG KHUẨN CỦA CAO CHIẾT LÁ TRẦU (*Piper betle* L.) TƯƠI VÀ KHÔ. Võ Thị Phước, Nguyễn Thị Tâm, Chế Thị Cẩm Hà..... 460

73. ĐỊNH DANH VÀ ĐÁNH GIÁ HOẠT TÍNH KHÁNG KHUẨN, KHÁNG NẤM CỦA HAI CHỦNG VI KHUẨN LAM DL1 VÀ DL12. Ngô Thị Trang, Nguyễn Thị Thu Hà, Bùi Lê Đăng Minh, Nguyễn Thị Thu Huyền, Trần Thị Thùy Anh, Phạm Thị Lương Hằng..... 467

74. DETERMINATION OF α-MANGOSTIN, TOTAL PHENOLIC CONTENT AND ANTIOXIDANT, ANTIBACTERIAL ACTIVITIES OF MANGOSTEEN (*Garcinia mangostana* L.) PERICARP EXTRACTS. Le Thi Tuoi, Van Thai Than, Nguyen Thi Hien, Trinh Minh Quan, Pham Bui My Linh, Bui Minh Quang, Nguyen Thi Len 474

75. OPTIMIZATION OF EXTRACTION OF TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITIES FROM *Ganoderma lucidum* (Leyss ex. Fr.) Karst. MUSHROOM AT NGHE AN, VIETNAM. Nguyen Tan Thanh, Le Thi My Chau, Dao Thi Thanh Xuan, Tran Phuong Chi, Nguyen Thi Huyen, Dinh Thi Kim Hao, Le Thi Mai Trang, Dinh Thi Phuong An, Nguyen Duc Dien..... 481

76. OPTIMIZATION OF MICROWAVE ASSISTED HYDRO DISTILLATION OF *Houttuynia cordata* LEAVES. Nguyen Tan Thanh, Pham Minh Trang, Tran Dinh Thang, Doan Manh Dung 487

77. TỐI ƯU HÓA QUÁ TRÌNH SẤY BƠM NHIỆT ĐỂ CHIẾT XUẤT TỔNG FLAVONOID VÀ PHENOLIC TỪ LÁ CÂY XUYÊN TÂM LIÊN. Lê Thị Mỹ Châu, Nguyễn Tân Thành, Trần Phương Chi, Nguyễn Thị Huyền, Đào Thị Thanh Xuân, Đặng Thị Ngọc, Đoàn Mạnh Dũng, Nguyễn Đức Anh..... 494

78. HOẠT TÍNH ỨC CHẾ TẾ BÀO UNG THƯ GAN HEPG2 CỦA SeNPs/OLIGOFUCOIDAN CHẾ TẠO BẰNG PHƯƠNG PHÁP CHIẾU XẠ TIA GAMMA (Co-60). Nguyễn Văn Linh, Trần Đức Trọng, Nguyễn Trọng Nghĩa, Nguyễn Thanh Vũ, Lê Quang Luân 501

79. ĐỊNH TÍNH HỢP CHẤT HÓA HỌC THỰC VẬT VÀ ĐÁNH GIÁ HOẠT TÍNH KHÁNG KHUẨN *Staphylococus aureus* CỦA CAO CHIẾT ETHANOL TỪ LÁ VÀ THÂN CÂY TÍA TÔ HAI MÀU (*Perilla frutescens* L.). Trương Quốc Tất, Phùng Thị Thúy Liễu, Nguyễn Duy Khánh..... 507

80. THÀNH PHẦN HÓA HỌC VÀ KHẢ NĂNG KHÁNG KHUẨN CỦA CAO CHIẾT ACETONE LY TRÍCH TỪ LÁ CỦA HAI LOÀI *Amorphophallus opertus* VÀ *Amorphophallus lanceolatus*. Thái Thị Kim Tuyền, Nguyễn Ngọc Ẩn, Nguyễn Thị Diệu Hạnh, Phạm Tấn Việt, Ngô Ngọc Hưng, Lê Văn Sơn, Lê Thành Thọ, Nguyễn Quốc Hùng, Văn Hồng Thiện 513

81. VAI TRÒ CỦA MỘT SỐ THẢO DƯỢC TRONG ĐIỀU TRỊ VÀ DỰ PHÒNG ĐỘT QUỴ THIẾU MÁU NÃO. Đinh Hữu Hùng, Nguyễn Hoàng Anh Thư, Nguyễn Anh Dũng..... 518

82. THÀNH PHẦN HOÁ HỌC, KHẢ NĂNG KHÁNG OXY HOÁ VÀ KHÁNG VIÊM CỦA TINH DẦU NGHỆ VÀNG (*Curcuma longa* L.*)* ĐƯỢC CHƯNG CẤT TỪ NƯỚC ÉP CỦ NGHỆ ĐÃ QUA XỬ LÝ. Nguyễn Thị Thanh, Trần Thị Kim Thi, Đoàn Mạnh Dũng, Phạm Bằng Phương, Nguyễn Thị Thu 527

83. ẢNH HƯỞNG CỦA KHỐI LƯỢNG PHÂN TỬ CỦA CHITOSAN ĐẾN KHẢ NĂNG KHÁNG KHUẨN *Streptococcus pneumoniae* CỦA PHỨC HỢP NANO CHITOSAN-AMOXICILLIN TẠO BẰNG PHƯƠNG PHÁP GEL ION VÀ SẤY PHUN. Nguyễn Vĩnh Nghi, Nguyễn Thị Huyền, Nguyễn Anh Dũng..... 535

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OPTIMIZATION OF EXTRACTION OF TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITIES FROM *Ganoderma lucidum* (Leyss ex. Fr.) Karst. MUSHROOM AT NGHE AN, VIETNAM

Nguyen Tan Thanh[1], Le Thi My Chau[1], Dao Thi Thanh Xuan[1], Tran Phuong Chi[1], Nguyen Thi Huyen[1], Dinh Thi Kim Hao[1], Le Thi Mai Trang[2], Dinh Thi Phuong An[3], Nguyen Duc Dien[1]

[1]School of Chemistry, Biology and Environment, Vinh University
[2]K61, Food Technology, School of Chemistry, Biology and Environment, Vinh University
[3]K60, Biotechnology, School of Chemistry, Biology and Environment, Vinh University

SUMMARY

Ganoderma lucidum (Leyss ex. Fr.) P. Karst, a rich source of several bioactive compounds, has been utilized for its medicinal and nutraceutical purposes since earliest history. Among its bioactive constituents, the total phenolic content and antioxidant activities have been widely reported to be responsible for some its bioactive properties. Recent trends in bioactive compounds' recovery have been focused on finding the best techniques that maximally extract valuable compounds from natural matrices. This study is aimed at optimizing the extraction of total phenolic content (TPC) and antioxidant activities (DPPH) from *G. lucidum* using microwave assisted extraction (MAE) techniques. Response surface methodology (RSM) was applied using a circumscribed central composite design with three independent variables (microwave power, time extraction and ethanol concentration). The yield of TPC and DPPH were maximized and the optimal conditions were determined. The conditions that maximize the responses (total phenolic content and antioxidant activities) were: microwave power 200 W, extraction time 22 min and ethanol concentration 71% ethanol. The experimental values of TPC and DPPH radical scavenging activities were 30.05±0.5 mg GAE/g and 94.82±0.95%, respectively.

Keywords: Antioxidant activities, *Ganoderma lucidum*, microwave-assisted, phenolic, response surface methodology.

INTRODUCTION

Ganoderma lucidum (Leyss ex. Fr.) Karst. is a basidiomycete fungus belonging to the Polyporaceae family, which is known as a medicinal mushroom and as a Chinese traditional folk remedy for centuries. In the regions of China, Vietnam and other Asian countries, *G. lucidum* is used to treat many diseases, including chronic hepatitis, nephritis, hypertension, arthritis, bronchitis, hypercholesterolemia, asthma and gastric cancer [1], [2]. *G. lucidum's* beneficial properties are related to a broad variety of bioactive compounds present in the fruiting body, mycelium and spores. Polysaccharides, triterpens, phenols, steroids, amino acids, nucleosides and nucleotides can be found amongst such compounds. The first two have been most studied, more than 100 compounds have been identified as having biological activity; β-1-3 and β-1-6 D-glucans are the polysaccharides having the greatest biological activity and A and B ganoderic acids in the triterpens [3].

It has been reported that polyphenols are the principal antioxidant components in methanol extracts of *G. lucidum* (16.5 to 27.9 mg/g) [4] and of *G. tsugae* (24.0 to 35.6 mg/g) [5], such values being similar to those reported for other macromycetes such as *Antrodia camphorata* (38.0 ± 0.7 mg/g) [6], *Ramaria botrytis* (20.32 ± 0.7 mg/g), *Hypholoma fasciculare* (17.67 ± 0.27 mg/g) and significantly higher than those found in other species, such as *Agaricus bisporus* (4.49 ± 0.16 mg/g), *Lactarius deliciosus* (3.40 ± 0.18 mg/g) and *Cantharellus cibarius* (1.75 ± 0.50 mg/g) [7].

Many reports on the beneficial effects of microwave assisted extraction (MAE) with respect to medicinal plants have been published, with significant improvements over conventional extraction methods offering much lowered extraction time and enhanced efficiency [8]. Compared with the traditional methods, MAE has many advantages, such as shorter extraction time, lesser solvent consumption, higher extraction rate and better products with lower cost. Direct interaction of microwaves with the free water molecules presents in the glands and vascular systems, causes a tremendous increase in internal pressure inside the plant cell due to evaporation of the internal moisture content which result in the subsequent rupture of the plant tissue and the release of the active compounds into the organic solvent [8]. Therefore, MAE is an interesting alternative to conventional extraction methods, especially in the case of botanical extractions. Using RSM to optimized Microwave - assisted extraction process with A Box-Behnken Design consisting fifteen experimental points with three replicates at the central point. The independent variables were the microwave power, extraction time and ethanol concentration while dependent variables were yields of TPC and DPPH radical scavenging activity of *G. lucidum* (Leyss ex. Fr.) P. Karst at Nghe An, Vietnam.

MATERIAL AND METHODS

Material

Ganoderma lucidum (Leyss ex. Fr.) Karst. mushroom was collected at the Quy Hop district of Nghe An province, Vietnam in September 2020 and identified by Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The material is dried, grounded and stored at 4^{o}C for further experiments.

Methods

Total Phenolic Content (TPC)

The TPC of the *G. lucidum* extract was measured according to the method reported by Singleton *et al*. [9] with a little modification. This method is based on measuring color change caused by reagent by phenolates in the presence of sodium carbonate. 1 mL of sample was mixed with 5 mL of Folin-Ciocalteu's solution. After 3 min, 4 mL of 7.5% sodium carbonate solution was added to a mixture and adjusted to 10 mL with deionized water. The mixture was kept at room temperature in a dark environment for 60 min. The color change was determined by scanning the wavelength at 765 nm. The TPC of the *G. lucidum* extract was determined as mg gallic acid equivalent using the standard curve prepared at different concentrations of gallic acid and reported as mg GAE/g dry weight (DW).

DPPH radical scavenging assay

The antioxidant activity of the *G. lucidum* extract was determined using the DPPH radical scavenging assay. The DPPH radical scavenging assay [10] measures the capacity of a compound or a sample to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl radical. DPPH of each extract was measured as follows: 0.5 mL of 0.5 mM DPPH liquid was put into a test tube in which 1 mL ethanol, 10 µL sample and 990 µL of 100 mM sodium acetate buffer (pH 5.5) were agitated, which remained in a darkroom for 5 minutes in order to induce responses. After that, a UV spectrometer was used to measure the concentration of the remaining radical in 517 nm.

All determinations were determined by replicate experiments with triplicate analysis. The radical scavenging activity was calculated according to the Eq. 1) below:

$$\text{Radical scavenging activity (\%)} = [1 - (Abs_{sample}/Abs_{control\ blank})] \times 100 \quad (1)$$

with Abs_{sample} being the absorbance of sample extract and $Abs_{control\ blank}$ being the absorbance of blank control.

Box-Behnken Design (BBK) of Response surface methodology

Microwave - assisted extraction optimized the experimental design using RSM. A Box-Behnken Design consisting fifteen experimental points with three replicates at the central point. The independent variables were the microwave power (X_1), extraction time (X_2) and ethanol concentration (X_3) while dependent variables were yields of TPC (Y_1) and DPPH radical scavenging activity (Y_2). Experiments were performed in replicate and the average values were used as the response. Statistical analysis on the means of triplicate experiments was carried out using the ANOVA procedure of the design expert software, version 7.0.

The effects of the extraction parameters were evaluated using the program Design-Expert®, version 7.0.0. The response variable was fitted to be a second-order polynomial model as follows (2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response; β_o is the intercept coefficient; β_i is the linear coefficient; β_{ii} is the squared coefficient; β_{ij} is the interaction coefficient; X_i and X_j are the coded independent variables; term of X_iX_j and X_i^2 are the interaction and quadratic terms, respectively.

RESULTS AND DISCUSSION

Fitting the models

Based on single factor research, choose the optimal area as microwave power 100-300W, extraction time 10 – 30 min and ethanol concentration 50-80% (table 1).

Three variables were selected to find the optimized condition for antioxidants extraction using Box - Behnken design. This design consisted of 15 experimental points with three replicates at the central point. The independent variables studied were microwave power (W), extraction time (min) and ethanol concentration (% v/v), and while response variables were total phenolic content and DPPH radical scavenging activity. The range and the levels of the experiments variables used in the coded and uncoded form in this study are given in table 1.

Table 1. Coded level of independent variables used in the RSM design

Independent variables	Units	Coded symbols	Coded variable levels		
			-1	0	+1
Microwave power	W	X_1	100	200	300
Extraction time	min	X_2	10	20	30
Ethanol concentration	%	X_3	50	65	80

Table 2. Experimental design and response values

RUN	X_1 (W)	X_2 (min)	X_3 (%)	TPC Y_1 (mgGAE/g)	DPPH Y_2 (%)
1	200	20	65	31.14	95.97
2	100	10	65	23.81	88.89
3	100	20	80	26.52	91.82
4	200	30	50	28.72	95.21
5	200	20	65	31.04	96.12
6	200	10	80	26.15	93.98
7	200	30	80	29.92	95.14
8	100	30	65	28.18	92.25
9	100	20	50	24.61	90.32
10	300	30	65	29.16	93.85
11	300	10	65	26.74	91.89
12	300	20	50	27.24	92.85
13	200	10	50	25.02	91.76
14	300	20	80	27.83	93.35
15	200	20	65	31.19	96.16

The final empirical regression model of their relationship between responses and the two tested variables for total phenolic content and DPPH could be expressed by the following quadratic polynomial equation [Eqs. (3-4)]:

$$Y_1 = 31.12 + 0.98X_1 + 1.78X_2 + 0.60X_3 – 0.49X_1X_2 - 0.33X_1X_3 + 0.018X_2X_3 – 2.53X_1^2 – 1.62X_2^2 – 2.05X_3^2 \quad (3)$$

$$Y_2 = 96.08 + 1.08X_1 + 1.02X_2 + 0.52X_3 – 0.35X_1X_2 - 0.25X_1X_3 – 0.57X_2X_3 – 3.18X_1^2 – 1.21X_2^2 – 0.85X_3^2 \quad (4)$$

Where Y_1 is total phenolic content, Y_2 is the DPPH radical scavenging activity, X_1 is the microwave power, X_2 is the time extraction. and X_3 is the ethanol concentration.

Table 3. Analysis of variance (ANOVA) for the model

Source	Y_1 – TPC		Y_2 – DPPH	
	F- value	p- value	F- value	p-value
Model	607.43	<0.0001[c]	304.81	<0.0001[c]
Linear				
X_1	526.21	<0.0001[c]	383.13	<0.0001[c]
X_2	1736.43	<0.0001[c]	503.74	<0.0001[c]
X_3	199.21	<0.0001[c]	87.98	0.0002[c]
Quadratic				
X_1^2	1610.28	<0.0001[c]	1497.72	<0.0001[c]
X_2^2	665.38	<0.0001[c]	222.00	<0.0001[c]
X_3^2	1056.58	<0.0001[c]	108.49	0.0001[c]
Interaction				
X_1X_2	64.94	0.0005[c]	20.03	0.0065[b]
X_1X_3	29.76	0.0028[b]	10.22	0.0241[b]
X_2X_3	0.084	0.7840[NS]	53.58	0.0007[c]
Lack of Fit	3.52	0.2293[NS]	3.40	0.2356[NS]
R^2	0.9991		0.9982	
C.V.%	0.43		0.17	

[a]: $p < 0.05$; [b]: $p < 0.01$; [c]: $p < 0.001$; NS: non-significant;
R^2 = Coeficient of multiple determination; C.V.% = Coeficient of variance.

Table 3 show the results of the quadratic polynomial model, one way analysis of variance was used. Good fits were achieved and the coefficients of multiple determinations (R^2) for the quadratic regression model were 0.9991 and 0.9982 for the yields of total phenolics content and antioxidant activity respectively. The R^2 of these values were

higher than 0.7, indicating that the model was suitable for use in the experiment [11]. In addition, the coefficients of the variance (C.V. %) were calculated to be 0.43 %, and 0.17 % for total phenolics and antioxidant activity respectively, indicating a good reliability for the experiment values [12].

No statistical significance in the lack of fit of any of the two models was found ($p > 0.05$), meaning that the lack of fit was not significant compared to the pure error. Therefore, the models can be used not only to adequately fit the experimental data but also to predict the response of tested dependent variables.

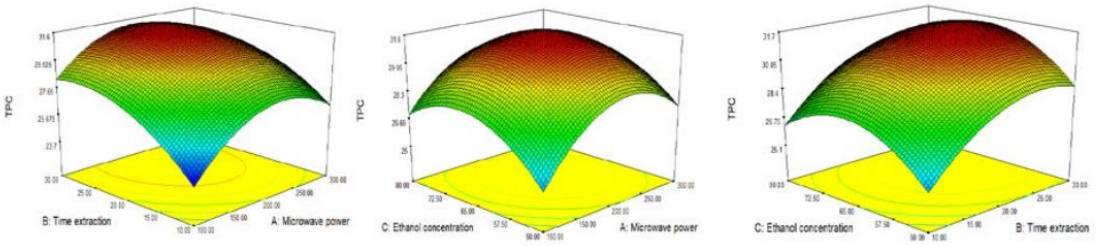
Response surface analysis

Three factors that microwave power, ethanol concentration and time extraction effects the extraction condition of the maximum total phenolics content and DPPH radical scavenging activity. This section discusses how these conditions work on natural antioxidants extraction. Three dimensional model graphs were plotted as shown in the respective figures. The response surface plots of the model were done by varying two variables, within experimental range under investigation and holding the other variables at its central level. Two dimensional response surfaces were constructed as depicted in figure 1 and figure 2.

Response surface analysis of total phenolic content

The response surface plots and contour plots shown in figure 1a-c demonstrated the effect and interaction of independent variables on the yields of total phenolics. At figure 1 and table 3, microwave power, extraction time and ethanol concentration showed negative quadratic effects ($p < 0.001$) while extraction time exhibited positive linear effects on the yields of total phenolics ($p < 0.001$).

In figure 1a, the yields of total phenolics increased with the increase of microwave power in the range of 100 to 220 W, and then when microwave power increased from 220 to 300 W, the TPC decreased. The yields of total phenolics increased with the rise of extraction time in the range of 10 to 30 min when microwave power were in the range of 200 to 300 W. The TPC also increased with the increase of ethanol concentration from 50%v/v to 70%v/v, and thereafter decreased (Fig.1b). This indicated that low to medium concentrations of ethanol were favorable for extracting phenolic compounds from such plants. This was due to the addition of ethanol in water which improved the breakage degree of cell membranes of plant raw material [13] and improved the solubility of phenolic substances [14]. However, a decline in the TPC when a high level of ethanol was used was probably due to the increased diffusion resistance by the coagulation of proteins in the plant leaves [15].

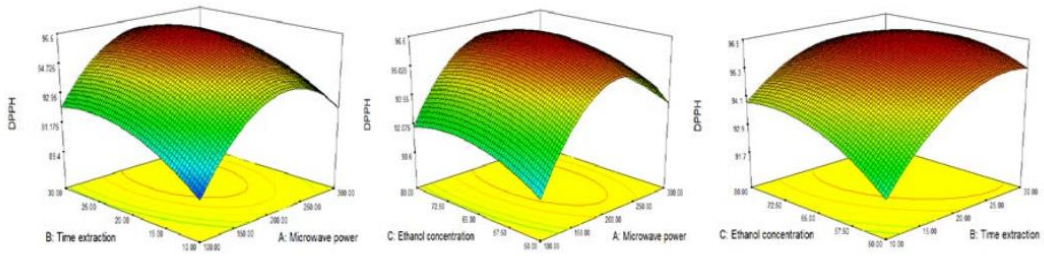


a b c

Figure 1. The response surface plot of TPC

Response surface analysis of antioxidant activity (DPPH)

Figure 2a-c shows three-dimension surface plots of the relationship between independent and dependent variables for antioxidant activity from the *G. lucidum* extract. Extraction temperature, time and ethanol concentration showed negative quadratic effects on the yields of antioxidant activity from the *G. lucidum* extract (table 3) ($p < 0.05$). The DPPH increased when microwave power increase from 100 to 230 W and thereafter decreased. The DPPH increased with the increase of extraction time and ethanol concentration in the range research. The addition of medium levels of ethanol into extraction solvent led to an increase in the antioxidant activity of the extract due to improved solubility of antioxidant compounds. The presence of suitable water in the extraction solvent also enhanced swelling of plant material, followed by increased extraction yield. Rises in extraction temperature and time improved the antioxidant activity of the extracts. On the contrary, declined antioxidant activity was observed at higher temperatures and time extraction. This might be attributed to the thermo sensitive phenolic compounds degrading with a rise of extraction temperature and longer extraction time, in particular chlorogenic acid. These results supported the important role of phenolic compounds and chlorogenic acid (main phenolic compound) in the antioxidant activity of *G. lucidum.*



a b c

Figure 2. The response surface plot of DPPH.

Optimization and model verification

The optimal values of the independent variables were obtained by solving second - order regression equations using a numerical optimization method. Experimental data suggested the existence of optimization of yield of TPC and DPPH radical scavenging activity with microwave power 200 W, extraction time 22 min and ethanol concentration 71% v/v.

Table 4. Optimum conditions, predicted and experimental values of responses on extraction of *G. lucidum* extract[a]

Independent variables			Dependent variables (Response)	Optimum value		% Difference (CV)
X_1 (W)	X_2 (min)	X_3 (%)		Experimental	Predicted	
200	22	71	Y_1 (mgGAE/g)	30.05±0.5	31.3851	4.24
			Y_2 (%)	94.82±0.95	96.3436	1.58

[a]X_1, microwave power (W); X_2, extraction time (min); X_3, ethanol concentration (% v/v); Y_1, total phenolic content (mg GAE/g); Y_2, DPPH radical scavenging activity (%). [b]Mean ± standard deviation (SD) of three determinations (n= 3).

CONCLUSION

In this study, RSM is a successful tool to describe the microwave-assisted extraction process yield of the total phenolic content and DPPH radical scavenging activity from *Ganoderma lucidum* (Leyss ex. Fr.) Karst. Mushroom at Nghe An, Vietnam for the following the optimal parameters: microwave power 200 W, extraction time 22 min and ethanol concentration 71% v/v. Under the optimum conditions, the experimental values of total phenolic content and antioxidant activity were 30.05±0.5 mg GAE/g and 94.82±0.95 %. With these optimum conditions, the experimental values of total phenolic content and antioxidant activity were agreed with those predicted, thus indicating suitability of the model employed.

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TỐI ƯU HÓA CHIẾT XUẤT TỔNG HÀM LƯỢNG PHENOLIC VÀ CÁC CHẤT CHỐNG OXY HÓA TỪ QUẢ THỂ NẤM *Ganoderma lucidum* (Leyss ex. Fr.) Karst. THU HÁI TẠI NGHỆ AN, VIỆT NAM

Nguyễn Tân Thành[1], Lê Thi Mỹ Châu[1], Đào Thị Thanh Xuân[1], Trần Phương Chi[1], Nguyễn Thị Huyền[1], Đinh Thị Kim Hào[1], Lê Thị Mai Trang[2], Đinh Thị Phương An[3], Nguyễn Đức Diện*[1*]

[1]Viện Công nghệ Hóa, Sinh, Môi trường, Đại học Vinh
[2]K61, Công nghệ Thực phẩm, Viện Công nghệ Hóa, Sinh, Môi trường, Đại học Vinh
[3]K60, Công nghệ Sinh học, Viện Công nghệ Hóa, Sinh, Môi trường, Đại học Vinh

TÓM TẮT

Ganoderma lucidum (Leyss ex. Fr.) P. Karst, là một nguồn giàu các hợp chất hoạt tính sinh học, đã được sử dụng cho các mục đích y học và dinh dưỡng từ lâu đời. Trong số các thành phần hoạt tính sinh học của nó, tổng hàm lượng phenolic và các chất chống oxy hóa đã được xác định là thành phần có hoạt tính sinh học chính. Xu hướng trong những năm gần đây là chiết xuất các hợp chất có hoạt tính sinh học và tập trung vào việc tìm ra các kỹ thuật tốt nhất nhằm chiết xuất tối đa các hợp chất có giá trị từ tự nhiên. Nghiên cứu này nhằm mục đích tối ưu hóa việc chiết xuất tổng hàm lượng phenolic (TPC) và hoạt tính chống oxy hóa (DPPH) từ *G. lucidum* bằng kỹ thuật chiết xuất có hỗ trợ vi sóng (MAE). Sử dụng phương pháp bề mặt đáp ứng (RSM) với thiết kế thí nghiệm theo Box - Bnhken với ba biến số độc lập (công suất vi sóng, thời gian chiết xuất và nồng độ ethanol). Hàm lượng TPC và DPPH thu được tối đa với các điều kiện tối ưu đã được xác định. Các điều kiện tối ưu để các hàm mục tiêu đạt giá trị lớn nhất (TPC và DPPH) là: công suất vi sóng 200 W, thời gian chiết 22 phút và nồng độ etanol 71%. Giá trị thực nghiệm tại các thông số tối ưu thu được hàm lượng TPC và DPPH là 30,05±0,5 mg GAE/g và 94,82±0,95%.

Từ khóa: Ganoderma lucidum, phenolic, chống oxy hóa, chiết vi sóng, đáp ứng bề mặt.

* Author for correspondence: Tel: 0988573768. Email: ducdienbio78@gmail.com