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RESEARCH ARTICLE

Microwave-assisted puffing of *Dendrobium officinale* for higher extraction rate of polysaccharides

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ABSTRACT

In order to increase the extraction rate of *Dendrobium officinale* polysaccharides, microwave puffing was used to process its fresh stems and the processing parameters were optimized. Response surface methodology was used to analyze the effects of sample length (1–5 cm), moisture content (15%–25%), microwave power (550–790 W), and microwave processing time (20–40 s) on the expansion ratio. The results showed that the optimized expansion ratio of 371.7% was achieved at the following conditions, i.e., sample length of 3.5 cm, moisture content of 23%, microwave power of 706 W, and processing time of 35 s. It was found that polysaccharides were extracted more readily from puffed *D. officinale* than from non-puffed *D. officinale* by 41.8%. Moreover, the bioactivities of polysaccharides from puffing *D. officinale* and non-puffing *D. officinale* were evaluated and compared in lipid peroxidation inhibition and anti-hyperglycemic assays.

Keywords: *Dendrobium officinale*; Microwave puffing; Polysaccharides; Lipid peroxidation; Anti-hyperglycemic activity

INTRODUCTION

As one of the largest genera in the Orchidaceae family, *Dendrobium* contains over 1400 species around the world, with many of them used as valued health foods and nutrients in traditional Chinese medicine because of various bioactive properties (Chen et al., 2021a). *Dendrobium officinale* Kimura et Migo, belonging to the national secondary protected medicinal plants, have been widely used in traditional Chinese and folk medicine for centuries. According to the Compendium of Materia Medica written by Shizhen Li, a famous medical scientist in the Ming Dynasty of China, *D. officinale* could nourish the stomach, promote the production of body fluid, clear away heat-evil, moisten the lung, relieve a cough, eliminate fright, strengthen intelligence, maintain strong muscles and prolong life. However, wild herbs are in serious danger of extinction because of excessive collection for medicinal use and health foods in China. To promote sustainable development of the *Dendrobium* industry, Chinese government has legislated against destructive activities to the herbs and set up a project to fund their artificial cultivation. The herbs are grown in many regions in China to satisfy the market demand. Yunnan is the province with the most *Dendrobium* plantations in China, accounting for about 70% of the national planting area (Li, 2021). The *Dendrobium* industry in China is now expanding and flourishing due to good industrial policies and government support.

Dendrobium polysaccharides, the major bioactive materials in *Dendrobium* species, were reported to have potential as immunomodulatory, anti-cancer, antioxidant, and anti-hyperglycemic agents (Chen et al., 2021b, Lee et al., 2018, Ye et al., 2021). Among *Dendrobium* species, *D. officinale* has one of the highest polysaccharide contents reported in the literature. The polysaccharide content of *D. officinale* from different regions of China ranges from 25.2% to 36.5%, and the average content in plants from Yunnan province is approximately 33.2%, which is greater than that specified as the standard in the Chinese Pharmacopoeia (Gong et al., 2013, Liu et al., 2013). Although the polysaccharide content in *D. officinale* stems is considerable, the extraction yield is relatively low. Nowadays, the traditional extraction by solvent refluxing method is often used to extract *Dendrobium* polysaccharides. It is difficult to make the *Dendrobium* polysaccharides fully cross the cell walls, so it has not been well developed and utilized. Recent methods for the extraction of polysaccharides, such as enzyme-assisted,

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ultrasonic-assisted, high pressure-assisted, and subcritical water extraction, are still described as inadequate (He et al., 2018, Liu et al., 2019, Liu et al., 2015).

Recent researches revealed that the puffing technology has obvious advantage in terms of extraction speed and yield of effective ingredients in traditional Chinese medicine (Kaur et al., 2023). At present, application of the extrusion puffing technology in the processing of traditional Chinese medicines is very extensive due to characteristics of simple operation process, high production efficiency, and unlimited types of raw materials (Song et al., 2020, Li et al., 2021). However, microwave puffing technology was mainly applied in the field of food processing (Kantrong et al., 2022, Ma et al., 2022, Yang et al., 2022). There are few researches in the processing of traditional Chinese medicines (Huang et al., 2022, Li et al., 2020). The application of microwave puffing technology in the extraction of *Dendrobium* polysaccharides has been one of the most important researches by our team (Huang et al., 2019; Li et al., 2018; Tian et al., 2018). Hereto, microwave puffing technology was investigated for its potential for increasing the extraction yield of *D. officinale* polysaccharides. The bioactivities of polysaccharides from puffing *D. officinale* and non-puffing *D. officinale* were evaluated and compared in lipid peroxidation inhibition and anti-hyperglycemic assays.

MATERIALS AND METHODS

Sample preparation

Fresh *D. officinale* was obtained from Honghe Jufeng Biotechnology Co. Ltd., China. We washed and sliced the fresh *D. officinale* into strips of 1 cm, 3 cm, 5 cm, 7 cm, and 9 cm in length. The fresh strips with the required moisture content were obtained through drying at 100 ºC in a hot air cabinet.

Microwave treatment of samples

Microwave puffing of samples was performed in an M-NJL07-3 microwave oven equipped with an output power of 0~800 W continuously adjustable (Beijing Zhongxi Yuanda Science and Technology Co. Ltd, China). The puffed strips were naturally cooled and sealed in airtight bags for subsequent analysis.

Moisture content of samples

The moisture content of the *D. officinale* strips was measured using the method reported by Lee et al. (Lee et al., 2000). The *D. officinale* strips were dried in a hot air oven at 105°C for different periods of time, and calculated moisture content from the mass-loss data.

Volume expansion ratio of samples

The volume of the puffed strips was recorded using an interesting method to measure the volume of objects with irregular shapes (Li et al., 2003), and the expansion ratio was calculated using the below formula.

$$Expansion\ ratio = \frac{V_2}{V_1} \times 100\% \quad (1)$$

Where V_1 is the volume of the non-puffed strips (NPS); V_2 is the volume of the puffed strips (PS).

Experimental design

A series of single-factor tests revealed that strips with 3 cm sample length, 20% moisture content, 670 W microwave power, and 30 s processing time had the highest quality (Fig. 1). Based on the results, the ranges of independent variables were determined, i.e., sample length of 1–5 cm, moisture content of 15%–25%, microwave power of 550–790 W, and processing time of 20–40 s.

We used Response Surface Methodology (RSM) to analyze the impact of the processing conditions on the expansion ratio of fresh *D. officinale* stems, such as sample length, moisture content, microwave power, and processing time. Code and factor levels of the independent variables are displayed in Table 1. The second-order polynomial response surface model was used to construct the function relationship between the independent variables and the response values. Fit each response variable according to the following regression equation.

$$Y_k = b_{0k} + \sum_{i=1}^{4} b_{ki} X_i + \sum_{i=1}^{4} b_{kii} X_i^2 + \sum_{i \neq j=1}^{4} b_{kij} X_i X_j \quad (2)$$

Where b_{k0}, b_{ki}, b_{kii} and b_{kij} are the regression coefficients for the intercept, linearity, square, and interaction terms; Y_k is the response variable; X_i and X_j are the coded independent variables.

Scanning electron microscopy analysis

For internal microstructure analysis, samples were examined and images were recorded on a scanning electron microscope (VEGA 3 SBH, TESCAN China, Ltd.,

Table 1: Coded levels of independent variables used in the RSM design

Variable	Symbol	Coded levels of variables		
		-1	0	+1
Sample length (cm)	X_1	1	3	5
Moisture content (%)	X_2	15	20	25
Microwave power (W)	X_3	550	670	790
Processing time (s)	X_4	20	30	40

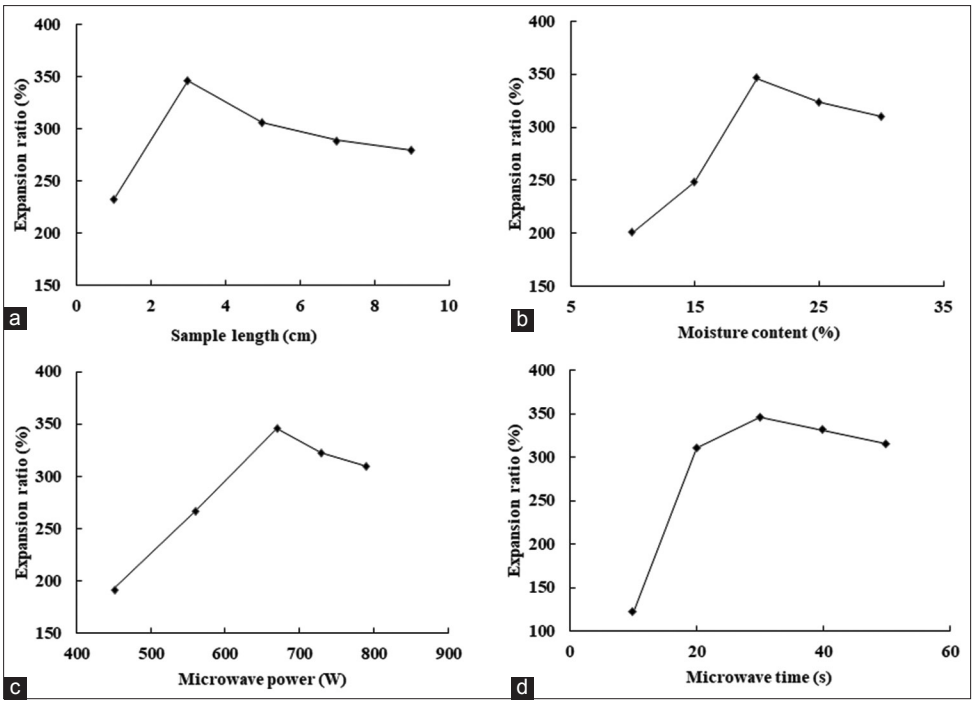

Fig 1. Relationship between the expansion ratio and four single factors. (a) The expansion ratio and sample length at same levels of other factors. (b) The expansion ratio and moisture content at same levels of other factors. (c) The expansion ratio and microwave power at same levels of other factors. (d) The expansion ratio and processing time at same levels of other factors.

Shanghai, China). The microstructure differences between the PS and the NPS were examined at 100× magnification.

Measurement of polysaccharide yield

The method previously described was applied to extract polysaccharides from the PS and the NPS (Li et al., 2018; Zhang et al., 2009). Using the following formula, the yields of polysaccharides from the PS and the NPS were calculated.

$$\text{polysaccharides yield}(\%) = \frac{\text{polysaccharides weight}}{\text{sample weight}} \times 100\% \quad (3)$$

Lipid peroxidation inhibition assays

The lipid peroxidation inhibition activity of polysaccharides from *D. officinale* was evaluated through the thiobarbituric acid method that has been reported in the literature (Yen & Hsieh, 1988). We used vitamin C as a positive reference substance.

The α-glucosidase inhibition assays

The inhibitory assay has been conducted spectrophotometrically using a method that has been previously described, in order to evaluate anti-α-glucosidase activity of polysaccharides (Li et al., 2018).

The glucose uptake assays

The L6 rat skeletal myoblast cells (Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) were grown in the Dulbecco's modified Eagle's medium (DMEM, Shanghai Basalmedia Technologies Co., Ltd., China) containing 20% fetal bovine serum (FBS, Zhejiang Tianhang Biotechnology Co., Ltd., China) at 37 ºC under 5% CO_2 atmosphere and maintained below 70% confluence. A density of 1×10^6 cells/well was used in 96-well multiwell plates (Costar, Thermo Scientific) to differentiate cells into L6 myotubes. The 2% (v/v) FBS/DMEM medium was switched every second day until myotubes were visible. The differentiated L6 cells were cultivated by DMEM serum-free medium with 0.5% bovine serum albumin (BSA, Thermo Scientific) for another 4 hours culture under starved condition. Cells in the experimental group were pretreated with polysaccharides at doses of 0.78, 3.13, 12.50, and 50.00 g/mL, and their supernatants were obtained after 48 hours of cultivation. Wells with equivalent 20% DMEM were used as a blank control, and metformin was used as a positive control. Wells with pure DMEM and without cells were used as negative control. The glucose concentrations in the supernatant were determined using a glucose assay kit (Shanghai Rongsheng Biotech Co., Ltd., China). The glucose consumption was calculated by the formula:

$$\textit{Glucose consumption}\ (\text{mmol/L}) = \frac{A_n - A_e}{A_b} \times 5.55 \quad (4)$$

where A_n refers to the absorbance of the negative control group, and A_e refers to the experimental group, and A_b refers to the calibration solution.

Statistical analysis

Statistical analysis was conducted using Design Expert (version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA). RSM was employed to obtain the optimal process parameters for microwave puffing. One-way ANOVA with the application of Duncan's tests was used to calculate significant differences ($P \leq 0.05$) between mean values. A triplicate sample was used for all assays.

RESULTS AND DISCUSSION

Experimental design and analysis of variance

Based on the RSM, we attempted to discuss the effects of sample length, moisture content, microwave power, and processing time on the expansion ratio of *D. officinale* strips. Presented in Table 2 are the results on a Box-Behnken experiment for microwave heating at each point. The results indicate that there is an empirical relationship of the responses and the independent variables. The afterward second-order polynomial equations can be used to express the relationship.

Table 2: Experimental design for microwave drying

Run order	X_1[a]	X_2[b]	X_3[c]	X_4[d]	Expansion ratio (%)
1	0	1	-1	0	226
2	0	0	0	0	336
3	0	0	1	-1	300
4	-1	0	1	0	141
5	-1	0	-1	0	108
6	0	-1	0	-1	251
7	-1	0	0	-1	214
8	0	0	0	0	356
9	0	0	0	0	335
10	1	0	1	0	280
11	1	-1	0	0	228
12	-1	0	0	1	248
13	0	0	-1	1	228
14	0	-1	-1	0	164
15	0	1	0	-1	307
16	1	0	0	1	308
17	0	1	0	1	330
18	-1	-1	0	0	132
19	0	0	-1	-1	170
20	0	1	1	0	317
21	0	0	1	1	313
22	0	0	0	0	355
23	1	0	-1	0	122
24	0	-1	1	0	232
25	0	0	0	0	355
26	1	1	0	0	303
27	1	0	0	-1	305
28	-1	1	0	0	250
29	0	-1	0	1	242

[a]Sample length. [b]Moisture content. [c]Microwave power. [d]Processing time.

$$Y_1 = 347.40 + 37.75X_1 + 40.33X_2 + 47.08X_3 + 10.17X_4 - 10.75\ X_1X_2 + 31.25X_1X_3 - 7.75X_1X_4 + 5.75X_2X_3 - 8.00X_2X_4 - 11.25X_3X_4 - 82.12X_1^2 - 39.24X_2^2 - 86.87X_3^2 - 9.99X_4^2 \quad (5)$$

Where X_1, X_2, X_3, and X_4 are sample length, moisture content, microwave power, and processing time, respectively, whereas Y_1 represent the expansion ratio.

In Table 3, ANOVA yields highly significant results ($P < 0.01$) for every response, as indicated by the corresponding regression coefficients and F values. It can be concluded from the high R^2 values that a large percentage of the variability was explained by the variables tested. CVs for all responses in this study were <10%, indicating satisfactory agreement between the model and the actual data. Having a "Lack of Fit F-value" of 3.92 in the model implies that it was not significant. A non-significant lack of fit is desirable.

Process parameters affecting the expansion ratio

The impacts of process parameters and their interactions on the expansion ratio were assessed using Design Expert in conjunction with a three-dimensional response surface of the regression model. With the exception of processing

Table 3: Analysis of ANOVA for the second-order polynomial model for the response variables

Source	SS[a]	df[b]	MS[c]	*F*-value	*P*-value
Model	1.51×10^5	14	10436.01	29.63	<0.0001[d]
X_1	17100.75	1	17100.75	46.85	<0.0001[d]
X_2	19521.33	1	19521.33	53.49	<0.0001[d]
X_3	26602.08	1	26602.08	72.89	<0.0001[d]
X_4	1240.33	1	1240.33	3.40	0.0865[e]
X_1X_2	462.25	1	462.25	1.27	0.2793[e]
X_1X_3	3906.25	1	3906.25	10.70	0.0056[d]
X_1X_4	240.25	1	240.25	0.66	0.4308[e]
X_2X_3	132.25	1	132.25	0.36	0.5568[e]
X_2X_4	256.00	1	256.00	0.70	0.4164[e]
X_3X_4	506.25	1	506.25	1.39	0.2585[e]
X_1^2	43739.33	1	43739.33	119.84	<0.0001[d]
X_2^2	9988.60	1	9988.60	27.37	0.0001[d]
X_3^2	48945.85	1	48945.85	134.10	<0.0001[d]
X_4^2	647.57	1	647.57	1.77	0.2041[e]
Residual	5109.78	14	364.98		
Lack of fit	4636.58	10	463.66	3.92	0.1000[e]
Pure error	473.20	4	118.30		
Cor Total	1.57×10^5	28			
R^2	0.9674				
Adj. R^2	0.9347				
CV%	7.43				

[a]Sum of squares. [b]Degree of freedom. [c]Mean square. [d]Highly significant at P<0.01. [e]Not significant.

time, the expansion ratio first increased with an increase in the other factors and subsequently decreased after a certain value. In addition, the influence of interaction between sample length and microwave power on the expansion ratio was highly significant, while the effects of other interactions were insignificant (Table 3).

As depicted in Fig. 2a, the interaction between sample length and microwave power significantly affected the expansion ratio. As the sample length increased up to 3.5 cm, the expansion ratio of *D. officinale* strips increased, and then decreased as the sample length continued to grow. According to Yang (2008), this trend is likely explained by the effect of sample length on the equilibrium of water vapor between inside and outside the strips. Strips that are too short or too long cannot produce enough water vapor to increase pressure within them and expand. Too-short strips evaporated rapidly, while too-long strips produced insufficient water vapor for puffing. For strips of the same length, the expansion rate fell when the microwave power exceeded 706 W because excessive microwave power caused rapid vaporization and bubble formation. Bubbles would burst if water vapor was continuously released from the strips. Excessive microwave power caused extreme damage not only to the appearance but also to the nutrient components in the strips.

As shown in Fig. 2b, the expansion ratio significantly increased to a maximum when the moisture content was about 23% and the microwave power was approximately 706 W. When the moisture content in the strips exceeded 23%, microwave power had no significant effect on the expansion ratio. Water in materials is the main driving force for microwave puffing. When the moisture content is very high, the microwave provides energy to produce more water vapor in the air. The increasing air pressure results in shrinking of the puffed strips and a decrease in the expansion ratio. Therefore, 23% moisture content and 706 W microwave power should be considered optimal for puffing.

As can be seen in Fig. 2c, the expansion ratio increased to a maximum value (372%) until the moisture content reached 23% and then decreased slightly. As moisture content increased, the expansion ratio decreased due to more microwave power was required to produce sufficient pressure to inflate strips with higher moisture content, which was independent of processing time. For strips with the same moisture content, the expansion ratio was unchanged with increasing processing time. Longer processing times did not increase the expansion ratio, but they were not economical, and the strips burned easily. In this case, the processing time of 35 s was therefore considered optimal for puffing.

Optimization of process parameters based on analysis and statistical data

The maximum expansion ratio was used as an evaluation indicator to discuss the optimal conditions for puffing *D. officinale* strips. We optimized the puffing parameters

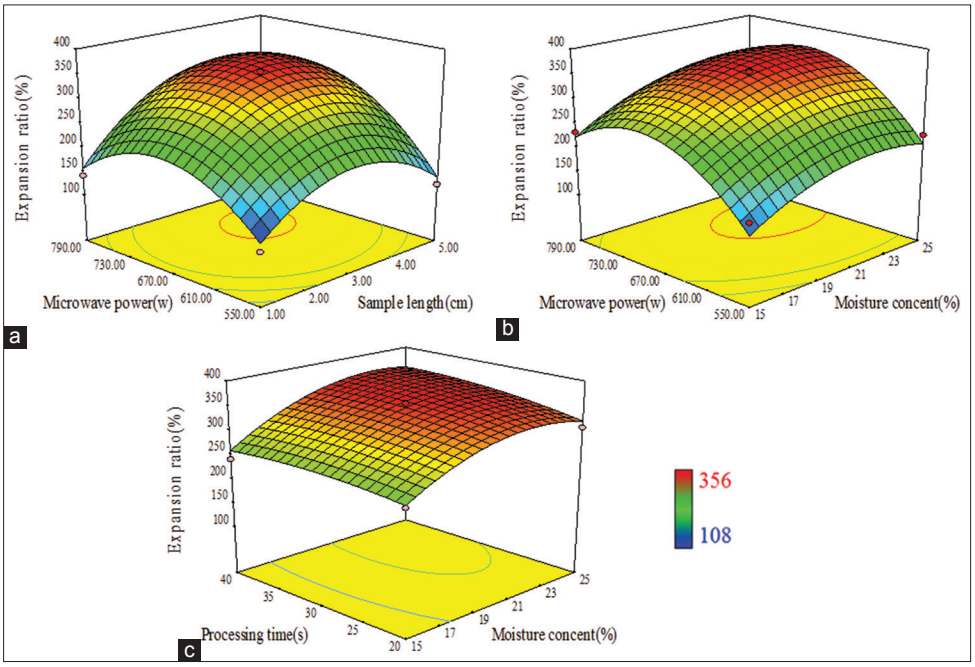

Fig 2. The comprehensive effects of processing parameters on the expansion ratio (Y_1) of puffed *D. officinale* strips in the response surface plots. (a) The impacts of sample length (X_1) and microwave power (X_3) on the expansion ratio (Y_1). (b) The impacts of moisture content (X_2) and microwave power (X_3) on the expansion ratio (Y_1). (c) The impacts of moisture content (X_2) and processing time (X_4) on the expansion ratio (Y_1).

based on the second-order polynomial models. As a result, sample length, moisture content, microwave power, and processing time were optimally 3.5 cm, 23%, 706 W, and 35 s, respectively. With the conditions, the puffed *D. officinale* strips showed maximum desirability, up to an expansion ratio of 371.7%. Results are consistent with predictions (Table 4). Consequently, the model could be adopted in order to confirm what the optimal conditions would be when microwave puffing takes place.

Microstructural and appearance characteristics

To demonstrate the effectiveness of microwave puffing, a comparison of the microstructure and appearance between the NPS and the PS was undertaken (Figs. 3 and 4). The SEM studies showed a difference in the microstructure between the NPS and the PS, where the puffed materials were characterized by the presence of expanded void spaces. The vapour pressure is critical to the formation of air cells in the expanded products, and this is provided by water and cytoplasm in parenchymal cells of the strips under microwave heating. Because of such effect of microwave, the strips were puffed by successive buildup of vapor pressure, which lead to a greater rupture of tissue. As a result, the "beehive" network with many large air cells was observed in microwave-treated strips as shown in Fig. 3b. The untreated strips have very compact structures as depicted in Fig. 3a. The NPS in Fig. 4a appear gray and wrinkled, whereas the PS in Fig. 4b have an expanded body and a glossy surface. As can be seen in the picture, the PS looked better and was more acceptable than those without puffing.

Influence of Expansion Ratio on the extraction yield of polysaccharides

The relationship between the extraction yield of polysaccharides and the expansion ratio of the strips is shown in Fig. 5. When the expansion ratio was between 132% and 280%, the increase in extraction yield was relatively significant. This might be because crude polysaccharides of the PS are more easily dissolved out and extracted than that of the NPS, resulting in the extraction yield of polysaccharides increased with increased expansion ratio. However, polysaccharides production reached a maximum at the expansion ratio of 372%, and then no longer changed as the expansion ratio increased. This indicated that the expansion ratio of 372% was the most favorable for extracting the polysaccharides from puffed products, resulting in a 42.1% extraction yield. In terms of polysaccharide extraction yield, the PS was 41.8% greater than the NPS. As a general rule, microwave process is accompanied by the rupture of the cell wall. During microwave heating, the water in the strips vaporizes, which causes the pressure in the strips to increase, causing the cell wall rupture. As a result of the effect, polysaccharides were released from parenchymal cells more readily and passed through the cell membrane more easily, resulting in a higher yield of polysaccharides. It proved that microwave puffing technology effectively enhanced the extraction rates of *D. officinale* polysaccharides.

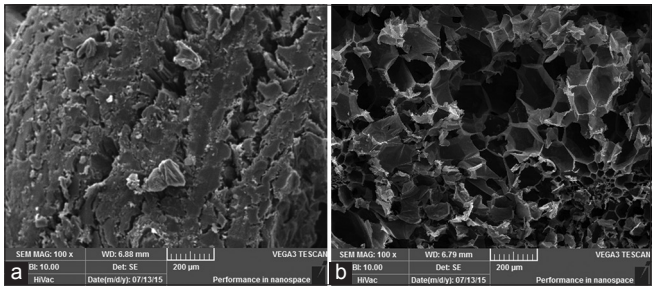

Fig 3. 100× magnification SEM micrographs of the NPS (a) and the PS (b). SEM: scanning electron microscopy; NPS: non-pufed strips; PS: puffed strips.

Inhibition effect on lipid peroxidation of polysaccharides

The inhibitory effect of polysaccharides from the NPS and the PS on $FeCl_2$-induced lipid peroxidation is shown in Fig. 6. There was a dose-dependent relationship between their inhibition rates and polysaccharide concentrations, which ranged from 0.1 to 2.0 mg/mL. The 45.6% inhibition yield of polysaccharides from the PS was found to be achieved at 2.0 mg/mL, almost the same as the 42.5% inhibition rate of the NPS. These results revealed that *D. officinale* polysaccharides were still able to inhibit lipid peroxidation after puffing (Kim et al., 2020).

Anti-α-glucosidase activity of polysaccharides

As shown in Table 5, the polysaccharides from the NPS and the PS were evaluated for their inhibitory activity against the α-glucosidase. Acarbose, as positive control, has an inhibition ratio of 98.3% at a dose of 1.0 mg/mL. However, polysaccharides derived from the NPS and the PS showed 34.9% inhibition and 36.7% inhibition, respectively, at same concentration. These results indicated that anti-α-glucosidase inhibitory of polysaccharide has little changed during microwave puffing process.

Table 4: Predicted and experimental values for responses under optimal conditions

Sample length (cm)	Moisture content (%)	Microwave power (W)	Processing time (s)	Expansion ratio (%)	
				Predicted value	Experimental value[a]
3.5	23	706	35	372.3	371.7±0.58

[a]Actual values represent average of three measurements±SD.

Fig 4. Microwave oven (a), non-pufed strips (b), and puffed strips (c).

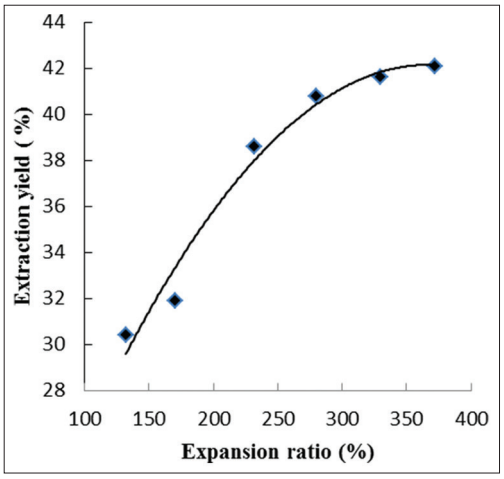

Fig 5. Relationship between the extraction yield of polysaccharides and the expansion ratio of the strips. Points represent mean values of three measurements ($n = 3$).

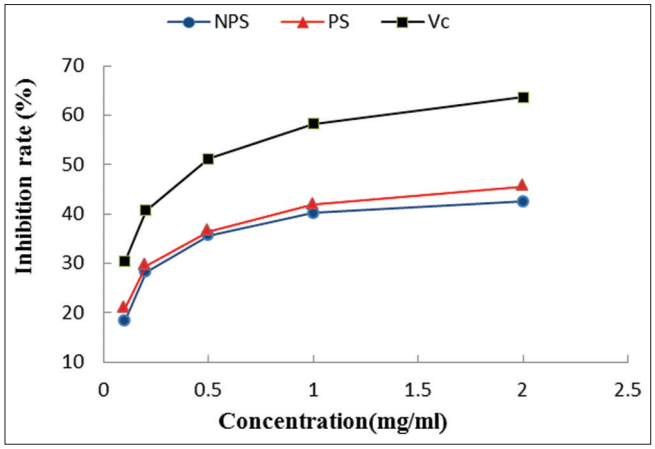

Fig 6. The lipid peroxidation inhibition activities of polysaccharides from the NPS and the PS. NPS: non-pufed strips; PS: puffed strips; Vc: vitamin C.

The effects of polysaccharides on glucose consumption in L6 myotubes

The effects of polysaccharides from the NPS and the PS on glucose consumption in L6 myotubes were evaluated and the results are shown in Fig.7. The effect of the NPS polysaccharides on glucose consumption in L6 cells was very low with a value of 3.70 mmol/L at a concentration of 0.78 μg/mL, compared to metformin as positive control with an effect of 7.92 mmol/L. However, the glucose consumption of the PS polysaccharides with the value of 6.05 mmol/L was higher than that of the NPS polysaccharides. The results showed that the effects of *D. officinale* polysaccharides on glucose consumption in L6 cells were increased during the puffing process.

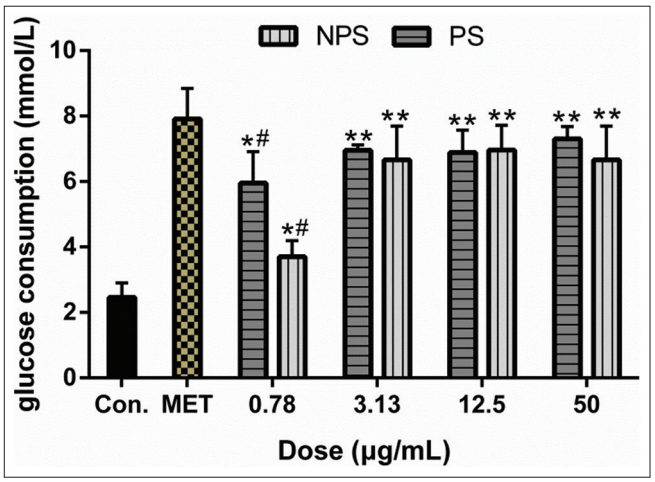

Fig 7. The effects of polysaccharides from the NPS and the PS on glucose consumption in L6 myotubes. NPS: non-pufed strips; PS: puffed strips; K: the blank control group; M: metformin (10 nmol/L) was used as positive reference substance. Each value represents the mean ± S.E.M., n=6. **Highly significant difference in comparison with the blank control group (p<0.001); *Significant difference in comparison with the blank control group (p<0.01); #Significant difference in comparison with the positive control group (p<0.05).

Table 5: Anti-α-glucosidase activities of NPS and PS polysaccharides.

Name of samples	Concentration (mg/mL)	Inhibition of α-glucosidase (%)
Polysaccharides from NPS[a]	1.0	34.9
Polysaccharides from PS[b]	1.0	36.7
Acarbose[c]	1.0	98.2

[a]Non-puffed strips. [b]Puffed strips. [c]Positive control.

CONCLUSION

In this study, the expansion ratio of *D. officinale* strips was analyzed and optimized using RSM by changing microwave

puffing parameters. As a result, sample length, moisture content, microwave power, and processing time were optimally 3.5 cm, 23%, 706 W, and 35 s, respectively. Under the above conditions, the puffed *D. officinale* strips had an optimal expansion ratio of 371.7%. The extraction rate of polysaccharides from the PS was significantly higher than that from the NPS. In terms of lipid peroxidation inhibition and anti-α-glucosidase assays, polysaccharides from the NPS and the PS showed similar activities. However, the PS polysaccharides exhibited a greater effect in L6 cells assays than the NPS polysaccharides. The research has developed a basis for the utilization of *Dendrobium* polysaccharides, further suggesting that microwave puffing technology has a broad prospect to increase the extraction of active components from medicinal plants.

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Author's contributions

Yanhong Li: formal analysis, writing original draft, funding acquisition

Wang Zuoqiang: collection and analysis of experimental

Li Tingting: performing the experiments, analysis of the data

Bai Xishan: writing-review, formal analysis

Tian Kai: conceptualization, formal analysis

Huang Xiangzhong: design of the experiment, revision of the manuscript, funding acquisition

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