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Extraction optimization, characterization and bioactivity of crude polysaccharides from *Herba Moslae*

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ABSTRACT

Response surface methodology (RSM), based on a Box–Behnken design (BBD), was used to optimize the extraction conditions of crude polysaccharides from *Herba Moslae*. Three independent variables, extraction temperature, extraction time and water to solid ratio were investigated. Based on the RSM analysis, the optimal extraction conditions were determined to be at a temperature 86.9 °C, time 4.1 h and water to solid ratio 17.7:1 (mL/g). The crude polysaccharides produced under these optimal conditions contained 37.84% neutral carbohydrates, 6.14% proteins, 5.18% uronic acids, 8.1% moisture and 4.6% ash. The result of monosaccharide composition by gas chromatography (GC) showed that the crude polysaccharides consisted of eight kinds of monosaccharides: rhamnose, ribose, fucose, arabinose, xylose, mannose, glucose and galactose, with the molecular ratio of 3.72:2.45:0.92:6.00:2.76:5.09:13.53:9.58. Results of pinocytic activity and nitric oxide assays of mouse peritoneal macrophage suggested that the crude polysaccharides had potential immunomodulatory properties.

1. Introduction

Herba Moslae, also known as *E. ciliata (Thunb) Hyland*, is a famous traditional medicinal herb in China with an early historical record in "MING YI BIE LU". *Herba Moslae* belongs to *Labiatae* family, mainly distributed in the southern parts of China, as well as Vietnam, India and Japan. It grows vigorously in summer and autumn, and is usually used for both food and medicine. Its fruit is warm in nature and acrid in taste. It was reported to contain abundant essential oil and flavonoids (Liu, Lee, Wang, & Du, 2007). The whole grass of *Herba Moslae* is commonly used for the treatment of many diseases, such as cold, fever, diarrhea, dysentery, digestion disorder, vomiting, stroke and edema (Pharmacopoeia of People's Republic of China, 2005). In the past several years, medicinal plant polysaccharides have been widely studied for their chemical properties and biological activities (Chen, Zhang, Qu, & Xie, 2008), including antitumor (Wasser, 2002), immunostimulation (Li et al., 2003; Yamada, 1994), antioxidation (Li et al., 2003; Liu, Ooi, & Chang, 1997). However, little information is available about the polysaccharides from *Herba Moslae*.

Plant polysaccharides have been used in the food industry and in medicine for a long time, and have attracted much attention in recent years. In general, hot-water treatment has been used as a classical method to extract crude polysaccharides from plants. Our preliminary studies have shown that extraction temperature, extraction time and water to solid ratio might have significant effects on the yield of the crude polysaccharides. Thus, it is important to optimize the extraction process in order to obtain a high yield.

The classical method of studying one variable at a time may be effective in some processes, but fails to consider the combined effects of several factors involved. Moreover, it requires greater amounts of reagent and time to accomplish. Therefore, it is necessary to use an optimization method that can be used to determine all the factors as well as the possible interactions among these independent variables (Cui, Mazza, Oomah, & Biliaderis, 1994).

Optimization through factorial design and response surface analysis (RSM) particularly fulfills this requirement. The RSM is a multivariate technique, which fits mathematically the experimental domain studied in the theoretical design through a response function (Santelli, Bezerra, SantAna, Cassella, & Ferreira, 2006). RSM is an empirical modelization technique devoted to the evaluation of the relationship of a set of controlled experimental factors and observed results. It requires a prior knowledge of the process to achieve a statistical model. Basically, this optimization process involves four major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model. The main advantage of RSM is that the number of experimental trials can be reduced, and it is less laborious and time-consuming than other approaches (Wu, 2002).

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Extraction of polysaccharides is an important step for its application or further research and development. The objective of the present work was to optimize the extraction conditions of the crude polysaccharides from *Herba Moslae* in order to obtain a high extraction yield. The chemical composition of the crude polysaccharides was analyzed. To determine the biological activity of the crude polysaccharides, we assayed pinocytic activity and nitric oxide levels in mouse peritoneal macrophage.

2. Materials and methods

2.1. Samples

Herba Moslae plants were collected from Jiangxi Province, China, during the vegetative stage (July, 2009). The species was identified by Dr. Zhi-hong Fu, Jiangxi University of Traditional Chinese Medicine, Nanchang, China. The whole plants were cut into smaller pieces and further ground into a fine powder in a high speed disintegrator (Model DFY-500, Da De Chinese Traditional Medicine Machine Co., Ltd, Zhejiang, China), and passed through a 20 mesh sieve.

2.2. Chemicals and reagents

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA). RPMI-1640 medium was from Gibco (NY, USA). Bovine calf serum was from Hyclone (Utah, USA). Trypan blue was from Sangon Biotech Co. Ltd. (Shanghai, China); Griess Reagent Kit was from Beyotime Chemical Reagent Co. (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.3. Animals

Male BALB/c mice between 6 and 8 weeks old (weight: 19.0 ± 1.0 g) were purchased from the Experimental Animal Center, Medical College, Nanchang University, China. Mice were randomly assigned to cages containing sawdust bedding, with free access to food and water.

2.4. Extraction of crude polysaccharides

The samples were defatted in a Soxhlet apparatus with petroleum ether at 30–60 °C, and soaked with 80% ethanol twice to remove some colored materials, oligosaccharides and some small molecules. The organic solvent was volatilized, and pretreated powder was obtained, as described previously (Chen, Cao, & Song, 1996).

The pretreated powder (10.0 g) was extracted with deionized water [water to solid ratio (mL/g) ranging from 12:1 to 18:1], while the temperature of the water bath ranged from 80 to 90 °C (±1.0 °C). The mixture was boiled in a 2.5 L stainless steel boiler in the water bath for 3.5–4.5 h. The mixture was centrifuged (4800 rpm, 10 min), then the supernatant was separated from insoluble residue with four-layer filter cloth. The extracts were precipitated by the addition of ethanol to a final concentration of 75% (v/v) and incubated overnight. The precipitates were then collected by centrifugation (4800 rpm, 10 min), solubilized in deionized water and dried by vacuum to get the crude polysaccharides.

2.5. Analytical methods

2.5.1. Experimental design of RSM

To explore the effects of independent variables on the response within the range of investigation, we performed a Box–Behnken Design (BBD) with three independent variables (X_1, extraction temperature; X_2, extraction time; X_3, ratio of water to solid) at three levels. BBD is a second-order multivariate design based on three-level incomplete factorial design. This method has been widely used to assess critical experimental conditions, that is, maximum or minimum of response functions.

Table 1
The ranges and corresponding levels of independent variables used for experimental design.

Factors	Actual values	Coded values	Levels of coded factors		
			−1	0	1
Extraction temperature (°C)	X_1	x_1	80	85	90
Extraction time (h)	X_2	x_2	3.5	4.0	4.5
Water to solid ratio (mL/g)	X_3	x_3	12	15	18

For statistical calculation, the variables were coded according to the following formula:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the independent variable coded value, X_i is the actual value, X_0 is the independent variable actual value on the centre point and ΔX_i is the step change value. The range of independent variables and their levels were presented in Table 1. The independent variables and their ranges were determined from our preliminary experiments. Three experiments of each condition were carried out and the mean values were stated as observed responses. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

2.5.2. Statistical analysis

The quadratic model for predicting the optimal point was expressed according to the following formula:

$$Y_k = \beta_{k_0} + \sum_{i=1}^{n} \beta_{k_i} x_i + \sum_{i=1}^{n} \beta_{k_{ii}} x_i^2 + \sum_{i<j=2}^{n} \beta_{k_{ij}} x_i x_j \quad (2)$$

where Y_k is the response function, β_{k_0} is the centre point of the system, β_{k_i}, $\beta_{k_{ii}}$ and $\beta_{k_{ij}}$ represent the coefficients of the linear, quadratic and interactive terms, respectively; x_i, x_i^2 and $x_i x_j$ represent the linear, quadratic and interactive terms of the coded independent variables, respectively. The fitted polynomial equation is expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimal conditions (Triveni, Shamala, & Rastogi, 2001). The analysis of variance table was generated (Table 2), and the effects and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of all terms in the polynomial was judged statistically by computing the Fishers variance ratio (F-value) at a probability (P) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculation to generate dimensional and contour maps from the regression models.

Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (version 7.6.1, LASTStat-Ease Inc., Washinton, USA). Additional confirmation experiments were subsequently conducted to verify the validity of the experimental design.

2.5.3. Analysis of chemical properties

The yield of crude polysaccharides was calculated as a percentage of the total weight of sample used. The neutral carbohydrate content was determined by the phenol-sulfuric acid method (Chaplin & Kennedy, 1986). The uronic acid content was measured by a modified hydroxydiphenyl assay (Huang, Lin, Tian, & Ji, 1998) with glucuronic acid as the standard. Protein concentration

Table 2
The Box–Behnken design matrix and the results for extraction yield of crude polysaccharides.

Run numbers	X_1 Extraction temperature (°C)	X_2 Extraction time (h)	X_3 Water to solid ratio (mL/g)	Yiled (%)
1	90.00	4.50	15.00	2.94
2	90.00	3.50	15.00	2.9
3	85.00	4.50	12.00	2.84
4	85.00	4.00	15.00	3.33
5	85.00	4.00	15.00	3.42
6	80.00	3.50	15.00	2.56
7	85.00	4.00	15.00	3.38
8	80.00	4.00	18.00	2.76
9	85.00	3.50	12.00	2.64
10	85.00	4.00	15.00	3.40
11	90.00	4.00	12.00	3.04
12	80.00	4.50	15.00	2.86
13	90.00	4.00	18.00	3.27
14	80.00	4.00	12.00	2.51
15	85.00	4.50	18.00	2.97
16	85.00	4.00	15.00	3.38
17	85.00	3.50	18.00	2.63

was determined using the Bradford assay method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard to construct the calibration curve. Moisture and ash contents were determined according to AOCS and AOAC methods (AOAC 934.01; AOAC 942.05). The analysis of monosaccharide compositions was performed by gas chromatography (GC, Agilent 6890, USA) equipped with a DB-1701 capillary column (30 m × 0.32 mm, film thickness 0.25 μm) (Yin, Nie, Zhou, Wan, & Xie, 2009). The crude polysaccharides from *Herba Moslae* were hydrolyzed by 2 M trifluoroacetic acid at 100 °C for 12 h into monosaccharides, and were detected with a flame ionization detector (FID).

2.5.4. Bioactivity assay

2.5.4.1. Isolation of peritoneal macrophages. Peritoneal fluid from male BALB/c mice was harvested from peritoneal cavities by infusing 10 mL ice-cold sterile PBS (pH 7.2–7.4). After centrifugation at 1000 rpm for 5 min, the cell pellets were suspended with RPMI-1640 cell culture media with 10% (v/v) bovine calf serum and cultivated in a 96-well plate at a density of 5×10^5 cells/mL, and allowed to adhere for 3 h at 37 °C in a 5% CO_2 humidified incubator. After 3 h incubation, non-adherent cells were removed by washing twice with PBS and freshly prepared medium was added (Moretão, Buchi, Gorin, Iacomini, & Oliveira, 2003). The viability of the adherent cells was assessed by trypan blue exclusion test, and the percentage of macrophages was determined by cell morphology under a microscope.

2.5.4.2. Phagocytosis assay. Macrophages (5×10^5 cells/mL) were cultured in the presence of various concentrations of crude polysaccharides in a 96-well plate in a total volume of 200 μL per well for up to 48 h at 37 °C. 100 μL/well of 0.1% neutral red was added and incubated for another 4 h. Media were discarded and macrophages were washed twice with PBS (pH 7.2-7.4). Then, 100 μL/well of cell lysing solution [50% ethanol:50% acetic acid (v/v) = 1:1] was added and kept for 2 h. The absorbance at 540 nm was measured using an ELISA plate reader (Cheng, Wan, Wang, Jin, & Xu, 2008).

2.5.4.3. Nitric oxide assay. Adherent macrophages (5×10^5 cells/well) were placed in a 96-well plate and incubated in complete RPMI medium alone or media containing various concentrations of crude polysaccharides or LPS (10 μg/mL, as a positive control) for 48 h. Nitrite content in the culture medium was determined by Griess reaction (Green et al., 1982). At the end of the culture period, a total of 50 μL/well of cell culture medium was incubated with equal volume of Griess Reagents at room temperature for 10 min. The absorbance was read at 540 nm, and the concentrations of NO_2^- were determined by a standard curve with $NaNO_2$.

Table 3
Analysis of variance for response surface quadratic model obtained from experimental results.

Source	Sum of squares	df	Mean square	*F* value	Probability > *F*
Model	1.55	9	0.17	16.36	0.0007
x_1	0.27	1	0.27	25.30	0.0015
x_2	0.097	1	0.097	9.19	0.0191
x_3	0.045	1	0.045	4.27	0.0776
x_1x_2	0.017	1	0.017	1.60	0.2458
x_1x_3	0.0001	1	0.0001	0.0095	0.9251
x_2x_3	0.0049	1	0.0049	0.47	0.5171
x_1^2	0.21	1	0.21	19.52	0.0031
x_2^2	0.50	1	0.50	47.86	0.0002
x_3^2	0.30	1	0.30	28.28	0.0011
Residual	0.074	7	0.011		
Lack of Fit	0.069	3	0.023	20.61	0.0068
Pure error	0.0045	4	0.0011		
Cor total	1.62	16			

Std. dev.: 0.10; R^2: 0.9546; Mean: 2.99; Adj R^2: 0.8963; C.V. %: 3.43; Pred R^2: 0.3136; Adeq precision: 11.745.

3. Results and discussion

3.1. Statistical analysis and the model fitting

The experimental design and results were shown in Table 2. The whole design consisted of 17 experimental points carried out in a random order, which included 12 factorial points and 5 centre points. Five replicates (runs 4, 5, 7, 10, 16) at the centre of the design were used to allow for estimation of a pure error sum of squares. The results were fitted with a second order polynomial equation. The values of regression coefficients were calculated, and the fitted equation (in terms of coded values) for predicting yield (Y) was given as below:

$$Y = 3.38 + 0.18 \times x_1 + 0.11 \times x_2 + 0.075 \times x_3 - 0.065 \times x_1 \times x_2 - 0.005 \times x_1 \times x_3 + 0.035 \times x_2 \times x_3 - 0.22 \times x_1^2 - 0.35 \times x_2^2 - 0.27 \times x_3^2 \quad (3)$$

where x_1, x_2 and x_3 represent extraction temperature, extraction time and, water to solid ratio, respectively.

The statistical significance of the regression model was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model was shown in Table 3.

ANOVA is required to test the significance and adequacy of the model. The mean squares were obtained by dividing the sum of squares of each of the two sources of variations, the model and the error variance, by the respective degrees of freedom. The *F*-value = (Sr^2/Se^2) is the ratio of the mean square owing to regression to the mean square owing to error. It is the measure of variation in the data about the mean. Here the ANOVA of the regression model demonstrates that the model is highly significant as evident from the calculated *F*-value (16.36) and a very low probability value (0.0007). The Lack of Fit *F*-value of 20.61 implies that the Lack of Fit is significant. There is only a 0.68% chance that a"Lack of Fit *F*-value" could occur due to noise. The Pred R^2 of 0.3136 is not as close to the Adjusted R^2 of 0.8963 as one might normally expect. This may indicate a large block effect or a possible problem with model and/or data. Things to consider are model reduction, response transformation, outliers, etc. "Adequate Precision" measures the signal to

Table 4
Chemical analysis of the crude polysaccharides from *Herba Moslae.*

Main components	Protein	Neutral carbohydrates	Moisture	Ash	Uronic acid
Contents (%)[a]	6.14 ± 0.01	37.84 ± 0.04	8.10 ± 0.42	4.61 ± 0.11	5.18 ± 0.16

[a] Means ± S.D. (S.D. = standard deviation; $n = 3$, number of replicates.).

noise ratio. A ratio greater than 4 is desirable. Here the ratio of 11.745 indicates an adequate signal.

The *P*-values were used as a tool to check the significance of each of the coefficients, which in turn, may indicate the patterns of the interaction among the variables. The larger magnitude of T and smaller value of *P* indicate that the corresponding coefficient is more significant. Values of "Prob > *F*" less than 0.05 indicate model terms are significant. In this case X_1, X_2, X_1^2, X_2^2, X_3^2 are significant model terms. Values greater than 0.10 indicate that the model terms were not significant. This implies that the linear effects of extraction temperature ($P = 0.0015$), extraction time ($P = 0.0191$) and the square effects of extraction temperature ($P = 0.0031$), extraction time ($P = 0.0002$), water to solid ratio ($P = 0.0011$) are more significant. Table 3 also indicates that all the interactive effects have less significant influence on the yield of crude polysaccharide from *Herba Moslae*.

The goodness of fit was manifested by the determination coefficient (R^2). The closer the value of R^2 to the unity, the better the empirical model fits the actual data (Lee, Yusof, Hamid, & Baharin, 2006). In this case, the R^2 value of 95.5% indicated that the response model can explain 95.5% of the total variations. In general, a regression model having a R^2 value higher than 0.9 is considered to have a very high correlation (Haaland, 1989). The value of the adjusted R^2 ($R^2_{Adj} = 89.6\%$) was also high enough to indicate the significance of this model.

The 3D response surfaces plots were employed to determine the interaction of the extraction conditions and optimal levels that have the most significant effect on the yield. The response surfaces plots based on the model are shown in Fig. 1a–c. Fig. 1a showed both extraction temperature and extraction time had a quadratic effect on the yield. It was clear from Fig. 1a that the minimum response of yield (2.51%) occurred when temperature was at its lowest level. The yield increased considerably as temperature increased, indicating that temperature for the yield has a significant effect on the responses. As the temperature increased, the responses were maximal nearly at the middle of extraction time. Fig. 1b showed the effects of water to solid ratio and extraction temperature on the yield. Clearly, both of them have quadratic effects on the yield. Similarly Fig. 1c showed the effects of water to solid ratio and extraction time. Considering all the responses, it is evident that extraction temperature, extraction time and the square effects among them had a significant effect on product yield while the effect of water to solid ratio was more limited. The results agree well with Table 3.

The optimal extraction conditions, obtained by differentiation of the quadratic model, for achieving maximal yield, were $X_1 = 86.9\,^{\circ}C$, $X_2 = 4.1$ h and $X_3 = 17.7$ mL/g. The predicted optimal yield corresponding to these values was 3.28%. To confirm the goodness of the model for predicting maximal yield, we performed additional experiments in quintuplicate using these optimized extraction conditions.

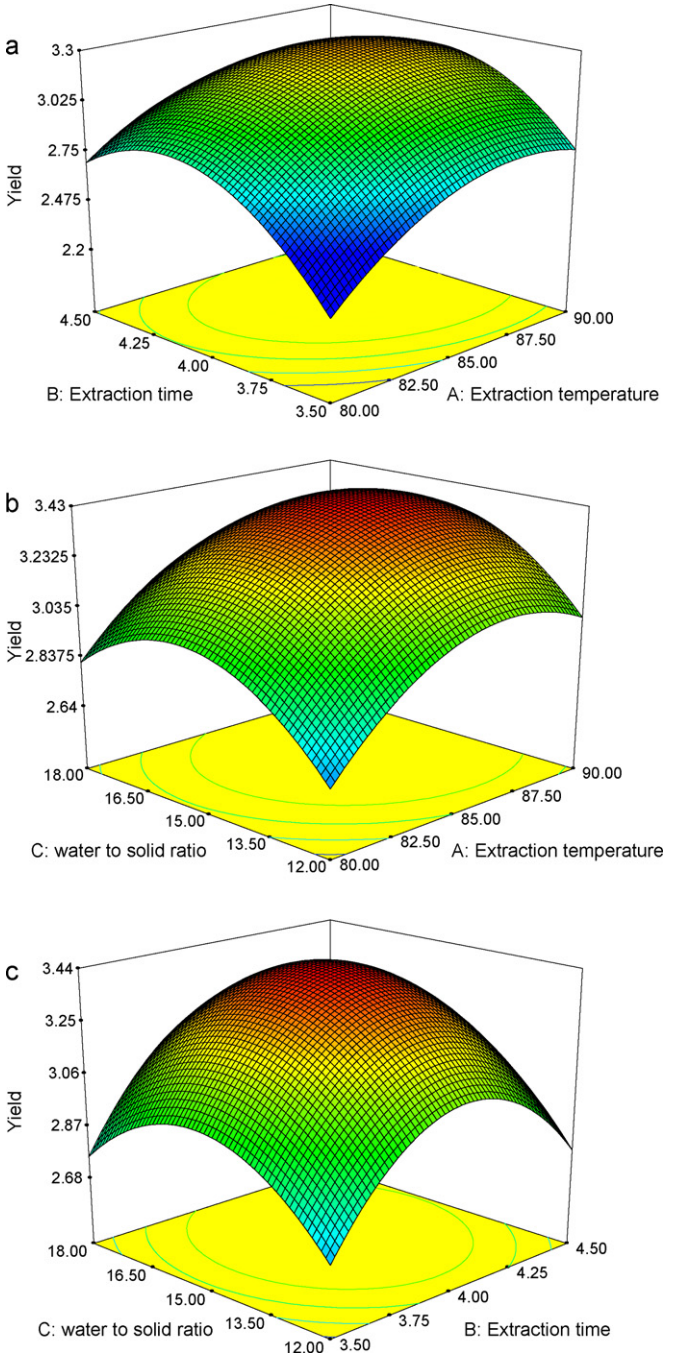

Fig. 1. Surface plots for crude polysaccharides yield of *Herba Moslae.* (a) Effect of extraction time and extraction temperature on the yield of crude polysaccharides with water to solid ratio 17.7 mL/g. (b) Effect of water to solid ratio and extraction temperature on the yield of crude polysaccharides with extraction time 4.1 h. (c) Effect of water to solid ratio and extraction time on the yield of crude polysaccharides with extraction temperature 86.9 °C.

3.2. Chemical compositions

The crude polysaccharides extracted under the optimal conditions were further analyzed for chemical and monosaccharide compositions as shown in Tables 4 and 5, respectively.

Table 5
Monosaccharide compositions of the crude polysaccharides.

Monosaccharide	Rhamnos	Ribose	Fucose	Arabinose	Xylose	Mannose	Glucose	Galactose
Content (wt%)	0.60	0.36	0.15	0.89	0.41	0.91	2.66	1.71
Molecular ratio	3.72	2.45	0.92	6.00	2.76	5.09	13.53	9.58

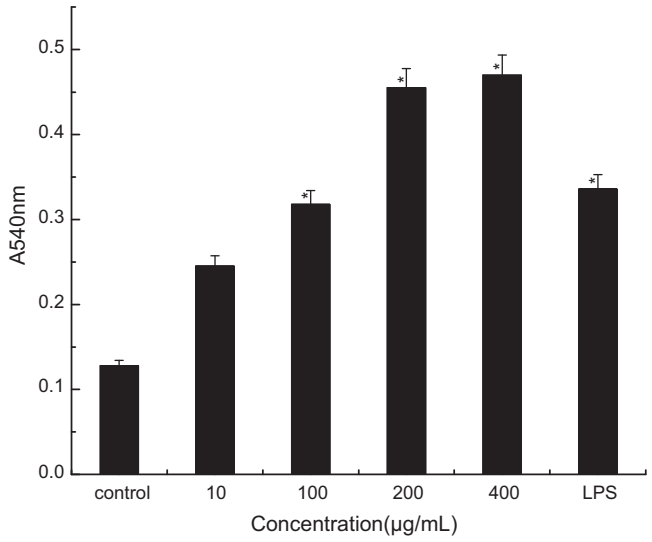

Fig. 2. Effects of the crude polysacchrides from *Herba Moslae* on pinocytic activity of macrophages. Values are means ± S.D. ($n = 3$), $^*P \leq 0.01$ vs. control.

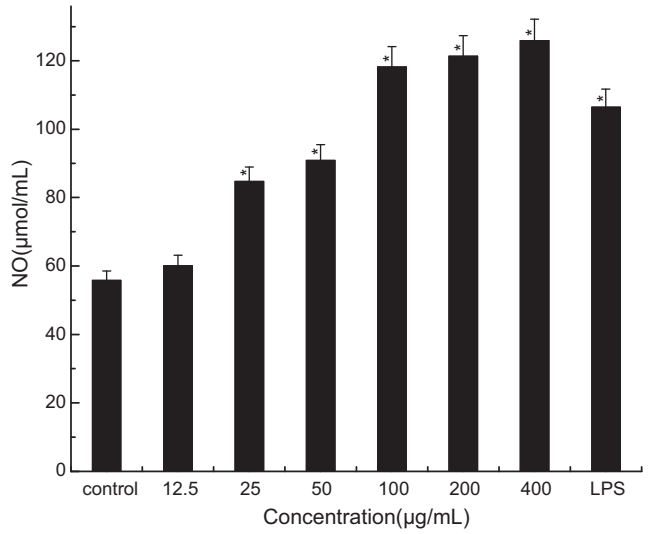

Fig. 3. Effects of the crude polysacchrides from *Herba Moslae* on inducing NO production of macrophages. Values are means ± S.D. ($n = 3$), $^*P \leq 0.01$ vs. control.

3.3. Pinocytic activity assay

A dose-dependent enhancement of pinocytic activity was observed in macrophages treated with 10–400 μg/mL of crude polysaccharides from *Herba Moslae* (Fig. 2). Crude polysaccharides appeared to enhance the pinocytic activity of macrophages, which was greater than LPS (10 μg/mL), the positive control.

3.4. Nitric oxide assay

We investigated whether nitric oxide (NO) production was increased in the macrophages of mice stimulated with crude polysaccharides for 48 h. As shown in Fig. 3, a minimal amount of NO was released when macrophages were exposed to medium alone, whereas the crude polysaccharides induced NO production in a dose-dependent manner. Furthermore, the levels of NO at 100–400 μg/mL concentrations of the crude polysaccharides were comparable to or even higher than that elicited by 10 μg/mL LPS.

4. Conclusions

RSM was used to determine the optimal process parameters that gave a high extraction yield. ANOVA showed that the effects of extraction temperature and extraction time were significant and quadratic models were obtained for predicting the responses. The optimal conditions were: extraction temperature 86.9 °C, extraction time 4.1 h and water to solid 17.7:1.

The chemical constitution and bioactivity assay of the crude polysaccharides from *Herba Moslae* was conducted. One of the most distinguished features of macrophage activation would be an increase in pinocytic activity. NO is a gaseous molecule synthesized from L-arginine by NO synthase (NOS). NO is known to play a key role during the course of infections (MacMicking, Xie, & Nathan, 1997). In this study, we demonstrated that the crude polysaccharides from *Herba Moslae* have immunomodulatory properties that enhance macrophage functions, and that this may contribute, at least in part, to the therapeutic potential of *Herba Moslae*. The further studies on the purification, structure and immunocompetence are currently in progress in our laboratory. It is also necessary to establish the structure–bioactivity relationship of the polysaccharides from *Herba Moslae* in order to expand its applications.

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