Induction of apoptosis and G1 phase cell cycle arrest by *Aster incisus* in AGS gastric adenocarcinoma cells

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Abstract. In recent decades, various bioactive compounds from plants have been investigated for their potential use in the treatment of diseases in humans. *Aster incisus* extract (AIE) is the extract of a common plant that is mostly found in Asia. It has traditionally been used for medicinal purposes in South Korea. In this study, we evaluated the potential anticancer effects of a methanolic extract of *Aster incisus* in a normal human cell line (HaCaT keratinocytes) and in 4 different types of human cancer cell lines (A549, lung cancer; Hep3B, liver cancer; MDA-MB-231, breast cancer; and AGS, gastric cancer). The HaCaT, A549, Hep3B, MDA-MB-231 and AGS cells were treated with various concentrations of AIE and following treatment, cell survival was evaluated. Additional analyses, such as WST-1 assay, western blot analysis, DAPI staining, flow cytometry, immunofluorescence staining and wound healing assay were performed to elucidate the mechanisms and pathways involved in the cell death induced by AIE. Treatment with AIE induced morphological changes and considerably reduced the viability of the both normal and cancer cell lines. Further analysis of the AGS gastric cancer cells revealed that AIE led to the induction of apoptosis and a high accumulation of cells in the G1 cell phase following treatment with AIE in a dose-dependent manner. The results also revealed that AIE successfully suppressed the migration of the AIE-treated AGS cells. The results of western blot analysis indicated that AIE increased the expression of pro-apoptotic proteins, particularly Bid, Bad, Bak, cytochrome *c*, apoptosis inducing factor (AIF), cleaved caspase-3, -8 and -9 and cleaved poly(ADP-ribose) polymerase (PARP). Additionally, AIE decreased the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL. On the whole, the findings of this study demonstrate that AIE induces apoptosis through the activation of the caspase-dependent pathway mediated by the mitochondrial pathway and by arresting the cell cycle in AGS cells.

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Introduction

In South Korea, cancer has been a leading health issue since the 1980s. Currently, lung cancer is the most commonly diagnosed type of cancer in Korean males followed by gastric, colorectal, liver and prostate cancer (1). In females, however, the 5 most commonly diagnosed types of cancer are breast, thyroid, colorectal, gastric and lung cancer. In 2015, cancer was the leading cause of death in the Republic of Korea (2).

The World Health Organization (WHO) report cites that approximately 80% of the population of developing countries in Asia and Africa still use traditional herbs and plants as medicine (3,4). Pharmaceutical companies now rely more on experiments conducted on various traditionally known medicinal plants to discover novel molecules and bioactive substances that are more efficient against disease targets (5,6). There has been an increase in the concern associated with the fatal side-effects of some of the medicine available on the market and this has led researchers to focus more on studying traditional medicinal plants. Considering the fact that only 1% of >500,000 known plant species has been investigated, the need for novel medicinal bioactive compounds is substantial (7,8).

Gastric cancer is the fourth most commonly diagnosed type of cancer globally and the second most fatal in cancer patients (9-11). The incidence rate of gastric cancer is markedly high in Eastern Asia, Eastern Europe and South America (12,13). At present, the treatment of gastric cancer involves surgery, chemotherapy, radiotherapy, chemoradiation and targeted therapy; however, the prognosis remains poor (14-17). In addition, the effects of current chemotherapeutic drugs are not particularly satisfactory and these drugs have various side-effects. Therefore, it is important to search for novel agents from natural sources, which have the ability to treat gastric cancer (7,18-21).

Cancer is characterized by the loss of cell cycle regulation followed by uncontrolled cell growth (22,23). Cancer cells grow and constitute tumors in certain types of cancer. After a period of time, cancer cells are able to leave the primary site of establishment and invade other organs of the body. This process

is termed metastasis and is one of the reasons cancer is difficult to cure. The control of the cell cycle by the induction of cell death through the activation of cell cycle arrest or the activation of apoptosis is the major goal of cancer experiments. Various proteins regulate the cell cycle. The interaction between cyclins and specific cyclin-dependent kinases (CDKs) allow the cells to progress from one phase to another (24). The complexes which play a significant role in G1/S cell phase transition are cyclin D1,3/CDK4,6 and cyclin E/CDK2. These cyclin/CDK complexes are activated and regulated by phosphorylation.

Apoptosis is an extraordinarily controlled cell death mechanism through which cells are eliminated without inducing inflammation around the dying cell (25,26). Apoptotic cell death is triggered by the interaction between ligands and membrane extrinsic death receptors or by the intrinsic mitochondrial-mediated pathway (27,28). While the extrinsic pathway is triggered by ligand-receptor interaction, the intrinsic pathway is initiated by intracellular processes, such as cell stress and leads to the release of cytochrome *c* from the mitochondria. The cytochrome in turn activates apoptosis through a number of cascade reactions in the cell. The activation of downstream caspases results in the formation of apoptotic bodies, DNA fragmentation and chromatin condensation. Dying cells ultimately shrink and are removed by phagocytic cells (29,30).

Aster incisus, also known as *Kalimeris incisa*, is a flowering plant of the Asteraceae family. It is an aromatic plant and mostly grows in many regions of the Northern Hemisphere. Other plants belonging to the Asteraceae family have been used for traditional medicinal purposes and most recently, plants from the *Aster* genus were studied and reported for their biological activities. *Aster* tataricus can reduce the damage of neurons in epileptic rats (31), inhibit oxidative stress (32), regulate the expression of inflammatory cytokines in THP-1 cells (33) and inhibit the proliferation of SCC-9 human oral squamous carcinoma (34). *Aster yomena* can inhibit adipogenesis in 3T3-L1 preadipocytes (35) and inflammation in RAW 264.7 macrophages (36). *Aster tripolium* and *Aster spathulifolius* both have anti-obesity effects (37,38).

In the current study, we evaluated the anticancer effects, as well as the possible signaling pathways involved in the cell death of human gastric adenocarcinoma AGS cells treated with methanolic extract of *Aster incisus* referred to as AIE, *Aster incisus* extract.

Materials and methods

Cell culture and cell viability assay. HaCaT cells (normal human keratinocytes) were obtained from AddexBio (San Diego, CA, USA) and A549 (lung adenocarcinoma), Hep3B (hepatocellular carcinoma), MDA-MB-231 (breast adenocarcinoma) and AGS (gastric adenocarcinoma) cells were purchased from ATCC (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) and Roswell Park Memorial Institute 1640 Medium (RPMI-1640; HyClone) Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 1% antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin; PAA Laboratories GmbH, Pasching, Austria) and incubated at 37˚C in 5% CO_2 in a humidified air atmosphere. For the cell viability assay, all the cells were separately plated in 96-well plates at a final concentration of 10^4 cells per well and were incubated for 24 h. All the cells were later treated with various concentrations (50, 100 or 150 μg/ml) of AIE and incubated at 37˚C for a further 24 h. AIE (voucher no. 016-001) was purchased from Korean Plant Extract Bank (KPEB, Cheongju, Korea) with 99.9% HPLC purity. Following treatment for 24 h, the medium was replaced with fresh medium and 10 μl of WST-1® solution were added to each well followed by 3 h of additional incubation at 37˚C. Cell viability was determined by reading the absorbance using an ELISA microplate reader (Molecular Devices, Silicon Valley, CA, USA) at 460 nm and the percentages of inhibition were calculated. Additionally, all cells were separately plated in a 96-well plate and incubated at 37˚C. Following 24 h of incubation, the cells were incubated with 40 μM of the caspase inhibitor, z-VAD-fmk (Sigma-Aldrich, St. Louis, MO, USA), for 1 h at 37˚C. Following incubation with z-VAD-fmk, the medium was removed and the cells were treated with 150 μg/ml of AIE for 24 h. Following treatment for 24 h, the medium was replaced with fresh medium and 10 μl of WST-1® solution were added to each well followed by 3 h of additional incubation at 37˚C. Cell viability was determined by reading the absorbance using an ELISA microplate reader (Molecular Devices) at 460 nm and the percentages of inhibition were calculated.

4,6-Diamidino-2-phenylindole (DAPI) staining. The HaCaT, A549, Hep3B, MDA-MB-231 and AGS cells were plated on coverglass bottom dishes and incubated at 37˚C for 24 h. The following day, the cells were challenged with 150 μg/ml of AIE and were incubated at 37˚C again for an additional 24 h. After the additional incubation, the cells were washed once with phosphate-buffered saline (PBS) buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, and 1.4 mM potassium dihydrogen phosphate) and stained with 1 μg/ml of DAPI solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) diluted in methanol. Following incubation in the dark at 37˚C for 20 min, the dishes were rinsed with PBS and fixed with 4% formaldehyde for 15 min at room temperature. The fixed cells were mounted on the slide with Prolong Gold Antifade Reagent and observed under a ZEISS LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Western blot analysis. The AGS cells seeded in 100-mm dishes and treated with the various concentrations (80, 100 or 140 μg/ml) were harvested and lysed in ice-cold lysis buffer. Proteins were quantified using CBB solution and were separated on a 12% SDS-PAGE gel by electrophoresis. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes which were blocked using PBST buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium dihydrogen phosphate and 0.5% Tween-20) containing 5% skim milk powder. The blots were probed overnight at 4˚C with the following primary antibodies: Cleaved caspase-8 (Cat. no. 9496), cleaved caspase-9 (Cat. no. 20750), caspase-3 (Cat. no. 9662), cleaved caspase-3 (Cat. no. 9661), FLIP (Cat. no. 8510), cleaved PARP (Cat. no. 5625), cytochrome *c* (Cat. no. 4272), Bcl-xL

(Cat. no. 2762), Bcl-2 (Cat. no. 2872), Bad (Cat. no. 9292), Bak (Cat. no. 3814), Bid (Cat. no. 2002), AIF (Cat. no. 5318), cyclin D1 (Cat. no. 9932), cyclin D3 (Cat. no. 9932), cyclin E2 (Cat. no. 9870), CDK2 (Cat. no. 9932), CDK4 (Cat. no. 9932), CDK6 (Cat. no. 9932), p-p53 (Cat. no. 9919), p-Chk2 (Cat. no. 9931), p16 (Cat. no. 80772), p18 (Cat. no. 9932), p21 (Cat. no. 2947), p27 (Cat. no. 3686), E2F-1 (Cat. no. 3742), pRb (Cat. no. 9969), cdc25A (Cat. no. 3652) and β-actin (Cat. no. 4970) obtained from Cell Signaling Technology (CST; Danvers, MA, USA) and diluted according to the manufacturer's instructions (1:1,000 in 1X PBS, 5% BSA and 0.1% Tween-20). The blots were then washed 3 times in PBST, followed by incubation at room temperature for 1 h with HRP conjugated anti-rabbit (Cat. no. 7074) or anti-mouse IgG secondary antibody (Cat. no. 7076) obtained from CST and diluted according to the manufacturer's instructions (1:1,000 in 1X PBS, 5% BSA and 0.1% Tween-20). The immunoblots were then washed in PBST and visualized using ECL detection solutions.

Immunofluorescence staining. The AGS cells were plated in coverglass bottom dishes, incubated at 37˚C and treated with 140 μg/ml of AIE for 24 h. The cells were first stained with 1 μg/ml of a solution of DAPI for 20 min at room temperature. After staining with DAPI, the cells were then fixed with 4% formaldehyde for 15 min at room temperature and blocked for 1 h in a blocking solution, including 5% rabbit and mouse normal sera (Santa Cruz Biotechnolgy Inc., Santa Cruz, CA, USA) with 0.3% Triton X-100. The fixed and blocked cells were incubated with the primary antibodies: β-actin (CST, Cat. no. 4970) and cleaved caspase-3 (CST, Cat. no. 9661) for 3 h and washed 3 times with PBS buffer. After washing, the cells were treated with 0.1 μg/ml of anti-mouse IgG (H+L), F(ab')2 fragment (Alexa Fluor® 555 Conjugate) (CST, Cat. no. 4409) and anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor® 488 Conjugate) (CST, Cat. no. 4412) for 1 h. The stained cells were mounted on the slide with Prolong Gold Antifade Reagent and observed in a ZEISS LSM 710 confocal laser scanning microscope (Carl Zeiss).

Flow cytometric analysis. Following treatment with 80, 100 or 140 μg/ml of AIE for 24 h, the cells were harvested by trypsinization and fixed with 70% ethanol at 4˚C overnight. Subsequently, the cells were resuspended in PBS buffer containing 0.2 mg/ml RNase A and incubated for 1 h at 37˚C. The cells were stained with 40 μg/ml propidium iodide for 30 min at room temperature in the dark. The distribution of sub-G1 DNA was analyzed using a BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA).

Wound healing assay. For the wound healing assay, the AGS cells were seeded in a culture-Insert (ibidi, Planegg/Matrinsied, Germany) and the cells were cultured until they reached 80-90% confluency. The culture insert was removed and the cells were then washed with PBS to remove the non-adherent cells. Fresh medium containing 140 μg/ml of AIE was provided to the cells prior to photographing the plates with an Olympus CKX41 inverted microscope (Olympus Corp., Tokyo, Japan) at 0, 12 and 24 h to capture two different fields at each time point for each plate. The average wound width was measured between the two lines representative of cell migration determined by the mean of the furthest and the nearest cells at the leading edge.

Cell invasion assay. The invasion of the AGS cells was investigated using 24-well plate Transwell inserts (8 μm pore size; Corning Inc., New York, NY, USA). The inserts were first coated with 100 μl of Matrigel (BD Biosciences). RPMI-1640 medium containing FBS in the lower compartment was used as a chemoattractant. During and after treatment with AIE, the media in the inserts were replaced with FBS-free media and the plate was incubated at 37˚C in 5% CO_2 for 24 h. Following incubation, the non-invading cells were removed from the inner side of the inserts using a cotton swab and the invading cells in the lower surface were stained with 1% of crystal violet (Junsei Chemical Co., Tokyo, Japan) for 20 min at 37˚C. Images were taken using an Olympus CKX41 inverted microscope (Olympus Corp.).

Statistical analysis. Data are presented as the means ± standard deviation (SD) for the indicated number of separate experiments. The mean of the control was compared with the mean of each individual treatment group using Tukey's multiple comparisons analysis following two-way ANOVA by GraphPad Prism 7 software. (Graphpad Software Inc., La Jolla, CA, USA), and statistical significance was set at $P<0.05$.

Results

AIE suppresses the proliferation of human cancer cell lines. We examined the viability of the HaCaT, A549, Hep3B, MDA-MB-231 and AGS cells treated with various concentrations of AIE and found that AIE effectively suppressed the growth of both the treated cancer cell lines, but did not affect the proliferation of the non-cancerous keratinocytes, HaCaT cells, up to a concentration of 150 μg/ml. These results determined that AIE was safe at concentrations from 200 μg/ml and below. The treatment of the human cancer cell lines with AIE demonstrated that 150 μg/ml was the most active concentration for the suppression of cancer cell growth without affecting human non-cancerous cells. In order to verify whether the effects of AIE were dose-dependent, we therefore selected 3 different concentrations ≤150 μg/ml for use in further experiments. The results revealed that AIE gradually suppressed the viability of the cancer cells in a dose-dependent manner (Fig. 1A). Furthermore, the shrinkage of cells in all the treated cancer cells was observed under an Olympus CKX41 inverted microscope and the obtained images revealed that AIE significantly reduced the growth of the AGS cells and affected their morphology (magnification, x100) (Fig. 1D).

AIE induces apoptotic changes in treated cancer cell lines. The A549, Hep3B, MDA-MB-231 and AGS cells were incubated with the caspase inhibitor (z-VAD-fmk) followed by treatment with 150 μg/ml of AIE. The results of cell viability assay with WST1 revealed that treatment with the caspase inhibitor significantly increased the proliferation of both the cancerous and non-cancerous treated cells (Fig. 1B), demonstrating that the caspase-related cell death pathway may play a major role in the anti-proliferative effects of AIE. Additionally, changes in nuclear morphology indicative of apoptosis were

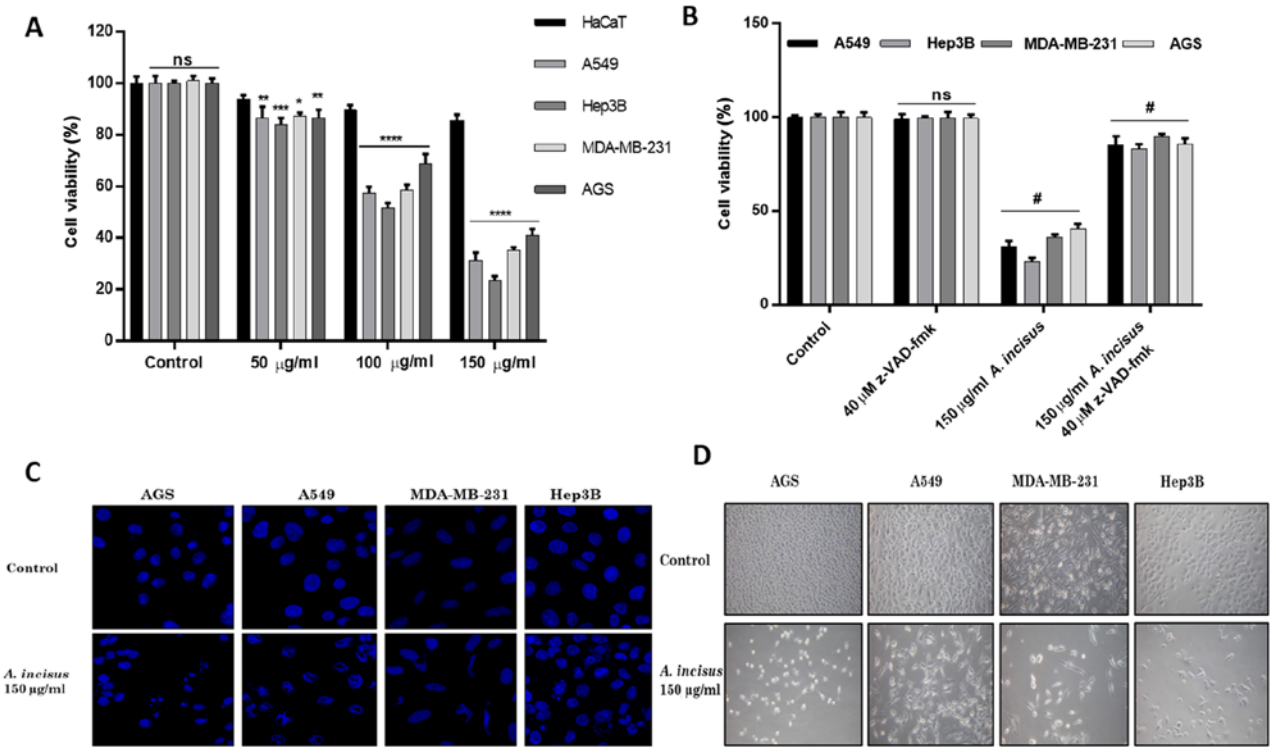

Figure 1. (A) Viability of cancer cells treated with *Aster incisus* extract (AIE). (B) Viability of cancer cells challenged with caspase inhibitor and treated with AIE. (C) Morphology of cancer cells treated with AIE. (D) DAPI staining of cancer cells treated with AIE. Statistical significance difference was set at *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and $^{\#}$P<0.0001. ns, not significant.

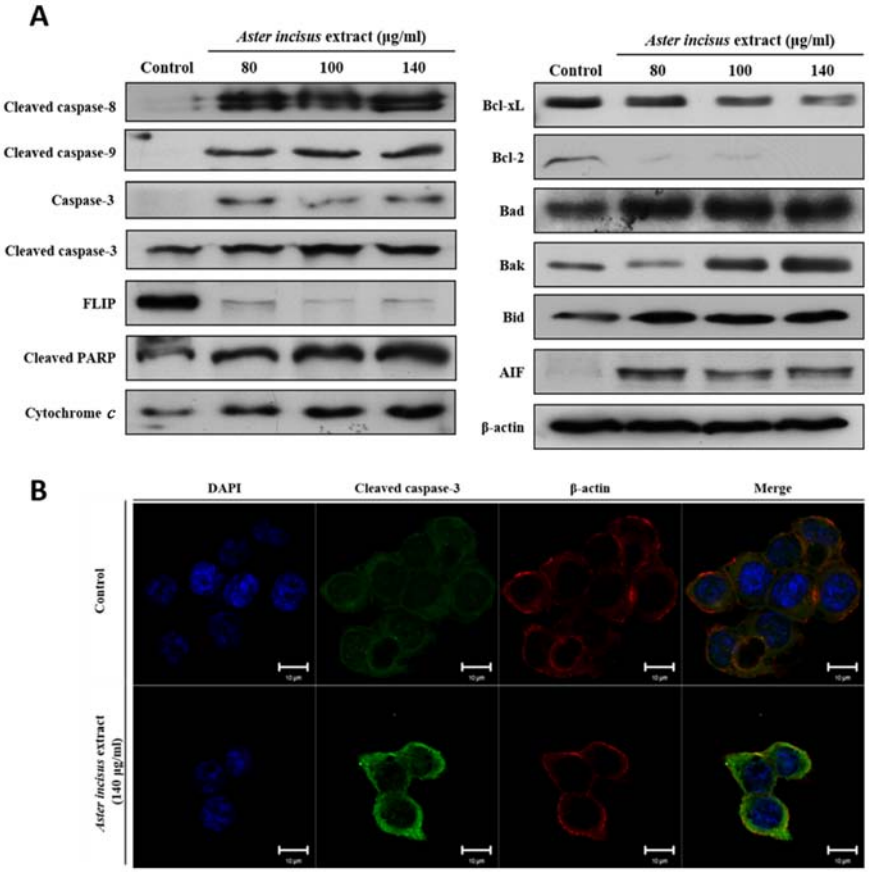

Figure 2. (A) Apoptotic proteins examined by western blot analysis in AGS gastric cancer cells treated with *Aster incisus* extract (AIE). AGS cells were treated with 80, 100, and 140 μg/ml of AIE for 24 h followed by the extraction of proteins. Following quantification, proteins were separated by gel electrophoresis and the results revealed the level of expression of different analyzed apoptotic proteins. (B) Immunofluorescence staining caspase-3 for in cells AGS treated with AIE. AGS cells were treated with 140 μg/ml of AIE and stained with DAPI, caspase-3 antibody and β-actin. Images were obtained using a confocal microscope.

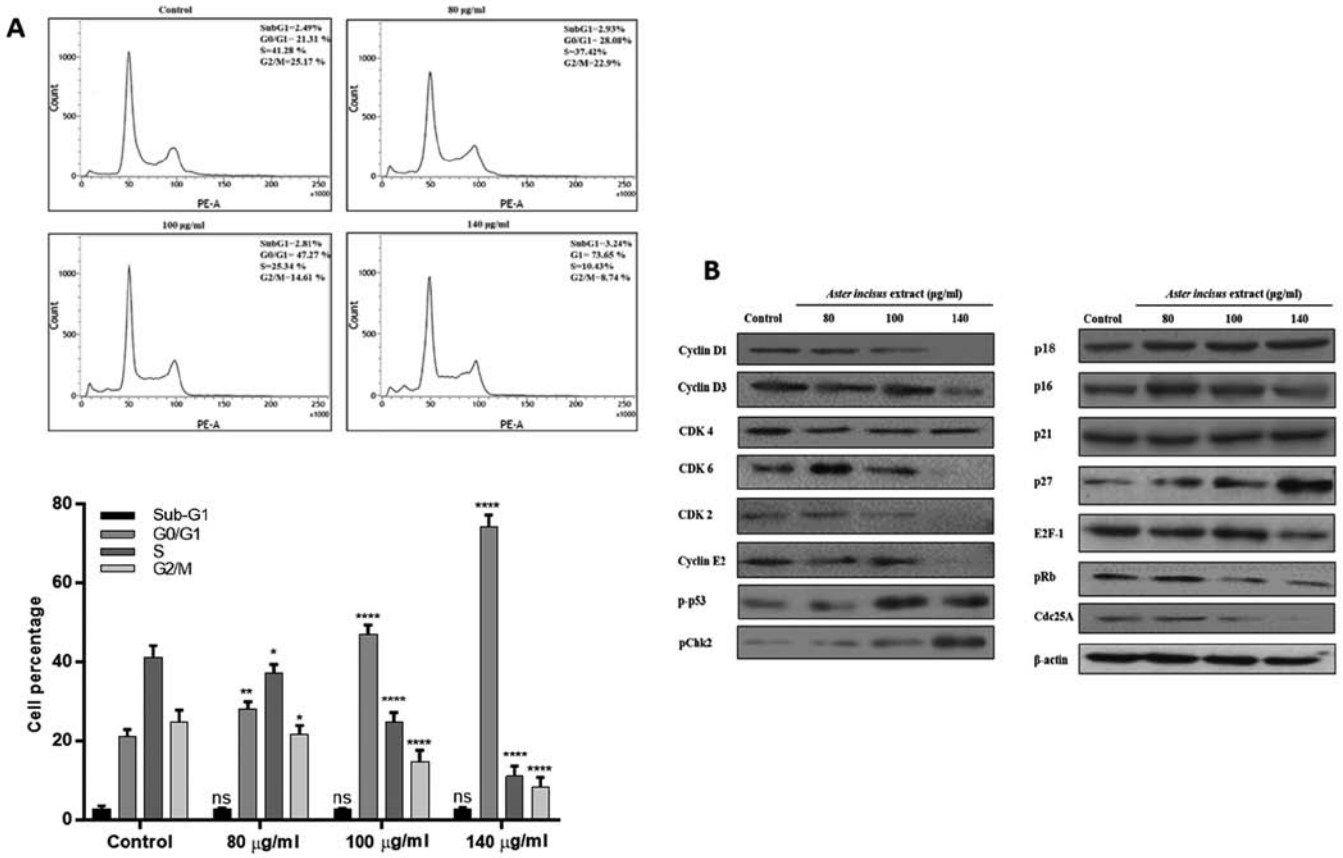

Figure 3. (A) Flow cytometric analysis of AGS cells treated with various concentrations of *Aster incisus* extract (AIE). AGS cells were treated with 80, 100, and 140 μg/ml of AIE and later stained with propidium iodide. Percentages of each cell cycle phase were obtained by flow cytometric analysis. Statistically significant differences between the control group and the treated groups were determined using GraphPad prism 7 software and the differences between the group were considered significant at *P<0.05, **P<0.01 and ****P<0.0001. ns, not significant. (B) Expression of G1 phase-related proteins examined by western blot analysis.

observed after the cancer cells were treated with 150 μg/ml of AIE and stained with DAPI solution. Images were obtained and analyzed under a ZEISS LSM 710 confocal laser scanning microscope (magnification, x1,000). The results revealed that the AIE-treated cells were characterized by apoptotic morphological changes, such as the nuclear condensation and the formation of apoptotic bodies, whereas the untreated cells exhibited normal round-shaped nuclei (Fig. 1C).

AIE induces the apoptosis of AGS gastric cell lines. After the initial investigation of the 4 different cancer cell lines, we then focused on AGS cell lines specifically. Following the results mentioned above, we examined the expression of apoptosis-related proteins by western blot analysis. The results revealed that the expression of proteins from both the intrinsic and extrinsic pathways varied in a dose-dependent manner. The expression levels of pro-apoptotic proteins, such as Bak, Bid, Bad, AIF, cytochrome *c*, cleaved caspase-8,-9 and -3, and cleaved PARP were significantly upregulated, while the expression levels of anti-apoptotic proteins, such as FLIP, Bcl-2 and Bcl-xL were lower in the AIE-treated AGS cells when compared with the untreated cells. These results confirmed that AIE induced the apoptosis of the AGS cells (Fig. 2A). To further confirm these results, we conducted an immunofluorescence staining assay of the AGS cells using caspase-3 antibody. The results revealed that caspase-3 protein was highly expressed in the AGS cells treated with AIE when compared with the controls (Fig. 2B). Taking all these results into consideration, it can be concluded that AIE suppresses the proliferation of the AGS gastric cell line by inducing apoptosis.

AIE induces G1/S phase cell cycle arrest in the AGS gastric cancer cell line. We investigated the effects of AIE on cell cycle phases by using PI staining in FACS analysis. This assay allowed us to quantify the percentage of cells in the different phases of the cell cycle. For this analysis, the AGS cells were treated with 80, 100, 140 μg/ml of AIE for 24 h and later stained with PI. The results of flow cytometric analysis revealed the accumulation of cells in the G0/G1 phase in a dose-dependent manner. The number of cells in the G0/G1 phase were 21.3% in the untreated cells, and 28.08, 47.2 and 73.6% in the cells treated with AIE at 80, 100 and 140 μg/ml, respectively (Fig. 3A). To validate these results, we conducted a western blot analysis of proteins that are involved in the G1/S phase, particularly cyclins, CDKs and CDK inhibitors. The results revealed that AIE downregulated the expression levels of proteins involved in the G1/S phase progression, including cyclin D1, cyclin D3, cyclin E2, CDK2, CDK4 and CDK6, while it increased the expression levels of CDK-related inhibitors, such as p16, p18, p21 and p27 (Fig. 3B). Taking these results into consideration, it can be concluded that AIE successfully induced cell cycle arrest in the treated AGS cells, leading to the accumulation of cells in the G1/S phase (please also see Fig. 5).

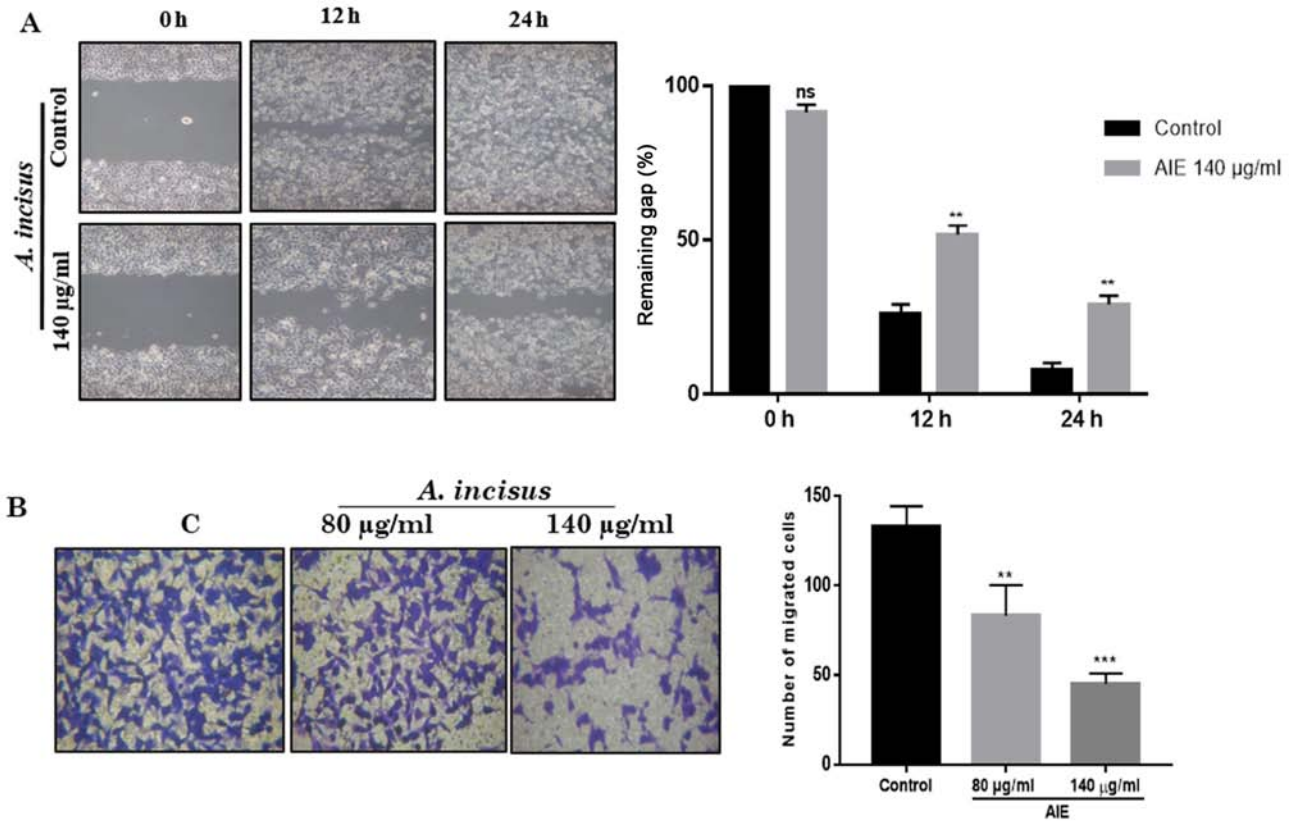

Figure 4. (A) Wound healing assay of AGS cells treated with *Aster incisus* extract (AIE). AGS cells were treated with 140 μg/ml of AIE and regularly monitored to capture the migration of cells. Images were acquired using an inverted microscope following treatment then at 12 h and finally after 24 h. The assay was repeated 3 times and statistically significant differences were set at, **P<0.01. ns, not significant. (B) Cell invasion assay of AGS cells treated with AIE. AGS cells were plated in inserts coated with Matrigel, treated with AIE, incubated for 24 h. Non-invading cells were removed and invading cells stained and observed under an inverted microscope. 'C' indicates the control group. The experiment was performed 3 separate times and the results were statistically analyzed and significant difference was set at, **P<0.01 and ***P<0.001.

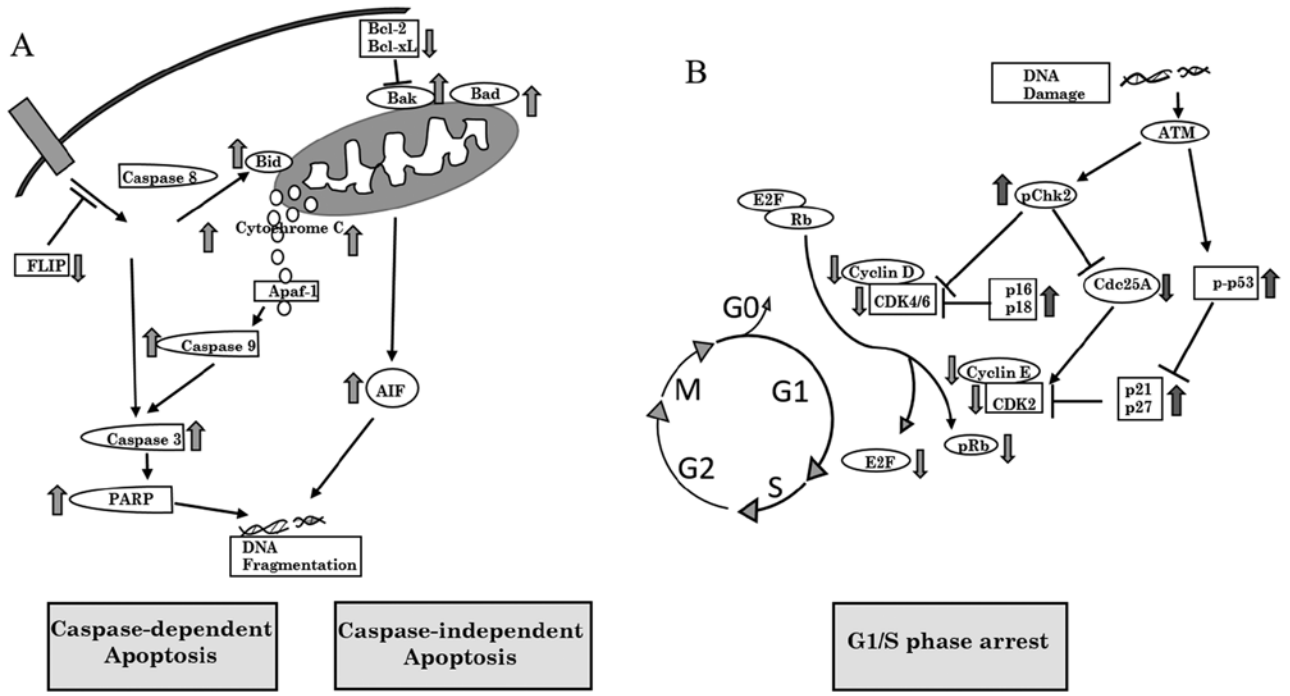

Figure 5. (A) Schematic representation of pathways involved in the activation of apoptosis by *Aster incisus* extract (AIE) in AGS gastric cancer cells. (B) Graphic representation of the pathways involved the cell cycle arrest by AIE in treated AGS cells. AIE induced apoptosis by increasing the levels of pro-apoptotic proteins, such as caspases and mitochondrial pro-apoptotic proteins. The anti-apoptotic proteins, Bcl-2, Bcl-xL and FLIP were downregulated following AIE treatment. AIE treatment additionally induced cell cycle arrest in treated AGS cells. Cyclins and cyclin-dependent kinases were significantly downregulated, while inhibitors proteins were increased.

AIE suppresses the migration and invasion of AGS cells. To explore the effects of AIE on cell migration, a wound healing assay was conducted. For the wound healing assay, images were acquired after 24 h of treatment using an inverted microscope to record the progression of cells from the beginning of the treatment up to 24 h. The results shown in Fig. 4A indicatd that AIE was suppressed the migration of the treated AGS cells. The treated AGS cells were not able to fill the gap between the cells, while the untreated cells filled the surface within 24 h. These results confirm that AIE suppresses the migration of AGS gastric cancer cells.

From the invasion assay, we observed that AIE was able to successfully prevent the invasion of AGS cells through the Matrigel layer. These results suggest that AIE has the ability to inhibit the degradation of extracellular Matrigel by proteases from the cancer cells, and thus, prevent the spread of cancer cells to other organs (Fig. 4B).

Discussion

AIE belongs to the Asteraceae family and has been used for medicinal purposes in South Korea. However, to date, and at least to the best of our knowledge, there is no report on the use of AIE for the treatment of cancer cells. In this study, we investigated the potential anticancer effects of AIE in 4 different cancer cell lines, A549, Hep3B, MDA-MB-231 and AGS cells. The results suggested that AIE reduced cell viability in a dose-dependent manner (Fig. 1A).

Apoptosis involves well-defined morphological changes, resulting from the activation of specific signaling pathways. Apoptotic cells exhibit DNA fragmentation, nuclear condensation and the blebbing of the plasma membrane, leading to the release of small membrane-enclosed particles, known as apoptotic bodies (39,40). In this study, we found that AIE was able to initiate nuclear condensation in both the cancer cell lines when treated with 150 μg/ml (Fig. 1D). The results of WST-1 assay for the cells challenged with the caspase inhibitor before treatment clearly demonstrated that the inhibitor significantly increased the survival of AGS gastric cancer cells that were treated with AIE (Fig. 1B)

The two major most common pathways involved in apoptosis are the extrinsic and the intrinsic pathways. The first pathway (extrinsic) is initiated through the interaction between cellular death receptors and their respective ligands. Following the interaction, intracellular caspase-8 is recruited by the intracellular part of the death receptor and is activated. Activated caspase-8 then initiates apoptosis through the cleavage of effector caspases or cleavage of Bid into tBid (41). The results of this study revealed that AIE increased the expression of cleaved caspase-8 and Bid in the AGS cells (Fig. 2A).

The second pathway (intrinsic pathway) is initiated by different stimuli that do not require a transmembrane receptor, such as cellular stresses, in which Bcl-2 family proteins play a crucial role. Bcl-2 family proteins are a large group of proteins that include anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1 and Bcl-w), pro-apoptotic proteins (Bax and Bak), and finally the BH3-only proteins (Bad and Bid). BH3-only proteins monitor cellular processes and transduce both extrinsic and intrinsic death signals to the Bcl-2 family of proteins at the outer mitochondrial membrane (OMM) to modulate mitochondrial outer membrane permeability (MOMP). The increase in MOMP leads to the release of cytochrome *c*, AIF and EndoG in the cytosol. Cytochrome *c* and the Apaf-1 complex cleave procaspase-9 and cleaved caspase-9 cleaves procaspase-3 to activate caspase-3 (42). AIF and EndoG translocate to the nucleus where they participate in DNA fragmentation (43). A number of *in vitro* studies have confirmed that the induction of apoptosis leads to the death of cancer cells (44-46).

In this study, we observed the morphological changes and nuclear condensation in the cells. The results revealed that AIE induced mitochondrial dysfunction via the increased expression of Bak and pro-apoptotic proteins, and by the decreased expression of Bcl-2 and Bcl-xL, anti-apoptotic proteins, in the treated AGS cells specifically in a dose-dependent manner (Fig. 2A). The results of western blot analysis and immunofluorescence staining of caspase-3 as shown in these figures, clearly indicate a significant increase in the levels of pro-apoptotic proteins and the inhibition of anti-apoptotic proteins in the treated cells when compared with the untreated cells. The results were corroborated with the results of immunofluorescence staining for caspase-3 expression in the treated AGS cells (Fig. 2B).

The cell cycle is the most biologically conserved process throughout the years. The cell cycle is regulated by numerous proteins and variations in the cell regulation usually result in cancer. Cancer development can be due to various causes, including a dysregulation in cellular functions. The human cell cycle is divided into 4 phases: The G1 phase, S phase, G2 phase and M phase. Cyclins and CDKs are crucial for cell division; therefore, the expression and regulation of these proteins play a crucial role in the initiation of cancer or in cancer treatment. Each cell cycle phase is regulated by specific cyclins and CDKs. The interaction between cyclins and CDKs allow the cells to progress with their division. Genetic alterations in cells, such as a mutation can lead to the loss of cell regulation. Components of the cell cycle machinery are frequently altered in human cancer. A group of inhibitory proteins, cyclin-dependent kinases inhibitors or CDK inhibitors, control the cyclin-CDK activity thereby restraining cell cycle progression in response to extracellular and intracellular signals (29). The crucial role of CDKs has prompted great interest in the development of specific kinase inhibitors that could be expected to block cell cycle progression and induce growth arrest. CDK inhibitors are divided into two major groups: The CDK4 inhibitors named due to their ability to inhibit CDK4 and CDK6 by competing with cyclin D, include p16 and p18. The second family is Cip/Kip family (for CDK interacting protein/kinase inhibitory protein) which include p21 and p27 (47). As other studies have reported previously, the activation of cyclin-dependent kinases inhibitors is a very effective method with which to inhibit the proliferation of cancer cells (48,49). The G1 phase is a very important part of the cell cycle as it provides the signal to the cell to allow it to enter the cell division stage.

The cyclin D-CDK4/6 complex initiates the phosphorylation of Rb during G1 to S phase transition, which leads to the release of E2F-1 by Rb allowing the initiation of transcription and translation. Later in the G1 phase, the complex cyclin A-CDK2 and the cyclin E-CDK2 complex in the early S phase regulate the transition from the G1 phase to S phase. Cyclin-dependent kinases inhibitors, by interfering in the interaction of cyclins with CDKs, trigger the arrest of cell

cycle in G1 phase. Many plants used as traditional medicine have been reported to arrest the cell cycle of various cancer cells lines during the G1/S phase transition (50-53).

One of the other most important tumor suppressors is tumor protein p53, which plays a key role in the cellular response to DNA damage. Similar to cyclin-CDK complexes, p53 is very important in the transition of cells during division from the G1 to S phase. p53 also arrests the cells in the G1 phase after DNA damage by activating the cyclin-dependent kinases inhibitors (54-56).

Chk2 protein is also phosphorylated following DNA damage, which in turn inactivates the cell division cycle 25A phosphatase (Cdc25A). The deactivation of Cdc25A results in the elevation of the phosphorylated (inactive) form of Cdk2; therefore, cells cannot progress into the S phase and replicate their DNA (57,58).

The results from the flow cytometric analysis in the current study demonstrated the accumulation of treated AGS cells in the G1 phase. Additional results from western blot analysis revealed that AIE reduced the expression of cyclins and CDKs involved in the G1 phase (more specifically, cyclin D1, cyclin D3, cyclin E, CDK2 CDK4 and CDK6), but increased the expression of the cyclin-dependent kinases inhibitors, p16, p18, p21 and p27 (Fig. 3B).

To deepen our understanding, we analyzed the effects of AIE on cell migration and cell invasion. Migration and invasion are very important steps in metastasis. In cancer patients, some cancer cells can spread from the initial site to secondary sites. This process is known as metastasis and is made possible by the ability to migrate, degrade the extracellular matrix and spread to other parts of the body through the bloodstream. Once cancer cells start to diffuse, the prognosis for the patient worsens and reduces the chances of survival considerably. The results of this study demonstrated that AIE successfully suppressed the migration of the AIE-treated AGS gastric cancer cell lines in a wound healing assay. AGS cells plated in inserts coated with Matrigel when treated with AIE were not able to migrate. These results revealed that AIE considerably reduced the ability of AGS gastric cancer cells to invade tissues by degrading the extracellular matrix components. We thus concluded that AIE has the ability to inhibit the migration of AGS cells and their invasion (Fig. 4).

In conclusion, the findings of this study demonstrate that AIE induces the apoptosis of AGS gastric cancer cells via both the intrinsic mitochondrial-mediated cell death pathway and the caspase-dependent pathway via the activation of caspases. AIE also significantly induced cell cycle arrest in G1/S phase and furthermore, AIE suppressed the migration and invasion of AGS cells. The results of this study, at least to the best of our knowledge, are the first report of any biological activity of *Aster incisus*. Further studies are warranted for the isolation and the identification of the active single compounds responsible for the anti-proliferative activities of AIE in gastric cancer cells. A schematic representation of the pathways through which AIE induces apoptosis and cell cycle arrest in AGS cells is shown in Fig. 5.

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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

DN conducted the experiments and wrote the manuscript; YAS provided the plant extract and advice in the conception and design of the study; YBS and GDK conducted parts of the experiments, MPP and IN contributed to the analysis of the data.

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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