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Original Research Article

Anti-tumor effect of polysaccharides isolated from Taraxacum mongolicum Hand-Mazz on MCF-7 human breast cancer cells

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Abstract

Purpose: *To optimize the extraction conditions for the ultrasound-assisted extraction of polysaccharides from T. mongolicum (PTM) and investigate their anti-tumor effect on human breast cancer MCF-7 cells.*

Methods: *To optimize the extraction conditions of PTM, response surface methodology (RSM) was performed. The effects of extraction temperature, liquid-solid ratio and extraction time on the yield of PTM were investigated using a Box-Behnken design (BBD). The in vitro anti-tumor effect of PTM on MCF-7 cells was investigated by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, while the mechanism of PTM-induced apoptosis was assessed by evaluating the expressions of p53, Bax and Bcl-2 proteins using western blot analysis. Furthermore, the in vivo anti-tumor effect of PTM on MCF-7 cells was studied in mice.*

Results: *The optimal conditions for the extraction of PTM were as follows: extraction temperature, 58.2 °C; liquid-solid ratio, 15 mL/g; and extraction time, 44.12 min. Under these optimal conditions, the yield of PTM was 4.84 ± 0.13 %. PTM showed significant anti-tumor effect on MCF-7 cells in vitro. The expressions of pro-apoptotic proteins, p53 and Bax, were significantly upregulated ($p < 0.05$), while the expression of anti-apoptotic protein, Bcl-2, was significantly down-regulated ($p < 0.05$) after treatment with PTM. PTM also showed significant inhibitory effect ($p < 0.05$) on MCF-7 cells in vivo in a dose-dependent manner.*

Conclusion: *RSM is effective in optimizing the extraction conditions of PTM by ultrasonic extraction. PTM possesses significant anti-tumor effect on MCF-7 human breast cancer cells, both in vitro and in vivo.*

Keywords: *Polysaccharides, Taraxacum mongolicum, Human breast cancer, MCF-7 cells, Apoptosis, Box–Behnken design, Response surface methodology*

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INTRODUCTION

Although diagnosis and treatment have been improved, breast cancer still remains the second leading cause of cancer-related death in women worldwide [1]. Currently, breast cancer is the most common cancer among women in China, and approximately 0.03 % women will develop

breast cancer in their lifetime, and the proportion is rising as this disease becomes more and more common in younger patients [2,3]. However, the conventional treatments (chemotherapy and radiation) have harmful side effects [4,5]. Thus, it is very necessary to develop new effective drugs for the treatment of breast cancer.

Recently, more and more polysaccharides from natural plants have been proved to have various biological activities. More importantly, a growing amount of researches have shown that polysaccharides could resist tumors by inducing tumor apoptosis [6,7]. *Taraxacum mongolicum* Hand.-Mazz., a member of genus *Taraxacum*, is a folk medicine which was used to treat viral infectious diseases and inflammatory disorders, etc. [8]. Furthermore, *T. mongolicum* has gained wide attention because of its favorable therapeutic effect for many diseases, especially jaundice gonorrhoea, pneumonia and mastopathy [8,9]. However, there have been few reports regarding the anti-tumor effect of polysaccharides extracted from *T. mongolicum* on breast cancer. Therefore, the present study was aimed to investigate the optimum extraction of polysaccharides from *T. mongolicum* (PTM) and explore their anti-tumor effect on breast cancer.

EXPERIMENTAL

Chemicals and reagents

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Sigma Chemical (St. Louis, MO, USA). Minimum Essential Medium (MEM) was obtained from Gibco (Grand Island, NY, USA). p53, Bcl-2, Bax, β-actin monoclonal primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other reagents and chemicals used in the experiment were of analytical grade.

The preparation of PTM

The whole plant of *T. mongolicum* was purchased from the Traditional Chinese Medicine Market of Nanyang (Nanyang, China), and authenticated by the Department of Traditional Chinese Medicine in Qilu Hospital (Jinan, China). The voucher specimen (FTCM no. 20160321) was deposited in the hospital herbarium of Qilu Hospital. The powder of dried whole plant of *T. mongolicum* (10 g) was put into conical flask with stopper, and then extracted by an AS3120A ultrasonic device (Tianjin Automatic Science Instrument Co., Ltd, Tianjin, China) with designed extraction time (30, 40 and 50 min), liquid–solid ratio (6, 12 and 18 mL/g) and extraction temperature (40, 50 and 60 °C). After extraction, the solutions were concentrated under reduced pressure to 20 mL by a rotary evaporator. The concentrated solutions were mixed with anhydrous ethanol (1:3, v/v) and left overnight at 4 °C. Then the precipitates were obtained by centrifugation at 5000 rpm for 10 min, and washed respectively with anhydrous ethanol and acetone. Finally, the precipitates were dried to a constant weight at 45 °C. The extraction yield (%) was calculated as in Eq 1.

$$Y\ (\%) = (W_1/W_0)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

where Y is the yield of polysaccharides extracted from *T. mongolicum*, W_1 is the weight of polysaccharides extracted from *T. mongolicum* (g), and W_0 is the weight of the dried powder of *T. mongolicum* (g).

Experimental design of RSM

Design-Expert trial version 8.0.5 software (Stat-Ease Inc., Minneapolis, USA) was used to analyze the experimental data. To determine the optimal levels for extraction conditions of extraction temperature, liquid–solid ratio and extraction time, a Box-Behnken design (BBD) (three-level and three factors) was applied. As shown in Table 1, the complete experimental design carried out in random order, and was consisted of 17 experimental points.

Cell culture

MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in MEM supplemented with 10 % fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 U/mL). Then cells were incubated at 37 °C with 5 % CO_2 and 95 % air in humidified atmosphere.

Cell proliferation assay

MCF-7 cells were harvested and seeded into 96-well plates at the concentration of 1×10^5 cells/mL, and incubated for 12 h at 37 °C. Then the cells were treated with different concentrations of PTM (0, 25, 50, 100, 200 and 400 μg/mL for 24 h) and also incubated for different time points at the concentration of 200 μg/mL for 12, 24 and 48 h. At the end of the cultivation, cells were treated with 20 μL MTT (5 mg/mL) in each well and then incubated for 4 h (37 °C). Then DMSO (100 μL) were added in each well to dissolve the formazan crystals. The

absorbance was measured using a microtiter plate reader (Bio-Rad Laboratories, CA, USA) at 570 nm. The cell viability was calculated as the percent values compared with the control group.

Western blot analysis

After the indicated treatments, MCF-7 cells were collected and the protein was extracted. Equal amounts of 30 μg solubilized proteins were separated on 12 % SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5 % skimmed milk, and then incubated with primary antibodies overnight at 4 °C. Subsequently, the membranes were incubated with corresponding HRP conjugated secondary antibodies. Detection of proteins was carried out using a Bio-Rad enhanced chemiluminescence detection system (Bio-Rad Laboratories, Hercules, CA).

Animals and *in vivo* tumor xenograft study

Female BALB/c nude mice (18 - 22 g) were obtained from Shandong Laboratory animal center (Ji'nan, China). Animals were maintained in a specific pathogen-free (SPF) environment (21 ± 2 °C and 55 ± 5 % humidity) under a 12 h light/12 h dark cycle. The feed and water were supplied *ad libitum*. All experiments were carried out in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [10] and approved by Animal Ethics Committee of Qilu Hospital (approval no. ERK/2016/32).

Breast cancer xenograft models were established by subcutaneously injecting MCF-7 cells (5×10^6 /mouse) into the right flank of the nude mice. When the tumors reached about 100 mm^3, the mice were randomly divided into 5 groups (n = 10): positive group (capecitabine, 60 mg/kg), negative group (0.9 % saline) and three PTM groups (20, 40 and 60 mg/kg). All groups were treated by intragastric administration for 21 days. Micrometer calipers were used to measure tumor sizes were every 3 days. Tumor volume was calculated using the formula as follows: V = (length×width^2) /2. At the end of the experiment, the mice were sacrificed, and the tumor were segregated and measured.

Data analysis

All data are presented as mean ± standard deviation (SD, n = 3) and were evaluated by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant. RSM data were analyzed by Design-Expert trial version 8.0.5 (Stat-Ease Inc, Minneapolis, USA).

RESULTS

Model fitting

The effects of three variables (extraction temperature, liquid-solid ratio and extraction time) on the yields of PTM were examined using the BBD design. The complete design matrix together with the response values was shown in Table 1. The yield of PTM were 3.12 % - 4.65 %, and reached maximum with the extraction temperature of 60 °C, liquid-solid ratio of 18 mL/g and extraction time of 40 min. The response variable (yield) and the test variables (extraction temperature, liquid-solid ratio and extraction time) can be related by the following equation (Eq 2).

$$\text{Yield (\%)} = 4.53 + 0.46\,A + 0.27\,B + 0.27\,C + 0.13\,AB + 0.043\,AC + 0.045\,BC - 0.3\,A^2 - 0.39\,B^2 - 0.3\,C^2 \quad \text{..... (2)}$$

The results of statistical analysis indicated that the established model was highly significant ($p < 0.0001$, $F = 573.3965$). The model showed a good fit with the high R^2 value of 0.9986 and adjusted determination coefficient (R^2_{adj}) of 0.9969. The C.V. was 0.7652, indicating that the experimental values were of a high degree of precision and a good deal of reliability. In addition, the independent variables of A, B and C, the interaction terms of AB, AC and BC, and all two quadratic terms A^2, B^2, and C^2, significantly affected the yield of PTM ($p < 0.05$).

Optimization of extraction conditions

The response surface plots and contour plots are shown in Fig. 2. One variable kept constant at middle level while the other two variables within experimental range were depicted in one response surface plot. It has been reported that the shapes of the contour plots were circular or elliptical, indicating the mutual interactions between the variables were significant or not [11]. The results in Fig 2 indicate that the interactions between the test variables were significant and the optimal extraction conditions by Design-Expert software were: extraction temperature of 58.17 °C, liquid–solid ratio of 15.03 mL/g and extraction time of 44.12 min. The predicted yield of PTM at the optimal extraction condition was 4.84 %.

Table 1: BBD and the response values for PTM yield

Run	A: Extraction temperature (°C)	B: Liquid–solid ratio (mL/g)	C: Extraction time (min)	Yield (%)
1	40.00	18.00	40.00	3.47
2	50.00	12.00	40.00	4.53
3	40.00	12.00	50.00	3.59
4	60.00	12.00	50.00	4.58
5	40.00	6.00	40.00	3.23
6	40.00	12.00	30.00	3.12
7	50.00	18.00	30.00	3.73
8	50.00	12.00	40.00	4.55
9	60.00	12.00	30.00	3.94
10	50.00	6.00	50.00	3.67
11	60.00	6.00	40.00	3.88
12	50.00	12.00	40.00	4.54
13	50.00	6.00	30.00	3.25
14	50.00	12.00	40.00	4.48
15	50.00	12.00	40.00	4.55
16	50.00	18.00	50.00	4.33
17	60.00	18.00	40.00	4.65

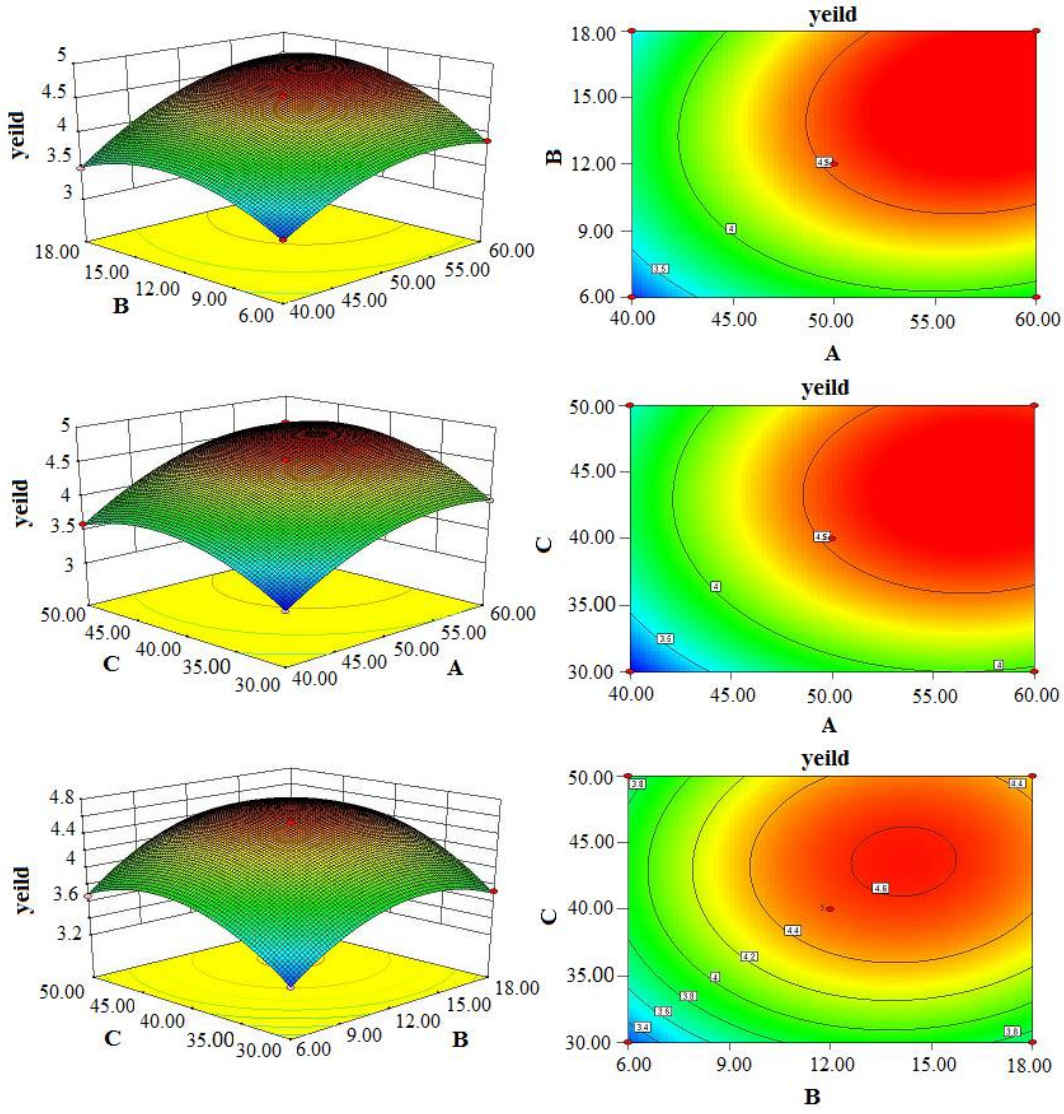

Figure 1: Response surface and contour plots showing the effects of variables and their mutual effects on the extraction yield of PTM

Validation of the models

To validate the adequacy of the model, a verification experiment was performed under the following conditions: extraction temperature of 58.2 °C, liquid-solid ratio of 15 mL/g and extraction time of 44.12 min. Under these conditions, the yield of PTM was 4.86 ± 0.13 %. The results indicated that the RSM was effective and appropriate for optimizing the conditions for extracting PTM.

PTM inhibited the viability of MCF-7 cells

MTT assay was performed to study the inhibitory effects of PTM on MCF-7 cells in the present study. As shown in Figure 2A, PTM inhibited the viability of MCF-7 cells at the concentrations of 50, 100, 200 and 400 µg/mL in concentration-dependent manners. The relationship between the inhibitory effect and time was also investigated within 48 h, and the results showed that PTM inhibited the viability of MCF-7 cells in time-dependent manners (Figure 2B).

Expression of p53, Bax and Bcl-2 proteins

The expression of apoptosis regulatory proteins were examined to study the effect of PTM on MCF-7 cells apoptosis. As can be seen from Figure 3, the expression of pro-apoptotic proteins p53 and Bax were significantly upregulated by treating with PTM compared with control cells at the concentrations of 100, 200 and 400 µg/mL. Furthermore, the expression of anti-poptotic protein Bcl-2 was significantly down-regulated after treatment of PTM compared with cells in the control group at the tested concentrations.

PTM suppressed tumor growth in mouse

From Figure 4, the results showed that the tumor growth inhibitory effects were increased with the dose increase of PTM. The treatment of PTM significantly reduced the tumor volume at the doses of 20, 40 and 80 mg/kg compared with the control group ($p < 0.01$). The tumor weights of PTM-treated mice (20, 40 and 80 mg/kg) were also significantly less than that of the control group ($p < 0.01$).

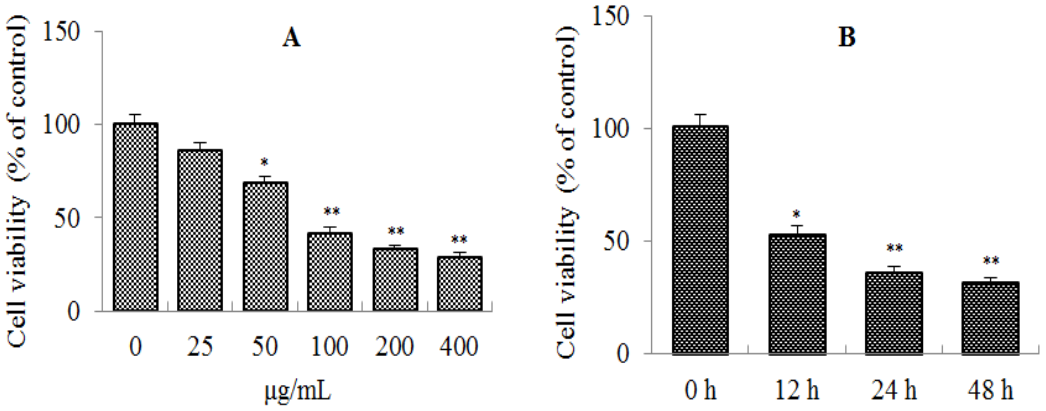

Figure 2: Effect of PTM on the proliferation of MCF-7 cells

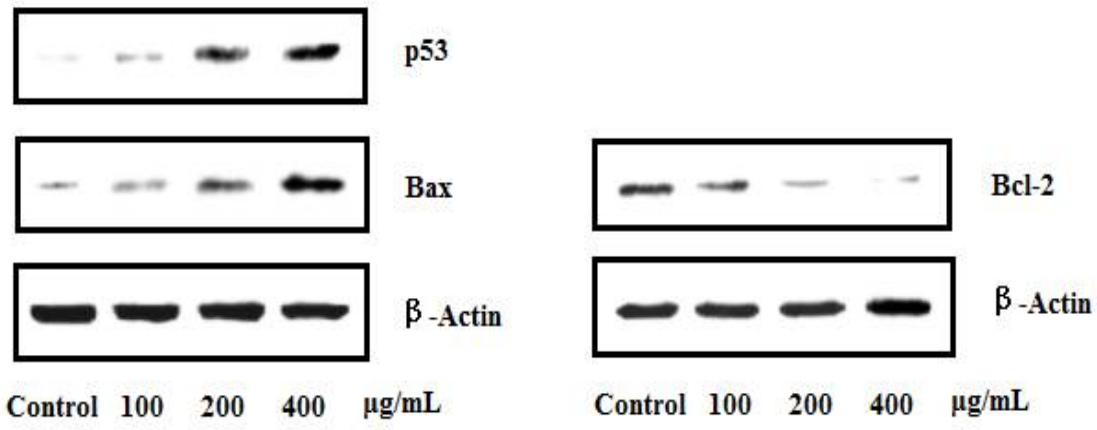

Figure 3: p53, Bax and Bcl-2 protein expression in MCF7 cells treated with PTM

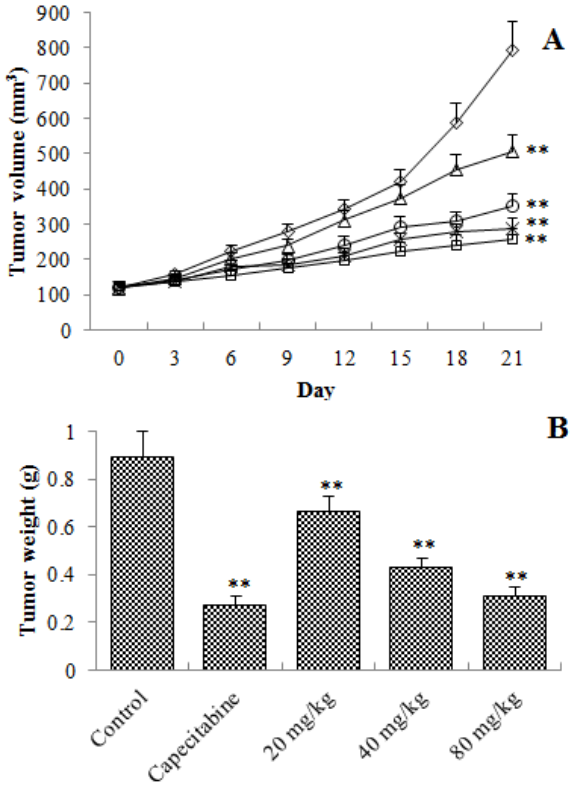

Figure 4: The effects of PTM treatment on MCF-7 cells *in vivo.* (A) Tumor volume, measured once every 3 days; (B) Tumor mass weight; ◇ control, □ Capecitabine, ○ 40 mg/kg, △ 20 mg/kg, × 80 mg/kg

DISCUSSION

Ultrasonic extraction is a new technology that attracts much attention in the extraction of natural products and it is considered as one of the most inexpensive, simple and efficient techniques [12]. Response surface methodology (RSM) is an effective model for optimizing complex processes, and it saves more labor and time than other methods [13]. RSM is easy to carry out, and it has already been applied to optimize the extraction conditions of polysaccharide in previous researches [14,15].

Ultrasonic extraction technique was used in the present study for extracting PTM and the related conditions of extraction were optimized by BBD. As a result, the optimal extraction conditions for PTM were obtained, and the validation experiments indicated that the RSM was effective.

Apoptosis is the process of programmed cell death and it is very important in the control of cell development and proliferation [16]. Apoptosis may result in abnormal expression of Bcl-2 family members including Bcl-2 and Bax, which are key regulators of cell apoptosis and play pivotal roles in anti-apoptotic and pro-apoptotic, respectively [17]. Furthermore, p53 is a very important tumor suppressor protein that mediates the stress-induced apoptosis cascade [18,19].

In this study, a significant decrease of Bcl-2 expression and a marked increase of Bax and p53 expression were observed in MCF-7 cells after treating with PTM. Therefore, the results indicate that the anti-tumor effect of PTM is closely associated with induction of apoptosis.

The present study showed that polysaccharides from *T. mongolicum* can inhibit the growth of MCF-7 cells *in vitro* and *in vivo*. Thus, polysaccharides might be one of the effective components of *T. mongolicum* for the treatment of mastopathy.

CONCLUSION

RSM has been shown to be effective for optimizing the extraction conditions of PTM by ultrasonic extraction. PTM possesses significant anti-tumor effect on MCF-7 cells in vitro and in vivo by inducing apoptosis. Thus, PTM have the potential to develop into anti-tumor drugs for the treatment of breast cancer in the future.

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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