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*Full Length Research Paper*

# Extraction, characterization and antitumor effect of the polysaccharides from star anise (*Illicium verum* Hook. f.)

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**Polysaccharides production from star anise was carried out. The effects of extraction temperature, time, ratio of water to raw material and number of extraction were optimized by using an orthogonal L9(3)4 test design. The optimum extraction conditions were determined as follows: extraction temperature 100°C, time 4 h, number of cycle 2 and ratio of water to raw material at 11. Under optimized conditions, the experimental yield 10.50 ± 0.08% agreed closely with the predicted yield. High Performance Liquid Chromatography (HPLC) and Infrared (IR) methods were used for qualitative and quantitative determination of the polysaccharides. HPLC analysis showed that the polysaccharides were composed of three kinds of monosaccharide, namely xylose, arabinose and glucose in molar ratios of 1:4.8:18.3. Pharmacological studies revealed star anise polysaccharides could inhibit the growth of Sarcoma 180 tumor *in vivo*. The tumor inhibition ratio of high dose polysaccharides (720 mg/kg) was 30.92%. No significant difference of spleen and thymus index was observed at three doses of the polysaccharides while compared with model control.**

**Key words:** Star anise, polysaccharides, extraction, characterization, antitumor effect.

## INTRODUCTION

Polysaccharides of plant sources have drawn the attention of biochemical and nutritional researchers in recent years due to their various biological activities. The value of polysaccharides and derivatives in food, agriculture and medicine has been well documented (Vierhuis et al., 2003; Shi et al., 2007; Cai et al., 2008). Polysaccharides offer healthy benefits, such as anti-cancer effects, immuno modulation, anti-bacterial and anti-cardiovascular disease effects (Sonoda et al., 1998; Deters et al., 2005). Similarly, polysaccharide-based treatment regimens have also been shown to be potent immune modulators (Han et al., 2009; Sun et al., 2009) and potentially new options for combating oxidative stress-mediated disorders. Therefore, polysaccharides from various sources have recently emerged as an important class of bioactive natural products (Dourado et al., 2004). Some plant polysaccharides have been commercially developed into important components of therapeutic drugs and skin care products (Deters et al., 2001).

Star anise (*Illicium verum* Hook. f.) fruits are produced on a medium-sized evergreen tree, native to southern China that is an important essential oil tree cultivated in China. The fruit is star-shaped and consists of eight to thirteen carpel joined centrally and is commonly used as a condiment in Chinese cuisine. Star anise is an ingredient of the traditional five-spice powder of Chinese cooking. The essential oil from star anise fruits is used in the confectionery trade to flavor licorice and other candies, in the baking trade to flavor cakes, cookies and biscuits and in the liqueur industry for flavoring anisette. It is a common flavouring for medicinal teas, cough mixtures and pastilles. Except for its usage in food, star anise is still a natural nutrient and a traditional Chinese medicine which may be especially useful for combating colic and rheumatism (Claus and Tyler, 1965). It also has

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carminative, stomachic, stimulant and diuretic properties. More recently, shikimic acid, extracted from star anise, is one of the chief ingredients in the antiviral Tamiflu drug used to fight avian influenza. Due to its many health effects, great attention has been paid to star anise. Recent studies have shown that star anise possesses antioxidant activities (Kim and Kim, 2003) and significant anti-carcinogenic potential (Yadav and Bhatnagar, 2007). Star-anise powder, its ethanol/water (80:20)-soluble fraction and essential oil showed strong antioxygenic activity (Padmashree et al., 2007). The treatment with star anise can reduce the tumor burden, lower oxidative stress and increase the level of phase II enzymes, which may contribute to its anti-carcinogenic potential. The extensive modern pharmacological studies on this plant are mainly on its essential oil (Stahl and Gerard, 1985; WHO and Institute of Materia Medica, 1990; Dang and Sarath, 1997; Liu et al., 1997) and shikimic acid (Treanor et al., 2002; Richard and Michael, 2005) portion. However, so far there is not any information published about star anise polysaccharides extraction technology and its antitumor activity research. Thus, extraction, characterization, evaluation and pharmacologically activities of polysaccharides from star anise, have become a challenge. In this study, we took orthogonal experiment to optimize the best star anise polysaccharides extraction conditions for getting high yield and quality bioactive polysaccharides. Chemical characterization and antitumor activity in S180 tumor mice of the complex water-soluble polysaccharides were evaluated.

## MATERIALS AND METHODS

### Plant materials

Star anise fruits were purchased from Nanning (Guangxi, China) in May, 2009. The plant was authenticated by College of Pharmacy, Guangxi traditional Chinese Medical University, where voucher specimen has been deposited.

### Extraction procedure

Dried star anise fruits was ground in a high speed disintegrator to obtain a fine powder (Particle diameter size: 300 – 400 μm) and then was extracted with petroleum ether at 60°C for 4 h to remove some colored materials, oligosaccharides and some small molecule materials under reflux in the apparatus. The organic solvent was separated by centrifugation (4000 r/min, 20 min) and pretreated powder was obtained. Each dried pretreated sample (20 g) was extracted with water (star anise–water (g /ml) ranging from 1:9 to 1:14), while the temperature of the water bath ranged from 60 to 100°C and was kept steady (within ±1.0°C), extraction times ranged from 1 to 5. The water-star anise slurry was stirred with magnetic force for a given time (extraction time ranging from 1 to 5 h) during the entire extraction process. After centrifuging to remove insoluble residue (4000 r/min, 20 min), the water extraction solution was concentrated in a rotary evaporator under reduced pressure at 50°C. The concentrated extract was precipitated at 4°C for 24 h by the addition of anhydrous ethanol to a final concentration of 75% (v/v). The precipitate was collected by centrifugation (4000 r/ min, 20 min) and then vacuum-dried at 40°C to afford crude polysaccharides.

### Estimation of polysaccharides amount

Polysaccharides amount was measured by employing sulphuric acid–phenol method (Dubois et al., 1956). Thirty milligrams polysaccharide of star anise was weighed and dissolved in a capacity bottle with a small amount of water first and then to the final scale of 100 ml. Exactly 1.0 ml of the stored solution was taken out and mixed with 1 ml 5% phenol and 5 ml concentrated H2SO4. The mixture was placed at room temperature for 5 min and then kept in boiling water for 15 min. After cooled with running tap water, absorbance was measured at 486 nm by using a SHIMADZU UV-2550 UV-visible spectrophotometer. Then content of sample extract was calculated by the calibration curve of glucose. Extraction yield of polysaccharides was calculated as follow:

Extraction yield (%) = (polysaccharides weight / raw material weight) × 100%.

### Optimization of polysaccharides extraction

An orthogonal L9 (3)4 test design was used to investigate the optimal extraction condition of polysaccharides from star anise. As seen from Table 1, the extraction experiment was carried out with 4 factors and 3 levels, namely extraction temperature (80, 90 and 100°C), extraction time (3, 4 and 5 h), number of extraction (1, 2 and 3) and water to raw material ratio (11, 12 and 13). The range of each factor level was based on the results of preliminary experiments. The yield (%) of polysaccharides was the dependent variable.

### Infrared (IR) analysis

The polysaccharides were characterized by the FTIR spectrum. Infrared analysis of the sample was obtained by grinding a mixture of polysaccharides with dry KBr and then pressing in a mold. The IR spectrum was determined using a SHIMADZU FTIR-8400S Fourier transform infrared spectrophotometer. Spectra were run in the 4000 – 400 $cm^{-1}$ region.

### Hydrolysis of polysaccharide

174 mg of polysaccharide sample was dissolved in 10 ml of 2 M H2SO4 in an ampoule. The ampoule was sealed under a nitrogen atmosphere and kept in boiling water bath to hydrolyze the polysaccharides into component monosaccharide for 10 h. After being cooled to room temperature, the reaction mixture was neutralized with $BaCO_3$ and then centrifugated at 3000 rpm for 5 min. The supernatant was collected. 200 μl hydrolyzed and neutralized sample solutions were added with 5 ml distilled water and filtrated through a 0.45 μm syringe filter then ready for High Performance Liquid Chromatography (HPLC) experiments.

### Preparation of high performance liquid chromatography monosaccharide standard solution

Standard solutions (5.0 mg/ml) were prepared by dissolving each standard monosaccharide in deionized water. The solutions were

**Table 1.** $L_9(3)^4$ orthogonal design and results.

| No. | A, extraction temperature (℃) | B, extraction time (h) | C, ratio of water to raw material | D, number of extraction | Extraction yield (%) |
|---|---|---|---|---|---|
| Level 1 | 80 | 3 | 11 | 1 | |
| Level 2 | 90 | 4 | 12 | 2 | |
| Level 3 | 100 | 5 | 13 | 3 | |
| 1 | 1 | 1 | 1 | 1 | 5.62 |
| 2 | 1 | 2 | 2 | 2 | 7.26 |
| 3 | 1 | 3 | 3 | 3 | 6.86 |
| 4 | 2 | 1 | 2 | 3 | 8.40 |
| 5 | 2 | 2 | 3 | 1 | 6.61 |
| 6 | 2 | 3 | 1 | 2 | 8.68 |
| 7 | 3 | 1 | 3 | 2 | 9.65 |
| 8 | 3 | 2 | 1 | 3 | 9.85 |
| 9 | 3 | 3 | 2 | 1 | 7.46 |
| K1 | 6.580 | 7.890 | 8.050 | 6.563 | |
| K2 | 7.897 | 7.907 | 7.707 | 8.530 | |
| K3 | 8.987 | 7.667 | 7.707 | 8.370 | |
| R | 2.407 | 0.240 | 0.343 | 1.967 | |

K1, K2, K3 is the average extraction yield of level 1, 2 and 3, respectively. R refers to the result of extreme analysis.

filtered through a 0.45 μm syringe filter and were degassed using an ultrasonic bath for 2 min prior to use. All the solutions prepared were stored in the dark at 4℃ until being used.

### High performance liquid chromatography conditions for monosaccharide compositions analysis

The monosaccharide compositions were analyzed by HPLC system equipped with a waters 510 HPLC pump, a Rheodyne7725i injector, a 20 μl sample loop, a SHIMADZU RID-6A refractive index detector and a waters temperature control module. The analytical column used was a NH2 column (4.6 mm i.d.× 250 mm, 5 μm, Kromasil, China). Elution was carried out at a flow rate of 1.0 ml/min at 30℃. The mobile phase was CH3CN: H2O=72:28 (v/v). The injection volume was 15 μl. Data processing was performed using WMAD9010 software. Each monosaccharide was identified using the standard monosaccharide and quantified by the calibration curve.

### Tumor transplanted animals and treatment

Fifty six-week-old Kunming mice weighing 20 ± 2 g were provided by the Laboratory Animal Center of Guangxi Medical University (Nanning, China. Quality Certificated Number: SCXK2009－0002). The mice were housed under normal laboratory conditions, that is, room temperature, 12/12 h light –dark cycle with free access to standard rodent chow and water. Fifty mice were randomly divided into five groups, each group consisting of 10 animals. Seven-day-old Sarcoma180 (S180) ascites (0.2 ml, 1×1010cells) were transplanted subcutaneously into the left axilla of each mouse. Three of the S180-inoculated groups were administered by gavage with 80, 240, or 720 mg/kg b.w. star anise polysaccharides daily for 14 days. A fourth model control group received 0.4 ml saline alone. The fifth group of S180-bearing mice served as the cytoxan positive group (cytoxan 50 mg/kg b.w. intraperitoneal injection).

### Tumor inhibition ratios, the index of thymus and spleen

Twenty-four hours after the last drug administration, mice were sacrificed by cervical dislocation. Tumor weights in the mice were measured. The antitumor activity *in vivo* was expressed as an inhibitory rate calculated as [(A-B)/A] ×100%, where A and B were the average tumor weight of the model control and experimental groups, respectively. The spleen and thymus of the mice were also removed and weighed to obtain the index of the spleen and thymus. The thymus and spleen indices were assayed according to the method (Zhang et al., 2003) and calculated according to the following formula:

Thymus or spleen index = weight of thymus or spleen (mg) / body weight (g)

### Statistical analysis

All values are expressed as mean ± SD. Data were analyzed with ANOVA followed by Dunnett's test using SPSS/13.0 software. The results were evaluated in 95% confidence interval and significant differences set at $P < 0.05$.

## RESULTS AND DISCUSSION

### Effect of different temperature on extraction yield of polysaccharides

The increase of the polysaccharides diffusion coefficient and the enhanced solubility of the polysaccharides in the extracting solvent at higher temperatures caused the increase of the polysaccharides mass going out from the star anise particles into the solution (Li et al., 2006). The

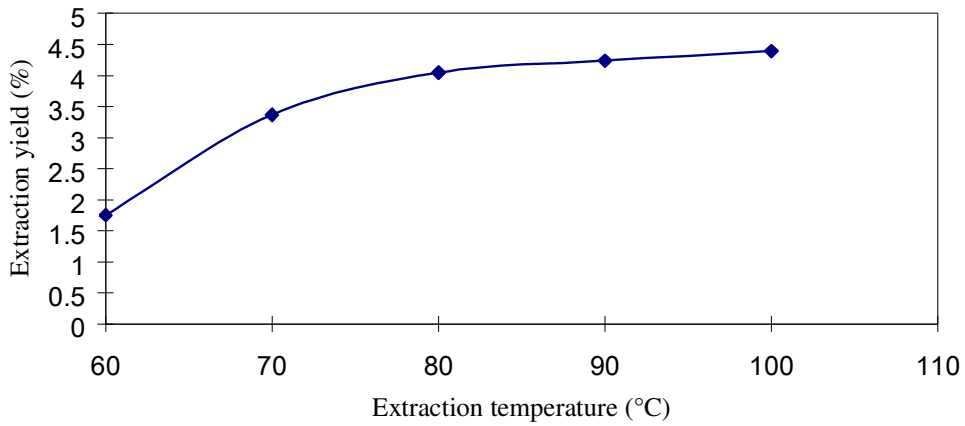


**Figure 1.** Effect of different extraction temperature on extraction yield of polysaccharides.

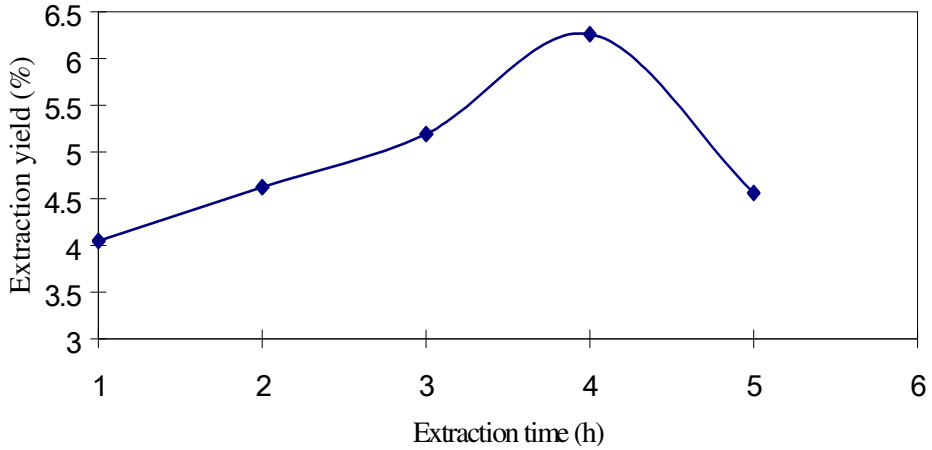


**Figure 2.** Effect of extraction time on extraction yield of polysaccharides.

extraction coefficient increased with increasing the extraction temperature due to the increase of the polysaccharides solubility (Braga et al., 2006). The yield of star anise polysaccharides affected by different extraction temperature (60 - 100℃) was seen in Figure 1, when other three factors (extraction time, number of extraction and water to raw material ratio) were fixed at 2 h,1 and 9. The extraction yield of polysaccharides increased with the increasing extraction temperature and reached the maximum value (4.24 ± 0.15%) when extraction temperature ranging from 80 to 100℃.

## Effect of different time on extraction yield of polysaccharides

Extraction time is another factor that would influence the extraction efficiency and selectivity of the fluid. A longer extraction time also presents a positive effect on the yield of polysaccharides. It was reported that a long extraction time favors the production of polysaccharides (Liu et al., 2006). The extraction yield of polysaccharides affected by different extraction time (1 - 5 h) was seen in Figure 2, when other three factors (extraction temperature, number of extraction and water to raw material ratio) were fixed at 100℃, 1 and 9. The extraction yield of polysaccharides increased with the increasing extraction time and reached the peak value 6.26% at 4 h and then dropped from 4 to 5 h.

## Effect of different ratio of water to raw material on extraction yield of polysaccharides

The effect of different ratio of water to raw material on extraction yield of polysaccharides was shown in Figure 3. Extraction was carried out at different ratio of water to raw material (9 -14) conditions, when other three factors (extraction time, temperature and number of extraction) were fixed at 4 h, 100℃ and 1. The extraction yields of the polysaccharides significantly increased from 3.14 to 6.11% as the ratio of water to raw material increased from 9 to 12 shown in Figure 3, due to the increase of the

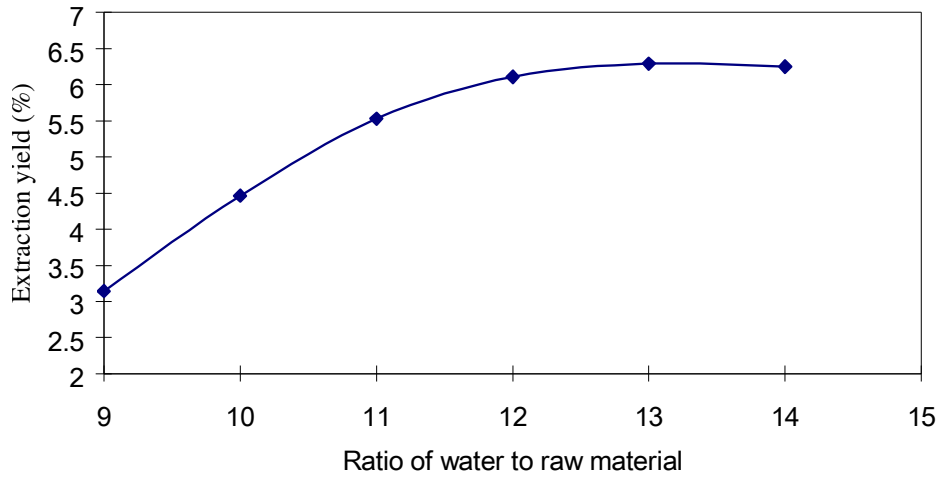


**Figure 3.** Effect of ratio of water to raw material on extraction yield of polysaccharides.

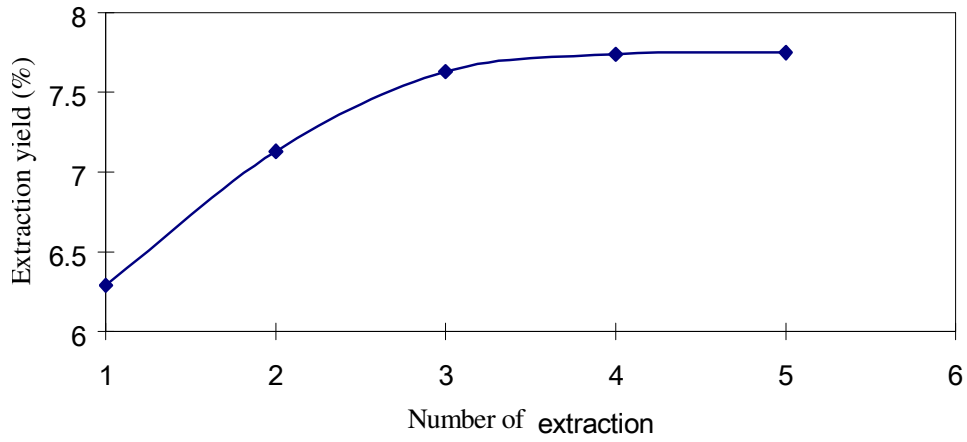


**Figure 4.** Effect of number of extraction on extraction yield of polysaccharides.

driving force for the mass transfer of the polysaccharides (Bendahou et al., 2007). However, when the ratio continued to increase, the extraction yields no longer changed.

## Effect of number of extraction on extraction yield of polysaccharides

The effect of number of extraction on extraction yield of polysaccharides was shown in Figure 4. Extraction was carried out at different number of extraction (1 - 5) conditions, when other three factors (extraction time, temperature and water to raw material ratio) were fixed at 4 h, 100℃ and 13. The extraction yield of the polysaccharides got the critical value 7.63% when the samples were extracted for 3 times. And then there was a little increase when extracted exceeded 3 times. So in this study, we adopted extraction time of 3–5 h, extraction temperature of 80 -100℃, extraction number of 1 - 3 times and water to raw material ratio of 11 - 13 for further study objects in the orthogonal test design experiment.

## Optimization of the extraction parameters

The first step in the extraction parameters of polysaccharides from star anise is to optimize the operating conditions to obtain an efficient extraction of the target compounds and avoid the co-extraction of undesired compounds such as fatty acids and their esters. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a solvent extraction method. In fact, the extraction temperature, extraction time, ratio of water to raw material and number of extraction are generally considered to be the most important factors. Optimization

**Table 2.** Effect of star anise polysaccharides on tumor growth.

| Group | Dose (mg/kg) | Body weight (g)[a] | | Tumor weight (g)[a] | Inhibition ratios (%) |
|---|---|---|---|---|---|
| | | Before treatment | after treatment | | |
| Model group | - | 20.61 ± 1.74 | 27.16 ± 3.70 | 1.52 ± 0.46 | - |
| Cytoxan | 50 | 20.51 ± 1.75 | 25.58 ± 1.63 | 0.85 ± 0.22* | 44.08 |
| Polysaccharides | 80 | 20.38 ± 1.68 | 26.44 ± 2.12 | 1.28 ± 0.58 | 15.79 |
| Polysaccharides | 240 | 20.16 ± 1.98 | 28.08 ± 3.52 | 1.24 ± 0.42 | 18.42 |
| Polysaccharides | 720 | 20.30 ± 1.73 | 27.43 ± 4.49 | 1.05 ± 0.55* | 30.92 |

a: The data were presented as means ± S.D. ($n$ = 10), *P < 0.05, significantly different from the model group.

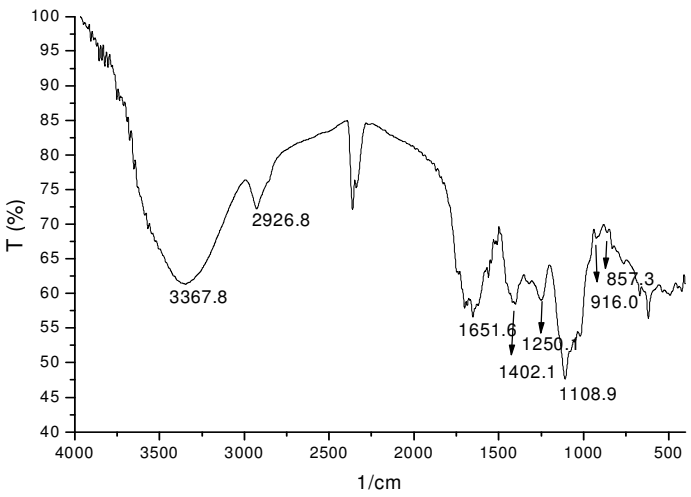


**Figure 5.** IR of polysaccharides.

of the suitable extraction conditions in the polysaccharides extraction can be carried out by using an experimental design. In the present study, all selected factors were examined using an orthogonal L9(3)4 test design. The results of orthogonal test and extreme difference analysis were presented in Table 1. In accordance with the results of the orthogonal layout, taking all the influencing factors and the results into consideration, we can find that the influence to the extraction yields decreases in the order: A > D > C > B according to the R values. The extraction temperature was found to be the most important determinant of the yield. The optimum conditions were considered as A3B2C1D2 according to the K values of each level, namely, extraction temperature 100℃, extraction time 4 h, ratio of water to raw material 11 and number of extraction 2 times. Through confirmatory test, we get the high yield polysaccharides, with a yield of 10.50 ± 0.08%.

## Infrared (IR) spectrum

Figure 5 showed the IR spectrum of the polysaccharides in the range of 4000 - 400 $cm^{-1}$. A broad stretching characteristic peak was shown at around 3367.8 $cm^{-1}$ for hydroxyl (OH) group, and a C-H stretching band was observed at 2926.8 $cm^{-1}$ (Santhiya et al., 2002). The peaks between 1400~1200 $cm^{-1}$ also indicated the bending vibration of aliphatic C-H bonds. The absorption bands at 1651.6 and 1108.9 cm-1 was attributed to the stretching vibration of the C–O bond of carboxyl group. The band at 857.3 $cm^{-1}$ was ascribed to α-type glycosidic linkages in the polysaccharide (Barker et al., 1954). The bands at 857.3 and 916.0 $cm^{-1}$ were characteristic of (1→4)-α-glucan (Li et al., 2008).

## Analysis of monosaccharide compositions

The chemical composition of the polysaccharides was measured by a HPLC. On the basis of HPLC of standard samples, HPLC analysis showed the polysaccharides was composed of three kinds of monosaccharide, namely xylose, arabinose and glucose in molar ratios of 1:4.8:18.3.

## Effect of star anise polysaccharides on tumor growth

The inhibitory effect of star anise polysaccharides on the growth of transplantable S180 tumor in mice was shown in Table 2. The average tumor weights of the three polysaccharides groups ranged from 1.05 to 1.28 g lower than that of the model control group (1.52 g). As a positive control, CTX had significant inhibitory effect on the growth of S180 compared with the model control group (P < 0.05) and showed high inhibitory rate of 44.08%. The growth of transplantable S180 in mice was also significantly inhibited by high dose star anise polysaccharides (720 mg/kg, P < 0.05), with the inhibitory rate being 30.92%. However, the treatment with low and middle dose (80 and 240 mg/kg) had no significant effect on the inhibitory response (P > 0.05) with inhibitory rate of 15.79 and 18.42%, respectively. In addition, no signs of toxicity were observed in the mice treated with star anise polysaccharides on base of body weight (P > 0.05).

**Table 3.** Effect of star anise polysaccharides on thymus and spleen index.

| Group | Dose (mg/kg) | Spleen weight (mg) | Thymus weight (mg) | Spleen index (mg/g) | Thymus index (mg/g) |
|---|---|---|---|---|---|
| Model group | - | 243 ± 83 | 119 ± 36 | 8.95 ± 2.39 | 4.38 ± 1.15 |
| Cytoxan | 50 | 133 ± 46 | 27 ± 17 | 5.19 ± 1.83* | 1.05 ± 0.23* |
| Polysaccharides | 80 | 227 ± 91 | 100 ± 38 | 8.44 ± 3.11 | 3.76 ± 1.40 |
| Polysaccharides | 240 | 272 ± 91 | 78 ± 43* | 9.97 ± 3.69 | 3.06 ± 1.46 |
| Polysaccharides | 720 | 218 ± 106 | 99 ± 24 | 7.71 ± 2.52 | 3.72 ± 1.22 |

The data were presented as means ± S.D. (n = 10), *P < 0.05, significantly different from the model group.

### Effect of star anise polysaccharides on thymus and spleen index

As was shown in Table 3, significantly decreased thymus and spleen index were observed at the cytoxan positive group (P < 0.05). No significant difference of spleen and thymus index was observed at three doses of the polysaccharides while compared with model control group (P > 0.05).

## Conclusion

No report is so far available in the literature regarding the polysaccharides extraction from star anise. In this work, the performance of the polysaccharides extraction from star anise was studied with a statistical method based on orthogonal L9(3)4 test design. The optimal conditions include the following parameters: extraction temperature 100°C, extraction time 4 h, ratio of water to raw material 11and number of extraction 2. Results of HPLC indicated that the polysaccharide was composed of xylose, arabinose and glucose in molar ratios of 1:4.8:18.3. Preliminary pharmacological tests suggested that the polysaccharides could inhibit the growth of Sarcoma 180 tumor in vivo. The tumor inhibition ratio of high dose polysaccharides (720 mg/kg) was 30.92%. No significant difference of spleen and thymus index was observed at three doses of the polysaccharides while compared with model control. The results obtained in the study will provide basic data for further development and utilization of star anise.

## ACKNOWLEDGEMENTS

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