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OPTIMIZATION AND KINETICS OF THE SUPERCRITICAL FLUID EXTRACTION OF TRITERPENOIDS FROM *GANODERMA LUCIDUM* WITH CO_2 AND ETHANOL AS COSOLVENT

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ABSTRACT

This research aims to optimize ethanol-modified supercritical carbon dioxide extraction (SC-CO_2) conditions for extracting triterpenoids from *G. lucidum* using a response surface methodology (RSM). A central composite face-centered design (CCF) was employed to investigate the influences of three independent variables, including ethanol concentration in SC-CO_2 (X_1), extraction pressure (X_2), and temperature (X_3) on the response, triterpenoid content (Y). The results showed that the optimal RSM-based SC-CO_2 conditions were 380 bar, 7%v/v, and 60°C, achieving the maximum value of 1.49g/100g. Under these conditions, the predicted values for triterpenoids agreed well with the experimental results, confirming the validity of the generated model. The SC-CO_2 extraction technique showed clear advantages over conventional maceration extraction and soxhlet extraction in terms of high triterpenoid recovery and antioxidant activity. The kinetics of the solvent-based triterpenoid extraction processes were subsequently assessed via the first-order and second-order kinetic models. The second-order kinetic model was more sufficient to describe the extraction mechanism of triterpenoids from *G. lucidum* in comparison to the first-order kinetic extraction model. According to these findings, SC-CO_2 extraction is a promising and efficient method for triterpenoid extraction from *G. lucidum*.

1. Introduction

Ganoderma lucidum (*G. lucidum*) is a traditional medicinal mushroom in China, Japan, and other Asian countries, which has been utilized for more than 2000 years (Li et al., 2020; Zhu et al., 2020). The *G. lucidum* spore contains many bioactive compositions such as triterpenoids, steroids, phenolics, and nucleotides (Mau et al., 2001; Zhu et al., 2020; Li et al., 2016). Among these compounds, triterpenoids have been known as strong antioxidant compounds in *G. lucidum* (Taofiq et al., 2017; Zhang et al., 2008). Triterpenoids have many effective therapeutic actions including antitumor, anti-inflammatory, anti-hepatitis, antimetastatic, and antihyperlipidemic (Cai et al., 2016; Zhu et al., 2020; Pan et al., 2013).

In the last few decades, the conventional extraction methods (i.e., maceration, soxhlet, and heat reflux extraction) are commonly used for industrial extraction of triterpenoids from *G. lucidum* (Taofiq et al., 2017; Plazas et al., 2020). The main disadvantages of conventional soxhlet extraction are very time-consuming and require large volumes of solvents (Rodrigues et al., 2021; Xu et al., 2017; Ibáñez and Cifuentes,

2015; Blicharski and Oniszczuk, 2017). In comparison, supercritical carbon dioxide (CO_2) fluid extraction has obvious advantages such as low operation temperature, low cost, and no solvent residue. However, CO_2 is not a suitable solvent for the extraction of triterpenoid compounds because triterpenoids are usually non-polar compounds. Therefore, the use of ethanol as a co-solvent extraction has been considered for enhancing the solubility of the triterpenoid compounds in the solvent (Herrero et al., 2010; Pieczykolan et al., 2019).

Ethanol, a green solvent, is suggested for the extraction of triterpenoids from *G. lucidum* due to its availability, low cost, and environmentally friendly solvent (Rodrigues et al., 2021; Li et al., 2017). Some previous studies showed that various extraction factors such as co-solvent extraction, extraction temperature, and extraction pressure significantly influence the extraction efficacy of triterpenoids (Zhu et al., 2020; Yim et al., 2019). However, to the best of our knowledge, there are limited or no studies, available in the literature on optimization and kinetic extraction of triterpenoid from *G. lucidum*. In this study, CO_2 solvent with the addition of ethanol was used for triterpenoid extraction from the Vietnamese *G. lucidum*. The optimization of different SC-CO_2 extraction conditions (extraction pressure, ethanol content in SC-CO_2, and extraction temperature) on the recovery of triterpenoids was investigated. A response surface methodology (RSM) was used to find the optimal extraction conditions and interpret the interactions among these independent variables by establishing a model from experimental data. Furthermore, the first and second-order kinetic models were utilized to explain and describe the kinetic behavior of the SC-CO_2 process extracting triterpenoids with a mixture of SC-CO_2 and ethanol. Then the radical scavenging activity of the extracts was compared with the soxhlet extraction (SE) and maceration extraction (ME). It is expected that the developed SC-CO_2 processes will contribute to improving the efficiency of the overall extraction process and be applied in the *Ganoderma* industry.

2. Materials and methods

2.1. Materials

The dried *G. lucidum* was obtained from a L'ang farm store (Da Lat city, Lam Dong province, Vietnam) as dry material. All samples were ground in a Waring blender and passed through a 20-mesh sieve before extraction.

Ursolic acid, perchloric acid 70 %, glacial acetic acid, and ethanol (99.8%) were obtained from Merck (Dam-stadt, Germany). Carbon dioxide (99.9%) was purchased from the Daxing Gas Co., Beijing, China. All other chemical reagents used were of analytical grade.

2.2. Extraction procedure

The SC-CO_2 method was experimented using a supercritical fluid system (SFE-500F2-C50, Waters, USA). In this study, SC-CO_2 with ethanol addition was used to investigate the effect of extraction parameters on the recovery of triterpenoid from *G. lucidum*. Firstly, the effect of the addition of ethanol as a polar cosolvent extraction on the triterpenoid content was analyzed. In each run, approximately 10 g *G. lucidum* powder was introduced into the extraction vessel, and the extraction was performed at 50°C, with the different ethanol concentrations in SC-CO_2 (0-12%v/v). The extraction pressure was set at the desired value of 400 bar. Secondly, the influence of extraction pressure on the recovery of triterpenoids was experimented on between the range of 200 and 500 bar at 50°C using 6%v/v of ethanol in SC-CO_2. Finally, the influence of temperature extraction on the recovery of triterpenoids was performed. The temperature extraction was in a range of 30 to 80°C, and the extraction process was performed with 6%v/v of ethanol at 400 bar. All the extractions were performed with a constant CO_2 flow rate of 0.45 mL/min for 2 hours. After extraction, the extracts were collected and the solvent was then evaporated in a rotatory evaporator (Buchi R210, Flawil Switzerland). The extracts were kept refrigerated at -20°C for further analysis.

2.3. Experimental design

Central composite face-centered design (CCF) was used to determine the optimum conditions for cosolvent extraction of triterpenoid content. Three independent variables were ethanol concentration in SC-CO_2 (X_1, %v/v), extraction pressure (X_2, bar), and extraction temperature (X_3, °C).

Table 1. Coded and actual levels of three variables

Variables	**Coded levels of variables**		
	Low (-1)	Medium (0)	High (+1)
Ethanol content (X_1, %v/v)	2	7	12
Pressure extraction (X_2, Bar)	200	350	500
Extraction temperature (X_3, °C)	30	55	80

Table 2. Experimental and predicted gamma oryzanol recovery under variable ethanol content (X_1, %v/v), pressure extraction (X_2, Bar), and extraction temperatures (X_3, °C)

Run	Coded variable						Experimental value	Predicted value
	X_1	X_2	X_3	X_1	X_2	X_3	g/100g	g/100g
1	-1	-1	-1	2	200	30	0.513±0.01	0.506±0.02
2	-1	-1	+1	2	500	30	0.772±0.02	0.735±0.01
3	-1	+1	-1	2	200	80	1.050±0.01	1.033±0.01
4	-1	+1	+1	2	500	80	0.938±0.01	0.928±0.03
5	+1	-1	0	12	200	30	0.791±0.01	0.794±0.01
6	+1	-1	+1	12	500	30	1.098±0.02	0.986±0.02
7	+1	+1	0	12	200	80	0.596±0.01	0.578±0.01
8	+1	+1	+1	12	500	80	0.810±0.01	0.811±0.02
9	0	0	0	7	200	55	1.231±0.02	1.128±0.01
10	0	0	+1	7	500	55	1.510±0.02	1.507±0.01
11	0	-1	0	7	350	30	1.281±0.01	1.260±0.02
12	0	+1	0	7	350	80	1.539±0.01	1.460±0.03
13	-1	0	0	2	350	55	1.196±0.01	1.068±0.01
14	+1	0	0	12	350	55	1.356±0.02	1.308±0.01
15	0	0	0	7	350	55	1.511±0.01	1.450±0.02
16	0	0	0	7	350	55	1.512±0.01	1.516±0.01
17	-1	-1	-1	2	200	30	0.513±0.01	0.510±0.02

The triterpenoid content was selected as the response (Y) for the combination of the independent variables. Statistic MODDE software (version 13; Umetri, Umeå, Sweden) was used for the regression analysis of the experimental data. The variation of these factors was varied at three levels (low, moderate, and high), and coded as -1, 0, and +1, respectively (Table 1), and the experimental design consisted of 17 experimental runs (Table 2). A second-order polynomial equation was established to predict for optimization of the triterpenoid content.

$$Y = \beta_0 + \sum_{i-1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X^2 \qquad (1)$$

where Y is the predicted response; β_0 is a constant; X_1, X_2 and X_3 are independent variables and βi, βij and βii are the linear

coefficients, interaction coefficients, and quadratic coefficients, respectively. The model adequacy was evaluated based on an F-test and the coefficient of determination (R^2) using analysis of variance (ANOVA).

2.4. Kinetic extraction

In this study, the first- and second-order kinetic models were employed to explain the extraction behaviors. The kinetic analysis was conducted based on two scenarios: (i) variable extraction temperature (30°C, 55°C, 80°C), given 350 bar SC-CO_2 pressure; and (ii) variable SC-CO_2 pressure (200 bar, 350 bar, 500 bar), given 55°C extraction temperature. Ethanol was used as cosolvent extraction (the RSM-based optimal ethanol concentration was 7%v/v), while the extraction time was varied between 0 min, 20 min, 40 min, 60 min, 80 min, 100 min, and 120 min.

2.4.1. First-order kinetic extraction

The first-order kinetic extraction was described well by Hobbi et al. (2021) as follows:.

$$\frac{dC_t}{dt} = k.(C_e - C_t) \quad (2)$$

where C_t is the extraction capacity at different extraction time t, and C_s is the triterpenoid content of G. lucidum at saturation. k (min^{-1}) is the extraction rate constant. Eq. 2 was then integrated at the boundary conditions Ct = 0 at t = 0 and Ct = Ct at t = t to obtain Eq. 3.

$$\ln\frac{C_e}{C_e - C_t} = k.(C_e - C_t) \quad (3)$$

2.4.2. Second order kinetics

In this study, the second-order model was employed to describe the kinetics behavior of solid-liquid extraction, and can be written as follows:

$$\frac{dC_t}{dt} = k_s.(C_e - C_t)^2 \quad (4)$$

where k_s is the second-order rate constant (100g/g min), C_t is the weight of the triterpenoids in the SC-CO_2 extraction at a given time t (g/100g), and Cs is the equilibrium concentration of the triterpenoids extracted by the SC-CO_2 (g/100g). Eq. 4 can be integrated at the initial and boundary condition (t = 0 to t, and C = 0 to C), and then Eq. 5 can be re-arranged as:

$$\frac{t}{C_t} = \frac{1}{k.C_e^2} + \frac{t}{C_e} \quad (5)$$

When *t* approaches 0, the initial extraction rate, *h*, is written as in Eq. 6.

$$h = k.C_e^2 \quad (6)$$

The initial extraction rate (h), the extraction capacity (C_S), and the second-order extraction rate constant (k) can be determined from the slope and intercept by plotting t/C_t versus t.

2.5. Conventional extraction

The efficiency of extraction of soxhlet extraction (SE) and ethanol maceration extraction (ME) was carried out to compare with the SC-CO_2 extraction method. For soxhlet extraction, 10 g *G. lucidum* powder was performed with 500 mL of ethanol 99.8% for 6 hours (Rodrigues and Silva 2021). For ethanol maceration, 100g G. lucidum powder was extracted with 1 L ethanol 99.8% for 24 hours in a shaking water bath set at 160 rpm and 32±0.5°C, based on the preliminary experiment, showing a suitable extraction condition. Then, the solution was filtered through a Whatman No. 1 filter paper to collect the extracts. The solvents were removed using a rotary evaporator (Buchi R210, Flawil Switzerland), and the extracts were then stored at −20 °C for further analysis. The experiments were performed in triplicate.

2.6. Determination of triterpenoid content

The determination of total triterpenoids was performed following the method of Wei et al., 2015 with minor modifications. Specifically, a 0.16ml extract was mixed with 0.4 mL of 5% vanillin/glacial acetic acid (w/v) in the screw cap test tube. After that, 1.0 mL of perchloric acid solution was added and incubated at 60°C for 30 min using a water bath (Memmert WNB, GmbH & Co. KG, Germany). The mixture was rapidly cooled and added 5.0 mL of glacial acetic acid, and then measured at 573 nm using a UV spectrophotometer/NIR (Shimazu, UV-2600,

Japan). For triterpenoid analysis, ursolic acid was used as the standard solution. To construct the calibration curves, the standard solutions of triterpenoid (0.1–1.0 g/100mL in methanol) were used. The results were calculated in mg of ursolic acid equivalents per g of dw.

2.7. Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) (JEOL Model JSM-6490LV, Peabody, MA, USA) was used to depict the effect of different extraction methods on the morphologic structure of *G. lucidum*. The dried samples were placed on an adhesive carbon tab and coated with a thin layer of gold (Cressington 108 auto, Ted Pella, Redding, CA, USA) by sputtering. The most representative SEM images were obtained at 1000x magnifications with an accelerating voltage of 15kV.

2.8. Statistical analysis

The statistical analyses were conducted using Stagraphic Centrution XV (Statsoft Inc., Umeå, Sweden), and the results were expressed as mean ± SD, and. The data from the response surface methodology (RSM) were analysed using Modde (version 10; Umetri, Umeå, Sweden) by F-test and ANOVA.

3.Results and discussions

3.1. The effect of ethanol as a polar cosolvent extraction on the triterpenoid content

Triterpenoids are less soluble in pure SC-CO_2 (Yang and Wei, 2015; Domingues et al., 2013). The addition of a polar cosolvent is thus necessary to increase the solubility of triterpenoids in the SC–CO_2 solvent. Fig 1. illustrates the effect of ethanol content in SC-CO_2 on the recovery of triterpenoids extracted from G. lucidum using the SC-CO_2 method, where the ethanol contents in SC-CO_2 varied between 0%v/v, 2%v/v, 4%v/v, 6%v/v, 8%v/v, 10%v/v, and 12%v/v.

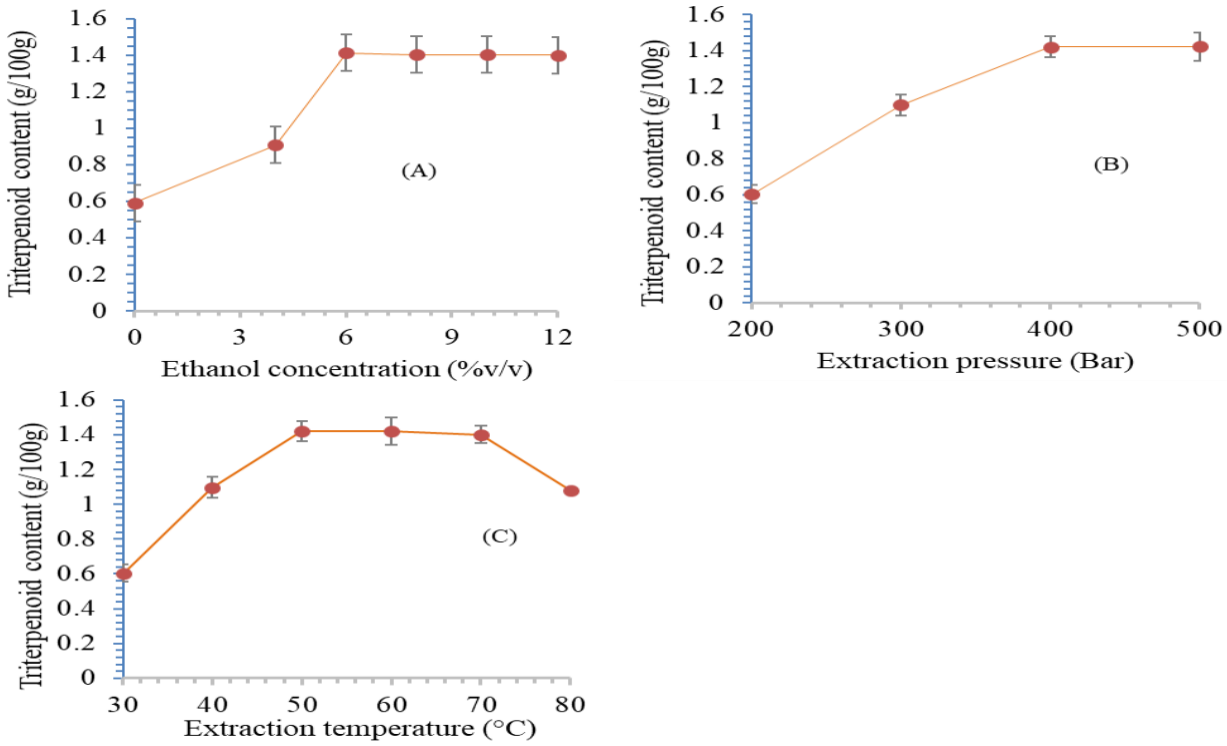


Figure 1. The effects of extraction variables on triterpenoid content: (A) effect of ethanol concentration in SC-CO_2 on the triterpenoid content; (B) effect of extraction pressure on the triterpenoid content; (C) effect of extraction temperature on the triterpenoid content.

As shown in Fig 1A, it can be seen that the triterpenoid contents obtained from the extracts significantly increased when the ethanol content varied from 2%v/v to 8 %v/v. At 6%v/v of ethanol, the triterpenoid content achieved the maximum values of 1.411g/100g, approximately 1.3 times higher than that of the extracted sample without ethanol (0%v/v) and 2%v/v. The results could be attributed to the interaction of ethanol with the matrix of the cell plant, which enhanced the solubilization of triterpenoids into the extraction solvent. Its finding is similar to Pieczykolan et al., 2019, who documented that the addition of ethanol enhanced the SC-CO_2 solvent power, and caused the swelling of the matrix, thus increasing the extraction yield. However, the addition of ethanol in SC-CO_2 was higher than 6%v/v, and the dissolution of triterpenoids was slightly decreased, as shown in Fig 1A. Taking all of the results into consideration, within the ranges of the parameters studied, the best ethanol concentration in SC-CO_2 for extraction was 6%v/v.

3.2. The effect of extraction pressure on triterpenoid content

The SC-CO_2 pressure can help triterpenoids dissolve out of cells. As presented in Fig 1B, it can be seen that the triterpenoid contents obtained from the SC-CO_2 extracts significantly increased when the extraction pressure ranged from 200 to 500 bar. The highest recovery of triterpenoids was 1.422 g/100g as the extraction pressure was in a range of 400-500 bar. This could be explained that an increase in pressure extraction at a constant temperature leads to a higher density of solvent extraction, resulting in the enhancement of the release of triterpenoids into the solvent, thus producing a higher yield of the triterpenoids. A similar result was reported by Yim et al., 2019, who showed that the higher the pressure used for extraction, the more solvent entered inside the cells and more bioactive compounds dissolved into the solvent. In 2015, Yang and Wei reported that an increase in pressure extraction caused changes in the mass liquid transfer. Consequently, the bioactive compounds are easily released into the solvent. Nevertheless, high pressure was unsafe and expensive, and the extract purification and analysis were more complicated. Thus, the best extraction pressure in this study was 400bar.

3.3. The effect of extraction temperature on triterpenoid content

Experiments were conducted to evaluate the effect of extraction temperature on the recovery of triterpenoids. The extraction was performed with 400bar extraction pressure and 6%v/v ethanol at different extraction temperatures (30, 40, 50, 60, and 70°C, respectively). In Fig 1C, the content of triterpenoids markedly increased as the temperature increased from 30 to 70°C, and achieved the maximum content at 50°C (1.421 g/100g). However, the content of triterpenoids slightly decreased when the extraction temperature continued to increase (exceeded 70 °C). It could be attributed to an increase in temperature (over 60°C) that destroyed the triterpenoid molecular structure of the five rings (Cai et al., 2019). Additionally, an increase in extraction temperature elevated the volatility of the solvent, reducing the diffusivity of the solutes to be extracted. Therefore, the extraction temperature of 50 °C was selected for supercritical fluid extraction of triterpenoid from *G. lucidum*.

3.4. Model fitting

In this study, RSM design (based on CCF) was used to investigate the effects of three independent factors on the recovery of triterpenoids. These parameters included ethanol content in SC-CO_2 (X_1), extraction pressure (X_2), and extraction temperature (X_3). Y is the response for the triterpenoid content. The 17 experiments of the design matrix and the measured average of triterpenoid are shown in Table 2. The experimental and predicted triterpenoid values were in the range of 0.513–1.512g/100g. The ANOVA results were used to check the adequacy of the suggested model and shown in Table 3.

Table 3. Results of the ANOVA for the response surface quadratic model

Tritepenoid content	Degree of free dom	Sum of squares	Mean square	F-value	p-value
Total Corrected	17	20.3167	1.19510		
Regression	9	1.80772	0.200857	38.6237	0.000
Residual	7	0.03640	0.005200		
Lack of Fit	5	0.03602	0.007203	37.5195	0.056
Pure Error	2	0.00039	0.000192		

R^2 = coefficient of determination = 0.983; adjusted R^2 = 0.957; Model predictive ability Q^2 = 0.819; $p < 0.05$ indicates statistical significance.

The model's F-value of 38.46 and the p-value of 0.0001 implied that the model is significant. The lack of fit with the p-value of 0.07 was not significant, which indicated the suitability of the model to predict the variations.

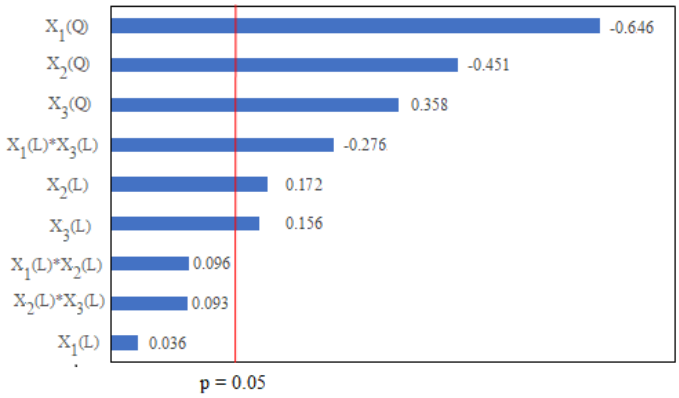


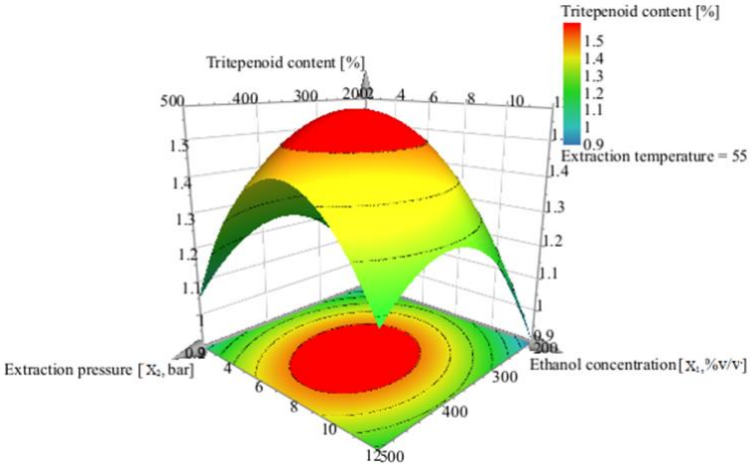
Figure 2. Pareto chart of the data analysis ($p<0.05$)

The fitness and predictive ability of the model are evaluated by the coefficient of determination (R^2), the adjusted R^2, and model predictive ability (Q^2) (Phan et al., 2020). According to our results, the R^2 and adjusted R^2 were 0.983 and 0.957, respectively, showing that 98.3% and 95.7% of the variability in the response was explained by the model. The Q^2 was 0.819, and the difference between R^2 (0.957) and Q^2 (0.819) was less than 0.3, which confirmed the validity of the predicted model (Phan et al., 2020). Furthermore, to evaluate the interactions of each variable on the recovery of triterpenoids, the Pareto analysis was used. As shown in Fig 2, the linear terms (X_2, X_3), the interaction terms (X_1*X_3), and the quadratic terms (X_2^2, X_3^2) had a considerable influence on the recovery of triterpenoid. Meanwhile, the linear term (X_1), the interaction terms (X_1*X_2, X_2*X_3) did not affect the triterpenoid recovery and were eliminated. A polynomial equation was then generated as follows.

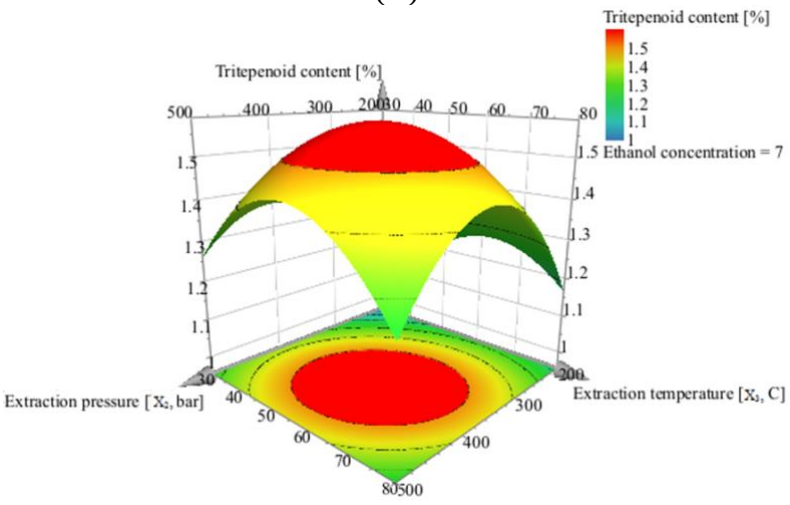
$$Y = 1.476 - 0.323X_1^2 + 0.229X_2^2 - 0.179X_3^2 + 0.086X_2 + 0.078X_3 - 0.138X_1 * X_3 \quad (6)$$

where the negative sign in front of the terms indicates the antagonistic effects and the positive sign indicates the synergistic effects of the factors. The relation between different variables and responses is elucidated by the 3D response surface plots as a function of two variables by maintaining the other variable at a central level. Fig 3A presents the interaction between the ethanol content in SC-CO_2 (X_1) and extraction pressure (X_2) on the recovery of

triterpenoids, given a 55 °C temperature. The results showed that an increase in X_1 (2-7%v/v) and X_2 (200-400bar) enhanced the recovery of triterpenoids.



(A)



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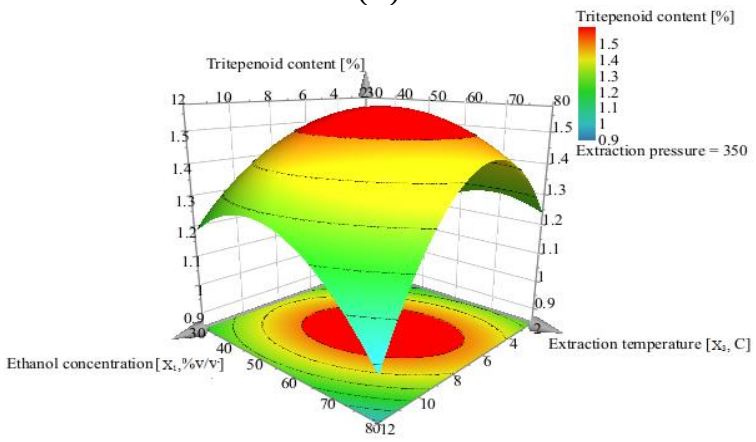

(C)

Figure 3. Response surface plots of relationships between triterpenoid recovery and ethanol contentration in SC-CO_2 (X_1, %v/v), extraction pressure (X_2, Bar), and extraction temperature (X_3, °C), given: (A) Extraction temperature of 55 °C; (B) ethanol concentration of 7%v/v; (C) extraction pressure of 350 bar

The maximum triterpenoid content (1.48g/100g) was achieved with X_1 at 6.9%v/v and X_2 at 375 bar. The triterpenoid content was not significantly increased as the extraction pressure and ethanol content increased from 375 to 500bar and 7%v/v to 12%v/v, respectively. Similar results were also reported for the extraction of triterpenoids from *Eucalyptus globulus* using the SC-CO_2 with the addition of ethanol as co-solvent extraction (Domingues et al., 2013). These authors suggested that an increase in ethanol content and extraction pressure lead to the change of the solvent polarity, which enhanced the interactions between ethanol and the triterpenoids within the cell, thus improving the recovery of triterpenoids. Nevertheless, in Fig 3A, a circular shape shows a low significant interaction between X_1 and X_2.

Fig. 3B indicates the simultaneous effects of X_2 and X_3 on the recovery of triterpenoid. With a fixed level of X_1 (7%v/v), the triterpenoid content increased and achieved the maximum value (1.59g/100g) at X_2 (374bar) and X_3 (60°C). The increase in extraction temperature was attributed to differences in the values of solvent density, which promoted a considerable increase in extraction yield (Yang and Wei, 2015; Marinho et al., 2019). However, when X_2 and X_3 were higher than 374bar and greater than 60 °C the triterpenoid recovery decreased. This could be attributed to the degradation of triterpenoids by the high extraction temperature and pressure. In addition, an increase in pressure and temperature causes a decrease in the effective diffusivity, thus reducing the triterpenoid recovery (Yang and Wei, 2015; Uwineza and Waśkiewicz, 2020) suggested that the SC-CO_2 extraction temperature of bioactive compounds should be fixed between 35 and 60°C to avoid degradation, and the pressure should be around 400 bar.

Figure 3C depicts the relation between X_1 and X_3. It can be seen that the increase in both X_1 and X_3 resulted in greater solubility of triterpenoids in the extract. The triterpenoid content reached its highest value with X_1 at 7%v/v and X_3 at 60 °C. The triterpenoid content was slightly reduced when X_1 rose from 7%v/v to 12%v/v and X_3 increased from 60 to 80°C. The high extraction temperature facilitates solvent volatilization, thus reducing the triterpenoid recovery. In addition, higher temperatures might cause thermal degradation of triterpenoid compounds. The result was according to other studies (Tran et al., 2021; Yang and Wei, 2015).

3.5. Optimization of reaction and model validation

Optimal extraction conditions for maximum recovery of triterpenoids from *G. lucidum* were further predicted using RSM mathematical models. The optimal extraction conditions (i.e, cosolvent content, extraction pressure, and extraction temperature) for triterpenoid extraction from the *G. lucidum* were 380 bar, 60°C, and 7%v/v, achieving 1.49g/100g. Under the optimal conditions, the experimental triterpenoid content was 1.51/100g which was very near to the predicted value, illustrating that the models fitted very well.

Table 4. Extraction conditions and triterpenoid recovery of the SC-CO_2, SE, ME methods

Methods	Extraction conditions				Triterpenoid content (g/100g)
	Pressure (bar)	Extraction time (hours)	Ethanol content (%v/v)	Temperature (°C)	
SC-CO_2	379	2	7	57.3±0.5	1.21^{b}±0.01
SE	-	6	-	70±1.0	1.51^{a}±0.02
ME	-	24	-	32±0.5	0.45^{c}±0.01

Different letters in each column denote statistically significant differences between treatments ($p < .05$). The values are the mean of three replications SD.

In this study, the SE and ME methods were compared for their efficiency in extraction recoveries of triterpenoids. As shown in Table 4, it can be seen that there was a significant difference in the recovery of triterpenoids among the SC-CO_2 (1.51g/100g), SE (1.21g/100g), and ME methods (0.45g/100g). It could be attributed to the morphological changes of the *G. lucidum* cell wall during the extraction processes.

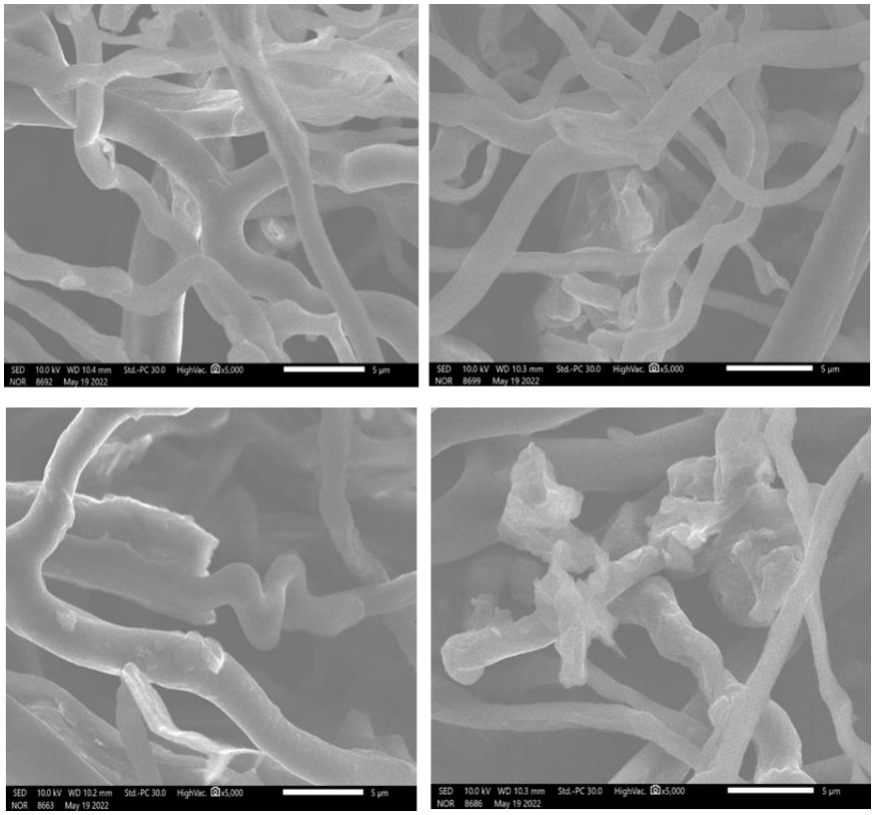

Figure 4. SEM images of untreated *G. lucidum* sample (a), SE treated sample (b), ME-treated samples (c), SC-CO_2 treated sample (magnification 5000) (d).

In Fig 4A, the surface of the native sample had a smooth surface. In Figs 4B-C, some cracks on the SE and ME-treated *G. lucidum* cell walls resulted in the enhancement of the solution of triterpenoids in the extracts. Meanwhile, the surface of the samples treated with SC-CO_2 modified with ethanol exhibited numerous cells that were completely broken (Fig 4C). Therefore, significant benefits in terms of extraction conditions indicated that the SC-CO_2 method is a useful extraction method for triterpenoids from *G. lucidum.*

3.6. Kinetic of SC-CO_2 extraction process

In this study, both the first- and second-order kinetic models were used to describe the effects of the SC-CO_2 parameters on the extraction of triterpenoids from *G. lucidum*, given an extraction time of 0-120min. For the first-order kinetic model, the plot of log (C_s/(C_s-C_t)) versus t gave a slope and intercept that was used to determine k (min^{-1}) and C_s. Based on Table 5, it can be seen that the k values increased with increasing SC-CO_2 extraction parameters and obtained values are 0.799 to 2.470 (100g/g.min). However, the first-order model presented low coefficients of determination (R^2) values, which can not represent well the experimental results of triterpenoid extraction. Compared with the first-order extraction models, the second-order model presented very high R^2 values, thus the second-order kinetic is a more suitable model for describing the kinetic extraction process of triterpenoids. As shown in Table 6, the

parameters hs and C_s values appreciably increased with increasing extraction temperature (30-55°C) or pressure extraction (200-350 bar), and then C_s and h slightly decreased.

Table 5. The first-order kinetic parameters of tritepenoid extraction from *G. lucidum* at various temperatures and extraction pressures

Extraction conditions		k (100g/g.min)	C_s (g/100g)	R^2
Temperature (°C)	30	1.548724	1.21	0.8115
	55	2.388239	1.58	0.8621
	80	1.998606	1.09	0.8634
Extraction pressure (bar)	200	0.798944	1.21	0.8435
	350	2.470431	1.55	0.8348
	500	2.339356	1.49	0.8648

These values were obtained from Microsoft Excel1. Cs= triterpenoid concentration at saturation; *k* = extraction rate constant; R^2 = coefficient of determination

Table 6. Second order kinetic parameters of triterpenoid recovery under different SC-CO_2 extraction pressures and extraction temperatures, given 120 min extraction time

Extraction conditions		*h* (g/100g)	*k* (100g/g.min)	C_e (g/100g.min)	R^2	RSME
Temperature (°C)	30	0.408	0.377	1.04	0.984	0.040
	55	1.829	0.802	1.51	0.989	0.079
	80	0.987	0.697	1.19	0.991	0.081
Extraction pressure (bar)	200	0.554	0.466	1.09	0.945	0.031
	350	1.758	0.792	1.49	0.966	0.045
	500	1.083	0.752	1.20	0.985	0.063

These values were obtained from Microsoft Excel1. Cs= triterpenoid concentration at saturation; *k* = extraction rate constant; *h* = initial extraction rate; R^2 = coefficient of determination; RMSE = root mean square error

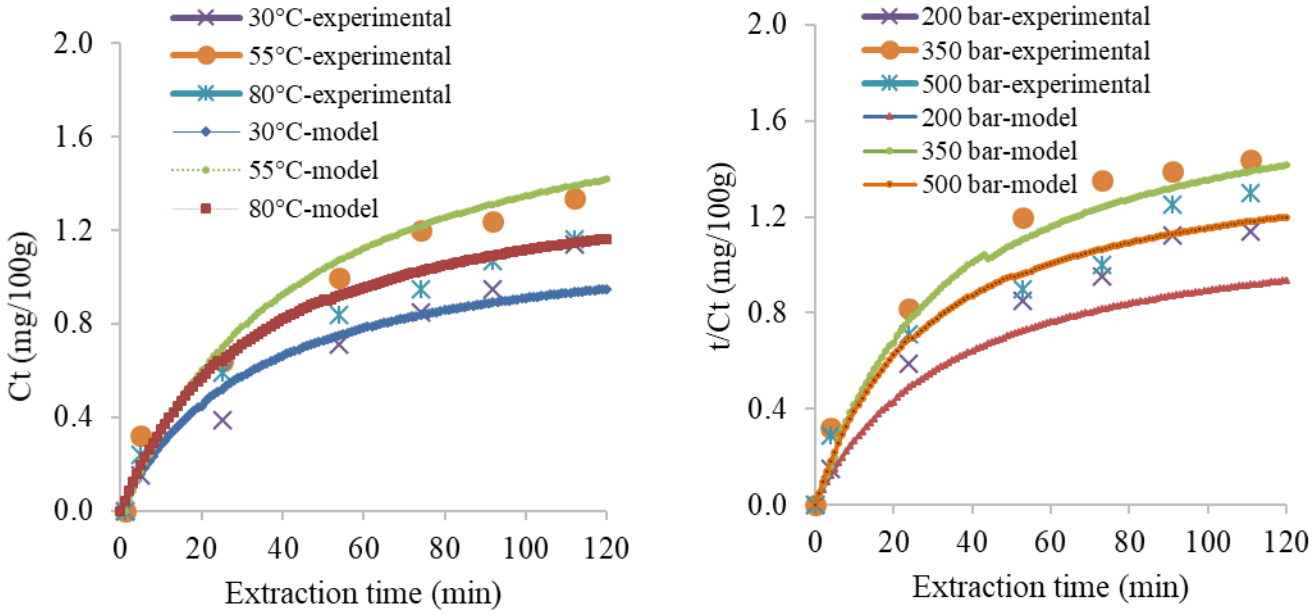

Figure 5. Second-order kinetic models (A–B) of extraction of triterpenoids from *G. lucidum* at different extraction temperatures and pressures

In Figs 5A-C, it also can be seen that the two- thirds of the triterpenoid content was recovered in the early SC-CO_2 extraction stage (0 to 100 min), and reached equilibrium after 100 min extraction. Specifically, the triterpenoid content increased from 0.036 to 1.43 mg/100g as the temperature raised from 30-55°C, or pressure extraction from 200-350 bar, respectively. The results could be explained that rising temperature and pressure enhanced the density of ethanol concentration in SC-CO_2, which thereby improves the solubility of triterpenoid in the solvent. Nevertheless, the recovery of triterpenoids declined with the increasing temperature to 55°C and pressure to 500 bar, consistent with the RSM results (Fig. 3A-C). The high temperature and pressure extraction lead to heat exposure, and other compounds were extracted more, reducing the triterpenoid content (Hobbi et al., 2021). According to our results, the second-order extraction models are correlated to the RSM results. Therefore, it can be concluded that the second-order kinetic extraction model was suitable for characterizing the effect of SC-CO_2 parameters on triterpenoid recovery.

3.7. Comparison of antioxidant activity of the triterpenoid-rich extracts among optimized SC-CO_2, SE, and ME processes

The DPPH and ABTS methods have been widely used to determine the free radical-scavenging activity of natural products. In this study, the $DPPH^{\bullet+}$and $ABTS^{\bullet+}$ scavenging activities of triterpenoids obtained by the optimized SC-CO_2 were compared with the ascorbic acid (AA) and conventional processes (ME and SE). The $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging activities of the extracts and ascorbic acid are shown in Figs 5A-B. As shown in Figs 6A-B, it can be seen that the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging activities of the extracts correlated positively with their concentration in the medium. In addition, the antioxidant activity of the extracts at the optimal SC-CO_2 conditions was higher than ME and SE in the same range of concentrations. When the concentrations of triterpenoids were 0.15 and 0.25 g/ml, the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ values of SC-CO_2 increased from 15.15% to 92.50% and 13.71% to 73.56%, respectively.

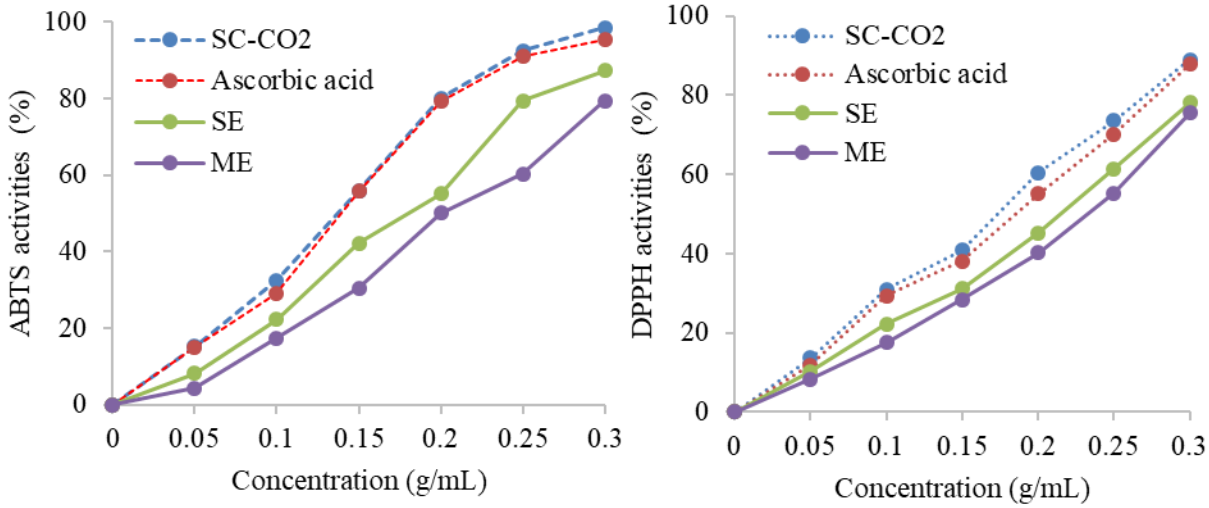

Figure 6. (A) ABTS and (B) DPPH radical scavenging activities of the extract and ascorbic acid

Meanwhile, the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ values of ME and SE extracts were in a range of 4.35 to 79.45% and 8.35% to 61.45%. Based on the relationship curve between the triterpenoid concentration, ascorbic acid, and the percentage of antioxidant activity, the value of IC50 was

determined. The results showed that the measured IC50 values of the optimized SC-CO_2 (0.141-0.178 g/ml) resembled the antioxidant capacity of ascorbic acid (0.14-0.17g/ml). Meanwhile, the SE and ME extracts were in a range of 0.20-0.22g/ml. A reasonable explanation is that the SC-CO_2 allows the extraction of different triterpenoid fractions responsible for the high antioxidant activity of the extracts. Our findings were similar to Porto et al for extracting proanthocyanidins from grap marc. However, this study only indicated the total content of triterpenoids which was responsible for the antioxidant activity. Thus, further studies should be conducted to investigate the bioactive compounds from triterpenoid fractions to establish the biochemical mechanisms.

4. Conclusions

In this work, the influences of the SC-CO_2 process parameters (ethanol concentration, extraction temperature, and extraction pressure) on the recovery of triterpenoids were investigated. The RSM was applied to determine the optimal extraction conditions (i.e., ethanol concentration in SC-CO_2, extraction temperature, and extraction pressure) for the maximum recovery of triterpenoids from *G. lucidum*. The first- and second-order kinetic extraction models were used to interpret the kinetic extraction behavior. According to our results, the optimal condition for triterpenoid extraction was 380 bar, 7%v/v, and 60°C, achieving the highest triterpenoid content (1.49 mg/100g). These results agreed with the experimented value of 1.51 mg/100g, indicating the success of RSM in the optimization of extraction parameters in the prediction of triterpenoid from *G. lucidum*. The second-order gave better fitting to the experimental data than the first-order kinetic model. The fit of the second order model is satisfactory and it was able to predict in reasonably way the recovery of the extraction process. Compared with the other two conventional methods, the SC-CO_2 method is faster, cleaner, and more efficient. The antioxidant activity of *G. lucidum* triterpenoids extracted by the SC-CO_2 method had a stronger ability to remove DPPH free radicals and ABTS free radicals than the two conventional extraction methods. Thus, the SC-CO_2 method is recommended for the extraction of triterpenoids from *G. lucidum*. However, in this study, the triterpenoids responsible for the antioxidative activity are not explained. Thus, further investigation is needed to clarify the antioxidant compounds present in the *G. lucidum* extract.

5. References

Blicharski, T. and Oniszczuk, A. (2017). Extraction Methods for the Isolation of Isoflavonoids from Plant Material. *Open Chemistry,* 15.

Cai, C., Ma, J., Han, C., Jin, Y., Zhao, G. and He, X. (2019). Extraction and antioxidant activity of total triterpenoids in the mycelium of a medicinal fungus, Sanghuangporus sanghuang. *Scientific Reports,* 9**,** 7418.

Cai, Z., Wong, C., Dong, J., Jiao, D., Chu, M., Chung, L., Lau, C., Lau, C.-P., Tam, L.-S. and Lam, C. (2016). Anti-inflammatory activities of Ganoderma lucidum (Lingzhi) and San-Miao-San supplements in MRL/lpr mice for the treatment of systemic lupus erythematosus. *Chinese Medicine,* 11.

Domingues, R., Melo, M., Oliveira, E., Neto, C., Silvestre, A. and Silva, C. (2013). Optimization of the supercritical fluid extraction of triterpenic acids from Eucalyptus globulus bark using experimental design. *The Journal of Supercritical Fluids,* 74**,** 105-114.

Herrero, M., Mendiola, J., Cifuentes, A. and Ibáñez, E. (2010). Supercritical Fluid Extraction: Recent Advances and Applications. *Journal of Chromatography A,* 1217**,** 2495-2511.

Hobbi, P., Okoro, O., Delporte, C., Alimoradi, H., Podstawczyk, D., Nie, L., Bernaerts, K. and Shavandi, A. (2021). Kinetic modelling of the solid–liquid extraction process of polyphenolic compounds from apple pomace: influence of solvent composition

and temperature. *Bioresources and Bioprocessing,* 8.

Ibáñez, E. and Cifuentes, A. (2015). Green extraction techniques 2015. *TrAC Trends in Analytical Chemistry,* 71.

Li, J., Zhang, X. and Liu, Y. (2016). Supercritical carbon dioxide extraction of G. lucidum Spore Lipids. *LWT - Food Science and Technology*, 70.

Li, K., Na, K., Sang, T., Wu, K., Wang, Y. and Wang, X. (2017). The ethanol extracts of sporoderm-broken spores of Ganoderma lucidum inhibit colorectal cancer in vitro and in vivo. *Oncology Reports,* 38.

Li, Z., Shi, Y., Zhang, X., Xu, J., Wang, H., Zhao, L. and Wang, Y. (2020). Screening Immunoactive Compounds of Ganoderma lucidum Spores by Mass Spectrometry Molecular Networking Combined With in vivo Zebrafish Assays. *Frontiers in Pharmacology,* 11**,** 287.

Marinho, C., Lemos, C., Arvelos, S., Barrozo, M., Hori, C. and Watanabe, E. (2019). Extraction of corn germ oil with supercritical CO2 and cosolvents. *Journal of Food Science and Technology,* 56.

Mau, J.-L., Lin, H.-C. and Chen, C.-C. (2001). Non-volatile components of several medicinal mushrooms. *Food Research International - FOOD RES INT,* 34**,** 521-526.

Pan, D., Zhang, D., Wu, J., Chen, C., Zhixue, X., Yang, H. and Zhou, P. (2013). Antidiabetic, Antihyperlipidemic and Antioxidant Activities of a Novel Proteoglycan from Ganoderma Lucidum Fruiting Bodies on db/db Mice and the Possible Mechanism. *PloS one,* 8**,** e68332.

Phan, M., Junyusen, T., Liplap, P. and Junyusen, P. (2020). Optimization and kinetics of ultrasound-assisted solvent extraction of gamma oryzanol from dried rice bran soapstock. 309-316.

Pieczykolan, A., Pietrzak, W. and Rój, E. (2019). Effects of Supercritical Carbon Dioxide Extraction (SC-CO2) on the content of tiliroside in the extracts from Tilia L. flowers. *Open Chemistry,* 17**,** 302-312.

Plazas, D., Soccol, C., Noseda, M., Tanobe, V., Marin, O., Karp, S., Pereira, G., De Carvalho, J. and Thomaz-Soccol, V. (2020). A comparative study of extraction techniques for maximum recovery of bioactive compounds from Ganoderma lucidum spores Short title: Recovery of bioactive compounds from Ganoderma lucidum spores. *Revista Colombiana de Ciencias Químico Farmacéuticas,* 49.

Rodrigues, V., Melo, M., Portugal, I. and Silva, C. (2021). Extraction of Added-Value Triterpenoids from Acacia dealbata Leaves Using Supercritical Fluid Extraction. *Processes,* 9**,** 1159.

Taofiq, O., Barros, L., Prieto Lage, M., Heleno, S., Barreiro, M. and Ferreira, I. (2017). Extraction of triterpenoids and phenolic compounds from Ganoderma lucidum: optimization study using the Response Surface Methodology. *Food & Function,* 9.

Tran, D., Nguyen, D.-V., Thao, L., Linh, N., Vuong Hoai, T., Linh, N., Ngan, N., Linh, N., Nam, H., Mai, P. and Nguyen, H. H. (2021). The Application of Ethanolic Ultrasonication to Ameliorate the Triterpenoid Content Extracted from Vietnamese Ganoderma lucidum with the Examination by Gas Chromatography. *ChemistrySelect,* 6**,** 2590-2606.

Uwineza, P. and Waśkiewicz, A. (2020). Recent Advances in Supercritical Fluid Extraction of Natural Bioactive Compounds from Natural Plant Materials. *Molecules,* 25**,** 3847.

Wei, L., Zhang, W., Yin, L., Yan, F., Xu, Y. and Chen, F. (2015). Extraction optimization of total triterpenoids from Jatropha curcas leaves using response surface methodology and evaluations of their antimicrobial and antioxidant capacities. *Electronic Journal of Biotechnology,* 16.

Xu, C., Wang, B., Pu, Y., Tao, J. and Zhang, T. (2017). Techniques for the analysis of pentacyclic triterpenoids in medicinal plants. *Journal of Separation Science,* 41.

Yang, Y.-C. and Wei, M.-C. (2015). Ethanol solution-modified supercritical carbon

dioxide extraction of triterpenic acids from Hedyotis corymbosa with ultrasound assistance and determination of their solubilities. *Separation and Purification Technology,* 150**,** 204-214.

Yim, S., Chan, Y., Suzana, Y., Johari, K., Quitain, A. and Joe Dailin, D. 2019. Supercritical Extraction of Value-Added Compounds From Empty Fruit Bunch: An Optimization Study by Response Surface Methodology.

Zhang, C.-R., Yang, S.-P. and Yue, J.-M. (2008). Sterols and triterpenoids from the spores of Ganoderma Lucidum. *Natural product research,* 22**,** 1137-42.

Zhu, L., Wu, M., Li, P., Zhou, Y., Zhong, J., Zhang, Z., Li, Y., Yao, W. and Xu, J. (2020). High-Pressure Supercritical CO2 Extracts of Ganoderma lucidum Fruiting Body and Their Anti-hepatoma Effect Associated With the Ras/Raf/MEK/ERK Signaling Pathway. *Frontiers in Pharmacology,* 11.

Conflicts of interest

The authors declare that they have no conflicts of interest.