Composition and bioactivity of polysaccharides from tea seeds obtained by water extraction

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In this paper, the composition and biological activities of polysaccharides from tea seed (TSPS) obtained by water extraction were investigated. The properties and chemical compositions of TSPS were analyzed with HPGPC, IC, and IR methods. The results showed that TSPS consisted of three kinds of polysaccharides with the molecular weight of 500 kDa, 130 kDa, and 5 kDa. TSPS consisted of rhamnose, xylose, arabinose, glucose and galactose, GalA, GaLa, with a molar ratio of 4.9:1.7:11.1:27.2:14.0:3.4:1. sugar backbone of TSPS might consist of glucose, but branched chain may consist of rhamnose, xylose, arabinose, and galactose. The IR spectrum of TSPS revealed the typical characteristics of polysaccharides and protein. TSPS significantly inhibited the growth of K562 cells, especially, at the concentration of 50 μg/ml; the inhibition activity of TSPS was the highest with an inhibition ratio beyond 38.44 ± 2.22% (P<0.01). TSPS with high concentrations (100, 200 and 400 μg/ml) had higher proliferation effect on lymphocyte. Results of these studies demonstrated that the polysaccharide had a potential application as natural antitumor drugs.

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1. Introduction
Green tea (Camellia sinensis) has been used as the second most consumed beverage for thousands of years in the world next to water and has caused great interests among researchers [1–3]. Tea polysaccharides from different sources, such as tea flowers and tea leaves, have been found to be an important water soluble polysaccharide with certain bioactivities in the late 1980s [4–6]. Including immunostimulation [7,8], antitumor [9], antioxidant activities [10], anti-inflammatory [11], hypoglycemic activities [12], etc. All these activities are due to their contribution to enhance immune function of the human body.

Tea seed polysaccharides (TSPS) are the main effective components in tea seeds, accounting for a comparative large proportion. In this study, tea seeds contained many nutrition compounds, such as protein, sugar, sucrose, vitamin, amino acid, tea polyphenols and caffeine. From this point, tea seeds are also of important application value as leaves and flowers. For a long time, however, there are very few studies about the tea seed polysaccharides (TSPS). Recently, the tea leaves polysaccharide (TPS) and tea flower polysaccharide (TFPS) has attracted great interest among researchers [13–15]. The studies and application of TSPS are also becoming valuable.

2. Materials and methods
2.1. Materials and reagents
Tea seeds were obtained commercially from Hubei province of China. D-Ribose (Rib), L-rhamnose (Rha), D-arabinose (Ara), L-fucose (Fuc), D-xylose (Xyl), D-mannose (Man), D-glucose (Glc), D-galactose (Gal), D-galacturonic acid (GalA) and bovine serum albumin (BSA) were purchased from Sigma (MO, USA). 1640 cell culture medium, Coomassie brilliant blue G-250 were provided from Sinopharm Chemical Reagent Co. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double distilled water.

2.2. Analytical methods of components in tea seed polysaccharides
The total sugars were determined by the phenol–sulphuric acid method [16] with d-glucose as standard. The soluble protein (SP) was determined by the Coomassie brilliant blue G-250 method [17] with bovine serum albumin as a standard. Uronic acid content was determined according to a meta-hydroxydiphenyl colorimetric method [18] with galacturonic acid as standard.

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2.3. Preparation of crude polysaccharides

The outer covering of tea seeds was removed and the kernels of tea seeds were ground into powder. The dry ground tea seeds (80 g) were treated by aqueous vapor for 20 min and extracted with Na-citric acid buffer (pH 4.0) in bath at 55 °C, and the solution were extracted with 0.5% (m/v) enzyme solution at 55 °C for 6 h. After centrifugation, the oil phase was extracted by petroleum ether and the extraction phase was demulsificated. After vacuum distillation, the oil and the tea seeds meal were obtained, respectively. Tea seeds meal and distilled water were put into Beaker at a proportion of 1:6 (v/v). The extraction performed at 70 °C for 1.5 h, respectively. Then the combined filtrate solution was centrifuged to remove the contaminants. The supernatant was concentrated and precipitated with 75% alcohol, and then the precipitation was dissolved with water and dialyzed to remove the small molecules. The dialyzed solution was freeze-dried to yield polysaccharides powder.

2.4. Determination of molecular weight—HPGPC

The molecular weight of TSPS was determined by HPGPC method, which was performed on a Shodex SB-804 HQ GPC column (300 mm × 8 mm) with a Shodex SB-G guard column (50 mm × 6 mm) from Showa Denko K.K. (Tokyo, Japan). The mobile phase was 0.02 M phosphate buffer solution and the flow rate was 0.3 ml/min. The column temperature was 50 °C. Samples (10 mg) were dissolved in 1 ml of 0.02 M phosphate buffer solution and centrifuged at 16,000 r/min for 10 min to give the supernatant, then 20 μl supernatant was injected for HPGPC analysis. The molecular weight was calculated by the calibration curve obtained by using various standard dextrins with different molecular weight (T3, T6, T10, T40, T100, T500, and T1000).

2.5. Analysis of monosaccharide composition

Polysaccharides samples (2 mg) were dissolved in 4 ml of 2 mol/l trifluoroacetic acid solution (TFA) and hydrolyzed at 110 °C for 6 h. The hydrolysate of TFA was evaporated by drying under reduced pressure. Then, TFA was removed by washing with methanol (3 ml) four times in order to remove TFA absolutely. The dried hydrolysates were dissolved with ultra-pure water and diluted to 100 ml, and then measured by diluting 10-fold again [19]. Ion chromatography (IC) was used for the identification and quantification of monosaccharide. IC experiment was performed on a Dionex ICS2500 chromatographic system (CA, USA) with a Dionex pulsed amperometric detector equipped with an Au electrode, a Dionex Carbobap PA20 column (150 mm × 3 mm). The temperature was kept at 30 °C and the injection volume was 25 μl. NaOH solution (2 mmol/l) were used as eluents at a flow rate of 0.45 ml/min. D-Fuc, D-GalA, D-GluA, D-Man, D-Xyl, D-Rib, D-Glu, D-Gal, D-Fru, L-Rha, and L-Ara were used as references.

2.6. IR spectroscopy

The IR spectrum of TSPS was recorded with a Nicolet 5700 IR spectrometer. The sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FT-IR measurement in the frequency range of 4000–400 cm⁻¹ (mid infrared region).

2.7. The inhibition ratio of K562 tumor cells

The inhibition effects of TSPS on the K562 human leukemic cells were evaluated in vitro using MTT assay [20]. The K562 cells were incubated on a 96-well cultivation plate at a concentration 1 × 10⁶ cells/ml. Each well was inoculated with 100 μl RPMI1640 media supplemented with 10% fetal bovine serum solution containing the K562 cells and 20 μl samples (at concentrations of 0.5, 5, 25, 50, 100, 200, and 400 μg/ml, respectively) under an atmosphere of 5% CO₂ at 37 °C for 24 h. The tumor cells were continuously incubated for another 4 h after 10 μl MTT (5 mg/ml) had been added. The supernatant was removed by centrifugation, and then 100 μl of DMSO was added to terminate the reaction. Then MTT colorimetric method was used to observe the effect of growth inhibition of K562 tumor cell induced by TSPS. The sample groups were compared with control groups in the absence of the tested samples. All in vitro results were expressed as the inhibition ratio (A) of tumor cell proliferation as $A = (1 - Nt/Nc) \times 100\%$, where Nc and Nt are the average number of viable tumor cells of the control group and test group, respectively.

2.8. Determination of immunological activity in vitro

The spleen was removed under aseptic condition, chopped and washed through screen mesh (200 meshes) with sterile normal saline and centrifuged at 2000 r/min for 5 min for three times. The precipitation of spleen cells were suspended with 1 ml of complete culture medium and seeded at 2 × 10⁶ cell/ml per well into 96 well plates. Some wells of the cell culture were added with tea seed polysaccharides. The cells were incubated at 37 °C for 72 h in a humidified atmosphere of 5% CO₂ in air. 10 μl of MTT (5 mg/ml) was added into the cell culture per well at the 68th h, incubated continuously for the rest 4 h. After incubation for 72 h, 100 μl of 10% SDS was added in per well and mixed thoroughly to dissolve the dark blue crystals. The plate was kept overnight at room temperature. On the next day, the plate was read with an ELISA reader, using test wavelength of 570 nm.

3. Results and discussion

3.1. Main chemical components of TSPS

The main chemical components in tea seeds, tea leaves and tea flowers were determined and shown in Table 1. As shown in Table 1, the tea seeds contained similar nutrients as tea leaves and tea flowers. However, the total tea polyphenols in seeds were less than that of leaves and flowers.

The tea seeds same as leaves can be used as medical and health food.

3.2. Molecular weight determination of TSPS

The distribution of molecular weight of TSPS was studied. As shown in Fig. 1, the components of TSPS mainly consisted of three kinds of polysaccharides with the molecular weight of 500 kDa, 130 kDa, and 5 kDa, respectively. The average molecular weights of homogeneous polysaccharides from tea were about 10–130 kDa according to most of the previous studies. Chen et al. [21] also found that the molecular weight distributions of polysaccharides were decreased from 3.8 to 251.5 kDa with the fermentation of the tea from green tea. The molecular weight of TSPS obtained here was much larger than tea polysaccharides according to above literatures, the difference could come from the different extraction methods or raw materials with different source.
3.3. Monosaccharide composition of TPS

The monosaccharide composition of TPS was shown in Fig. 2. Ion chromatography of TPS showed that TPS was consisted of rhamnose, xylose, arabinose, glucose and galactose, GaLA, GulA, with a molar ratio of 4.9:1.7:11.1:27.2:14.0:3.4:1, but the contents of glucose was very higher. As shown in Fig. 2, the main monosaccharide of TPS was glucose.

3.4. Infrared spectra of TPS

The IR spectrum of TPS was shown in Fig. 3. The absorption bands within the range of 3600–3200 cm⁻¹, 3000–2800 cm⁻¹, 1800–1500 cm⁻¹ and 1200–1000 cm⁻¹ were the characteristic absorption peaks of polysaccharides. IR spectrum of TPS exhibited a broadly stretched intense peak at 3298.99 cm⁻¹ which was hydroxy stretching vibration and that the peaks at 2960.98 cm⁻¹, 1408.61 cm⁻¹, and 1243.60 cm⁻¹ corresponded to a weak C–H stretching vibration. The peak at around 1660 cm⁻¹ was attributed to N–H vibration or C–O asymmetric vibration of carboxyl group, which was also the characteristic IR absorption of polysaccharide.

3.5. Effect of TPS on K562 cell inhibition

After incubated with TPS for 24 h at the concentrations from 0.5 to 400 μg/ml, the inhibition ratio of K562 cells was observed and compared with control. The inhibition ratios of TPS in different concentration against human leukemic K562 cells were summarized in Fig. 4. At the concentrations from 0.5 to 400 μg/ml, TPS significantly inhibited the growth of K562 cells, the inhibition activity of TPS at the lower concentration less than those at higher concentration.

Especially, at the concentration of 50 μg/ml, the inhibition activity of TPS was the highest with an inhibition ratio beyond 38.43 ± 2.22%. From above, TPS significantly inhibited the growth of human leukemic K562 cells.

Fig. 1. The molecular weight of TPS.

Fig. 2. (A) Ion chromatograms of 11 neutral monosaccharides (1) fucose, (2) rhamnose, (3) arabinose, (4) galactose, (5) glucose, (6) xylose, mannose, (7) mannose, (8) fructose, (9) d-ribose, (10) d-GaLA, (11) d-GluA. (B) Ion chromatogram of TPS.

Fig. 3. The IR spectrum of TPS.

Fig. 4. The inhibition ratio of human leukemic K562 cells in vitro by TPS at different concentrations (X ± S).
The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular mass, structure, conformation, even the extraction and isolation methods [22,23]. The results suggested the antitumor activity of polysaccharide might act through the activation of host immune response to stimulating T-cell subsets and cytokine (TNF-α and IFN-γ) production to participate in the antitumor effects. In addition, it might be achieved by enhancing the immune system functions. However, further investigation about the relationship between immunomodulation and antitumor activity is needed.

3.6. Immunological activity of TSPS

Cell culture method was adopted to determine the effect of TSPS on mice lymphocyte in vitro. The lymphocytes were seeded into 96 well plates divided into three experimental groups of blank control group, TSPS group, and PSP group. Each experimental group was added with the sample solution at gradient final concentrations of 0.5, 5, 25, 50, 100, 200, and 400 µg/ml and repeated for six wells. The results were represented in the form of ‘mean number ± standard deviation’. The proliferation effect of TSPS and PSP on the mice lymphocyte was shown in Fig. 5. TSPS with high concentrations (100, 200, and 400 µg/ml) had higher proliferation effect on lymphocyte than that of PSP. However, the results also indicated that the TSPS had stronger proliferation effect on lymphocyte at the concentration of 400 µg/ml (P < 0.05) than that of PSP. The pharmacological effect of PSP as immunomodulator has broadly been accepted by many researchers. Fig. 5 shows that TSPS had the ability to directly promote the mice splenic lymphocyte as PSP.

4. Conclusion

The components, molecular weight and bioactivity of tea seed polysaccharides were studied. The total sugars, protein and uronic acid contents in TSPS were 47.58%, 6.7%, and 12.77%, respectively. TSPS consisted of three kinds of polysaccharides with the molecular weight of 500 kDa, 130 kDa, and 5 kDa. TSPS consisted of rhamnose, xylose, arabinose, glucose and galactose, GalA, GluA, with a molar ratio of 4.9:1.7:11.1:27.2:14.0:3.4:1. The main monosaccharide of TSPS was glucose. The IR spectrum of the TSPS revealed also typical characteristics of polysaccharides and protein.

In this paper, these results indicated that TSPS significantly inhibited the growth of K562 cells, especially, at the concentration of 50 µg/ml; the inhibition activity of TSPS was the highest with an inhibition ratio beyond 34.05 ± 5.22% (P < 0.01). TSPS with high concentrations (100, 200 and 400 µg/ml) had higher proliferation effect on lymphocyte than that of PSP. However, the results also indicated that the TSPS had stronger proliferation effect on lymphocyte at the concentration of 400 µg/ml (P < 0.05) than that of PSP.

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