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Research Article

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Microwave-assisted extraction and effect of *Radix Rehmanniae* Preparata on osteoblast *in vitro*

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ABSTRACT

A native polysaccharides (RRP) was isolated from Radix Rehmanniae Preparata by microwave-assisted extraction (MAE). In the method, the optimized conditions were: the ratio of raw material to water was 1:50 (g/ml); microwave irradiation power was 550W; extraction temperature was 60 ℃; extraction time was 30min. As a result, the average yield of RRP using MAE was 9.35%, and R.S.D. was 2.15%. Furthermore, RRP which was extracted by MAE and was purified (RRP_{MAE}) and (PRP_{HWE}), PRP_{HWE} can stimulate the proliferation and differentiation of osteoblasts in vitro.

Key words: Radix Rehmanniae Preparata; polysaccharide; microwave-assisted extraction; osteoblast

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INTRODUCTION

Plant polysaccharides have been widely studied for their chemical properties and biological activities in food and pharmaceutical industry. *Radix Rehmanniae* Preparata (RR) is a traditional Chinese medicinal herb and noted in traditional Chinese medicine (TCM) as a drug[1]. Polysaccharide of *Radix Rehmanniae* Preparata (RRP) was a heterosaccharide with a molecular mass of 3.5×10^4Da. The four monosaccharides, glucose, galactose, fructose and stachyose were identified in the hydrolysate of RRP, and their mol ratio was 0.71:1.21:1.35:1.56 [2]. RRP was the main effective ingredient with many biological activities, such as enhancing immunity, anti-oxidation, anti-cancer, nourishing effect of the body.

Many papers aimed at investigating the influence of extraction parameters of plant polysaccharides, such as particle size, ratio of raw material to solvent, extraction time, extraction temperature, pH value and number of extraction [3,4,5,6]. Initially classical hot water extraction (HWE) of polysaccharides had been carried out just to compare with microwave-assisted extraction (MAE). It should be noted that HWE was associated with long extraction time and high temperature.

MAE was a process that used microwave energy and solvents to extract target compounds from various matrices. The spectral frequency of microwave ranged from 300 to 300,000MHz [7]. MAE had many advantages on extracting bioactive compounds, such as lower solvent consumption, shorter extraction time and higher selectivity of target molecules [8,9]. Microwave energy acted as a nonionising radiation that caused rotation of the dipoles. The highly localized temperature of MAE could cause selective migration of target compounds from the material to the surroundings at more rapid rate. In previous studies, MAE had been used to extract bioactive compounds from a wide variety of plants [10,11,12], and many products had been obtained.

To the best of our knowledge, the investigation of the microwave effected on polysaccharides structure and corresponding treatment of osteoporosis was rather limited. In our study, we attempted to study the total extraction yield of RRP using MAE in aqueous solution. Orthogonal design was employed to optimize the extraction parameters (ratio of raw material to water, microwave irradiation power, extraction temperature and extraction time) of crude RRP by MAE. In this study, we observe the effect of RRP on the proliferation and differentiation of osteoblasts *in vitro* to provide a preliminary experimental basis for PRP on prevention and treatment of osteoporosis.

RESULTS AND DISCUSSION

1.1 Extraction optimization of RRP

2.1.1 Drawing of standard glucose curve

In this paper, the method of phenol-sulfate acid [13] was adapted and a linear regression of absorbance value (Y) and glucose concentration (X) was made, from which the regression equation Y= 0.0087X+0.0056 and coefficient R^2 =0.997 were obtained. The standard glucose curve is shown in Figure 1.

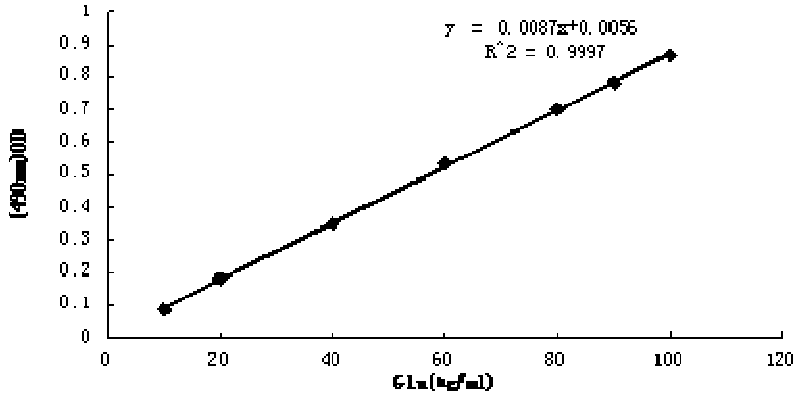

Fig 1 Standard curve of glucose

Table 1 Factors and levels of orthogonal test

Level	A extraction time (min)	B extraction temperature (℃)	C ratio of raw material to water ($g \cdot ml^{-1}$)	D microwave irradiation power (W)
1	20	50	1:30	500
2	30	60	1:40	550
3	40	70	1:50	600

Table 2 The analysis and results of orthogonal test of RRP extracted with microwave.

Number	A(min)	B（℃）	C（$g \cdot ml^{-1}$）	D(W)	Extracion yield Y（%）
1	20	50	1:30	500	6.84
2	20	60	1:40	550	13.25
3	20	70	1:50	600	10.25
4	30	50	1:40	600	7.34
5	30	60	1:50	500	9.05
6	30	70	1:30	550	14.42
7	40	50	1:50	550	17.86
8	40	60	1:30	600	9.45
9	40	70	1:40	500	7.45
K1	30.33	32.07	30.72	23.34	
K2	30.81	31.74	28.05	45.57	
K3	34.8	32.13	37.20	27.03	
k1	10.11	10.69	10.24	7.78	
k2	10.27	10.58	9.35	15.19	
k3	11.60	10.71	12.40	9.01	
R	1.48	0.12	3.05	7.41	
Sequence of factor	D>C>A>B				
optimal level	A_3	B_3	C_3	D_2	
optimumconditions	$A_3B_3C_3D_2$				

2.1.2 Analysis of orthogonal L_9 (3^4) test results

The optimization of extraction parameters was investigated by an orthogonal design L_9 (3^4). On the basis of single-factor experiment, four factors were extracted: time (A), temperature (B), proportion of spice (C), microwave

power (D), and three levels. Please refer to table 1 for factor-level. Analysis of variance was performed by statistical software SPSS 13.0. In this paper L9（3^4）table was adopted. Please refer to table 2 for orthogonal design and statistical analysis—the results and related analysis of the orthogonal test of RRP extracted with microwave. The extraction yields of RRP were obtained and the relationships among the factors were calculated.

Table3 Variance analysis of the experiment results

Source of variance	sum of squares of deviations	Degree of freedom	F	F critical-values（0.10）	significance
A	3.93	2	155.50	4.32	0.006
C	14.656	2	580.06	4.32	0.002
D	94.22	2	3728.96	4.32	0.000
Deviation		2			

According to the largest donating rule, as far as each investigated factor, the largest value which affected the extraction yield of RRP should be the selected value. Thus, the K and R values were calculated and listed in Table 2. As seen from Table 2, we could find that the influence to the mean extraction yields of RRP in the order: D>C>A>B according to the R values. Therefore, the optimized experimental conditions were as follows: microwave irradiation power was 550W;the ratio of raw material to water was 1:50 (g/ml)**;** extraction time was 40 min; the extraction temperature was 70℃. This indicated that the extraction yield of RRP could be enhanced by a combination of those factors at different levels in the preparation process.

In order to study which factors had more significant effect on extraction yield, a further orthogonal analysis was necessary. As shown in Table 3, Sig. of A, C, and D were 0.006, 0.002 and 0.000, respectively. When Sig. value of a factor was <0.05, we thought that it would have significant effect on test. So the orthogonal analysis indicated that the microwave irradiation power in MAE had more obvious influence on extraction yield than other factors.

2.1.3 Stability test of the optimum conditions

To study the stability of the optimum conditions and equipment, the experiments were repeated six times. The results were shown in Table 4. The average yield of RRP was9.35% and the relative standard deviation (R.S.D.) was 2.15%. Therefore, it was demonstrated that the optimum conditions were highly reproducible.

Table 4 Stablility test of RRP by microwave extraction

No	Extraction yield (%)	Mean	R.S.D
1	9.56	9.35	2.15%
2	9.42		
3	9.18		
4	9.14		
5	9.22		
6	9.60		

2.2 Effect of RRP on proliferation and differentiation of osteoblasts in vitro

2.2.1 Effect of RRP on proliferation of osteoblasts in vitro

The influence of RRP on the proliferation and differentiation of osteoblast can be found in Table 5. From it, we can see, compared to control group, positive drug group had a proliferation function on osteoblast after 48 hours use of NaF and the difference owned statistical significance ($p<0.05$), which means positive drug NaF promotes osteoblast proliferation. Furthermore, after 48 hours use of RRP, when the concentration was 3.5ug/mL-0.035ug/mL, the RRP group stimulated on osteoblasts compared to control group and the difference owns statistical significance ($p<0.05$), when concentration was 3.5ug/mL-0.035ug ug/mL, the difference owned statistical significance ($p<0.05$) compared to positive drug group NaF, which means RRP has a worse proliferation effect on osteocyte than NaF.

Table 5 Effect of RRP on proliferation of osteoblasts in vitro

Group	48h OD
Control	0.27±0.014
NaF	0.58±0.015$^{\bullet}$
RRP（35*ug/mL*）	0.29±0.008$^{\blacktriangle}$
RRP（3.5*ug/mL*）	0.36±0.001$^{\bullet\blacktriangle}$
RRP（0.35*ug/mL*）	0.39±0.003$^{\bullet\blacktriangle}$
RRP（0.035*ug/mL*）	0.42±0.001$^{\bullet\blacktriangle}$

$^{\bullet}p<0.05$ *vs Control group;* $^{\blacktriangle}p<0.05$ *vs NaF Group*

2.2.2 Influence of RRP on osteoblasts alkaline phosphatase

The influence of RRP on osteoblasts alkaline phosphatase is shown in Table 6, from which we can see that the positive drug group obtained a difference of statistical significance ($p<0.05$) after using NaF for 3 days compared to the control group. The result indicates that NaF can boost the secretion of alkaline phosphatase by osteocyte. The RRP group obtained a difference of statistical significance ($p<0.05$) when the concentration of the drug group was3.5ug/mL-0.035ug/mL after 5-day function on the osteocytes, compared to the control group, meaning RRP can boost the secretion of alkaline phosphatase by osteocyte, when the concentration is 3.5ug/mL-0.035ug/mL, Stimulation reached its peak in D7. The concentration of alkaline phosphatase of the drug group was less than that of the positive drug group ($p<0.05$) when the concentration was 3.5ug/mL-0.035ug/mL, it means RRP own worse function than NaF in promoting osteocytes to secrete alkaline phosphatase.

Table 5 Effect of RRP on osteoblasts alkaline phosphatase

Group	D3	D5	D7	D9
Control	383.6±55.1	352.9±33.7	283.8±12.4	181.3±10.1
NaF	717.9±29.3$^{\bullet}$	1111.8±31.9$^{\bullet}$	1212.5±55.0$^{\bullet}$	862.8±69.4$^{\bullet}$
RRP（35*ug/mL*）	393.1±44.2$^{\blacktriangle}$	384.2±78.2$^{\blacktriangle}$	488.2±123.2$^{\blacktriangle}$	296.1±70.5$^{\blacktriangle}$
RRP（3.5*ug/mL*）	400.8±51.3$^{\blacktriangle}$	520.0±8.7$^{\bullet\blacktriangle}$	744.6±10.2$^{\bullet\blacktriangle}$	669.6±18.2$^{\bullet\blacktriangle}$
RRP（0.35*ug/mL*）	412.3±61.2$^{\blacktriangle}$	555.8±12.6$^{\bullet\blacktriangle}$	896.6±14.2$^{\bullet\blacktriangle}$	142.6±84.23$^{\bullet\blacktriangle}$
RRP（0.035*ug/mL*）	450.0±82.2$^{\blacktriangle}$	600.2±39.6$^{\bullet\blacktriangle}$	911.2±19.3$^{\bullet\blacktriangle}$	758.3±47.2$^{\bullet}$

$^{\bullet}p<0.05$ vs Control group; $^{\blacktriangle}p<0.05$ vs NaF Group

EXPERIMENTAL SECTION

Radix Rehmanniae Preparata (produced in Henan Province, lot number: 110201), provided by Zhanjiang Hongfeng Chinese Herbal Medicine Company; They were placed in an oven for 30 h at 50℃, then dried further for 24 h in a thermostatic vacuum drier at 50℃, -0.08 MPa. After vacuum drying, the materials were milled and sieved. The powder was 60 mesh.Phenol, 98% concentrated sulfuric acid and absolute ethyl alcohol were analytical reagent from Guangzhou Chemical Reagent Factory; macroporous resin DM130 from Tianjin Haiuang Chemical Company.

Extraction solvent used distilled water. Papain was from Beijing Huamei Biotechnology Co., Ltd. (Beijing, China). SephadexG-100 was from Pharmacia Co., (Sweden). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was from Sigma Co., (France). The required concentration of RRP_{HWE} was made with DMEM; newly updated 1-day old SPF rat from Guangdong Medical Experiment Animal Center. DMEM media from Gibo Company; fetal calf serum from Hyclone; Alkaline phosphatase Kit from Nanjing Jiancheng Bioengineering Institute.

3.2 Microwave-assisted extraction (MAE) of RRP

3.2.1 MAE procedure

MAE was carried out using microwave experiment equipment (2450 MHz, Nanjing, China) with adjustable power set tings ranging from 100 to 700W. It was equipped with one closed vessel, a power sensor, a temperature sensor, a temperature controller and cooling system.

50g of the powder were extracted by MAE using distilled water. Ratio of raw material to water, microwave irradiation power, extraction temperature and extraction time were calculated for MAE. When the extraction process was accomplished, the samples were cooled to room temperature and were filtered through filter paper. The above procedure was repeated three times. After extraction, the extract solution was evaporated under reduced pressure at 55℃ and was precipitated with 95% ethanol, freeze-dried to obtain the crude polysaccharide and weighed.

3.2.2 Optimization of RRP extraction

Orthogonal design was employed to statistically optimize the formulation parameters, and was evaluated main effects on the yields of polysaccharide. The levels of the main factors that were ratio of raw material to water, microwave irradiation power, extraction temperature and extraction time using the orthogonal design were shown in Table 1. Nine experiments were performed in the different conditions as shown in Table 2. All of experiments were repeated three times.

3.3 Purification of RRP

Sevage method was introduced to remove superfluous protein polysaccharide according to the report[14]. After centrifugation, the supernatant was dialyzed against distilled water (24h). Then through SephadexG-100, the purified RRP was obtained. RRP was marked as RRP_{MAE} after it was extracted by MAE and was purified, and was marked as RRP_{HWE} after it was extracted by hot water extraction and was purified.

3.4 Estimation of total polysaccharide in Radix Rehmanniae Preparata

The content of total polysaccharide was measured by Vitriol–Phenol with anhydrous glucose as standard control[15]. An exactly weighed amount L (g) of crude polysaccharide was mixed with a volume V (cm^3) of distilled water, and then the content of total polysaccharide was determined by colorimetry.

The RRP yield (Y) was calculated as the polysaccharides content of extraction divided by dried sample weight.

$Y\ (\%) = (CV/m) \times 100\%$

Where C was the concentration of the solution used for colorimetric analysis (g/cm^3), V was the total volume of crude polysaccharide solution (cm^3) and m was the mass of dried sample (g).

3.5 *Cell culture*

Preparation and culture of the skull osteoblasts from a newly born rat was accomplished in the same way in the report by Yuyu Liu et al[16].

3.5.1 *Experiment of osteoblast Proliferation*

Take some osteoblasts, and make cell suspension with 0.25% trypsinization. Inoculate it to a 96-holes board, 3 x 10^4/ m L or 100uL for each hole, and set a null hole, After 24-h normal culture of the cells, add RRP_{HWE} in different concentrations and test it in the set time points. Add 100 uL culture medium without blood serum and 10 uL 0.5%MTT, remove the culture medium after 4-hour incubation, add 100uL DMSO, shake out until the crystal is completely dissolved, then measure the OD value on 490nm with a microplate reader.

3.5.2 *Experiment of alkaline phosphatase*

Inoculate 3 x 10^4/ m L cells in each hole on the 24-hole board, after 24 h culture, add RRP_{HWE} in different concentrations and change the solution every other day; after 7-day culture, clean them with PBS and dry them, add 250 uL distilled water, repeat freeze thawing 2 times and have a ultrasound, then operate according to the instruction of the kit.

CONCLUSION

A systematic extraction and purification process were set up for separating RRP from *Radix Rehmanniae* Preparata. When the extraction of RRP using MAE was investigated in this study, the method was more efficiently than classical methods. In the new method, the optimized experimental conditions were as follows: the ratio of raw material to water was 1:50 (g/ml); microwave irradiation power was 550W; extraction temperature was 60℃; extraction time was 30min. The average yield of RRP was 9.14% and R.S.D. was 2.63%. The proposed technique was a green, simple, rapid and effective extraction method for separation of RRP from *Radix Rehmanniae* Preparata. Purified RRP can boost osteoblast proliferation, and the secretion of alkaline phosphatase, but they are affected by various factors. For this reason, it's necessary to make animal experiments toward them. As for the preventive and treatment function of RRP on osteoporosis in the animal experiment mode, it still needs further studies.

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REFERENCES

[1] Zhang, R.X., Zhou, J.H., Jia, Z.P., et al.Hypoglycemic effect of Rehmannia glutinosa oligosaccharide in hyperglycemic and alloxan-induced diabetic rats and its mechanism. *J Ethnopharmacol*. 90(12), pp.39–43. **2004**.

[2] Chen, C.F., Zhang, P.Z.. Two different extraction methods of polysaccharide from Radix Rehannia Preparate. *Food and Nutr.* in China. 11, 42-44.**2008**

[3] Jiang，YY., Wang, L., Zhang L., et al. Characterization, antioxidant and antitumor activities of polysaccharides from Salvia miltiorrhiza Bunge.*Int J Biol Macromol*.70C:pp.92-99,Jun，**2014**

[4] Tian S^1, Zhou X, Gong H, et al. Orthogonal test design for optimization of the extraction of polysaccharide from Paeonia sinjiangensis K.Y. Pan. *Pharmacogn Mag.* Vol. 7(25),pp.4-8, Jan,**2011**

[5] Yongguang Y, Yuzhu H, Yong H. Pulsed electric field extraction of polysaccharide from Rana temporaria chensinensis David. *Int. J Pharm.* Vol.312(1-2), pp.33–36, Apr,**2006**.

[6] Wang, Z.J., Luo, D.H., Ena, C. Optimization of polysaccharides extraction from Gynostemma pentaphyllum Makino using Uniform Design. *Carbohyd. Polym.* Vol.69,pp.311–317,**2007**

[7] Mahesar, S.A., Sherazi, S.T.H., Abroa, K., et al. Application of microwave heating for the fast extraction of fat content from the poultry feeds. Vol. *Talanta* 75(5), pp. 1240–1244, Jun,**2008**.
[8] Pan, X.J., Niu, G.G., Liu, H.Z. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. *Chem. Eng. Process.*Vol. 42(2), pp. 129-133.,Feb,**2003**.
[9] Tahmouzi S. Extraction, antioxidant and antilisterial activities of polysaccharides from the flower of viper's bugloss. *Int J Biol Macromol.* Vol.69,pp.523-31, Aug, **2014**
[10] Song JZ ,Mo SF, Yip YK,et al. Development of microwave assisted extraction for the simultaneous determination of isoflavonoids and saponins in radix astragali by high performance liquid chromatography. *J Sep Sci.* Vol.30(6),pp,819-24,Apr,**2007**
[11] Zhou,T.W.,C.J.,Study on Technology of Microwave- assisted Extraction of Solanesol from Eggplant Leaves. *Journal of Anhui Agri. Sci.*Vol.38(14),pp.7266-7267,7290,**2010**
[12] Monica Gallo, Rosalia Ferracane, Giulia Graziani ,et al. Microwave Assisted Extraction of Phenolic Compounds from Four Different Spices. *Molecules* .Vol. *15*(9),pp. 6365-6374,**2010**
[13]Zhao,P.G.,Liu,X. Study onextraction and puification of rehmnnia glutinosa polysaccharide .*China Pharmacy.*Vol.21(23) ,pp.2146-2148,**2010**
[14]Tan YF, Li HL, Lai WY,,et al. Crude dietary polysaccharide fraction isolated from jackfruit enhances immune system activity in mice. *J Med Food.*Vol. 16(7),pp.663-668,Jul,**2013**
[15] Xu, G.Y., Yan, J., Guo, X.Q.,et al. The betterment and apply of phenol-sulphate acid method. *Food Sci.* Vol.26(8),pp.342-345,**2005**
[16]Liu,Y.Y.,Cui,L.,Wu,T.,et al. Effects of emodin on the proliferati on and differentiati on of osteoblast isolated from neonatal rat calvarium in vitro.*Chinese Pharmacological Bulletin.*Vol.21(2),pp.235-240,Feb,**2005**