Antitumor activity of the *Ailanthus altissima* bark phytochemical ailanthone against breast cancer MCF-7 cells

RUXING WANG1,2, YANJIE LU3, HONG LI4, LIXIN SUN4, NING YANG4, MINGZHEN ZHAO3, MANLI ZHANG1 and QINGWEN SHI1

1Department of Medicinal Natural Product Chemistry, School of Pharmaceutical Sciences, Hebei Medical University, Shijiazhuang, Hebei 050017; 2Hebei Province Key Laboratory of Research and Development for Chinese Medicine, Institute of Chinese Materia Medica; 3Department of Pathology, Institute of Cancer Research, Chengde Medical College; 4Department of Central Laboratory, The Affiliated Hospital of Chengde Medical College, Chengde, Hebei 067000, P.R. China

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**Abstract.** Ailanthone is isolated from the bark of *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae). The mechanism that underlies the activity of ailanthone on MCF-7 cells was investigated by MTT assay. Breast cancer MCF-7 cells were treated with 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml ailanthone for 24, 48 and 72 h. The inhibition of proliferation induced by treatment with ailanthone was assessed by MTT assay. Apoptosis and cell cycle distribution in MCF-7 cells with the same doses of ailanthone for 48 h were determined by flow cytometry. Expression of apoptosis-associated genes and proteins were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis, respectively. The results revealed that ailanthone inhibited MCF-7 cell proliferation. Flow cytometry assay demonstrated that ailanthone induced apoptosis and G0/G1 cell cycle arrest in MCF-7 cells. Western blotting and RT-PCR assays demonstrated that upregulation of pro-apoptotic caspase-3 and Bcl-associated X, and downregulation of anti-apoptotic apoptosis regulator B-cell lymphoma-2 in MCF-7 cells may be associated with the induction of apoptosis and inhibition of proliferation. To the best of our knowledge, the present study is the first to investigate the antitumor activity of ailanthone from *A. altissima* on MCF-7 cells and to attempt to elucidate the underlying mechanism. The present study revealed the presence of ailanthone-mediated antitumor effects, indicating that ailanthone may be a novel phytomedicine with potential use in breast cancer therapy.

**Introduction**

Breast cancer is one of the most common cancers and the leading cause of cancer-associated mortality among women, causing significant morbidity worldwide (1). Nearly 5.8/100,000 new breast cancer cases are diagnosed in developed countries and >10.5 new breast cancer cases per 100,000 individuals occur worldwide each year (1). In the early stages of the disease, breast cancer, as in case of a number of other cancers, tends to spread asymptotically throughout the body and at the point of diagnosis there is an increased likelihood of identification of metastatic lesions (2). Conventional therapies, including surgery, radiotherapy and chemotherapy, have served roles in treatment of breast cancer, but there remain numerous problems in tumor therapy. For example, cyclophosphamide, methotrexate and 5-fluorouracil chemotherapy for breast cancer have various side effects (3,4).

Despite progress in anticancer therapeutics, there are few efficient drugs with low toxicity available to treat cancer. Plants have been previously used in cancer therapy (5). Traditional medical phytotherapy has been used for cancer treatment for several millennia in Asia, including in China, Japan and Thailand, and is currently being used for cancer prevention and therapy worldwide (6-8). Throughout the centuries, certain plant extracts have been tested for antitumor potential (9). Plants are a source of a number of effective anticancer agents in current use, including taxanes, irinotecan, topotecan, vincristine and vinblastine (10-12). Plant products such as these can aid in discovery and development of novel anticancer drugs. In addition, plant products demonstrate fewer side effects compared with chemical drugs. There has been an increasing interest in identifying and isolating natural compounds from medicinal plants with an aim to develop novel anticancer drugs (13).

*Ailanthus altissima* is an example of a plant that has been used in tumor therapy (14). *A. altissima*, which grows in Northeast and Central China, can also be present in Europe and the USA and has been used as a traditional Chinese medicine for a number of years (15). The bark of *A. altissima* has been used for the treatment of ascariasis, diarrhea, spermatorrhea, bleeding and gastrointestinal diseases in China (16).
The antitumor effect that has enabled the use of this plant in the treatment of colonic, cervical, and rectal cancer has been previously described (17). In the present study, ailanthone was successfully isolated from *A. altissima*. Ailanthone has a wide spectrum of biological activities, including anti-allergic, anti-malarial, anti-HIV, anti-inflammatory, anti-ulcer and anti-microbial activities (18,19). Ailanthone has been demonstrated to exhibit in vitro growth-inhibitory effects against several cancer cell lines, including HepG2, Hep3B, R-HepG2, HeLa and A549 cells (18,20). Rosati (20) demonstrated that ailanthone could induce mitochondrial membrane depolarization and caspase-3 activation in Jurkat cancer cells. However, the antitumor activity in breast cancer treatment and the underlying molecular mechanism remain to be elucidated. In the present study, MCF-7 cells were used to evaluate the antitumor activity of ailanthone and the underlying molecular mechanism.

**Materials and methods**

**Plant material and extraction.** The bark of *A. altissima* (catalog no., NMC-2012-2) was purchased from Anguo Zhong Sheng Medicinal Material Co., Ltd. (Angoushi, China). The bark of *A. altissima* was powdered, a 10-fold volume of methanol was added to the powdered sample and allowed to soak for 12 h. Hot reflux was performed two times serially, each time using a rotary evaporator with 95% ethanol at 80°C for 2 h, filtered, and the filtrate was merged. The combined filtrates were concentrated in vacuo, and then treated with saturated salt solution at concentration of 20%. It was extracted with petroleum ether and dichloromethane for 2 h, and dried in vacuo at 40°C respectively. The extract with separated with 50 times the amount of 200-300 mesh silica gel column (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). The mobile phase was a solution of dichloromethane and methanol in ratios of 50:1, 30:1, 15:1, 10:1, 5:1, and 500 ml was collected using each ratio. A total of 211 fractions were collected, and similar fractions were combined on the basis of TLC to obtain 35 major fractions. Following further purification by crystallization, ailanthone was purified as previously described (21).

**Cell preparation.** Breast cancer MCF-7 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.). DMEM was supplemented with 100 µg/ml streptomycin, penicillin (Sigma-Aldrich; Merck KGA, Darmstadt, Germany) and 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C and 5% CO₂ in a humidified environment.

**Cell viability assay.** Cell viability was assessed by MTT colorimetric assay (22). The cells were treated with various concentrations (0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml) of ailanthone for 24, 48 and 72 h. At the end of the treatment, 50 µl MTT (5 mg/ml) was added to each well (1x10⁵ cells/ml) and the samples were incubated for an additional 1 h. The culture medium was removed and dissolved by adding 100 µl dimethyl sulfoxide. The absorbance was measured at a wavelength of 490 nm.

**Cell apoptosis.** The level of cell apoptosis was determined by flow cytometry with annexin V (BD Biosciences, Franklin Lakes, NJ, USA) labeling and propidium iodide (PI) exclusion staining. MCF-7 cells were treated with 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml ailanthone for 48 h, collected, washed with PBS and suspended in binding buffer (BD Biosciences). Subsequently, the cells (1x10⁶ cells/ml) were stained with 10 µl annexin V-fluorescein FITC and 5 µl PI, incubated in dark at room temperature for 15 min according to the manufacturer's protocol and subjected to flow cytometry using a flow cytometer (ModFit 6.0, BD Biosciences, USA). Cells undergoing apoptosis were stained with annexin V and treated as described above.

**Cell cycle distribution.** Cells were seeded at a density of 1x10⁶/ml in 6-well microplates and treated with 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml ailanthone for 48 h. A total of 1x10⁵ cells were collected for each treatment, washed with PBS, trypsinized with 0.25% trypsin and harvested by centrifugation for 5 min at 70 x g at room temperature. Cells were re-suspended with 0.5 ml PBS, fixed overnight with cold 70% ethanol at -20°C and stained with PI solution containing 10 µg/ml RNase A and 50 µg/ml PI for 60 min at room temperature. Cells were analyzed by flow cytometry, as previously described (23).

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** MCF-7 cells were treated by ailanthone (0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml), and following extraction of total cellular RNA from the MCF-7 cells using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 µg of total RNA was reverse transcribed to generate cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.). The following thermocycling conditions were used for RT-PCR: Initial denaturation at 94°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec, and the final extension at 72°C for 8 min. The PCR products were resolved in 2% agarose gels and visualized by staining with ethidium bromide (24). To semiquantify the PCR products, the bands representing the amplified products were analyzed by Quantity One analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative level of the target mRNA expression was defined as the ratio of the absorbance of the target band to that of the β-actin band. The following primer sequences were used for PCR: (Takara RNA PCR Kit AMV, Ver.3.0) Caspase-3 (442 bp), forward 5’-ATCTCGGTCTTGTAGCAAGTGCGAT-3’, reverse 5’-TGAATTTCGCCAAAATCAGATACCA-3’; Bcl-associated X (Bax; 258 bp), forward 5’-CAACACGATGACGTTGA-3’, reverse 5’-GTCACGTTACGATCCATTCA-3’; B-cell lymphoma 2 (Bcl-2; 383 bp), forward 5’-TACAGTGTTGGATGCGGAGATGT-3’, reverse 5’-CCAAGCTTCAAGAGAGCC-3’; GAPDH (135 bp), forward 5’-CAATGACCCCCCTCTTTGACC-3’, reverse 5’-TGGAAGATGGTGATGGATT-3’.

**Western blot analysis.** MCF-7 cells were treated by ailanthone (0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml), and lysed in lysis buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitor]. Determination of total protein content was performed using a BCA assay (Beijing Solarbio Science and Technology, Co., Ltd., Beijing China). The proteins were separated by 12%
SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk in Tris-buffered saline-0.05% Tween-20 (TBST) for 3 h at room temperature. Following washing in TBST, the membranes were incubated for 2 h at room temperature with 1:1,000 dilutions of rabbit monoclonal antibodies for BCL-2, (cat no. 2872; Cell Signaling Technology, Inc., Danvers, MA, USA), Bax (cat no. 2772; Cell Signaling Technology, Inc.) and Caspase-3, (cat no. 9662; Cell Signaling Technology, Inc.), and a mouse monoclonal antibody for β-actin (ab8226; Abcam, Cambridge, UK). Following washing in TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (rabbit monoclonal antibody; mouse monoclonal antibody, 1:3,000; Abcam) for 1 h at room temperature. The signal was detected using an ECL Western Blotting Substrate kit (Applygen Technologies, Inc., Beijing, China), and exposed to X-ray film at room temperature. Protein expression levels were quantitated using Quantity-one software (v 4.62; Bio-Rad Laboratories, Inc.).

Statistical analysis. SPSS software (version 16; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. Data are presented as the mean ± standard deviation. One-way analysis of variance followed by Dunnett’s test was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effect of ailanthone on proliferation of tumor cells. The effect of ailanthone on viability of MCF-7 cells was measured by MTT assay. Compared with the untreated control, the viability of MCF-7 cells treated with 0.5, 1.0, 2.0, 4.0 or 8.0 µg/ml of ailanthone was 92.62, 88.46, 86.36, 73.74 and 64.05%, respectively, following 24 h of treatment; 77.27, 75.65, 69.89, 61.40 and 52.40%, respectively, following 48 h of treatment; and 64.36, 62.48, 57.64, 50.24 and 43.24%, respectively, following 72 h of treatment (Fig. 1). The data indicated that ailanthone inhibits MCF-7 cell proliferation.

Effects of ailanthone on apoptosis of MCF-7 cells. Following 48 h of treatment with ailanthone, 22.28, 27.99, 35.88, 49.77 and 75.51% of cells were apoptotic at doses of 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml, respectively (Fig. 2). In total, 2.13% of control cells were apoptotic at 48 h, which is significantly different to all treatment groups (P<0.01).

Effects of ailanthone on cell cycle of MCF-7 cells. Following treatment with 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml ailanthone for 48 h, all treatment groups exhibited statistically significant differences compared with the control. Following treatment with ailanthone, the proportion of cells in the G0/G1 phase increased and percentage of cells in S and G2/M phases decreased significantly compared with the control group (Fig. 3).

Alteration of expression levels of Bcl-2 and Bax mRNA. Following 48 h of treatment with different doses of ailanthone, RT-qPCR demonstrated that in the MCF-7 cells, expression of the Bax and caspase-3 genes increased, whereas expression levels of the Bcl-2 gene decreased. Doses of 1.0, 2.0, 4.0 and 8.0 µg/ml of ailanthone results in significantly altered expression, compared with the control group (Fig. 4).

Discussion

Breast cancer is the primary cause of mortality among women in the world, according to statistics from 2009 (2). Currently, certain cytotoxic drugs are used for the treatment of breast cancer, including daunorubicin, doxorubicin, cisplatin and bleomycin (25). However, these agents are costly and have been demonstrated to induce several side effects, including emesis, anemia, myelosuppression and cellular resistance (3). Therefore, it is necessary to identify alternative drugs or therapies to minimize side effects (26). As a result, natural medicines that cause fewer side effects have been attracting substantial attention (27). Previous research has demonstrated that natural antitumor drugs may serve important roles in the future. A phytochemical study demonstrated the presence of quassinoids in A. altissima and these compounds have been previously demonstrated to exhibit potent antitumor properties (28). To the best of our knowledge, the present study is the first to report that ailanthone isolated from A. altissima has inhibits proliferation of MCF-7 cells.

The majority of drugs achieve antitumor effects by inducing apoptosis in tumor cells (29). Cell cycle analysis is used to determine the distribution of cells in different phases of the cell cycle and enables investigation of tumor proliferation as opposed to apoptosis. There are three cell cycle regulation points in G1, S and G2 phases, which can modulate cell cycle progression. Induction of tumor cell cycle arrest in G1/G0 phase of tumor cells may be an important mechanism of antitumor activity of ailanthone.
Figure 2. Apoptosis of MCF-7 cells following treatment with different doses of ailanthone (0.5, 1, 2, 4 and 8 µg/ml) for 48 h, measured by flow cytometry. (A) Control (0 µg/ml) and (B) 0.5 µg/ml, (C) 1.0 µg/ml, (D) 2.0 µg/ml, (E) 4.0 µg/ml and (F) 8.0 µg/ml ailanthone. (G) Histogram of apoptosis of MCF-7 cells. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. the respective control group. 1, normal cells; 2, early apoptosis; 3, late apoptosis; 4, dead cells.

Figure 3. Cell cycle of MCF-7 cells following treatment with different doses of ailanthone. MCF-7 cells were treated with different doses of ailanthone for 48 h and the cell cycle distribution was measured by flow cytometry. (A) Control (0 µg/ml) and (B) 0.5 µg/ml, (C) 1.0 µg/ml, (D) 2.0 µg/ml, (E) 4.0 µg/ml and (F) 8.0 µg/ml ailanthone. (G) Cell cycle distribution of MCF-7 cells. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. the respective control group.
Certain molecules, including tumor protein p53, serve a role in cell cycle inhibition and induction of apoptosis; cells may be arrested in the G\textsubscript{1} phase and apoptosis may be induced by p53 (31). In the present study, the results of flow cytometry revealed that, following treatment with ailanthone, apoptosis in MCF-7 cells increased. Analysis of the cell cycle by flow cytometry demonstrated an increase in the number of MCF-7 cells in G\textsubscript{0}/G\textsubscript{1} phase following treatment with ailanthone and a decrease in the number of cells in S phase, indicating that the cells were arrested in the G\textsubscript{0}/G\textsubscript{1} phase so that the cells could not enter S phase or perform DNA synthesis, thus inhibiting proliferation. This checkpoint may be involved in the effects of ailanthone on the cell cycle of MCF-7 cells (32), but this potential mechanism remains to be further investigated. Caspases are mediators of apoptosis, of which caspase-3 are frequently activated death protease that catalyzes specific cleavage of numerous cellular proteins (33). Tumor inhibition and apoptosis, as well as the expression of caspase-3, Bcl-2 and Bax were determined by flow cytometry; the results demonstrated that ailanthone can induce apoptosis in tumor cells. Apoptosis is an active cell suicide process that is regulated by p53 (34). The effect of Bcl-2 depends on the ratio of its expression with Bax; this ratio determines whether cells undergo apoptosis or survival upon signal stimulation (35). Excessive Bax expression in cells promotes apoptosis, whereas excessive Bcl-2 expression promotes survival (36).

Western blot analysis was used to detect levels of Bax and Bcl-2 protein expression; it was demonstrated that following treatment of MCF-7 cells with 0.5, 1.0, 2.0, 4.0 or 8.0 µg/ml ailanthone for 48 h, Bax expression increased, whereas that of Bcl-2 decreased markedly. The results indicated that the mechanism underlying ailanthone-induced MCF-7 cell apoptosis may be associated with the adjusting of the Bax and Bcl-2 family proteins. Experimental results demonstrated that ailanthone exhibited an inhibitory effect on cellular proliferation and induced apoptosis. The promotion of Bax and the inhibition of Bcl-2 proteins may further enhance the antitumor effect.
pathway, demonstrating that the ailanthone treatment of Huh7 cells resulted in a decrease in the expression of PI3K and AKT phosphorylation at threonine-408 and serine-473.

Treatment of MCF-7 cells with ailanthone resulted in cell apoptosis. In the present study, the antitumor effect of ailanthone indicated that this compound may be beneficial for the treatment of breast cancer. Further investigation is required to identify the mechanism underlying the antitumor activity.

In conclusion, the present study demonstrated that ailanthone, isolated from A. altissima, exhibited an inhibitory effect on MCF-7 cells and promoted cell apoptosis by upregulating Bax protein and mRNA. Ailanthone inhibited the protein and mRNA expression of Bcl-2, indicating that it has potential antitumor activity. Ailanthone may be a novel phytomedicine for tumor therapy.

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Competing interests

The authors declare that they have no competing interests.

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