

Original Research Article

Pectolinarigenin - A Flavonoid Compound from *Cirsium Japonicum* with Potential Anti-proliferation Activity in MCF-7 Breast Cancer Cell

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Abstract

Purpose: To isolate high purity pectolinarigenin from *Cirsium japonicum* and evaluates its anti-proliferative potential and induction of apoptosis activity in MCF-7 breast cancer cell.

Methods: Pectolinarigenin was purified by the combination of silica gel and Sephadex LH-20 column chromatography. The structure was identified by ¹H-nuclear magnetic resonance (NMR), ¹³C-NMR and electrospray ionization tandem mass spectrometry (ESI-MS) analyses. The anti-proliferation activity of pectolinarigenin was also evaluated by methylthiazolyldiphenyl-tetrazolium bromide (MTT) and colony formation assays in MCF-7 breast cancer cell.

Results: Pectolinarigenin showed potent anti-proliferation activity by inducing apoptosis and downregulation of Bcl2 expression in MCF-7 breast cancer cell. Protein expression of Bcl2 was almost completely inhibited upon the treatment with 25 μM pectolinarigenin.

Conclusion: A rapid method to isolate high purity pectolinarigenin from *C. japonicum* and as well as evaluate its anti-proliferative potential and induction of apoptosis in MCF-7 breast cancer cells is presented for the first time. Induction of apoptosis of MCF-7 is via downregulation of Bcl2 expression.

Keywords: *Cirsium japonicum*, Anti-proliferation activity, Pectolinarigenin, Breast cancer, Apoptosis.

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INTRODUCTION

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 [1]. Pharmacological studies show that the extract of *C. japonicum* and its major constituents possess anti-tumor [1, 2], anti-diabetic [3], antioxidant [4], anti-inflammatory [4], and antifungal [5]. *C. japonicum* contains significant amounts of flavonoid compounds, the

principal characteristic of the flavonoid compounds are pectolinarin and pectolinarigenin (Figure 1), which exhibited powerful anti-diabetic, hepatoprotective and anticancer activities *in vitro* [3,6,7].

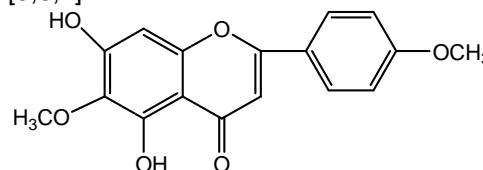


Figure 1: Chemical structure of pectolinarigenin (1)

A previous study has shown that the methanol extract of *C. japonicum* inhibits MCF-7 cell growth at both proliferation and apoptosis levels [8]. However, the active components have not been isolated and identified. Bcl2 gene is an important regulator of apoptosis, which is an over-expression in many solid organ tumours including breast cancer [9]. Increasing evidence supports their modulated expression in breast cancer cells and in many cases, their relation to chemotherapy response, outcome, and overall prognosis, as well as their value are important potent therapeutic targets [10].

The aim of this study was to determine a quick method to isolate high purity pectolinarigenin from *C. japonicum* and assess the biological activity of pectolinarigenin in breast cancer.

EXPERIMENTAL

Plant material and equipment

The aerial parts of *C. japonicum* were purchased from the Chinese herbal medicine market in Zhang Shu (Jiang Xi, China) in July 2010 and authenticated by Qingzhi Kong of Hubei University of Chinese Medicine. A voucher specimen (2010-07) has been deposited in the Hubei University of Chinese Medicine. The 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 carried 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., LtdS (Shanghai, China). Bcl2 and Bax were purchased from Santa Cruz Biotechnology, Inc. Caspase 3 was obtained from Cell Signaling Technology. β-actin and Anti-poly (ADP-ribose) polymerase (PARP) were obtained from Sigma Chemical Company (Sigma-Aldrich). The cell culture medium Dulbecco's Modified Eagle Medium (DMEM); high glucose, without sodium pyruvate) and fetal bovine serum (FBS) were purchased from Hyclone Company (Logan, UT, USA). Acrylamide-bis solution (30 %; 29:1) was bought from Bio-Radlaboratories (Hercules, CA, USA), Inc. Protein content was tested using a BCA kit (Thermo Scientific).

Cell line and culture

The human breast cancer cells line, MCF-7, was purchased from American Type Culture Collection. MCF-7 Cells were cultured in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin

and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5% CO₂ (all reagents from Invitrogen, Carlsbad, CA). The cells were maintained in log phase by seeding twice a week at a density of 1 × 10⁵/ml, and the experiments were performed 1 day after trypsinization.

Extraction and isolation of pectolinarigenin

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 vacuo* 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 growth determination by MTT assay

MTT assay measures the ability of viable cells to reduce yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan by mitochondrial succinate dehydrogenase (SDH). Cells (1×10³) were seeded onto a 96-well plate in triplicate with 1, 5, 10, 25 and 50 µM pectolinarigenin for 24 h, and then the cells were washed three times with fresh medium and cultured for an additional 7 days. The medium was removed, and the MCF-7 cells were then incubated in DMEM medium containing 0.5 mg/ml MTT for an additional 3 h. The medium was removed, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan. Absorbance was read at 490 nm in an ELISA reader (Bio-Rad iMARK™ Microplate Reader). The absorbance of cultures treated with 0.1 % DMSO was regarded as 100% cell viability.

Colony forming assay

For colony expansion assay, MCF-7 cells were plated in 6-well plates at a density of 1000 cells/well and allowed to adhere overnight. The culture medium was replaced with media containing indicated concentrations of pectolinarigenin. After exposure for 24 h, the cells were washed twice with phosphate buffered saline (PBS). Fresh medium was then added, and the cells cultured at 37 °C in humidified 5 % CO₂ atmosphere for 8 - 10 days. The cells were viewed regularly under the microscope for colony formation. At termination, the culture medium was decanted, the cells rinsed with PBS and stained with crystal violet (0.5 % in 95 % ethanol) for 5 min after fixation with 10 % formaldehyde for 5 min.

Western blotting assay

The cells were washed twice with cold PBS and harvested in 150 μ l of Lysis Buffer RIPA (150 mM NaCl; 50 mM Tris pH 8; 0.1 % SDS; 0.5 % sodium deoxycolate; 1 % Nonidet P40; 5 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 2 mM DTT). The cell homogenates were centrifuged at 10,000 g for 20 min at 4 °C. The resulting supernatant was used as a cellular protein for Western blotting analysis. The protein content in the lysates was measured by BCA protein assay. Equal amounts of proteins were electrophoresed in SDS-PAGE and the proteins transferred to polyvinylidene difluoride (PVD) membranes. The detection was achieved using the immobilon western chemiluminescent HRP Substrate kit (Millipore). The primary antibodies used are: PARP (Santa Cruz Biotechnology, Inc.), Caspase 3 (Cell Signaling Technology), Bcl2 (Santa Cruz Biotechnology, Inc.), Bax (Santa Cruz Biotechnology, Inc.) and β -actin (Sigma-Aldrich).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software. Values are expressed as mean \pm SEM. Pair-wise comparisons were performed using Student's t test (two-tailed). $P < 0.05$ was set as significant different.

RESULTS

NMR and ESI-MS spectral data

Pectolinarigenin (1): yellow crystal (methanol), $C_{17}H_{14}O_6$, UV (MeOH) λ_{max} : 336, 273 nm, ESI-MS m/z : 313.1[M-H]⁻. ¹H-NMR (400 MHz, DMSO- d_6 , δ , ppm, J/Hz): 6.86 (1H, s, H-3), 6.61 (1H, s, H-8), 8.02 (2H, d, J=8.8, H-2', 6'), 7.10 (2H, d, J=8.8, H-3', 5'), 12.97 (1H, s, OH-5), 10.78 (1H, s, OH-7), 3.94 (3H, s, 6-OCH₃), 3.86(3H, s, 4'-OCH₃). ¹³C-NMR (100 MHz, DMSO- d_6): 163.8 (C-2), 103.5 (C-3), 182.6 (C-4), 153.2 (C-5), 131.8 (C-6), 157.8 (C-7), 94.7 (C-8), 152.9 (C-9), 104.6(C-10), 123.3 (C-1'), 128.7 (C-2', 6'), 114.9 (C-3', 5'), 162.7 (C-4'), 60.4 (6-OCH₃), 55.9 (4'-OCH₃). The NMR data were consistent with the literature [11].

Effect of pectolinarigenin on MCF-7 cell growth

Treatment with pectolinarigenin inhibited cell proliferation in MCF-7 (Figure 2). The inhibitory effect on MCF-7 treated with 1, 5, 10, 25 and 50 μ M pectolinarigenin were 16, 31, 61, 82, and 89, respectively, compared with controls. This result suggested that pectolinarigenin caused a

sustained growth arrest of MCF-7 in dose-dependent manner.

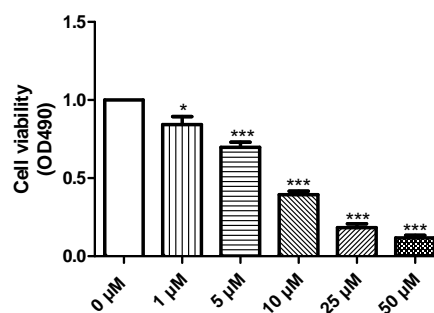


Figure 2: Inhibition of proliferation MCF-7 cells. MCF-7 cells were exposed to different concentrations of pectolinarigenin. Data represent mean normalized optical densities \pm SEM (n = 3); * $p < 0.05$, *** $p < 0.001$ compared control

Colony formation

To further investigate the inhibitory effect of pectolinarigenin on MCF-7 growth, colony formation assay was carried out. The results also revealed that pectolinarigenin elicited anti-proliferation activity in MCF-7 in a dose-dependent manner (Figure 3).

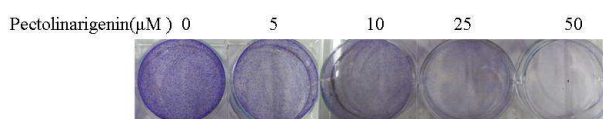


Figure 3: Inhibition of colony formation in MCF-7 breast cancer cells by pectolinarigenin

Pectolinarigenin modulates apoptosis-related protein expression

The results show that 5 and 25 μ M pectolinarigenin efficiently induced the cleavage of PARP and Caspase 3 in MCF-7 cells after 24-h treatment (Figure 4). At the same time, a significant difference was found in the expression of Bcl2 and Bax. MCF-7 treated with 5 and 25 μ M pectolinarigenin as it resulted in the downregulation of anti-apoptotic protein Bcl2 expression. However MCF-7 treated with 5 μ M pectolinarigenin, even upto 25 μ M pectolinarigenin there was no change ($p > 0.05$) in pro-apoptotic protein Bax expression (Figure 4).

DISCUSSION

Pectolinarigenin has been reported as an anti-tumor agent in mice with S180 and H22 tumor cells [1] and many other cancer cell lines, including lung cancer, colon cancer and renal cancer cells [2,7]. In the present study, we found that 5, 10, 25 and 50 μ M pectolinarigenin significantly inhibit MCF-7 breast cancer cell

proliferation. Pectolinarigenin, at a concentration of 50 μM , almost completely inhibited MCF-7 colony formation.

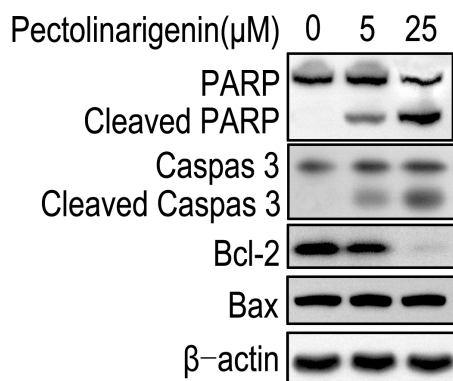


Figure 4: Modulation of apoptosis related protein expression in MCF-7 cells by pectolinarigenin.

Induction of apoptosis by pectolinarigenin is an important way to inhibit cancer cell growth. Caspase 3 is a member of the cysteine-aspartic acid protease family and a critical executioner of apoptosis [12]. PARP is a DNA repair enzyme, and cleavage of PARP facilitates cellular disassembly. Cleavage of Caspase 3 and PARP serves as markers of cell undergoing apoptosis [13]. Our findings indicate that 5 and 25 μM pectolinarigenin induced cleavage of Caspase 3 and PARP and that pectolinarigenin inhibits MCF-7 cell proliferation via induction of MCF-7 apoptosis. The apoptosis-preventing effect of Bcl2 is counteracted by the pro-apoptotic protein, Bax; furthermore, the ratio of Bax/Bcl-2 mixture determines sensitivity to different apoptotic stimuli. While 5 and 25 μM pectolinarigenin resulted in downregulation of Bcl2 protein expression, the isolate had no effect on Bax protein expression. Thus, downregulation of Bcl2 protein expression is the probable mechanism of induction of apoptosis by pectolinarigenin.

CONCLUSION

Induction of apoptosis of MCF-7 via downregulation of Bcl2 expression is the probable mechanism of action of pectolinarigenin. Pectolinarigenin inhibition of breast cancer cell MCF-7 proliferation not only an addition to the current knowledge of the compound's properties

but also presents a novel potential therapeutic agent for the treatment of breast cancer.

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