JBUON 2015; 20(6): 1487-1496
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Antiproliferative and apoptotic effects of the ethanolic herbal extract of *Achillea falcata* in human cervical cancer cells are mediated via cell cycle arrest and mitochondrial membrane potential loss

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Summary

Purpose: *Cervical carcinoma is the second most common malignancy in females and most of the cases are found in developing countries. The objectives of the present study were (a): to demonstrate the antiproliferative and apoptotic effects of Achillea falcata (A.falcata) extract in human cervical cancer cells (HeLa), and (b): to study the effect of the extract on cellular morphology, cell cycle phase distribution and mitochondrial membrane potential.*

Methods: *MTT assay was used to evaluate the anticancer effect of the extract on HeLa cells. Phase contrast, fluorescence microscopy and transmission electron microscopy (TEM) were used to investigate the morphological changes in these cancer cells after extract treatment. Flow cytometry was used to evaluate the effects of the extract on cell cycle and mitochondrial membrane potential.*

Results: *The results revealed that A. falcata extract led to a significant antiproliferative effect in HeLa cancer cells. The extract induced cellular shrinkage, chromatin condensation and appearance of apoptotic bodies which are the hallmarks of cellular apoptosis. TEM results showed that extract-treated cells had nuclear membrane which was hemispherical and the nuclear chromatin was concentrated and bundled on the inner border of karyotheca. The endoplasmic reticulum also became enlarged in the inner segment. The extract also induced G2/M phase cell cycle arrest along with loss of mitochondrial membrane potential.*

Conclusion: *Achillea falcata extract induced potent antiproliferative and apoptotic effects in HeLa cells. This was accompanied by cellular shrinkage, chromatin condensation, G2/M phase cell cycle arrest and a loss of mitochondrial membrane potential in these cancer cells.*

Key words: *Achillea falcata, anticancer activity, apoptosis, cell cycle, cervical cancer*

Introduction

Cervical carcinoma, the second most common cancer in females globally, is the seventh foremost cause of cancer-related mortality in women and more than 80% of cases are found in developing countries [1]. Although cervical cytology screening has helped decrease mortality rates, managing preinvasive and invasive cervical lesions is still a challenge. Cervical cancer is mostly linked with the high-risk human papillomavirus (HPV) infection [2]. Initially cervical cancer patients do not exhibit any visible symptoms, however, later symptoms may comprise of pelvic pain or pain during sexual intercourse, abnormal vaginal bleeding etc. It is well-established that during the development of cervical cancer, a series of abnormal events is induced, including disturbance

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Received: 25/04/2015; Accepted: 17/06/2015

of cell cycle, disturbance of antitumor immune response, alteration of gene expression, and deregulation of microRNA expression. Even though surgery and chemo-radiotherapy can cure 80-90% of women with early-stage cervical carcinoma, the recurrent and metastatic malignancy remains a main cause of cancer deaths all across the globe. Many advances have been made to design novel drugs and develop gene therapies to treat cervical cancer [3,4]. Imiquimod and gemcitabine (GEM), which are the immunomodulatory agents, have been recently used for cervical cancer treatment. The specific mechanism of action through which imiquimod and its analogs activate the immune system is still not clear [5,6].

There is a number of treatments used for cervical carcinoma; nonetheless, each of them has apparent drawbacks. Although radiotherapy is an effective treatment choice, one third of patients will develop advanced or recurrent cancers, the pelvis being the most common site of failure [7]. Currently, cervical cancer treatment consists of a combination of surgery, chemotherapy and radiotherapy. In the United States, 5-year survival is around 65% and this rate largely depends on how early the tumor is detected. Bigger size early-stage tumors may be treated with a combination of radiotherapy and cisplatin-based chemotherapy, hysterectomy (plus adjuvant radiation therapy), or cisplatin chemotherapy followed by hysterectomy [8,9]. Recently, for patients with late stage cervical carcinoma, hycamtin and cisplatin have been prescribed. But this treatment strategy is not without side-effects including neutropenia, anemia, and thrombocytopenia [8,9].

Clinical studies as well as experimental approaches have discovered the anticancer properties of a large number of medicinal herbs that are acting through different mechanisms, including altered carcinogen metabolism, induction of DNA repair systems, immune activation and apoptosis [10]. The objective of the present research work was to evaluate the antiproliferative and apoptotic effects of *A. falcata* herbal extract in HeLa cervical cancer cells and to study the effect of the extract on cell cycle arrest and mitochondrial membrane potential.

Methods

Plant material and extraction procedure

A. falcata aerial parts were collected in July 2014 in Guangzhou Province, China, and identified by Prof. Liu Xu; a voucher specimen (Voucher specimen number: 14-09-766-02) was deposited in the Herbarium of Southeast University, Nanjing, China. The aerial parts of the plant were thoroughly washed with water, shade-dried and then grinded into small pieces. Ethanol (90%) was used for cold extraction which was carried out for 72 hrs in an extraction apparatus. The extract was then concentrated under reduced pressure in a rotary evaporator at 45°C and was then kept in a refrigerator at 4 °C prior to use.

Cell line and culture condition

HeLa (human cervical cancer cells) were procured from the Shanghai Institute of Cell Biology (Shanghai, China). MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Lonza Biologics, Singapore) and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells were treated with different doses (0, 5, 15, 30, 60, 100 and 120 µg/ml) of the ethanol extract of *A. falcata* at three different time intervals (12, 48 and 72 hrs).

Cell viability testing by MTT assay

The HeLa cells were seeded in different 96-well plates containing $2x10^6$ cells/100 µL/well. The cultured cells were treated with the addition of different doses of the extract (0, 5, 15, 30, 60, 100 and 120 µg/ml) of *A. falcata* at two different time intervals (12 and 48 hrs). In addition, the DMSO alone was added to another set of cells as the solvent control (DMSO). The cells were then incubated for another 48 hrs prior to the addition of 20 µL of 2 mg/mL solution of MTT into each well. The incubation was continued for another 3 hrs before the media were removed. A mixture of DMSO (150 µL) was added to each well and mixed to ensure dissolving of the formazan crystals before the absorbance at 570 nm was measured. Each experiment was performed in triplicate and the 50% inhibitory concentration (IC_{50}) of the extract was calculated.

The cell viability ratio was calculated by the following formula:

Inhibitory ratio (%) = (OD/optical density control – OD treated) / OD control × 100.

Cytotoxicity was expressed as the concentration of extract inhibiting cell growth by 50% (IC_{50} value).

Morphological study of HeLa cells with phase contrast microscopy after treatment with the extract

For morphological changes, HeLa cells were cultured in 12-well plates at a density of $1x10^6$ cells/well and treated with or without different doses (0, 15, 60 and 120 µg/ml) of the *A. falcata* extract for 24 hrs. Morphological changes in extract-treated cells were examined and photographed using phase-contrast light

microscopy [11]. All results were obtained from three independent experiments.

Morphological and apoptotic study of HeLa cells by fluorescence microscopy after treatment with the extract using Hoechst staining

Morphological observation of nuclear change was assayed with Hoechst 33258 using fluorescence microscopy. HeLa cells (1x10^6 cells/ml) were seeded in 6-well plates and were exposed to different concentrations (0, 15, 60 and 120 µg/ml) of *A. falcata* extract for 48 hrs at 37°C. The cells were collected, washed, fixed in 3% paraformaldehyde for 20 min and then stained with Hoechst solution (Hoechst, 20 µg/mL). The mixture was then washed once with PBS and the pellet resuspended in 100 µL of PBS/glycerol (1:1). The solution (20 µL) was poured onto the slide and observed for nuclear morphology alterations under fluorescence microscope (Olympus X 70, magnification x 200).

Morphological and apoptotic study of HeLa cells by fluorescence microscopy after treatment with the extract using acridine orange/ethidium bromide staining

Morphological evidence of apoptosis was also obtained by means of acridine orange and ethidium bromide staining as previously described [12]. In brief, after extract treatment at various doses (0,15,60 and 120 µg/ml), cells in 8-well chamber slides were stained with acridine orange and ethidium bromide (5 µg/ml each). Cells were examined by fluorescence microscopy (Olympus BX51 microscope, NY, USA), and photographed using a SPOT camera with SPOT RT software (Olympus). Acridine orange permeates throughout the cells and renders the nuclei green. Ethidium bromide is taken up by the cells only when cytoplasmic membrane loses its integrity, and stains the nuclei red. Normal cells appear as green while apoptotic cells appear as red fluorescence.

Transmission electron microscopy study of HeLa cells after treatment with the extract

HeLa were seeded and grown at 2x10^5 cells/ml. After treatment with different concentrations (0,15,60 and 120 µg/ml) of the extract for 48 hrs cells were harvested and washed twice with PBS and then fixed in 3% gluteraldehyde/25 mM sodium phosphate, pH 7.2, at 37°C. Analysis was then performed with a Transmission Electron Microscope (JEM-2000EX; JEOL Co; Japan).

Cell cycle analysis by flow cytometry

HeLa cells (2×10^5) were seeded in 60-mm dishes and treated with various doses (0, 15, 60 and 120 µg/ml) of A. falcata extract for 48 hrs. Floating and adherent cells were trypsinized and washed three times with PBS. Cells were incubated in 70% ethanol at -20°C for 12 hrs, treated with 10µg/mL RNase A, then stained with 10 µg/mL of propidium iodide. Finally the stained cells were analyzed and studied using FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software in the Fluorescence-activated Cell Sorting (FACS) machine at a wavelength of 488 nm.

Evaluation of mitochondrial membrane potential ($\Delta\Psi m$) in HeLa cells

HeLa cells were seeded at 2x10^5 cells/well into 6-well plates. After 24-hr incubation, cells were treated with varying doses (0, 15, 60 and 120 µg/ml) of *A. falcata* extract for 48 hrs. Untreated and treated cells were harvested and washed twice with PBS. The cell pellets were then resuspended in 10 mL of fresh incubation medium containing 4.0 µM rhodamine-123 and incubated at 37°C in a thermostatic bath for 30 min with mild shaking and then analyzed using FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software in the Fluorescence-activated Cell Sorting (FACS) machine.

Statistics

The experiments were performed in triplicate. Data were expressed as means ± standard deviation (SD). Statistical correlation of data was checked for significance by ANOVA and Student's t-test. A p value <0.05 was considered to indicate a statistically significant difference.

Results

A. falcata extract induced potent antiproliferative effects in HeLa cells

The anticancer activity of the extract against HeLa cells was evaluated by MTT assay. Different concentrations of the extract (0, 5, 10, 30, 60, 100 and 120 µg/ml) were tested against the cancer cells. The cervical cancer cells showed substantial and dose-dependent susceptibility to the treatment of different concentrations of the extract. The extract also displayed time-dependent inhibition of the cancer cell growth as shown in Figure 1 at 24 and 48 hour time intervals. The number of viable cancer cells exposed to the extract treatment reduced significantly as the dose increased. The IC_{50} value of the extract after 24 and 48-hrs intervals was 87.2 and 53.1 µg/ml respectively.

Morphological study of HeLa cells using phase contrast microscopy

Morphological study using phase contrast microscopy revealed that the extract induced growth

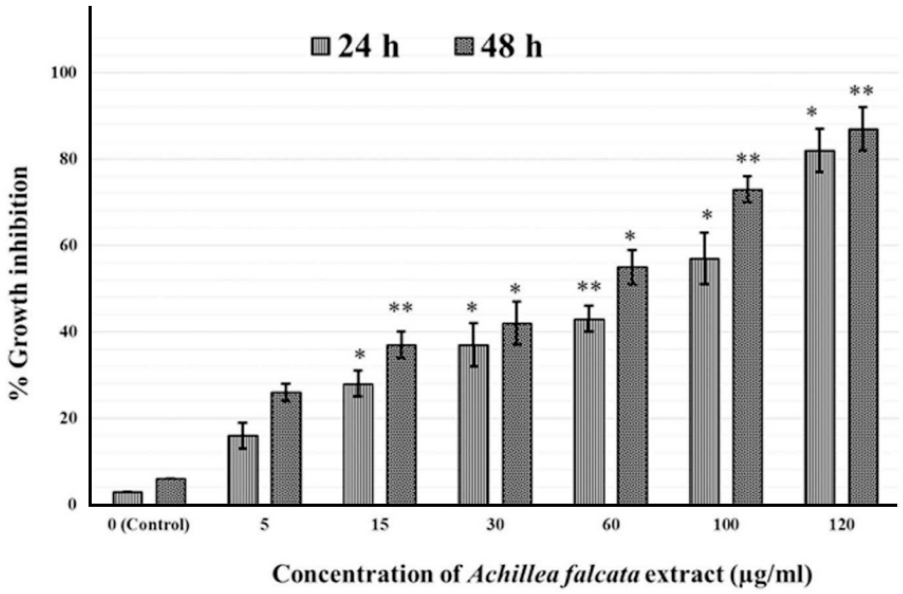

Figure 1. Cytotoxic effect of Achillea falcata extract in human cervical cancer cells (HeLa). Data are shown as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs 0 µg/ml (control).

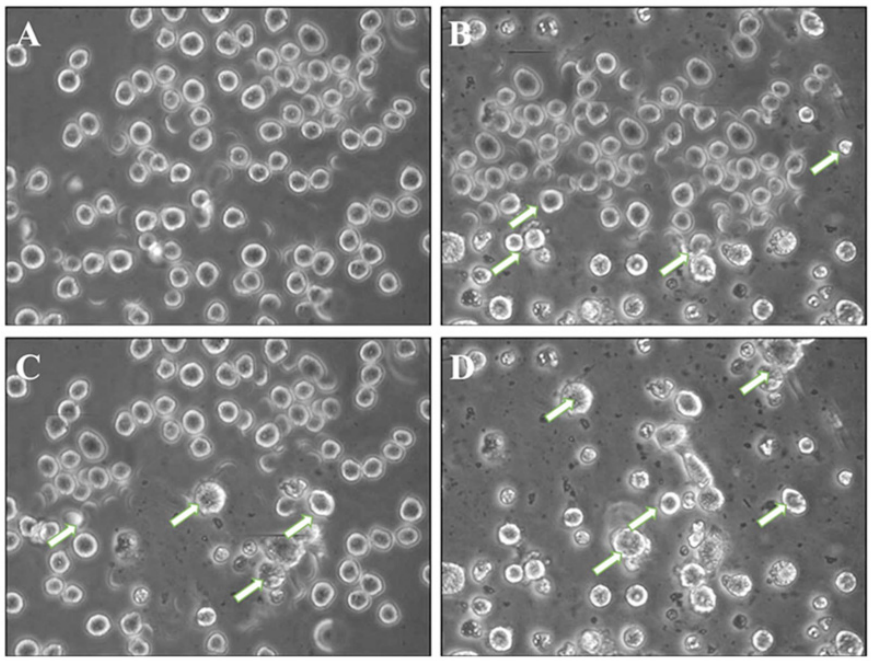

Figure 2. Morphological evaluation of the cervical cancer (HeLa) cells by phase contrast microscopy after treatment with different concentrations of the *A.falcata* extract. Photomicrographs of extract-treated or untreated cells were taken by an inverted microscope at 200x magnification. Representative images are taken from three independent experiments. Cell shrinkage was observed in citronellol-treated cells (arrows). **A** represents untreated cells, **B**,**C** and **D** represent effect of 15, 60 and 120 µg/ml of *A. falcata* extract on cellular morphology of HeLa cells.

inhibition and cellular shrinkage in HeLa cells. As shown in Figure 2 A-D, the morphological changes designate that the cells in the extract-treated and the control group differed considerably. Some of the cells detached from the surface and debris were also detected in the plate of the extract-treat-

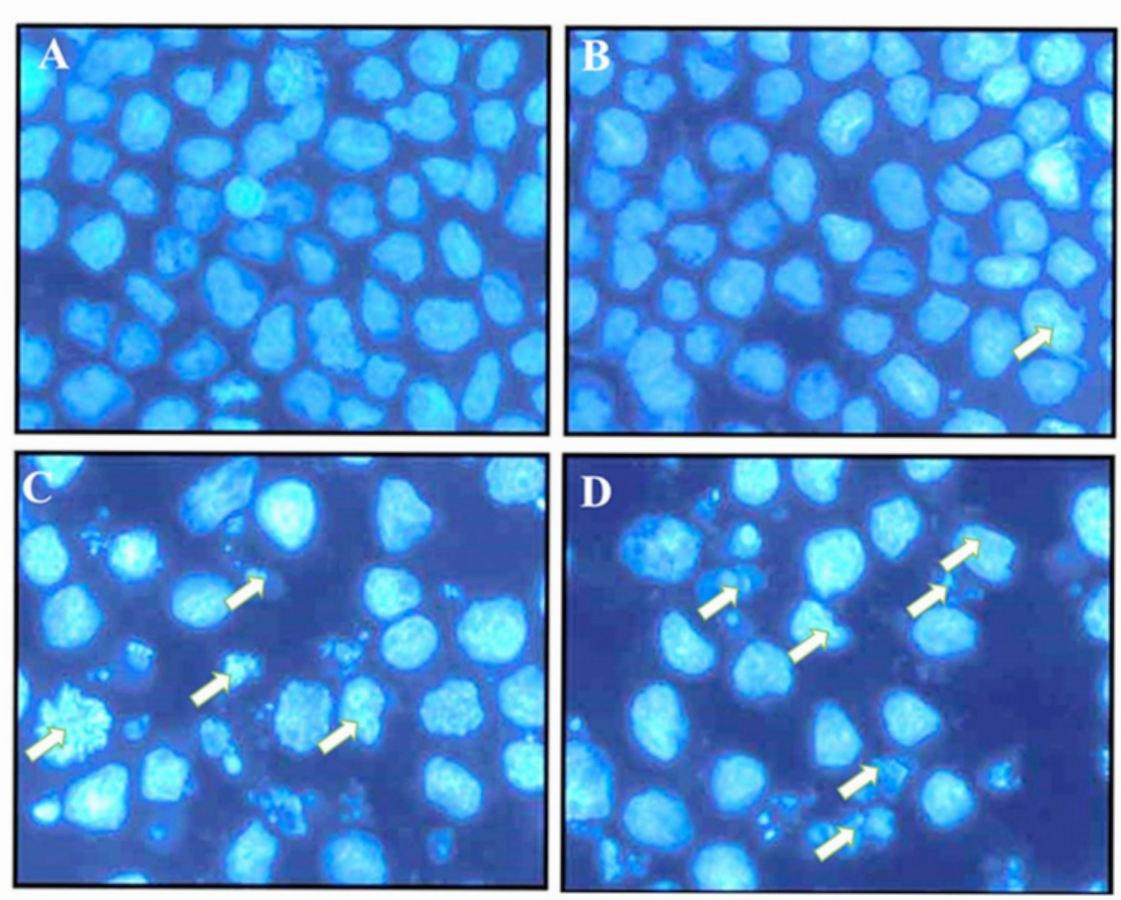

Figure 3. Fluorescence microscopic study of the morphological evaluation of human cervical cancer cells (HeLa) after treatment with various doses of the *A. falcata* extract. HeLa cells were treated with (**B**, **C**, **D**) and without extract (**A**) for 48 hrs. The arrows show the dead cells showing bright fluorescence indicating chromatin condensation has occurred.

ed group, but the control cells were well spread with flattened morphology. The number of cells with shrinkage increased with increase in extract concentration.

Morphological study by fluorescence microscopy using Hoechst and acridine orange with ethidium bromide

In case of fluorescence microscopy, HeLa cells were stained and evaluated for nuclear shape using a fluorescence microscope with Hoechst staining (Figure 3 A-D). The results showed that the extract-treated cells exhibited significant chromatin condensation or dense staining fragmentation (apoptotic bodies), which is an indication of an early apoptotic event. The presence of such apoptotic bodies was related with the extract dose. The results revealed nuclear condensation, membrane blebbing, nuclear fragmentation and apoptotic bodies (the halmarks of apoptosis) in cells that had been incubated with *A. falcata* extract at various doses. The control cells did not exhibit any of the above morphological changes, the nuclei were less stained in bright blue and the color was homogeneous (Figure 3A). Chromatin condensation and other apoptotic features were observed only in the treated cells (Figure 3B-D).

Further, the effects of the extract on HeLa cells' morphology were verified by acridine orange/ethidium bromide staining using fluorescence microscopy. The results of this experiment (Figure 4 A-D) were similar to the other two experiments, indicating that the extract induces cell death through mediation of apoptosis. In this case, normal cells appeared as green while apoptotic cells appeared as red fluorescence.

Morphological study of HeLa cells after extract treatment using transmission electron microscopy

The cells were treated with 0 (A), 15 (B), 60 (C) and 120 (D) µg/ml of A. falcata extract for 48 hrs and were then analyzed by Transmission Electron Microscope (JEM-2000EX; JEOL Co; Japan). HeLa cells were round and regular, with abundant organelles and normal double-membrane nuclei (Figure 5A). After treatment with different doses

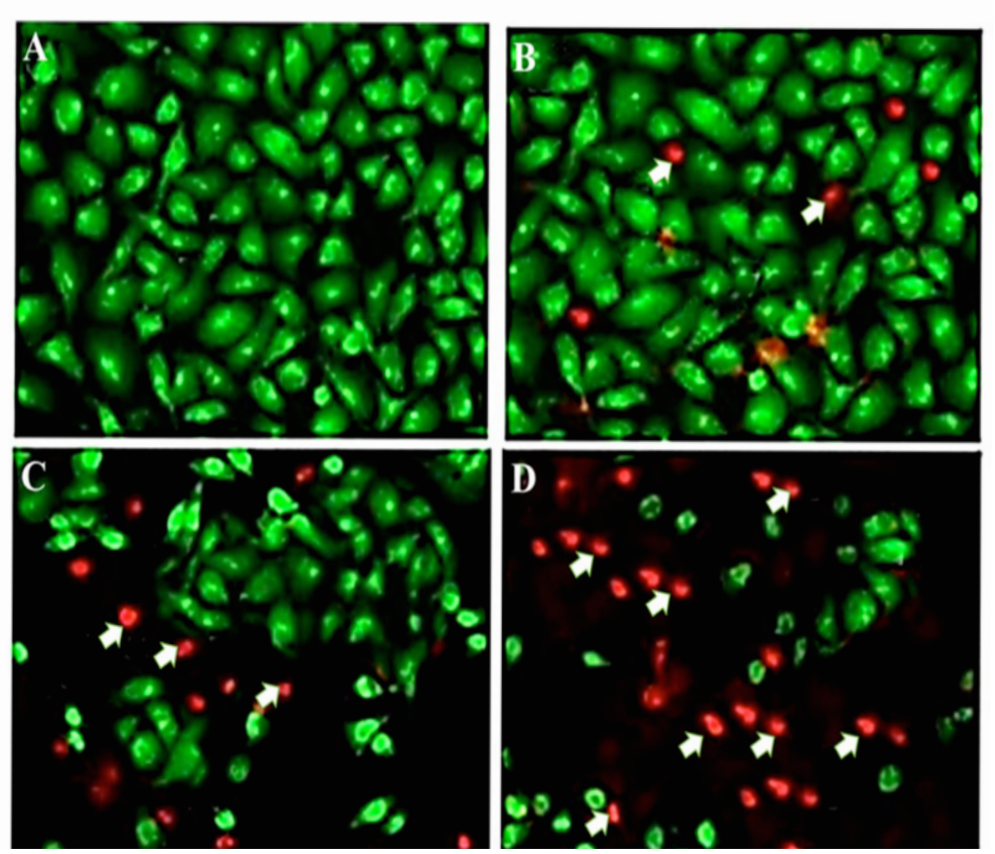

Figure 4. Fluorescence microscopic study of human cervical cancer cells (HeLa) stained with a combination of acridine orange:ethidium bromide (1:1 ratio). **A** shows untreated control cells, **B** shows HeLa cells treated with 25 µg/ml of extract, **C** shows cells treated with 60 µg/ml and **D** shows cells treated with 120 µg/ml of the *A. falcata* extract. Normal cells appear as green while apoptotic cells appear as red fluorescence.

of the *A. falcata* extract for 48 hrs, early-stage apoptosis could be seen (Figure 5 B-D). This effect increased with increasing dose of the extract. Nuclear membrane was hemispherical, with a sharp angle outward, and the nuclear chromatin was concentrated and bundled on the inner border of karyotheca. The endoplasmic reticulum became enlarged in the inner segment.

A. falcata extract induced G2/M phase cell cycle arrest in HeLa cells

The effect of different concentrations of the *A. falcata* extract on the cell cycle phase distribution in HeLa cells is shown in Figure 6. The cells were treated with 0,15,60 and 120 µg/ml of the A. falcata extract for 48 hrs and were then analyzed by flow cytometry to evaluate cell cycle phase distribution after drug treatment. The percentage of G2/M phase cells increased gradually from 6.23% in control cells (untreated cells) to 12.2, 42.1 and 53.7% after treatment with 15,60 and 120 µg/ml of the extract, respectively. This increase in G2/M population was accompanied by a decrease in S-phase population of the cells.

Effect of A. falcata extract on mitochondrial membrane potential loss (ΔΨm) in HeLa cells

The fluorescent dye rhodamine-123 (Rh-123) is a specific probe for the detection of alterations in mitochondrial membrane potential in viable cells. The results of this study revealed that different doses of the *A. falcata* extract induced a potent and dose-dependent loss in ΔΨm after 48-h treatment. Compared to the control, the extract-treated cells displayed a decreased mitochondrial membrane potential. The effect of the extract on ΔΨm is shown in Figure 7.

Discussion

The genus Achillea consists of over 150 species globally, which are mostly distributed in the northern hemisphere, most indigenous to Europe and the Middle East [13]. Many of the *Achillea* species are widely used in traditional medicine of several cultures due to many therapeutic proper-

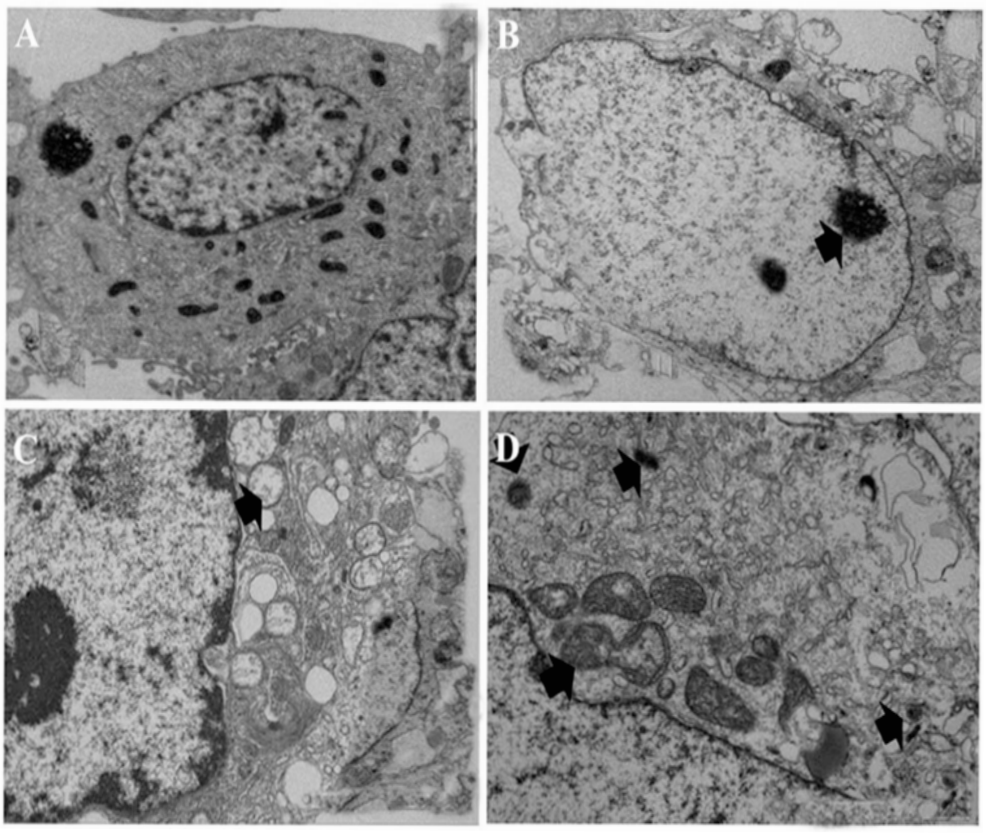

Figure 5. Effect of *A. falcata* extract on the cell apoptosis induced in HeLa cervical cancer cells observed by transmission electron microscopy. The cells were treated with 0 (**A**), 15 (**B**), 60 (**C**) and 120 (**D**) μg/ml of the *A. falcata* extract for 48 hrs and then analyzed by Transmission Electron Microscope (JEM-2000EX; JEOL Co; Japan) (magnification x8000).

ties, such as antioxidant, antispasmodic, anti-inflammatory, antihemorrhoidal, stomachic, emmenagogue and antiseptic [14,15]. Contemporary studies have revealed that many *Achillea* species possess antioxidant and anticancer properties as well [16,17]. In particular, *A. falcata* has been described to have useful effects on internal hemorrhages, stomach ailments, gastritis, and bladder stones [18]. Many other reports have shown anti-proliferative activity of isolated constituents from *A. falcata* [19-21]. Infusion of *A. falcata* has been proved to possess antioxidant activities [22]. Phytochemically, *A. falcata* has been reported to contain a diversity of chemical constituents, most of them being volatile organic compounds present in its essential oil. The various chemical constituents that have been reported in *A. falcata* are monoterpenoids like cineole, camphor and borneol [23]. Flavonoids have also been reported from the plant. Sesquiterpene lactones including 3-β-methoxy-iso-seco-tanapartholide (β-tan) which exhibit potent antitumor properties have also been reported from *A. falcata*. β-tan which was purified from *A. falcata*, differentially inhibited the growth of the epidermal human HaCaT cells at non-cytotoxic concentrations to primary epidermal keratinocytes [24].

However, there are no reports on the antitumor effect of the ethanol extract of *A. falcata* on cervical cancer cells nor is there any report on the effect of the *A. falcata* extract on apoptosis induction, cell cycle phase distribution or mitochondrial membrane potential. Therefore, our aim was to evaluate the antitumor properties of the ethanol herbal extract of *A. falcata* on the HeLa human cervical cancer cells along with demonstrating its mode of action by assessing its effect on apoptosis, cell cycle arrest and mitochondrial membrane potential loss. The extract exhibited both dose-dependent as well as time-dependent growth inhibitory effects on the HeLa cancer cells. Phase contrast and fluorescence microscopic investigations using Hoechst and acridine orange/ethidium bromide staining agents revealed that the *A. falcata* extract induced potent morphological changes in cervical cancer cells including cell shrinkage,

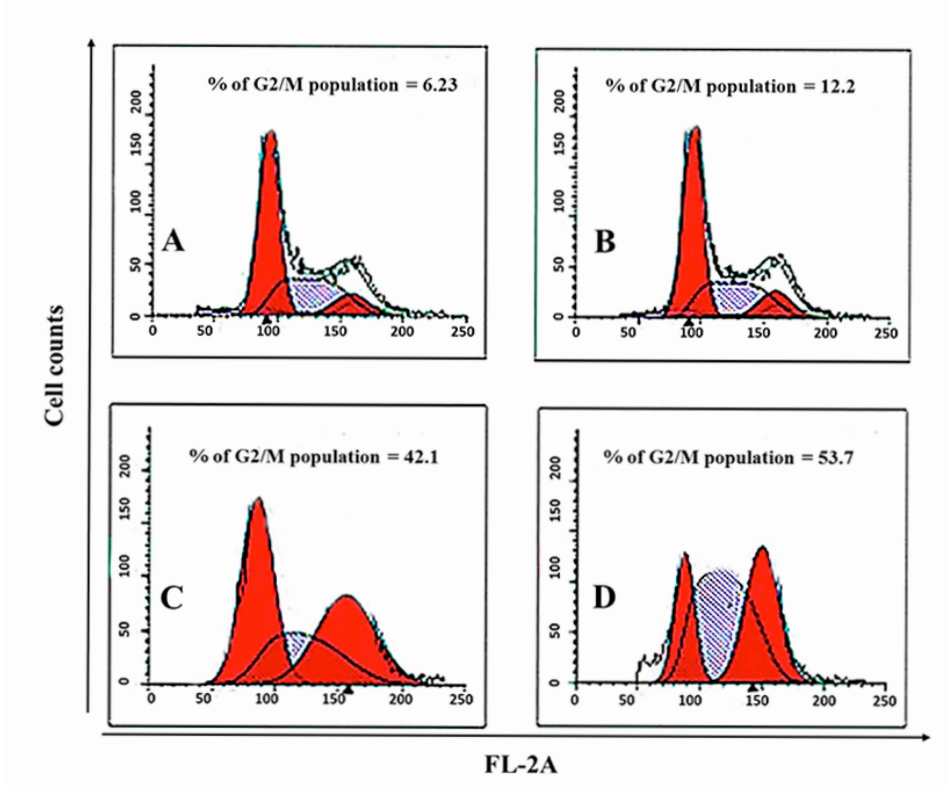

Figure 6. *A. falcata* extract induced G2/M cell cycle arrest in HeLa cervical cancer cells. HeLa cells were treated with 0 (**A**), 15 (**B**), 60 (**C**) and 120 (**D**) μg/ml of the *A. falcata* extract for 48 hrs and then analyzed by flow cytometry. The population of G2/M cells increased significantly from **A**-**D** as the dose of the extract increased.

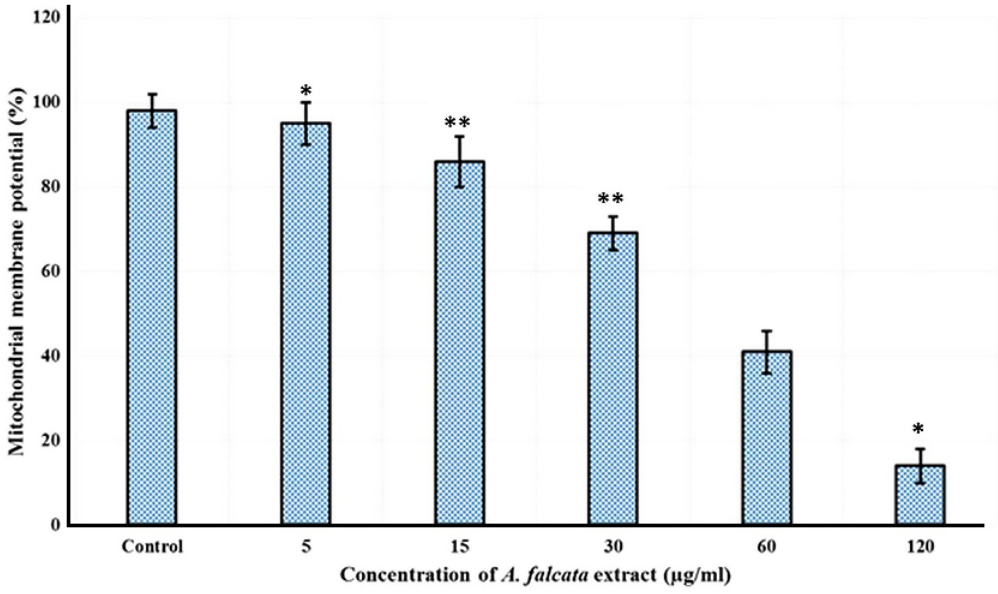

Figure 7. *A. falcata* extract induced loss of mitochondrial membrane potential in HeLa cervical cancer cells. The number of cells with decreased mitochondrial membrane potential increased with increasing *A. falcata* extract dose. Flow cytometry was used in combination with rh-123 fluorescent probe to determine changes in mitochondrial membrane potential. Data are the mean±SD of 3 independent experiments.
*p<0.05, **p<0.01 vs 0 μg/ml (control).

membrane blebbing, chromatin condensation, and appearance of apoptotic bodies. The fraction of apoptotic cells which showed red fluorescence in acridine orange/ethidium bromide staining, increased with increasing doses of the extract (Figure 4). Transmission electron microscopy results also indicated that the extract could induce potent apoptosis in the HeLa cells (Figure 5). In addition, *A. falcata* extract led to G2/M cell cycle arrest and this effect was shown to increase with increasing extract dosing. Mitochondrial membrane potential in HeLa cells was measured after extract treatment. The results of this experiment revealed that different doses of the *A. falcata* extract induced a potent and dose-dependent loss in ΔΨm after 48-h treatment. As compared to the control, extract-treated cells led to a decreased mitochondrial membrane potential (Figure 7).

In conclusion, *A. falcata* extract exhibited potent antitumor effects in HeLa human cervical cancer cells and this antitumor effect was shown to be time-dependent and dose-dependent. The extract also induced significant apoptosis along with inducing cell cycle arrest in G2/M phase of the cell cycle. Mitochondrial membrane potential loss occurred following *A. falcata* extract treatment. Thus the extract induces antitumor effects mainly through disturbing cell cycle and mitochondrial membrane potential.

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