BIOMEDICAL RESEARCH JOURNAL

Volume 35, 2020

Ultrasonic extraction and determination of polysaccharide from *Smilacina japonica*

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Original Research

Abstract: Study the ultrasonic extraction and determination of polysaccharide from *Smilacina japonica* by orthogonal array design. The polysaccharide content was determined by phenol-sulfuric acid coloration, using ultraviolet-visible spectrophotometry, the polysaccharide extraction condition was optimized by ortho-gonal array design. The results showed that the optimum technological parameter for ultrasonic extraction was 1: 20, temperature was 30°C, ultrasonic time was 20 min, ultrasonic 1 times. The polysaccharide content was determined by phenol-sulfuric acid colorimetric method, using glucose as reference substance, the maximum absorption wavelength was 490 nm. The precision, reproducibility, recovery and stability of the proposed method were 3.80%, 4.43%, 0.04%, 0.16% respectively.

Key words: *Smilacina japonica*; polysaccharide; ultrasonic; phenol- sulfuric acid coloration.

Introduction

Polysaccharide is one of the essential biological macromolecules for the human. As the informational molecule, Carbohydrate are important effect on cell reco-gnition, growth and development, biological fertilization, nervous system and balance immune system(1). So far there are about 300 kinds of polysaccharides have been etracted and separated from the natural. Part of item are clinical treatment and rehabilitation treatment for cancer, hepatitis, cardiovascular disease products(2). The chemi-cal structure, pharmacological action and mechanism of active polysaccharides have become one of the most ac-tive fields in the research of life science(3,4) .

Smilacina japonica A. Gray (*S.japonica*) is a perennial plant, belongs to the family Liliaceae *Smilacina*. The dis-tribution and abundant of wild *S.japonica* is very exten-sive in China. The reserves of wild resources are enor-mous in the northeast of Jilin Province. Tt's named from its one of the nine grasses that are often eaten by deer that according to ancient Chinese medicine records, It's alias are Shan mi zi, Pian tou qi, Pan long qi, Pang xie qi, Tu fei qi, Little *Smilacina*(5,6). *S.japonica* is medici-nal and edible herbal, the young stems and leaves can be eaten from seedling to flowering stage with bittersweet, delicious fragrance. Its nutritional value is that it contains the necessary amino acids and inorganic elements of the human body(7,8). Andamino acids contents ishigh, its edible value has been utilized and developed(9,10). The rhizome, as a folk traditional medicine, used for the treat-ment of lung ailment, impotence, migraine, headache, rheumatism, cuts and bruises, mastitis and menstrual di-sorder with taste of «sweet, bitter, warm, non-toxic» (11). Modern research shows that there are saponin, flavone, polysaccharide active components in the rhizome of the *S. japonica*(12-16). Recent years, the study is increasing on it day by day(17,18). For improve the quality of meat and enhance the feeding animal's immune system, the far-mer often crushing the rhizome of *S. japonica* with the feed(19).

Preliminary study showed the content of polysaccha-ride in *S. japonica* (SJP) was high, and it hasantioxidation and inhibition of tumor cell proliferation activity.So the research on the ultrasonic extraction technology and de-termination content will provide the theoretical basis for the development and utilization of *S. japonica* resources.

Materials and reagents

Materials

S. japonica plant samples were collected from Mahu-tou mountain where in Jiutai city Jilin Province, China. The roots were washed, dried under shade, crushed.

Equipment and reagents

The model of DL-1000E Intelligent ultrasonic cleaner, made of Shanghai Zhisun Equipment Co.,Ltd; the mo-del of ME203E electronic balance, made of METTLER TOLEDO company; the model of DJ-10A grinder, made of Shanghai Tuolong Equipment Co.,Ltd; the model of T6 ultraviolet spectrophotometer, made of Beijing Purkinje General Instrument Co.,Ltd; the model of HH-6 digital display thermostat water bath, made of Changzhou Aohua Co.,Ltd. Glucose, fructose, mannose, phenol and concen-trated sulfuric acid were all analytically pure.

Methods

Extraction method and process design

Extration method and sample solution preparation Weighted the *S. japonica* rhizome crude powder drying to constant at 1.0 g, the sample was extracted with water by ultrasonic according to the orthogonal experiment de-sign of Table 1 for 1 times under the condition of 70 KHz, then was be filtrated. Filtrated volume to 50 ml, amount them of 2 ml, added absolute ethyl alcohol to 10ml, and mixed them fully, centrifuge the for10min at 5 000 r/min speed, dissolved the precipitate in water, filtrated volume to 100ml, get the SJP sample.

Orthogonal test of ultrasonic extraction

The optimum process of ultrasonic extraction SJP was studied using absorbance value of SJP coloration as in-dex. Based on previous work about the single factor test, selected the following conditions to test: ultrasonic tempe-rature, ratio of material to liquid, ultrasonic time and ultra-sonic frequency, each of them was tested at three levels. The ultrasonic extraction was carried out according to the $L_9(34)$orthogonal design scheme (Table 1). The extracting solution was made by «3.3.1» method. Determination of polysaccharide content in each extract according to the determination of polysaccharide content determined by "4.5"the methods for determination of determining the content.

Content determination

Selection of control article and maximum absorption wavelength

Putted each kind of Glucose, fructose, mannose and SJP solution (2.0 mg/ml) 2ml in test tube, added 4% phe-nol 1ml, then shake them, added concentrated sulfuric acid 7ml in them and shake well. Putted them in 40 de-grees Celsius water bath heat for 30 min, then puttd the in ice water bath for 5 min. Full wavelength scanned in 300-700 nm, used the corresponding reagent blank to eliminate the disturbance.

Optimization of color rendering conditions

Putted SJP solution 2ml in test tube, developed them by phenol and sulfuric acid, measured absorbance at the maximum absorption wavelength, the content of L9(34) was determined by spectrophotometry (Table 2). Opti-mized the reagent amount of phenol and concentrated

Table 1. Orthogonal experiment design and result analysis of ultrasonic extraction (n=3).

No	Factors and levels				Absorbance (average value)	Polysaccharide amount/%
	A Temperature/ degrees Celsius	B Ratio of material to liquid /g: ml	C Time /min	D Times		
1	1(30)	1(1:10)	1(20)	1(1)	1.095	20.90
2	1	2(1:15)	2(25)	2(2)	1.135	21.67
3	1	3(1:20)	3(30)	3(3)	1.329	25.39
4	2(40)	1	2	3	0.735	14.01
5	2	2	3	1	1.095	20.91
6	2	3	1	2	1.209	23.09
7	3(50)	1	3	2	0.559	10.64
8	3	2	1	3	1.103	21.06
9	3	3	2	1	1.000	19.09
k_1	1.186	0.796	1.136	1.063		
k_2	1.013	1.111	0.957	0.968		
k_3	0.887	1.179	0.994	1.056		
R	0.299	0.383	0.179	0.096		

Table 2. Orthogonal experiment design and result analysis of color developing condition (n=3).

No	Factors and levels				Absorbance (average value)
	A Amount of phenol/ml	B Amount of sulfuric acid /ml	C Water bath temperature /degrees Celsius	D Water bath time /min	
1	1(0.5)	1(6)	1(35)	1(20)	1.223
2	1	2(7)	2(40)	2(25)	1.069
3	1	3(8)	3(45)	3(30)	0.917
4	2(1.0)	1	2	3	1.661
5	2	2	3	1	1.481
6	2	3	1	2	1.190
7	3(1.5)	1	3	2	1.761
8	3	2	1	3	1.675
9	3	3	2	1	1.436
k_1	1.070	1.548	1.362	1.379	
k_2	1.443	1.408	1.389	1.340	
k_3	1.623	1.180	1.386	1.416	
R	0.553	0.368	0.027	0.076	

sulfuric acid, water temperature and time, determined the best color conditions for polysaccharide.

Making standard curve

First, weighted the anhydrous glucose which drying to constant in 105 degrees Celsius at 100 mg, dissolved with distilled water to 100 ml, shooked them, obtained refe-rence solution. Precised amount of the reference solution 0.5, 1, 1.5, 2, 2.5, 3,and 3.5 ml input 50 ml volumetric flask, respectively, added distilled water to 50 ml, shook them. Precision measurement 2.0 ml of the above stan-dard solution in a test tube, respectively, then determined the absorbance according to the best color conditions. Standard curves were plotted using the concentration of glucose solution C(mg/ml) as abscissa, the absorbance va-lue of A as ordinate.

Data processing

Each experiment was repeated three times, analysis of all data by Excel 2003, calculated the standard error and graphics. The data obtained on orthogonal experiment were analysis by range analysis and variance analysis by DPS.

Results and Analysis

Process of ultrasonic extraction

The content of SJP was positively correlated with the absorbance of the coloration solution. Through analysis the value of absorbance of SJP, the result of orthogonal array design for ultrasonic extraction were shown in table 1.Through intuitive analysis and Anova, the order of in-fluence of various factors on ultrasonic extraction of SJP was B>A>C>D. The ratio of material to liquid has the most obvious effect on the extraction percentage, the next factor were ulatrasonic temperature and ultrasonic time. The impact of them on the extraction rate reached a very significant level(P<0.01). The extraction times affected the extraction rate to the minimum. There was no statis-tical significance because of the difference between the 3 levels was not significant. The best process of ultrasonic extraction was A1B3C1D1, specifically, 1:20 of the ratio of material to liquid, 30 degrees Celsius ultrasonic tempera-ture, 20 min ultrasonic time, 1 times of ultrasonic.

Control article and maximum absorption wavelength Figure 1 shows phenol-sulfuric acid color reaction

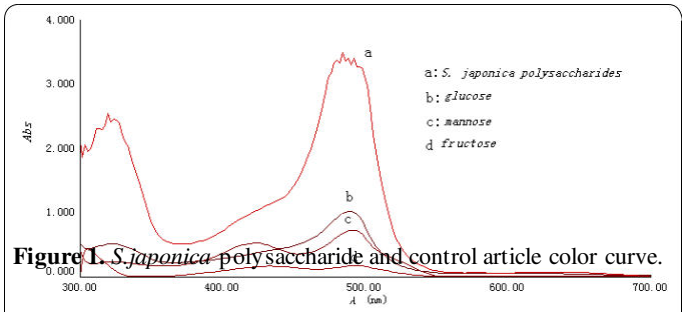

Figure 1. *S.japonica* polysaccharide and control article color curve.

curve of SJP, glucose, fructose and mannose. The co-lor-developing curve of polysaccharide basically the same as Glucose's, the maximum absorption peak is at 490 nm. But fructose's and mannose's appears double absorption peak after coloration, not only the maximum absorption peak at 492 nm and 494 nm respectively, but also another absorption peak at 424 nm and 434 nm respectively. *S. japonica* polysaccharide extracts no absorption at 490nm, so choose glucose as control article in the determination of SJP in which the method of phenol- sulfuric acid, the detection wavelength was 490 nm.

The best color-developing condition

Table 2 and Table 3 shows the orthogonal test results and analysis of SJP color-developing condition. The best color scheme was A3B1C2D3, the order of influence of all factors is A>B>C>D. Anova analysis shows that the amount of phenol and concentrated sulfuric acid reached a significant level of effecting absorbance. There was no significant difference effect on water temperature and time for water bath. So the best color-developing condition is A3B1C2D3, specifically, 1.5 ml of phenol doses, 6 ml of concentrated sulfuric acid, 40 degrees Celsius of water bath temperature, 30 min of water bath time.

Standard curve

The regression equation of the standard curve was A=13.049C+0.003 7, $r2$=0.999 8.

Content determination method

Precise amount of SJP solution of obtained by the best process of ultrasonic extraction 2 ml in test tube, added 4% phenol 1.5ml, then shake them, added concentrated sulfuric acid 7ml in them and shake well. Putted them in 40 degrees Celsius water bath for 30 min, then putted in

Table 3. Analysis of variance of color development conditions.

Variation source	Sum of deviation square	Freedom	Variance	*F*	*P*
A	0.9541	2	0.47706	161.15446	<0.01
B	0.4124	2	0.20618	69.65012	<0.01
C	0.0029	2	0.00143	0.48151	>0.05
D	0.0173	2	0.00867	2.92864	>0.05
Error	0.0237	8	0.00296		

Table 4. Recovery test

Polysaccharide /mg	Standard product /mg	Theoretical value/mg	Estimated value/mg	Percent recovery/%	Average recovery/%	RSD/%
10.00	0.02	10.02	9.89	98.67		
10.00	0.04	10.04	9.91	98.66	98.64	0.04
10.00	0.06	10.06	9.92	98.59		

ice water bath for 5 min. Then test the ultraviolet visible absorption value of the solution at 490 nm which corres-ponding reagent was blank. The resulting absorbance va-lues were inputted regression equation of standard, calcu-lation of the content of SJP.

Verification experiment and methodology study

Three validation experiments were carried out accor-ding to the optimum technological conditions. The results showed that the extraction rate was high, the average content of polysaccharide was 23.79± 0.02%, and it had better repeatability. These practically results show that this method is available and accurate.

Methodology study the SJP content determinant in the which the method of phenol-sulfuric acid.

Precision measurement 3 samples of glucose standard solution, each of the 2ml, measured absorbance after colo-ration.The result showed RSD was 3.80%, which showed good precision.

Precision measurement 3 samples of SJP solution, each of them 2ml, measured absorbance after coloration. The result showed RSD was 4.43%, which showed good reproducibility.

Precision measurement 9 samples of SJP extract of known content, added glucose standard solution with different volumes, determined the recovery ratio. The re-sult showed(Table 4)that the mean recovery was 98. 64%, RSD was 0.04%.

Precision measurement one sample of SJP extract, fol-lowing experiment with the best coloured condition of polysaccharide, the absorbance was measured every 10 min within 1.5 h from the start of the coloration. The result showed the sample solution was stable within 1.5 hours, RSD was 0.16%.

The methodological evaluation showed that the me-thod of coloration for determined the content of the poly-saccharide in the *Smilacina japonica* was stable and fea-sible.

Conclusions and perspectives

In this study, SJP content determination was finally determined by using phenol-sulfuric acid method with glucose as standard. Also determined SJP content by using the method of the «Chinese Pharmacopoeia» in *Po-lygonatum* Polysaccharide content was determined by the anthrone-sulfuric acid method. The results showed that the two color developing methods of determination poly-saccharide content, there was no significant difference for the same sample. It was further proved that it was feasible to determine the content of SJP.

Ultrasonic assisted extraction of natural products with convenient, time saving, high efficiency and other charac-teristics. This study determined the optimal process of the ultrasonic extraction of SJP by orthogonal test, the best optimal process is 1: 20 of solid-liquid ratio, 30 degrees Celsius of ultrasonic temperature, 20 min of ultrasonic time, ultrasonic frequency 1 times. The content of crude polysaccharide in the rhizome of *S. japonica* is 20% or so, it can be inferred that *S. japonica* has a broad prospect of research and development.

Acknowledgments

The study was supported by Natural Science Foundation of Jilin Province (No. 20140101128JC).

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