International Journal of Pharmacology

ISSN 1811-7775

science alert

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2016.754.759

Short Communication
Optimization of Ultrasonic-assisted Extraction of Polysaccharides from *Scutellaria barbata* and Determination of their Anticancer and Antioxidant Activities

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Abstract

Background and Objective: The polysaccharides of herbal *Scutellaria barbata* (PSB) are a group of active components with multiple bioactivities. Though PSB is thought to constitute the main medicinal properties of *Scutellaria barbata* (*S. barbata*) the current study on the isolation of PBS and their anticancer and antioxidant activities are rare and thus, remain as an ongoing challenge. Therefore, the purpose of this study is to further develop a new isolation technique for industrial production of PSB and also determine the anticancer and antioxidant properties of PBS *in vitro*. **Methodology:** This study optimized an ultrasonic-assisted method for PSB isolation and led to the yield rate at 2.96±0.24%. The isolated PSB were able to induce the apoptosis of the human esophageal cancer EC109 cell line and human liver cancer HepG2 cell line in Annexin-V staining apoptotic assay. **Results:** The IC_{50} of PSB on EC109 and HepG2 were determined as 43.62±1.51 μg mL^{-1} and 60.44±2.31 μg mL^{-1}, respectively using MTT cytotoxic test. Moreover, PSB demonstrated the dose-dependent antioxidant and free radical scavenging activities in the total antioxidant activity and ABTS free radical scavenging assay. Its highest effect was comparable to that of the positive control butylated hydroxytoluene (BHT). **Conclusion:** In this study, the method of PSB isolation is significantly developed and characterized with higher yield rate and less time/resources consuming which greatly facilitate the current and future study of PSB. Meanwhile, the growth inhibitory and apoptotic effects of PSB are firstly determine in esophageal cancer cell and human liver cancer cell *in vitro*. The PSB is confirmed as an active anticancer and antioxidant component of *S. barbata* 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 *S. barbata* and support its current use in clinic of China.

Key words: *Scutellaria barbata*, polysaccharides, isolation, antioxidant, anticancer

Received: March 24, 2016 Accepted: August 09, 2016 Published: September 15, 2016

Citation: Jiayu Gao, Xiao Yang, Pu Liu and Weiping Yin, 2016. Optimization of ultrasonic-assisted extraction of polysaccharides from *Scutellaria barbata* and determination of their anticancer and antioxidant activities. Int. J. Pharmacol., 12: 754-759.

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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

Scutellaria barbata D.Don (*S. barbata*) commonly known as Ban Zhi Lian is a perennial herb widely used in Traditional Chinese Medicine (TCM) through the centuries. Used either alone or as a part of the herbal formula *S. barbata* is given to patients to treat symptoms associated with carbuncle, scrofula, haematemesis, epistaxis, ascites, traumatic injuries and tumours in the TCM hospitals of China[1,2]. The multiple classes of bioactive photochemicals such as flavonoids, polysaccharide, terpenoid alkaloids and essential oils have been identified from *S. barbata*[3]. Anticancer and antioxidant activities are the most reported pharmacological effects rooted in these active compounds[4].

Previously, flavonoids and terpenoids were considered as the most important active compounds of *S. barbata*. However, an increasing number of studies have been reported regarding to its pharmacological activities of polysaccharides isolated from *S. barbata* (PSB) recently, especially in last 5 years. Yang and his colleagues firstly studied that PSB treatment can significantly inhibit the invasion and metastasis of human lung cancer 95-D cells *in vitro* through the regulation of C-MET and E-CAD molecules[5]. In the next study of 2014, Yang's group further studied that PSB could inhibit the growth of 95-D cells *in vitro* with IC_{50} at as low as 35.2 μg mL^{-1}. *In vivo*, PSB also inhibited tumor growth in the 95-D subcutaneous xenograft model in a dose-dependent manner. The treatment of once-daily intraperitoneal injection (100 mg kg^{-1}) for 3 weeks was able to inhibit 42.72% tumor growth[6]. Moreover, another study further revealed that PSB potently inhibited the proliferation of lung adenocarcinoma Calu-3 cells and human epidermal growth factor receptor 2 (HER2) phosphorylation *in vitro* and also downregulated the expression of the downstream signaling molecules including phospho-Akt and phospho-extracellular signal-related kinase. Similarly, PSB demonstrated significant antitumor activity in a Calu-3 subcutaneous xenograft model *in vivo*. Taken together, the suppression of the HER2 pathway was identified to account for the anti-tumor effects of PSB[7]. Polysaccharide compounds were also reported to constitute the antioxidant and immune modulatory activities of *S. barbata*[8,9]. Exposed to PSB at 1.0 mg mL^{-1}, the radical scavenging rates of superoxide, DPPH and hydroxyl achieved as much as 67.1, 60.3 and 74.5%, respectively[9]. This demonstrated the potential anticancer and antioxidant activity for polysaccharides of *S. barbata*. However, the study of PSB isolation and their bioactive activities are still rare and remain as an ongoing challenge.

To further develop the isolation techniques for industrial production and determine the anticancer and antioxidant properties of PBS, the isolation of polysaccharides from *S. barbata* was optimized using an ultrasonic-assisted extraction method in this study. The anticancer activities of PSB were also determined in human esophageal (EC109) and liver (HepG2) cancer cell lines *in vitro* for the first time. Both type of the cancer cells were found to be apoptosis-induced by PSB. The isolated PSB also demonstrated the dose-dependent antioxidant activity and free radical scavenging in the total antioxidant activity and ABTS free radical scavenging tests and providing a potential natural antioxidant for further development.

MATERIALS AND METHODS

Cell lines and chemicals: The human esophageal cancer cell line (EC109) and human liver cancer cell line (HepG2) 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. Experiments were performed when cells were approximately 80% confluent. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), butylated hydroxytoluene (BHT), cisplatin and camptothecin were purchased from Sigma (Sigma-Aldrich, Sternhein, Germany).

Sample preparation: *Scutellaria barbata* was purchased from Hao Yi Sheng Chinese herbal medicine store, Jinghua Street, Luoyang. The original plant was collected in Shandong province, China (July, 2015) and the identity kindly confirmed as *S. barbata* D. Don 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: JGS2015-039A).

The dry herb of *S. barbata* was minced and extracted with light petroleum twice for 24 h at room temperature. The solvent were completely removed at 35°C under reduced pressure and the residue was dried in the fume cupboard. The dried herbal residues were then extract with distilled water at proposed ratio (1:6, 1:10, 1:14, 1:18 and 1:24) in the ultrasonic cleaning machine for proposed minutes (10, 20, 30, 40 and 50 min) filtered and collected supernatants. Added the same amount of distilled water as before and repeated proposed times (1, 2, 3, 4 and 5). The collected supernatants were then

combined and centrifuged at 1500 rpm for 8 min. The solvent were reduced to 1/4 volume at 35°C under reduced pressure. The condensed extracts were mixed with chloroform/butanol (5:2) at volume ratio of 1:1. 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 proposed percentage (50, 60, 70, 80 and 90%). 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.

Cell proliferation assay: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as follows: briefly, 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 PSB (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 using cytotoxicity:

$$\text{Percentage} = \left[\frac{\text{Control O.D-Sample O.D}}{\text{Control O.D}}\right]\times100$$

Apoptosis assay: The cell suspension (800 μL) was seeded at a concentration of 1.5×10^4 cells mL^{-1} in a 24-well plate with 30 mm cover slide plated in the bottom of the well. After overnight incubation, 50 μg mL^{-1} (Value close to the IC_{50} value) PSB were added in 40 μL aliquots. The negative control was 40 μL 0.01% DMSO in Hank's solution. Camptothecin (50 μg mL^{-1}) was used as positive control. The cell-seeded cover slides were removed from 24-well plates at 24 h after treatment. The cells were then stained using the Annexin-V-FLUOS staining kit (Roche Applied Science, Mannheim, Germany). Labelling solution (20 μL Annexin-V-FLUOS-labelling reagent in 1 mL HEPES buffer with 20 μL propidium iodide reagent) was used to stain apoptotic cells which were then visualized by green fluorescence microscopy with excitation wavelength at 488 nm.

Total antioxidant activity test: Prepared PSB and BHT at concentration of 0.24, 0.48, 0.72, 0.96, 1.20 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 PSB and BHT at concentration of 0.24, 0.48, 0.72, 0.96, 1.20 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 100 times using absolute ethanol to produced $ABTS^+$ working solution. The 4 mL $ABTS^+$ working solution was mixed with 1 mL sample and 1 mL absolute ethanol. The mixtures were then shake for 1 min and measure the absorbance at 734 nm as follows:

$$\text{ABTS free radical scavenging rate}(\%) = \frac{A_0\text{-}A}{A_0}\times100$$

where, A_0 is absorbance of $ABTS^+$ working solution+ absorbance of absolute ethanol and A is absorbance of $ABTS^+$ working solution+ absorbance of samples.

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 AND DISCUSSION

Optimization of PSB isolation: The water extraction following alcohol precipitation is the regular principle used for herbal polysaccharides isolation. In this study, optimized the PSB isolation method through adjusting the material/water ratio, ultrasonic parameters and ethanol percentage. In the single factor experiment, the content of PSB was found to decrease along the *S. barbata* (g)/water (mL) (1:6, 1:10, 1:14, 1:18 and 1:24) decrease. The isolation with shorter ultrasonic time (10, 20 and 30 min) didn't produce less PSB than that of longer time (40 and 50 min) but repeat ultrasonic times (2, 4 and 5 times) could lead to a higher polysaccharides content. About 60, 80 and 90% ethanol resulted in the higher amount precipation of PSB than 50 and 70% ethanol. Based on these,

a L9 (3^4) orthogonal experiment was designed to optimized the ultrasonic-assisted PSB isolation. Four factors including material/water ratio; (A) Ultrasonic time, (B) Repeat times, (C) Ethanol percentage and (D) Under three conditions (A:1:6, 1:10 and 1:14, B: 10, 20 and 30 min, C: 2, 4 and 5 and D: 60, 80 and 90%) were used as parameters. The analytical results indicated that the best isolation condition was $A_3B_1C_3D_2$. The optimized method parameters for PSB isoaltion were thus determined as material/water ratio at 1:14, 10 min ultrasonic, repeat 5 times and 80% ethanol usage. This set of parameters was further used to validate experimentally. A mean value of PSB yield rate was at 2.96±0.24% (n = 4) indicating that the optimized extraction process was adequate for the PSB isolation. Compared to the traditionally used PSB isolation, this ultrasonic-assisted PSB isolation method is characterized with higher yield rate and less time and resources consuming[10]. Moreover, the ultrasonic employed in this method is characterized of fast, clean and efficiency. As the whole isolation was performed in the room temperature and the bioactive compounds were remained in the maximum amounts of their original structure without heat degradation. The significant of this development is to greatly facilitate the gathering of large amounts of PSB which would promote the current and future study and finally transform PSB to the valuable medicinal products.

Anticancer activities of PSB *in vitro*: The anticancer activities of *S. barbata* have been widely reported in both of the *in vitro* and *in vivo* models[11-13]. Previously, the flavonoids and diterpenoid alkaloids were recognized as the major active anticancer components of *S. barbata*[3,14]. Recently, the inhibitory effects of PSB were found in 95-D cancer cell lines and xenograft tumours[5-7]. However, the current studies were only limited in just lung cancer cells or animal models. In this study, the anticancer activities of PSB were further determined in human esophageal cancer cell EC109 and human liver cancer cell HepG2 *in vitro*. As shown in Fig. 1, PSB inhibited the proliferation of both of the cancer cell lines *in vitro* on a dose-dependent manner. The IC50 values of PSB on EC109 and HepG2 were 43.62±1.51 μg mL^{-1} and 60.44±2.31 μg mL^{-1}, respectively. Moreover, the PSB (50 μg mL^{-1}) could induce the apoptosis of EC109 and HepG2 cells under 24 h treatment. As these results were highly in agreement with the data reported in previous study, PSB could be confirmed as an effective inhibitor for the growth of cancer cells through apoptosis-inducing mechanisms[6,7].

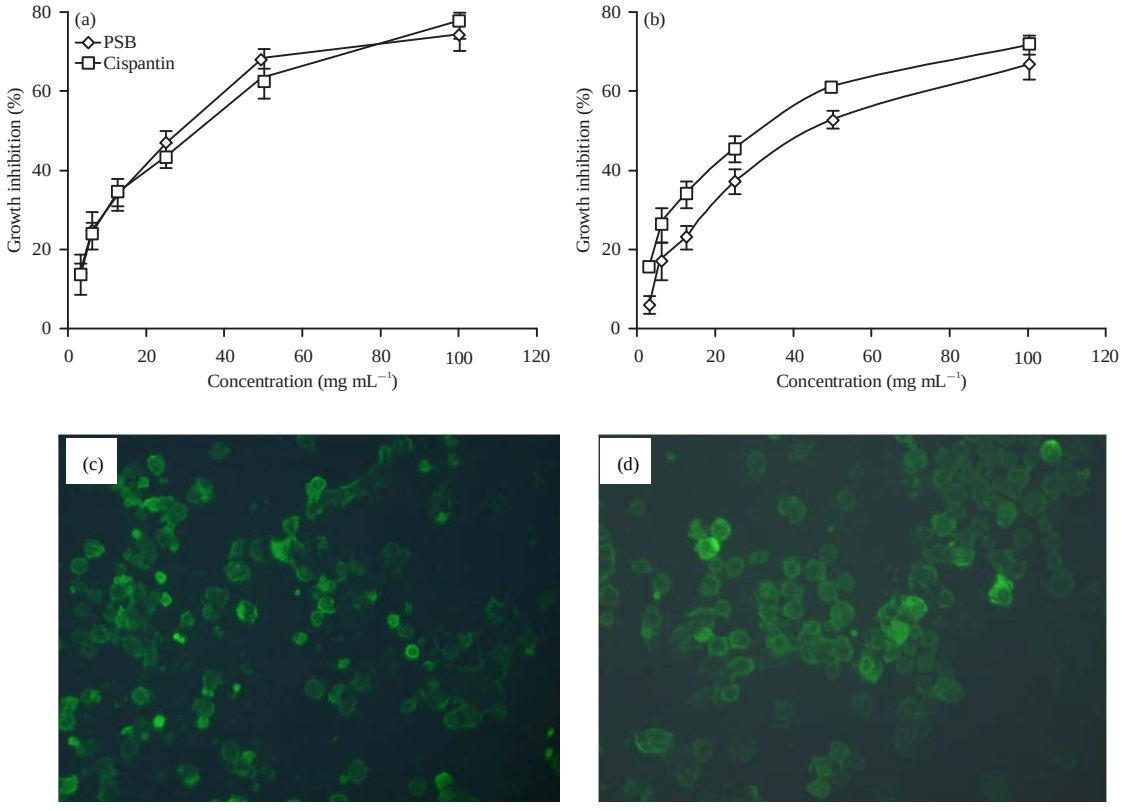

Fig. 1(a-d): (a) Growth inhibition of PSB on EC109, (b) HepG2, (c) Apoptosis induced by PSB on EC109 and (d) HepG2

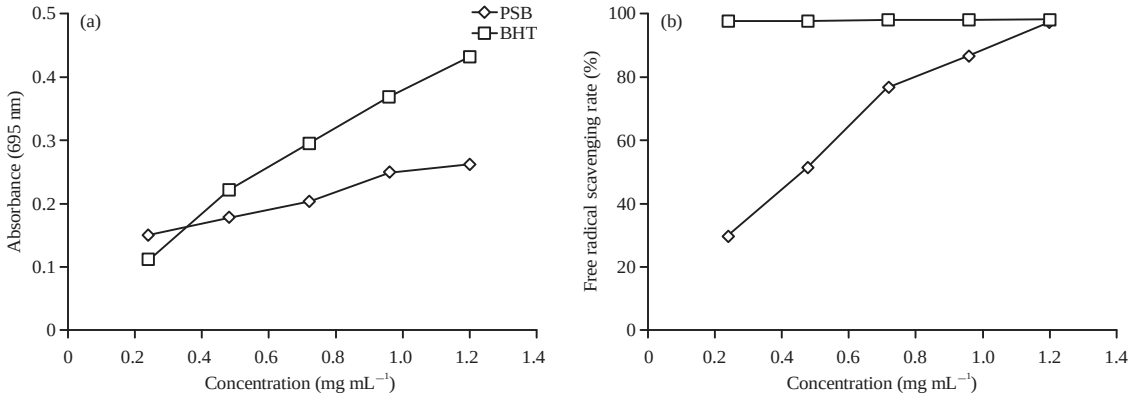

Fig. 2(a-b): (a) Total antioxidant activity of PSB and BHT (Left) and (b) ABTS free radical scavenging rate of PSB (Right)

The more detailed mechanisms may include regulation the CDK/cyclin cascade and apoptotic proteins such as Bcl-2, Bax, Cyclin D1, CDK4 and p21[11,15]. As reported by Lin *et al.*[11] the ethanol extract of *S. barbata* could significantly suppress the activation of STAT3, Erk and p38 signalings in color cancer tissues through regulation of multiple critical genes expression such as Bcl-2, Bax, Cyclin D1, CDK4 and p21. The chloroform fraction of *S. barbata* was reported to increase the ratio of the pro-apoptotic Bax/Bcl-2 and decreased the expression of the pro proliferative cyclin D1 and cyclin-dependent kinase 4 in human colon cancer cells[15]. According to a previous genomics screening study reported that the *S. barbata* induced apoptotic death of cancer cells need multiple genes involved in mechanisms including control of DNA damage, cell cycle, nucleic acid binding and protein phosphorylation[12]. The PSB may also involve in this complex regulation network. Taken together, this significant of current results is to firstly evident the anticancer activities and apoptosis-inducing abilities of PSB on human esophageal and liver cancer cells *in vitro* which expand the fundamental understanding of the anticancer activity of *S. barbata* and support its clinical use. However, the detailed underlying mechanisms of anticancer effects of PSB still remain as an ongoing challenge and thus are highly suggested to be further studied.

Antioxidant activities of PSB: Oxidative stress has been known as a causative factor for progression of diseases as well as cancer. In this study, the antioxidant activities of isolated PSB were examined since, the wide use of *S. barbata* in medicine. Characterization of antioxidant effects of PSB was determined and compared to standard butylated hydroxytoluene (BHT). Two different assays including total antioxidant activity test and ABTS free radical scavenging assay were employed for the comparison. As shown in Fig. 2, PSB presented a dose-dependent antioxidant activity and this activity could achieve similar effects as BHT did at lower concentration level (0.24 and 0.48 mg mL^{-1}). In the ABTS free radical scavenging assay, the different concentration of BHT demonstrated a constant high scavenging rate (over 95%) while, PSB's effects increased gradually and reach BHT's value at concentration of 1.2 mg mL^{-1}. Taken together, the isolated PSB presented a great antioxidant and free radical scavenging activity which was in good agreement with the research reports in previous study[8]. The results above could confirmed PSB as the major active group constitutes the antioxidant activities of herbal *S. barbata* thus, providing a valuable natural antioxidant for further investigation and development. Moreover, current study of antioxidant activities of PSB were all conducted to test *in vitro* assays and no *in vivo* experiments reported so far. Therefore, *in vivo* efficacy, side effects and safety of PSB remain unknown. The proper designed *in vivo* models, comprehensive well-controlled and double-blind clinical trials are urgently needed.

CONCLUSION

Overall, the herb *S. barbata* is widely used in TCM hospitals of China and its medicinal values are gradually accepted worldwide. Therefore, the exploration of bioactivities of its major components is important to underpin the fundamental understanding of the medicinal activities of *S. barbata* and support its clinical use. In this study, this study has optimized an ultrasonic-assisted method to extract the polysaccharides from *S. barbata*. The PSB's anticancer and antioxidant activities which are two of most used medicinal properties of *S. barbata* were then determined in *in vitro* experimental models. The significant of this results to current

knowledge of the research field include; (1) The developed PSB isolation method provides higher yield rate and consumes less time/resources compared to the method already published which would promote the current and future research in relevant fields, (2) The growth inhibitory and apoptotic effects of PSB are firstly determine in those two cancer cell lines (EC109 and HepG2) *in vitro*. It determined that PSB was a significant bioactive group of components constitute the anticancer and antioxidant effects of *S. barbata* providing a potential natural medicinal resource for further investigation and development. The further studies are strongly suggested to explore its underlying mechanisms of bioactivities and develop the PSB-based drugs and health products or addictives.

ACKNOWLEDGMENT

The authors gratefully acknowledge the University Scientific Research Funding (2014QN012) from Henan University of Science and Technology and the Research Fund of Returned Overseas Personnel (13530001) from Ministry of Education of the People's Republic of China.

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