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Isolation and extraction of glansreginin A from walnut meal and its effect on the proliferation of 3T3-L1 cells

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Abstract

Glansreginin A is an indicative component in walnut and is abundant in walnut meal. The aim of this study was to isolate and purify glansreginin A from the walnut meal, and to investigate the weight loss and lipid-lowering potential of glansreginin A by studying the effect of glansregin A on the proliferation of 3T3-L1 preadipocytes. Firstly, the response surface methodology was used to effectively improve the extraction yield of glansreginin A. The maximum extraction rate of glansreginin A was 0.363%, and the optimal extraction process conditions were determined. In addition, the lipid-lowering activity of glansreginin A was investigated by cell experiments. The results showed that glansreginin A could inhibit the proliferation of 3T3-L1 preadipocytes in a dose-dependent manner. And cell cycle of different groups of cells treated with glansreginin A was also measured using flow cytometry. The results showed most of the cells were blocked in G0/G1 phase and significantly decreased in S phase. These results suggest that glansreginin A could inhibit the proliferation of 3T3-L1 preadipocytes by causing cell cycle arrest. These findings provided a theoretical basis for the future research of glansreginin A and the development of slimming and fat-reducing foods.

Keywords: glansreginin A; walnut; response surface methodology; 3T3-L1 preadipocyte; growth inhibition; lipid-lowering.

Practical Application: It not only improve the utilization value of walnut meal, a by-product of walnut, but also provide a theoretical basis for the development of functional foods for fat reduction and weight loss.

1 Introduction

Over the last few decades, obesity has become a global epidemic in both developed and developing countries (Aleman et al., 2023; Geng et al., 2022). Obesity causes or exacerbates many health problems, both independently and in association with other diseases (Park et al., 2020; Yin et al., 2022). Obesity has been associated with the development of type II diabetes mellitus, coronary heart disease, an increased incidence of certain forms of cancer, and respiratory complications (Hwang et al., 2019; Santos & Sinha, 2021). The growth of adipose tissue is caused by both an increase in the number of fat cells and an increase in size (Zhang et al., 2016). Pluripotent stem cells in adipose tissue can be directed to differentiate into preadipocytes, and the proliferation and differentiation of preadipocytes also leads to an increase in the number of adipocytes (Ko & Ku, 2022; Poglio et al., 2010). In conclusion, controlling the proliferation of preadipocytes, the differentiation of preadipocytes into adipocytes, and the lipid metabolism in adipocytes is the main way to control adipose tissue and obesity.

Glansreginin A is a dicarboxylic acid derivative (Hsu & Yen, 2006). This substance is a unique component of walnut compared to other nuts, so it may be an indicative component of walnut (Haramiishi et al., 2020). Most studies have shown that the crude extract of walnut meal contains glansreginin A, which is high and stable (Xu et al., 2022; Zu-kun et al., 2020). Previous studies on crude extracts of walnut meal showed that glansreginin A may have antioxidant, anti-inflammatory, anti-atherosclerotic, analgesic, cholesterol absorption reduction and antibacterial properties (Bati et al., 2015; Berryman et al., 2013; Ho et al., 2018; Papoutsi et al., 2008; Raafat, 2018; Ren et al., 2018a; Slatnar et al., 2014). In terms of hypolipidemic activity, walnut meal extract mainly contained glansreginin A, ethyl gallate and ellagic acid, which could significantly regulate blood lipids in liver and blood (Ren et al., 2018b; Liang et al., 2017). The spectral effect relationship between the ethanolic extract of walnut meal and the lipid-lowering effect was analyzed by fingerprinting, and it was hypothesized that glansreginin A might play a role in the spectral effect relationship between the ethanolic extract of walnut meal and the lipid-lowering effect, which provided a basis for determining the lipid-lowering substance basis of walnut meal extract, and glansreginin A is a unique component of walnut that deserves further study (Zu-kun et al., 2020). In addition, the utilization value of walnut by-product walnut meal is low (Multescu et al., 2022). At present, due to the difficulties in the separation and purification of glansreginin A and the low extraction rate, the research on the monomer component in walnut is insufficient.

Response Surface Method (RSM) is a method for optimizing experimental conditions, which is suitable for solving problems related to nonlinear data processing (Du et al., 2022). It includes many test and design techniques, such as experimental design,

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modeling to check the suitability of the model, and seeking the best combination conditions. Based on the response value of each factor level, the optimal predicted response value and corresponding experimental conditions can be found (Dong et al., 2022).

In this work, the RSM were used to optimize the operational parameters (Material-liquid ratio, extraction temperature, extraction time) to obtain the maximum yield of glansreginin A from walnut meal. RSM was designed through employing a Box–Behnken design (BBD) to systematically analyze the effects of extraction parameters on the yields of glansreginin A from walnut meal and their interactions. In addition, the effect of glansreginin A on the proliferation of 3T3-L1 preadipocytes was evaluated by using various methods, including Cell Counting Kit 8 (CCK-8) cell proliferation assay, Hoechst 33258 cell nuclear staining assay, flow cytometry to detect cell cycle. It provide a basis for the application of glansreginin A and it also provides some reference for the utilization of walnut by-product walnut meal.

2 Materials and methods

2.1 Materials and reagents

Walnut meal (Northern Xiangling walnut) was provided by Hebei Yangyuan Zhihui Beverage Co., Ltd (Hebei, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other cell-culture reagents were obtained from Gibco (Gibco, Oklahoma, USA). Cell Counting Kit 8 Assay Kit, Hoechst33258, Cell Cycle Analysis Kit and Annexin V/propidium iodide (PI) staining kit were purchased from Biorigin Inc (Beijing, China). Lactate dehydrogenase (LDH) Assay Kit was purchased from Biyuntian Biotechnology Co., Ltd (Beijing, China). RNA Easy Fast Tissue/Cell Kit, FastKing RT Kit (With gDNase), SuperReal PreMix Plus (SYBR Green) were purchased from Tiangen Biotech CO., Ltd (Beijing, China). All other chemicals were analytical grade unless otherwise stated.

2.2 Preparation of walnut meal extract

The walnut meal was pulverized, passed through a 60-mesh sieve, extracted in 60% ethanol for 50 min by an ultrasonic-assisted method according to a material-to-liquid ratio of 1:10, vacuum filtered, the supernatant was rotary evaporated, and then freeze-dried to obtain a crude extract of walnut meal.

2.3 Apparatus and chromatographic conditions

The ethanol extract of walnut meal was prepared into a solution of 100 mg/mL with water, centrifuged at 12000 r/min for 10 min, and the supernatant was taken and passed through a 0.22 μm microporous membrane, the glansreginin A was further purified by UV double-guided mass spectrometry analysis and preparation of chromatograph (RP-HPLC; Waters 2767, Waters, USA) using an XSelect® Peptide CSH™ C18 reverse-phase column (19 mm × 150 mm, 130 Å, 5 μm, 1/pkg). The ultraviolet wavelength was 265 nm and the mass spectrometry was in negative ion mode. The mobile phases for flash chromatography and RP-HPLC were 1mL/L trifluoroacetic acid (TFA) aqueous solution (solvent B) and acetonitrile (solvent A). The gradient settings for the elution program were as follows: 0-5 min, 10-22% A; 5-10 min, 22-23% A; 10-20 min, 23-60% A; 20-21 min, 60-90% A; 21-25 min, 90% A; 25-26min, 90-10% A; 26-30 min, 10% A. Mass spectrometry conditions are in negative ion mode. The injection volume was 1000 μL at 15 mL/min by preparative high performance liquid chromatography. Finally, the purified glansreginin A was concentrated by rotary evaporation and dried using vacuum freeze dryer.

2.4 RSM optimization test design

On the premise that the optimal scheme of three factors was obtained according to the single factor test results, the optimal scheme was set as 0, and the data under the premise of the optimal scheme was set as -1, 1, respectively. The selected factor levels and codes are shown in Table 1.

2.5 Cell culture

The 3T3-L1 cells (Procell, Wuhan, China) (Cui et al., 2021) were cultured in DMEM supplemented with 10% newborn calf serum and 1% penicillin-streptomycin. All operations are strictly aseptic, and cells were cultured in a sterile environment at 37 °C and 5% CO_2.

2.6 Cell viability assay

Glansreginin A was prepared in DMSO at the concentration of 100 mg/mL and diluted by cell culture medium to various working concentrations. The proliferation of all cell lines were examined using a CCK-8 Assay Kit. Briefly, 100 μL of cells (3×10^4 cells/mL) in the logarithmic growth phase were seeded on 96-well plates and cultured for 24 h. Next, cells were treated with a wide range of glansreginin A concentrations (0, 50, 100, 200, 300, 400, 500 μg/mL) for 24 h and 48 h respectively, while the culture medium served as vehicle control. Subsequently, the mixtures were dyed with CCK-8 solution for 4 h, and their absorption at 450 nm was measured by a Microplate Reader (INFINITE Spark 10 M; TECAN).

2.7 Lactate dehydrogenase (LDH) assay

3T3-L1 preadipocytes in logarithmic growth phase were inoculated in 96-well plates at a density of 3×10^4 cells/mL. After overnight incubation, the complete culture medium was discarded and 200 μL of complete culture medium containing different concentrations of glansreginin A (0, 50, 100, 200, 300, 400, 500 μg/mL) was added to the drug-treated group, the blank group without cells and glansreginin A, the control group with maximum enzyme activity without drug treatment (containing cells), and the control group without glansreginin A were replaced

Table 1. Experimental Design Data of RSM Optimization.

Factors	Levels		
	-1	0	1
A-Material-liquid ratio (g/mL)	1:15	1:20	1:25
B-Temperature (°C)	30	40	50
C-Time (min)	30	40	50

with equal volumes of culture medium. Six replicate wells of each group were incubated in the incubator for 24 h and 48 h. At 1 h before the scheduled time, add 20 μL of LDH release reagent to the untreated maximum enzyme activity control, and after reaching the scheduled time, centrifuge at 400*g* for 5 min and take 120 μL of supernatant to a new 96-well plate, adding 60 μL of LDH working solution. Mix well and incubate for 30 min at room temperature, and measured the absorbance value at 490 nm on the Microplate Reader.

2.8 Hoechst 33258 staining

3T3-L1 preadipocytes in logarithmic phase were inoculated into 12 well cell culture plate at the density of 2×10^4 cells per well and cultured overnight. The medium was aspirated from the plates after overnight incubation, and then added 1.5 mL of different concentrations (0, 300, 400 and 500 μg/mL) glansreginin A solution, the control group was added 2 mL DMEM complete medium, cultured in the CO_2 incubator at 37 °C for 48 h. Cells were treated and the medium was discarded, then washed the cells with PBS three times, added 500 μL 4% methanol to fix 30 min at 4 °C. After removing the fixed solution, washed two times with PBS and 500 μL Hoechst 33258 staining solution was added to dye 10 min. After washing with PBS for three times, it was observed under fluorescence microscope.

2.9 Analyses of Cell Cycle (PI Staining Method) by Flow Cytometry

The cells in logarithmic growth phase were inoculated with 4×10^4 cells per well on a 6-well cell culture plate. After being cultured overnight, samples of different concentrations (0, 300, 400 and 500 μg/ mL) were added to the centrifuge tube for 48 h. The treated cell culture medium was collected and washed once with PBS buffer. The cleaning solution was added to the tube. The cells were collected by trypsin digestion and collected in the tube. It was centrifuged at 1000*g* for 5 min and removed supernatant, then 1mL PBS buffer was added to resuscitate the supernatant. Added 75-80% ethanol precooled by 1 mL ice bath, gently blow and mix, fixed overnight at -20 °C, centrifuged 5 min about 1000*g*, precipitated cells, added 1 mL ice bath precooled PBS, resuspended cells, centrifuged precipitate cells again, discarded supernatant. Next, added 0.5 mL propidium iodide staining solution to each tube cell sample, slowly and fully resuspend cell precipitation, incubated 15-30 min at room temperature away from light, and detected by BD C6 flow cytometer from BD Bioscience.

2.10 Real-time PCR detection cell cycle marker genes

Total RNA was extracted from the 3T3-L1 cells by an RNA Easy Fast Tissue/Cell Kit from the different concentrations (0, 300, 400 and 500 μg/ mL), and then reversed transcribed using the FastKing RT Kit (With gDNase), and quantitative real-time PCR were performed by a SuperReal PreMix Plus (SYBR Green). Table 2 shows the primer sequences that were synthesized by Sino-American Taihe Biotechnology Co., Ltd. (Beijing, China). To enable comparison of Ct (cycle threshold) values between groups, quantities of all target genes in the test samples were normalized by GAPDH. The results of relative mRNA expression were used in Formula 1:

$$2^{-\Delta\Delta Ct}, \Delta Ct = Ct\ value(target\ gene) - Ct\ value(GAPDH)$$
$$\Delta\Delta Ct = \Delta Ct(treatment) - \Delta Ct(control\ group) \quad (1)$$

2.11 Statistical analysis

Data were expressed as mean ± SD and subjected to one-way analysis of variance (ANOVA; SPSS version 26.0) to determine significant differences. Each experiment was analyzed in triplicate and one-way analysis of variance was performed. A value of $p < 0.05$ and $p < 0.01$ was considered statistically significant.

3 Results

3.1 Separation and identification of glansreginin A

In order to investigate the hypolipidemic activity of glansreginin A, it first needs to be extracted to obtain it. In this study, the ethanol extract of walnut meal was first obtained by using 60% ethanol extraction with sonication assistance, and then separated using RP-HPLC with mass spectrometry analysis. It was found that the target peak with molecular weight consistent with that reported in the literature appeared at a retention time of 9.8 min (Medic et al., 2021), and the substance was tentatively identified as glansreginin A (Figure 1). After comparison with the literature (Ito et al., 2007), the substance was identified as glansreginin A.

3.2 Analysis of results of single factor test

According to the data in Figure 2A, the extraction yield of glansreginin A was the highest when the material-liquid ratio was 1:20, and the extraction yield decreased when the material-liquid ratio continued to extend. Therefore, the 1:20 extraction material-liquid ratio was chosen as the best extraction time. Figure 2B shows that when the temperature was 40°C, walnut meal have the highest yield of glansreginin A extraction, and the extraction rate dropped as the temperature

Table 2. Primer sequences used for real-time PCR.

gene	Forward Primer	Reverse Primer
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGGCCATGAGGTCCACCAC
Cyclin D1	ATGGAACACCAGCTCCTGTGCTGC	TCAGATGTCCACGTCCCGCACGT
Cyclin E1	GGATTATTGCACCATCCAGAGGCT	CTTGTGTCGCCATATACCGGTCAA
CDK2	AACACAGAGGGGGCCATCAAGC	CAGGAGCTCGGTACCACAGGGTC
CDK4	CAATGGAGGAGGAGGTGGAG	CCATCAGCCGGACAACATT
CDK6	CGTGGTCAGGTTGTTTGATGT	CGGTGTGAATGAAGAAAGTCC

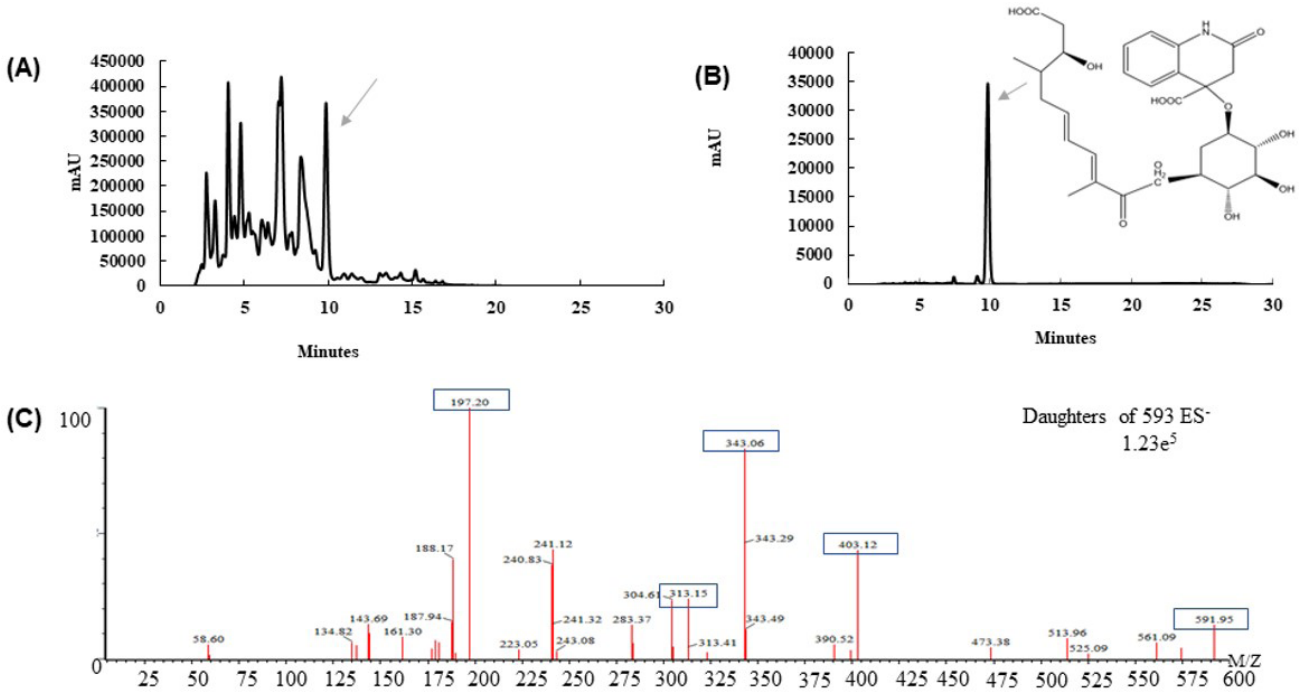

Figure 1. Separation and purification of glansreginin A. (A) HPLC chromatogram (265 nm) of Walnut Meal extract; (B) HPLC chromatogram (265 nm) of glansreginin A; (C) MS/MS chromatogram of glansreginin A.

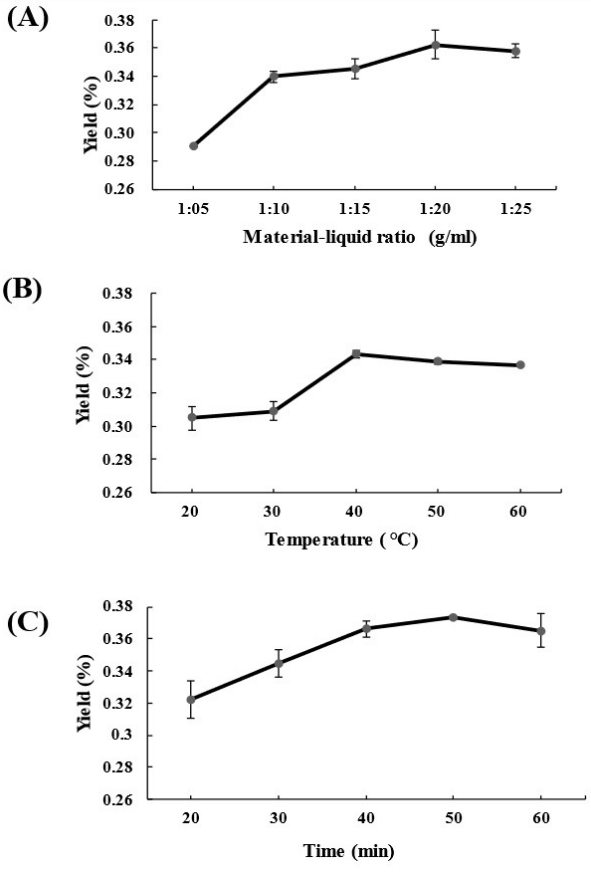

Figure 2. Effects of different extraction parameters on extraction yield of glansreginin A from walnut meal (A) material-liquid ratio, g/mL; (B) extraction temperature, °C; (C) extraction time, min.

continued to rise. Therefore, the optimal temperature was 40°C. Figure 2C shows that the extraction yield of glanareginin A in walnut meal was the highest when the extraction time was 50 min, and the extraction yield decreased with the increasing temperature. Therefore, 50 min was selected as the optimal extraction temperature.

3.3 Data Identification by RSM

According to the Box-Behnken principle, an experimental program was designed to investigate the effects of three factors: liquid-solid ratio, extraction temperature, and extraction time on the extraction rate of walnut meal glansreginin A. A total of 17 experimental points were designed for the RSM test. The design table and results were shown in Table 3. The p-value of the sample in Table 4 is 0.0073 ($p < 0.05$), which is significant, indicating that the model is fitting. The Lack of Fit data is 0.1970, which is proven meaningful. By applying multiple regression analyses on the experimental data, the response variable and the test variables were associated with the following second-order polynomial Equation 2:

$$Y = 0.35986 + 0.00042A + 0.01779B + 0.00441C - 0.00421A^2 - 0.01898B^2 - 0.01228C^2 + 0.00367AB - 0.00333AC - 0.00315BC \quad (2)$$

3.4 Response surface analysis

The response surface curve can be drawn through the software Design Expert 12.0 to analyze the interaction of three factors, namely, the material liquid ratio, ultrasonic temperature and ultrasonic time, on the extraction yield of glansreginin A. The results are shown in Figure 3.

Table 3. Box–Behnken design and observed responses.

Test Number	Material-liquid ratio	Temperature	Time	Yield of Glansreginin A
1	-1	1	0	0.3523
2	1	0	-1	0.3408
3	-1	0	-1	0.3403
4	-1	-1	0	0.3132
5	0	0	0	0.3684
6	0	0	0	0.3523
7	0	-1	-1	0.3071
8	0	1	1	0.3438
9	1	-1	0	0.3137
10	0	1	-1	0.3381
11	0	0	0	0.3536
12	0	0	0	0.3641
13	0	0	0	0.3609
14	-1	0	1	0.3526
15	1	0	1	0.3398
16	0	-1	1	0.3254
17	1	1	0	0.3675

Table 4. Variance analysis table of glansreginin A extraction yieid of walnut meal optimized by Response Surface Method.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	0.0052	9	0.0006	7.5	0.0073 significant
A	1.44×10^{-6}	1	1.44×10^{-6}	0.0186	0.8952
B	0.0025	1	0.0025	32.65	0.0007
C	0.0002	1	0.0002	2.01	0.1993
AB	0.0001	1	0.0001	0.6969	0.4314
AC	0.0000	1	0.0000	0.5705	0.4747
BC	0.0000	1	0.0000	0.5120	0.4974
A^2	0.0001	1	0.0001	0.95820	0.3602
B^2	0.0015	1	0.0015	19.56	0.0031
C^2	0.0006	1	0.0006	8.18	0.0243
Residual	0.0005	7	0.0001		
Lack of Fit	0.0004	3	0.0001	2.52	0.1970 not significant
Pure Error	0.0002	4	0.0000		
Cor Total	0.0058	16			

Figures 3A and 3B could illustrate that the curves are relatively smooth as the liquid to solid ratio increases, indicating that the liquid to solid ratio has little effect on the extraction rate of glansreginin A, while as can be seen from Figures 3A and 3C, the curves are very steep as the temperature increases, indicating that temperature has a significant effect on the extraction of glansreginin A, while the effect of extraction time on the extraction of glansreginin A lies somewhere in between. Based on the analysis of the response surface regression model equation, the optimal extraction conditions were: material-liquid ratio 1:21.136 (g/mL), extraction temperature 44.836 °C, extraction time 40.867 min, and extraction yield 0.364%. The extraction conditions were modified to 1:21 (g/mL), 45 °C and 41 min, and the average value was 0.363%, which was similar to the predicted value.

3.5 Effect of glansreginin A on the proliferation of 3T3-L1 cells

The inhibitory effect of different concentrations of glansreginin A on 3T3-L1 preadipocytes is shown in the Figure 4. After treatment with glansreginin A for 24 h and 48 h, it showed significant a dose-dependent anti-proliferation activity on 3T3-L1 preadipocytes. With the increase of glansreginin A concentration from 50 μg/mL to 500 μg/mL, the survival rate of 3T3-L1 decreased significantly from 94% to 75% (Figure 4), and the inhibition rate increased with the increase of action time.

3.6 Effect of glansreginin A on membrane permeability of 3T3-L1 preadipocytes

In order to study the specific effect of membrane permeability, the effect of glansreginin A on the membrane permeability of 3T3-L1 preadipocytes was measured by the release rate of LDH (Figure 5). When the concentration of glansreginin A was from 50 μg/mL to 500 μg/mL, the LDH release rate of 3T3-L1 preadipocytes did not change significantly, indicating that glansreginin A had no significant effect on the membrane permeability of 3T3-L1 preadipocytes. It can also indicate that the inhibitory effect of glansreginin A on 3T3-L1 preadipocytes

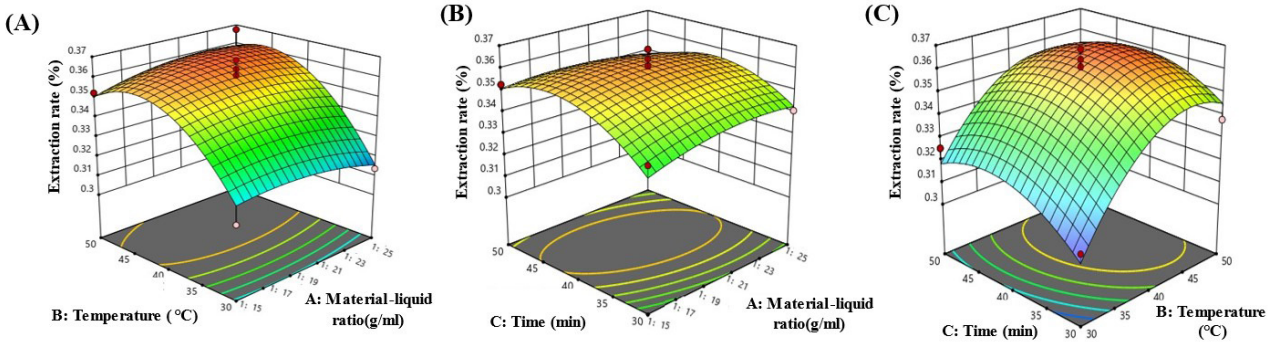

Figure 3. Response surface methodology interaction of three factors affecting the extraction yield of glansreginin A from walnut meal. (A) The effect of the interaction between liquid-solid ratio and extraction temperature on the yield of glansreginin A; (B) The effect of the interaction of liquid- solid ratio and extraction time on the yield of glansreginin A; (C) The effect of the interaction between extraction time and temperature on the yield of glansreginin A.

was not achieved by altering the cell membrane permeability pathway.

3.7 Effects of glansreginin A on the nucleus in 3T3-L1 preadipocytes

To study specific changes in cell state and nuclei, the effect of glansreginin A on 3T3-L1 preadipocyte state and nuclei was examined by Hoechst 33258 staining. Compared with the control group, the number of 3T3-L1 preadipocytes in the experimental group decreased, However, no nuclear staining was evident.

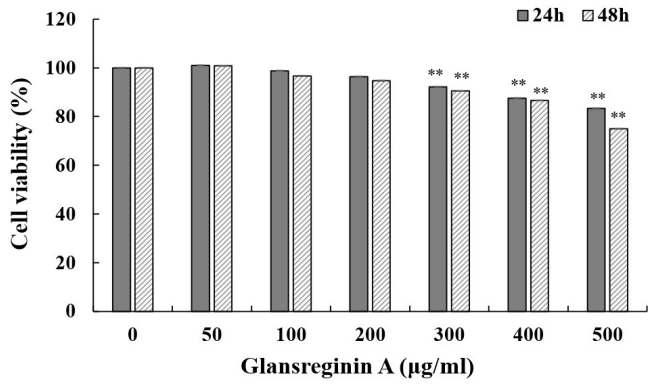

Figure 4. Cell growth inhibition effect of glansreginin A on 3T3-L1 preadipocytes (**p < 0.01).

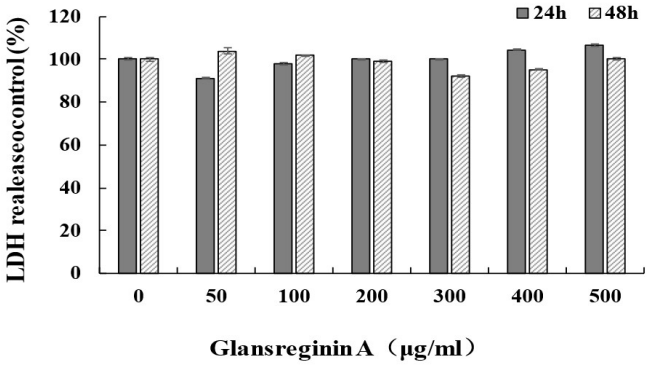

Figure 5. Changes of LDH release rate in 3T3-L1 preadipocytes treated by glansreginin A. LDH, lactate dehydrogenase.

The results indicate that glansreginin A inhibits the increase in the number of glansreginin A preadipocytes but may not cause apoptosis (Figure 6).

3.8 Effect of glansreginin A on the cell cycle arrest of 3T3-L1 preadipocytes

To further explore the mechanism responsible for glansreginin A induced growth inhibition, flow cytometry, a fast and precise method of evaluating the physical and chemical properties of individual cells, was used to detect the DNA distribution.

Treatment with different concentrations glansreginin A in 3T3-L1 preadipocytes for 48 h led to a dose-dependent increase of G0/G1 cell fraction, in which the G0/G1 cell percentages were 53.6% (control), 64.6% (300 μg/mL), 69% (400 μg/mL), and 69.2% (500 μg/mL), respectively (Figure 7). However, there was no significant change in the G2/M phase, and the S phase percentages were decreased from 35% to 21.2%. The cell cycle distribution was measured by flow cytometry and the results indicated that glansreginin A effectively inhibited the transition from G1-Phase to S-phase. Thus, glansreginin A inhibited cell proliferation and decreased cell number.

3.9 Effect of glansreginin A on the expression of cell cycle marker genes in 3T3-L1 cells

In order to further study the effect of glansreginin A on G0/G1 phase of 3T3-L1 preadipocytes, we detected the key genes of Cyclin D1, Cyclin E1, CDK2, CDK4 and CDK6. The results showed that Cyclin D1 was significantly down-regulated at glansreginin A concentration of 500 μg/mL, CDK6 was significantly down-regulated at glansreginin A treatment concentration of 400 μg/mL, and Cyclin E1, CDK2 and CDK4 were down-regulated at an action concentration of 300 μg/mL. (Figure 8) These results suggest that glansreginin A can inhibit cell proliferation by down-regulating these G0/G1 phase key genes and blocking cell proliferation in G0/G1 phase.

4 Discussion

Glansreginin A is an indicator component of walnuts and has many biological activities, but has been less studied due to problems of isolation and purification and extraction yield. Preparation of the liquid phase is a method of isolation and

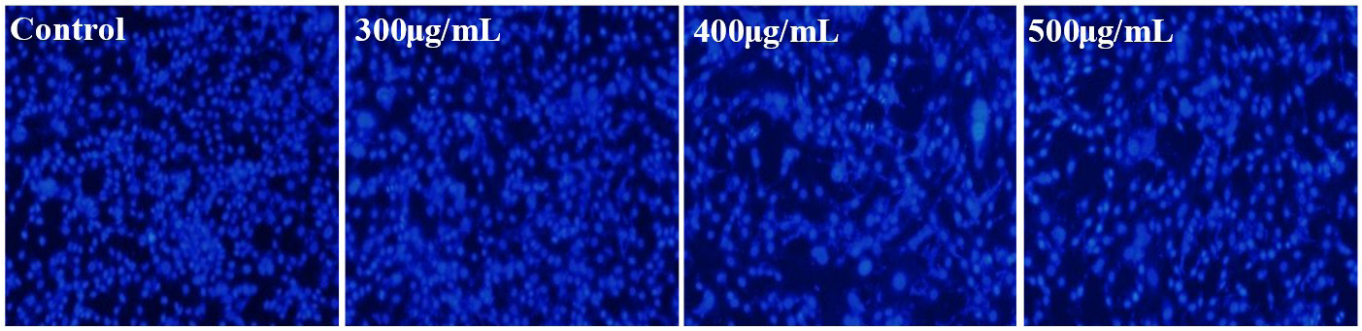

Figure 6. Effects of glansreginin A on the nucleus in 3T3-L1 preadipocytes (enlargement factor: ×100).

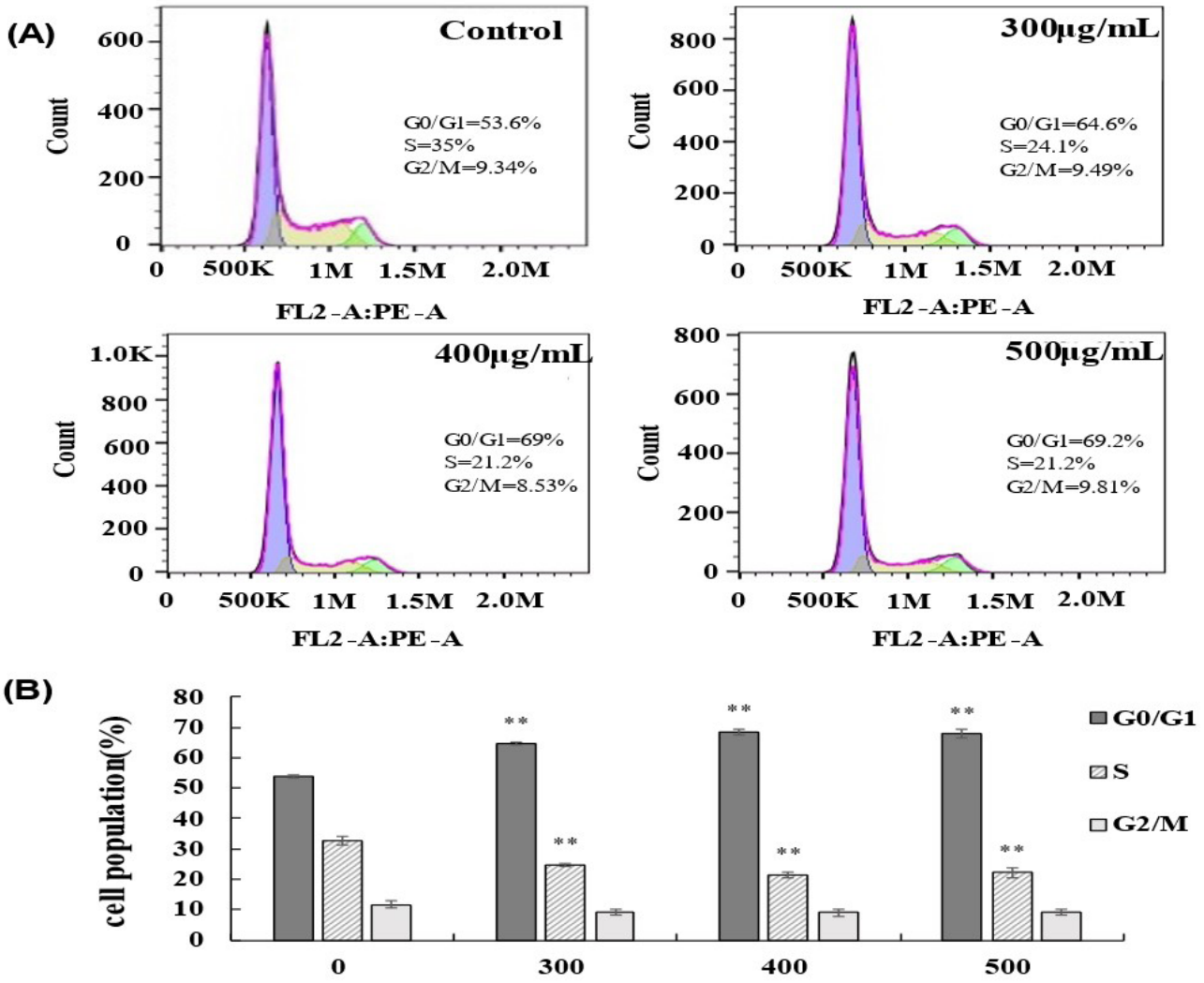

Figure 7. Cell cycle arrested of 3T3-L1 preadipocytes induced by glansreginin A. (A) FlowJo analysis of 3T3-L1 preadipocytes treated with glansreginin A; (B) Cell cycle quantification of the cells from (A). Date are averages of three independent experiments (**p < 0.01).

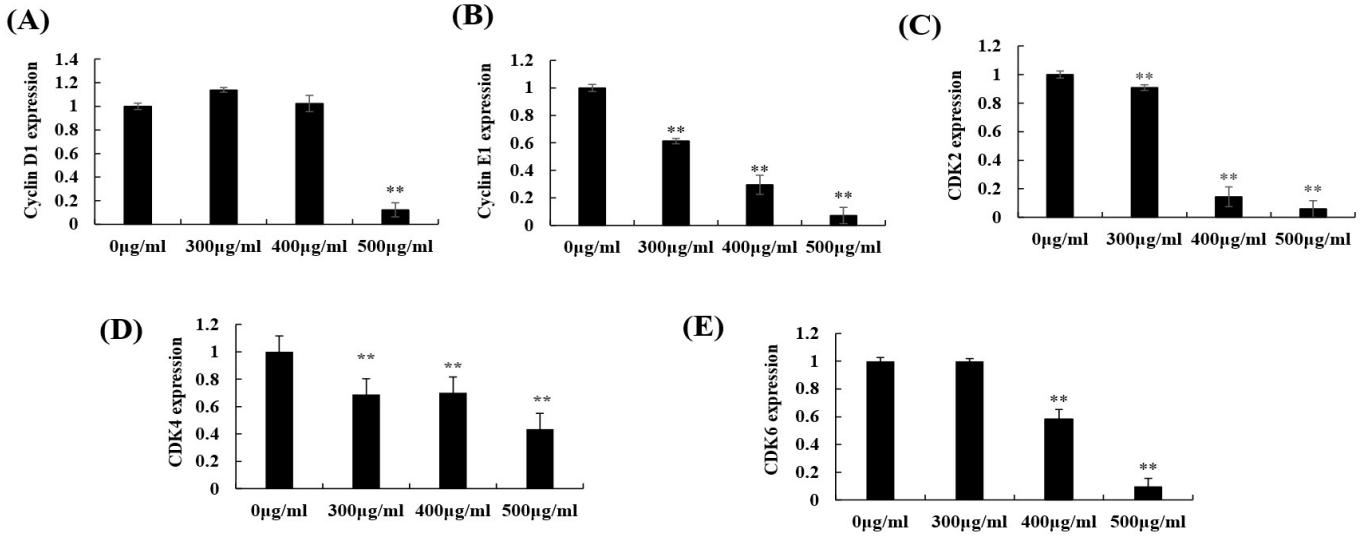

Figure 8. Cell cycle related gene in different concentrations of glansreginin A. (A) Cyclin D1 expression (B) Cyclin E1 expression (C) CDK2 expression (D) CDK4 expression (E) CDK6 expression (**p <0.01).

preparation that can yield higher purity. RSM is a well-established method for the optimization of extraction processes and can be used to develop, improve and optimize processes. In addition, the method can minimise the number of experiments and has been successfully applied to the extraction of bioactive components from plant sources (Savic et al., 2015). We obtained the monomer glansreginin A with a purity close to 98% using a semi-preparative high performance liquid chromatograph, and further determined the optimal extraction process parameters using single-factor combined with response surface optimization.

Obesity can be defined as a disease, because of excessive body fat accumulation can also lead to other physical diseases, such as nonalcoholic fatty liver disease, type 2 diabetes, osteoarthritis and several cancers, resulting in a decline in quality of life and life expectancy (Alsheridah & Akhtar, 2018; Matar et al., 2020; Srisawat et al., 2017; Tillman & Rolph, 2020). Obesity is characterized by an increase in the number and size of adipocytes differentiated from fibroblast preadipocytes in adipose tissue at the cellular level (Jeffery et al., 2015; Petrus et al., 2018). 3T3-L1 preadipocytes were isolated from Swiss 3T3 cells of mouse embryos (17-19d) and could be specifically induced to differentiate into mature adipocytes (Poulos et al., 2010). Because it is convenient to obtain mouse preadipocytes and the culture technology of mouse preadipocytes is more mature, 3T3-L1 preadipocytes have become an internationally recognized cell model for the study of fat metabolism.

There is increasing evidence that some natural products can inhibit the growth of 3T3-L1 preadipocytes, such as rutin, naringin, hesperidin, resveratrol and genistein (Hsu & Yen, 2006; Harmon & Harp, 2001; Popovich et al., 2010). Previous studies on glansreginin A are mainly based on walnut meal crude extract. Studies have shown that walnut meal crude extract plays an important role in improving anti-metabolic syndrome, preventing weight gain and improving glucose and lipid disorders. In addition, walnut meal crude extract inhibits fat production of 3T3-L1 cells in vitro (Ren et al., 2018b; Liang et al., 2017). In this study, glansreginin A was isolated and purified from the ethanolic extract of walnut meal and its role in fat reduction and weight loss was investigated. Therefore, we selected 3T3-L1 preadipocytes to study its anti-proliferation effect. Various concentrations of glansreginin A exhibited excellent antiproliferative activity on 3T3-L1 preadipocytes in a dose-dependent manner (Figure 4). By hoechst 33258 staining, it was found that the nuclei did not show obvious intense staining, but the number of cells decreased with the increase of glansreginin A concentration (Figure 6), On this basis, we designed various experiments to further verify the antiproliferative activity of glansreginin A.

To verify that glansreginin A blocks 3T3-L1 preadipocytes in GO/G1 phase, we validated the major regulatory genes in G0/G1 phase. Cyclin D1 is expressed early in G1 and is a cell cycle initiator and growth factor sensor (Qie & Diehl, 2016). Overexpression of Cyclin D1 accelerates the transition from G1 to S phase, with cells rapidly passing the G1/S checkpoint and shortening S phase (Mohamed et al., 2018). Studies have shown that blocking or knocking down Cyclin D1 causes cells to undergo cell cycle arrest. Cyclin D1 could bind to CDK4 or CDK6, forming a complex that phosphorylates the 172-position tyrosine residue of CDK4/6 and activates the kinase activity of CDK (Cai et al., 2023). Cyclin E1 synthesis begins in mid-G1 and is not expressed in G2 and M. Cyclin E1 functions mainly in late G1 and is the rate-limiting factor for the transition between G1 and S phases (Kim et al., 2022). In G1 and S phases, Cyclin E1 forms a complex with CDK2, which inactivates Rb by phosphorylation, and the phosphorylated RB separates from E2F, losing its inhibition of E2F transcriptional activation, allowing the cell to move from G1 to S phase (Aziz et al., 2021). Our study found that glansreginin A suppressed the expression level of cyclinD1-CDK4-CDK6-cyclinE1 and CDK2. These findings demonstrated that glansreginin A causes cell cycle arrest of 3T3-L1 preadipose in G0/G1 phase.

5 Conclusion

In this study, the optimal process parameters for glansreginin A extraction were determined by Box-Behnken response surface experiments. Glansreginin A was firstly purified by semi-preparative high performance liquid chromatography and quantitatively analyzed by analytical high performance liquid chromatography. The optimum parameters for extraction of glansreginin A were 1:21 (g/mL), 45 °C and 41 min. In addition, glansreginin A was found to inhibit the proliferation of 3T3-L1 preadipocytes and the proliferation of 3T3-L1 preadipocytes by blocking at G0/G1 phase. Based on these findings, the results suggested that glansreginin A in walnuts has weight loss and fat loss potential, which provided a scientific basis for the development of functional foods.

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