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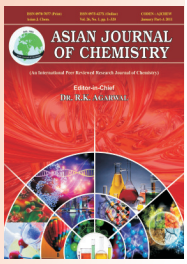

Optimization of Extraction Process and Antibacterial Activity of *Bletilla striata* Polysaccharides

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The aim of the study was to optimize the optimal process conditions for extraction of *Bletilla striata* polysaccharides and its antibacterial activity *in vitro*. Conventional water extraction and ethanol precipition method with polysaccharide content as an index through single factor investigation and $L_9(3^4)$ orthogonal table was used to optimize extraction conditions, respectively. The test tube double dilution method was chosen for preliminarily screening of the antibacterial activity and the determination of antibacterial mechanism was according to bacterial state changes in electron microscopy observation. The optimal extraction condition was obtained using cold soaking for 6 h prior to extraction, two times 3 h extraction technology, at 90 °C, with a extract-water ratio of 1:15, for purification using concentration of extract to 1:5 (w/v), addition of a 3-fold volume of 95 % ethanol, ethanol content of up to 80 % and polysaccharide content of 53.72 %, in addition, the MIC to *S. aureus* was 6.25 mg/mL and MBC could reach 12.50 mg/mL, the mechanism may be related with the permeability of cell membrane. The extraction and purification process for *Bletilla striata* polysaccharides established in this experiment is simpler, more scientific and suitable and *Bletilla striata* polysaccharides has a obvious inhibition against *S. aureus*.

Keywords: Extraction, *Bletilla striata*, Polysaccharides, Antibacterial activity.

INTRODUCTION

Bai Ji is a perennial herbaceous plant *Bletilla striata* (Thunb.) Reichb. f. of family Orchidaceae, which is also known as Xiao Bai Ji, Lian Ji Cao, *etc*[1]. Its medicinal part is dried tuber and its medicinal composition is *Bletilla striata* gum, which contains polysaccharides, starches, glucose, volatile oils, *etc*. It has less adverse reactions and has lung tonifying, hemostatic, astringent and antibacterial actions[2], which has all along been used as a traditional Chinese medicine clinically, mainly for the treatment of lung injury, hemoptysis, surgical wounds, ulcers, pyogenic infections, *etc*.[3,4]. Meanwhile, *Bletilla striata* gum can also be used as thickeners, emulsifiers and moistouring agents in the petroleum, food, pharmaceutical and cosmetic industries, which has a bright prospect[5].

Bletilla striata polysaccharides are a kind of sticky polysaccharides in the plant obtained by water extraction and ethanol precipitation, their chemical components are glucose and mannose, with a ratio of 1:4, it contents are extremely rich[6], which account for about 55-60 % of the plant. Therefore, as one important direction for development of novel drugs, biologically active *Bletilla striata* polysaccharides have gained increasing attention of scholars at home and abroad, in order to find a more reasonable and feasible production process. The present experiment studied the extraction process of total polysaccharides from *Bletilla striata* and antibacterial activity *in vitro*.

EXPERIMENTAL

ZHJH-CH09B clean bench (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.); RT-08 herb pulverizer (Beijing Huanya Tianyuan Machinery Technology Co., Ltd.); JJ500 electronic high precision balance (G&G GmbH, in USA), U-3010 UV-visible (Hitachi, in Japan); HH-8 digital thermostat water bath (Changzhou Guohua Electric Appliance Co., Ltd.); Rotary evaporator RE-52AA (Shanghai Yarong Biochemical Instrument Factory); TFFD-27 freeze dryer (Beijing Zhongyi Instruments Co., Ltd.); CT15RE high speed refrigerated centrifuge (Hitachi, in Japan); ZD-85A dual-function air bath thermostat shaker (Jintan Jieruier Electric Appliance Co., Ltd.); Hitachi H-7500 transmission electron microscope (Hitachi, in Japan); JSM-6360LV scanning electron microscope (JEOL, in Japan).

Bletilla striata decoction pieces (purchased from Dalian Baidu Medicine Co., Ltd., which was identified by Professor Diao Yun-peng from the Department of Pharmacognosy, College of Pharmacy, Dalian Medical University as the tubers of *Bletilla striata* (Thunb.) Reichb. f. of the family Orchidaceae); Anhydrous D-glucose reference substance (AR) (Panjin Tianyuan Pharmaceutical Co., Ltd.); 95% ethanol, concentrated sulfuric

acid, phenol, anhydrous ethanol, acetone, *etc.* (the above reagents were all of analytical grade) (Tianjin Kermel Chemical Reagent Co., Ltd.); Nutrient broth (Beijing Land Bridge Technology Co., Ltd.); Agar (Sigma, USA), Cefoperazone sodium for injection (General Pharm. Factory, Harbin Pharmaceutical Group Co., Ltd.).

S. aureus (ATCC25923) was provided by the Department of Pharmacology, Liaoning University of Traditional Chinese Medicine.

Determination of *Bletilla striata* polysaccharides extraction technology

Plotting of standard curve[7]: 50 mg of glucose, which was dried to constant weight, was accurately weighed, placed into a 50 mL volumetric flask, dissolved in water and diluted to the mark to obtain 1 mg/mL glucose stock solution. 1, 2, 3, 4, 5 and 6 mL of the solution were accurately pipetted, respectively, placed into 50 mL amber volumetric flasks, diluted to the mark with water and shaken well to obtain a series of glucose standard solutions with the concentrations of 20, 40, 60, 80, 100 and 120 µg/mL, respectively. 1 mL of each glucose standard solution was accurately pipetted and 1 mL of distilled water was served as the blank control, the above were separately placed into 10 mL stoppered test tubes, added with 1 mL of 5 % phenol reagent and shaken vigorously, then quickly added with 6 mL of concentrated sulfuric acid, mixed well and allowed to stand at room temperature for 0.5 h, followed by measurement of absorbance at 490 nm. Standard curve was plotted with the absorbance value as the ordinate and the concentration (µg/mL) of standard glucose solution as the abscissa[8].

Preparation process of *Bletilla striata* polysaccharides: The process for extraction of *Bletilla striata* polysaccharides was as follows: crude material was rinsed, dried, pulverized and then dried to constant weight. 5 g of *Bletilla striata* powder was accurately weighed, added with 75 mL of distilled water and extracted under reflux at 90 °C for 3 h twice, then filtered, the two filtrates were combined and then concentrated under reduced pressure to a volume of 30 mL. The concentrated solution was then precipitated with 95 % ethanol, ethanol volume was three times that of the concentrated solution so that the ethanol content could reach 80 % and allowed to stand overnight. After suction filtration and removal of protein by Sevage method[9], the 95 % ethanol precipitated extract was washed three times sequentially with anhydrous ethanol and acetone, followed by removal of organic solvent and freeze drying to get *Bletilla striata* polysaccharides.

Preparation of test solution: 100 mg of *Bletilla striata* polysaccharides was accurately weighed, diluted to a volume of 100 mL and shaken wells, 1 mL of the above solution was precisely drawn and diluted to a volume of 25 mL and shaken well to obtain the test solution. Experiment was continued as per "1 mL of each standard test solution was placed into 10 mL stoppered test tubes. Preparation of standard curves, next the concentration of test sample solution was obtained from the standard curve and the percentage content of *Bletilla striata* polysaccharides was calculated.

Investigation of soaking time prior to extraction of *Bletilla striata* polysaccharides: 5 g of *Bletilla striata* powder, which was dried to constant weight, was accurately weighed and extracted twice under reflux at 90 °C by addition of distilled water with a ratio of 1:15, each time lasted 3 h, under the same conditions as described above. *Bletilla striata* was soaked at room temperature for 1 h, 6 h and 12 h, respectively and polysaccharide content was separately determined.

Investigation of extraction temperature during the extraction of *Bletilla striata* polysaccharides: 5 g of *Bletilla striata* powder, which was dried to constant weight, was accurately weighed and refluxed twice by addition of distilled water with a ratio of 1:15 at different temperatures of 70, 80 and 90 °C, respectively, each time lasted 3h, the two filtrates were combined and polysaccharide content was separately determined.

Optimization of orthogonal test during the extraction of *Bletilla striata* polysaccharides: The present experiment used the conventional water extraction and ethanol precipitation method to extract *Bletilla striata* polysaccharides. The results of preliminary experiments, factors influencing the extraction effect of *Bletilla striata* polysaccharides were determined to be: solvent volume (a), extraction time (b) and extraction times (c), each factor had three levels and test was arranged according to $L_9(3^4)$ orthogonal table. A total of 9 portions of 5 g of *Bletilla striata* powder were used in the test, which were extracted under reflux at 90 °C, comprehensive scoring was performed with extraction yield and polysaccharide content as the indices and optimal process was screened out.

Determination of *Bletilla striata* polysaccharides purity

Influence of concentration multiple on extraction yield when added with the same volume of ethanol during the purification: To remove impurities and maximize the retention of active components, purification process is necessary after the water extraction. At first, the concentration multiple of water extract was investigated. *Bletilla striata* water extract was prepared according to the optimal extraction process optimized as above. The same amounts of water extracts were separately concentrated under reduced pressure, concentration multiples were 2, 3, 4, 5 and 6 folds, respectively. The concentrated water extracts were then added with equivalent amount of ethanol and mixed, followed by ethanol precipitation. The ethanol precipitates were dried, weighed and the yield of *Bletilla striata* polysaccharides was calculated[10].

Influence of ethanol content on extraction yield when the concentration multiple of water extract was constant during the purification: When the *Bletilla striata* water extracts were concentrated into a 3-fold volume, they were added with different concentrations of ethanol to make their ethanol contents 30, 40, 50, 60, 70, 80 and 90 %, respectively, then mixed and precipitated. The ethanol precipitates were then freeze-dried, weighed precisely and the yield of *Bletilla striata* polysaccharides was calculated.

Determination of minimum inhibitory concentration (MIC) of *Bletilla striata* polysaccharides[11]: Eight sterile test tubes were taken and marked with numbers 1-8, respectively. Tubes #1-6 were sample tubes, tube #7 was the positive control tube and tube #8 the negative control tube. 1.8 mL of sterile culture medium and 0.2 mL of drug solution were added to tube #1 and tubes #2-6 were separately added with 1 mL of

sterile culture medium, 1 mL of 500 mg/mL sample solution was added to tube #1 and tubes #1-6 were diluted by test tube double dilution method; tube #7 was added with 0.2 mg/mL cefoperazone sodium solution and tube #8 with 1 mL of sterile culture medium. Next, each tube was added with 1 mL of logarithmic phase bacterial solution with a concentration of 5×10^5 CFU/mL. The test tubes were than stoppered and placed in a thermostat shaker set at 37 °C, after static incubation for 24 h, the tubes were removed and visually observed, the lowest concentration of culture medium in the test tube at which no turbidity was observed was considered as the MIC of the drug against the tested bacteria.

Determination of minimum bactericidal concentration (MBC) of *Bletilla striata* polysaccharides[12]: Drug concentrations were prepared as MIC, 2 MIC, 4 MIC, 8 MIC *etc*., and test was carried out according to the method for MIC determination. After culturing for 18 or 24 h, a 100 µL aliquot was taken from each tube and inoculated on agar plates, after inoculation, the plates were again placed into a 37 °C thermostat shaker and statically cultured for 24 h, then the number of colonies on the plates was visually observed and the lowest drug concentration at which the number of colonies was less than 5 was considered as the MBC against the tested bacteria[13,14].

Drawing of the time-killing curves: The drug of 1/2 MIC, MIC, 2 MIC was taken in the test tube, meanwhile in the control group, drug was replaced by sterile distilled water and other conditions in the control group remain unchanged. Next, each tube was joined with *Staphylococcus aureus* bacteria solution with a concentration of 5×10^5 CFU/mL, then were placed in static culture at 37 °C. Make the sample tubes mixed sufficiently and dilute at 0, 2, 4, 6, 8, 12, 18, and 24 h, draw the bacteria-time curve with the number of viable bacteria with the plate method[14].

Scanning electron microscope (SEM) determination: Three conical flasks containing 30 mL of culture medium were taken and added with samples until the final concentrations of the samples in each flask were 12.50, 6.25 and 3.125 mg/mL, *i.e*., MBC, 1/2 MBC and 1/4 MBC, respectively, then 30 µL of logarithmic phase bacterial solution (concentration of 5 × 105 CFU/mL) was added to each conical flask, a fourth conical flask was added only with 30 µL of bacterial solution and 30 mL of culture medium, after incubation at a constant temperature incubator set at 37 °C for 18 h, the flasks were taken out and centrifuged (4000 r/min) at 4 °C for 10 min, the supernatant was discarded and the precipitate was washed with saline and centrifuged (conditions as above) three times, next, the precipitate was taken and mixed with 2.5 % glutaraldehyde (pH = 7.2, prepared with 0.1 mol/L phosphate buffer), pre-fixed, then fixed with 1 % osmic acid, dehydrated with gradient ethanol and isoamyl acetate, respectively, replaced with isoamyl acetate, critical point dried and sputter coated with gold, finally, the coated samples were observed under SEM and photographed[15,16].

Transmission electron microscope (TEM) determination: 1 mL of different concentrations of samples were added separately to 3 conical flasks containing 30 mL of culture medium until their final concentrations reached 12.50, 6.25 and 3.125 mg/mL, respectively, *i.e.*, MBC, 1/2 MBC and 1/4 MBC, then 30 µL of logarithmic phase bacterial solution (concentration of 5×10^5 CFU/mL) was added to each conical flask and a bacterial solution without drug was served as a negative control, the flasks were statically incubated at 37 °C for 18 h, then centrifuged. The supernatant was discarded and 2.5 % glutaraldehyde was added slowly along the centrifuge tube wall, followed by fixation at 4 °C for 1 h. After fixation, the samples were washed three times with 0.1 mol/L PBS buffer with each time lasting 10 min, fixed again with 1 % osmic acid at 4 °C for 1 h, then washed with 0.1 mol/L PBS buffer three times with each time lasting 10 min. The samples were dehydrated sequentially with 70, 80, 90 and 100 % acetone at 4 °C, dehydration lasted for 10 min for each concentration, after dehydration, the samples were replaced with propylene oxide for 10 min, soaked in Epon812 epoxy resin, embedded, polymerized, sliced, stained with uranyl acetate-lead citrate and finally, placed on TEM, observed and photographed[17,18].

Statistical Analysis: Data were analyzed Graphpad software (version 5.0). Differences among groups were performed using One-way ANOVA followed by Fisher's Least Significant Difference test. Differences of p values < 0.05 were considered statistically significant. Results are expressed as mean and standard deviation (SD).

RESULTS AND DISCUSSION

Standard curve (Fig. 1) was plotted with the absorbance value A as the ordinate and the concentration (µg/mL) of standard glucose solution as the abscissa and the regression equation is found to be: A = 0.0095C - 0.0078, (R^2 = 0.9992, n = 6), which indicated a good linear relationship within a range of 20-120 µg mL^{-1}.

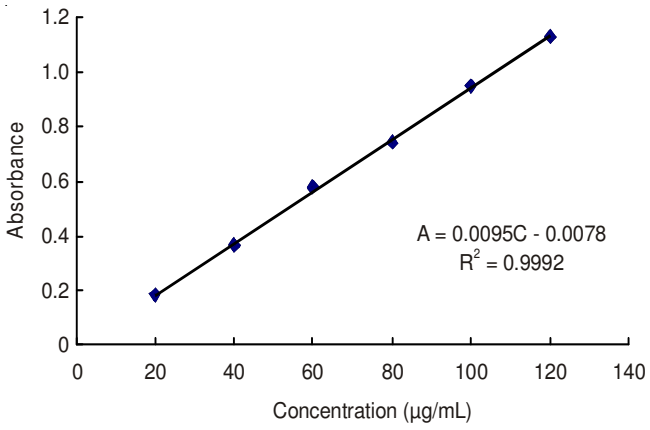

Fig. 1. Result of standard curve of glucose (UV)

Investigation of soaking time during the extraction of crude *Bletilla striata* polysaccharides: As can be seen from Table-1, polysaccharide content obtained was higher when soaking for 6 h prior to extraction, the content did not increased significantly when the soaking time was extended further, so taking into account the time, human production and other factors, pre-soaking time of 6 h should be adopted in the extraction.

TABLE-1
INVESTIGATION OF SOAKING TIME

Soaking time (h)	Content (%)	Extraction yield (%)
1	5.48	16.36
6	6.85	18.23
12	6.78	18.65

Investigation of extraction temperature during the extraction of crude *Bletilla striata* polysaccharides: As can be seen from Table-2, polysaccharide content was the highest at 90 °C; therefore, 90 °C was the optimal extraction temperature for crude *Bletilla striata* polysaccharides.

TABLE-2
INVESTIGATION OF EXTRACTION TEMPERATURE

Extraction temperature (°C)	Content (%)	Extraction yield (%)
70	6.27	16.21
80	6.64	16.72
90	7.54	19.48

Optimization of orthogonal test during the extraction of crude *Bletilla striata* polysaccharides: It can be seen from Tables-3 and 4 the intuitive analysis that the factors influencing the extraction of crude *Bletilla striata* polysaccharides in descending order were: A > B > C > D, *i.e.*, solvent amount > extraction time > extraction times; ANOVA results Table-5 showed that: factors A, B, *i.e.*, the influences of solvent amount and extraction time were significantly different. Integrating the three factors and taking into account the extraction efficiency, time consumption, costs and other factors, the extraction process conditions were optimized as $A_2B_3C_2$, *i.e.*, a 15-fold amount of water, two times of extraction under reflux, with each time lasting 3 h.

TABLE-3
LEVELS AND FACTORS OF EXTRACTION PROCESS

Factor	A	B	C
	Solvent amount (folds)	Extraction time	Extraction times
Level	10	1	1
	15	2	2
	20	3	3

TABLE-4
ORTHOGONAL TEST OF EXTRACTION PROCESS

Test No.	1 (A)	2 (B)	3	4 (C)	Extraction yield (%) y1	Total polysaccharide content (%) y2	Compreh. Scoring z
1	1	1	1	1	18.44	5.05	55.23
2	1	2	2	2	22.56	6.69	69.00
3	1	3	3	3	26.28	7.70	78.98
4	2	1	2	3	22.75	7.20	82.58
5	2	2	3	1	25.80	7.98	100.00
6	2	3	1	2	22.95	6.39	92.11
7	3	1	3	2	28.43	8.79	63.16
8	3	2	1	3	22.14	6.67	65.30
9	3	3	2	1	25.80	7.21	76.04
K1	67.403	66.657	70.547	74.757	–	–	–
K2	91.563	78.100	75.873	76.757	–	–	–
K3	68.167	82.377	80.713	75.620	–	–	–
R	24.160	15.720	10.166	2.000	–	–	–

Note: Z = (y1/y1max) × 30 + (y2/y2 max) × 70

TABLE-5
RESULT OF ANOVA

Factor	Sum of squares	Degrees of freedom	F ratio	Significance
Solvent amount	1131.692	2	187.459	*
Extraction time	396.358	2	65.655	*
Extraction times	6.037	2	1.000	

Note: $F_{0.05(2,2)} > 19.00$, $^*P < 0.05$

Influence of concentration multiple on extraction yield (%) during the purification of *Bletilla striata* polysaccharides: As can be seen from Fig. 2, the influence of the concentration multiple of *Bletilla striata* water extract on the yield of *Bletilla striata* polysaccharides increased with the increase of concentration multiple and has a sharply rise at 1:5. When the concentration multiple reached 1:6 of the original extract, the increase in polysaccharides yield slowed down, which was probably due to peeling on the surface of the concentrate, while the gum surface was hardly soluble and thus wrapped with floccules during the ethanol precipitation, thereby affecting the yield of *Bletilla striata* polysaccharides. So after comprehensive evaluation, the yield of *Bletilla striata* polysaccharides was found to be relatively high when the concentration ratio was 1:5.

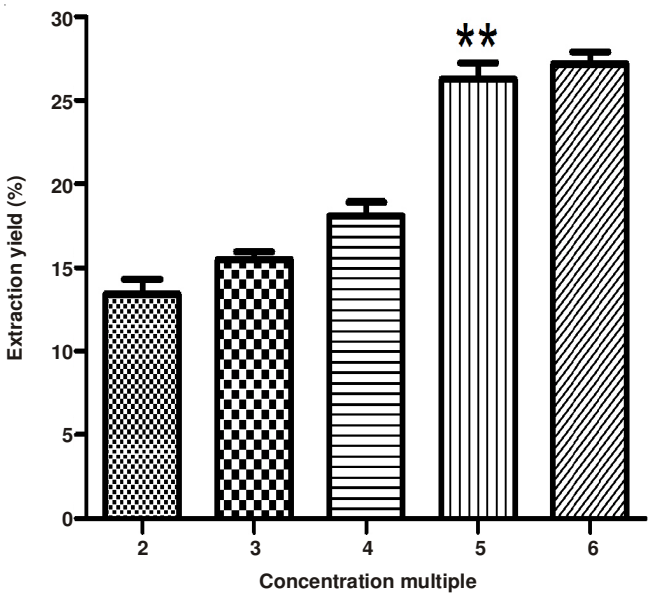

Fig. 2. Res ult of concentration multiple on the yield of *Bletilla striata* polysaccharides, **P < 0.01, *vs.* prior multiple

Influence of ethanol content on extraction yield (%) during the purification of *Bletilla striata* polysaccharides: As can be seen from Fig. 3, during the ethanol precipitation, the yield of *Bletilla striata* polysaccharides increased significantly when the ethanol content of *Bletilla striata* water extract concentrate was between 30-80 %, while little difference was noted between 80 and 90 %. After analysis and taking into account economic factors, an ethanol content of 80 % was considered most appropriate during the ethanol precipitation of *Bletilla striata* water extract concentrate.

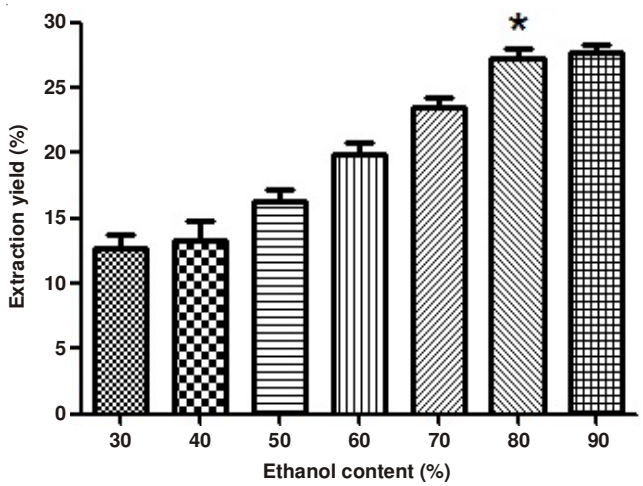

Fig. 3. Result of ethanol content on the yield of *Bletilla striata* polysaccharides, *P < 0.05, *vs.* prior content

MIC and MBC results of *Bletilla striata* polysaccharides: As can be seen from Table-6, the MIC, MBC of *Bletilla striata* polysaccharides of water extrat was 25.00, 50.00 mg/mL, meanwhile after ehanol precipition was 6.25, 12.50 mg/mL. against *S. aureus*, The result shows that the purified polysaccharides inhibitory effect was visibly stronger than the water extract. The smaller the MBC, the better the antibacterial activity.

TABLE-6
MIC AND MBC DETERMINATION RESULTS

Final drug concentration (mg/mL)	50.00	25.00	12.50*	6.25#	3.125	1.5625	0.78	0.39
Water extract	–	+	+	+	+	+	+	+
After ethanol precipitation	–	–	–	+	+	+	+	+

"–" indicates no colonies; "+" indicates the presence of colonies
"#" indicates MIC; "*" indicates MBC

Time-killing curves: The result showed that the more drug concentration, the better of the inhibition effect, the growth of the bacteria had a sharp increase at 4 h and entered into the platform stage at 6 h, then the drug became efficient (Fig. 4). The inhibition effect of the drag with the concentration of MIC and 2 MIC were evident and the bacteria would be killed by 2 MIC at 18 h.

SEM: The bacteriostatic effects of different concentrations of polysaccharides against *S. aureus* were observed by SEM, the result is shown in Fig. 5.

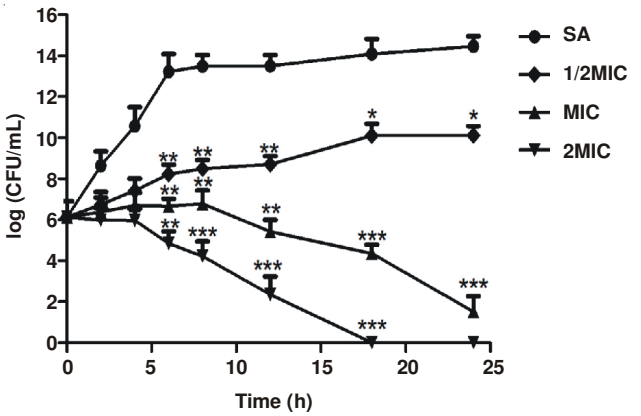

Fig. 4. Result of time-killing curves, *P < 0.05, **P < 0.01, ***P < 0.001, *vs.* SA

As can be seen from the SEM pictures, the surface of normal *S. aureus* was smooth and mellow and looked full, boundaries between cells were clear and cells were arranged in distinct grape-like clusters. After treatment with 1/4 MBC, the grape-like arrangement of bacteria became loose and the surface became rough. After treatment with 1/2 MBC, the *S. aureus* still maintained original grape-like shape, cells were swollen and cell surface was uneven. After treatment with MBC, the cell morphology was completely destroyed, cell walls began to break down and cell wall degradation products were bonded into blocks.

TEM: The bacteriostatic effects of different concentrations of polysaccharides against *S. aureus* were observed by TEM (Fig. 6).

The normal *S. aureus* were full in shape and uniform in size, cell walls and membranes were intact and smooth and cytoplasm was relatively uniform. After treatment with 1/4 MBC, septa began to form in the cells of *S. aureus*, cell wall structure was indistinct and cytoplasmic density was uneven, cells were swollen and enlarged probably due to the changes in cell membrane permeability. After treatment with 1/2 MBC, the cell wall surface of *S. aureus* was uneven, structure was partially destroyed and bacteriolysis occurred. After treatment with MBC, the cell wall structure of *S. aureus* was completely destroyed and cell structure collapsed. It shows that the antibacterial effect of drugs strengthened as the concentration increased, have a significant dose-dependent[19].

Bletilla striata polysaccharides were extracted by conventional water extraction and ethanol precipitation method. This method has followed advantages: Simple operation steps,

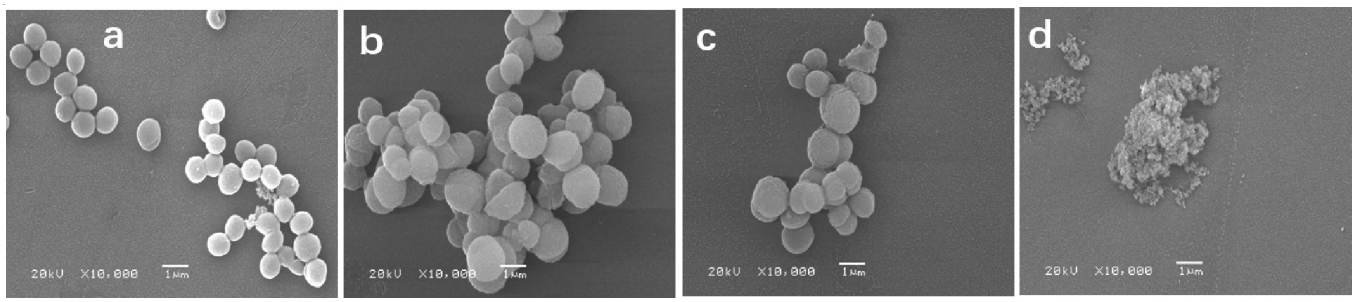

Fig. 5. SEM results: (a) Normal morphology of S. aureus 18 h (20 KX); (b) Morphology of bacteria after treatment (20 KX), the concentration of drug was 1/4 MBC; (c) Morphology of bacteria after treatment (20 KX), the concentration of drug was 1/2 MBC; (d) Morphology of bacteria after treatment (20 KX), the concentration of drug was MBC

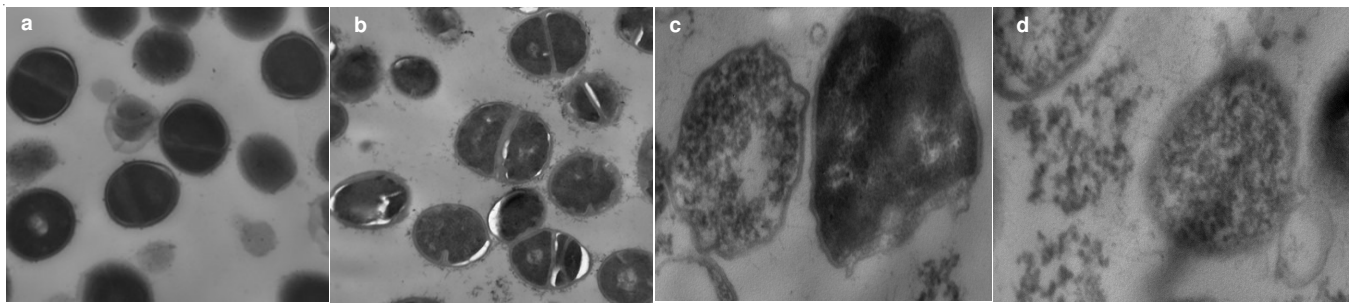

Fig. 6. TEM results: (a) Normal morphology of *S. aureus* 18 h (20 KX); (b) Morphology of bacteria after treatment (20 KX), the concentration of drug was 1/4 MBC; (c) Morphology of bacteria after treatment (20 KX), the concentration of drug was 1/2MBC; (d) Morphology of bacteria after treatment (20 KX), the concentration of drug was MBC

equipment costs are relatively inexpensive and can significantly improve the clarity of liquids, so it is widely applied in recent years. It is one of the best separation and purification technologies applied by the medicine industry in China. However there are a number of factors could have an impact on result of experiment. Therefore, integrally considering various factors, it ultimately determines that the experiment use the influence of single factor acted on soaking time and extraction temperature. Solvent volume, extraction time and extraction times were applied in the orthogonal design choice, as well as the influence of the solution concentration multiple and alcohol content were inspected during the purification experiment. The established extracting method made the *Bletilla striata* polysaccharides content relatively high, in addition the operation is simpler, more convenient and accurate.

In common, antibacterial activity screening methods include disk diffusion method, dilution method (micro dilution method and macro dilution method), E test, flow cytometry (FCM), *etc.*[20]. Chemical constituents of traditional Chinese medicine extract are complex, in addition ionic strength, pH, solubility, extracted color, *etc*. They have vital influence on the results[21]. Therefore the Chinese medicine determination is achieved by diffusion or dilution method. However, compared with diffusion method, dilution method is more effect in antibacterial effect caused by the deep penetration of the ingredients of Chinese medicine on agar[22]. Because of the broth microdilution method tested by a microplate reader, it is more suitable for herbal extracting, the samples which are water-soluble, lighter colorful[23] after dissolving. This method has multiple advantages as time-saving, low cost, high-throughput screening, automation, low detection limit.

In the study, test tubes dilution method and agar plate method were used in combination. It can get rid of the influence of the precipitation and color and measure MIC and MBC of tradition Chinese medicine extract effectively.

The plate count method was used by the time-killing curves, which can accurately distinguish the bacterial be inhibited or killed in a certain concentration at some time. As can be seen from Fig. 4, the drug restrained the growth of bacterial in the 1/2 MIC. However, bacterial restored and grew because of adaptation of the drug. The drug of MIC can inhibit the sustained growth of bacterial, but it cannot kill the bacterial completely. The 2 MIC of the drug, not only can inhibit the growth of bacterial, but also has bacterial action at 18 h. According to the result before, it proves that the drug concentration is the key factor for inhibit effect of bacterial and has a time-dependent. The bacterial activity would be more efficient caused by higher concentration and longer period.

The main mechanism of antimicrobial drugs mainly affect structure and function of the bacteria through interfere with the bacterial metabolism process and make it lose the ability of normal growth and reproduction. Eventually bacteria would be inhibited or killed. Currently, the key mechanism includes the inhibition of the synthesis of bacterial cell wall, the influence of the permeability of cell membrane, the suppression of the synthesis of DNA and RNA and the effect of metabolism of folic acid[24]. The related literature states that the antibacterial mechanism of traditional Chinese medicine extract is generally affect the permeability of cell membrane, destroy the structure of cell wall, so as to achieve antimicrobial activity[25]. The ultrastructure of bacteria can be observed more intuitive by means of the electron microscopy (SEM) pictures, in which a variety of ultrastructure changes of inside thallus like the cell wall, cell membrane and other organelles happening before and after medical treatment can be observed: the thickness of the bacteria cell wall, the extent of damage, the overflow volume of cytoplasm, the change of cell shape, etc. That it can recognize that the drug antibacterial mechanism further, which is a possibility that certain medicine induce to release of intracellular lysozyme ultimately resulting of cell autolysis[26,27]. Further investigation of its antibacterial mechanisms will be performed in future studies.

Staphylococcus aureus is the most important opportunistic pathogen for traumatic infection[28]. Drug resistance mechanisms are generated by inactivated enzyme of drug, biological membrane preventing drug penetration, active efflux, the change of drug targets and other mechanisms[29-34]. Therefore, searching and discovering new compounds to inhibit the drug resistance of bacteria become a problem to be solved urgently. Extracts from traditional Chinese medicine (TCM) is one of the common and effective methods. It commonly extracts and purifies the effective component of TCM. Then, the advanced compounds would be identified through the structure of the modification and synthesize and high-throughput screening which are useful element to develop into the new drugs.

Conclusion

In this study, conventional water extraction and ethanol precipition method was used to optimize the extraction process of *Bletilla striata* polysaccharides with polysaccharide content as an index through single factor investigation and $L_9(3^4)$ orthogonal table. The optimum extraction process for *Bletilla*

striata polysaccharides established in the experiment is simpler, more scientific and suitable. Additionally, antibacterial activity showed that *Bletilla striata* polysaccharides had an obvious bacteriostatic effect against *S. aureus*. The antibacterial mechanism may be related with the permeability of cell membrane.

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