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Preparation and application of phosphorylated *Lotus* root polysaccharide

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Abstract

In this study, we extracted *lotus* root polysaccharide (LRP) and synthesised phosphorylated *lotus* root polysaccharide (PLP) using response surface methodology (RSM). RSM analyses revealed that the optimal conditions for PLP synthesis were a reaction duration of 7 h, temperature of 70 °C and pH of 11.38. Under these conditions, the predicted degree of substitution (DS%) was determined to be 9.96%. The structure of the LRP1 was examined by ultraviolet (UV) spectroscopy scan, Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) (^{1}H and ^{13}C). The monosaccharide composition of LRP1 was determined to be mannose (0.12%), ribose(0.18%), glucuronic acid(0.60%), galacturonic acid(0.09%), glucose(98.79%) and galactose(0.21%). The number average molecular weight (Mn) and the weight average molecular weight (Mw) of LRP1 were 10236 and 251783 g/mol. LRP1 exhibited high antioxidant activities in scavenging ABTS radicals, Superoxide anion radicals and Metal ion scavenging activity. PLP exhibited strong antioxidant activity *in vitro*. In addition, PLP inhibited Skov3 cancer cell proliferation and induced reactive oxygen species (ROS) production. Our data revealed that PLP is a promising natural antioxidant with potential value as a food supplement and for the treatment of cancer.

Keywords: *Lotus* root polysaccharide; phosphorylation; antioxidant; antitumor.

Practical Application: The work shows a complete study about the method of extraction of *lotus* root polysaccharide (LRP) and synthesised phosphorylated *lotus* root polysaccharide, and their contribution to show the potential value of natural antioxidant with potential value as a food supplement and for the treatment of cancer.

1 Introduction

Nelumbo nucifera Gaertn. is a perennial aquatic herb of the water lily family, which is mainly distributed throughout China, India and Japan, among other countries. *Lotus* root is commonly consumed as a vegetable with high nutritional value, and it is also widely used for its therapeutic effects. *Lotus* root contains high amounts of starch, protein, fat, carbohydrate, calcium, phosphorus, iron and other minerals. In Chinese traditional medicine, *lotus* root has some pharmacological effects, such as immune regulation, antioxidant, antidiabetic and antiobesity activities, as well as a protective effect against liver injury.

Sugars are biological macromolecules with many biological activities. Polysaccharides are sugar chains linked by many glycoside bonds. Polysaccharide side chains can have a variety of active groups, allowing a wide range of molecular modifications. Polysaccharides are important components of cells, and they are the main active component of *lotus* roots. They have been reported to present good antioxidant (Chen et al., 2015; Jeddou et al., 2016), antitumor (Wang et al., 2016), hypoglycaemic (Chen et al., 2016) and antiviral activities (Zheng et al., 2016), and also play a role in immune function regulation (Liu et al., 2016). The activity of polysaccharides is directly and indirectly affected by its molecular structure. Choosing a suitable polysaccharide biomolecule and modifying its structure can improve its physicochemical properties and biological activity to a certain extent. The most common molecular modifications of polysaccharides include carboxymethylation, selenium methylation, sulfation and alkylation (Wang et al., 2017b), among which phosphorylation modification has become an important polysaccharide modification method due to its simple synthesis method, safety and simple and controllable reaction conditions. Through molecular modification, the biological activity of polysaccharide can be improved to a certain extent, and new functions can be added. Natural lentinan can inhibit tumour growth, and lentinan sulphate shows high anti-HIV activity (Yamamoto et al., 1988). Furthermore, phosphorylated *Codonopsis pilosula* polysaccharide has anti-duck hepatitis a virus activity (Ming et al., 2020). Chen et al. (Chen & Huang, 2019) extracted *garlic* polysaccharide from *garlic* by hot water extraction and modified it by phosphorylation. The experimental results showed that both *garlic* polysaccharide and phosphorylated *garlic* polysaccharide showed superoxide anion and hydroxyl radical scavenging abilities, and the antioxidant activity of phosphorylated *garlic* polysaccharide was higher than that of *garlic* polysaccharide. The porphyrin extracted by Zhang et al. from the red alga of *Begonia* is a type of plant polysaccharide. After phosphorylation and modification, it was found to have enhanced scavenging ability of hydroxyl radicals (Zhang et al., 2009). With more and more studies focussing on the structure-activity relationship of polysaccharides, the chemical modification of polysaccharides has become increasingly important.

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In this study, *lotus* root polysaccharide was extracted by water extraction and alcohol precipitation, then neutral polysaccharide was separated and purified. A response surface optimisation design was used to establish the synthesis process of phosphorylated *lotus* root polysaccharide, and its antioxidant and antitumor activities were studied before and after modification. This study will effectively support the utilisation and development of *lotus* root and *lotus* polysaccharide.

2 Materials and methods

2.1 Materials

Lotus root powder was purchased from the Hubei Province of China. All other chemicals and solvents were of analytical grade.

2.2 Extraction of lotus root polysaccharide and preparation of phosphorylated lotus root polysaccharide

Lotus root powder (20 g) was mixed with 800 mL of 80% ethanol in a 1000-mL flask. After mixing evenly, the flask was placed in a water bath and allowed to have condensation and reflux at 55°C for 2 h to remove lipids. The mixture was centrifuged to separate the supernatant, which was removed, then 800 mL of purified water was added and further to have condensation and reflux at 80 °C for 2 h. The supernatant was collected and the Sevage method was used to remove the proteins, then alcohol was added to precipitate polysaccharides to obtain crude polysaccharides. DEAE-52 and Sephadex G-25 columns were used for further purification.

Firstly, the *lotus* root polysaccharide (LRP1) alkali solution was prepared. LRP1 (0.2 g) was mixed with 100 mL of sodium hydroxide solution (1 mol/L) in a flask, then placed in a water bath at 50 °C for 20 min to obtain the LRP1 alkali solution. 3 g sodium tripolyphosphate and 0.3 g sodium trimetaphosphate dissolved in 100 mL of purified water and mixed with the LRP1 alkali solution, the pH was adjusted to 7 with hydrochloric acid, which was allowed to react for 3 h at 50°C in a water bath. After the reaction was finished, phosphorylated *lotus* root polysaccharide (PLP) was obtained from the reaction solution by dialysis and alcohol precipitation. The degree of substitution (DS%) of PLP was determined following the published method (Chen & Huang, 2019).

2.3 Optimisation of phosphorylated lotus root synthesis and experimental design

DS% was the detection index. The extraction time, temperature and pH were selected as the three variables for optimisation of PLP synthesis. Each variable was tested individually. The extraction time was tested in the range of 3 to 7 h, temperature was tested in the range of 50 °C to 90 °C, and the pH was tested in the range from 7 to11. Each experiment was repeated three times, and the results were showed as the average of three independent trials.

Response surface methodology (RSM) was used to investigate the effects of the three variables. The levels and codes of DS% used in the Box-Behnken design (BBD) are shown in Table 1. The BBD and the results for PLP synthesis are shown in Table 2.

Table 1. Levels and code of extraction variables used in Box-Behnken design.

Variables	Symbols	Coded levels		
	Coded	-1	0	1
Reaction time (h)	X_1	5	6	7
Reaction temperature (°C)	X_2	70	80	90
pH	X_3	10	11	12

Table 2. Experimental Design and Results of PLP Box-Behnken.

Serial numbers	Level			
	X_1	X_2	X_3	DS (%)
1	1	-1	0	9.68562
2	0	0	0	7.73625
3	0	0	0	8.17375
4	0	0	0	8.17375
5	0	0	0	8.17375
6	0	1	1	6.34875
7	-1	0	-1	6.62500
8	-1	-1	0	7.33125
9	-1	1	0	6.95000
10	1	0	1	8.44375
11	0	1	-1	6.69375
12	1	0	-1	7.41875
13	1	1	0	7.40125
14	0	0	0	8.36125
15	0	-1	1	8.34375
16	-1	0	1	5.95625
17	0	-1	-1	8.2125

2.4 Characterisation of lotus root polysaccharide and phosphorylated lotus root

(1) Ultraviolet spectrum analysis

10 mg/mL aqueous polysaccharide solutions of LRP1 and PLP were prepared. Ultraviolet spectrum scan was carried out in the ultraviolet wavelength range of 190–600 nm.

(2) Fourier transform infrared spectroscopy analysis

20 mg dried LRP1 and PLP was fully mixed with 200 mg of KBr and uniformly ground, then the correct amount of fine powder was placed into a circular mould and pressed into a transparent circular sheet. The tableted powder was analysed by Fourier transform infrared spectroscopy (FTIR; Spectrum400; PerkinElmer, USA) within the wave number range of 4000 to 500 cm^{-1}.

(3) NMR analysis

About 40 mg LRP1 was dissolved in 0.5 mL D-DMSO. The ^{13}C NMR and ^{1}H NMR spectra were recorded at 25°C with a Bruker Advance III HD 500 MHz spectrometer (USA).

(4) Composition analysis and molecular weight determination of monosaccharide

Monosaccharide composition of purified LRP1 was analysed using a Promosil C18 column (250 mm × 4.6 mm × 5 μm). Monosaccharides were identified based on comparing their mass spectra with those in the Wiley mass spectral library, and the relative abundance of each monosaccharide was calculated using the peak area normalisation method (Yuan et al., 2020). The LRP1 molecular was measured with HPSEC-MALLS-RID on the basis of the dn/dc method according to previous reported methods (Yao et al., 2020).

2.5 Antioxidant activity of lotus root polysaccharide 1 and phosphorylated lotus root

The ABTS radical, superoxide anion and metal ion scavenging activity were investigated as described by Yao et al. (2020). The appropriate solutions of LRP1 and PLP were prepared at concentrations of 1, 2, 4, 8 and 10 mg/mL for analysis of antioxidant activities.

2.6 MTT assay and reactive oxygen species assay

The cytotoxicity of LRP1 and PLP were calculated based a method reported previously (Yao et al., 2020). Human ovarian carcinoma cells (Skov3) were cultured in DME/F12 medium (Hyclone, Logan, UT, USA) supplemented with foetal bovine serum (10%) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C and 5% CO_2 in a humidified incubator. Cells were treated with different concentrations of LRP1 and PLP (0, 100, 200, 300, 400 and 500 μg/mL) and incubated for 24 h. An MTT assay was used to evaluate cell proliferation and the cytotoxicity of LRP1 and PLP. The experiment was repeated three times.

Skov3 cells were inoculated on a 24-well cell culture plate and cultured for 24 h, then treated with 0, 100, 200, 300, 400 and 500 μg/mL LRP or PLP for 24 h. An ROS assay kit was used to detect ROS production with a fluorescent enzyme labelling instrument following the manufacturer's instructions.

2.7 Statistical analyses

Data from triplicate assays were subjected to ANOVA to identify significant changes in the response to treatments. Differences were considered obviously at $P < 0.05$ and highly significant at $P < 0.01$. Data are presented as the mean ± SEM unless otherwise stated.

3 Results and discussion

3.1 Separation and purification of lotus root polysaccharide 1

As shown in Figure 1A and B, crude LRP was purified with DEAE-52, revealing five peaks. The major peak (neutral polysaccharide) corresponded to LRP1 (Figure 1A). Then, LRP1 was further purified using SephadexG-100 column chromatography and eluted using deionised water. LRP1 was unimodal and peaks were symmetrical, indicating that the sample was relatively uniform (Figure 1B).

The most complete structural information for carbohydrates can be obtained with or without background knowledge of the structure by using NMR technology. This overcomes the limitation of using a chemical method to determine the structure of a polysaccharide and provides a favourable tool for complex structure analysis. Through comprehensive consideration of one-dimensional and two-dimensional maps and mutual verification, more accurate polysaccharide structure information can be obtained (Barb et al., 2011; Chen et al., 2013). Polysaccharides are a repeated structural unit composed of monosaccharides in a certain order, and they can be composed of one or more types of monosaccharide, so it is very important to determine the residual number of polysaccharides. In general, the residual number of sugars is determined by heterocarbon and heterohydrogen of polysaccharide. The monosaccharides that making up polysaccharides can generally be divided into a furan configuration or pyranose configuration (Hou et al., 2017; Yan et al., 2014). The 1H NMR spectra of LRP1 showed that the heterocephalic hydrogen signals of *lotus* polysaccharides are δ 5.43, 5.42, 5.33, 5.04 and 4.51, with the chemical shifts at δ 5.43, 5.42, 5.33 and 5.04 representing hydrogen on α-heterocephalic carbon, and the chemical shift at δ 4.51 is the hydrogen on β-heterocephalic carbon. Therefore, there are two kinds of glycoside structures in LRP1 (Figure 1C). The results showed that the LRP1 contained both five-carbon and six-carbon, and LRP1 was α and β heterocephalic polysaccharides.

The molecular weight of LRP1 was determined by HPSEC-MALLS-RID, which has advantages of simple operation, high sensitivity and high precision. As shown in Figure 1E, the number average molecular weight (Mn) and the weight average molecular weight (Mw) were 10236 and 251783 g/mol.

Monosaccharides are the basis of polysaccharide chemical structure, which contribute to the biological activity of polysaccharides. The monosaccharide composition of LRP1 (Figure 1F) implied the dominance of mannose (0.12%), ribose (0.18%), glucuronic acid (0.60%), galacturonic acid (0.09%), glucose (98.79%) and galactose (0.21%).

3.2 Response surface analysis of phosphorylated lotus root

The results of 17 trial points tested in a random order based on the BBD design, including design and experimental values, are presented in Table 2. The predicted response (Y) for the CPP of PLP can be fitted into the following second-order polynomial Equation 1:

$$Y = -93.86836 + 3.31523\,X_1 + 0.33188\,X_2 + 14.45016\,X_3 - 0.047453\,X_1X_2 + 0.42344\,X_1X_3 - 0.011906\,X_2X_3 - 0.28461\,X_1^2 + 4.14063E-005\,X_2^2 - 0.72820\,X_3^2 \quad (1)$$

Experimental results were further analysed using Design-Expert 8.0.6 software. The results are shown in Table 3. The results indicated that X_1, X_2, X_1X_2, X_1X_3 and X_3^2 significantly impacted the response value ($P < 0.01$). The judgment coefficient ($R^2 = 0.9736$) showed that the model was reliable and that experimental factors influenced the response value. The calibration coefficient of R^2_{Adj} = 0.9396 indicated that 93.96% of experimental data variability could be explained by this regression model. The *F* value of the model was 28.96, which indicated that the model reached a

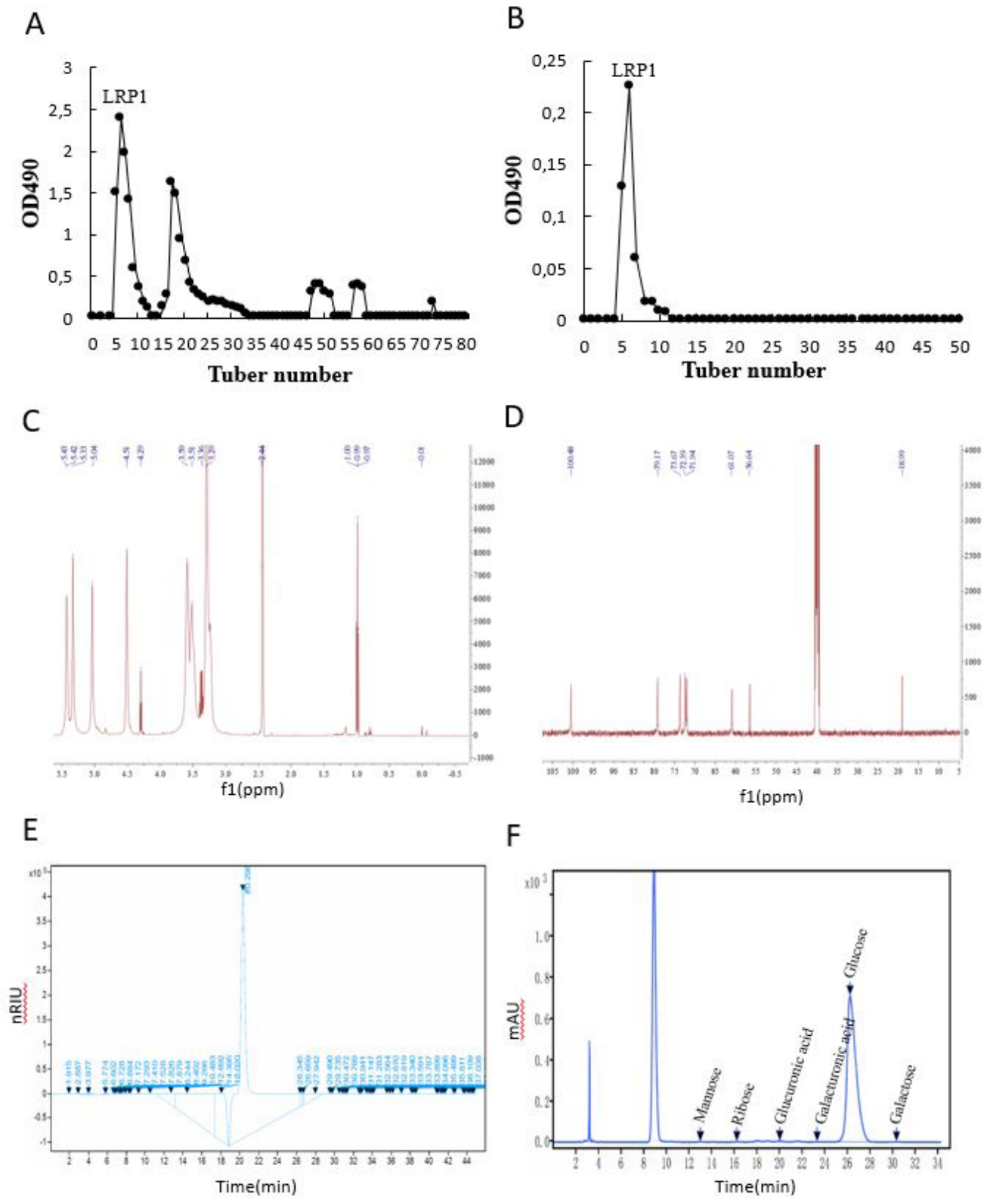

Figure 1. Analysis of lotus root polysaccharide (LRP) and LRP1. (**A**) Elution curve of LRP using the DEAE-52 ion-exchange column. The eluent was H_2O and NaCl (0.1, 0.2, 0.3 and 0.5 mol/L) with a flow rate of 0.5 mL/min (10 min/tube); (**B**) Elution curves of LRP1 using the Sephadex column. The eluent was H_2O with a flow rate of 0.4 mL/min (20 min/tube); (**C**) ^{1}H NMR spectrum of LRP1 in D-DMSO; (**D**) ^{13}C NMR spectrum of LRP1 in D-DMSO; (**E**) Molecular weight determination of LRP1 with HPSEC-MALLS-RID system; (**F**) The monosaccharide composition of LRP1.

Table 3. Variance Analysis Table.

Varibles	Sum of squares	df	Mean square	*F*-alue	*p*-Value
model	13.77	9	1.53	28.68	0.0001***
X_1	4.64	1	4.64	86.98	<0.0001***
X_2	4.77	1	4.77	89.35	<0.0001***
X_3	2.538E-003	1	2.538E-003	0.048	0.8335
$X_1 X_2$	0.90	1	0.90	16.89	0.0045**
$X_1 X_3$	0.72	1	0.72	13.45	0.0080**
X_2X_3	0.057	1	0.057	1.06	0.3368
X_1^2	0.34	1	0.34	6.39	0.0393*
X_2^2	7.219E-005			1.354E-003	0.9717
X_3^2	2.23			41.86	0.0003***
Residual	0.37				
Lack of fit	0.16			0.99	0.4817
Pure error	0.21				
Correlation total	14.14	R^2=0.9736	R^2_{Adj}=0.9396		

p:Significance test; F: F test; df: Degree of Freedom; * p<0.05; ** p<0.01; *** p<0.001.

significance level of $P < 0.0001$. In addition, the lack of fit was determined to be 0.99 ($P = 0.4817 > 0.05$), which showed that there was no significant relationship between the missing value and pure error.

As shown in Figure 2, the two-factor effect of the model was carried out. As shown in Figure 2A and a, the interaction between reaction time and reaction temperature was significant and the influence of reaction time and reaction temperature on the DS(%) was significant. According to Figure 2B and b, the interaction between reaction time and reaction pH was significant, the slope of the three-dimensional response surface of reaction time was steep, and the influence on the DS(%) was more significant. The interaction between reaction temperature and reaction pH was significant, and the graph slope of reaction temperature was significantly larger than that of reaction pH, indicating that the effect of reaction temperature on the DS(%) was more significant than that of reaction pH (Figure 2C and c).

Analysis from Design Expert 8.0.6 revealed that the optimal conditions for PLP synthesis were a reaction duration of 7 h, temperature of 70 °C and pH, 11.38. Under these conditions, the predicted DS% was determined to be 9.96%. In order to verify the feasibility of response surface results, the conditions were optimised and validated. The DS% of five repeated experiments were 9.76%, 9.68%, 9.50%, 9.61% and 9.77%, with an average value of 9.67% and standard deviation of 2.98%. The conclusions of verification and the error of response surface model prediction met the requirements, indicating that the prediction equation fit well with the actual situation and verified the correctness of the response surface model.

3.3 Characterisation and analysis of lotus root polysaccharide and phosphorylated lotus root

The UV absorption spectra of LRP1 and PLP from 190 to 600 nm wavelength are shown in Figure 3A. The UV scanning curves of LRP1 and PLP have a similar shape in the range of 200–300 nm, while no UV absorption peak was observed at 280 nm for LRP1 and PLP, indicating that the two deproteinization processes were successful and that there were almost no foreign protein residues in LRP1 and PLP.

The FTIR spectra of LPR1 and PLP are shown in Figure 3B. LRP1 and PLP presented many characteristic absorption peaks of polysaccharide chemical groups in the infrared spectra. The infrared absorption peak with the wave number of 3400 cm^{-1} was the O-H stretching vibration, the absorption peak at 1100 cm^{-1} was the O-H deformation vibration, and the absorption peak at 710 cm^{-1} was the O-H out-of-plane bending vibration. Combinedly, this was determined to be the alcohol hydroxyl characteristic absorption peak of polysaccharide molecules. The double absorption peaks with wave number 2900 cm^{-1} are the stretching vibrations of methyl or methylene. There was no double absorption peak at 2820 and 2720 cm^{-1} in LRP, which indicates that there is no aldehyde group in the molecular structure of LRP. A weak absorption peak at 1250 cm^{-1} was the P=O stretching vibration, while infrared absorption peaks at 1030 and 995 cm^{-1} of PLP may be caused by the P-O-C stretching vibration (Deng et al., 2020; Udchumpisai & Bangyeekhun, 2019).

3.4 Antioxidant activities of lotus root polysaccharide 1 and phosphorylated lotus root

As one of the main components of traditional food and traditional Chinese medicine, polysaccharides have attracted a great deal of attention due to their important biological activities and functions, one of which is their antioxidant effect. Many studies have found that polysaccharides have a significant scavenging effect on DPPH, ABTS, superoxide, metal ions and hydroxyl radicals (Wang et al., 2017a; Yao et al., 2020; Zhong et al., 2019). Also, polysaccharide derivatives have antioxidant activity, and phosphorylated polysaccharides have stronger antioxidant

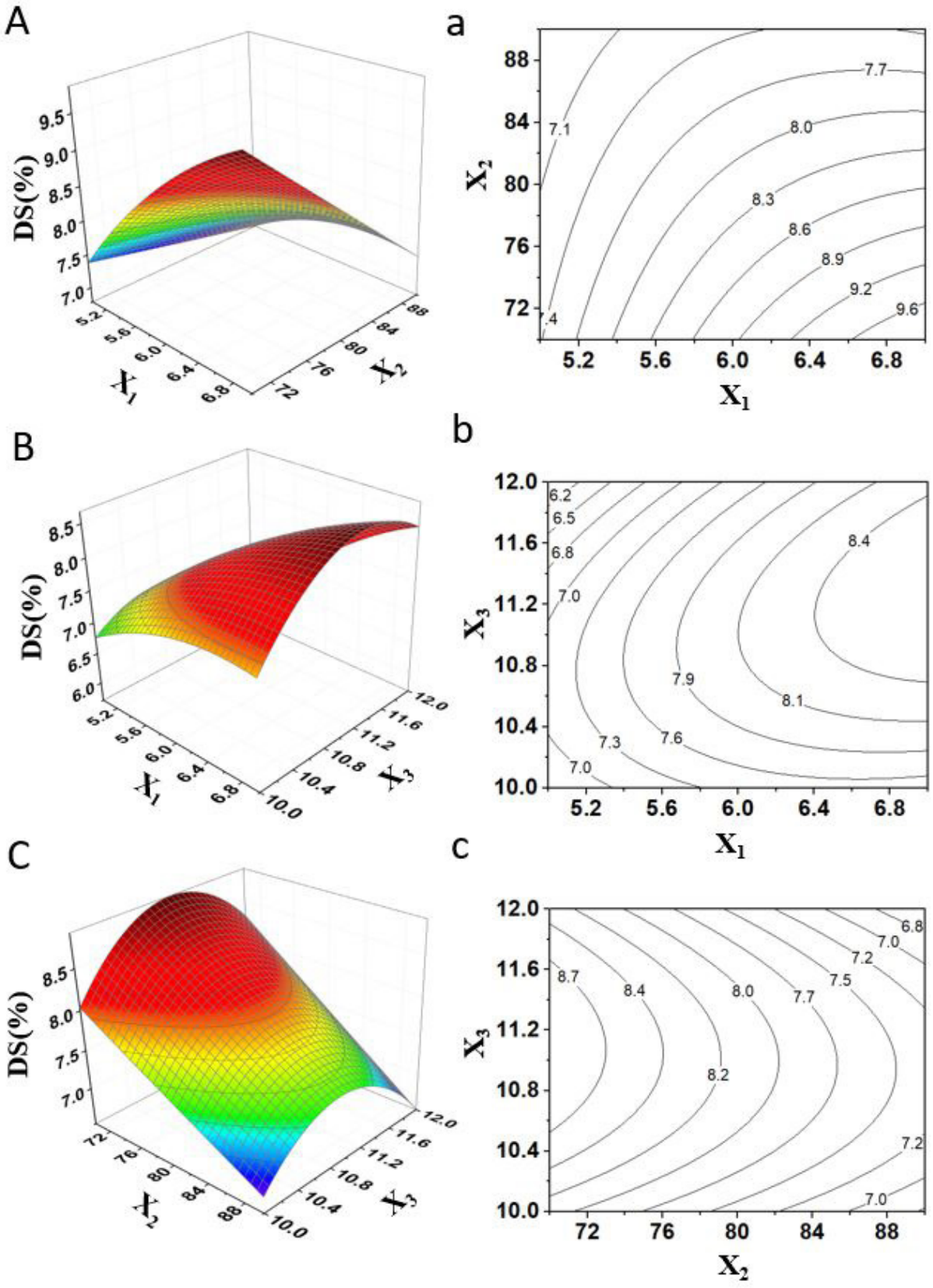

Figure 2. Response surface plots showing the effect of DS% on phosphorylated lotus root (PLP). **(A, a)** Response surface map and contour map of time (X_1) and temperature (X_2). (**B, b**) Response surface map and contour map of time (X_1) and pH (X_3). (**C, c**) Response surface map and contour map of temperature (X_2) and pH (X_3).

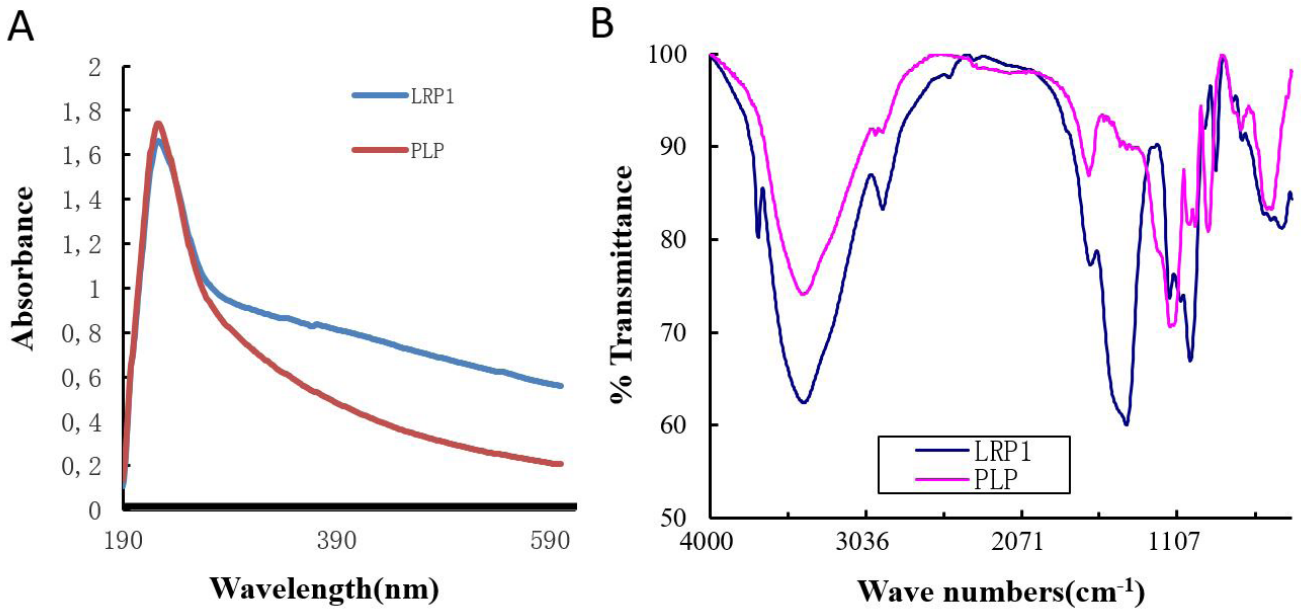

Figure 3. Ultraviolet spectroscopy and Fourier transform infrared spectroscopy (FTIR) spectra of lotus root polysaccharide (LRP) 1 and phosphorylated lotus root (PLP). (**A**) Ultraviolet spectra of LRP1 and PLP; (**B**) FTIR spectra of LRP1 and PLP.

activity (Qi et al., 2005; Shao et al., 2013; Suzuki & Terasawa, 2020; Wei et al., 2012).

As shown in Figure 4, LRP1 and PLP both presented ABTS radical, hydroxyl radical, superoxide radical ion and metal ion radical scavenging activities. After the phosphorylation modification, the antioxidant capacity of PLP improved significantly. The ABTS scavenging rates of LRP1 and PLP in aqueous solutions both show an upward trend with polysaccharide aqueous solutions of increasing concentration (Figure 4A). The ABTS radical scavenging ability of PLP was stronger than LRP1. The results of scavenging superoxide ions are shown in Figure 4B. LRP1 had the highest scavenging rate for superoxide ions at the concentration of 2 mg/mL, while the scavenging rate for superoxide ions decreased from 2 to 10 mg/mL. However, the superoxide radical ion clearance rate of PLP increased as the concentration increased. Aside from 2 mg/mL, the superoxide radical ion clearance rate of PLP was higher than that for LRP1. Detecting the scavenging effect of samples on ferrous ions is a common indicator for detecting the antioxidant activity of chemical substances in laboratories, which is characterised by a fast and simple experimental operation. The experiment not only verified the activity of natural *lotus* root polysaccharide in removing metal ions, but also confirmed the effect of LRP1 on removing ferrous ions after acidification modification by measuring and comparing the clearance rates of different concentrations of LRP1 and PLP to metal ions. The experimental results are shown in Figure 4C. PLP was found to have a significant scavenging effect on metal ions, and the scavenging rate of PLP on metal ions was positively correlated with the concentration of PLP. When the concentration of PLP was 10 mg/mL, the scavenging rate of ferrous ions reached 71.9%.

2.5 Antitumor activities of lotus root polysaccharide 1 and phosphorylated lotus root

Polysaccharides can promote the immune function. At the same time, it promotes the release of some cytokines in the body, which can inhibit tumour cells. Polysaccharides have been demonstrated to exhibit good anticancer activity across a range of cancer cell lines, exhibit selective cytotoxicity toward tumour cells, and damage cancer cells without producing the commonly associated adverse effects. Polysaccharides can inhibit or kill cancer cells indirectly by improving the host's immune function, or they can play a direct antitumour role by inducing tumour cell differentiation or apoptosis, affecting the expression of oncogenes and so on. Lentinan is a typical T cell activator which has been found to enhance the immune function of the body by activating the activity of immune cells such as T cells, macrophages and natural killer cells, as well as to enhance the phagocytic ability of immune cells (Matsuo et al., 1982). Polysaccharides can activate macrophages, enhance the phagocytic activity of macrophages, promote the synthesis and release of monocytes, activate lymphocytes and activate the immune response to tumour cells. *Lycium barbarum* polysaccharide can trigger IL-1 and IL-2 secretion by lymphocytes and TNF release from macrophages (Hu et al., 2016). LMPAB was found to decrease lung metastatic foci in mouse B16 melanoma and inhibit the growth of double-grafted SW180 tumour cells (Niu et al., 2009). The mechanistic classification of anticancer polysaccharides includes cell cycle arrest, depolarization of the mitochondrial membrane, nitric oxide pathway and immunomodulation (Khan et al., 2019). Polysaccharide derivatives have antitumor activity. Phosphorylated *Achyranthes bidentata* polysaccharide (P-ABPS) showed antisarcoma activity in a mouse model of Lewis lung cancer (Chen et al., 2002; Huang & Huang., 2017).

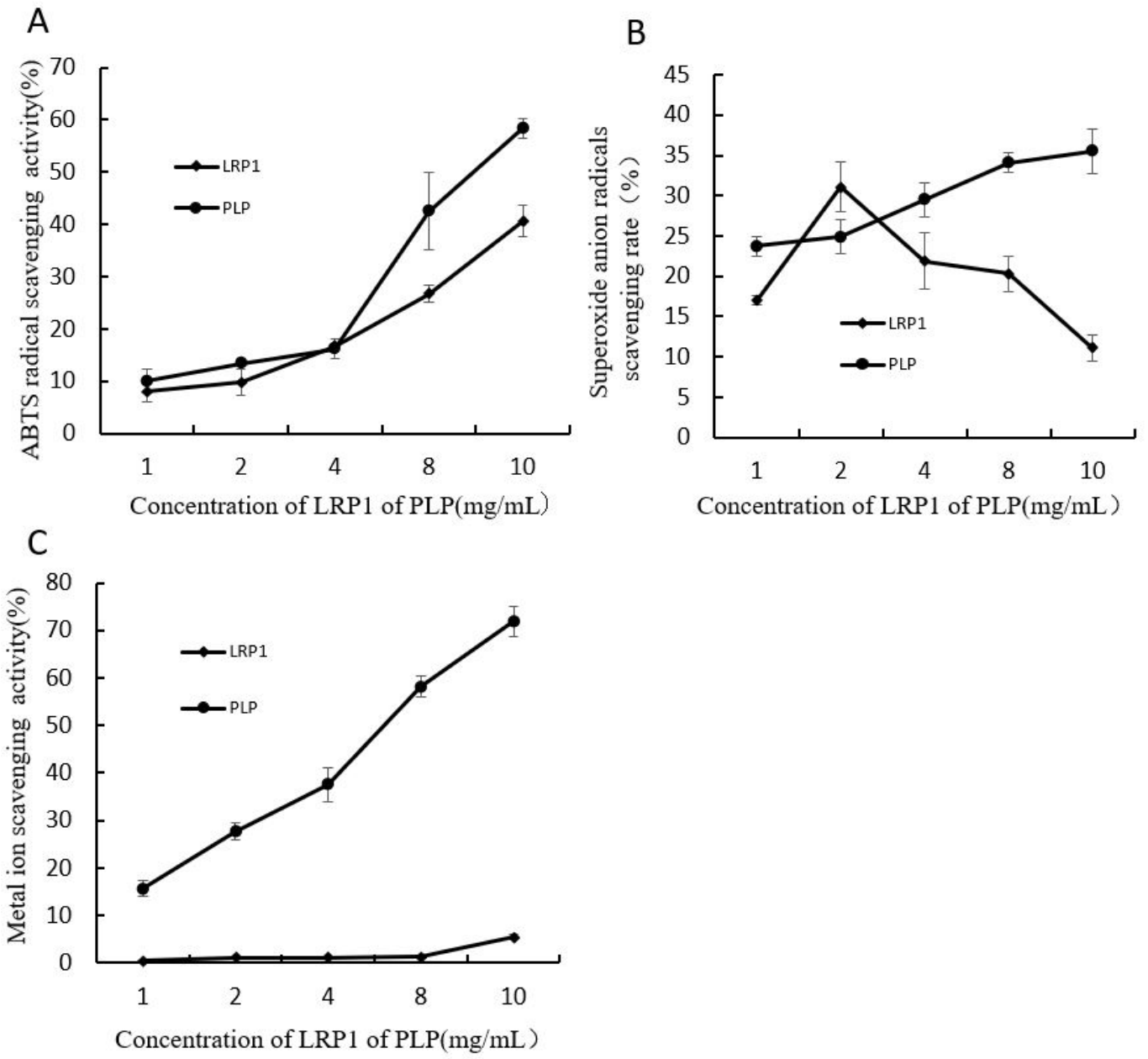

Figure 4. Antioxidant capacity of lotus root polysaccharide (LRP) 1 and phosphorylated lotus root (PLP). (**A**) Scavenging of ABTS radicals by LRP1 and PLP; (**B**) Scavenging of superoxide anions by LRP1 and PLP; (**C**) Metal chelating activity of LRP1 and PLP.

The exopolysaccharide isolated from *Rhizopus nigricans* has been described to have antitumor and proapoptotic activities against colorectal cancer (Lu et al., 2020).

LRP1 and PLP have an inhibitory effect on Skov3 cells, and this inhibitory ability increases with increasing polysaccharide concentrations (Figure 5A). However, LRP1 had stronger inhibitory ability on Skov3 cells than that of PLP at a low concentration. When the concentrations of LRP1 and PLP were 500 μg/mL, the survival rates of Skov3 tumour cells were 87.04% and 87.28%, respectively. The effect of LRP1 and PLP on ROS content in Skov3 cells is shown in Figure 5B. With increasing polysaccharide concentrations, the ROS content in Skov3 cells firstly increases and then decreases. The ROS content in Skov3 cells was highest when treated with LRP1 and PLP at 400 μg/mL. The overall ability of PLP to induce ROS production was higher than that of LRP, especially at the concentration of 400 μg/mL.

3 Discussion

In this experiment, LRP was extracted and purified, and PLP was modified. This single-factor experiment revealed that the optimal PLP synthesis conditions established using RSM were a temperature of 70°C, duration of 7 h and pH of 11.3. Under

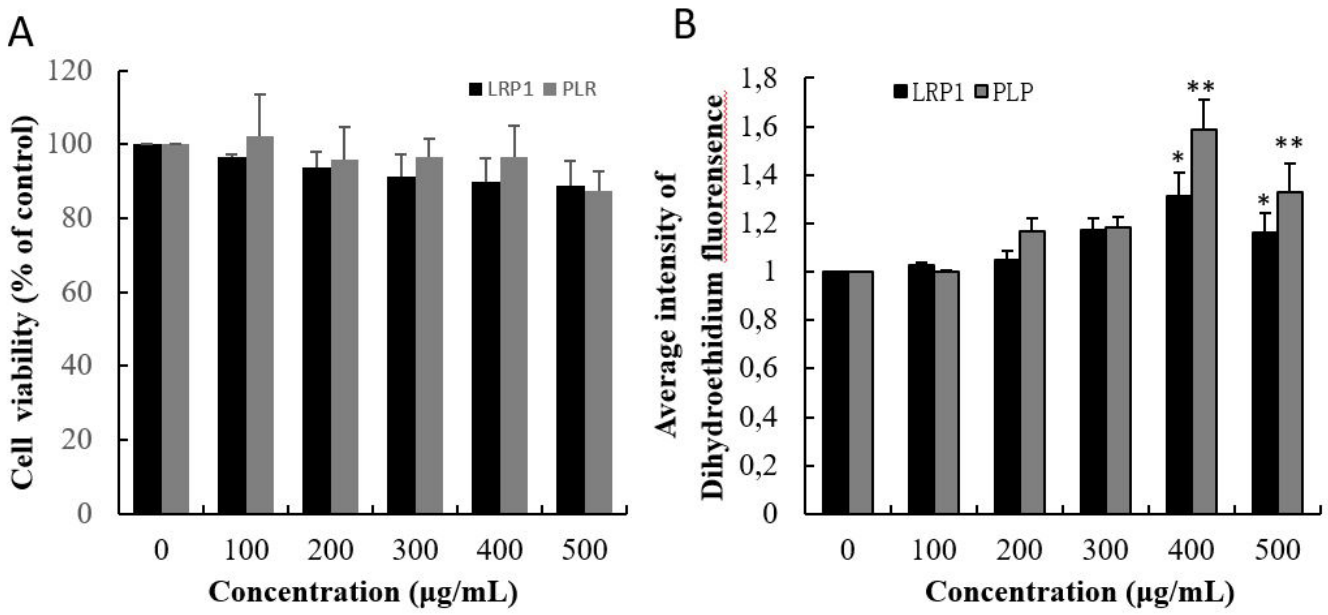

Figure 5. Effect of lotus root polysaccharide (LRP) 1 and phosphorylated lotus root (PLP) on cellular viability and reactive oxygen species (ROS) level in Skov3 cells. (**A**) Skov3 cells were incubated with different LRP1 and PLP concentrations for 24 h and cell viability was determined using a cell counting kit-8 (CCK-8) assay. Viability and morphological features of Skov3 cells are shown; (**B**) Average intensity of fluorescence in Skov3 cells. Data are expressed as the mean ± SD of three independent experiments; *P < 0.05, **P < 0.01.

these conditions, the DS% was 9.96% and polysaccharide purity reached 90%. The antioxidant activity of PLP was higher than that of LRP, PLP had an inhibitory effect on the proliferation of Skov3 cells, and it induced greater ROS production in Skov3 cells than LRP1.

Conflict of interest

The authors have declared that no competing interest exists.

Availability of data and material

The data used to support the findings of this study are included within the article.

Author contributions

Dr. Zhang XF designed the study. Yan YY, Yuan S, Zhao S, Xu CY and Zhang XF collected data. All authors agreed the final version.

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References

Barb, A. W., Freedberg, D. I., Battistel, M. D., & Prestegard, J. H. (2011). NMR detection and characterization of sialylated glycoproteins and cell surface polysaccharides. *Journal of Biomolecular NMR*, 51(1-2), 163-171. http://dx.doi.org/10.1007/s10858-011-9550-0. PMid:21947924.

Chen, J., & Huang, G. (2019). Antioxidant activities of garlic polysaccharide and its phosphorylated derivative. *International Journal of Biological Macromolecules*, 125, 432-435. http://dx.doi.org/10.1016/j.ijbiomac.2018.12.073. PMid:30537497.

Chen, P. X., Wang, S. N., Nie, S. P., & Marcone, M. (2013). Properties of Cordyceps Sinensis: a review. *Journal of Functional Foods*, 5(2), 550-569. http://dx.doi.org/10.1016/j.jff.2013.01.034. PMid:32288794.

Chen, R. Z., Tan, L., Jin, C. G., Lu, J., Tian, L., Chang, Q. Q., & Wang, K. (2015). Extraction,isolation,characterization and antioxidant activity of polysaccharides from Astragalus membranaceus. *Industrial Crops and Products*, 77, 434-443. http://dx.doi.org/10.1016/j.indcrop.2015.08.006.

Chen, T. L., Zhang, M., Li, J. L., Surhio, M. M., Li, B. B., & Ye, M. (2016). Structural characterization and hypoglycemic activity of Trichosanthes peel polysaccharide. *Lebensmittel-Wissenschaft + Technologie*, 70, 55-62. http://dx.doi.org/10.1016/j.lwt.2016.02.024.

Chen, X.-M., Zhang, J., & Han, G.-Y. (2002). Studies on synthesis and antitumor activity of phosphorylated achyranthes bidentate polysaccharide(P-ABPS). *Chinese Journal of Chemistry*, 20(11), 1406-1410. http://dx.doi.org/10.1002/cjoc.20020201142.

Deng, Q., Wang, X., Chen, H., Zhao, C., Gong, X., & Zhou, X. (2020). Structural characterization, modification and hepatoprotective effects of polysaccharide from Mori Fructus. *International Journal of Biological Macromolecules*, 153, 357-363. http://dx.doi.org/10.1016/j.ijbiomac.2020.02.300. PMid:32112846.

Hou, Y. L., Ding, X., Hou, W. R., Song, B., & Yan, X. H. (2017). Structure elucidation and antitumor activity of a new polysaccharide from Maerkang Tricholoma matsutake. *International Journal of Biological Sciences*, 13(7), 935-948. http://dx.doi.org/10.7150/ijbs.18953. PMid:28808425.

Hu, B., An, H. M., Wang, S. S., Chen, J. J., & Xu, L. (2016). Preventive and therapeutic effects of chinese herbal compounds against hepatocellular Carcinoma. *Molecules (Basel, Switzerland)*, 21(2), 142. http://dx.doi.org/10.3390/molecules21020142. PMid:26828466.

Huang, G., & Huang, H. (2017). The derivatization and antitumor mechanisms of polysaccharides. *Future Medicinal Chemistry*, 9(16), 1931-1938. http://dx.doi.org/10.4155/fmc-2017-0132. PMid:29076350.

Jeddou, K. B., Chaari, F., Maktouf, S., Nouri-Ellouz, O., Helbert, C. B., & Ghorbel, R. E. (2016). Structural, functional, and antioxidant properties of water-soluble polysaccharides from potatoes peels. *Food Chemistry*, 205, 97-105. http://dx.doi.org/10.1016/j.foodchem.2016.02.108. PMid:27006219.

Khan, T., Date, A., Chawda, H., & Patel, K. (2019). Polysaccharides as potential anticancer agents—A review of their progress. *Carbohydrate Polymers*, 210, 412-428. http://dx.doi.org/10.1016/j.carbpol.2019.01.064. PMid:30732778.

Liu, J. Y., Feng, C. P., Li, X., Chang, M. C., Meng, J. L., & Xu, L. J. (2016). Immunomodulatory and antioxidative activity of Cordyceps militaris polysaccharides in mice. *International Journal of Biological Macromolecules*, 86, 594-598. http://dx.doi.org/10.1016/j.ijbiomac.2016.02.009. PMid:26853825.

Lu, Y., Zhang, X. J., Wang, J. Y., & Chen, K. S. (2020). Exopolysaccharides isolated from Rhizopus nigricans induced colon cancer cell apoptosis in vitro and in vivo via activating the AMPK pathway. *Bioscience Reports*, 40(1), BSR20192774. http://dx.doi.org/10.1042/BSR20192774. PMid:31894839.

Matsuo, T., Kurahashi, Y., Nishida, S., Onodera, C., Izawa, M., & Hamuro, J. (1982). Effect of lentinan, a specific T-cell adjuvant, on murine granulopoiesis and its roles on antitumor effect. *International Journal of Immunopharmacology*, 4(4), 269. http://dx.doi.org/10.1016/0192-0561(82)90133-3.

Ming, K., He, M., Su, L. L., Du, H. X., Wang, D. Y., Wu, Y., & Liu, J. G. (2020). The inhibitory effect of phosphorylated Codonopsis pilosula polysaccharide on autophagosomes formation contributes to the inhibition of duck hepatitis A virus replication. *Poultry Science*, 99(4), 2146-2156. http://dx.doi.org/10.1016/j.psj.2019.11.060. PMid:32241500.

Niu, Y. C., Liu, J. C., Zhao, X. M., & Cao, J. (2009). A low molecular weight polysaccharide isolated from Agaricus blazei Murill (LMPAB) exhibits its antimetastatic effect by down-regulating metalloproteinase-9 and up-regulating Nm23-H1. *The American Journal of Chinese Medicine*, 37(5), 909-921. http://dx.doi.org/10.1142/S0192415X09007351. PMid:19885951.

Qi, H. M., Zhao, T. T., Zhang, Q. B., Li, Z. E., Zhao, Z. Q., & Xing, R. G. (2005). Antioxidant activity of different molecular weight sulfated polysaccharides from Ulva pertusa Kjellm (Chlorophyta). *Journal of Applied Phycology*, 17(6), 527-534. http://dx.doi.org/10.1007/s10811-005-9003-9.

Shao, P., Chen, X. X., & Sun, P. L. (2013). In vitro antioxidant and antitumor activities of different sulfated polysaccharides isolated from three algae. *International Journal of Biological Macromolecules*, 62, 155-161. http://dx.doi.org/10.1016/j.ijbiomac.2013.08.023. PMid:23994786.

Suzuki, K., & Terasawa, M. (2020). Biological activities of rhamnan sulfate extract from the Green Algae Monostroma nitidum (Hitoegusa). *Marine Drugs*, 18(4), 228. http://dx.doi.org/10.3390/md18040228. PMid:32344720.

Udchumpisai, W., & Bangyeekhun, E. (2019). Purification, structural characterization, and biological activity of polysaccharides from Lentinus velutinus. *Mycobiology*, 48(1), 51-57. http://dx.doi.org/10.1080/12298093.2019.1693482. PMid:32158606.

Wang, L., Li, Y. L., Zhu, L. D., Yin, R., Wang, R., Luo, X. H., Li, Y. F., Li, Y. N., & Chen, Z. X. (2016). Antitumor activities and immunomodulatory of rice bran polysaccharides and its sulfates in vitro. *International Journal of Biological Macromolecules*, 88, 424-432. http://dx.doi.org/10.1016/j.ijbiomac.2016.04.016. PMid:27064087.

Wang, M., Zhu, P., Zhao, S., Nie, C., Wang, N., Du, X., & Zhou, Y. (2017a). Characterization, antioxidant activity and immunomodulatory activity of polysaccharides from the swollen culms of Zizania latifolia. *International Journal of Biological Macromolecules*, 95, 809-817. http://dx.doi.org/10.1016/j.ijbiomac.2016.12.010. PMid:27939270.

Wang, Y., Su, N., Hou, G., Li, J., & Ye, M. (2017b). Hypoglycemic and hypolipidemic effects of a polysaccharide from *Lachnum* YM240 and its derivatives in mice, induced by a high fat diet and low dose STZ. *MedChemComm*, 8(5), 964-974. http://dx.doi.org/10.1039/C6MD00697C. PMid:30108811.

Wei, D., Cheng, W., Wei, Y., & Zhang, L. (2012). Phosphorylated modification and in vitro antioxidant activity of Radix Hedysari polysaccharide. *Glycoconjugate Journal*, 29(4), 167-172. http://dx.doi.org/10.1007/s10719-012-9377-2. PMid:22535466.

Yamamoto, N., Yoshida, O., Nakashima, H., Yoshida, T., Kaneko, Y., Yamamoto, L., Matsuzaki, K., & Uryu, T. (1988). Sulfation of the immunomodulating polysaccharide lentinan: a novel strategy for antivirals to human immunodeficiency virus (HIV). *International Journal of Immunopharmacology*, 10, 34. http://dx.doi.org/10.1016/0192-0561(88)90243-3.

Yan, J. K., Wang, W. Q., & Wu, J. Y. (2014). Recent advances in *Cordyceps sinensis* polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: a review. *Journal of Functional Foods*, 6, 33-47. http://dx.doi.org/10.1016/j.jff.2013.11.024. PMid:32362940.

Yao, Y. L., Shu, C., Feng, G., Wang, Q., Yan, Y. Y., Yi, Y., Wang, H. X., Zhang, X. F., & Wang, L. M. (2020). Polysaccharides from Pyracantha fortuneana and its biological activity. *International Journal of Biological Macromolecules*, 150, 1162-1174. http://dx.doi.org/10.1016/j.ijbiomac.2019.10.125. PMid:31794823.

Yuan, S., Xu, C. Y., Xia, J., Feng, Y. N., Zhang, X. F., & Yan, Y. Y. (2020). Extraction of polysaccharides from Codonopsis pilosula by fermentation with response surface methodology. *Food Science & Nutrition*, 8(12), 6660-6669. http://dx.doi.org/10.1002/fsn3.1958. PMid:33312549.

Zhang, Z. S., Zhang, Q. B., Wang, J., Shi, X. L., Song, H. F., & Zhang, J. J. (2009). In vitro antioxidant activities of acetylated, phosphorylated and benzoylated derivatives of porphyran extracted from Porphyra haitanensis. *Carbohydrate Polymers*, 78(3), 449-453. http://dx.doi.org/10.1016/j.carbpol.2009.04.026.

Zheng, Y., Zhang, S., Wang, Q., Lu, X., Lin, L., Tian, Y., Xiao, J., & Zheng, B. (2016). Characterization and hypoglycemic activity of a β-pyran polysaccharides from bamboo shoot (Leleba oldhami Nakal) shells. *Carbohydrate Polymers*, 144, 438-446. http://dx.doi.org/10.1016/j.carbpol.2016.02.073. PMid:27083836.

Zhong, Q., Wei, B., Wang, S., Ke, S., Chen, J., Zhang, H., & Wang, H. (2019). The antioxidant activity of polysaccharides derived from marine organisms: an overview. *Marine Drugs*, 17(12), 674. http://dx.doi.org/10.3390/md17120674. PMid:31795427.