

Original Research Article

Anti-tumor effect of polysaccharides from rhizome of *Curculigo orchioides* Gaertn on cervical cancer

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Abstract

Purpose: To investigate the anti-tumor effects of polysaccharides from *Curculigo orchioides* (PDC) on cervical cancer and the possible mechanisms involved.

Methods: A Box–Behnken design (BBD) was employed to optimize extraction conditions for PDC. The anti-tumor effect of PDC on cervical cancer was investigated *in vivo* in mice injected with HeLa cells. The parameters measured were tumor volume and weight. *In vitro* anti-tumor effects of PDC were assessed by measuring expressions of caspase-3, caspase-9 and P53 proteins in HeLa cells via ELISA assay. Thymus and spleen indices were calculated for assessment of PDC effect on immune function.

Results: The optimum extraction conditions predicted by the response surface methodology (RSM) were: extraction time = 1.58 h, ratio-of-water-to-sample = 30.05 mL/g and extraction number = 1.95. PDC showed significant anti-tumor effect on cervical cancer in mice. It significantly increased thymus and spleen indices in mice; and significantly up-regulated expressions of caspase-3, caspase-9 and P53 proteins in HeLa cells.

Conclusion: PDC has significant anti-tumor effect on cervical cancer *in vivo* and *in vitro*, most probably through mechanisms involving enhancement on immune function and induction of apoptosis.

Keyword: *Curculigo orchioides*, Polysaccharides, Cervical cancer, HeLa cells, Apoptosis

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INTRODUCTION

Cervical cancer is one of the most common cancers in women in developing countries, with about 530,000 new cases and 275,000 deaths yearly [1]. Patients with early-stage cervical cancer can be cured by simple surgery, but advanced stage or relapsed cervical cancers are often given subjected to a combined treatment of radiation and chemotherapy [2]. However, long-term and high-dose chemotherapy usually cause a variety of adverse reactions and drug resistance [3]. Therefore, it is very important to find novel, safer and less toxic therapeutic strategies for effective treatment of cervical

cancer. Attention has focused on natural products as potential sources of anti-tumor drugs with high efficiency and low toxicity.

Curculigo orchioides Gaertn, which belongs to the family Amaryllidaceae, is a tiny herbal plant widely distributed in China, India, Malaya, Japan and Australia [4]. Its rhizome, known as Xianmao in China, is a common traditional Chinese medicine (TCM) used as alterative, demulcent, diuretic and restorative agent [5]. *C. orchioides* is reported to possess numerous pharmacological activities including anti-osteoporotic [6], estrogenic [7], antioxidant [8], antibacterial and neuro-protective effects [9]. Interestingly, it has been reported that

polysaccharides extracted from *C. orchioides* have anti-tumor effects [10]. However, not much is known about optimization of extraction conditions of these polysaccharides, or their anti-tumor effects on cervical cancer.

The present study was designed to optimize the extraction of PDC, and to investigate its anti-tumor effect on cervical cancer *in vivo* and *in vitro*. In addition, the mechanisms of the anti-tumor effect were investigated.

EXPERIMENTAL

Chemicals and reagents

MTT [3-(4,5)-dimethylthiazolium (-z-y1)-3,5-diphenyltetrazoliumromide] and DMSO (dimethyl sulfoxide) were purchased from Sigma Aldrich Co. LLC. (St. Louis, MO, USA); RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco BRL Co. Ltd. (USA); caspase-3, caspase-9 and P53 ELISA kits were obtained from R&D systems China Co. Ltd. (Shanghai, China). All the other chemicals and reagents used in the experiment were of analytical grade.

Extraction of PDC

Whole herb of *C. orchioides* was collected from Bozhou Chinese herbal medicine market (Bozhou, China), and authenticated in the Department of Traditional Chinese Medicine in Fudan University Shanghai Cancer Center. A voucher specimen (YCB no. 2015-105) was deposited in the herbarium of the Department. The dried herb was powdered and extracted by refluxing with distilled water. Extraction time, ratio-of -water-to-sample and extraction number were designed and used in the extraction.

The aqueous extracts were filtered and concentrated under reduced pressure in a rotary evaporator (RE-2000A, Shanghai Lihua Equipment Co., Ltd.). The polysaccharides were then precipitated by addition of anhydrous ethanol (final concentration was 80 %, v/v). The extract-ethanol solution was left overnight at 4 °C, and centrifuged at 5000 rpm for 20 min. The precipitate was washed with acetone and anhydrous ethanol, and dried at 40 °C to a constant weight. The yield of the crude polysaccharides was then calculated.

Experimental design of RSM

Based on the results of single factor analysis, three major factors including extraction time (A),

ratio-of-water-to-sample (B) and extraction number (C) were confirmed, and then a response surface methodology (RSM) was conducted. As shown in Table 1, a total of 17 experiments were carried out in triplicate according to the BBD matrix.

Animals

Female nude mice, aged 6 - 8 weeks old and weight (20 ± 2 g) were obtained from Shanghai Laboratory Animal Research Center (Shanghai, China). They were maintained at a 12 h light/12 h dark cycle under a temperature regulated environment (20 ± 1 °C); and housed with free access to feed and water. All procedures used for the animal experiments were in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [11] approved by the Animal Ethics Committee of Fudan University Shanghai Cancer Center (approval no. SCC-AU-2015-19).

Cell culture

HeLa (human cervical cancer) cells were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI 1640 culture medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin/streptomycin and incubated in the presence of 5 % CO₂ at 37 °C.

Animal model and administration

HeLa cells (1×10^7 cells/mouse) were subcutaneously injected into the lower abdominal region of the mice, and the animal model was established as previously described [12], with some modifications. The mice were randomly divided into 4 groups: control group (normal saline), low-dose PDC group (10 mg/mL/day), medium-dose PDC group (20 mg/mL/day) and the high-dose PDC group (40 mg/mL/day). All treatments were given daily for 15 days.

In vivo anti-tumor effect of PDC

Tumor volumes in the cervical cancer bearing mice were measured using a caliper [tumor volume = $0.5 \times \text{length} \times (\text{width})^2$] at the 6, 8, 10, 12 and 15 days after transplant. At the end of the experiment, the animals were weighed and sacrificed. Subsequently, tumors were dissected out for measurement of the tumor weights, while thymus and spleen were removed and weighed. The thymus index and spleen index were

expressed as the thymus or spleen weight relative to body weight (mg/g).

In vitro anti-tumor effect of PDC

The *in vitro* anti-tumor effect of PDC on HeLa cells were evaluated by MTT assay as the previously described [13]. The cells were plated onto a 96-well plate at a density of 1×10^5 cells/mL and incubated with PDC (5, 10, 20, 40 and 80 $\mu\text{g/mL}$) for 24 h. Subsequently, MTT (5 mg/mL) was added to each well and the well incubated for another 4 h at 37 °C. The resultant formazan crystals were dissolved in 150 μL of DMSO, and the absorbance was read in plate reader (Bio-Tek, USA) at 570 nm. The % inhibition was calculated using Eq 1.

$$\% \text{Inhibition} = \{(A_c - A_t)/A_c\}100 \dots\dots\dots (1)$$

where A_c and A_t are the absorbance of control and treated samples, respectively.

Determination of casepase-3, casepase-9 and P53

HeLa cells were seeded onto a 96-well plate at a density of 1×10^5 cells/mL. PDC at concentrations of 10, 20 and 40 mg/mL were added to separate wells. After incubating for 24 h, concentrations of casepase-3, casepase-9 and P53 proteins were analyzed by ELISA using commercial kits according to the manufacturer instructions.

Statistical analysis

All data are expressed as means \pm standard deviations. The RSM data analysis was performed by using Design Expert Version 7.0.0 software (Stat-Ease, Inc., USA). Analysis of Variance (ANOVA) was used to analyze the fitness of the polynomial model equation. The significance of difference between groups was determined by student's t-test. *P*-value less than 0.05 was considered statistically significant.

RESULTS

BBD design

RSM optimization was more advantageous than single parameter optimization because it saves time, space and raw material [14]. In present study, BBD design was performed and the experimental data was analyzed by the multiple regression analysis (Table 1). The response variable and the test variables were related by the following second-order polynomial equation:

$$Y = -14.255 + 16.889 A + 0.440 B + 0.107 C + 0.003 AB + 315 AC + 0.016 BC - 0.572 A^2 - 7.93 \times 10^{-3} B^2 - 0.281 C^2 \dots\dots\dots (2)$$

As shown in Table 2, the high F-value ($F = 72.14$) and very low *p* value ($p < 0.0001$) indicated that the model was highly significant. The lack of fit F-value ($F = 0.81$) was very low and the associated *p*-value (0.5494) was very high, implying that the lack of fit F-statistic was insignificant. These indicate that the model equation was adequate for predicting the yield of PDC under any combination of values of variables. R^2 and R^2_{Adj} values of the established RSM were 0.9893 and 0.9756 respectively, indicating a high degree of correlation between the observed and predicted values. The coefficient of variation (C.V.) value 3.18% suggests good precision and higher reliability of the models in predicting experimental results.

Table 1: Box-behnken design (BBD) with the independent variables

| Run | A | B | C | Yield (%) |
|-----|------|----|---|-----------|
| 1 | 1.50 | 30 | 2 | 5.89 |
| 2 | 2 | 30 | 1 | 4.50 |
| 3 | 2 | 40 | 2 | 4.01 |
| 4 | 1.5 | 30 | 2 | 5.54 |
| 5 | 1.5 | 20 | 1 | 4.93 |
| 6 | 2 | 20 | 2 | 3.87 |
| 7 | 1 | 40 | 2 | 3.24 |
| 8 | 2 | 30 | 3 | 4.68 |
| 9 | 1.5 | 30 | 2 | 5.69 |
| 10 | 1 | 30 | 3 | 3.35 |
| 11 | 1 | 30 | 1 | 3.80 |
| 12 | 1.5 | 30 | 2 | 5.91 |
| 13 | 1 | 20 | 2 | 3.13 |
| 14 | 1.5 | 20 | 3 | 4.52 |
| 15 | 1.5 | 30 | 2 | 5.75 |
| 16 | 1.5 | 40 | 1 | 4.52 |
| 17 | 1.5 | 40 | 3 | 4.76 |

Optimization of extraction conditions of PDC

Response surface models were plotted to study the effects of the variables (extraction time, ratio-of-water-to-sample and extraction number); and their interactions on yield of PDC. Based on ANOVA and response surface plots, the predicted optimum extraction conditions were: extraction time of 1.58 h, ratio-of-water-to-sample of 30.05 mL/g and extraction number of 1.95. The predicted yield of PDC at these optimal conditions was 5.79 %. In order to facilitate the extraction process, the optimal conditions were modified to by using 1.6 h, 30 mL/g and 2 for extraction time, ratio-of-water-to-sample and extraction number, respectively. Under these conditions, the actual experimental yield of PDC

was 5.58 ± 0.34 % which is in agreement with the predicted model value.

Effect of PDC on tumor volume and tumor weight

Results of PDC effect on tumor growth and tumor weight are shown in Figure 2A and Figure 2B,

respectively. Tumor volume of PDC groups at the doses of 10, 20 and 40 mg/kg were significantly decreased ($p < 0.01$) relative to the control group. Tumor weight in the PDC treated groups were also significantly and dose-dependently decreased ($p < 0.01$) when compared with the control group.

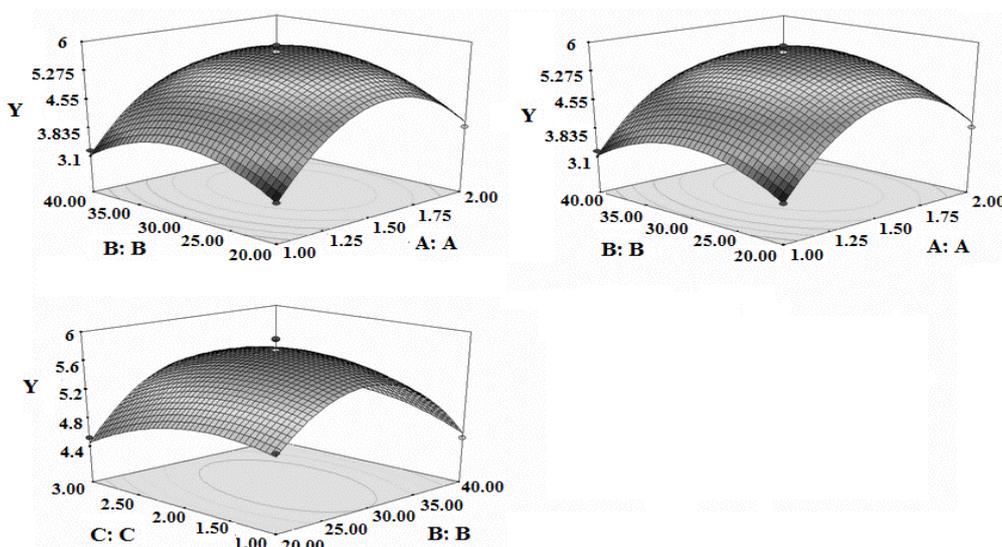


Fig 1: Response surface methodology assay. A = extraction time, B = ratio-of-water-to -sample; C = extraction number

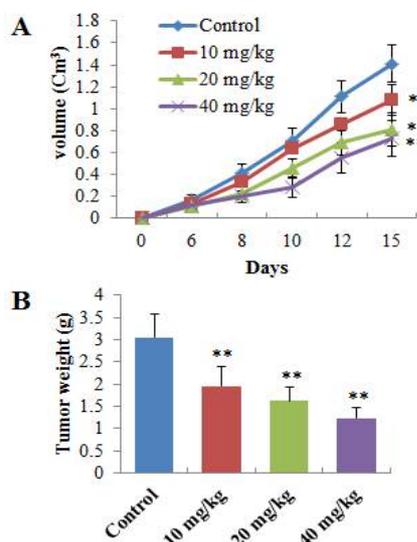


Fig 2: Antitumor effect of PDC against Hela cells. (A = effect on tumor growth; B = effects on tumor weight). ** $p < 0.01$ compared with control mice

Effect of PDC on spleen index and thymus index

To evaluate the effect of PDC on immune function on the cervical cancer bearing mice *in*

vivo, the thymus index and spleen index were examined. As shown in Figure 3, thymus index

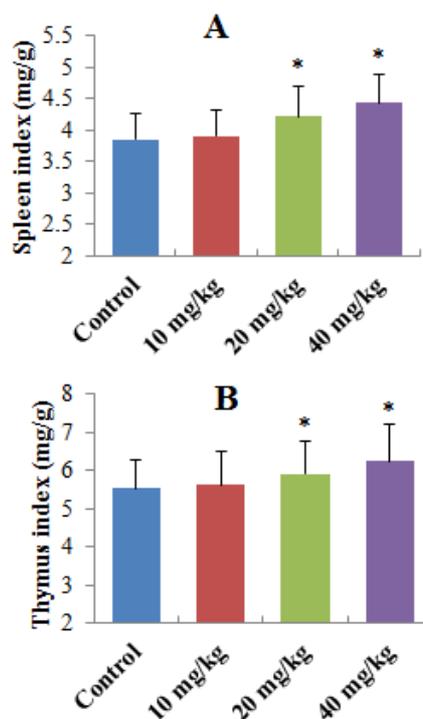


Figure 3: Effect of PDC of Spleen (A) and Thymus (B). * $p < 0.05$, compared with control mice

and spleen index were significantly increased in PDC-treated groups ($p < 0.05$) at the doses of 20 and 40 mg/kg compared with the control group. The results indicate that PDC enhanced immune function of the cervical cancer-bearing mice.

Anti-tumor effect of PDC on HeLa cells

MTT assay was used to investigate the inhibitory effect of PDC on HeLa tumor cells. PDC had no significant inhibitory effect on HeLa tumor cells at the lowest concentration (5 mg/mL; Figure 4). However, it exhibited significant and dose-dependent inhibitory effects at higher concentrations of 10, 20, 40 and 80 mg/mL ($p < 0.05$, $p < 0.01$) relative to the control group.

Effect of PDC on caspase-3, caspase-9 and P53

To understand whether the inhibitory effect of PDC on HeLa cells occurred through apoptosis, the expressions of caspase-3, caspase-9 and P53 were tested by ELISA and the results were shown in Figure 5 and Figure 6.

The expressions of caspase-3 and caspase-9 were significantly and concentration-dependently up-regulated after treatment with PDC at the concentrations of 10, 20 and 40 mg/mL ($p < 0.05$ and $p < 0.01$) compared with control group (Figure 5). The results in Figure 6 also indicated that PDC at low concentration of 10 mg/mL could not up-regulate the expression of P53. However, it had significant up-regulating effect on the expression of P53 at high concentrations of 20 and 40 mg/mL compared with the control group.

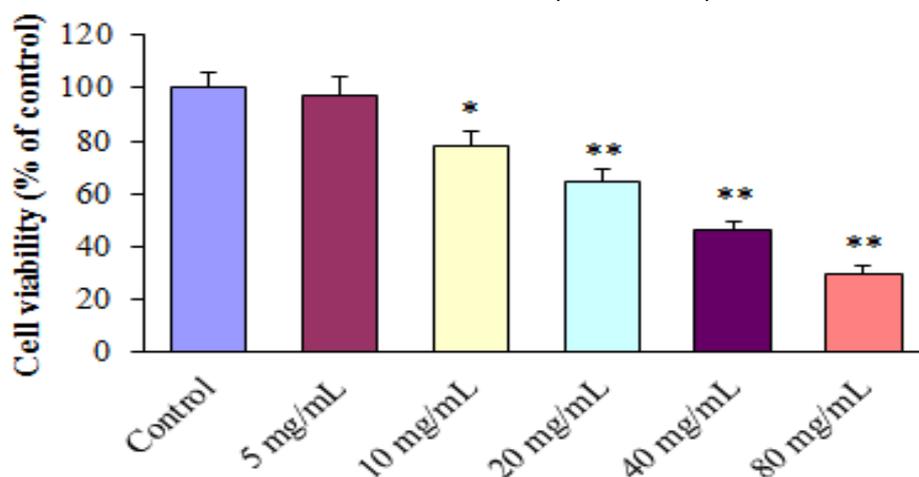


Figure 4: Anti-proliferative effect of PDC on HeLa cells; * $p < 0.05$, ** $p < 0.01$, compared with control mice

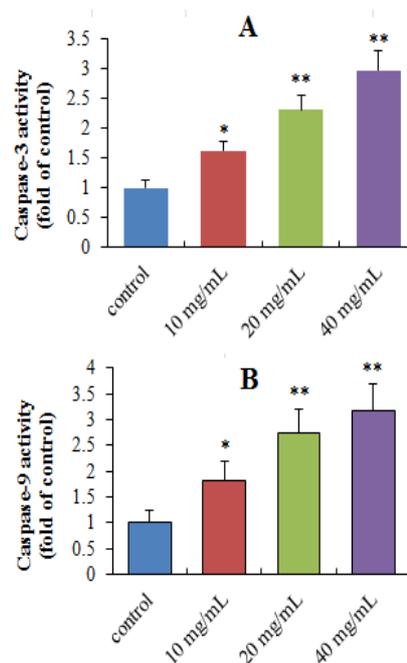


Figure 5: Up-regulating effects of PDC on caspase-3 and 9 in HeLa cells; * $p < 0.05$, ** $p < 0.01$, compared with control mice

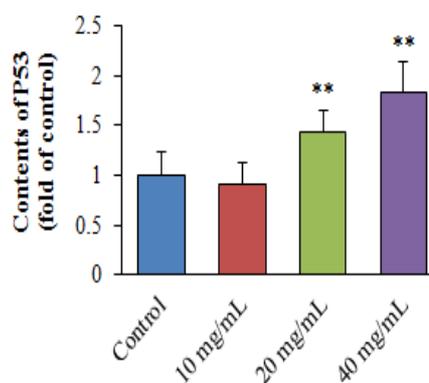


Fig 6: Up-regulating effects of PDC on P53 in HeLa cells; ** $p < 0.01$, compared with control mice

DISCUSSION

Traditional Chinese medicine plays an important role in the discovery of new drugs, including anticancer drugs [15]. Recent reports have shown that polysaccharides resist tumors by improving the immune system and inducing apoptosis [16,17].

Response surface methodology (RSM) is an effective tool for optimizing extraction conditions [18], and BBD is a popular type of RSM used for optimization due to advantages such as cheapness, reduced extraction time and use of less raw materials [19]. BBD was employed in the present study to optimize the effects of extraction time, ratio-of-water-to-sample, and extraction number on the yield of polysaccharides obtained from *C. orchoides* (PDC). The results showed that BBD was an appropriate method for assessing combined effect of independent variables on the extraction yield of PDC.

Thymus and spleen are the most important immune organs, and their immunologic function are suppressed during tumorigenesis. Thymus index and spleen index (expressed as the thymus or spleen weight relative to body weight) are important indicators for immunologic function [20,21]. Our results showed that PDC significantly increased the thymus index and spleen index, suggesting its anti-tumor effect was related to the enhancement on immunologic function.

It has been reported that sequential activation of caspases (including caspase-3 and caspase-9) plays a central role in the execution-phase of apoptosis [22]. More importantly, P53 is an important tumor suppressor protein that mediates stress-induced apoptosis cascade; restoration of p53 function is critical for effective therapeutic targeting and management of cervical cancer [23]. The present study demonstrated that PDC up-regulated the expressions of caspase-3, caspase-9 and P53, indicating that its anti-tumor effect was related to induction of apoptosis.

CONCLUSION

RSM is a useful tool for the optimization of PDC extraction, and that PDC has significant *in vivo* and *in vitro* anti-tumor effect on cervical cancer, the mechanisms of which might be related to enhancement of immune function and induction of apoptosis.

DECLARATIONS

Acknowledgement

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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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