



Optimization of flavonoid compound extraction from *Ganoderma lucidum* and its antioxidant activity on PC₁₂ cells exposed to H₂O₂

Linxiu Wu¹ · Xiaoyan Liu³ · Siyi Wu² · Fan Wang² · Mengyao Lu² · Fanghua Wang⁴ · Rongkang Hu^{2,3} 

Received: 7 April 2023 / Accepted: 11 September 2023 / Published online: 28 September 2023
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2023

Abstract

Ganoderma lucidum has a high concentration of flavonoid compounds. The extraction procedure was optimized using a response surface methodology to achieve a high concentration of flavonoids from *G. lucidum* using ethanol as the extracting solvent, and the antioxidant activities were investigated. The highest flavonoids content was 0.56 ± 0.03 mg/g when the solid-liquid ratio, extraction temperature, extraction time and ethanol concentration were 1:25 mg/L, 75 °C and 1.5 h and 65%, respectively. The most influential factor was shown to be the solid-liquid ratio. FT-IR and ¹H NMR analysis of the purified ethanol extracts (PEE) revealed that it was primarily a 5, 7-Dihydroxyflavone. PEE exhibited significant DPPH• and hydroxyl radical scavenging activity, as well as stronger reducing capacity. Notably, in the presence of PEE, cell viability increased by 13.76% when compared to H₂O₂ treatment ($p < 0.05$), reaching 72.5%. In addition, when treated with the PEE, the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH-Px) had significantly increased when compared to the H₂O₂-treated group ($p < 0.01$), which were 36.93, 15.01 and 15.11 U/g-protein. More importantly, PEE treatment could upregulate the expression of PI3K, Akt and downregulate the expression of Caspase-3 in PC₁₂ cells stimulated with H₂O₂. As a result, PEE could be used as a novel source of natural antioxidants.

Keywords Antioxidant activity · Extract · Flavonoid · *Ganoderma lucidum* · Process optimization

Introduction

Ganoderma lucidum, a traditional edible mushroom, has been widely utilized as a health food and valuable drug [1]. *G. lucidum* extracts are rich in biologically active components such as triterpenoids, polysaccharides, phenolics

and flavonoids content, all of which are easily absorbed by humans [2, 3]. Thus, it has been used for a long history to treat several types of diseases, e.g. antioxidant, antiviral, hepatoprotective activities and other medicinal properties.

Polyphenolics are one of the major contributors to the antioxidant properties of *G. lucidum* [4, 5]. Flavonoids are naturally occurring phenolic compounds, and *G. lucidum* flavonoids such as catechin and myricetin have important pharmacological effects. Currently, research is being conducted to determine whether enrichment of flavonoids during *G. lucidum* growth and extraction improves the antioxidant properties of *G. lucidum*. According to research, increasing total flavonoids during cultivation with the *G. lucidum* mycelium can increase the antioxidant and anti-inflammatory activity and cytotoxicity of cancer cells [6]. In addition, *G. lucidum* were extracted with chloroform, methanol and water by maceration method, it was found that total flavonoids content in chloroform extracts of *G. lucidum* can reach 38.00 ± 0.75 mg quercetin equivalents/g, and it has stronger antioxidant activities [7]. Similarly, after solvent fractionation with ethyl acetate in ethanol extracts of *G. lucidum* mycelium, its fraction has

✉ Xiaoyan Liu
354271455@qq.com

✉ Rongkang Hu
Rongkang_Hu@163.com

¹ Translational Medicine Center, The Second Affiliated Hospital of Wannan Medical College, Wuhu 241000, Anhui, PR China

² College of Life Sciences, Anhui Normal University, Wuhu 241000, Anhui, PR China

³ Beijing Engineering and Technology Research Center of Food Additives, School of Food and Health, Beijing Technology and Business University, Beijing 100048, PR China

⁴ School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, PR China

the highest total flavonoids content and antioxidant activities [8, 9]. However, *G. lucidum* in the different regions and using different extraction solvents have significantly affected the biologically active ingredients and content [10, 11]. At present, the most commonly used solvents for extraction include ethanol, methanol and hot water, etc. Use of methanol, the extraction rate is higher as compared to that with ethanol [12], but the toxicity of methanol is stronger and its pollution to the environment is more serious. In hot water extracts system, *G. lucidum* extracts are mainly crud polysaccharides. Using as ethanol solvent, it is beneficial to increase the solubility and flavonoids content [13–15]. Furthermore, the concentration of ethanol has also dramatically influenced on flavonoids content [16]. Hence, it is of great importance to increase the levels of flavonoids and their antioxidant activities by improving the ethanol extraction process.

Recent study has shown that flavonoids have a wide spectrum of biological effects under normal and pathological situations [17]. Most flavonoids appear to have biological activity primarily due to their capacity to enter cells and inhibit enzymes of signaling pathways and transcription factors, including those involved in the activation, proliferation, and fulfillment of effector functions of immune system cells [18]. Flavonoids have been implicated in the regulation of critical signaling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, mitogen-activated protein kinase (MAPK) pathway, Janus kinase and signal transducer and activator of transcription proteins (JAK/STAT) pathway, Toll-like receptors (TLR) pathway, nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and cAMP response element-binding protein (CREB) pathway [19]. Mitochondria are multifunctional organelles that participate in almost all cellular processes. Mitochondria are involved in reactive oxygen species (ROS) signaling because they are a major source of cellular ROS [20]. Mitochondria contain important components of cellular ROS scavenging systems. These include superoxide dismutase (SOD2), glutathione peroxidases (GPX 1 and GPX4), peroxiredoxins (PRX3, PRX5), thioredoxin 2, and thioredoxin reductase 2 (TRR2) [21]. Mitochondria are also involved in a number of pathways that lead to cell death. However, it is unknown whether *G. lucidum* flavonoids exert its antioxidant activities through activating the ROS scavenging systems in mitochondria.

In this study, response surface methodology (RSM) was used to optimize the extraction procedures of extraction time, ethanol concentration, extraction temperature, and solid-liquid ratio of *G. lucidum* flavonoids. We also analyzed the ingredients of the purified ethanol extracts (PEE) by FT-IR spectroscopy and ^1H NMR spectroscopy, and investigate its antioxidant activity. Finally, we used H_2O_2 -induced oxidative stress in PC_{12} cells as an in vitro model to further

evaluate the effects of PEE on the prevention mechanism of antioxidants.

Materials and methods

Materials and chemicals

Ganoderma lucidum was obtained from the National Engineering Research Center of JUNCAO Technology, Fuzhou, Fujian, China. Rutin, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous sulfate (FeSO_4), potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], trichloroacetic acid (TCA), 3-[4,5-dimethylthiazol-2-yl] – 2,5-diphenyltetrazoliumbromide (MTT) and H_2O_2 were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). The kits for Glutathione (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) were all obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-Caspase-3 was from Abcam (Cambridge, MA, USA). Anti-PI3K and anti-Akt, were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The other reagents used were of analytical grade.

Designing experiments to improve the extraction procedure

Ganoderma lucidum was dried by hot air in an oven at 60 °C. Prior to further experimentation, dried materials were powdered and filtered through a sieve of 30 meshes to obtain a homogeneous powder. The extraction process was optimized using Design-Expert 8.0.6.1 software, and the effect of each factor on flavonoids content was evaluated. Before the response surface method was established, the central level of the main effect factors was screened by single factor experiments, including ethanol concentration, solid-liquid ratio, extraction time and extraction temperature. The rutin equivalent method was used to determine the flavonoids content [22]. After the extraction, the sample was centrifuged at 4,500 g for 20 min. The liquid supernatant was concentrated using a rotating evaporator and then freeze-dried to obtain ethanol extracts.

Purification of ethanol extracts

The ethanol extracts were dried once the best extraction conditions were determined, followed by separation and purification with polyamide resin. The polyamide resin was initially immersed in a 90–95% ethanol solution while being repeatedly stirred to force the bubbles out. The resin was then packed into the glass column and washed down with a 90–95% ethanol solution until the eluent was clear and there was little residue left after the eluent was dried. The resin

was then washed with 2–2.5 bed volumes of 5% sodium hydroxide, 1 bed volume of distilled water, and 2–2.5 bed volumes of 10% acetic acid solution in that order. Finally, it was rinsed with distilled water until the pH of the eluent was neutral. The resin was then cured and stored for future use.

Purified ethanol extracts (PEE) were collected in a glass column (11 mm × 400 mm) wet-packed with 5.0 g (dry weight basis) pretreated polyamide resin using the previously described method with minor modifications [23]. The bed volume (BV) and length were 30 mL and 30 cm, respectively. In all cases, the flow of samples was downward and was monitored using a HD-3 UV-detector (Shanghai Huxi Analyses Instrument Company, Shanghai, China). The flavonoids concentration in the eluate was determined using the colorimetric method after sample solutions flowed through the glass column at 2 mL/min. Following adsorption equilibrium, the adsorbate-laden column was washed with deionized water and then ethanol solution at a flow rate of 1.5 mL/min. The colorimetric method was used to measure the flavonoids content of the eluate. The flavonoids concentration and the volume of the eluate were used to calculate the loading curve. The flavonoid components were also collected using repeated chromatography. The rotary evaporator concentrated the eluate, and the vacuum oven dried it. The residue was the purified product, and the flavonoids content was determined by weighing it.

Identification of PEE

A Fourier-transform infrared (FT-IR) spectrophotometer system (Nicolet Instruments Corp, Madison, WI, U.S.A.) within the range between 4,000 and 400 cm^{-1} was used to examine dried PEE. The sample (10 mg) was mixed with KBr powder (100 mg) [24]. Deuterium was suspended in CD₃OD samples and freeze-dried three times to replace exchangeable protons. NMR spectra of PEE were obtained by an AVANCE-600 NMR spectrometer (Bruker, Rheinstetten, Germany). The relaxation delays time, the ionization potential and the temperature of the NMR ion source experiment were set as 1.0 s, 70 eV, and 220 °C, respectively. ¹H-NMR experiments were conducted at room temperature in CD₃OD [25].

Antioxidant assay in vitro

The antioxidant activity of PEE was assessed. Three complementary methods were used, as explained further below. 60 M DPPH• was dissolved in 3 mL ethanol, and then 0.5 mL of various PEE concentrations were added. There were also blank experiments with 0.5 mL of 65% ethanol instead of the extract. At room temperature, the absorbance was measured at 517 nm [26]. Two milliliters of various concentration samples were mixed with 2 mL of FeSO₄ (6

mmol/L) and 2 mL of H₂O₂ (6 mmol/L). After being shaken, the mixture was placed at room temperature for 10 min, and followed by the addition of 2 mL salicylic acid (6 mmol/L). The mixture was shaken and incubated for 30 min at room temperature in the dark. At 510 nm, the absorbance of the reaction mixture was measured. Extra pure water was used as a control solution [27]. The reducing power of PEE was determined using the Oyaizu method with some modifications [28]. In brief, 1.0 mL of various concentration samples were mixed with 2.5 mL of 0.2 M PBS buffer (pH 6.6), followed by 2.0 mL of 0.1% (w/v) K₃Fe(CN)₆ solution. For 20 min, the mixture was incubated in a water bath at 50 °C. The mixture was then centrifuged at 3000 rpm for 10 min with 2.5 mL of a 10% (w/v) TCA solution added. Following the absorbance analysis at 700 nm, 2.0 mL of the upper layer was combined with 2.0 mL of distilled water and 0.5 mL of 0.1% (w/v) FeCl₃ solution. Increased absorbance of the reaction mixture indicates increased reducing power.

The effect of PEE on cell viability in rat pheochromocytoma (PC₁₂)

The rat pheochromocytoma (PC₁₂) cells (ATCC CRL-1721) were obtained from the American Type Culture Collection (ATCC). PC₁₂ cells were seeded into 96-well plates (0.5 × 10⁴ cells/well), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Every other day, the medium of culture was changed. In 96-well plates, cell viability was evaluated using the MTT test. Cells were exposed to various concentrations of PEE or H₂O₂ for 24 h at 37 °C. After incubation, PC₁₂ cells were treated for 4 h at 37 °C with 10 μl of 5 mg/mL MTT and the medium was carefully removed. To dissolve the formazan crystals, the MTT medium was replaced with DMSO (250 μl). A microplate reader was used to measure absorbance at 570 nm [29].

Assay for antioxidant defense system in PC₁₂ cells

To investigate the effect of PEE on oxidative stress in PC₁₂ cells treated with H₂O₂, the activities of SOD, CAT, and GSH-Px were measured using a commercial assay kit according to the manufacturer's protocols (Nanjing Jiancheng BioENG, Co., Nanjing, China). Protein concentration was determined using the BCA protein assay reagent kit (Sangon Biotech Co., Ltd., Shanghai, China).

Western blot analyses

The expression of Caspase-3, PI3K and Akt were assessed by western blot analysis. Cell samples were lysed on ice

with lysis buffer containing cocktail proteinase inhibitors and protein phosphatase inhibitors (Thermo Fisher Scientific, Inc., Rockford, USA). The protein concentration was quantified using a bicinchoninic acid (BCA) assay kit (Sangon, Shanghai, China). Protein samples were separated by SDS-PAGE using 4–20% precast gradient polyacrylamide gels. After separation by SDS-PAGE, proteins were transferred to a PVDF membrane (Sangon, Shanghai, China). The blots were then incubated with primary antibodies and subsequently incubated with horseradish peroxidase-(HRP-) conjugated secondary antibodies. Immunoreactive proteins were detected using the ECL Western blotting detection system (Gene, Hongkong, China). The results of western blot were analyzed by the Image J program.

Statistical analysis

Statistical analysis was executed with GraphPad and Design Expert software statistical software program. The difference between groups with p values < 0.05 was considered as statistically significant.

Results

Box Behnken design response surface methodology test model.

The four main influencing factors of solid-liquid ratio (factor A), extraction temperature (factor B), extraction time (factor C), and ethanol concentration (factor D) were selected as the response factors. Table 1 shows the design and the corresponding response values. The total flavonoids content in group 15 was the highest, reaching 0.56 mg/g, and the extraction conditions were 1:25 mg/L solid-liquid ratio, 75 °C extraction temperature, 1.5 h extraction time and 65% ethanol concentration. Based on the given experimental results, multiple quadratic regression fitting equations were designed by software to fit the experimental data as shown in equation:

$$Y_{\text{ethanol extracts}} = 0.53 - 0.02A - 8.33E - 004B - 0.019C - 0.013D + 2.50E - 003AB - 0.012AC + 5.00E - 003AD - 1.00E - 002CD - 0.22A^2 - 0.055B^2 - 0.15C^2 - 0.15D^2$$

According to the results of ANOVA, the obtained model had a $p < 0.0001$, which indicated that the model built with the level of solid-liquid ratio, extraction temperature, extraction time, and ethanol concentration as independent variables

Table 1 Response surface design and experimental data

Experiment No.	A (mg/L)	B (°C)	C (h)	D (%)	Flavonoids content (mg/g)
1	35	75	1.5	45	0.17
2	25	65	1.5	85	0.36
3	25	65	1.0	65	0.34
4	25	75	1.5	65	0.52
5	25	75	1.0	85	0.21
6	25	85	1.5	85	0.33
7	15	75	1.0	65	0.22
8	15	75	2.0	65	0.23
9	35	75	1.0	65	0.18
10	15	85	1.5	65	0.20
11	35	65	1.5	65	0.20
12	25	75	1.0	45	0.19
13	25	75	2.0	45	0.17
14	25	85	1.5	45	0.38
15	25	75	1.5	65	0.56
16	35	85	1.5	65	0.18
17	25	75	1.5	65	0.53
18	25	75	1.5	65	0.55
19	25	65	2.0	65	0.28
20	25	85	1.0	65	0.39
21	35	75	2.0	65	0.14
22	35	75	1.5	85	0.15
23	25	65	1.5	45	0.41
24	15	75	1.5	45	0.21
25	15	65	1.5	65	0.23
26	25	75	1.5	65	0.50
27	15	75	1.5	85	0.17
28	25	85	2.0	65	0.33
29	25	75	2.0	85	0.15

A: solid-liquid ratio (mg/L). B: extraction temperature (°C). C: extraction time (h). D: ethanol concentration (% v/v).

(Table 2). Total flavonoids content as response value was extremely significant, the credibility was extremely high, and there was a significant relationship between the independent and dependent variables explored in this model. In this case, the value of R^2 was 0.9155, and R^2_{Adj} was 0.831, and the difference between the two is less than 0.2; indicating that the model has high fitting accuracy, and the error between the fitting result and the real result is small. In addition, a low coefficient of variation (C.V. %= 19.13) demonstrated reliability and precision. Both linear coefficients and cross coefficients had no significant effect on flavonoids content ($p > 0.05$). The flavonoids content was significantly affected by a quadratic term coefficient solid-liquid ratio (A^2), extraction time (C^2), and ethanol concentration (D^2) ($p < 0.05$).

Table 2 Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0.47	14	0.034	10.83	<0.0001	Significant
A	4.80E-03	1	4.80E-03	1.53	0.2359	
B	8.33E-06	1	8.33E-06	2.66E-03	0.9596	
C	4.41E-03	1	4.41E-03	1.41	0.255	
D	2.13E-03	1	2.13E-03	0.68	0.4228	
AB	2.50E-05	1	2.50E-05	7.99E-03	0.93	
AC	6.25E-04	1	6.25E-04	0.2	0.6618	
AD	1.00E-04	1	1.00E-04	0.032	0.8607	
BC	0	1	0	0	1	
BD	0	1	0	0	1	
CD	4.00E-04	1	4.00E-04	0.13	0.726	
A ²	0.32	1	0.32	103.55	<0.0001	
B ²	0.019	1	0.019	6.21	0.0258	
C ²	0.16	1	0.16	49.64	<0.0001	
D ²	0.14	1	0.14	44.19	<0.0001	
Residual	0.044	14	3.13E-03			
Lack of Fit	0.042	10	4.15E-03	7.29	0.0353	Significant
Pure Error	2.28E-03	4	5.70E-04			
Cor Total	0.52	28				
R-Squared	0.9155					
Adj R-Squared	0.831					
C.V. %	19.13					

** $p < 0.01$; * $p < 0.05$; A: solid-liquid ratio (mg/L). B: extraction temperature ($^{\circ}\text{C}$). C: extraction time (h). D: ethanol concentration (% v/v)

Besides, from the size of analytical comparison of mean square values, the magnitude of factors affecting flavonoids content is: A (solid-liquid ratio) > C (extraction time) > D (ethanol concentration) > B (extraction temperature).

Response surface analysis

The interactions between AB, AC, AD, BC, BD and CD factors, should be further analyzed in order to establish an ideal range level for each factor, based on the ANOVA results and model fitting data. The response surface plots and contour plots were thus still applied using the software. As shown in Fig. 1, the contours were elliptical and the response surface was steep with a large surface, and AB, AC, AD, BC, BD and CD factors had a strong interaction. More critically, the AC factor interaction was the strongest, with the greatest effect on total flavonoids content. The effect of the interaction of the extraction time and solid-liquid ratio on the flavonoids content of the extract at a constant extraction temperature and ethanol concentration is shown in Fig. 1A. The maximal flavonoids content value was attained by increasing the extraction time from 1.0 to 1.5 h. However, the flavonoids content reduced after 1.5 h. Similarly, the flavonoids content increased from 1:15 to 1:25 mg/L within the

solid-liquid ratio, and the maximum flavonoids content value was obtained before decreasing from 1:25 to 1:35 mg/L. Finally, the quadratic model predicted a maximum flavonoids content of 0.532 mg/g when the coded levels of solid-liquid ratio, extraction temperature, ethanol concentration, and extraction time were 1:25, 75 $^{\circ}\text{C}$ 1.5 h, and 65%, respectively. The experimental result had a flavonoids content of 0.56 ± 0.03 mg/g, which was an excellent agreement with the model prediction value.

Characterization of flavonoids from *G. lucidum*

The ethanol extracts of *G. lucidum* were isolated and purified using polyamide resin to investigate the presence of bioactive components, and the most effective fraction was screened to analyze composition. As shown in Fig. 2, peaks at approximately 3500 and 1000 cm^{-1} , are characteristic of flavonoids in Fourier transform infrared (FT-IR). Among all the spectra, the O–H in flavonoid compounds has a strong and broad absorption band at 3374.90 cm^{-1} . Another observation is related to the region above 2900 cm^{-1} , which can be assigned to the O–H and C–H bands. Peaks observed at 1650–1450 cm^{-1} are representative of an aromatic ring. Finally, the absorption 1086.34 cm^{-1} may be due to the

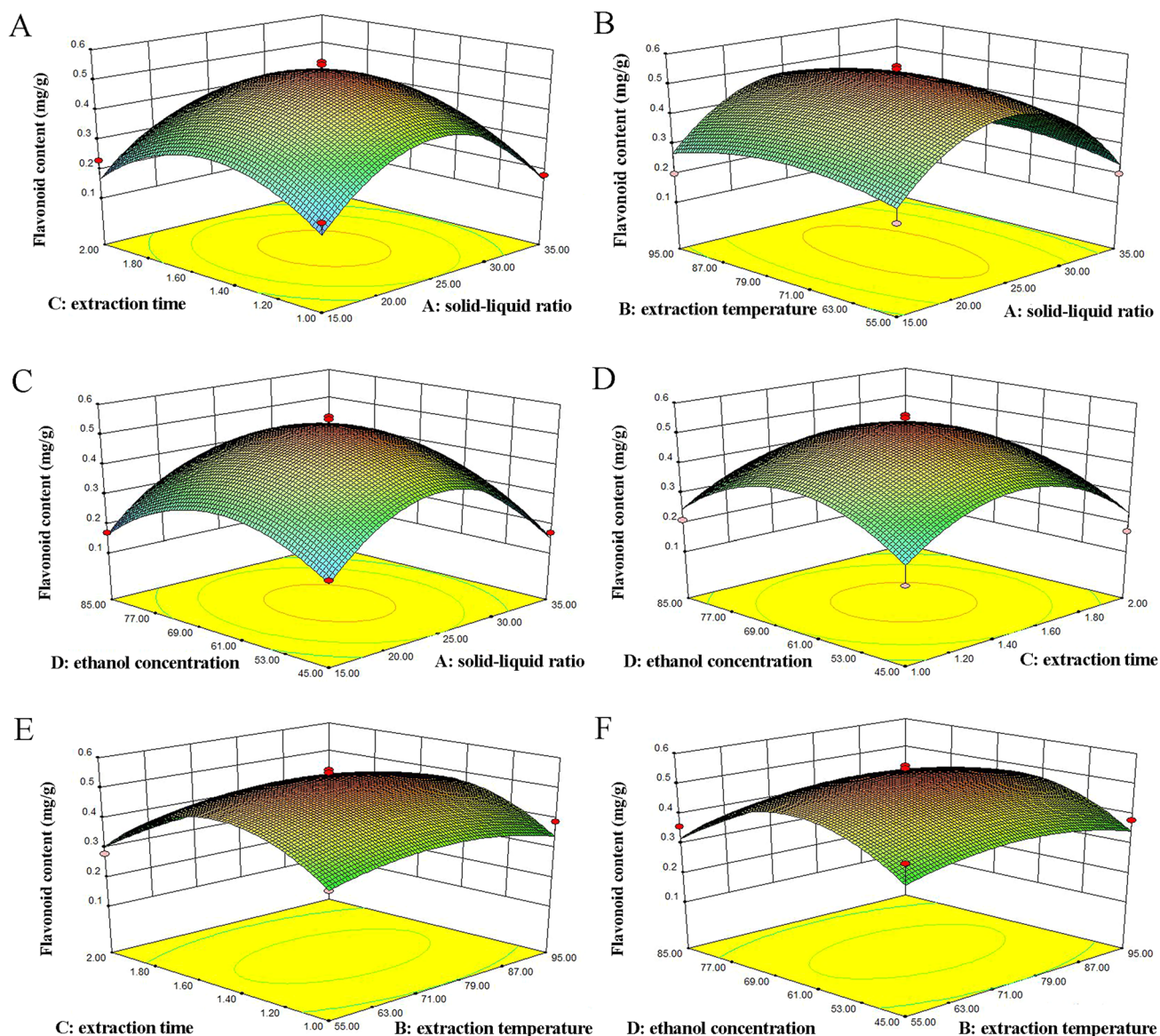


Fig. 1 Response surface plots for flavonoids content affected by **A** Solid-liquid ratio and extraction time. **B** Solid-liquid ratio and extraction temperature. **C** Solid-liquid ratio and ethanol concentra-

tion. **D** Extraction time and ethanol concentration. **E** Extraction time and extraction temperature. **F** Extraction temperature and ethanol concentration

carboxylic acid bands (CO stretching) [30–32]. These findings suggest that PEE contains functional groups such as alcohol, ketones, aldehydes, and carboxylic acid.

The nuclear magnetic resonance (^1H NMR) spectra of the flavonoids were mainly concentrated within the range of 5.5 to 9.0 ppm. The flavonoid protons signal was detected in the ^1H NMR spectra of the following data at δ 8.21 (H-5), 7.53 (H-3, 5, H-4), 7.19 (H-2), 6.74 (H-3), 6.73 (H-6), 5.71 (H-23), 4.65 (H-6), 4.64 (H-1), 2.09 (H-2), and 1.47 (H-5) [33–38]. 1,5,7-dihydroxyflavonoids were detected at 5.7–6.9 ppm. The bimodal signals of 2 H in the 4'-oxygenated flavonoids appeared at 5.0–6.5 ppm and 7.0–9.5 ppm. A single

peak of 3,3',4',5'-trioxy-substituted flavonoids appeared in the region of 6.5–7.5 ppm. The presence of a C=O bond at β -position of the carbonyl group led to a single peak at 7.60–7.8 ppm, which was lower than the average aromatic proton. The structure of the PEE was investigated using FT-IR spectroscopy and ^1H NMR spectroscopy, which revealed that it was primarily a 5,7-Dihydroxyflavone [39].

Antioxidant activity of *G. lucidum* purified ethanol extracts

The capability of scavenging the stable DPPH $^{\bullet}$ radicals and hydroxyl radicals were estimated by measuring the

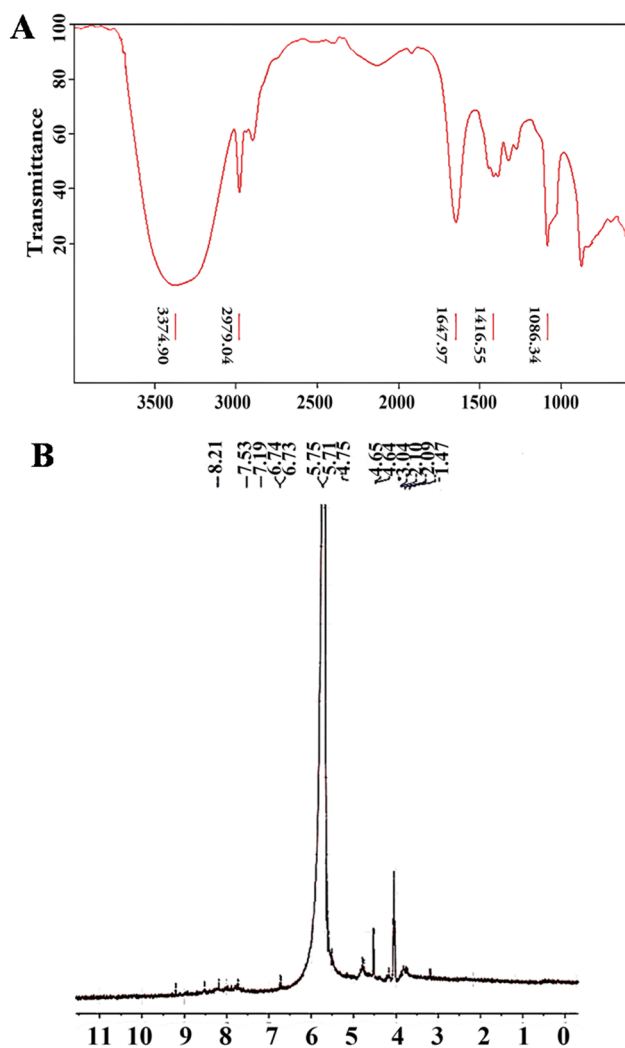


Fig. 2 Identification of PEE. **A** FT-IR spectra of PEE in the frequency range approximately 3500 and 1000 cm^{-1} . **B** ^1H NMR spectroscopy of PEE

decrease in its absorbance induced by antioxidants. As shown in Fig. 3, the tested samples dose-dependently exhibited a DPPH^\bullet -scavenging effect and hydroxyl radical scavenging activity at all the investigated concentrations ranged from 0.4 mg/mL to 3.8 mg/mL. At 3.8 mg/mL, the DPPH^\bullet -scavenging ability and the ability to scavenge hydroxyl radicals were 89.81% and 89.90%, respectively. In agreement with the scavenging effects on DPPH^\bullet radicals and hydroxyl radicals, PEE also showed stronger reducing capacity. The presence of antioxidants in this assay system causes the reduction of the $\text{Fe}^{3+}/\text{K}_3\text{Fe}(\text{CN})_6$ complex to the ferrous form (Fe^{2+}), and consequently, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. The absorbance of PEE at 700 nm increased from start to 3.5 mg/mL and tended to be equilibration from 3.5 mg/mL to 9 mg/mL.

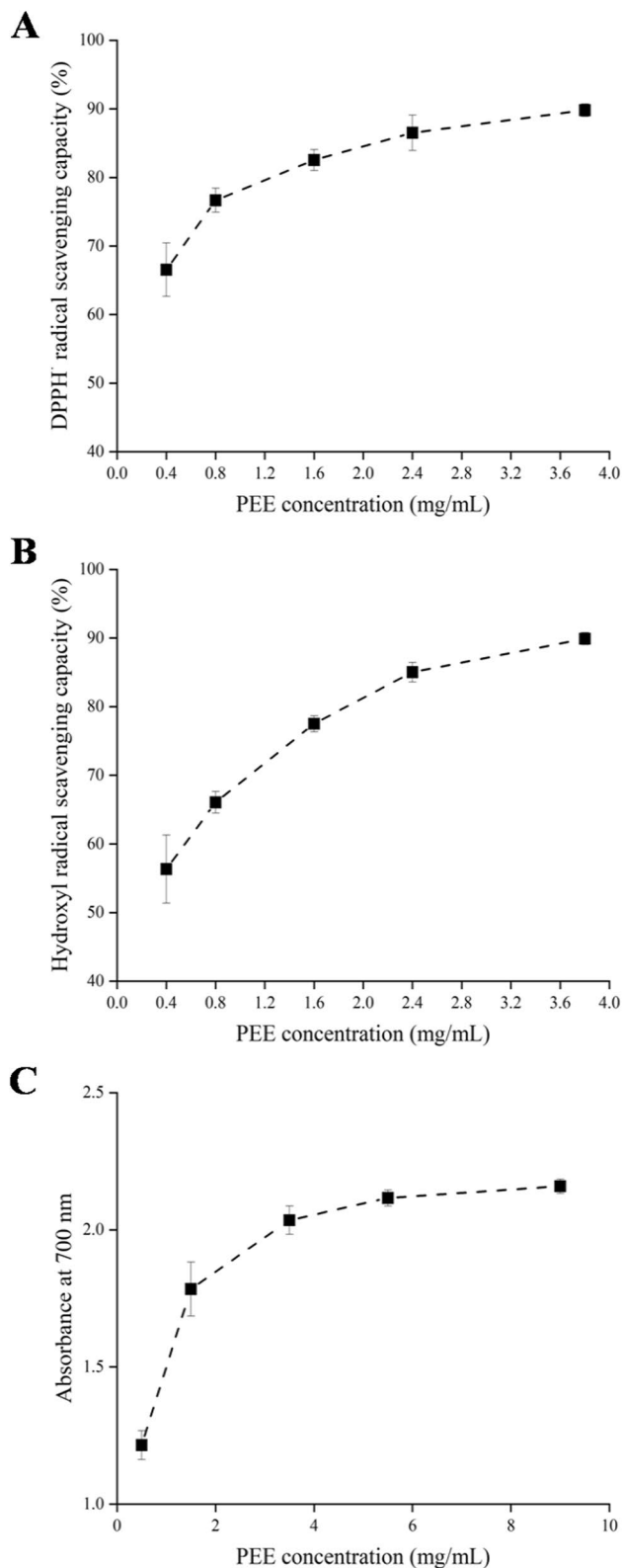


Fig. 3 Antioxidant activity of PEE of *G. lucidum*. **A** DPPH^\bullet radical-scavenging activity of PEE. **B** Hydroxyl radical-scavenging activity of PEE. **C** Iron(III) to iron(II) reducing power of PEE. Data are presented as mean \pm SD of three replicates ($n = 3$)

Fig. 4 Effects of PEE on viability of PC₁₂ cells. MTT assay was used for determining cell viability. Data are expressed as mean \pm S.D., * p < 0.05 vs. untreated or control group, ** p < 0.01 vs. untreated or control group, # p < 0.05 vs. H₂O₂-treated group, ## p < 0.01 vs. H₂O₂-treated group

Effects of PEE on viability of PC₁₂ cells

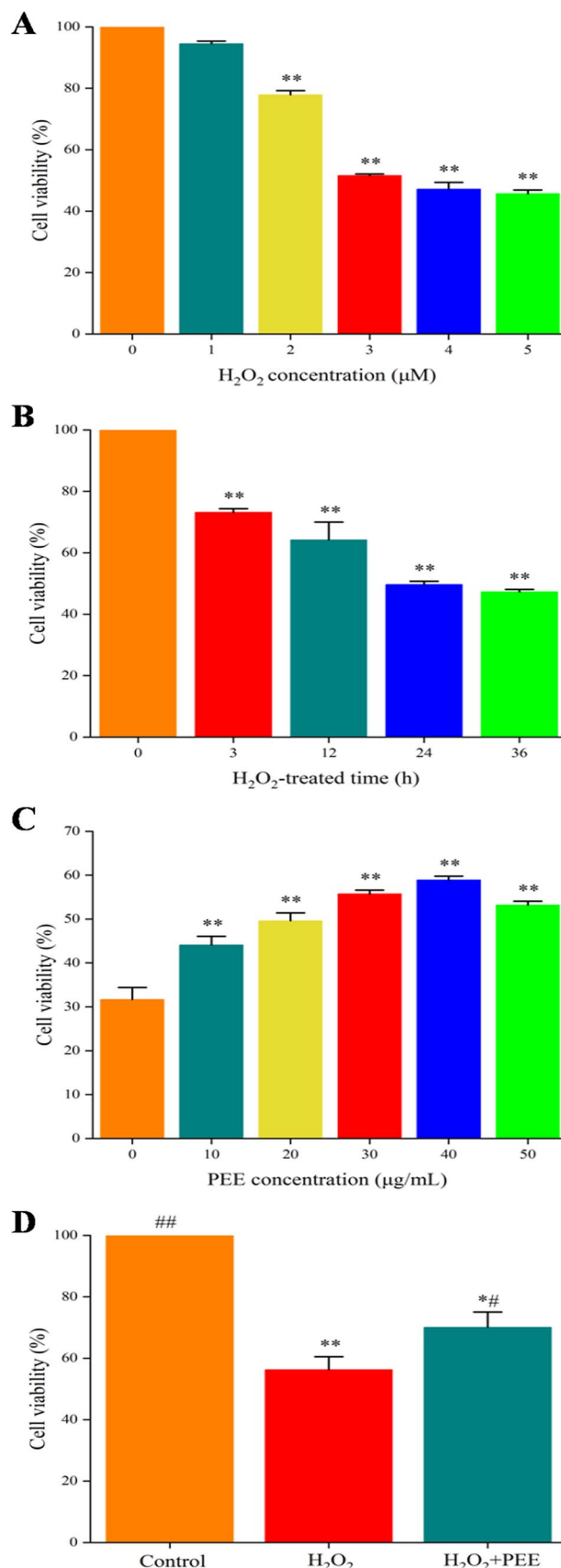
Figure 4 A showed the residual viability of PC₁₂ cells after 24 h incubation with H₂O₂. When the concentration of H₂O₂ was greater than 2 μ M, PC₁₂ cell viability was significantly (p < 0.01) decreased compared to the untreated group. The residual cell viability was only about 50% when the concentration of H₂O₂ was greater than 3 μ M. The cells were treated with 3 μ M H₂O₂, and cell viability decreased significantly (p < 0.01) as incubation time increased. Furthermore, the residual cell viability was around 50% after 24 h of incubation. As a result, we chose 3 μ M H₂O₂-treated PC₁₂ cells for 24 h in preparation for future experiments (Fig. 4B). As shown in Fig. 4C, compared with the untreated group, the residual cell viability of the PC₁₂ cells increased gradually with PEE concentration increasing to 40 μ g/mL (p < 0.01), but decreased at 50 μ g/mL. Thus, 40 μ g/mL PEE concentration was chosen to analyze PEE suppressing oxidative stress effects on H₂O₂-induced PC₁₂ cells. As shown in Fig. 4D, cell viability was reduced to 55.1% after H₂O₂ treatment when compared to the control group. Notably, in the presence of PEE, cell viability increased by 13.76% when compared to H₂O₂ treatment (p < 0.05), reaching 72.5%.

Effect of PEE on the antioxidant defense system in PC₁₂ cells

This work therefore sought to analyze whether PEE could reduce H₂O₂-induced oxidative damage of PC₁₂ cell through increasing enzymatic antioxidants (Fig. 5). Compared with the control group, SOD, CAT and GSH-Px activities decreased significantly (P < 0.01) to 19.97, 10.37 and 10.43 U/g-protein, respectively, after 24 h H₂O₂ treatment of PC₁₂ cells. Interestingly, when treated with the PEE, the activities of SOD and CAT and GSH-Px had significantly increased when compared to the H₂O₂-treated group (p < 0.01), which were 36.93, 15.01 and 15.11 U/g-protein.

Effect of PEE on the protein expression in PC₁₂ cells stimulated by H₂O₂

To explore the mechanisms underlying the effects of PEE treatment, the relative levels of PI3K, Akt and Caspase-3 protein in PC₁₂ cell were determined using western blot analyses. As shown in Fig. 6, the results of western blotting showed that compared with the control group, the



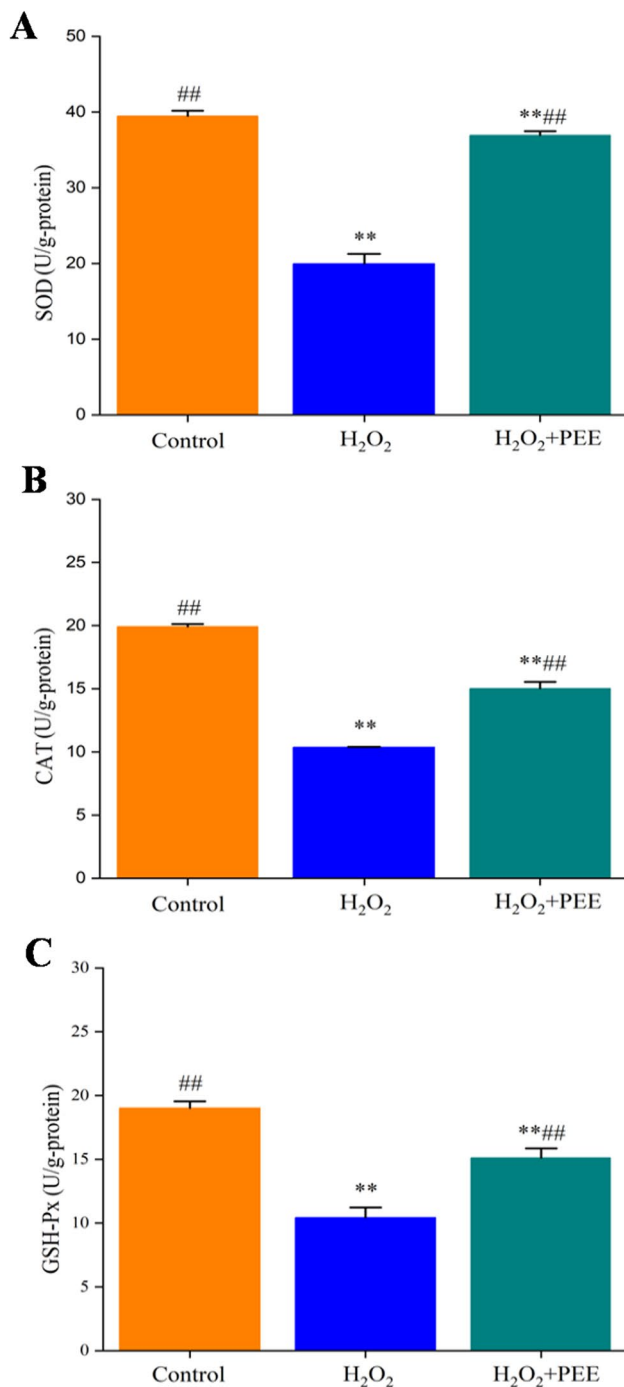


Fig. 5 Effects of PEE on the antioxidant enzymes activities in PC₁₂ cells. (A) SOD, (B) CAT and (C) GSH-Px. Results are expressed as a mean SD (n=3). Data are expressed as mean±SD., **p*<0.05 vs. control, ***p*<0.01 vs. control, #*p*<0.05 vs. H₂O₂-treated group, ##*p*<0.01 vs. H₂O₂-treated group

expression of PI3K and Akt were decreased, while the expression of Caspase-3 was increased in the H₂O₂-treated group. In the PEE group, PEE treatment could upregulate the expression of PI3K, Akt and downregulate the expression of Caspase-3 in PC₁₂ cells stimulated with H₂O₂.

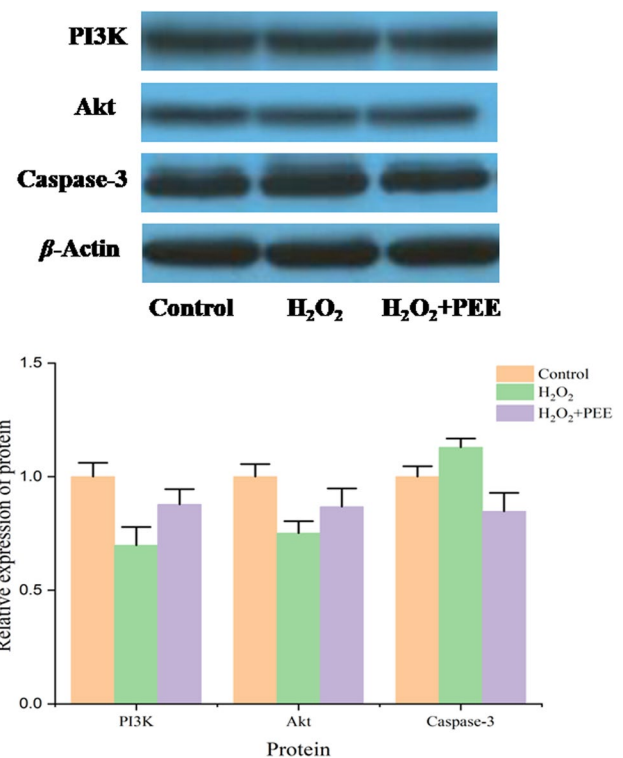


Fig. 6 The protein expressions levels as determined using Western blotting. The differences were assessed by ANOVA

Discussion

The antioxidant properties of *G. lucidum* extracts were related to its polyphenolic components in the extraction. Significantly, flavonoids are naturally occurring phenolic chemicals with exceptional performance in a wide range of food and health products as well as medical capabilities [40]. Optimizing the extraction procedure will aid in increasing the flavonoids content and antioxidant activity of *G. lucidum*. As a result, we hope to achieve a high content of *G. lucidum* flavonoids during the extraction process using a response surface methodology. The solid-liquid ratio was found to be the most influential factor in the extraction process in our study. In general, the solubility of flavonoids increases as the amount of solvent increases. When the liquid-solid ratio exceeded 1:25 mL/L, the declining trend appeared, indicating that the liquid-solid contact area had reached saturation. The extraction time is also an important factor in the extraction process. Extending the extraction time could result in flavonoids decomposition and a decrease in extraction rate [41]. We discovered that after 1.5 h of extraction time, the extraction rate of *G. lucidum* flavonoids decreased. Furthermore, increasing the polarity of ethanol by adding the appropriate amount of water could increase the solubility of the flavonoids [42]. To obtain high flavonoids content in *G. lucidum*, 65% ethanol was preferable. The

extraction temperature would also influence the flavonoids content during the extraction process. Temperature increases can affect molecular motion, penetration, dissolution, and diffusion, causing flavonoids to be released and dissolution to improve [43]. Thus, the highest flavonoids content was found when the solid-liquid ratio was 1:25 mg/L, the extraction temperature was 75 °C, the extraction time was 1.5 h, and the ethanol concentration was 65%.

The generation of free radicals is a major consequence of oxidative stress, which is ubiquitous during normal cell metabolism [44]. H₂O₂ can penetrate the cell membrane and can be converted into other free radicals, such as superoxide anions and hydroxyl radicals [45]. Previous research has shown that flavonoids have the ability to scavenge free radicals by breaking free radical chain reactions [40, 46]. When antioxidant defenses are unable to remove excess free radicals, natural antioxidant compounds can react in vitro in one-electron reactions with free radicals to prevent oxidative damage, which mediates the imbalance between intracellular antioxidant defenses and oxidative damage, and even has a protective effect on DNA damage caused by hydroxyl radicals [47]. In our research, PEE demonstrated strong DPPH• and hydroxyl radical scavenging activity, as well as stronger reducing capacity. Furthermore, the results of cell viability also showed that the viability of PC₁₂ cells decreased with approximately 50% after 24 h of 3 μM H₂O₂ stimulation, and PEE treatment could significantly increase the viability of PC₁₂ cells induced by H₂O₂. These may be due to a balanced system to neutralize the extra ROS by enzymatic antioxidants, such as SOD, CAT, and GSH-Px, to reduce oxidative state in the cell or as the first-line defense antioxidants, which can decompose superoxide radical, breakdown H₂O₂ and hydroperoxides to harmless molecules [48]. PEE could reduce oxidative damage in PC₁₂ cells by increasing the activities of SOD, CAT, and GSH-Px, thereby improving their antioxidant defenses. Our results confirmed the findings of earlier studies.

Compared with the H₂O₂-treated group, PEE treatment could upregulate the expression of PI3K and Akt in PC₁₂ cells. The PI3K/Akt signaling pathway regulates cell survival, differentiation, proliferation, and apoptosis [49, 50]. PI3Ks phosphorylates inositol phosphate at the D-3 position of the inositol head group, resulting in D-3 phosphate production [51]. When PI3K phosphorylation increases, signals are transmitted via inositol 3-phosphate dependent protein kinase-1 (PDK1), a serine/threonine kinase. After PI3K activation, PDK1 is recruited to the cell membrane, where it phosphorylates and activates Akt [51]. H₂O₂ treatment of PC₁₂ cells resulted in excessive production of intracellular ROS, and excessive production of ROS can inhibit PI3K phosphorylation. As a result, PEE treatment might inhibit the decrease in PI3K phosphorylation caused by H₂O₂. With the restoration of mitochondrial membrane

potential, mitochondria will reduce cytochrome release and inhibit caspase family activation [52]. PEE treatment also altered the expression levels of CAT, SOD, and GSH-Px, which helped to eliminate excess reactive oxygen species in PC₁₂ cells. These results showed that PEE may have a protective effect on H₂O₂-induced PC₁₂ cells via the PI3K/Akt pathway.

Conclusion

The solid-liquid ratio was found to be the most influential factor in the extraction process, and the highest flavonoids content was found when the solid-liquid ratio was 1:25 mg/L, the extraction temperature was 75 °C, the extraction time was 1.5 h, and the ethanol concentration was 65%. The structure of the PEE was investigated using FT-IR spectroscopy and ¹H NMR spectroscopy, which revealed that it was primarily a 5, 7-Dihydroxyflavone. PEE exhibited significant DPPH• and hydroxyl radical scavenging activity, as well as stronger reducing capacity. Moreover, PEE pretreatment could significantly increase the viability of PC₁₂ cells induced by H₂O₂. In addition, PEE could reduce oxidative damage in PC₁₂ cells by increasing the activities of SOD, CAT, and GSH-Px, thereby improving their antioxidant defenses. More importantly, PEE treatment could upregulate the expression of PI3K, Akt and downregulate the expression of Caspase-3 in PC₁₂ cells stimulated with H₂O₂.

Acknowledgements This work was supported by Scientific Research Plan Project of University in Anhui Province (2022AH050202), Outstanding Innovative Research Team for Molecular Enzymology and Detection in Anhui Provincial Universities (2022AH010012) and the National Natural Science Foundation of China (31972014).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

1. M.S. Swallah, P. Bondzie-Quaye, Y. Wu, A. Acheampong, F.L. Sossah, S.M. Elsherbiny, Q. Huang, Therapeutic potential and nutritional significance of *Ganoderma lucidum*—a comprehensive review from 2010 to 2022. *Food Funct.* **14**(4), 1812–1838 (2023)
2. S. Sudheer, I. Alzorqi, S. Manickam, A. Ali, Bioactive compounds of the wonder medicinal mushroom *Ganoderma lucidum*. *Bioact. Molecules Food* **11**, 1863–1893 (2019)
3. G. Tel, M. Ozturk, M.E. Duru, Antioxidant and anticholinesterase activities of five wild mushroom species with total bioactive contents. *Pharm. Biol.* **53**(6), 824–830 (2015)
4. H.T. Ha, H. Tran Van, T. Van Tran, H.T.N. Nguyen, D.T.A. Phan, Study on chemical compositions, antioxidants and intracellular anti-melanogenic activities of varieties of *Ganoderma lucidum* in

- vietnam. Int. J. Food Sci. Tech. (2022). <https://doi.org/10.1111/jifs.16019>
5. A.T. Bristy, T. Islam, R. Ahmed, J. Hossain, H.M. Reza, P. Jain, Evaluation of total phenolic content, HPLC analysis, and antioxidant potential of three local varieties of mushroom: a comparative study. Int. J. Food Sci. Tech. **2022**, 3834936 (2022)
 6. M.H. Park, M. Kim, Antioxidant and anti-inflammatory activity and cytotoxicity of ethanol extracts from *rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Prev. Nutr. Food Sci. **23**(4), 326–334 (2018)
 7. M.M. Farimani, Antioxidant activity, total flavonoid and phenolic contents of three different extracts of *Hircanian reishi*. Curr. Bioact Compd. **15**(1), 109–113 (2019)
 8. M. Park, M. Kim, Analysis of antioxidant and anti-inflammatory activities of solvent fractions from *rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Prev. Nutr. Food Sci. **22**(4), 365–371 (2017)
 9. F. Hidayat, S. Fatmawati, Antioxidant evaluation of *Ganoderma lucidum* extracts. Mater. Sci. Eng. **588**, 012042 (2019)
 10. J. Nie, S. Yang, M. Mo, Z. Hu, In vitro antioxidant activity of hot water extracts from 7 different sources of *Ganoderma Lucidum*. Med. Plant. **8**(4), 62–71 (2017)
 11. D. Cor, T. Botic, Z. Knez, A. Gregori, F. Pohleven, The effects of different solvents on bioactive metabolites and in vitro antioxidant and anti-acetylcholinesterase activity of *Ganoderma lucidum* fruiting body and primordia extracts. Maced J. Chem. Chem. En. **36**(1), 129–141 (2017)
 12. D. LoubnaFerchichia, Chohraa, K. Mellouk, Total phenolic content, total flavonoid and antioxidant activity of methanolic and ethanolic extract of the flowers of a fruit tree *cydoniaoblonga*: quince from *Eastern algeria*. Ann. Rom Soc. Cell. Bio **25**(7), 1078–1087 (2021)
 13. F.A. Badiuzaman, M.H.M. Amini, N.S. Sulaiman, M. Mohamed, S.A. Sobri, M.N. Masri, M.B.A. Bakar, Preliminary characterization of methanolic and ethanolic extract of *Musa acuminata* leaves. AIP Conf. Proc. **2454**(1), 1–5 (2022)
 14. E. Vamanu, Antioxidant properties and chemical compositions of various extracts of the edible commercial mushroom, *pleurotus ostreatus*, in romanian markets. Rev. Chim. **64**(1), 49–54 (2013)
 15. K.A. Lee, H. Park, K.-T. Kim, M.-S. Chung, H.-D. Paik, P.-S. Chang, H.J. Kim, Antioxidant activities of onion (*Allium cepa* L.) peel extracts produced by ethanol, hot water, and subcritical water extraction. Food Sci. Biotechnol. **23**(2), 615–621 (2014)
 16. N.S. Yamashita, W.K.H.O. Marise, H. Edgar Matias, Bach, E.E. Bach, Chemical, toxicological, anti-inflammatory and antimicrobial evaluation of *Ganoderma lucidum* extracts. J. Food Agric. **27**(7), 577–584 (2015)
 17. Rana, Gulliya, Chemistry and pharmacology of flavonoids-a review. Indian J. Pharm. Educ. Res. **53**(1), 8–20 (2019)
 18. S.I. Pavlova, D.Z. Albegova, Y.S. Vorob'eva, O.S. Laptsev, I.G. Kozlov, Flavonoids as potential immunosuppressants affecting intracellular signaling pathways. Pharm. Chem. J. **49**(10), 645–652 (2016)
 19. A.M. Hamsalakshmi, M. Alex, S. Arehally Marappa, Joghee, S.B. Chidambaram, Therapeutic benefits of flavonoids against neuroinflammation: a systematic review. Inflammopharmacology. **30**(1), 111–136 (2022)
 20. R. Zhao, S. Jiang, L. Zhang, Z. Yu, Mitochondrial electron transport chain, ROS generation and uncoupling (review). Int. J. Mol. Med. **44**(1), 3–15 (2019)
 21. A.A. Starkov, The role of mitochondria in reactive oxygen species metabolism and signaling. Ann. NY Acad. Sci. **1147**(1), 37–52 (2008)
 22. Y. Zhang, L. Zhang, J. Liu, J. Liang, J. Si, S. Wu, Dendrobium officinale leaves as a new antioxidant source. J. Funct. Foods. **37**, 400–415 (2017)
 23. H. Jin, H. Guo, X. Tan, M. Liu, F. Wang, W. Cai, Y. Zhang, J. Deng, Purification of total flavonoids from *Ampelopsis gros-sedentata* by combined use of macroporous adsorption resin and polyamide. Food Sci. **37**(12), 13–18 (2016)
 24. Z.M. Lima, L.S.D. Trindade, G.C. Santana, F.F. Padilha, M.L.H. Macedo, Effect of *Tamarindus indica* L. and *Manihot esculenta* extracts on antibiotic-resistant bacteria. Pharmacogn Res. **9**(2), 195–199 (2017)
 25. M.A. Hossain, M.S. Akhtar, S. Said, T. Hilal, Two new flavonoids from *adenium obesum* grown in oman. J. King Saud Univ. Sci. **29**(1), 62–69 (2017)
 26. Y. Wang, Y. Gao, H. Ding, S. Liu, X. Han, J. Gui, D. Liu, Subcritical ethanol extraction of flavonoids from *Moringa oleifera* leaf and evaluation of antioxidant activity. Food Chem. **218**, 152–158 (2017)
 27. H. Zhang, X. Li, K. Wu, M. Wang, P. Liu, X. Wang, R. Deng, Antioxidant activities and chemical constituents of flavonoids from the flower of *Paeonia ostii*. Molecules. **22**(1), 5 (2016)
 28. Q. Wei, Y. Zhan, B. Chen, B. Xie, T. Fang, S. Ravishankar, Y. Jiang, Assessment of antioxidant and antidiabetic properties of *Agaricus blazei murill* extracts. Food Sci. Nutr. **8**(1), 332–339 (2020)
 29. X. Ren, J. Zhang, Y. Zhao, L. Sun, Senegenin inhibits $\alpha\beta_{(1-42)}$ -induced PC₁₂ cells apoptosis and oxidative stress via activation of the PI3K/Akt signaling pathway. Neuropsych Dis. Treat. **18**, 513–524 (2022)
 30. T. Toledo, L. Silva, T.C. Botelho, R.J. Ramos, R. Bento, Characterization of flavonoid 3-Methoxyquercetin performed by FT-IR and FT-Raman spectroscopies and DFT calculations. J. Mol. Struct. **1029**(49), 22–27 (2012)
 31. B. Venkatadri, A. Khusro, C. Aarti, M.R. Rameshkumar, P. Agastian, Vitro assessment on medicinal properties and chemical composition of *Michelia nilagirica* bark. Asian Pac. J. Trop. Biomed. **7**(9), 782–790 (2017)
 32. M. Krysa, M. Szymańska-Chargot, A. Zdunek, FT-IR and FT-Raman fingerprints of flavonoids – A review. Food Chem. **393**, 133430 (2022)
 33. A.K. Khalafallah, S.A. Suleiman, A.H. Yousef, N.A. El-kanzi, A.E. Mohamed, Prenylated flavonoids from *Tephrosia apollinea*. Chin. Chem Lett. **20**(12), 1465–1468 (2009)
 34. H.H. Barakat, A.M. Souleman, S.A.M. Hussein, O.A. Ibrahim, M.A.M. Nawwar, Flavonoid galloyl glucosides from the pods of *Acaciafarnesiana*. Phytochemistry. **51**(1), 139–142 (1999)
 35. T. Hatano, S. Mizuta, H. Ito, T. Yoshida, C -Glycosidic flavonoids from *Cassia occidentalis*. Phytochemistry **52**(7), 1379–1383 (1999)
 36. G.V. Awolola, N.A. Koorbanally, H. Chenia, F.O. Shode, H. Baijnath, Antibacterial and anti-biofilm activity of flavonoids and triterpenes isolated from the extracts of *ficus sansibarica* varb. Subsp. *Sansibarica* (moraceae) extracts. Afr. J. Tradit Complement. **80**(10), 124–131 (2014)
 37. L. Wen, Y. Lin, R. Lv, H. Yan, J. Yu, H. Zhao, X. Wang, D. Wang, An efficient method for the Preparative isolation and purification of flavonoids from leaves of *crataegus pinnatifida* by HSCCC and Pre-HPLC. Molecules. **22**(5), 767 (2017)
 38. J. Peng, G. Yang, G. Fan, Y. Wu, Preparative isolation and separation of a novel and two known flavonoids from *Patrinia villosa* Juss by high-speed counter-current chromatography. J. Chromatogr. A **1092**(2), 235–240 (2005)
 39. M.-S. Shiao, K.R. Lee, L.-J. Lin, C.-T. Wang, Natural products and biological activities of the chinese medicinal fungus *Ganoderma lucidum*. Food Phytochem. Cancer Prev. **547**, 342–354 (1994)
 40. P. Karak, Biological activities of flavonoids: an overview. Int. J. Pharm. Sci. Res. **10**(4), 1567–1574 (2019)

41. K. Doldolova, M. Bener, M. Lalikoğlu, Y.S. Aşçı, R. Arat, R. Apak, Optimization and modeling of microwave-assisted extraction of curcumin and antioxidant compounds from turmeric by using natural deep eutectic solvents. *Food Chem.* **353**, 129337 (2021)
42. V.Z. Pinto, D. Pilatti-Riccio, E.S. Costa, Y.M.S. Micheletto, E. Quast, H. F. d. Santos, Phytochemical composition of extracts from yerba mate chimarro. *SN Appl. Sci.* **3**(3), 1–5 (2021)
43. J.O. Chaves, M.C. De Souza, L.C. Da Silva, D. Lachos-Perez, P.C. Torres-Mayanga, A.P.D.F. Machado, T. Forster-Carneiro, M. Vázquez-Espinosa, A.V. González-de-Peredo, G.F. Barbero, M.A. Rostagno, Extraction of flavonoids from natural sources using modern techniques. *Front. Chem.* **8**, 507887 (2020)
44. K. Jakubczyk, K. Dec, J. Kałduńska, D. Kawczuga, J. Kochman, K. Janda, Reactive oxygen species-sources, functions, oxidative damage. *Pol. Merkur Lekarski.* **48**(284), 124–127 (2020)
45. M.D. Jacobson, Reactive oxygen species and programmed cell death. *Trends Biochem. Sci.* **21**(3), 83–86 (1996)
46. Y. Zheng, G. Deng, Y. Zhang, Multiple free radical scavenging reactions of flavonoids. *Dyes Pigm.* **198**, 109–877 (2022)
47. S.B. Nimse, D. Pal, Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Adv.* **5**(35), 27986–28006 (2015)
48. O.M. Ighodaro, O.A. Akinloye, First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. *Alexandria J. Med.* **54**(4), 287–293 (2018)
49. W. Chen, H. Zhang, G. Liu, J. Kang, B. Wang, J. Wang, J. Li, H. Wang, Lutein attenuated methylglyoxal-induced oxidative damage and apoptosis in PC₁₂ cells via the PI3K/Akt signaling pathway. *J Food Biochem.* **46**(12), e14382 (2022)
50. N. Wen, B. Guo, H. Zheng, L. Xu, H. Liang, Q. Wang, D. Wang, X. Chen, S. Zhang, Y. Li, L. Zhang, Bromodomain inhibitor jq1 induces cell cycle arrest and apoptosis of glioma stem cells through the VEGF/PI3K/AKT signaling pathway. *Int. J. Oncol.* **55**(4), 879–895 (2019)
51. A. Carnero, Novel inhibitors of the PI3K family. *Expert Opin. Investig. Drugs.* **18**(9), 1265–1277 (2009)
52. X. He, X. Guo, Z. Ma, Y. Li, J. Kang, G. Zhang, Y. Gao, M. Liu, H. Chen, X. Kang, Grape seed proanthocyanidins protect PC₁₂ cells from hydrogen peroxide-induced damage via the PI3K/AKT signaling pathway. *Neurosci. Lett.* **750**, 135793 (2021)

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.