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Short communication

Isolation, purification and *in vitro* anti-tumor activity of polysaccharide from *Ginkgo biloba* sarcotesta

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ABSTRACT

Isolation and purification of polysaccharide from *Ginkgo biloba* sarcotesta by cellulose DEAE-52 and Sephadex G-100 column chromatography were performed in this study. Four water-soluble polysaccharides named GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were obtained and their anti-tumor activities *in vitro* were tested by MTT assay. GBEPP44 with molecular weight (MW) 9.5×10^5 Da, GBEPP33 with MW 3.1×10^5 Da and GBEPP22 with MW 4.8×10^4 Da were composed of rhamnose, glucose, galactose with a molar ratio of 16:10:0.96, 2.64:1:1.43 and 37.01:1:8.46, respectively. GBEPP11 with MW 3.4×10^3 Da was composed of rhamnose, glucose with a molar ratio of 1.9:1. MTT assay revealed that the polysaccharides had anti-tumor activity in a dose-dependent manner. The inhibitory rate of the polysaccharides on U937 was the highest among the three cancer cells, which increased as the MW decreased. Furthermore, the activity of acetylated GBEPP11 was higher than that of GBEPP11 and the inhibitory rate reached 78.97% at the concentration of 640 μg/mL.

1. Introduction

Ginkgo biloba, one of the most sold medicinal plants in the world, has been existed on the earth since 200 million years and is considered as a "living fossil" (Singh, Kaur, Gopichand, Singh & Ahuja, 2008). *G. biloba* sarcotesta, the epicarp of mature seeds, is regarded as the waste of gingko seeds, for there is no appropriate way to use them. It has become an urgent problem to the local enterprises and governments of the planting area for its poison to the environment (Wu, Mao, Yang, Wu, & Zhu, 2006). Most studies of *G. biloba* sarcotesta mainly focused on ginkgolic acid in recent years (Choi et al., 2009; Wu et al., 2007), while the active polysaccharide in *G. biloba* sarcotesta has always been ignored. It is found by Aihua Xu and Liuqing Yang (Xu, Chen, Sun, & Gu, 2003; Xu, Ren, Zheng, & Chen, 2008; Yang, Mao, Wu, Fan, & Zhu, 2009) that the crude polysaccharide of *G. biloba* sarcotesta had anti-tumor activity. To the best of our knowledge, no information has been reported about the purification of the polysaccharide and its activity. The present study was to investigate the purification of polysaccharide from *G. biloba* sarcotesta by a combination of cellulose DEAE-52 and Sephadex G-100 column chromatography, and to determine the monosaccharide composition, molecular weight of the purified fractions and their anti-tumor activity *in vitro*.

2. Materials and methods

2.1. Materials and reagents

G. biloba sarcotesta was collected from Jiangsu University (Zhenjiang, Jiangsu) in September 2005, and identified by Chen Li (Chief Pharmacist, Food and Drug Administration Bureau of Zhenjiang). The sarcotesta was preserved in a fridge at −18 °C. The sample was thawed, air-dried and then crushed into powders with a mill (10 meshes). The powders were placed into a Soxhlet apparatus with petroleum (boiling range 60–90 °C) at 80 °C to remove the fat, then dried at 60 °C for following use.

Dextrans of different molecular weight were purchased from Pharmacia Co. (Uppsala, Sweden), Cellouse DEAE-52 and Sephadex G-100 were purchased from Whatman Co. (Maidstone, Kent, UK), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). DMEM was purchased from GIBCO Co. (USA). All solvents and chemicals were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Isolation and purification of polysaccharides

100 g of defatted *G. biloba* sarcotesta powders were extracted with boiling water thrice in a ratio of 1:12 (w/v) for 2 h. The supernatant was collected, concentrated and precipitated with ethanol

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Table 1
Growth inhibition of *Ginkgo biloba* sarcotesta polysaccharide at different concentrations against the human stomach cancer cell lines AGS *in vitro*.

Samples	Concentrations (μg/mL)				
	40	80	160	320	640
Crude polysaccharide	7.42 ± 0.058^{bc}	10.70 ± 0.009^{a}	22.28 ± 0.070^{b}	28.56 ± 0.035^{b}	41.72 ± 0.012^{c}
GBEP	4.21 ± 0.011^{a}	9.92 ± 0.013^{a}	26.74 ± 0.018^{d}	39.04 ± 0.013^{d}	47.17 ± 0.016^{d}
GBEPP44	6.42 ± 0.003^{b}	13.00 ± 0.004^{b}	14.26 ± 0.001^{a}	15.87 ± 0.007^{a}	16.21 ± 0.001^{a}
GBEPP33	4.64 ± 0.011^{a}	25.10 ± 0.008^{e}	29.14 ± 0.013^{e}	37.42 ± 0.085^{c}	39.08 ± 0.070^{b}
GBEPP22	6.94 ± 0.042^{bc}	21.80 ± 0.021^{c}	30.8 ± 0.098^{f}	42.05 ± 0.011^{f}	46.03 ± 0.028^{d}
GBEPP11	15.3 ± 0.018^{d}	24.00 ± 0.021^{d}	26.03 ± 0.020^{c}	41.71 ± 0.085^{e}	51.16 ± 0.023^{e}
Acetylated GBEPP11	15.8 ± 0.010^{d}	26.03 ± 0.009^{e}	41.72 ± 0.003^{g}	51.47 ± 0.016^{g}	58.12 ± 0.004^{f}

Each value was the mean and standard deviation of sixth determinations. Values in the same column followed by a different letters (a–g) were significantly different ($p < 0.05$) according to least significant difference (LSD) test. GBEP, deproteinated crude polysaccharide. GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were obtained from DEAE-52 elution with distilled water, 0.05 M NaCl, 0.1 M NaCl and 0.15 M NaCl, and then purified by Sephadex G-100, respectively.

(1:4, v/v). After successively washing with ethanol, acetone and ether in that order for there times, the precipitate was lyophilized, giving the crude polysaccharide with a yield of 23%.

1 g crude polysaccharide was dissolved in 100 mL distilled water, then deproteinated by using trichloracetic acid (TCA) and obtained deproteinated polysaccharide (GBEP). GBEP was dissolved with distilled water and scanned from 190 to 440 nm to evaluate the effect of deproteination. GBEP (carbohydrate content 42.8%) was dissolved in distilled water, centrifuged and the supernatant was loaded onto a cellulose DEAE-52 column (1.6 cm × 50 cm). The crude polysaccharide was eluted with programmed from 0 M to 0.2 M NaCl and monitored for the presence of carbohydrate by using the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Four fractions GBEPP1 (distilled water), GBEPP2 (0.05 M, NaCl), GBEPP3 (0.1 M, NaCl) and GBEPP4 (0.15 M, NaCl) were obtained. The four factions were further fractionated on Sephadex G-100 column (1.6 cm × 50 cm) and eluted with 0.1 M NaCl. All of them presented as a single symmetrical peak, named GBEPP11, GBEPP22, GBEPP33 and GBEPP44 with carbohydrate content of 90.87%, 95.36%, 92.57% and 94.63%, respectively.

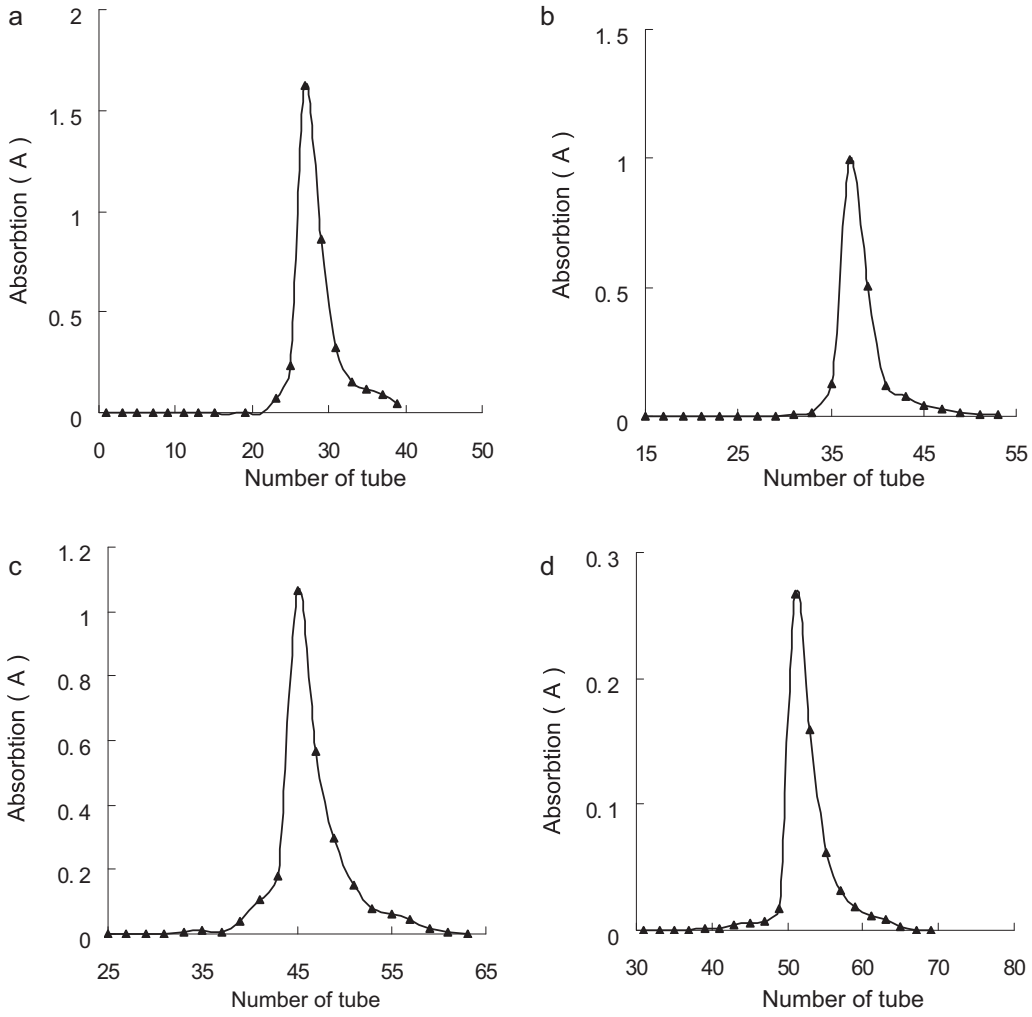

Fig. 1. Four fractions purified by Sephadex G-100, GBEPP11 (a), GBEPP22 (b), GBEPP33 (c) and GBEPP44 (d). GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were obtained from DEAE-52 elution with distilled water, 0.05 M NaCl, 0.1 M NaCl and 0.15 M NaCl, and then purified by Sephadex G-100, respectively.

Table 2
Growth inhibition of *Ginkgo biloba* sarcotesta polysaccharide at different concentrations against the human stomach cancer cell lines SGC *in vitro*.

Samples	Concentrations (μg/mL)				
	40	80	160	320	640
Crude polysaccharide	−6.24 ± 0.021[a]	−5.50 ± 0.099[a]	−2.01 ± 0.010[a]	−1.02 ± 0.014[a]	3.78 ± 0.004[a]
GBEP	−1.83 ± 0.226[b]	−1.65 ± 0.084[b]	0.46 ± 0.042[b]	13.3 ± 0.013[b]	15.43 ± 0.085[c]
GBEPP44	9.75 ± 0.009[e]	10.86 ± 0.030[d]	12.07 ± 0.010[c]	13.27 ± 0.006[b]	13.88 ± 0.001[b]
GBEPP33	6.45 ± 0.004[c]	7.03 ± 0.003[c]	15.08 ± 0.003[d]	19.25 ± 0.004[d]	20.34 ± 0.005[d]
GBEPP22	11.87 ± 0.007[f]	12.82 ± 0.042[e]	15.54 ± 0.028[e]	19.0 ± 0.011[c]	29.57 ± 0.038[e]
GBEPP11	8.00 ± 0.013[d]	10.95 ± 0.032[d]	17.05 ± 0.099[f]	21.72 ± 0.016[e]	32.2 ± 0.015[f]
Acetylated GBEPP11	8.00 ± 0.010[d]	12.83 ± 0.001[e]	19.25 ± 0.006[g]	21.72 ± 0.001[e]	34.09 ± 0.004[g]

Each value was the mean and standard deviation of sixth determinations. Values in the same column followed by a different letters (a–g) were significantly different ($p < 0.05$) according to least significant difference (LSD) test. GBEP, deproteinated crude polysaccharide. GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were obtained from DEAE-52 elution with distilled water, 0.05 M NaCl, 0.1 M NaCl and 0.15 M NaCl, and then purified by Sephadex G-100, respectively.

2.3. Homogeneity and molecular weight of purified polysaccharide fractions

The homogeneity and molecular weight of the polysaccharide fractions were determined by high-performance gel-permeation chromatography (HPGPC) using a LC-10ATvp Plus HPLC system (Shimadzu, Japan), equipped with a TSK-Gel G4000PW (TOSOH, Japan) column (7.5 mm × 300 mm) and a RID-10A detector (Shimadzu, Japan). 20 μL of sample solution was injected in each run, eluted with 0.003 M sodium acetate solution at a flow rate of 0.8 mL/min. The column temperature was 25 °C. The standard curve was established with T-series dextrans of known MW (T-10, T-40, T-70, T-500, T-2000 and blue dextran T-2000).

2.4. Monosaccharide composition

5 mg GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were each hydrolyzed with 3 mL, 2 M H_2SO_4 at 105 °C for 8 h. The hydrolysate was neutralized with an excess of $BaCO_3$, centrifuged and the supernatant was lyophilized. 5 mg ammonium hydrochloride and 0.5 mL pyridine were added and allowed to react in a 90 °C water bath for 30 min, then cooled to room temperature, then 0.5 mL acetic anhydride was added and the mixture was incubated in the 90 °C water bath for 30 min to allow the occurrence of acetylation reaction. The mixture was further analyzed by GC on an Agilent 6280 instrument equipped with FID and HP-5MS column (0.25 mm × 30 m × 0.25 μm). The temperature increasing procedure as follows: 130 °C kept for 10 min, 130–240 °C at 4 °C/min, and then kept for 5 min. The injector and detector heater temperatures were 280 °C and 300 °C, respectively. The rate of carrier gas (N_2) was 50 mL/min.

2.5. Acetylation of GBEPP11

The method of acetylation GBEPP11 was the same as 2.4. The degree of acetylation was 9.2%.

2.6. Cell lines and cell proliferation assay

Human stomach cancer cells (AGS, SGC) and human Leukemia cells (U937) were obtained from the School of Life Science of Jiangsu University (Zhenjiang, China). AGS, SGC and U937 were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C (Hsu, Kuo, & Lin, 2004; Tomatsu, Ohnishi-Kameyama, & Shibamoto, 2003).

The proliferation of AGS, SGC and U937 were determined using the colorimetric MTT assay described by Mosmann (1983). Briefly, cells were seeded at a density of 2×10^4 cells/well in a 90 μL volume of the medium in 96-well plates and allowed to attach for 24 h. The dosages of crude polysaccharides, GBEP, GBEPP11, GBEPP22, GBEPP33 and GBEPP44 on the selected cell lines were 40, 80, 160, 320, 640 μg/mL while the negative controls were treated with the medium. 10 μL MTT (5 g/L) was added after 48 h. After incubated at 37 °C for 4 h, the supernatant was aspirated and then 100 μL DMSO was added to each well. Absorbance was measured at 570 nm by a 96 well microplate reader (Mode 680, Bio-Rad, Tokyo, Japan).

2.7. Statistical analysis

Data were reported as mean ± SD (standard deviation) of sixth determinations. Statistical calculations were carried out by SPSS version 16.0 (SPSS Inc., Chicago, USA). ANOVA was applied for determining differences between the results of samples. Values of $p < 0.05$ were considered as significantly different.

3. Results and discussion

3.1. Homogeneity, MW and monosaccharide composition

GBEP appeared as brown powders, no absorption at 280 or 260 nm in the UV spectrum, which indicated absence of

Table 3
Growth inhibition of *Ginkgo biloba* sarcotesta polysaccharide at different concentrations against the human Leukemia cancer cell lines U937 *in vitro*.

Samples	Concentrations (μg/mL)				
	40	80	160	320	640
Crude polysaccharide	3.43 ± 0.003[a]	6.42 ± 0.042[a]	11.00 ± 0.044[a]	40.39 ± 0.004[a]	49.47 ± 0.002[a]
GBEP	9.9 ± 0.017[b]	18.74 ± 0.002[c]	21.18 ± 0.092[b]	44.06 ± 0.076[b]	50.3 ± 0.010[c]
GBEPP44	3.43 ± 0.004[a]	6.43 ± 0.006[a]	11.08 ± 0.003[a]	40.43 ± 0.009[a]	49.89 ± 0.003[b]
GBEPP33	15.21 ± 0.056[c]	17.11 ± 0.003[b]	47.83 ± 0.001[d]	49.30 ± 0.024[c]	57.42 ± 0.017[d]
GBEPP22	16.7 ± 0.099[d]	27.76 ± 0.084[d]	37.01 ± 0.063[c]	57.40 ± 0.085[d]	61.95 ± 0.041[e]
GBEPP11	30.74 ± 0.018[e]	44.78 ± 0.013[e]	53.74 ± 0.028[e]	65.23 ± 0.028[e]	69.2 ± 0.070[f]
Acetylated GBEPP11	48.46 ± 0.001[f]	56.7 ± 0.001[f]	60.25 ± 0.001[f]	71.70 ± 0.003[f]	78.97 ± 0.000[g]

Each value was the mean and standard deviation of sixth determinations. Values in the same column followed by a different letters (a–g) were significantly different ($p < 0.05$) according to least significant difference (LSD) test. GBEP, deproteinated crude polysaccharide. GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were obtained from DEAE-52 elution with distilled water, 0.05 M NaCl, 0.1 M NaCl and 0.15 M NaCl, and then purified by Sephadex G-100, respectively.

protein and nucleic acid. The four fractions obtained from Sephadex G-100 were shown in Fig. 1. The profile showed a single and symmetrical peak as well as the HPGPC profile, indicating that each purified fraction was a homogeneous polysaccharide. The HPGPC results revealed that the MW of GBEPP11, GBEPP22, GBEPP33 and GBEPP44 were 3.4×10^3 Da, 4.8×10^4 Da, 3.1×10^5 Da and 9.5×10^5 Da, respectively. GBEPP22, GBEPP33 and GBEPP44 were composed of rhamnose (Rha), glucose (Glu), galactose (Gal) with a molar ratio of 16:10:0.96, 2.64:1:1.43 and 37.01:1:8.46, respectively. GBEPP11 was composed of Rha, Glu with a molar ratio of 1.9:1.

3.2. Anti-tumor activity

In this study, the antitumor activity of the seven polysaccharide samples, crude polysaccharides, GBEP, GBEPP11, GBEPP22, GBEPP33, GBEPP44 and acetylated GBEPP11 were tested on AGS, SGC and U937. The results revealed that all samples had no or weak antitumor activity on SGC, whereas good antitumor activity on AGS and U937, especially on U937. The acetylated GBEPP11 presented significantly highest antitumor activity (Tables 1–3). Table 1 showed that the inhibition ability of the seven polysaccharide samples on AGS was dose-dependent. As for the GBEPP11, GBEPP22, GBEPP33, GBEPP44, the inhibitory rate increased as their molecular weight decreased. At the concentration of 640 μg/mL, the inhibitory rate of the seven polysaccharide samples were 41.72%, 47.17%, 16.21%, 39.08%, 46.03%, 51.16% and 58.12%, respectively. At the concentration of 40 and 80 μg/mL, the inhibitory rate of GBEP was lower than that of the crude polysaccharide, whereas the result was adverse at higher concentration. As shown in Table 3, the inhibition ability of all the polysaccharide on U937 was dose-dependent. The inhibitory rate of GBEP was higher than that of the crude polysaccharide and the inhibitory rate were 49.47% and 50.3%, respectively, at the concentration of 640 μg/mL. The inhibition activity of GBEPP11, GBEPP22, GBEPP33 and GBEPP44 on U937 were increased as molecular weight decreased. At the concentration of 640 μg/mL, the inhibitory rate of acetylated GBEPP11 on U937 was 78.97%, higher than that of GBEPP11 (69.20%).

4. Conclusions

The anti-tumor activity on U937 was the highest among the three cell lines U937, AGS and SGC. The antitumor activity of the purified fractions was higher than that of the crude polysaccharide on U937. The inhibitory rate of acetylated GBEPP11 was higher than that of GBEPP11 on U937. The results indicated that *G. biloba* sarcotesta was a potential natural antitumor agent, and its utilization could solve the urgent problem to the local enterprises and governments of the *G. biloba* area. However, further structural identifications are required to study and elucidate the antitumor mechanisms.

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