

Original Research Article

Optimization of polysaccharides extracted from *Verbena officinalis* L and their inhibitory effects on invasion and metastasis of colorectal cancer cells

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Abstract

Purpose: To investigate polysaccharides (PEV) extracted from the aerial part of *Verbena officinalis* L. and their inhibitory effects on the invasion and metastasis of colorectal cancer (CRC) cells.

Methods: PEV was extracted by water and the optimization of extraction conditions was performed using a Box-Behnken design (BBD). The cell viability was evaluated by 4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The effects of PEV on cell adhesion and invasion were evaluated by Transwell invasion, wound healing migration and adhesion assays *in vitro*. The effects of PEV on the expressions of matrix metalloproteinase-9 (MMP-9), mesenchymal-epithelial transition factor (C-met), E-cadherin (E-CAD), cyclooxygenase-2 (COX-2) and E-prostanoid 2 receptor (EP2) were investigated by western blot.

Results: The BBD model was established successfully for the optimization of PEV extraction ($p < 0.0001$). The results indicate that PEV (50, 100 and 200 $\mu\text{g/mL}$) had significant inhibitory effects on cell invasion and migration of SW480 cells ($p < 0.05$). PEV also significantly decreased cell adhesion of SW480 cells. By treating with PEV, the expressions of C-met, MMP-9, COX-2 and EP2 were decreased, whereas the expression of E-CAD increased in SW480 cells.

Conclusion: RSM is effective for optimizing the extraction conditions of PEV. More importantly, PEV significantly inhibits the invasion and metastasis of SW480 cells by regulating the expression of MMP-9, C-met, E-CAD, COX-2 and EP2. Thus, PEV has the potential to be developed into therapeutic drugs for CRC in the future.

Keywords: Polysaccharides, Colorectal cancer, *Verbena officinalis*, SW480 cell lines, Cell invasion, Metastasis

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INTRODUCTION

The incidence of colorectal cancer (CRC) is increasing and it has become the second most common cancer related deaths worldwide [1]. Although surgery, along with chemotherapy or radiotherapy, has been widely used for the treatment of CRC, the prognosis is still poor

when the tumor has spread to lymph nodes or other organs [2,3]. A recent report from World Health Organization (WHO) revealed that the five-year survival rate for CRC patients (stage IV) is only 6 % [4]. Therefore, finding agents to prevent the invasive progression of CRC would significantly benefit the patient outcomes.

Verbena officinalis L., also called “*mabiancao*” in Chinese, is used as a medicinal herb in traditional Chinese medicine (TCM). The aerial part of this herb is commonly used in the southern part of the Yellow River of China for the treatment of bronchitis and rheumatism [5,6]. Modern pharmacological studies have shown that *V. officinalis* possessed various activities, such as anti-inflammatory, analgesic, immunity reinforcement, nerve protection and exciting uterine smooth muscle effects [6,7]. Researches on phytochemistry also have proved that *V. officinalis* contained many active constituents, including flavonoids, terpenoids, phenylpropanoids and iridoids [5]. However, there is no investigation on its polysaccharides and their pharmacological activities.

In the present study, the polysaccharides from *V. officinalis* and their inhibitory effects on invasion and metastasis of CRC cells were investigated, in order to determine their prospects in the treatment of CRC.

EXPERIMENTAL

Chemicals and reagents

RPMI 1640 culture medium was from Gibco BRL® (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone™ (Logan, UT, USA). The primary antibodies against E-CAD, C-met, MMP-9, COX-2 and EP2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dimethylsulfoxide (DMSO) and MTT were purchased from Sigma (St. Louis, MO, USA).

Plant material

Aerial part of *V. officinalis* was purchased from an herbal market for Chinese medicine (Huangshi, China) in August 6, 2016, and identified by an expert in Traditional Chinese Medicine Department of Huangshi Central Hospital (Huangshi, China). A specimen (ST20150115) was stored at the laboratory of Huangshi Central Hospital (Huangshi, China).

Extraction of PEV

The dried powder (50 g) of the aerial part of *V. officinalis* was extracted by refluxing with distilled water. After extraction, the solutions were collected, filtered and concentrated to an appropriate volume under vacuum at 50 °C. The concentrated solutions were precipitated by adding anhydrous ethanol (final concentration of 80 %, v/v) and left overnight (4 °C). After centrifugation, the precipitates were collected

and then washed anhydrous ethanol, benzene and acetone, respectively. The crude polysaccharides (PEV) were vacuum dried and the yield was calculated as in Eq 1.

$$\text{Yield (\%)} = (P_0/P)100 \dots\dots\dots (1)$$

where P_0 (g) is the weight of dried PEV; P (g) is the weight of dried herbs.

Optimization design

A BBD with three independent factors (A, extraction time; B, number of extraction; C, water-solid ratio) at three levels coded with -1, 0, 1 was used. The tested range of the three factors (A, B and C) and their levels are shown in Table 1.

Table 1: Factors and levels of BBD

Factor	Level		
	-1	0	1
Extraction time (A, min)	60	90	120
Number of extraction (B)	1	2	3
Water-solid ratio (C, mL/g)	10	20	30

Cell culture

CRC (SW480) cell line was from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 (the medium contained 10 % FBS) at 37 °C with 5 % CO₂/95 % air.

Cell viability assay

SW480 cells were plated into a 96-well plate (5 x 10⁴/well) and maintained overnight (37 °C). Then they were exposed to PEV (at concentrations of 50 - 600 µg/mL), and then incubated for another 24 h. Cell viability of SW480 cells was measured using the MTT method as previously reported [8].

Cell invasion assay

Matrigel-coated transwell chambers were used to evaluate the cell invasion of SW480 cells using the method described by Yuan *et al* [9]. The upper chambers were coated with matrigel (diluted in serum-free RPMI-1640 culture medium) and the bottom chambers were added RPMI-1640 medium (contained 10 % FBS). SW480 cells (5 x 10⁴/200 µL) were seeded onto the upper chambers. The cells were removed through the aperture (8 µm) after 24 h-incubation (37 °C). After staining with crystal violet (30 min), the migrated cells were observed using a microscope.

Wound-healing assay

The method of wound-healing assay was reported by Huang *et al* [10]. SW480 cells were grown to confluence by incubating overnight. The wound was created manually by using the tip of a 200 μ L-pipette. The cells were treated with PEV after washing with PBS for three times. After incubation for 24 h (37 °C), the migration distance of the cells was observed under a microscope.

Adhesion assay

Cell adhesion assay was performed using the method reported previously with minor modifications [11]. Firstly, 96-well plates were pre-coated with fibronectin (0.25 μ g/well) overnight, and then blocked with 3 % BSA. Cells (5×10^5 /mL) were suspended in 0.1 % BSA, and seeded into each well coated with fibronectin. After incubation for 30 min, each well was washed with PBS for three times. Attached cells were stained with crystal violet, and the absorbance was then measured at 590 nm.

Western blotting

SW480 cells were cultured and pre-treated with of PEV for 48 h. The lysates of SW480 cells were prepared and then centrifuged (12,000 \times g, 4 °C). The obtained proteins (50 μ g) were separated on 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE), and then electrophoretically transferred to polyvinylidene Fluoride (PVDF) membranes. After blocking (5 % skimmed milk) and incubating with the specific antibodies overnight (4 °C), the membranes were then incubated with HRP-conjugated secondary antibodies (2 h) and blots

were visualized using enhanced chemiluminescence.

Statistical analysis

The data analysis of RSM experiments was performed with Design-Expert software (version V8.0.6) (Stat-Ease, Inc., Minneapolis, MN, USA) and one-way analysis of variance (ANOVA) was used to analyze the statistical significance. Statistical analysis was determined by performing the Student's *t* test, and $p < 0.05$ was considered statistically different.

RESULTS

Optimized data

The complete design matrix and PEV yield (response values) obtained are shown in Table 2. Multiple regression analysis was used to analyze the experiment data of BBD. The response value (Y) and the tested factors (A, B and C) are related by Eq 2, according to the coded values.

$$Y = + 1.44 + 0.005 A + 0.046 B + 0.091 C + 0.042 AB - 0.0025 AC - 0.030 BC - 0.052 A^2 - 0.16 B^2 - 0.059 C^2 \dots\dots\dots (2)$$

From the analysis (Table 3), the low model *p*-value (< 0.0001), indicates that the established RSM was highly significant. The high R^2 (0.9947) and adj- R^2 (0.9880) showed goodness-of-fit between the data from actual experiments and the data predicted by the established model. The value of the coefficient of variation (CV) (1.02) indicated low deviations between actual experimental data and predicted data.

Table 2: BBD design and the obtained PEV yield

Run	A	B	C	Yield (%)
1	0.00	0.00	0.00	1.45
2	1.00	0.00	-1.00	1.25
3	-1.00	0.00	1.00	1.41
4	0.00	-1.00	1.00	1.29
5	0.00	0.00	0.00	1.44
6	0.00	1.00	-1.00	1.21
7	0.00	-1.00	-1.00	1.05
8	0.00	1.00	1.00	1.33
9	0.00	0.00	0.00	1.42
10	0.00	0.00	0.00	1.45
11	1.00	1.00	0.00	1.31
12	1.00	-1.00	0.00	1.14
13	-1.00	-1.00	0.00	1.23
14	-1.00	0.00	-1.00	1.22
15	1.00	0.00	1.00	1.43
16	-1.00	1.00	0.00	1.23
17	0.00	0.00	0.00	1.43

The high degree of adequate precision (37.538) also showed good reliability of the established model. ANOVA results also indicated that the coefficients of B, C, A², B², C², AB and BC exhibited significant effects on the yield of PEV ($p < 0.01$), while the other coefficients of A and AC were found nonsignificant ($p > 0.05$). The results also demonstrated that the number of extraction (B) and the water-solid ratio (C) were significantly associated with the PEV yield.

Response surface analysis and verification of the model

Through contour plots and response surface plots (Figure 1), the relationship between the tested factors and PEV yield were evaluated. The optimum conditions for maximum PEV yield were obtained: extraction time of 91.98 min, number of extractions of 2.08, and solid-water ratio of 27.50 g/mL.

The adequacy of the BBD model was validated by performing a verification experiment under the modified optimum conditions (extraction time of 92 min, number of extractions of 2, and solid-water ratio of 27.50 g/mL). As a result, the obtained yield ($1.48 \pm 0.06\%$) of PEV under these conditions ($n = 3$) was well-matched with the predicted value from the model (1.47%).

Effects of PEV SW480 cell viability

Cell viability was evaluated by MTT to select a suitable PEV concentration for investigating the cell invasion and metastasis of SW480 cells. As shown in Figure 2, PEV inhibited the growth of SW480 cells at high concentrations (> 400 µg/mL). However, PEV showed no obviously inhibitory effect on the growth of SW480 cells at concentrations ranged from 50 to 300 µg/mL. Therefore, PEV of 50, 100 and 200 µg/mL were selected for further study.

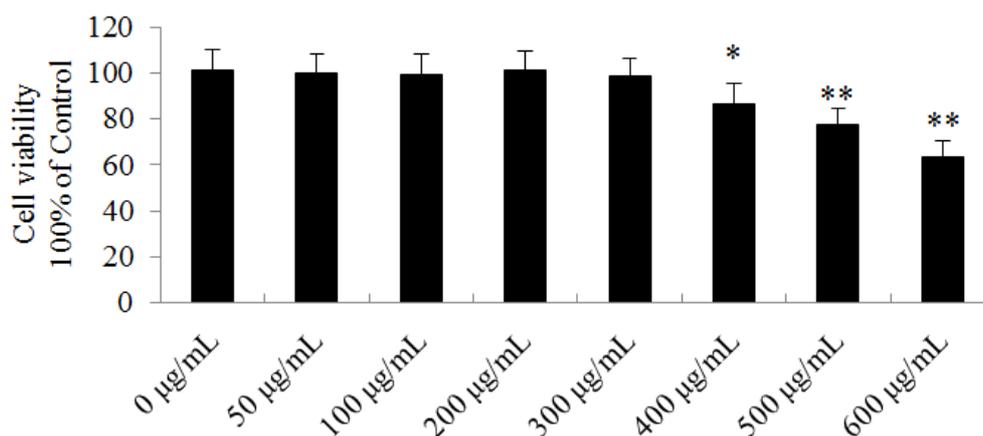


Figure 2: Effects of PEV on viability of SW480 cells. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group

Table 3: ANOVA for RSM of PEV extraction

Variables	Sum of squares	df	Mean square	F-value	P-value		
Model	0.24	9	0.026	147.20	< 0.0001		
A	2.00E-4	1	2.00E-4	1.12	0.3260		
B	0.017	1	0.017	95.45	< 0.0001		
C	0.067	1	0.067	371.54	< 0.0001		
A × B	7.23E-3	1	7.23E-3	40.30	0.0004		
A × C	2.50E-5	1	2.50E-5	0.14	0.7199		
B × C	3.60E-3	1	3.60E-3	20.08	0.0029		
A ²	0.011	1	0.011	62.29	< 0.0001		
B ²	0.11	1	0.11	593.72	< 0.0001		
C ²	0.015	1	0.015	81.75	< 0.0001		
Residual	1.26E-3	7	1.79E-4				
Lack of Fit	5.75E-4	3	1.92E-4	1.13	0.4378		
Pure Error	6.80E-4	4	1.70E-4				
Cor Total	0.24	16					
Standard Deviation	Mean	R ²	R ² _{Adj}	R ² _{Pred}	C.V.%	Press	Adequate precision
0.013	1.31	0.9947	0.9880	0.9570	1.02	0.010	37.538

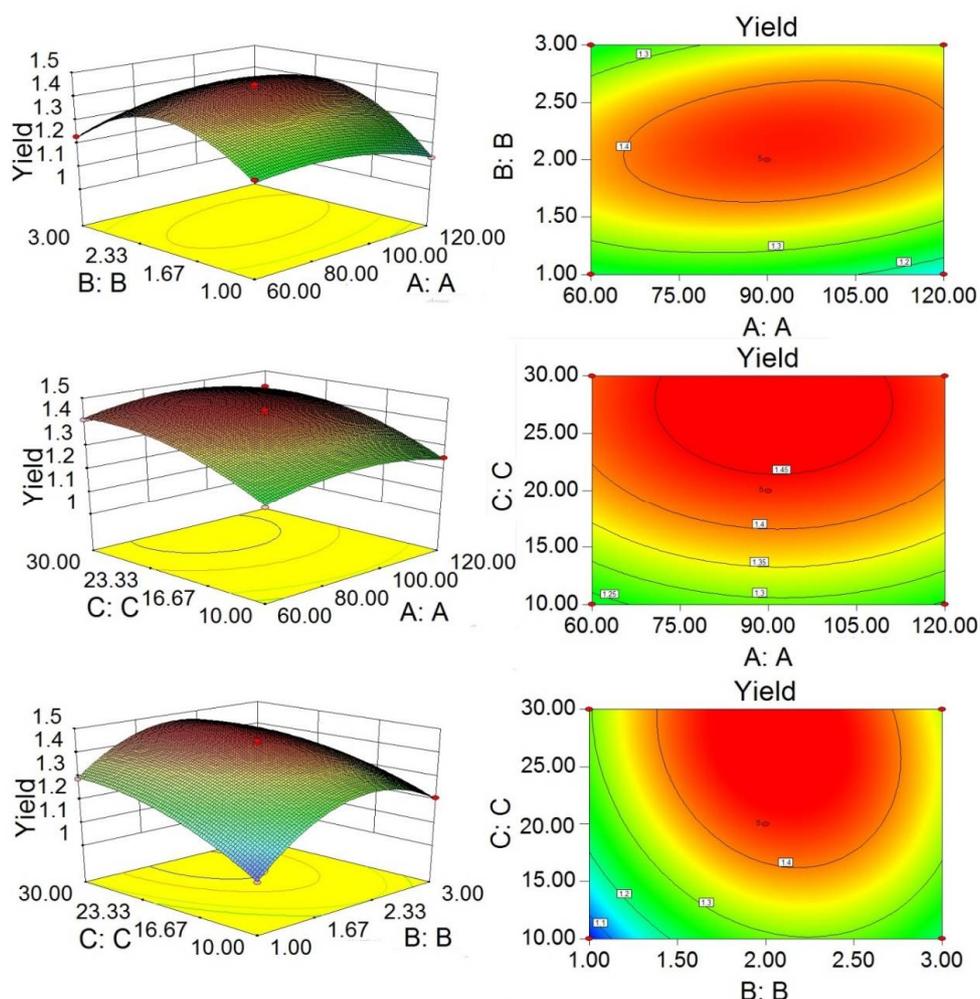


Figure 1: 3-dimensional response surface plots and 2-dimensional contour plots showing effects of the factors on PEV yield

***In vitro* invasion, wound healing and adhesion**

PEV effectively inhibited the invasion of SW480 cells at concentrations of 50, 100 and 200 $\mu\text{g}/\text{mL}$ ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) (Figure 3A). Wound healing assay indicated that the healing rates of PEV-treated cells were reduced with a concentration-dependent manner (Figure 3b). Furthermore, PEV also inhibited the adhesion of SW480 cells to fibronectin, and achieved the highest reduction (approximately 60 %) at 200 $\mu\text{g}/\text{mL}$ (Figure 3C).

Effects of PEV on expressions of E-CAD, C-met, MMP-9, COX-2 and EP2

As shown in Figure 4, the results indicated that treatment of PEV (100 and 200 $\mu\text{g}/\text{mL}$) significantly increased the expression of E-CAD, and decreased the expressions of C-met and MMP-9. Furthermore, the expressions of COX-2 and EP2 were significantly down-regulated in

SW480 cells by treating with PEV (100 and 200 $\mu\text{g}/\text{mL}$).

DISCUSSION

Response surface methodology (RSM) is reported to be an effective method widely used for optimizing parameters of extraction in food and pharmaceutical research [12]. As one type of RSM, BBD is popularly used to optimize the parameters in extraction process [13]. Compared with conventional methods, BBD can save materials, decrease expenses and reduce time, etc. in optimizing a process [14]. In this study, the conditions for PEV extraction were optimized by using a BBD, and the optimum conditions for PEV extraction were obtained: extraction time of 92 min, number of extractions of 2, and solid-water ratio of 27.50 g/mL.

MMPs are reported to be the pivotal extracellular matrix (ECM) degradation enzymes required for the invasion and metastasis of tumor cells.

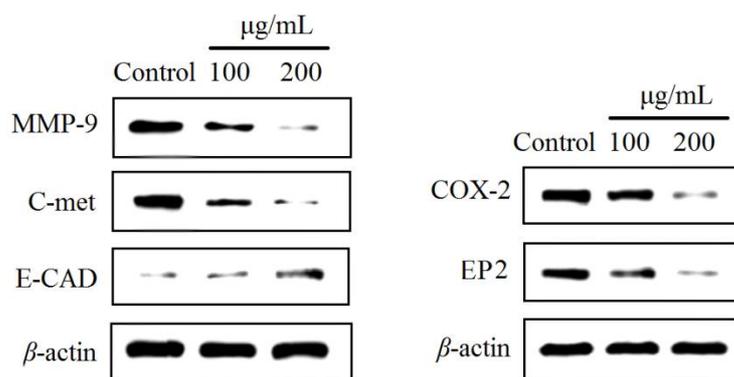


Figure 4: Effects of PEV on the expressions of E-CAD, C-met, MMP-9, COX-2 and EP2

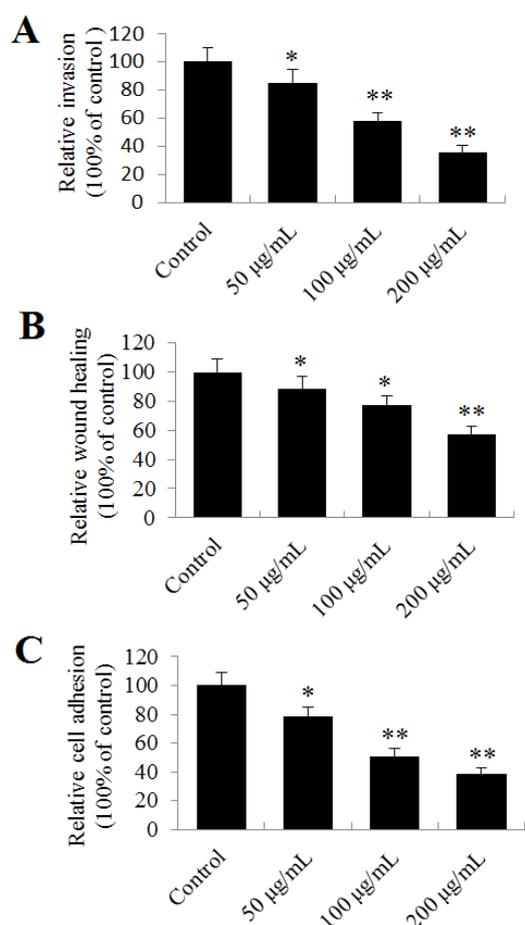


Figure 3: *In vitro* effects of PEV on invasion (A), wound healing (B) and adhesion (C) of SW480 cells; * $p < 0.05$ vs. the control group; ** $p < 0.01$ vs. control group

As one of the MMPs, MMP-9 is closely associated with metastasis of most cancers including CRC [15]. The function, activity and expression intensity of E-cadherin (E-CAD) play crucial roles in the separation and reattachment of tumor cells [16]. C-met, belongs to the receptor tyrosine kinase (RTK) family, is found to be abnormal in many types of tumors. Expression of C-met has been proved to be an important signaling pathway which is responsible

for proliferation, invasion, migration, and metastasis, etc. of tumor cells [17]. In this study, the effects of PEV on the expressions of MMP-9, E-CAD and C-met proteins were measured by using western blot, and the results demonstrated that PEV significantly increased the expression of E-CAD protein and decreased the expression of C-met and MMP-9 proteins.

Over-expression of COX-2 was found to be closely associated with colorectal tumorigenesis, and it can enhance the angiogenesis, attachment and migration/invasion of tumor cells [18]. In addition, prostaglandin E₂ (PGE₂) is the main catalyzed product of COX-2 from arachidonic acid, and PGE₂ produces its cellular signaling via activating G-protein coupled receptors (GPCRs). EP2 is one of the GPCRs which played vital roles in CRC [19, 20]. In this study, the effects of PEV treatment on COX-2 and EP2 expressions were also determined, and found that they were significantly down-regulated by treating with PEV.

CONCLUSION

RSM is an effective method for optimizing the extraction conditions of PEV. Furthermore, PEV significantly inhibits the invasion and metastasis of SW480 cells. The mechanisms operates probably via the regulation of the expression of MMP-9, C-met, E-CAD, COX-2 and EP2. Consequently, PEV can potentially be developed into therapeutic drugs for colorectal cancer.

DECLARATIONS

Acknowledgement

None.

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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