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# Silymarin induces cell cycle arrest and apoptosis in ovarian cancer cells

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ABSTRACT

The polyphenolic flavonoid silymarin that is the milk thistle extract has been found to possess an anti-cancer effect against various human epithelial cancers. In this study, to explore the regulative effect of silymarin on human ovarian cancer line A2780s and PA-1 cells, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay and flow cytometry were respectively used to determine the inhibitory effect of silymarin on the both cell lines, and to measure their cell cycle progression. Apoptosis induction and mitochondrial membrane potential damage were separately detected by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling assay and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide staining. Additionally, western blotting was applied to determine cytochrome C release and expression levels of p53, p21, p27, p16, CDK2, Bax, Bcl-2, procaspase-9, procaspase-3, cleaved caspase-9 and caspase-3 proteins. The activity of caspase-9 and caspase-3 was measured using Caspase-Glo-9 and Caspase-Glo-3 assay. The results indicated that silymarin effectively suppressed cell growth in a dose- and time-dependent manner, and arrested cell cycle progression at $G_1$/S phase in A2780s and PA-1 cells via up-regulation of p53, p21, and p27 protein expression, and down-regulation of CDK2 protein expression. Additionally, silymarin treatment for 24 h at 50 and 100 μg/ml resulted in a reduction of mitochondrial membrane potential and cytochrome C release, and significantly induced apoptosis in A2780s and PA-1 cells by increasing Bax and decreasing Bcl-2 protein expression, and activation of caspase-9 and caspase-3. Therefore, silymarin is a possible potential candidate for the prevention and treatment of ovarian cancer.





## 1. Introduction

Ovarian cancer is the most lethal of all gynecologic malignancies despite recent advances in treatment, and becomes the fifth most common cause of cancer-related death (Tierney et al., 2012). In 2013, there were estimated 22,240 new cases with about 14,030 deaths in the United States (Siegel et al., 2013). Because this disease is generally asymptomatic at the early stage of its progression, most of ovarian cancers are diagnosed at an advanced stage. The median five-year survival rates of stage III, and IV ovarian cancer patients have been reported to be approximately 37% and 25%, respectively (Zou et al., 2012). Generally, conventional treatment for ovarian cancer is cytoreductive surgery and followed by Q3 platinum or paclitaxel-based chemotherapy (Song et al., 2012a, 2012b), but development of acquired resistance results in a major limitation to successful treatment (Wang et al., 2013). Although Q4 recently it has been reported that some molecular-based novel agents such as epidermal growth factor receptor tyrosine kinase inhibitors for ovarian cancer treatment, their strong adverse effects including skin and gastrointestinal toxicity are still a tough problem to be resolved (Glaysher et al., 2013; Higgins et al., 2014). Therefore, development of new strategies and novel agents from herbal medicine with little side effects is an attractive avenue to prevention and treatment of ovarian cancer (Gao et al., 2011).

Silymarin, a flavonolignan isolated from the milk thistle (*Silybum marianum*), has been widely used as a natural remedy for liver diseases for several decades (Chen et al., 2009). Silymarin is the mixture of four isomeric flavonoids such as silibinin, isosilibinin, silydianin and silychristin. Silibinin is a major bioactive compound present in silymarin. In recent studies, silymarin has been reported to have cancer preventive and therapeutic effects against several human epithelial cancers including breast cancer, cervical cancer, skin cancer, bladder cancer, liver cancer, lung cancer and prostate cancer (Féher and Lengyel, 2012; Singh and Agarwal, 2002; Yu et al., 2012; Zhu et al., 2001). In addition, previous studies demonstrated that silymarin could decrease the secretion level of vascular endothelial growth factor in human prostate and breast cancer cells (Jiang et al., 2000), suppressing cell proliferation, modulating cell cycle progression and inducing apoptosis in human hepatic cancer cells (Ramakrishnan et al., 2009) or impeding the epithelial-to-mesenchymal transition in lung carcinoma

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cells (Cufí et al., 2013), which may contribute to the anti-cancer effects of silymarin.

The multiple bioactivities of silymarin especially its anti-cancer activities have excited many researchers' interests in exploring its new pharmacological action (Snima et al., 2014). However, to our knowledge, its effects on ovarian cancer cells have not been reported in the past years. Thus, in light of previous studies on silymarin in other cancer cells, we hypothesized that silymarin maybe is an effective candidate for anti-ovarian cancer cells. Therefore, to test this hypothesis in this study, we studied the inhibitory effects of silymarin on the human ovarian cancer A2780s and PA-1 cells and its underlying molecular mechanisms for silymarin-mediated cell cycle arrest and apoptosis in the two cell lines.

## 2. Materials and methods

### 2.1. Chemical and reagents

Silymarin and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), and silymarin was dissolved in dimethyl sulfoxide to a concentration of 1000 μg/ml as a stock solution and stored at 4 °C The desired final concentrations of silymarin were available by further dilution with culture medium, and the final content of dimethyl sulfoxide was kept to less than 0.1% in all cell cultures, which showed no effects on cell morphology and proliferation. The terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL) assay kit and Caspase-Glo-3/9 assay kit were obtained from Promega Corporation (Madison, WI, USA). The primary antibodies against p53, p21, p27, p16, CDK2, Bax, Bcl-2, cytochrome C, procaspase-9, cleaved caspase-9, procaspase-3, cleaved caspase-3, and β-actin were purchased from Cell Signal Technology (Beverly, MA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) mitochondrial membrane potential detection kit and ApoAlert Cell Fractionation Kit were purchased from Biotium company (Hayward, CA, USA) and Clontech Laboratories (Mountain View, CA, USA), respectively.

### 2.2. Cell viability assay

Human ovarian cancer A2780s and PA-1 cell lines were obtained from Shanghai Cell Bank of Chinese Academy of Science, and were separately cultured in RPMI (Roswell Park Memorial Institute) 1640 medium added with 10% fetal bovine serum in a humidified incubator with 5% $CO_2$ at 37 °C. The effects of silymarin treatment on the proliferation of A2780s and PA-1 cells were evaluated by MTT assay according to a published article (Sun et al., 2012), with some modifications. Briefly, A2780s and PA-1 cells were respectively seeded in 96-well plates at a density of $1 \times 10^4$ cells/well, and allowed for incubation overnight. The cells were then treated with silymarin at the following concentrations: 0, 25, 50, 100, 150, and 200 μg/ml for 24 h, or cells were treated with 100 μg/ml of silymarin for 24, 48 and 72 h. Subsequently, 20 μL of MTT solution was directly added into each well for another 4 h of incubation, and followed by addition of 150 μL dimethyl sulfoxide after removal of culture medium. Absorbance value at 570 nm was then determined with an automated spectrophotometric plate reader (PerkinElmer, USA). The experiments were carried out for duplicate at least three times.

### 2.3. Silymarin affects cell cycle progression in A2780s and PA-1 cells

The effects of silymarin treatment on cell cycle progression in ovarian cancer cells were performed as previously described (Zeng et al., 2013), with some modifications. Briefly, A2780s or PA-1 cells were inoculated in 6-well plates at a density of $1 \times 10^5$ cells/well, and allowed for incubation of 12 h, and then treated with silymarin at indicated concentrations (0, 50, and 100 μg/ml) for 24 and 48 h. 0 μg/ml of silymarin served as the control with culture medium of a same volume instead of silymarin. The cells were then harvested by centrifugation, and followed by fixation in cold 75% ethanol for 1 h, and centrifuged to remove ethanol. Subsequently, the cells were incubated in the staining solution containing 50 μg/ml of propidium iodide for 30 min in dark, and then measured for cell cycle distribution by flow cytometry (Becton Dickinson, NJ, USA).

### 2.4. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay for cellular apoptosis

To detect effects of silymarin on the apoptosis in ovarian cancer cells, TUNEL assay was performed as previously described (Song et al., 2012a, 2012b), with some modifications. Briefly, A2780s or PA-1 cells were seeded in the micro-well dishes at a density of $5 \times 10^5$ cells per dish, and allowed for incubation overnight, and followed by treatment with 50 and 100 μg/ml of silymarin for 24 h. The control was treated with culture medium with a same volume instead of silymarin. The cells were pretreated with 0.15% Triton X-100 in phosphate-buffered saline (pH 7.4) for 5 min after they were fixed in 4% paraformaldehyde for 30 min at 4 °C, and followed by incubation at 37 °C for 1 h in dark in a compound solution containing terminal deoxynucleotidyl transferase, deoxyadenosine triphosphate, fluorescein-2′-deoxyuridine 5′-triphosphate, ethylene diamine tetraacetic acid and Tris–HCl according to the instructions of TUNEL assay kit. After counterstaining with 4′, 6-Diamidino-2-Phenylindole for 5 min, the cells were observed with a laser confocal scanning microscope (Olympus FV1000, Japan) by a standard parameter for green fluorescence of fluorescein, and their micrographs were obtained. Apoptotic percentage of each sample was denoted by normalizing the apoptotic cell amount in five equal areas to the total cell amount in the corresponding areas.

### 2.5. Evaluation of mitochondrial membrane potential damage ($\Delta\psi_m$)

The extent of mitochondrial membrane potential damage was determined as previously described (Selvaraj et al., 2013), with some modifications. Briefly, A2780s or PA-1 cells were seeded in micro-well dishes (MatTek Corp., MA, USA) at a density of $1 \times 10^5$ cells/ml and allowed to adhere overnight, and followed by treatment with 50 and 100 μg/ml of silymarin for 24 h. The cells were then washed in sterile phosphate-buffered saline (PBS, pH 7.4), and incubated with 5, 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining solution for 20 min according to the instructions of manufacturer. Subsequently, the cells were re-suspended in fresh serum-free medium after washing with PBS, and then analyzed with a laser confocal scanning microscope (Olympus FV1000, Japan). It has been well-known that JC-1 can accumulate within intact mitochondria to produce JC-1 aggregates (red color), and the color changes from red to green fluorescence resulting from JC-1 monomer (green color) in apoptotic cells with depolarization of mitochondrial membrane potential. Therefore, the color alteration in A2780s or PA-1 cells was observed with a laser confocal scanning microscope after silymarin treatment, and the ration of red/green fluorescence intensity was calculated to obtain the mitochondrial membrane potential.



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