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Short Communication
Polysaccharides of Spina Gleditsiae, A Potential Treasure in Anticancer and Antioxidant Drug Discovery

[1]Jiayu Gao, [1]Xiao Yang, [1]Jiangxia Hu and [2]Xiao Yang

[1]School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology, Luoyang, Henan, China
[2]School of Clinical Medicine, Henan University of Science and Technology, Luoyang, Henan, China

Abstract

Background and Objective: Though polysaccharides are considered to constitute the main medicinal properties of herbal Spina Gleditsiae, the current research on their isolation and the anticancer/antioxidant activities remain as an ongoing challenge. The purpose of this study was to develop an isolation technique for polysaccharides of Spina Gleditsiae (PSG) and then determined their anticancer and antioxidant properties. **Materials and Methods:** This study developed an ultrasonic-assisted method for PSG isolation and then the anticancer activities of isolated PSG were determined using MTT cytotoxic test. Moreover, the antioxidant effects of PSG were evaluated in the cell antioxidant, total antioxidant activity, ABTS free radical scavenging and Iron ion reduction assays. **Results:** In this study, the method of PSG isolation was firstly developed and characterized with acceptable yield rate (1.85±0.12%) and time/resources consuming, which greatly facilitate the current and future study of PSG. Meanwhile, the growth inhibitory and antioxidant effects of PSG were firstly determine in esophageal/liver cancer and human embryonic kidney cells *in vitro*. The isolated PSG were able to inhibit the growth of EC109 and HepG2 cancer cells with IC_{50} at 32.19±2.07 and 34.17±3.3 μg mL^{-1}, respectively. Moreover, PSG demonstrated the dose-dependent antioxidant, free radical scavenging and reductive activities. **Conclusion:** The PSG was confirmed as an active anticancer and antioxidant component of Spina Gleditsiae, providing a potential natural medicinal resource for further investigation and development. Moreover, the results of this study underpin the fundamental understanding of the medicinal activities of Spina Gleditsiae and support its current use in clinic of China.

Key words: Spina gleditsiae, polysaccharides, isolation, antioxidant, anticancer

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Corresponding Author: Jiayu Gao, School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology, Luoyang, Henan, China Tel: +86 379 64231914

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gleditsia sinensis Lam (*G. sinensis*) is a perennial shrub native to China and widely grows in the basin of Yellow and Yangtze River, as well as southern provinces of China. Its dry thorns, known as Spina Gleditsiae, are the one of the most valuable herbs used in Traditional Chinese Medicine (TCM)[1]. Spina Gleditsiae has been traditionally used as a source of drugs for the detoxification, detumescence, apocenosis and anti-parasites in China. Currently, it is claimed to be used as the main active ingredient in many TCM anticancer and dephlogisticate formulae used in TCM hospitals[2]. Regarding to its medicinal uses, the functional compounds belonging to five major groups of secondary metabolites, including triterpene, sterol, flavonoid, phenolic and alkaloid, have been isolated and elucidated from Spina Gleditsiae[3-5]. However, an important bioactive group of phytochemical constitutes, polysaccharides were barely reported in Spina Gleditsiae research. Natural polysaccharides, the macro-molecules originated from many medicinal plants have been proven to possess many bioactive effects, including anti-cancer, anti-oxidant, anti-inflammatory, anti-microbes and immunoregulatory activities[6-9]. As Spina Gleditsiae has been reported to exhibit many of those pharmacological activities above, the biological properties of its polysaccharides thus attract reasonable attention for research[2].

As a very important bioactive group, the polysaccharides of Spina Gleditsiae (PSG) are still not well studied either regarding to the isolation technique or anticancer/antioxidant activities. This study, thus aimed to isolate the PSG using an ultrasonic-assisted extraction method. The anti-cancer activities of PSG were determined in human esophageal (EC109) and liver (HepG2) cancer cell lines *in vitro*. Antioxidant and free radical scavenging activities of PSG were also evaluated in both of cell and chemical models *in vitro*. The results firstly provided an efficient method of PSG separation as well as clarified the anti-cancer and anti-oxidant properties of polysaccharides isolated from Spina Gleditsiae.

MATERIALS AND METHODS

The work was performed during July 2017 to May 2018 in natural products research laboratory, School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology.

Cell lines and sample preparation: The human esophageal cancer cell line (EC109), human liver cancer cell line (HepG2) and human embryonic kidney 293 (HEK293) cell line were purchased from Beinglay Biotech (Wuhan, Hubei, China). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a 5% carbon dioxide humidified incubator at 37°C.

Spina Gleditsiae was purchased from Hao Yi Sheng Chinese herbal medicine store, Jinghua street, Luoyang. The original plant was collected in Henan province, China (December 2017) and the identity kindly confirmed as *Gleditsia sinensis* Lam by Professor Wenliang Li at the Department of Entomology, Henan University of Science and Technology. A voucher specimen has been deposited in natural products research laboratory, School of Chemical Engineering and Pharmaceutics (access number: JGS2017-078).

The adopted method of PSG isolation was developed based on the ultrasonic-assisted extraction of polysaccharides method reported before[10]. Briefly, the dry Spina Gleditsiae was minced. The dried herbal powder were then extract with distilled water at ratio of 1:30 (g mL^{-1}) in the ultrasonic cleaning machine for 40 min at 30 , filtered and collected supernatants. The solvent were reduced to 1/4 volume at 30 under reduced pressure. The condensed extracts were mixed with absolute ethanol at volume ratio of 1:5. The mixture was standstill overnight. The mixture was then centrifuged at 5000 rpm for 5 min. The supernatant was removed and the precipitates were then collected. The precipitates were washed twice by 80% ethanol and then acetone until the color was stable. The precipitates were again washed by diethyl ether twice and then dried in a vacuum freezing dryer to produce crude polysaccharides. The crude polysaccharides were dissolved in distilled water and mixed with chloroform/butanol (5:1) at volume ratio of 5:1. The mixture was standstill for 50 min. The water layer (upper) was then separated using separating funnel. The water extracts were mixed with absolute ethanol to achieve the final ethanol concentration at 70%. The mixture was standstill for 4 h and centrifuged to precipitate the polysaccharides. The isolated polysaccharides were then washed by distilled water and dried at -30°C under reduced pressure for 2 h.

Cytotoxicity assay: About 200 μL cell suspension was seeded at a concentration of 1.5×10^4 cells mL^{-1} in a 96-well plate. After overnight incubation, serial doubling dilutions of PSG (100-3.1 μg mL^{-1}) were added in 10 μL aliquots. The negative control was 10 μL 0.01% DMSO in Hank's solution. Cisplatin (100-3.1 μg mL^{-1}) was used as positive control. Each

concentration was tested three times. After 24 h incubation, 20 µL MTT solution (5 mg mL^{-1}, Sigma-Aldrich Ltd, Gillingham, Dorset, UK) was added and incubated at 37°C for 4 h to develop color. The optical density (OD) was measured at 590 nm using a Multiskan Spectrum Reader. The growth inhibition was determined by using:

$$\text{Cytotoxicity (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Cell antioxidant assay: About 200 µL cell suspension was seeded at a concentration of 1.5×10^4 cells mL^{-1} in a 96-well plate. After overnight incubation, H_2O_2 (1000, 10 and 0.1 µg mL^{-1}) and serial dilutions of PSG (1000, 500, 100 and 10 µg mL^{-1}) were added in 10 µL aliquots. After 6 h incubation, 20 µL MTT solution (5 mg mL^{-1}) was added and incubated at 37°C for 4 h to develop color. The optical density (OD) was measured at 590 nm using a Multiskan Spectrum Reader. The cell survive rate was determined by using:

$$\text{Cell survive rate (\%)} = \frac{\text{Sample OD}}{\text{Control OD}} \times 100$$

Total antioxidant activity test: Prepared PSG and BHT at concentration of 0.08, 0.16, 0.24, 0.32 and 0.40 mg mL^{-1}. Prepared the stock solution of phosphorus molybdenum reagent (0.6 mol L^{-1} sulfuric acid, 28 mmol L^{-1} sodium phosphate and 4 mmol L^{-1} ammonium molybdate). The 1.0 mL of each samples were mixed with 3.0 mL phosphorus molybdenum reagent solution. The mixtures were placed in 95 water bath for 90 min. The samples were cooled down until room temperature and then measured the absorbance at 625 nm. The distilled water was used as a blank and BHT was positive control.

ABTS free radical scavenging assay: Prepared PSG and BHT at concentration of 0.08, 0.16, 0.24, 0.32 and 0.40 mg mL^{-1}. The 0.8 mL of 7.4 mmol L^{-1} ABTS solution and 0.8 mL of 2.6 mmol L^{-1} $K_2S_2O_8$ were mixed and reacted in dark for 12 h. The prepared mixture was then diluted 50 times using absolute ethanol to produced $ABTS^+$ working solution. The 4 mL $ABTS^+$ working solution was mixed with 1 mL sample or 1 mL absolute ethanol. The mixtures were then shake for 1 min and measure the absorbance at 734 nm:

$$\text{ABTS}^+ \text{ free radical scavenging rate (\%)} = \frac{A_0\text{-}A}{A_0} \times 100$$

Where:

A_0 = Absorbance of $ABTS^+$ working solution+absolute ethanol

A = Absorbance of $ABTS^+$ working solution+samples

Iron ion reduction assay (FRAP): Prepared PSG and BHT at concentration of 0.08, 0.16, 0.24, 0.32 and 0.40 mg mL^{-1}. Prepared $FeSO_4$ solution at concentration of 400, 200, 100, 50 and 25 µmol L^{-1}. Dissolved 31.233 mg tripyridyl triazine in 40 mL 10 mmol L^{-1} HCL to produce 10 mmol L^{-1} TPTZ solution. Mixed 2.5 mL 10 mmol L^{-1} TPTZ solution, 2.5 mL $FeCl_3 \cdot 6H_2O$ and 25 mL 0.3 mmol L^{-1} natrium aceticum buffer solution to produce FRAP working solution. The 2.7 mL FRAP working solution (37) was mixed with 0.3 mL samples. The mixtures were shaken and measured the absorbance at 593 nm. Used series concentrations of $FeSO_4$ solution to produce the standard curve and then determined the $FeSO_4$ concentrations in the samples as FRAP values representing the antioxidant ability.

Statistical analysis: The data were shown as Mean ± Standard Deviation (SD). Statistical comparison among treatments was carried out using one-way analysis of variance (ANOVA). The statistical significances between control and sample groups were calculated by the Student's t-test. Data were taken as significant where $p<0.05$.

RESULTS

Isolation of PSG: In this study, an ultrasound-assisted method was successfully developed to extract the polysaccharides of Spina Gleditsiae. As described in the part of Materials and Methods above, the set of parameters developed in this study was used to isolate PSG for six times. As shown in Table 1, the current method could achieve a mean value of yield rates at 1.85 ± 0.12% (n = 6), indicating that the extraction process was stable and repeatable for isolation of PSG.

Anticancer activities of PSG: The PSG effects were determined in human esophageal cancer cell EC109 and human liver cancer cell HepG2 *in vitro*. The negative control didn't show any significant effects on both of cancer cell lines tested. As shown in Fig. 1, PSG inhibited the proliferation of both of the cancer cell lines *in vitro* on a dose-dependent manner. The higher of the concentration, the stronger of the inhibitory effects of PSG were observed. According to the calculation, the IC_{50} values of PSG on EC109 (Fig. 1a) and

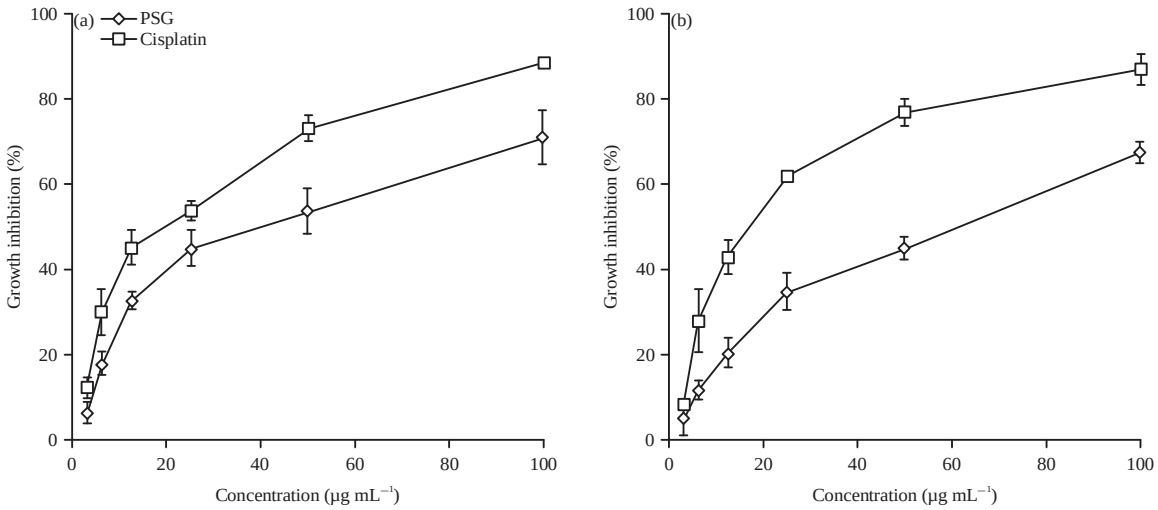

Fig. 1(a-b): Cytotoxic effects of PSG and cisplatin (positive control) on (a) EC109 and (b) HepG2 cancer cell lines

Table 1: Yield rates of the polysaccharides of spina gleditsiae (n = 6)

Raw materials of spina gleditsiae (g)	Produced PSG (g)	Yield rate (%)	Average of yield rate (%)	Standard variation of yield rate
200	3.90	1.95	1.85	0.12
200	4.02	2.01		
200	3.52	1.76		
200	3.44	1.72		
200	3.78	1.89		
200	3.54	1.77		

Table 2: IC_{50} values of PSG and cisplatin (positive control) tested on EC109 and HepG2 cancer cells

Cancer cell types	Materials	IC_{50} value (μg mL^{-1}, n = 3)			Average of IC_{50} values (μg mL^{-1})	Standard variation of IC_{50} value
EC109	PSG	52.16	49.64	61.43	54.41	6.21
	Cisplatin	32.53	29.97	34.08	32.19	2.07
HepG2	PSG	67.58	62.44	64.40	64.80	2.59
	Cisplatin	27.78	32.45	34.17	31.46	3.30

HepG2 (Fig. 1b) were 54.41±6.21 and 64.8±2.59 μg mL^{-1}, respectively. Meanwhile, as presented in Table 2, the IC_{50} values of Cisplatin (Positive control) were for EC109 (Fig. 1a) and HepG2 (Fig. 1b) were 32.19±2.07 and 34.17±3.3 μg mL^{-1} under 24 h treatment. Those results firstly provided the evidence for the anti-cancer activities of PSG on human esophageal and liver cancer cells *in vitro.*

Anti-oxidant activities of PSG: As shown in Fig. 2a, PSG presented dose-dependent anti-oxidant activities in both of cells and chemical model. Whether high (1000 μg mL^{-1}), mediate (10 μg mL^{-1}) or low (0.1 μg mL^{-1}) H_2O_2-treated groups, PSG could significantly increase the survive rates of oxidative damaged 293 cells *in vitro*. The 1000 μg mL^{-1} of PSG could completely turn back the damage of H_2O_2 oxidation. Moreover, anti-oxidant effects of PSG was determined and compared to standard butylated hydroxytoluene (BHT) in chemical models. Three different assays, including total anti-oxidant activity test, ABTS free radical scavenging assay and Iron ion reduction assay (FRAP) were employed for the comparison. As the results shown in Fig. 2b and d, the anti-oxidant and reductive activities of PSG were much stronger than that of BHT. In the ABTS free radical scavenging assay (Fig. 2c), the different concentration of BHT demonstrated a constant high scavenging rate (over 90%), while PSG's effects increased gradually and reach about 80% scavenging rate at concentration of 0.4 mg mL^{-1}.

DISCUSSION

Hot water extraction, heat reflux and acidic hydrolysis are the regular used for herbal polysaccharides isolation[11]. However, the traditional methods used above consume large amount of energy, big volumes of solvents and long extraction

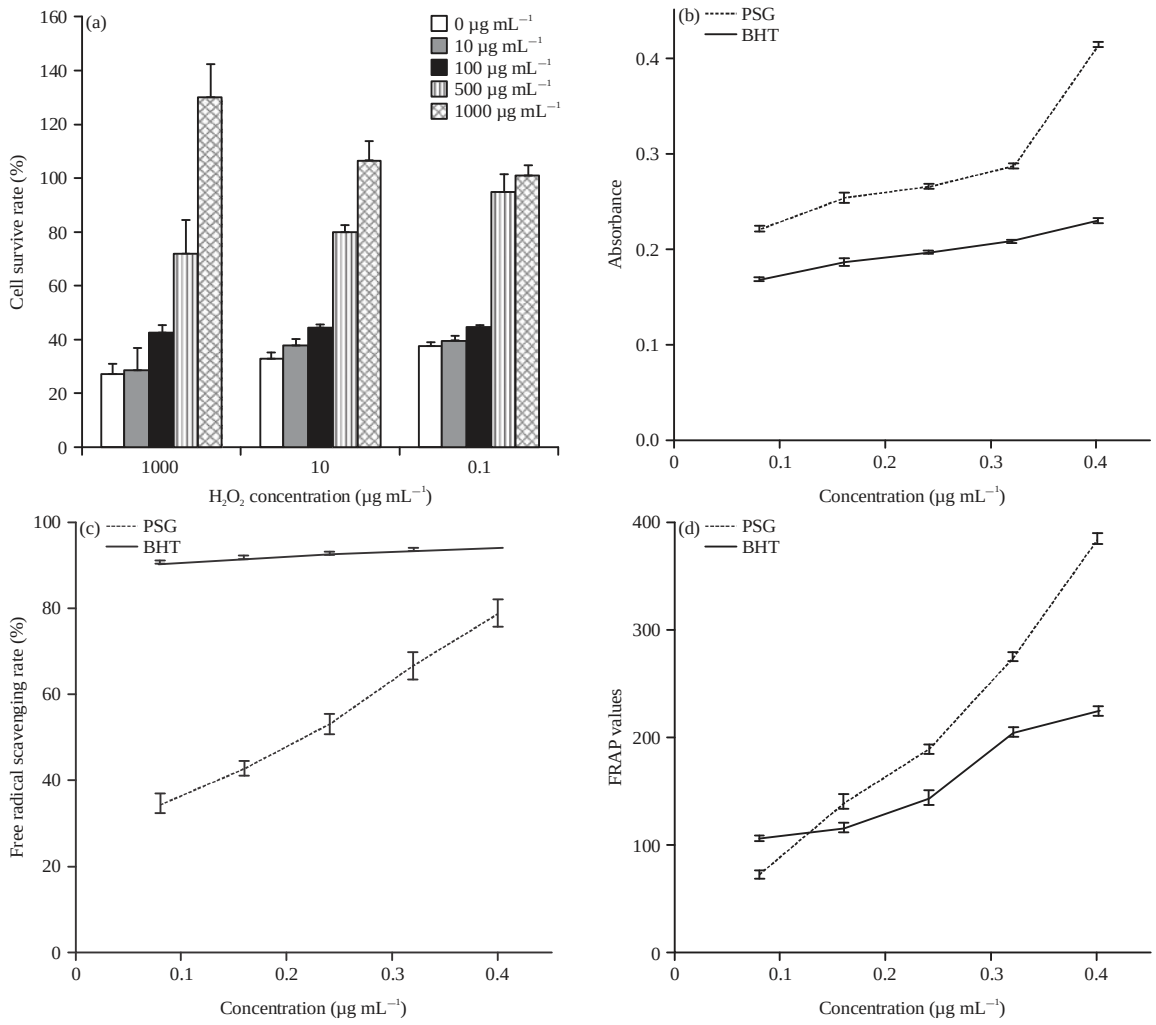

Fig. 2(a-d): Antioxidant activities of PSG (a) Cell antioxidant assay, (b) Total antioxidant activity test, (c) ABTS free radical scavenging assay and (d) Iron ion reduction assay (FRAP)

times and require expensive equipment as well. Moreover, the high temperature or acidic conditions can also damage the structure of the polysaccharides, thereby affecting the bioactive effects[12]. In this study, the method developed, characterizing of fast, clean and efficiency, is the first study regarding to the PSG isolation reported in the international literature. As the whole isolation was performed in the low temperature ($\leq$30), the polysaccharides molecules were thus remained in their original structure at maximum degree. The significant of this extraction is to greatly facilitate the stability and efficiency of PSG production, which would promote the current and future research and finally transform PSG to become a valuable additive agent for medicinal purposes.

The anticancer activities of Spina Gleditsiae have been widely reported in both of the *in vitro* and *in vivo* models[13-19]. Previously, the flavonoids and coumarin were reported as the major active anti-cancer constituents of Spina Gleditsiae[3,16]. In this study, PSG was proved to be another effective component for the anti-cancer uses of Spina Gleditsiae. It fundamentally expands the current understanding of the anti-cancer material basis of Spina Gleditsiae and thus supports its clinical uses in China. Moreover, the pharmacological mechanisms underlining the anti-cancer effects of PSG still remain as an ongoing challenge, thus highly suggested to be further studied.

Oxidative stress has been recognized as a causative factor for ageing and progression of various diseases[20]. In this work, it firstly confirmed the anti-oxidant activities of PSG in both cells and chemical models *in vitro*. On the oxidative damaged cells, PSG presented a significant recovery effects to enhance the cells' survive. This effect was proposed to be related to the antioxidant, reductive and radical scavenging abilities of

herbal polysaccharides[10]. The further series of chemical tests determined that PSG exhibited comparable effects to strong antioxidant agent, BHT, in total antioxidant activity, ABTS free radical scavenging and Iron ion reduction testes. Taken together, the results firstly determined PSG as the major active group constitute for the antioxidant activities of herbal Spina Gleditsiae, thus providing a valuable natural anti-oxidant for further investigation and development.

CONCLUSION

Overall, in this study, an ultrasonic-assisted method has been developed to extract the polysaccharides from Spina Gleditsiae. Its anti-cancer and anti-oxidant activities, which are two of most used medicinal properties of Spina Gleditsiae, were determined in *in vitro* experimental models, thus providing a natural resource for further investigation and development.

SIGNIFICANCE STATEMENT

This study firstly developed a PSG isolation method with acceptable productive rate and time/resources consuming and discovered the anti-cancer and anti-oxidant activities of PSG using cells and/or chemical models *in vitro*. It can be beneficial to underpin the fundamental understanding of the medicinal values of Spina Gleditsiae and support its claimed clinical uses in China. This study will help the researches to uncover the critical areas of bioactive polysaccharides of Spina Gleditsiae that many researches were not aware to explore yet. Thus a new theory on development of the PSG-based drugs, health products or addictives may be arrived at.

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