

Original Research Article

Evaluation of Anti-tumor and Chemoresistance-lowering Effects of Pectolarigenin from *Cirsium japonicum* Fisch ex DC in Breast Cancer

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Abstract

Purpose: To investigate the antitumor and chemoresistance-lowering effects of pectolarigenin on breast cancer cells.

Methods: Pectolarigenin was purified by a combination of silica gel and Sephadex LH-20 column chromatography from ethanol extracts of the aerial parts of *C. japonicum* DC. Breast cancer self-renewal properties were tested by colony formation and tumor sphere formation assays. Thereafter, real-time polymerase chain reaction (PCR) was used to detect breast cancer stem cell markers. Furthermore, the effect of pectolarigenin on breast cancer cell was evaluated by chemoresistance using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Finally, tumor formation in nude mice was used to test the effect of pectolarigenin on tumorigenicity of breast cancer cells in vivo.

Results: The results showed that pectolarigenin, extracted from *Cirsium japonicum* Fisch. ex DC., inhibited tumor cell self-renewal in MCF-7 breast cancer cells. Pectolarigenin (25 μ M) caused significant inhibition of colony formation (61.23 %, $p < 0.001$) and tumor sphere formation (59.49 %, $p < 0.01$) in MCF-7. The inhibitory effects were associated with changes in breast cancer stem cell markers. Treatment of breast cancer cells with pectolarigenin reduced the chemoresistance of the cells to doxorubicin. At the same time, mRNA expression of chemoresistance genes (ATP binding cassette subfamily G member 2, ABCG2 and ATP binding cassette subfamily B member 1, MDR1) was repressed by pectolarigenin. The inhibition efficiency of MDR1 and ABCG2 by 10 μ M pectolarigenin treatment was about 59.29 ($p < 0.01$) and 46.48 % ($p < 0.01$), respectively. Furthermore, pectolarigenin reduced tumor mass in nude mice xenograft model.

Conclusion: Pectolarigenin inhibits breast cancer stem cell-like properties and lowers the chemoresistance of the cancer cells to chemotherapy. The results provide an insight into the mechanism of the anti-breast tumor effects and an experimental basis for the use of pectolarigenin to enhance treatment of patients with breast cancer.

Keywords: Pectolarigenin, Cancer stem cells, Breast cancer, Chemoresistance, *Cirsium japonicum* Fisch. ex DC

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INTRODUCTION

Breast cancer accounts for 22.9 % of all cancers (excluding non-melanoma skin cancers) in women worldwide [1]. The death rate caused by breast cancer is 13.7 % of cancer deaths in women in 2008 [1]. However, survival rates are much poorer in developing countries compared with the western world. Recrudescence of breast cancer is still a great threat to the treatment of breast cancer [2].

Cancer stem cell (CSCs) hypothesis suggests that, they are rare, tumor-initiating cells that exhibit stem cell properties: capacity of self-renewal, pluripotency, highly tumorigenic potential, and resistant to therapy [2]. Cancer stem cells have been characterized and isolated from many cancers, including breast cancer. Cancer stem cells share many mechanisms described for tissue-specific stem cells, but due to oncogenic deregulations these cells have evolved even drug-intoxicating and antioxidant systems that may contribute to tumor recurrence and enhanced resistance to chemo- and/or radiotherapy. Thus, drugs that inhibit breast cancer cell self-renewal and reduce chemoresistance offer great promise for breast cancer treatment [3].

Considerable efforts have been made to search for specific surface and intracellular biomarker in recent years. CD44⁺, CD24⁻/low, and ESA⁺ (epithelial specific antigen) and lacking expression of specific lineage (ESA⁺CD44⁺CD24⁻/low Lin⁻) have been identified as breast cancer stem cell markers [4]. Interestingly, accumulating evidence indicates that the expression of Oct3/4, Nanog, and Sox2 transcription factors have a strong correlation with CSCs; knockdown of these genes inhibited tumor sphere formation and decreased tumor formation in xenograft tumor models [5,6].

Cirsium japonicum belongs to the family of Compositae and is widely used in traditional Chinese medicine (TCM) for the treatment of haemorrhage, hepatitis, hypertension and blood circulation [7]. Pharmacological studies show that the extract of *C. japonicum* and its major flavonoid constituents possess anti-tumor activity [7,8]

Pectolinarigenin is a hydrophobic agent widely distributed in a number of fruits, vegetables and plants with potential anticancer activity. Pectolinarigenin has been shown to have an

antioxidant activity, anti-inflammatory function and anti-aging properties [9,10].

Recently, we have confirmed that pectolinarigenin showed potent anti-proliferation activity by inducing apoptosis and downregulation of Bcl2 expression in MCF-7 breast cancer cell [11]. However, the effects of pectolinarigenin on breast cancer stem cell properties have rarely been investigated.

Therefore, the aim of this study was to investigate the effect and mechanism of action of pectolinarigenin on breast cancer stem cell properties.

EXPERIMENTAL

Plant material and equipment

The aerial parts of *C. japonicum* were purchased from the market of Chinese herbal medicine in Zhang Shu (Jiang Xi, China) in July 2010 and authenticated by Qingzhi Kong (a senior taxonomist at Hubei University of Chinese Medicine). A voucher specimen (2010-07) has been deposited in the Hubei University of Chinese Medicine. ¹H- and ¹³C-NMR spectra were recorded on a Bruker Avance-400 FT-NMR spectrometer, with TMS internal standard. ESI-MS were recorded on 3200 Q-trap ESI-MS spectrometer (ABI, American). Column chromatography was performed with silica gel (200-300 mesh) and Sephadex LH-20 (Pharmacia Co.). All the organic solvents used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Extraction and isolation

The extraction and isolation procedures had been reported previously [11]. Namely, the powdered air-dried aerial parts of *C. japonicum* (1 kg) were extracted three times with 6 L 80 % ethanol/10 % HCl (4:1, v/v) in a conical flask and refluxed at 80 °C for 2 h. The solvent was evaporated in vacuum and then the concentrated extract was partitioned with chloroform. The chloroform fraction was successively purified on silica gel (200-300 mesh) with CHCl₃-MeOH gradient and on Sephadex LH-20 with CHCl₃-MeOH (1:1) to yield compound 1 (61 mg).

Cell culture

Human breast cancer cell line, MCF-7, was purchased from Institute of Cell Biology (Shanghai, China, <http://www.cellbank.org.cn>).

Cells were maintained in Dulbecco's modified eagle medium (DMEM). All cell culture media were supplemented with 10 % fetal bovine serum (FBS), and 1 % of penicillin-streptomycin (all from Invitrogen (Carlsbad, CA, <http://www.invitrogen.com>)).

Soft agar colony formation assay

Triplicate samples of MCF-7 (1×10^3) were resuspended in 1 mL of DMEM containing 0.3 % low-melt agarose, 10 % fetal bovine serum, 1 % of penicillin-streptomycin. The cell mixture was plated on top of solidified layer with the same DMEM containing 0.6 % low-met agarose. Plates were incubated for 3 weeks at 37 °C in 5 % CO₂ in humidified incubator. Colony formation was stained with 0.01 % crystal violet, photographed and counted.

Tumorsphere culture

Tumorspere cultures were made in ultralow attachment six-well plate (Corning, Lowell, MA, USA) in suspension (500 cells/mL) in serum-free DMEM/F12 media, supplemented with B27 (1:50, Invitrogen, Carlsbad, CA, USA), 20 ng/mL human recombinant epidermal growth factor (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 4 µg/mL heparin (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich) and 1 % of penicillin-streptomycin in a humidified incubator at 37 °C in 5 % CO₂ [12,13]. Tumorsphere formation was tested by culturing MCF-7 cells in the presence or absence of pectolarigenin under the conditions mentioned above.

RNA extraction, RT-PCR and quantitative real-time PCR

Total RNA was extracted using TRIZOL Reagent (Invitrogen) and reverse transcribed with R-PCR Quick Master Mix (Toyoba) to produce cDNA. The primer sequences listed in Table 1 were used for quantitative real-time PCR. Real-time PCR was performed using SYBR Green-based detection in LightCycler^oR480 (Roche) according to the manufacturer's instructions. GAPDH (glyceraldehyde-3' phosphate dehydrogenase) levels were used as normalization controls.

Chemoresistance assay

The MTT assay (Cell titer 96^oR Aqueous One Solution Cell Proliferation Assay, Promega) was used to assess the rates of resistance to drugs. Briefly, breast cancer cells (2×10^3 /well) were seeded in 96-well plates. After 24 h in presence or absence of pectolarigenin, the indicated

concentration of doxorubicin (Ferrer Farma) was added and 72 h later, the MTT assay was performed using iMarkmicroplate Absorbance Reader (Bio-RAD, Richmond, CA) according to the manufacturer's instructions.

Table 1: Primers used for quantitative real-time PCR

Gene	Primer direction	Sequence
OCT4	Forward	GGTATTCAGCCAAACGAC CAT
	Reverse	ACGAGGGTTTCTGCTTTG CA
Nanog	Forward	GGTCCAGAACCAGAGAA TGAAA
	Reverse	GTTGCTCCACATTGGAAG GTT
SOX2	Forward	CTCGCCACCTACAGCAT
	Reverse	GACTTGACCACCGAACCC
CD44	Forward	CTCATGGATCTGAATCAG ATGGA
	Reverse	ACTGCAATGCAAACCTGCA AGA
CD24	Forward	CCTACCCACGCAGATTTA TT
	Reverse	TGGTGGCATTAGTTGGAT TT
MDR1	Forward	AGCTCGTGCCCTTGTTAG ACA
	Reverse	GTCCAGGGCTTCTTGGAC AA
ABCG2	Forward	GGACTCAATGCAACAGGA AACA
	Reverse	TCAGGTAGGCAATTGTGA GGAA
GAPDH	Forward	TCTCCTCTGACTTCAACAG CGA
	Reverse	GTCCACCACCCTGTTGCT GT

Tumor growth in xenografts

MCF-7 Cells (5×10^6) were injected subcutaneously in the right flank of each of 8 female 6-week old nude mice (Laboratory Animal Center, Xiamen University). All animals were maintained under controlled temperature, humidity, and 12 h light/dark cycles in the Xiamen University Laboratory Animal Center. The animals were fed standard rodent chow and allowed free access to water ad libitum. All experiments were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory. Animal care and use was approved by the Ethics Committee of Faculty of Medicine-Xiamen University-China. When the tumors developed at 7 days, the mice were randomly distributed into two groups, of untreated or treated by i.p. injection every day with pectolarigenin (20 mg/kg). Tumor volume (Tv) was measured every 3 days and calculated as in Eq 1.

$$T_v = (LW^2)/2 \dots\dots\dots (1)$$

where L and W are the length and breadth of the tumor, respectively.

Statistical analysis

The results are expressed as mean \pm SEM. Statistical significance was determined by Student's t-test or one-way or two-way analysis of variance (ANOVA) followed by Turkey's test, as appropriate, using Graphpad Prism statistics software (Graphpad Software). $p < 0.05$ was considered statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

RESULTS

Pectolarigenin inhibited BCSCs self-renewal properties

The isolated compound was identified by a combination of NMR and mass spectral data as reported previously [11]. Soft agar colony formation assay and tumor sphere assay have been widely used to identify stem cells in *in vitro* assays. The colony formation capacity in MCF-7 was firstly assayed under the treatment of pectolarigenin. The results showed pectolarigenin inhibited the colony formation capacity in MCF-7 cells in a dose dependent manner as shown in Fig 1A. Even 25 μ M concentration of pectolarigenin could cause significant inhibition of colony formation in MCF-7 (61.23 %, $p < 0.001$, Fig 1A).

Then the tumor sphere formation capacity was examined in MCF-7 under the treatment of pectolarigenin. Tumor spheres of MCF-7 were cultured as described elsewhere [12]. Tumor spheres with a tight appearance were observed in serum free medium (Fig 1B). Those results showed that the breast cancer cells sphere formation capacity was inhibited by pectolarigenin in a dose dependent manner as shown in Fig 1C, the inhibition efficiency of tumor sphere formation is 59.49 % when treated with 25 μ M pectolarigenin ($p < 0.01$, Fig 1C).

Moreover, BCSCs marker expression in monolayer MCF-7 cells, MCF-7 spheres and MCF-7 spheres treated with 25 μ M pectolarigenin were compared by real-time PCR. The results showed that the spheres expressed much higher levels of BCSCs markers, such as OCT-4, Sox2, Nanog, CD44, than the monolayer cells. At the same time, the spheres expressed much lower levels of CD24

than the monolayer cells. The induction and repression of BCSCs markers were greatly reversed in the spheres treated with 25 μ M pectolarigenin compared with the untreated spheres (Fig 1D).

Pectolarigenin reduced breast cancer cell chemoresistance

Studies in the past have suggested that chemoresistance is another characteristic of CSCs. Next studies were carried out to investigate whether pectolarigenin would influence the chemoresistance of MCF-7 cells. For this purpose, MCF-7 cells were pre-treated with or without pectolarigenin (10 μ M) for 24 h and then 0.25 μ M Doxorubicin was used to treat the cells. As showed in Fig. 2A, pectolarigenin enhanced chemosensitivity to Doxorubicin. Moreover, we detected chemoresistant genes (MDR-1 and ABCG2) expression in MCF-7 when treated with pectolarigenin (10 μ M). As shown in Fig. 2B, MDR1 and ABCG2 expression were repressed under treatment with pectolarigenin (10 μ M). Especially, the inhibition efficiency of MDR1 and ABCG2 were about 59.29 % ($p < 0.01$, Fig. 2B) and 46.48 % ($p < 0.01$, Fig. 2B) when treated with pectolarigenin as compared with the control, respectively.

Pectolarigenin reduced tumorigenicity *in vivo*

It has been reported that tumorigenicity *in vivo* correlates with the sphere formation ability of tumor cells *in vitro*. To test the effect of pectolarigenin on the tumorigenicity of breast cancer cells, MCF-7 cells (5×10^6) were inoculated into nude BALB/C mice. When the tumors developed in 7 days, the mice were randomly distributed into two groups, of untreated or treated with pectolarigenin (20 mg/kg). The size and weight of xenograft tumors treated with pectolarigenin were significantly smaller than control tumors (Fig. 3). These results thus demonstrated that pectolarigenin efficiently attenuated the tumorigenicity of breast cancer cells *in vivo*.

DISCUSSION

It is urgently required to develop an effective method of treatment of breast cancer, due to its high incidence and high recurrence rates in recent years. Hypothesis of cancer stem cells suggested that CSCs is the mainly responsible for causing relapse in cancer patients [2]. Bioflavonoids extracted from fruits and vegetables

have a variety of biological properties, including antibacterial, antioxidant, anti-inflammatory, antiviral, anticancer, cancer preventing activities [14,15]. Previously we have already demonstrated that the flavonoid pectolarigenin inhibits the proliferation of breast cancer cell [11]. We speculated that inhibiting CSCs properties may be a potential mechanism of preventing carcinogenesis by pectolarigenin. The tumor

sphere assay has been used to identify breast cancer stem cells, as shown in Fig. 1D, CSCs markers (OCT4, Nanog, SOX2 and CD44) were greatly induced in our experimental system. The results demonstrated that pectolarigenin inhibited breast cancer cell self-renewal capacity. Consistent with the inhibitory effects, pectolarigenin suppressed induction of BCSCs markers.

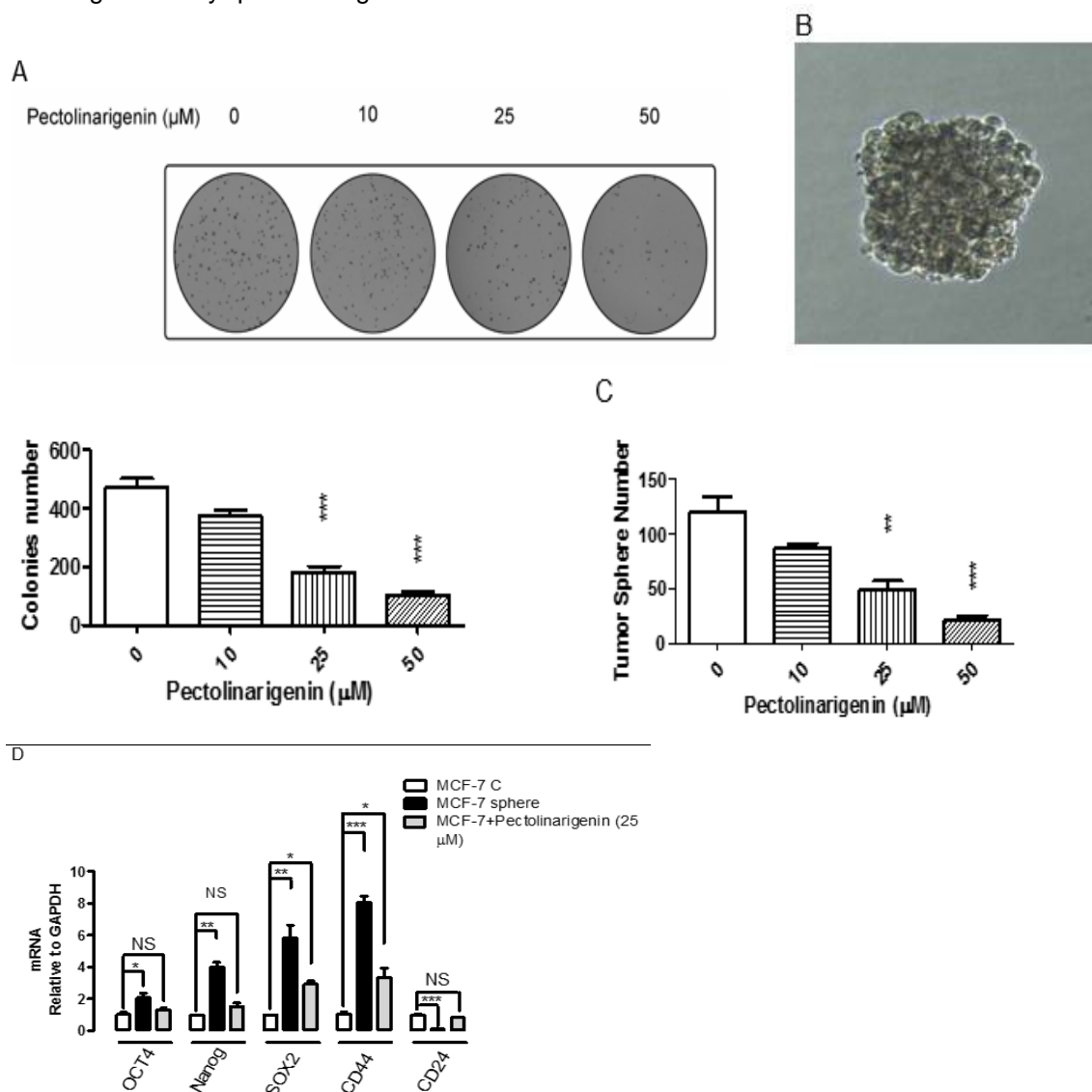


Figure 1: Pectolarigenin inhibited BCSCs self-renewal properties and negatively correlated with BCSCs characteristics. **(A)** Pectolarigenin inhibited the colony formation capacity in MCF-7 in a dose-dependent manner. MCF-7 cells treated with the indicated concentrations of pectolarigenin were plated in 0.3 % agarose, and colonies were counted 3 weeks later. Representative photograph of colony was shown on the top. Statistical analysis was shown on the bottom. **(B)** Representative photograph of tumor sphere. MCF-7 cells were cultured in the stem cell medium as described in the materials and methods. **(C)** Pectolarigenin inhibited the tumor sphere formation capacity in MCF-7 in a dose-dependent manner. MCF-7 cells treated with the indicated concentration of pectolarigenin were cultured in the stem cell medium as described in materials and methods. **(D)** Several breast cancer stem cell markers expression in tumor spheres and monolayer cells. OCT-4, Nanog, Sox2, CD44, and CD24 expression in monolayer MCF-7 cells, MCF-7 spheres and MCF-7 spheres treated with 25 μM pectolarigenin were detected by Real-Time PCR. Data are the mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control

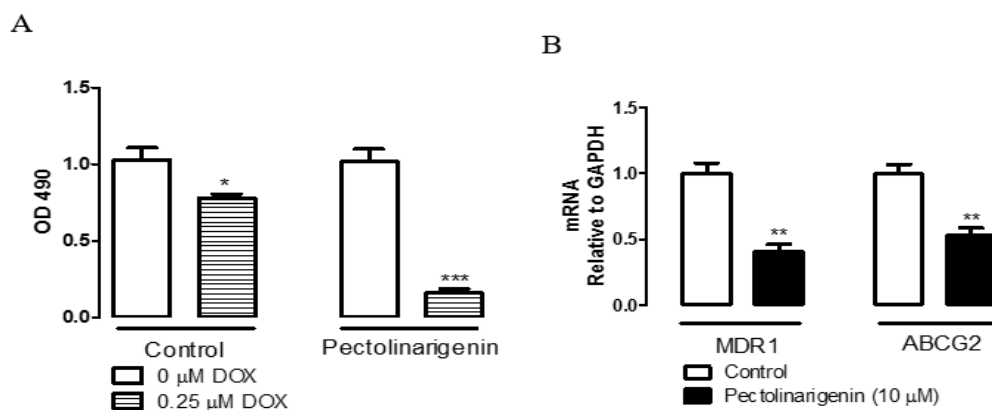


Figure 2: Pectolinarigenin reduced breast cancer cell chemoresistance. (A) Pectolinarigenin reduced MCF-7 chemoresistance. MCF-7 cells were pre-treated with or without pectolinarigenin (10 μ M) for 24 h, and then 0.25 μ M Doxorubicin was added to the MCF-7 cells. Cell viability was determined by MTT assay. (B) Drug resistant genes expression in MCF-7 when treated with pectolinarigenin. MDR1 and ABCG2 expression in MCF-7 cells treated with 10 μ M pectolinarigenin were detected by Real-Time PCR. Data are the mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control

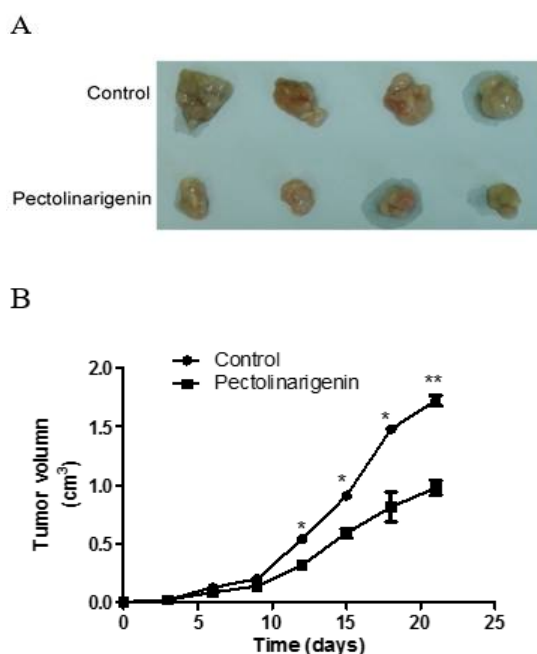


Figure 3: Pectolinarigenin reduced tumorigenicity *in vivo*. (A) Tumors produced by MCF-7. MCF-7 Cell (5×10^6) were injected subcutaneously in the right flank of nude mice ($n=4$). When the tumors developed in 7 days, the mice were randomly distributed into two groups, and were untreated or treated by i.p. injections every day with pectolinarigenin (20 mg/kg). (B) Tumor growth curves were monitored every 3 days during the experimental period ($n = 4$). Data represented the mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control

Another characteristic of CSCs is chemoresistance. MDR1 and ABCG2 play an important role in chemoresistance of breast cancer cells [15]. Our results showed that pectolinarigenin enhanced breast cancer cell

chemosensitivity to doxorubicin. The chemosensitivity is associated with downregulation of MDR1 and ABCG2 (Fig. 2B). Pectolinarigenin inhibited MDR1 and ABCG2 mRNA expression, and at the same time, repressed tumor sphere formation. Our results are consistent with other reports that MDR1 and ABCG2 not only play a major role in multidrug resistance but also are characteristics of CSCs markers.

CONCLUSION

The findings of this study indicate that pectolinarigenin inhibits BCSCs properties *in vitro*, reduces breast cancer cell tumorigenicity *in vivo*, and provides an experimental basis for the improvement of breast cancer therapy.

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