# Effects of an ethyl acetate extract of *Daphne altaica* stem bark on the cell cycle, apoptosis and expression of PPARγ in Eca-109 human esophageal carcinoma cells

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**Abstract.** *Daphne altaica* Pall. (*D. altaica*; Thymelaeaceae) has long been used in traditional Kazakh medicine for the treatment of cancer and respiratory diseases. Previous studies have demonstrated the *in vitro* anticancer effects of *D. altaica* extract and its constituents in certain cancer cell lines; however, the underlying molecular mechanisms are not completely understooD. The present study aimed to investigate the molecular mechanisms underlying the activity of an ethyl acetate extract of *D. altaica* (Da-Ea) by assessing its effects on cell morphology, cell apoptosis, cell cycle progression and the expression levels of peroxisome proliferator-activated receptor γ (PPARγ) in Eca-109 cells. Cell morphology was observed under a phase contrast microscope. Cell apoptosis and cell cycle progression were assessed by flow cytometry following Annexin V/propidium iodide (PI) double staining and PI single staining, respectively. The mRNA and protein expression levels of PPARγ were determined by reverse transcription-quantitative PCR and western blotting, respectively. Compared with the control group, the percentage of apoptotic cells, cell cycle arrest at S phase and apoptotic morphological cell characteristics were increased in Da-Ea-treated Eca-109 cells. Furthermore, Da-Ea treatment upregulated the mRNA and protein expression levels of PPARγ compared with the control cells. High-performance liquid chromatography with diode-array detection indicated that daphnetin-7-O-β-D-glucoside, daphnetin, demethyldaphnoretin-7-O-β-D-glucopyranoside and genkwanol A were the main constituents of Da-Ea. Collectively, the results suggested that Da-Ea displayed antiproliferative activities in Eca-109 cells by inducing apoptosis and S phase cell cycle arrest, as well as upregulating PPARγ expression levels.

## Introduction

Throughout history, natural products have served an important role in the treatment of human diseases. At present, natural products are the major source of pharmaceutical agents, particularly for cancer therapy. Natural products and their derivatives account for ~80% of all drugs approved for cancer therapy by the USA Food and Drug Administration during the last three decades (1,2).

*Daphne altaica* Pall. (*D. altaica*) is a medicinal herb used in traditional Kazakh medicine for the treatment of numerous diseases, including cancer of the digestive tract, tracheitis, common cold, sore throat, rheumatism and snakebite (3). The plant is endemically distributed in the North of the Jungar Basin

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of Xinjiang (Tacheng and Habahe areas), the Altai, Manrak and Tarbagatai Mountains of Kazakhstan, the Altai region of Russia and Northwest Mongolia (3). The medicinal use of D. altaica was first recorded in the Kazakh medical classic work Shipagerlik Bayan (3). However, the anticancer effects of D. altaica were reported for the first time by Kizaibek et al (3), who demonstrated that different D. altaica extracts, except for the aqueous extract, displayed moderate to significant in vitro cytotoxicity against several cancer cell lines, including Eca-109, AGS, SMMC-7721 and HeLa. Kizaibek et al (4) also identified antiproliferative activities of D. altaica in the human CCRF-CEM leukaemia and MDA-MB-231 breast cancer cell lines, and identified the constituents of the D. altaica $CH_2Cl_2$ extract using liquid chromatography (LC)-diode-array detection (DAD)-mass spectrometry and LC-DAD-high resolution electrospray ionization mass spectrometry in positive mode. Nugroho et al (5) reported that three new daphnane diterpenoids (Altadaphnans A-C) from the aerial parts of D. altaica significantly inhibited the proliferation of A549 cancer cells. However, the mechanisms underlying the antiproliferative activities of D. altaica have not been previously reporteD. Therefore, the present study aimed to identify the mechanism underlying the antiproliferative activity of an ethyl acetate extract of D. altaica (Da-Ea) by assessing cell apoptosis, cell cycle progression and the expression of peroxisome proliferator-activated receptor γ (PPARγ) in the human Eca-109 oesophageal squamous cell carcinoma cell line.

## Materials and methods

*Cell culture.* The human Eca-109 oesophageal cancer cell line was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and maintained at 37°C with 5% $CO_2$ in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (HyClone; GE Healthcare Life Sciences). At 90% confluency, cells were harvested using 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.). Cells in the exponential phase of growth were used for subsequent experiments.

*Plant materials.* The D. altaica plant was collected from Habahe County of Xinjiang Uyghur Autonomous Region, P.R. China in July 2017. The plant was identified by Dr Omirshat Tahan (College of Grassland and Environment Sciences, Xinjiang Agricultural University, Urumqi, China). A voucher specimen (no. HB-2017001) was deposited at the Traditional Kazakh Medicine Research Institute of Traditional Chinese Medicine Hospital of Ili Kazakh Autonomous Prefecture.

*Extraction of Da-Ea.* Da-Ea was extracted as previously described (3). Briefly, dried bark of the plant (150 g) was cut into small pieces and macerated with 95% EtOH for 2 weeks at room temperature in the dark. The extraction process was repeated twice. The extracted mixtures were combined, concentrated by distillation under vacuum and freeze-dried to yield the EtOH extract. Petroleum ether, chloroform and ethyl acetate (Da-Ea; 0.8243 g) extracts were obtained from the EtOH extract using a sequential liquid-liquid extraction with

solvents of increased polarity, including pure petroleum ether, pure chloroform and pure ethyl acetate. The only extract used in subsequent experiments was Da-Ea.

High performance liquid chromatography (HPLC) with diode-array detection (DAD) was used to analyse the main ingredients in the extracts. HPLC was conducted using a 1260 HPLC system (Agilent Technologies, Inc.) equipped with a 1260 Infinity Diode Array Detector (cat. no. G4212A; Agilent Technologies, Inc.) in gradient elution mode. An XBridge™ C18 column (4.6x250 mm; diameter, 5 $\mu$m; Waters Chromatography Europe BV) set at 25°C with a 0.5 ml/min flow rate was useD. For each experiment, a 5 $\mu$l injection volume was useD. The mobile phase consisted of 0.1% (v/v) acetic acid aqueous solution (solvent A) and acetonitrile (solvent B). The gradient conditions used were as follows: 10% solvent B for 0 min; 62% solvent B for 37 min; 62% solvent B for 39 min; and 10% solvent B for 40 min. The chromatogram was analysed using OpenLAB CDS Chemstation software (version A.01.05; Agilent Technologies, Inc.). To obtain stock solution, 22.7 mg Da-Ea was sonicated with 1,135 $\mu$l 80% MeOH-DMSO (1:1) at 40 MHz frequency and room temperature for 10 min. Subsequently, the stock solution was further diluted with methanol to generate 1.25 mg/ml sample solution for HPLC analysis. Standard compounds [daphnetin-7-O-β-D-glucoside, daphnetin, demethyldaphnoretin-7-O-β-D-glucopyrano side and genkwanol A; provided by Professor Zhengbing Gu (Jiangsu Yongjian Medical Technology Ltd., Co.)] were dissolved in methanol. Compounds were identified based on their retention time and UV spectra compared with reference standards.

*Evaluation of cell morphology.* Eca-109 cells were seeded ($2x10^5$ cells/well) into 6-well culture plates and incubated with Da-Ea (10, 20 or 50 $\mu$g/ml) at 37°C for 24, 48 or 72 h. Cells treated with DMSO (10, 20 or 50 $\mu$g/ml) were used as the control. For each test and control medium, the final concentration of DMSO was adjusted to 1%. Under x100 magnification, cell morphology was assessed using an IX71-12FL/PH phase contrast microscope (Olympus Corporation) with Olympus cellSens Standard imaging software, version 1.0 (Olympus Corporation).

*Detection of cell apoptosis.* Early cell apoptosis was examined by Annexin V-FITC Apoptosis Detection kit (BD Bioscience) according to the manufacturer's protocol. Cells ($2x10^5$ cells/well) were seeded into 6-well culture plates and allowed to grow overnight. Subsequently, cells were treated with Da-Ea (10, 20 or 50 $\mu$g/ml) for 24, 48 and 72 h. Following treatment, cells were collected and washed twice with PBS. Cells were incubated with 5 $\mu$l Annexin V and 5 $\mu$l PI (BD Biosciences; Becton, Dickinson and Company) for 15 min at room temperature in the dark. Following incubation, each sample was filtrated on a nylon membrane (pore size, 48 ìm). Flow cytometry was performed using a FACSAriaII flow cytometer (Beckman Coulter, Inc.) and FlowJo software, version 7.6 (Tree Star Inc.).

*Detection of cell cycle.* For cell cycle analysis, cells were seeded ($2x10^5$ cells/well) into 6-well culture plates and maintained overnight. Subsequently, cells were treated with Da-Ea

(10, 20 or 50 $\mu$g/ml) for 24 or 48 h. Cells were washed twice with PBS. Cells were fixed in 75% ethanol at 4°C for 2 h and subsequently washed three times with PBS. Cells were stained with 500 $\mu$l PI/RNase staining buffer (BD Biosciences; Becton, Dickinson and Company) at room temperature for 15 min in dark. Following staining, cells were filtrated on a nylon membrane (pore size, 48 ìm). Cells were analysed using a FACSAriaII flow cytometer (Beckman Coulter, Inc.) and ModFit LT software, version 3.2 (Verity Software House, Inc.).

*Assessment of PPARγ mRNA expression by reverse transcription-quantitative PCR (RT-qPCR).* Eca-109 cells in the logarithmic phase of growth were seeded ($3\times10^5$ cells/ml) into 6-well culture plates. Cells were incubated with Da-Ea (10, 20 or 50 $\mu$g/ml) for 24, 48 and 72 h at 37°C with 5% $CO_2$. Subsequently, cells were washed with PBS and total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, the cell lysate was incubated at room temperature for 5 min and transferred to a fresh 1.5 ml Eppendorf tube containing 200 $\mu$l chloroform. The tubes were vortexed for 15 sec, incubated at room temperature for 2-3 min and centrifuged at 15,400 x g for 15 min at 4°C until the liquid separated into three phases. The upper aqueous phase (400-500 $\mu$l) was transferred to a new RNase-free Eppendorf tube, mixed with an equal volume of isopropanol and incubated for 10 min at room temperature. Subsequently, the sample was centrifuged for 10 min at 15,400 x g and 4°C to precipitate the RNA. The supernatant was discarded and 75% ethanol was added to the RNA prior to centrifugation at 3,800 x g for 15 min at 4°C. Subsequently, the supernatant was discarded and the RNA was air-dried at room temperature for 5-10 min. Total RNA was stored at -20°C until further analysis. RNA purity was assessed by measuring the A260/A280 absorbance ratio using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). RNA with an A260/A280 ratio of 1.7-2.0 was considered high quality. RNA quality and integrity were verified by visualising 28S and 18S RNA bands on an ethidium bromide-stained 1.5% agarose gel using the Gel DOC XR imaging system (Bio-Rad Laboratories, Inc.). RNA samples with a clear and sharp 28S band that were twice as intense as the 18S band were used for reverse transcription. Total RNA was reverse transcribed to cDNA using RevertAid First strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). Reverse transcription was performed for a total volume of 20 $\mu$l consisting of 1 $\mu$g total RNA, 1 $\mu$l Oligo (dT)18 primer, 4 $\mu$l 5X Reaction Buffer, 1 $\mu$l Ribolock Rnase Inhibitor (20 U/$\mu$l), 2 $\mu$l dNTP mix (10 mm) and 1 $\mu$l RevertAid M-MuLV RT (200 U/$\mu$l). The following thermocycling conditions were used for reverse transcription: 3 min at 65°C; 60 min at 4°C; and 5 min at 70°C.

Subsequently, qPCR was performed using Fast Start Universal SYBR Green Master (ROX) kit (Thermo Fisher Scientific, Inc.) and a 7500 Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction system (20 $\mu$l) consisted of 10 $\mu$l Fast Start Universal SYBR Green Master (ROX), 2 $\mu$l cDNA, 8 $\mu$l ddH$_2$O, 0.5 $\mu$l forward primer and 0.5 $\mu$l reverse primer. The following primer pairs were used for qPCR: PPARγ forward, 5'-TACTGTCGGTTTCAGAAA TGCC-3' and reverse, 5'-GTCAGCGGACTCTGGATTCAG-3'; and β-actin forward, 5'-TGGCACCCAGCACAATGAA-3'

and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The following thermocycling conditions were used for qPCR: Initial denaturation for 3 min at 95°C; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 10 min; and melt curve analysis from 65-95°C at a heating rate of 20°C/sec. PPARγ mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (6).

*Assessment of PPARγ protein expression by western blotting.* Cells were treated with Da-Ea (10, 20 or 50 $\mu$g/ml) for 24, 48 or 72 h. Total protein was extracted from cells using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) containing PMSF. Subsequently, samples were centrifuged at 12,000 x g for 10 min at 4°C. Total protein was quantified using a bicinchoninic acid assay. Protein (20 $\mu$g per lane) was separated via 10% SDS-PAGE and transferred to PVDF membranes. Ponceau S staining was performed to confirm successful transfer of the proteins to the membrane, for 5 min at room temperature. Subsequently, the membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies targeted against: PPARγ (1:5,00; cat. no. sc-7273; Santa Cruz Biotechnology, Inc.) and β-actin (1:5,000; cat. no. 60008-1-Ig; ProteinTech Group, Inc.) After washing with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. SA00001-1; ProteinTech Group, Inc.) for 1 h at 37°C. Subsequently, the membranes were washed three times with TBST for 5 min. Protein bands were visualized using an ECL detection kit (7Sea Biotech; http://7seapharmtech.com/) and imaged using the ChemiScope 5300 Pro (Clinx Science Instruments Co., Ltd.) imaging system. β-actin was used as the loading control. The bands were then quantified using image analysis software (ImageJ2x; version 2.1.4.5; National Institutes of Health).

*Statistical analysis.* Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). Comparisons among groups were analysed using one-way ANOVA followed by Dunnett's post hoc test. Data are expressed as the mean ± standard deviation of three replicates. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*HPLC-DAD analysis.* The main components of Da-Ea were identified by HPLC-DAD. The HPLC chromatogram recorded at a wavelength of 320 nm is presented in Fig. 1. Based on their retention time and UV spectra, daphnetin-7-O-β-D-glucoside (Fig. 1C), daphnetin (Fig. 1D), demethyldaphnoretin-7-O-β-D-glucopyranoside (Fig. 1E) and genkwanol A (Fig. 1F) were detected in Da-Ea. The chemical structures of the four compounds are presented in Fig. 2.

*Cell morphological observation.* Cell morphology was examined using an inverted fluorescence microscope. Microscopic examination indicated that Eca-109 cells exposed to various
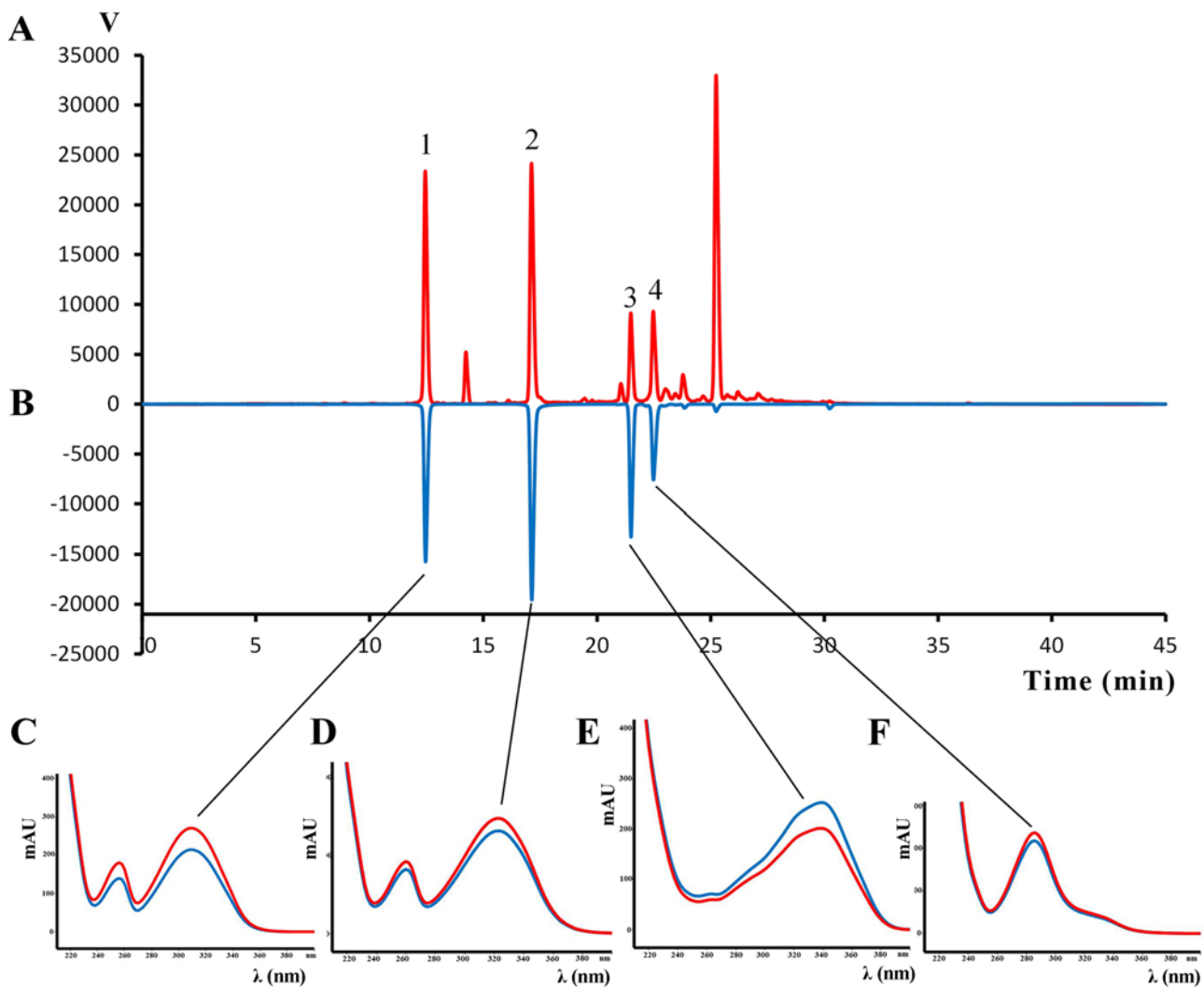
Figure 1. High performance liquid chromatography chromatograms. Compounds were identified by comparing the retention times and UV spectra of the (A) sample solution and (B) standard solution. The average UV spectrum of (C) daphnetin-7-O-β-D-glucoside, (D) daphnetin, (E) demethyldaphnoretin-7-O-β-D-glucopyranoside and (F) genkwanol A. The red line represents the sample solution and the blue line represents the standard solution.

concentrations of Da-Ea for 24-72 h underwent notable morphological alterations (Fig. 3). For example, round or polygonal-shaped cells became elongated, cells appeared to shrink, nuclei were destroyed and the number of floating cells increased in a time- and concentration-dependent manner. In addition, cellular integrity was increasingly damaged with longer incubations with Da-Ea.

*Cell apoptosis detection*. Cell apoptosis was assessed by Annexin-V and PI double staining using flow cytometry (Table I and Fig. 4). Following treatment with 10, 20 and 50 $\mu$g/ml Da-Ea, the percentage of apoptotic cells at 24 h was 1.756±0.040, 2.016±0.015 and 2.700±0.200%, respectively, which was significantly increased compared with that of the control group (1.463±0.055%; P<0.05). At 48 h, the percentage of apoptotic cells was 4.430±0.060, 5.800±0.010 and 6.876±0.025% in the 10, 20 and 50 $\mu$g/ml Da-Ea groups, respectively, which was also significantly increased compared with that of the control group (1.63±0.155%; P<0.05). Following incubation for 72 h, the percentage of apoptotic cells was significantly increased by ~4-fold to 7.173±0.251,

7.350±0.043 and 7.516±0.015% in the 10, 20 and 50 $\mu$g/ml Da-Ea groups, respectively, compared with the control group (1.763±0.045%; P<0.05). The results suggested that Da-Ea induced Eca-109 cell apoptosis in a dose- and time-dependent manner.

*Cell cycle detection*. Cell cycle distribution was detected by PI staining and flow cytometry (Table II and Fig. 5). Following treatment with Da-Ea for 24 h, the percentage of S phase cells significantly increased from 17.043±0.912% in the control group to 33.070±0.592% in the 50 $\mu$g/ml Da-Ea group, whereas the percentage of $G_0/G_1$ phase cells significantly decreased from 74.956±0.912% in the control group to 58.926±0.592% in the 50 $\mu$g/ml Da-Ea group. When exposed to Da-Ea for 48 h, the percentage of S phase cells significantly increased from 18.495±1.716% in the control group to 40.298±7.949% in the 50 $\mu$g/ml group, whereas the percentage of $G_0/G_1$ phase cells significantly decreased from 72.053±0.458% in the control group to 44.476±1.017% in the 50 $\mu$g/ml group. These effects were also observed in the 10 and 20 $\mu$g/ml Da-Ea groups. Da-Ea-induced
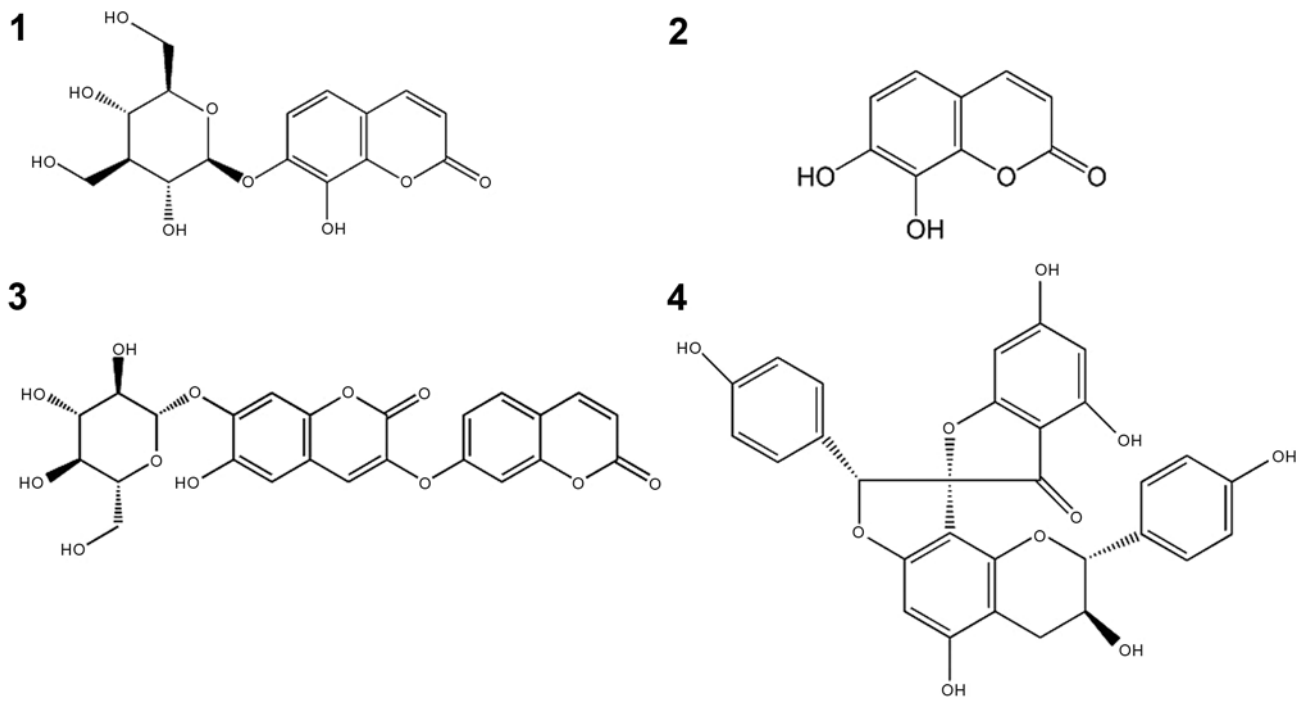
Figure 2. Chemical structures of the main components of an ethyl acetate extract of *Daphne altaica*. The chemical structure of (1) daphnetin-7-O-β-D-glucoside, (2) daphnetin, (3) demethyldaphnoretin-7-O-β-D-glucopyranoside and (4) genkwanol A.
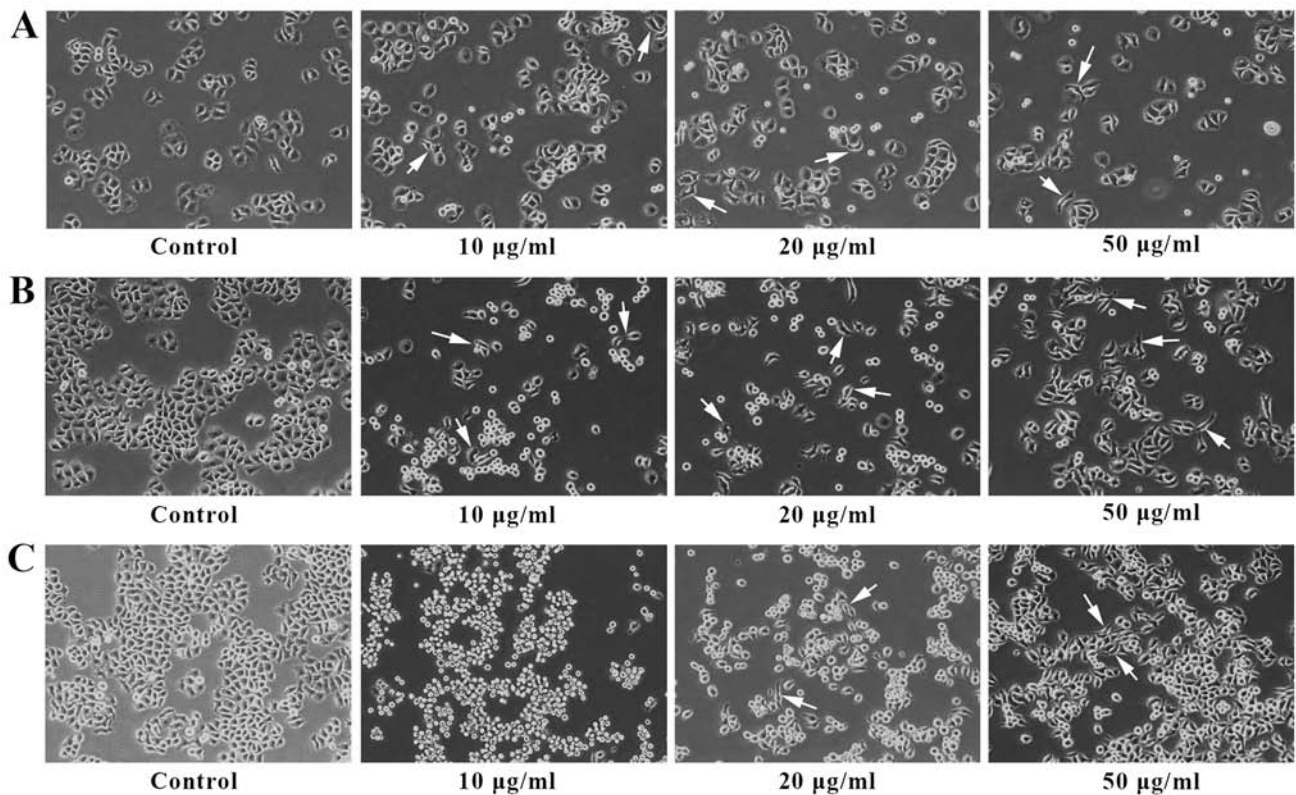


Figure 3. Effect of Da-Ea on Eca-109 cell morphology. Eca-109 cell morphology following treatment with 0, 10, 20, 50 µg/ml Da-Ea for (A) 24, (B) 48 and (C) 72 h (magnification, x100). The white arrows indicate cell shrinkage and elongation. Da-Ea, ethyl acetate extract of *Daphne altaica*.

alterations to the cell cycle distribution occurred in a time- and dose-dependent manner, which suggested that Da-Ea inhibited cell proliferation by inducing S phase cell cycle arrest in Eca-109 cells.

*PPARγ mRNA expression level*. Agarose gel electrophoresis demonstrated that the bands corresponding to 28S RNA and 18S RNA were sharp and clear. Furthermore, the intensity of the 28S RNA band was approximately twice as intense as the

Table I. Rate of Eca-109 cell apoptosis following treatment with ethyl acetate extract of *Daphne altaica* for 24, 48 or 72 h.

| Concentration ($\mu$g/ml) | Rate of apoptosis (%) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| Control | 1.463±0.055 | 1.630±0.155 | 1.763±0.045 |
| 10 | 1.756±0.040[a] | 4.430±0.060[a] | 7.173±0.251[a] |
| 20 | 2.016±0.015[a] | 5.800±0.010[a] | 7.350±0.043[a] |
| 50 | 2.700±0.200[a] | 6.876±0.025[a] | 7.516±0.015[a] |

[a]$P<0.01$ vs. the control group.

Table II. Proportion of cells in the G0/G1 and S phases of the cell cycle.

| Concentration | 24 h | | 48 h | |
|---|---|---|---|---|
| | $G_0/G_1$ phase | S phase | $G_0/G_1$ phase | S phase |
| Control | 74.956±0.912 | 17.043±0.912 | 72.053±0.458 | 18.495±1.716 |
| 10 | 69.096±0.382[b] | 24.570±2.542[b] | 57.857±0.236[b] | 29.390±5.520 |
| 20 | 61.410±1.260[b] | 30.590±1.260[b] | 50.843±0.458[b] | 35.873±5.849[a] |
| 50 | 58.926±0.592[b] | 33.070±0.592[b] | 44.476±1.017[b] | 40.298±7.949[b] |

[a]$P<0.05$ and [b]$P<0.01$ vs. the control group.
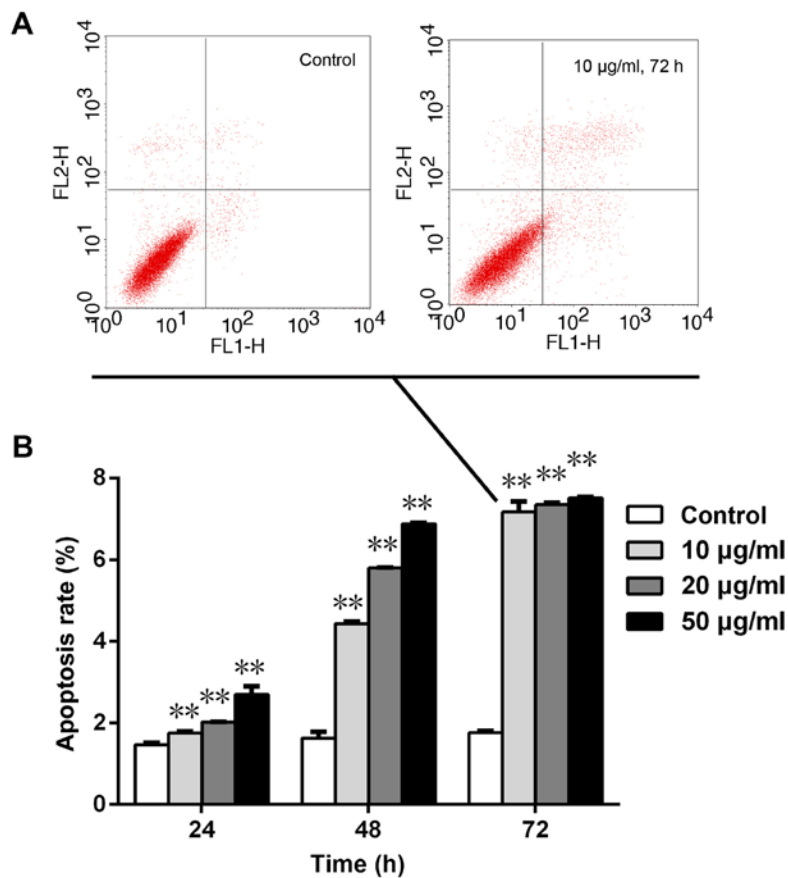


Figure 4. Effect of the ethyl acetate extract of *Daphne altaica* on the rate of Eca-109 cell apoptosis. Apoptotic cells were (A) detected by flow cytometry and (B) quantified. A representative flow cytometry plot was only presented for the 10 $\mu$g/ml Da-Ea group at 72 h. Cells in the upper and lower right quadrants were considered as apoptotic cells. [**]$P<0.01$ vs. the control group.
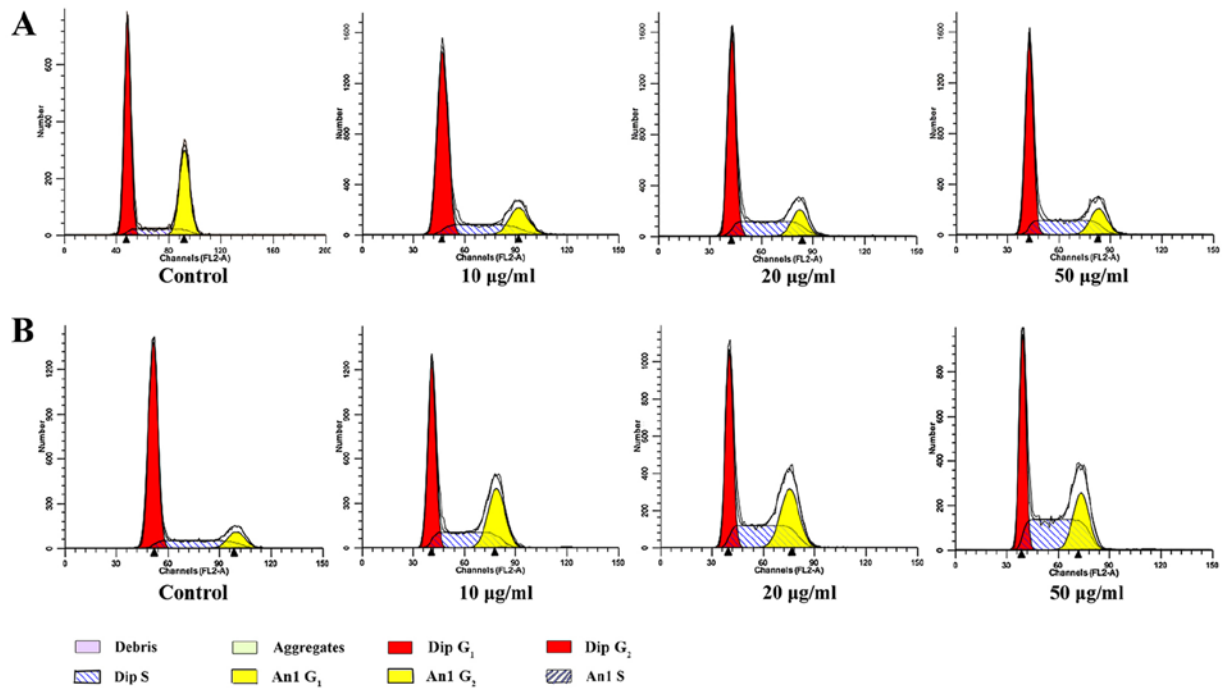
Figure 5. Da-Ea induces S phase cell cycle arrest in Eca-109 cells. The cell cycle distribution of Eca-109 cells treated with Da-Ea for (A) 24 and (B) 48 h was determined by flow cytometry. Da-Ea, ethyl acetate extract of *Daphne altaica*.
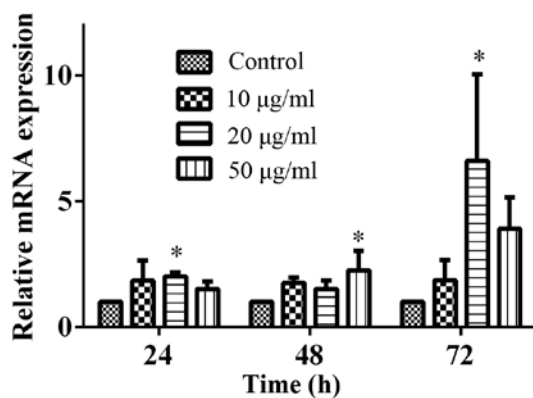


Figure 6. Effect of the ethyl acetate extract of *Daphne altaica* on the mRNA expression level of PPARγ in Eca-109 cells. *P<0.05 vs. the control group. PPARγ, peroxisome proliferator-activated receptor-γ.



Figure 7. Effect of the ethyl acetate extract of *Daphne altaica* on the protein expression level of PPARγ in Eca-109 cells. **P<0.01 vs. the control group. PPARγ, peroxisome proliferator-activated receptor-γ.

18S RNA band, demonstrating that the total RNA was intact and not degradeD. The A260/A280 ratios of the extracted RNA were 1.9-2.0, suggesting that the obtained RNA had a high purity. Additionally, only a single sharp peak was generated during the melting curve analysis, indicating high specificity of the products and absence of non-specific amplification products (data not shown). The mRNA expression levels of PPARγ were analysed by RT-qPCR. At 24, 48 and 72 h, PPARγ mRNA expression levels were increased in the 10, 20 and 50 µg/ml Da-Ea groups compared with the control group; however, the increases in expression were only significant for the 20 µg/ml Da-Ea group at 24 h, the 50 µg/ml group at 48 h and the 20 µg/ml Da-Ea group at 72 h (Fig. 6). The results suggested that Da-Ea increased the mRNA expression levels of PPARγ in Eca-109 cells in a time-dependent manner.
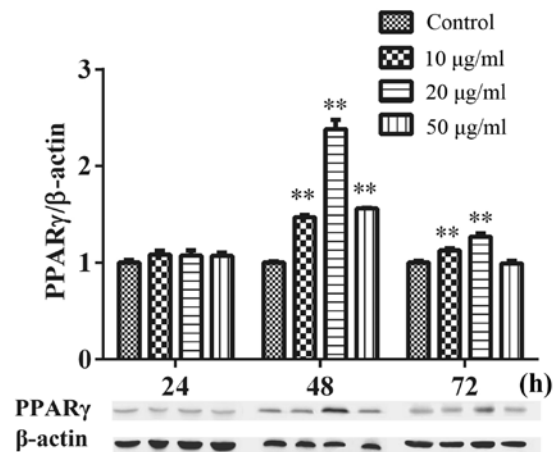
*PPARγ protein expression level.* Western blotting was performed to investigate the effect of DA-Ea on the protein expression levels of PPARγ in Eca-109 cells. A calibration curve was generated by plotting the absorbance of protein standards against their concentration and the linearity of the method was evaluated by analysing six working solutions. The binary linear equation for the calibration curve was determined as y=0.1056x-0.0018, with an R2 value of 0.9916, suggesting the method displayed good linearity (data not shown). Da-Ea (10, 20 and 50 µg/ml) treatment for 48 h significantly increased the protein expression level of PPARγ in Eca-109 cells compared with the control group. Following treatment for 72 h, Da-Ea (10 and 20 µg/ml) significantly increased PPARγ protein expression levels in Eca-109 cells compared with the control group (Fig. 7).

## Discussion

In the present study, the effects of Da-Ea on Eca-109 cell apoptosis and cell cycle distribution were analysed, and the underlying mechanisms were investigated by detecting the expression levels of PPARγ.

A previous study reported that petroleum ether, chloroform, DA-Ea and n-butanol extracts of *D. altaica* display moderate to significant *in vitro* cytotoxicity against several cancer cell lines (Eca-109, AGS, SMMC-7721 and HeLa). Moreover, Da-Ea inhibited Eca-109 cell proliferation to the greatest extent out of the four extracts; therefore, DA-Ea was used in the present study (3).

The present study demonstrated that the main constituents of Da-Ea were daphnetin-7-O-β-D-glucoside, daphnetin, genkwanol A and demethyldaphnoretin-7-O-β-D-glucopyranoside. Demethyldaphnoretin-7-O-β-D-glucopyranoside has been reported to display potent cytotoxicity against HepG2 and Hep3B cells (7), which indicates that this compound could be at least partially responsible for the antiproliferative activity of Da-Ea. To the best of our knowledge, the effects of the four identified compounds on PPARγ expression have not been previously reporteD. However, a previous study demonstrated that *D. gnidium*, which contains daphnetin-7-O-β-D-glucoside and daphnetin, activates PPARγ (8,9). Therefore, the effect of *Daphne*-derived chemical compounds, including the four compounds identified in the present study, on PPARγ expression requires further investigation.

In the present study, Da-Ea-treated cells displayed characteristic morphological features of apoptotic cells, including cell shrinkage, membrane blebbing, pyknotic cells with broken nuclei and floating cell formation. In addition, the rate of apoptosis increased in a time- and dose-dependent manner in Da-Ea-treated Eca-109 cells. Cancer is a disease that is associated with uncontrolled cell proliferation, which is mediated by antiapoptotic mechanisms. When cancer cells undergo apoptosis, no additional damage to surrounding normal cells and tissues is induced; therefore, enhancing apoptosis may serve as an effective therapeutic strategy for cancer (10). The results of the present study suggested that the anticancer effects of Da-Ea on Eca-109 cells were partly due to apoptosis induction. However, the rate of Da-Ea-induced apoptosis was not as high as expected, which may have been caused by low purity of the extract. Therefore, future studies investigating the active principles isolated from *D. altaica* are required.

Cell cycle progression dysregulation is also a common characteristic of cancer. The cell cycle is separated into four sequential phases, $G_1$, S, $G_2$ and M, which are regulated by a series of proteins, including cyclin-dependent kinases and cyclins, at a number of checkpoints. Cells can be arrested at a cell cycle checkpoint for a number of reasons, including DNA damage, which can ultimately result in apoptosis induction (10). Uncontrolled cell cycle progression is one of the most common causes of the transformation of normal cells to cancer cells (7); therefore, components of the cell cycle machinery may serve as molecular therapeutic targets for cancer (11). In the present study, Eca-109 cell S phase arrest was increased following treatment with Da-Ea for 24 and 48 h compared with the control cells, as determined by flow cytometry.

Additionally, the effects of *D. altaica* on the expression level of PPARγ in Eca-109 cells were investigateD. PPARs are ligand-activated transcription factors that regulate the expression of genes involved in lipid metabolism, glucose homeostasis, cell proliferation, differentiation and survival. PPARs are divided into three subfamilies: PPARα, PPARβ/δ and PPARγ, with the PPARγ subfamily being the most intensively investigated (12,13). A number of studies have demonstrated that natural bioactive compounds can exert chemopreventive effects by modulating PPARγ (14-16). According to the literature, triterpenoids, flavonoids, carotenoids and linoleic acid are cancer chemoprotective compounds that effectively activate PPARγ (13). Among these compounds, triterpenoids (17), flavonoids (18) and linoleic acid (19), which display antitumor activities, have been identified in *Daphne* species (20); therefore, investigating whether the bioactive extract of *D. altaica* can activate PPARγ expression is important.

In the present study, Da-Ea treatment for 48 h increased the protein expression level of PPARγ in Eca-109 cells compared with the control cells. Similarly, the mRNA expression levels of PPARγ in Eca-109 cells were increased following treatment with Da-Ea compared with the control cells, which indicated that *D. altaica* extract may inhibit cell proliferation and induce cell apoptosis by upregulating PPARγ gene expression. However, Da-Ea-induced PPARγ protein expression was not time- or concentration-dependent, which may be associated with the complexity of components present in the *D. altaica* extract. *Daphne* species contain various coumarins (21), diterpenes (22), triterpenes (23), flavonoids (24), biflavionoids (25), lignans (22,26), norlignans (27), simple phenylpropanoids (28) and steroids (26). Interactions, including synergism or antagonism, among the *Daphne*-derived components have been suggested (29); therefore, further investigation into the effects of *D. altaica* extract-derived purified compounds on PPARγ is required.

In summary, phase contrast microscopy was used to observe Da-Ea induced morphological alterations in Eca-109 cells. Flow cytometry was performed to investigate cell apoptosis and cell cycle arrest in the Eca-109 cells. RT-qPCR and western blotting were performed to detect the mRNA and protein expression levels of PPARγ, respectively. The results suggested that Da-Ea induced apoptosis and S phase cell cycle arrest, and also upregulated the mRNA and protein expression levels of PPARγ in Eca-109 cells. In conclusion, the results suggested that Da-Ea inhibited Eca-109 cell proliferation by inducing cell cycle arrest and apoptosis via PPARγ-mediated pathways.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

MK analysed the chemical constituents, performed the data analysis and drafted and revised the manuscript. AW performed the cell-based assays and data analysis. ZG provided the standard compounds and interpreted HPLC-DAD data. DB, LT and KN prepared the extract. DB also cooperated with AW on performing the cell-based assays. BC and JW provided the related materials and performed the HPLC-DAD assay. OT identified the plant taxonomically and collaborated with MK and AW on statistical analyses of the data. PC designed the study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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