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Original Research Article

Anti-tumor activity of polysaccharides extracted from *Senecio scandens* Buch, -Ham root on hepatocellular carcinoma

Chunqing Dou*, Bao Zhang, Mingming Han, Xin Jin, Liyuan Sun and Tao Li

Department of Hepatobiliary Surgery, The First Affiliated Hospital of Chinese PLA General Hospital, Beijing 100048, PR China

*For correspondence: **Email:** dhsdou@126.com; **Tel/Fax:** +86-010-66848631

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Abstract

Purpose: To optimize the extraction conditions of polysaccharides from the root of *Senecio scandens* Buch, -Ham. (PRS) and evaluate its anti-tumor effect on hepatocellular carcinoma.

Methods: Response surface methodology (RSM) applied with a Box-Behnken design (BBD, three levels and three factors) was employed to determine the effect of extraction time, number of extraction and ratio of water to raw material on the yield of PRS. The anti-tumor effect of PRS on A549, HL60, S180 and H22 cell lines was evaluated *in vitro* by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay, while *in vivo* anti-tumor effect was evaluated in H22 tumor transplanted mice. Furthermore, expressions of proteins including caspase-3, caspase-9, Bcl-2 and Bax were determined by western blotting assay.

Results: The established BBD model was highly significant and the optimal conditions were: extraction time, 3.06 h; number of extractions, 2; and ratio of water to raw material, 16.17 mL/g. PRS showed significant inhibitory effect on H22 cells ($IC_{50} = 42.4 \mu\text{g/mL}$), and significantly inhibited the growth of transplanted H22 tumors in mice at the doses of 20, 40 and 80 mg/kg ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively). Treatment with PRS (20, 40 and 80 $\mu\text{g/mL}$) significantly up-regulated the expressions of Bax, caspase-3 and caspase-9 in H22 cells, whereas Bcl-2 protein was significantly down-regulated.

Conclusion: The results suggest that PRS possesses significant anti-tumor activity on H22 cell line *in vitro* and *in vivo*, and the mechanism may be closely related to the induction of mitochondria-mediated apoptosis.

Keywords: *Senecio scandens*, Polysaccharides, Hepatocellular carcinoma, Response surface methodology, Anti-tumor activity, Apoptosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide with a high incidence and mortality [1,2]. Besides surgical management, chemotherapy is another important therapeutic option for HCC, but it would cause severe toxicities which are commonly difficult to tolerate by HCC patients [3,4]; for instance, fluorouracil and doxorubicin,

two drugs currently used for treating HCC, are not satisfactory since they can produce severe side effects and are not too effective [5]. Thus, searching for new candidate agents with better effectiveness and lower toxicity for treating HCC have received more and more attention.

Traditional Chinese Medicines (TCMs) have a long history in the treatment of various cancers, and they were reported to have low cost and less

side effects [6,7]. *Senecio scandens* Buch.-Ham. (Asteraceae), known as “*Qianliguang*” in Chinese, is one of the most popular species used as a Chinese folk medicine, and it is mainly distributed in southwest China [8]. It is used for the treatment for various ailments, such as enteritis, bacterial diarrhea, respiratory tract infections, and conjunctivitis in China [9]. However, there is no report on the anti-tumor effect of *S. scandens* and its related active components. In the present study, the antitumor effects of polysaccharides extracted from the root of *S. scandens* (PRS) against HCC were investigated, which could lead to further development of PRS as a potential drug for treating HCC.

EXPERIMENTAL

Chemicals and reagents

Fetal bovine serum (FBS) and Roswell park memorial institute (RPMI)-1640 culture medium were purchased from Thermo Fisher Scientific (Waltham, MA USA); 5-Fluorouracil (5-FU) and MTT were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA); primary antibodies of caspase-3, caspase-9, Bcl-2 and Bax were from R&D Systems, Ltd. (Minneapolis, USA). All the other chemicals and reagents were of analytical grade.

Extraction of polysaccharides from *S. scandens*

The dried whole plant of *S. scandens* was powdered, and defatted with 95 % ethanol for 24 h. The pretreated powder of *S. scandens* (50 g) were extracted by refluxing with distilled water at selected extraction times (2, 3 and 4 h), number of extraction (1, 2 and 3), and ratio of water to raw material (10, 15 and 20 mL/g). After filtration, the solution were concentrated and precipitated with ethanol to a final concentration of 80 % (v/v) overnight at 4 °C. After centrifugation (8000 rpm for 10 min), the precipitates were collected, and then washed respectively with 95 % ethanol, anhydrous ethanol and acetone. The crude polysaccharides were dried, and the percentage yield (%) was calculated as the following formula: PRS extraction yield (%) = Y_0/Y , where Y_0 (g) is the weight of dried crude polysaccharides (PRS); Y (g) is the weight of dried powder of *S. scandens*.

Experimental design of response surface methodology (RSM)

RSM with a Box-Behnken design (BBD, three levels and three factors) was applied to determine the effect of extraction time, number of

extractions, and ratio of water to raw material on the extraction yield of PRS. Based on the results of single factor experiment, the experimental range of each factor was determined. The experimental arrangement is shown in Table 1.

Cell culture

A549, HL60, S180 and H22 cell lines were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI medium supplemented with 10 % FBS, 100U/mL penicillin and 100 µg/mL streptomycin. The cell lines were cultured in a cell incubator (Thermo Scientific, Waltham, MA, USA) at 37 °C in 5 % CO₂/95 % air.

Animals

BALB/c mice (18-22 g) were obtained from the Experimental Animal Center of the First Affiliated Hospital of Chinese PLA General Hospital (Beijing, China) and acclimatized for 7 days before they were used for the experiment. The mice were housed under controlled environment (23 ± 1 °C and a 12-h light/12-h dark cycle) with free access to food and water. Research and animal experimental procedures were strictly in accordance with “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985) and approved by the Animal Care and Use Committee of the First Affiliated Hospital of Chinese PLA General Hospital (approval no. A 90-12-2015t09).

Determination of anti-tumor effect of PRS *in vitro*

A549, HL60, S180 and H22 cell proliferations were determined by using MTT assay as previously described [10]. Briefly, cells (2.0 × 10⁴/0.2 mL) were seeded into 96-well plates and allowed to attach for 24 h. A series of concentrations of PRS (10, 20, 40, 80, 160, 320 and 640 µg/mL) were added and incubated for 48 h. Subsequently, 5 mg/mL MTT (10 µL) solutions were added. After incubating at 37 °C for 4 h, the supernatant was discarded and dimethyl sulfoxide (100 µL) was added to each well. The plates were read at 570 nm with a Mode 680 96-well microplate reader (Bio-Rad, Tokyo, Japan). Inhibition rate (H) was calculated as in Eq 1.

$$H (\%) = \{(A_0 - A_x)/A_0\}100 \dots\dots\dots (1)$$

where A_0 is the absorbance of controls and A_x the absorbance of cells treated with PRS.

Determination of anti-tumor effect of PRS *in vivo*

The diluted tumor cells were subcutaneously inoculated (0.2 mL, 1×10^6 cells/mouse) into the right armpits of the mice to establish the animal model. After 24 h, mice were divided randomly into five groups. PRS was administered orally (*ig*) to each group at different dosages (20, 40, 80 mg/kg). The positive control group was treated with 5-FU at a dosage of 5 mg/kg intraperitoneally (*ip*), and the control group was treated with normal saline (*ig*). The weights of the mice were recorded before drug administration which lasted for 14 days. On the 15th day, all the mice were sacrificed and the segregated tumor weights were recorded.

Western blot assay

Western blot assay was used to investigate the apoptotic mechanisms of PRS on H22 cells [11]. Different concentrations of PRS (20, 40 and 80 $\mu\text{g/mL}$) were added to the H22 cells and incubated for 48 h. Then, the cells were harvested and total proteins were extracted. Total protein sample (40 μg) was separated using SDS/PAGE. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane, and subsequently subjected to immunoblotting analysis with corresponding primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotech, Haimen, China). After that, chemiluminescence reagents (Beyotime Biotech, Haimen, China) were added for visualization of the protein bands. Antibodies against β -actin was used to normalize for protein loading.

Statistical analysis

The data are expressed as mean \pm standard deviation (SD) and were analyzed by ANOVA. Significance of differences between groups was evaluated using Student's *t*-test. RSM was analyzed with Design Expert Version 8.0.5 software (State-Ease, USA). $P < 0.05$ was regarded as statistically significant.

RESULTS

Model fitting and statistical analysis

As shown in Table 1, independent variables (extraction time, number of extraction, and ratio of water to raw material) on the extraction yield of PRS were examined by BBD design (Table 1).

Multiple regression analysis was performed, and the predicted the yield (Y) of PRS could be obtained by the second-order polynomial equation as in Eq 2.

$$Y = 6.71 + 0.091 A + 0.61 B + 0.27 C - 0.005 AB - 0.033 AC - 0.16 BC - 0.72 A^2 - 1.05 B^2 - 0.48 C^2 \dots\dots\dots (2)$$

The analysis results of ANOVA are shown in Table 2. The "lack of fit F-value" (3.01) was not significant due to the relative pure error. Based on the validity analysis of the three variables, it showed that the independent variables of B and C, the interaction term of B \times C, and the two quadratic terms of A^2 , B^2 and C^2 significantly affected the yield of PRS ($p < 0.001$). The R^2 and R^2_{Adj} values (0.9890 and 0.9750, respectively) showed a high degree correlation between the observed values and the predicted values. In addition, the statistical analysis also showed a very low p -value (< 0.0001) and a very high F value (585.70), indicating the established model was highly significant.

Optimized PRS extraction conditions and validated models

By using the Design-Expert software, 3D graphic surface for the effects of three independent variables are shown in Figure 1. From the model, the optimal conditions for obtaining the highest PRS yield were: extraction time 3.06 h, number of extractions 2.27, and ratio of water to raw material 16.17 mL/g. The maximum yield of PRS predicted by the model was 6.83 %. To validate the accuracy of the model, the optimal condition was modified as follows: extraction time 2.11 h, number of extractions 2, and ratio of water to raw material 16.17 mL/g. A verification experiment was carried out under the modified conditions. As a result, the mean yield of PRS obtained from actual experiments was 6.85 ± 0.12 %. The results indicated that the RSM was effective for optimizing the extraction conditions for PRS.

Effect of PRS on cells growth *in vitro*

The cytotoxic effect of PRS on A549, S180, HL60 and H22 cell lines were evaluated by MTT assay. As a result, PRS significantly inhibited the cell proliferations of H22, A549, S180 and HL60 cell lines, and the IC_{50} values were shown in Table 3. PRS showed higher inhibitory effect against the proliferation of H22 cells (IC_{50} value of 42.4 $\mu\text{g/mL}$) than the other cell lines with the highest inhibition ratio of 73.3% at 320 $\mu\text{g/mL}$.

Table 1: Experimental design and test results of RSM

S/no.	A: Extraction time (h)	B: Number of extraction	C: Ratio of water to raw material(mL/g)	Yield (%)
1	3.00	3.00	10.00	5.72
2	3.00	2.00	15.00	6.73
3	4.00	2.00	10.00	5.32
4	2.00	1.00	15.00	4.22
5	3.00	1.00	20.00	4.98
6	2.00	2.00	10.00	5.11
7	4.00	3.00	15.00	5.66
8	3.00	2.00	15.00	6.72
9	3.00	1.00	10.00	4.18
10	3.00	2.00	15.00	6.75
11	2.00	3.00	15.00	5.45
12	2.00	2.00	20.00	5.78
13	3.00	2.00	15.00	6.66
14	3.00	3.00	20.00	5.87
15	3.00	2.00	15.00	6.69
16	4.00	2.00	20.00	5.86
17	4.00	1.00	15.00	4.45

Table 2: Test results of significance for regression coefficient

Source	Sum of Squares	df	Mean Square	F -Value	p-value
Model	12.26	9	1.36	585.70	< 0.0001
A	0.067	1	0.067	28.65	0.0011
B	2.96	1	2.96	1275.10	< 0.0001
C	0.58	1	0.58	250.84	< 0.0001
AB	1.000E-004	1	1.000E-004	0.043	0.8416
AC	4.225E-003	1	4.225E-003	1.82	0.2196
BC	0.11	1	0.11	45.43	0.0003
A ²	2.17	1	2.17	932.30	< 0.0001
B ²	4.62	1	4.62	1987.11	< 0.0001
C ²	0.95	1	0.95	408.60	< 0.0001
Residual	0.016	7	2.325E-003	-	-
Lack of Fit	0.011	3	3.758E-003	3.01	0.1576
Pure Error	5.000E-003	4	1.250E-003	-	-
Cor Total	12.27	16	-	-	-

C.V.%	R ²	R ² Adj	R ² Pred
0.85	0.9987	0.9970	0.9847

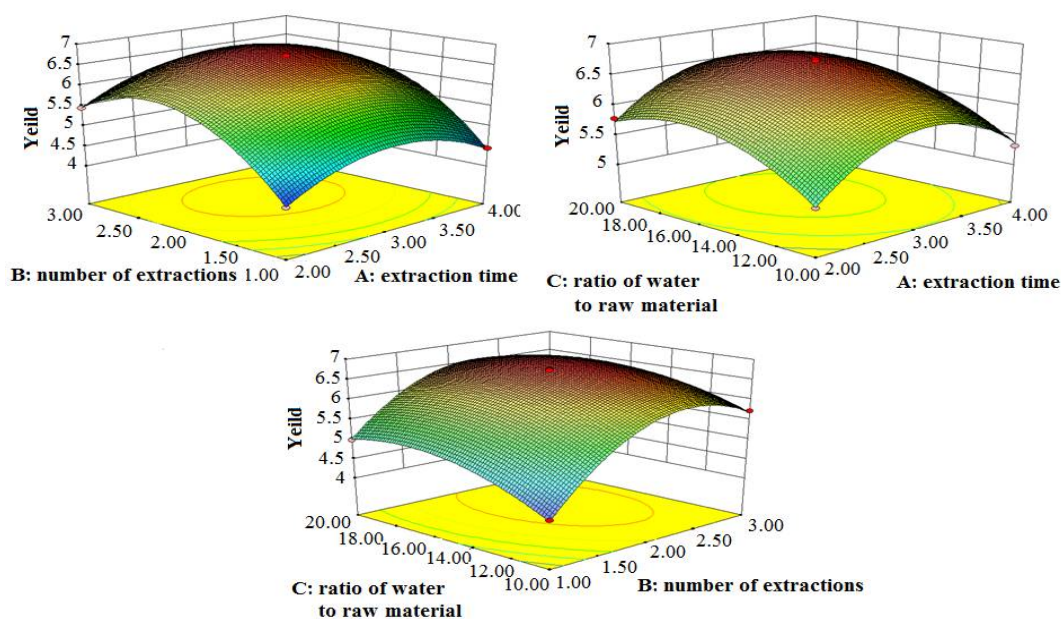


Figure 1: 3D graphic surface for t effects of extraction time, number of extraction, and ratio of water to raw material

Table 3: IC₅₀ values of PRS on tumor cell lines

Cell	IC ₅₀ value
H22	42.4
A549	102.8
S180	60.3
HL60	117.9

In vivo anti-tumor effect of PRS

The *in vivo* antitumor effect of PRS on transplanted H22 tumors in mice was showed in Figure 2. The results indicated that oral administration of PRS (20, 40 and 80 mg/kg; $p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) could significantly reduce the tumor weight in a dose-dependent manner. Also, the body weights of the mice treated with PRS were higher than that of control, especially at the doses of 40 and 80 mg/kg ($p < 0.05$, $p < 0.05$). The 5-FU could also significantly ($p < 0.01$) reduce the tumor weight, but the body weight of the mice was sharply decreased in this group ($p < 0.01$).

Effect of PRS on expressions of Bcl-2, Bax, caspase-3 and caspase-9

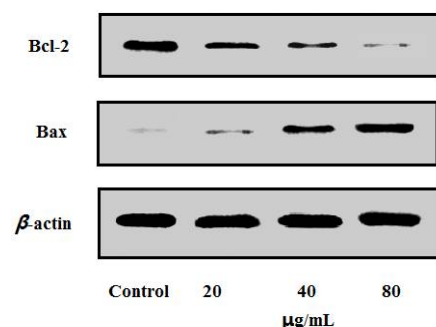


Figure 3: Changes in Bcl-2 and Bax proteins in H22 cells after treating with PRS

To study the pharmacological mechanisms for the anti-tumor effects of PRS, the effects of PRS

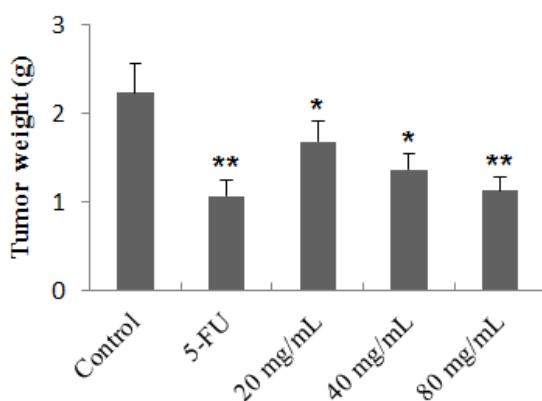


Figure 2: Effect of PRS on transplanted H22 tumor growth and body weight; * $p < 0.05$ vs control group; ** $p < 0.01$ vs control group

on apoptosis related proteins including Bcl-2, Bax, caspase-3 and caspase-9 were investigated in H22 cells. As shown in Figure 3, PRS treatment (20, 40 and 80 µg/mL) significantly up-regulated the expression of Bax, and down-regulated Bcl-2. Furthermore, similar to Bax, caspase-3 and caspase-9 were up-regulated following treatment with PRS (20, 40 and 80 µg/mL), compared with the control (Figure 4).

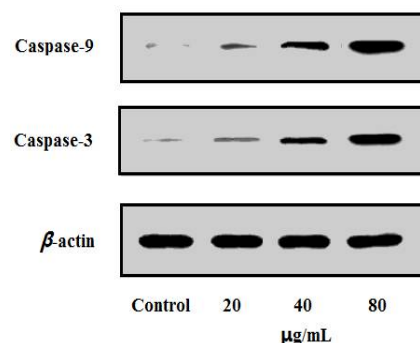
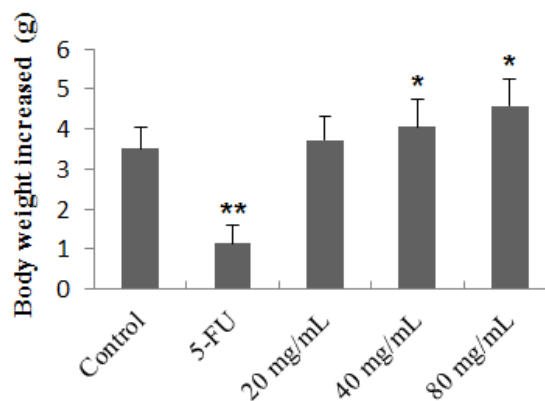


Figure 4: Changes in caspase-3 and caspase-9 proteins in H22 cells after treating with PRS.

DISCUSSION

Polysaccharides from various origins including wild plants, fungi and animals are often identified to be biological response modifiers. In recent years, researches on polysaccharides from medicinal plants have attracted much attention because of their potential biological and pharmacological activities, such as anti-tumor, immunomodulating, anti-inflammatory, cardiovascular, antidiabetic and antioxidant activities [12,13]. In the present study, polysaccharides from *S. scandens* (PRS) were extracted and the extraction conditions were optimized by employing response surface methodology (RSM), and furthermore the anti-tumor effects were investigated *in vivo* and *in vitro*.



It is well known that RSM is an effective method for optimizing the experimental conditions of complex processes [14]. The main advantage of RSM technique over other statistical techniques is that it can evaluate multiple parameters and their interactions, but the number of experiments needed is reduced [15,16]. Many researches have showed that RSM is an appropriate method used to optimize polysaccharide extraction processes [17,18]. RSM applied with a three-level three-factor BBD was employed in the present study, and the optimal conditions for PRS were as follows, extraction time, 2.11 h, number of extractions 2, and ratio of water to raw material, 16.17 mL/g.

There is increasing evidence showed that natural products and many antitumor chemotherapeutic agents can induce apoptosis of cancer cells [19]. It has also been indicated that the ratio of Bax/Bcl-2 is a key factor in the regulation of the apoptotic process, and a high ratio of Bax/Bcl-2 might promote apoptosis activity [19,20]. Furthermore, caspase-9 and caspase-3 are important activators in mitochondria-mediated apoptosis pathway, and caspase-3 can be activated by caspase-9 [21]. Thus, after treating with PRS, the expression of caspase-9 and caspase-3 proteins were up-regulated and Bax/Bcl-2 ratio increased in the H22 cells, indicating that PRS could induce mitochondrial-mediated apoptosis.

CONCLUSION

The findings of the present study indicate that PRS possesses significant anti-tumor effect on H22 tumor *in vitro* and *in vivo*, and that the mechanism of the anti-tumor effect is closely related to the induction of mitochondria-mediated apoptosis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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