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Ardisia gigantifolia ethanolic extract inhibits cell proliferation and targets cancer stem cells in gastric cancer

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ABSTRACT

Objective: To evaluate the effects of ethanol extract from *Ardisia gigantifolia* leaves on cell proliferation and cancer stem cell (CSC) number in gastric cancer.

Methods: The inhibitory effect of *Ardisia gigantifolia* extract on the proliferation of MKN45 and MKN74 gastric cancer cells was assessed using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay. Non-adherent culture (3D) model was used to evaluate the effect of the extract on tumorsphere size and number. Moreover, the expression of CD44, ALDH, and p21 was determined by immunofluorescence analysis. Flow cytometric analysis was performed to evaluate cell cycle arrest and the expression of gastric CSC markers CD44 and ALDH. Real-time PCR analysis was also carried out to assess the effect of the extract on the expression of cell cycle-regulated genes.

Results: *Ardisia gigantifolia* extract effectively inhibited cell proliferation with an IC_{50} of 55.7 μg/mL in MKN45 cells and 123.6 μg/mL in MKN74 cells. The extract also arrested cell cycle in the G_0/G_1 phase as well as significantly reduced the size and number of tumorspheres. The markedly increased expression of p21 was observed at both mRNA and protein levels in the extract-treated adherent cells and tumorspheres. In addition, *Ardisia gigantifolia* extract significantly reduced the number of CD44- and/or ALDH-expressing gastric CSC.

Conclusions: The development of gastric CSC can be inhibited by the ethanol extract of *Ardisia gigantifolia*.

KEYWORDS: *Ardisia gigantifolia*; Gastric cancer; Cancer stem cell markers; CD44; ALDH

1. Introduction

With 1 089 103 new cases and 76 879 fatalities recorded in 2020, gastric cancer is the fifth most frequent cancer globally[1]. One of the main reasons for the high death rates of stomach cancer is the difficulty in early diagnosis of this disorder since its symptoms resemble those of many other gastrointestinal diseases[2]. Therefore, the majority of gastric cancer cases are already metastatic at the time of diagnosis. The 5-year survival rate for this cancer is less than 30%, despite many advances in this cancer type's treatments[3]. Among the developed therapies, trastuzumab and ramucirumab

Significance

Currently, no studies have demonstrated the effect of *Ardisia gigantiflolia* extract on gastric cancer stem cells. This study shows that *Ardisia gigantiflolia* extract inhibited proliferation, induced cycle arrest, and significantly reduced the number of cancer stem cells in MKN45 and MKN74 gastric cancer cells. This finding suggests the potential of *Ardisia gigantiflolia* to inhibit gastric cancer stem cells.

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have been evaluated as having a good response to human epidermal growth factor receptor 2 (HER2) or/and epidermal growth factor receptor (EGFR) overexpressing gastric cancers[4,5]. However, less than 25% of gastric cancer cases were reported as HER2-positive, which is a challenge for the current target therapy[6]. On the other hand, numerous cases of HER2 overexpressing gastric cancer showed resistance to trastuzumab treatment through activation of the PI3K/AKT or IL-6/STAT3/Jagged-1/Notch signaling pathway[7,8]. The concept of cancer stem cell (CSC) and its role in tumor formation, development, and drug resistance, as well as in cancer targeting therapies, has been discussed in recent years in many types of solid and liquid cancers[9,10]. The presence of CSC in stomach tumors was first indicated in the study of Takaishi *et al*. in which CD44-expressing cells exhibited the characteristics of CSC, such as self-renewal, differentiation into other cancer cells, *in vitro* spheroid and *in vivo* tumor formation, and resistance to anti-cancer drugs 5-fluorouracil and paclitaxel[11]. The following study also showed that gastric CSC expressing CD44 and aldehyde dehydrogenase (ALDH) demonstrated resistance to cancer drugs such as 5-fluorouracil and doxorubicin[12]. These discoveries of CSC in tumors led to the development of therapies that target CSC, in order to prevent post-treatment recurrence and drug resistance.

The development of new drugs, especially anti-cancer drugs from natural herbs, is of particular interest at present[13]. *Ardisia gigantifolia* (*A. gigantifolia*) is a herb used to treat some stomach ailments in traditional Chinese medicine[14,15]. Several studies showed that *A. gigantifolia* can inhibit the growth of HepG2 live cancer cells and BCG gastric cancer cells[16]. However, the effects of *A. gigantifolia* extract on gastric CSC have not been studied. In this study, we evaluated the effect of *A. gigantifolia* extract on cell proliferation, cell cycle, tumorsphere formation, and the expression of gastric CSC markers.

2. Materials and methods

2.1. Preparation of A. gigantifolia extract

The *A. gigantifolia* leaves were collected at Dinh Hoa, Thai Nguyen Province, Vietnam (N21°49'48''; E105°32'20''). The indentification of the scientific name of the research sample was carried out by an expert of the Vietnam National Institute of Medicinal Materials. A voucher specimen of *A. gigantifolia* was deposited in the National Institutes of Medicinal Materials, Vietnam (NIMM-19110). *A. gigantifolia* leaves were washed several times with water, cut into small pieces, and dried at 50℃ for 48 h. Five grams of the dried *A. gigantifolia* leaves were crushed and put into falcon containing 30 mL absolute ethanol and shaken at 200 rpm at 37℃ for 24 h. The ethanolic extract was completely evaporated at 50℃ for 48 h and was then stored at −20℃ for the next experiments. The extract was then dissolved in dimethyl sulfoxide (DMSO ≥ 99.99%) (Thermo Fisher Scientific, Waltham, MA, USA) at a stock concentration of 100 mg/mL and stored at −20℃ for future experiments.

2.2. Cell culture and 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) assay

The MKN45 and MKN74 gastric cancer cell lines (ATCC, Manassas, MD, USA) were incubated in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (FBS) in 96-well plates at 37℃, 5% CO_2, with an initial density of 5×10^3 cells per well. After 24 h of culture, the cells were treated with *A. gigantifolia* extract at different concentrations ranging from 10 to 500 μg/mL. The control (0 mg/mL) was treated with an equivalent amount of DMSO. After 48 h of treatment, the cell phenotype was observed and evaluated using Ts2 inverted microscopy (NIKON, Tokyo, Japan). MTT (Thermo Fisher Scientific, Waltham, MA, USA) assays were conducted as follows: The old culture medium was completely removed, 100 μL of new culture medium containing 10% MTT agent at a concentration of 5 mg/mL was added, and cells were incubated at 37℃ for 4 h, and protected from light. Subsequently, the MTT-containing culture medium was completely replaced by 100 μL of DMSO, and cells were then incubated at 37℃ for 30 min to dissolve the purple formazan crystals that have formed. A Multiskan Sky spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the optical density (OD) at 570 nm. The cell proliferation rate was calculated by the formula:

Cell proliferation (%) = (OD of treated sample/OD of control) × 100

The IC_{50} value of each cell line was determined utilizing the GraphPad Prism 5.0 software, in accordance with the manufacturer's guidelines.

2.3. Tumorsphere culture and treatment

Tumorsphere culture was performed on a serum-free medium on a non-adherent plate. This is an important condition for tumorsphere formation from cells with CSC properties. The process was carried out as previously described[17].

The 96-well plate was treated with 10% poly-2-hydroxyethyl methacrylate (polyHEMA) to create a non-adhesive surface. A volume of 50 μL of 10% polyHEMA was added to each well of the 96-well plate, and then the plate was completely evaporated at 50℃ for 30 min. This step was repeated twice, and the plate surface was carefully washed three times with 1×phosphate-buffered saline (PBS).

An amount of 1 000 cells of MKN45 or MKN74 were seeded in non-adherent culture plates in a serum-free GlutaMAX-DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 20 ng/mL of epidermal growth factor (EGF) and basic

fibroblast growth factor (FGF) and cultured at 37 ℃, 5% CO_2. EGF, FGF, and polyHEMA were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). *A. gigantifolia* extract was mixed into the tumorsphere culture medium at a concentration of the IC_{50} mentioned above. An equivalent amount of DMSO was added to the tumorsphere culture medium of the control. The effects of *A. gigantifolia* extract on 3D tumorsphere formation were evaluated after five days of treatment by using an Eclipse Ti2 microscope at a ×20 objective. Five days are the necessary time for the formation of tumorspheres. The number and size of tumorspheres in each culture well were quantified using the NIS-Elements BR software (Nikon, Tokyo, Japan). A spherical cluster of cells with a diameter of 100 µm is considered a tumorsphere.

2.4. Cell cycle analysis

Gastric cancer cells (2×10^5 cells) were seeded on a 12-well plate. After 24 h of culture at 37 ℃ and 5% CO_2, the cells were treated with *A. gigantifolia* extract at the concentration of the IC_{50} value for 48 h. Control cells were treated with an equivalent amount of DMSO. Cells were then collected by treatment with trypsin/ethylenediaminetetraacetic acid and centrifugation at 1 300 rpm for 3 min. Cells were fixed with 95% ethanol at −20 ℃ for 24 h and then incubated in a fluorochrome solution containing 50 mg/L propidium iodide (PI) for 2 h at 4 ℃ to stain the cell nucleus. The distribution of cell cycle phases was determined by using a BD FACSCanto Ⅱ flow cytometer and BD FACSDiva™ software (BD Biosciences, San Jose, CA, USA). The laser used corresponds to the PI staining dye, and 20 000 events were analyzed.

2.5. Real-time PCR analysis

After 48 h of treatment with the IC_{50} concentration of *A. gigantifolia* extract or an equivalent amount of DMSO (control), the entire culture medium was removed by pipetting, and 1 mL of Trizol reagent (Invitrogen-Thermo Scientific, Waltham, MA, USA) was added into the cell-containing wells. Cells were then stored at −80 ℃ until RNA extraction.

The extraction of total RNA was performed according to the manufacturer's instructions. A volume of 500 µL of isopropanol was added to the aqueous phase for every 1 mL of TRIzol, and incubated for 10 min at room temperature. Then the mixture was centrifuged for 10 min at 10 000 rpm at 4 ℃. A white gel-like pellet of total RNA formed at the bottom of the tube. The supernatant was carefully removed using a pipette.

The RNA was dried for 5-10 min at 40 ℃, and 100 µL of deionized water was added. RNA was quantified at 260/280 nm using a NanoDrop spectrophotometer. Then, 1 µg RNA was used to synthesize cDNA using SuperScript™ Ⅲ Reverse Transcriptase (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Real-time PCR was performed using the Invitrogen™ Platinum™ SYBR™ Green qPCR SuperMix-UDG w/ROX (Invitrogen) in a qTower3 (Analytik Jena, Jena, Germany) in accordance with the manufacturer's protocol. Amplification was carried out using target primers (Table 1) with the following thermal profile: 95 ℃ for 2 min and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s, and their relative expression was determined by normalizing the expression using $2^{-\Delta\Delta Ct}$ method[18], and *HPRT1* was used as a housekeeping gene.

2.6. Flow cytometric analysis of CSC marker expression

Flow cytometry analyses were performed on 2D culture cells stained with gastric CSC markers CD44 and ALDH using a BD FACSCanto Ⅱ instrument and BD FACSDiva™ software. Cells were harvested using trypsin, then stained with a PE mouse anti-human CD44 antibody at 1:50 (BD Biosciences, Le Pont de Claix, France) in 1×PBS buffer for 20 min at 4 ℃. The cells were washed twice with 1×PBS by centrifuging at 1 300 rpm for 3 min. The cell nucleus was then stained with 3 µM of 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA) in PBS buffer during the final wash. One hundred thousand events were acquired by the flow cytometer.

To detect the ALDH activity, the ALDEFLUOR Kit (STEMCELL Technologies, Grenoble, France) was utilized for the ALDH marker, following the instructions provided by the manufacturer. Briefly, cells obtained from culture plates using trypsin were suspended in 1 mL of ALDEFLUOR assay buffer containing 5 µL of activated ALDEFLUOR™ reagent. As a negative control, for each sample of cells, an aliquot was treated with 5 µL of the diethylaminobenzaldehyde (DEAB, 3 µM), an inhibitor of ALDH. Cells were incubated at 37 ℃ for 30 min, then centrifuged at 500 rpm for 5 min. The cell pellets were suspended in 0.5 mL of ALDEFLUOR™ Assay Buffer and kept on ice for flow cytometric analysis.

Table 1. Primer sequences for real-time PCR analysis.

Gene	Primer sequences (5'–3')
CD44	F: CCAGAAGGAACAGTGGTTTGGC
	R: ACTGTCCTCTGGGCTTGGTGTT
ALDH	F: CGGGAAAAGCAATCTGAAGAGGG
	R: ATGCGGCTATACAACACTGGC
p21	F: AGGTGGACCTGGAGACTCTCAG
	R: TCCTCTTGGAGAAGATCAGCCG
p53	F: CCTCAGCATCTTATCCGAGTGG
	R: TGGATGGTGGTACAGTCAGAGC
CCND1	F: TCTACACCGACAACTCCATCCG
	R: TCTGGCATTTTGGAGAGGAAGTG
CCNE1	F: TGTGTCCTGGATGTTGACTGCC
	R: CTCTATGTCGCACCACTGATACC
PCNA	F: CAAGTAATGTCGATAAAGAGGAGG
	R: GTGTCACCGTTGAAGAGAGTGG
HPRT1	F: CATTATGCTGAGGATTTGGAAAGG
	R: CTTGAGCACACAGAGGGCTACA

F: forward; R: reverse; ALDH: aldehyde dehydrogenase; HPRT1: hypoxanthine phosphoribosyltransferase 1.

2.7. Immunofluorescence assay

After 48 h of treatment with *A. gigantifolia* extract at the IC_{50} concentration or an equivalent amount of DMSO (control), monolayer (2D) culture cells were rinsed with PBS twice, fixed with 4% formaldehyde for 5 min and treated with 0.5% Triton X-100 solution for 15 min at ambient temperature. Cells were then blocked in a tris-buffered saline solution containing 1% bovine serum albumin for 30 min. Cells were incubated with p21 primary antibodies 1:100 (Abcam, Cambridge, MA, USA) for 30 min. Subsequently, cells were rinsed with 1×PBS, and incubated with Alexa Fluor® 568 Goat Anti-Mouse IgG secondary antibodies 1:300 (Abcam, Cambridge, MA, USA). A 1×PBS solution containing DAPI (3 μM) was used for nuclear DNA staining (blue). For the CD44 and ALDH markers, 2D culture cells were directly incubated with PE mouse anti-human CD44 antibody or ALDEFLUOR reagent as described above in flow cytometric analysis. After washing with the ALDEFLUOR buffer, cells were recovered in a buffer containing 3 μM DAPI for nuclear DNA staining.

For tumorspheres, the tumorspheres (3D) formed on the 5th day of the culture process will be treated with *A. gigantifolia* extract for 48 h. Subsequently, the tumorspheres were collected by centrifugation at 500 rpm for 5 min and washed twice with 1×PBS. The immunofluorescence staining steps with p21, CD44, and ALDH were carried out on these tumorspheres as described above in 2D cultured cells. Images were captured and analyzed using NIKON Eclipse Ti2 Microscope and NIS-Elements BR software.

2.8. Statistical analysis

Statistics were performed using Mann-Whitney *U* test or one way ANOVA with *post hoc* analysis on GraphPad Prism 5 software, version 5.0 (GraphPad Software Inc., San Diego, CA). Quantification values were presented as mean ± standard deviation (SD).

3. Results

3.1. A. gigantifolia extract inhibits the proliferation and arrests the cell cycle of gastric cancer cells

To evaluate the effect of *A. gigantifolia* extract on cell proliferation, gastric cancer cells were treated with different concentrations of the extract (from 10 to 500 μg/mL) for 48 h and then determined by MTT assay (Figure 1A). Results showed that the proliferation of MKN45 and MKN74 gastric cancer cells was significantly reduced in a dose-dependent manner ($P<0.05$, $P<0.001$). The IC_{50} values against MKN45 and MKN74 cells were 55.7 μg/mL and 123.6 μg/mL, respectively. At the IC_{50} concentration, a marked change in cell phenotype was observed in MKN45 cells with oval cells compared to the control, and the ability to adhere to the culture surface was decreased. Although there was a marked decrease in cell density on the surface of the culture plate, no significant phenotypic difference was observed in the MKN74 cell line (Figure 1B). The IC_{50} concentration of *A. gigantifolia* extract was then used for further experiments. Figure 1C shows a marked change in the percentage of cells in each phase of the cell cycle after treatment with *A. gigantifolia* extract compared to the control. Treatment with *A. gigantifolia* extract caused cell cycle arrest at the G_0/G_1 phase ($P<0.05$) in MKN45 and MKN74 cells.

3.2. A. gigantifolia extract inhibits the formation and growth of tumorspheres

Tumor formation is one of the key features of CSC compared to other cancer cells. In this study, gastric cancer cell lines MKN45 and MKN74 were cultured in a serum-free medium and supplemented with growth factors EGF and FGF to give CSC the ability to produce tumorsphere. Figure 2A and 2B show that after treatment with *A. gigantifolia* extract, most MKN74 cells lost the ability to generate tumospheres. Whereas the number of tumorsphere formed from MKN45 cells decreased by about 50% compared with the control. In addition, the tumorspheres that were formed in the cell medium treated with *A. gigantifolia* extract were significantly smaller in size than the control ($P<0.05$) (Figure 2C). Thus, it can be seen that the *A. gigantifolia* extract significantly reduced the tumorpheres in both quantity and size.

3.3. Effects of A. gigantifolia extract on the expression of key genes related to cell cycle control

To evaluate the effect of *A. gigantifolia* extract on the expression of several genes that play an important role in cell cycle control, real-time PCR and immunofluorescence analyses were performed. Figure 3A shows that after 48 h of treatment, there was a significant increase in the expression level of *p21* ($P<0.001$) in MKN45 cells. There was no difference in the expression levels of the genes *p53*, *CCND1*, *CCNE1* and *PCNA* between *A. gigantifolia* extract-treated and non-treated cells. p21 was expressed in the nucleus. Alteration of p21 protein levels was measured by immunofluorescence analysis in both 2D culture cells and 3D tumorspheres. Cells treated with *A. gigantifolia* extract exhibited significantly higher p21 (red) levels compared to control cells (Figure 3B and 3C). The increase in the expression of *p21* gene was also confirmed in MKN74 cells treated with *A. gigantifolia* extract in 2D culture cells and 3D tumorspheres (Figure 4).

3.4. A. gigantifolia extract reduces gastric CSC marker expression

The effect of *A. gigantifolia* extract on the expression of gastric CSC marker genes and the number of gastric CSC in 2D culture cells was evaluated on the MKN45 cell line after 48 h of treatment. Real-time PCR analysis results on 2D cultured cells (Figure 5A) showed that *A. gigantifolia* extract reduced the expression level

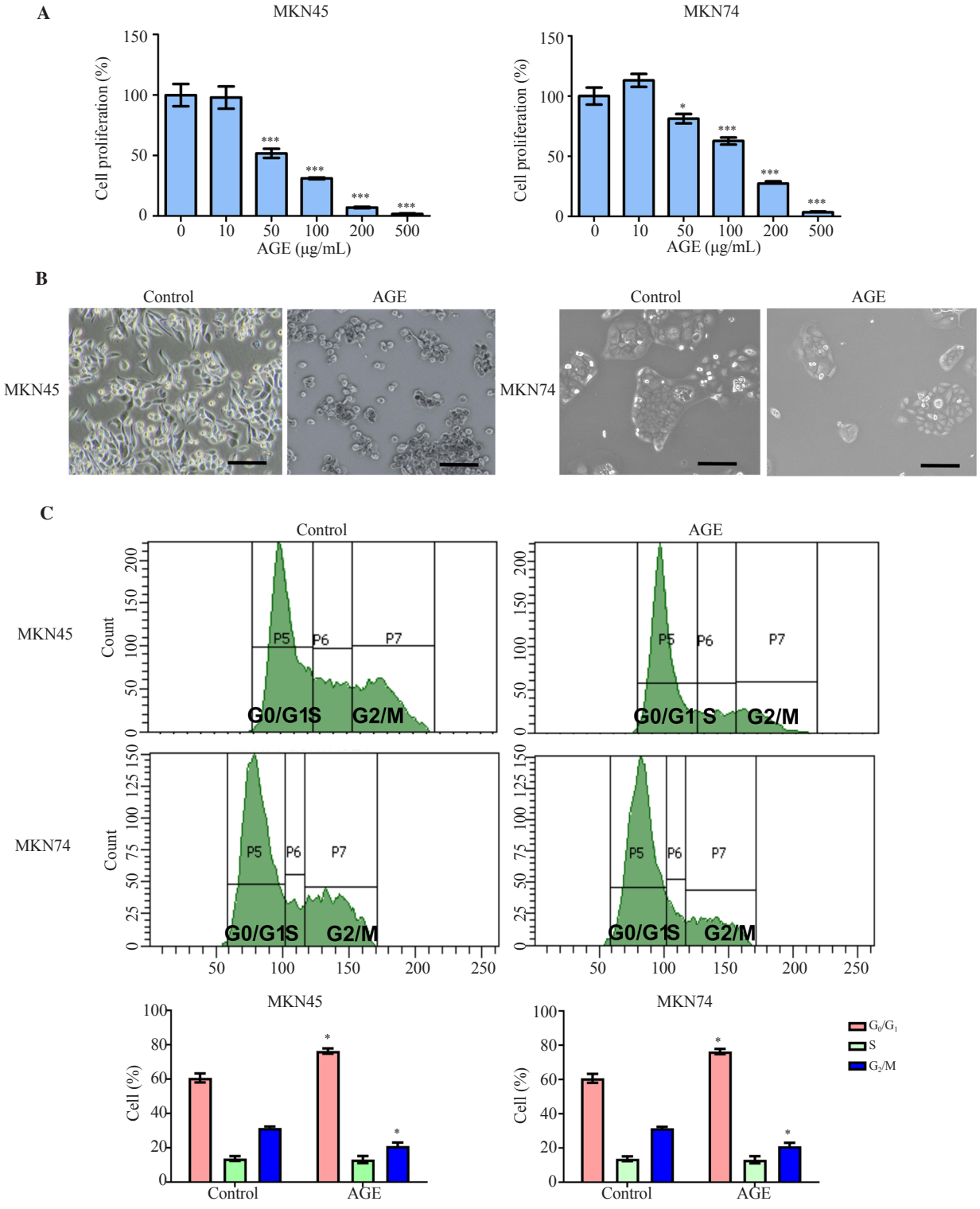

Figure 1. Effect of *Ardisia gigantifolia* extract (AGE) on the proliferation and cell cycle of MKN45 and MKN74 gastric cancer cells. (A) MKN45 and MKN74 cells were treated with AGE at concentrations from 0 to 500 μg/mL for 48 h, and cell proliferation was determined by MTT assay. The data are analyzed using one way ANOVA with Dunnett's test, $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the control. (B) Cell morphology was recorded using a microscope (Ts2, NIKON) at a magnification of ×200. Scale bar = 100 μm. (C) Phase changes in the cell cycle. The results are expressed as mean ± SD ($n = 3$) and analyzed using one way ANOVA with Bonferroni's multiple comparison test. $^{*}P < 0.05$ compared with the control.

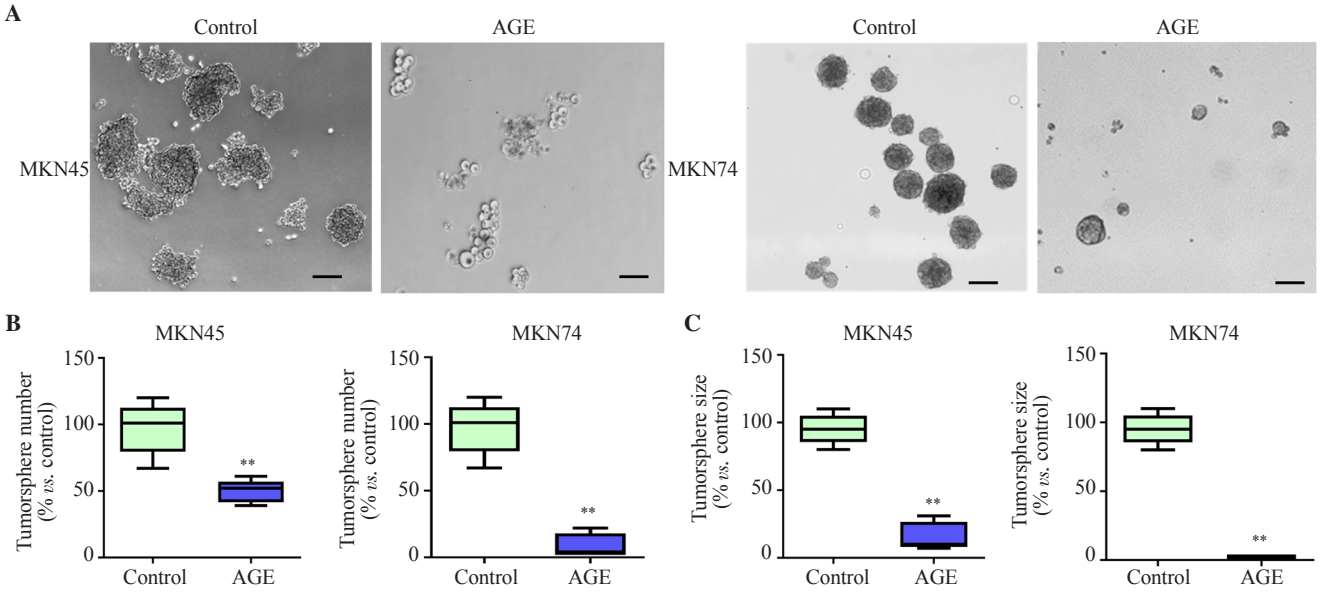

Figure 2. Effect of AGE on tumorsphere formation and growth. Cells were cultured in serum-free, non-adhesion conditions, and treated with AGE (at IC_{50} concentration) or DMSO (control). (A) Tumorsphere images were captured at ×200 magnification (scale bar = 100 μm). Change in (B) number and (C) size of tumorsphere, $10 < n < 30$. The results are analyzed by Mann-Whitney *U* test, $^{**}P < 0.01$ compared with the control.

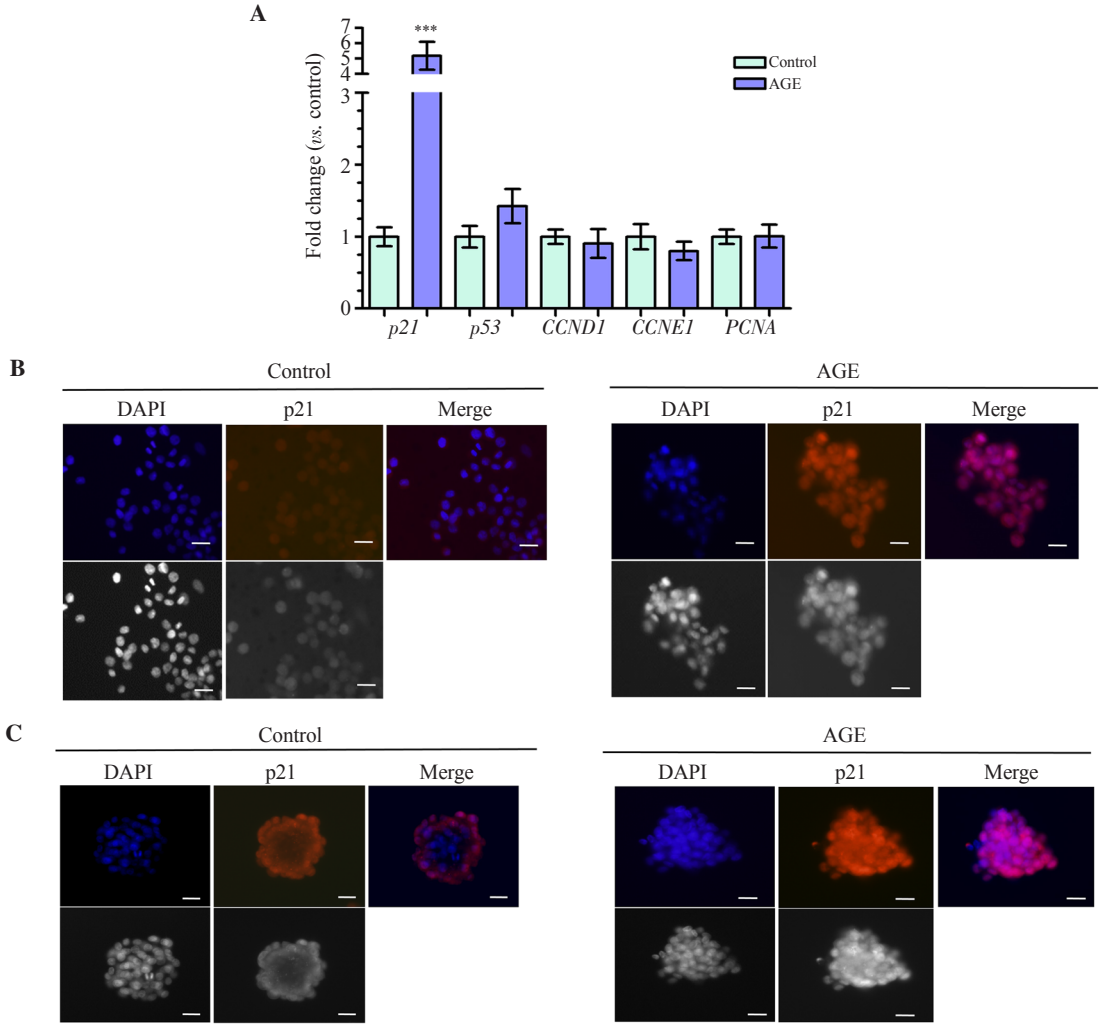

Figure 3. Effect of AGE on the expression of the genes that play an important role in cell cycle control of MKN45 cells. Cells were treated with AGE at concentrations of IC_{50} for 48 h. (A) The expression level of genes in AGE-treated MKN45 cells and control cells was determined using real-time PCR. The results are expressed as mean ± SD ($n = 3$) and analyzed by one way ANOVA with Bonferroni's multiple comparison test, $^{***}P < 0.001$ compared with the control. (B and C) The expression of p21 protein (red) in 2D culture cells and 3D tumorspheres. DAPI (blue) was used for cell nucleus staining. Magnification: ×200, scale bar = 50 μm.

of *CD44* gene to 0.57±0.10 compared to 1.00±0.28 in the control. Meanwhile, the expression level of *ALDH* was reduced to 0.36±0.08 compared to 1.00±0.23 in the control (P<0.05). A significant decrease in the CD44-positive cells (72.2±6.3)% and ALDH-positive cells (14.1±2.7)% after *A. gigantifolia* extract treatment compared to the control [(90.5±5.1)% for CD44 and (22.3±3.2)% for ALDH] was also shown in flow cytometry analysis (P<0.05) (Figure 5B). In addition, immunofluorescence analysis on 2D culture cells stained with both CD44 antibodies and the ALDEFLUOR Kit for ALDH activity showed that the number of cells expressing CD44 (red) and ALDH (green) was significantly decreased compared to the control (P<0.05) (Figure 5C). Especially, in the 3D culture model, after 48 h of treatment with *A. gigantifolia* extract, the expression of CD44 and ALDH in the cells was significantly decreased. A significant decrease in the expression of *CD44* and *ALDH* genes was also confirmed in *A. gigantifolia* extract-treated MKN74 cells by real-time PCR and immunofuorescent analyses (Figure 6).

4. Discussion

Evaluation of the effect of *A. gigantifolia* extract on cell proliferation by MTT assay was a critical selection to study the anticancer potential of this plant. In this study, *A. gigantifolia* extract effectively inhibited cell proliferation with an IC_{50} value of 55.7 μg/mL for MKN45 and 123.6 μg/mL for MKN74. The Lauren classification system divides gastric cancer into two main types: diffuse and intestinal. MKN45 used in this study belongs to the diffuse type, while MKN74 belongs to the intestinal type. The results of this study showed that *A. gigantifolia* extract had a stronger effect on the proliferation of MKN45 cells compared to MKN74 cells.

This may be related to the differences in biological characteristics between these two types of gastric cancer cells. Chemical composition analyses indicated that *A. gigantifolia* is a saponin-rich plant, and these saponins may have anticancer activity[19–21]. Subsequent studies by Mu *et al*. showed that the saponin-rich extract from *A. gigantifolia* can inhibit the proliferation of lung cancer cells A549 and colon cancer cells HCT-8 and arrest the cell cycle of MCF7 breast cancer cells in the G_2/M phase[22,23]. Cell proliferation is regulated by the progression of the cell cycle phases, and changes in these phases can lead to changes in cell proliferation rate[24]. However, the effects of extracts and chemical compounds isolated from *A. gigantifolia* on the cell cycle of gastric cancer cells have not been clearly understood. In this study, we found that *A. gigantifolia*

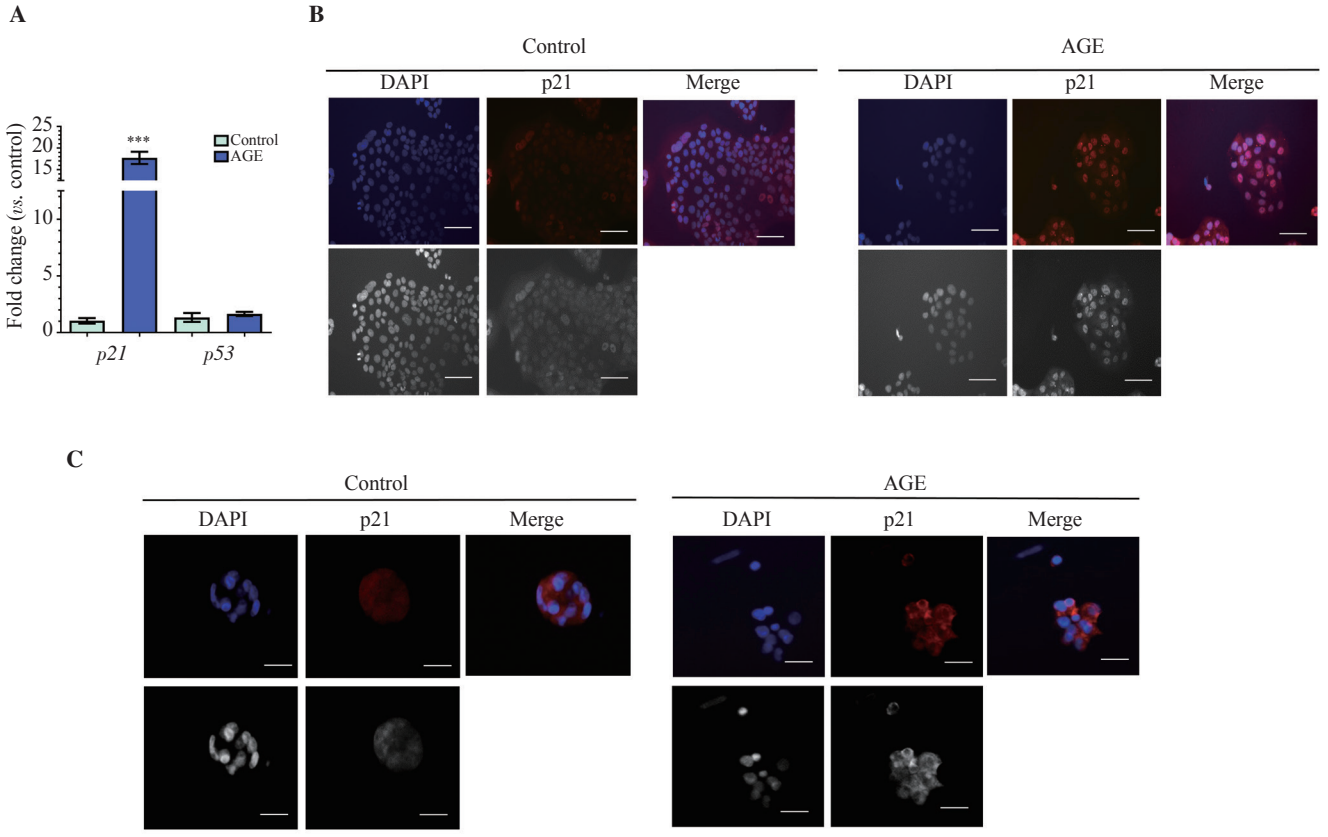

Figure 4. Effect of AGE on the expression of *p21* and *p53* genes in MKN74 cells. (A) The expression level of *p21* and *p53* genes in 2D culture cells. The results are expressed as mean ± SD (n = 3) and analyzed using one way ANOVA with Bonferroni's multiple comparison test, $^{***}P$ < 0.001 compared with the control. The immunofluorescence staining images of p21 protein in (B) 2D culture cells and (C) in 3D tumorspheres treated with AGE or DMSO. Scale bar = 50 μm.

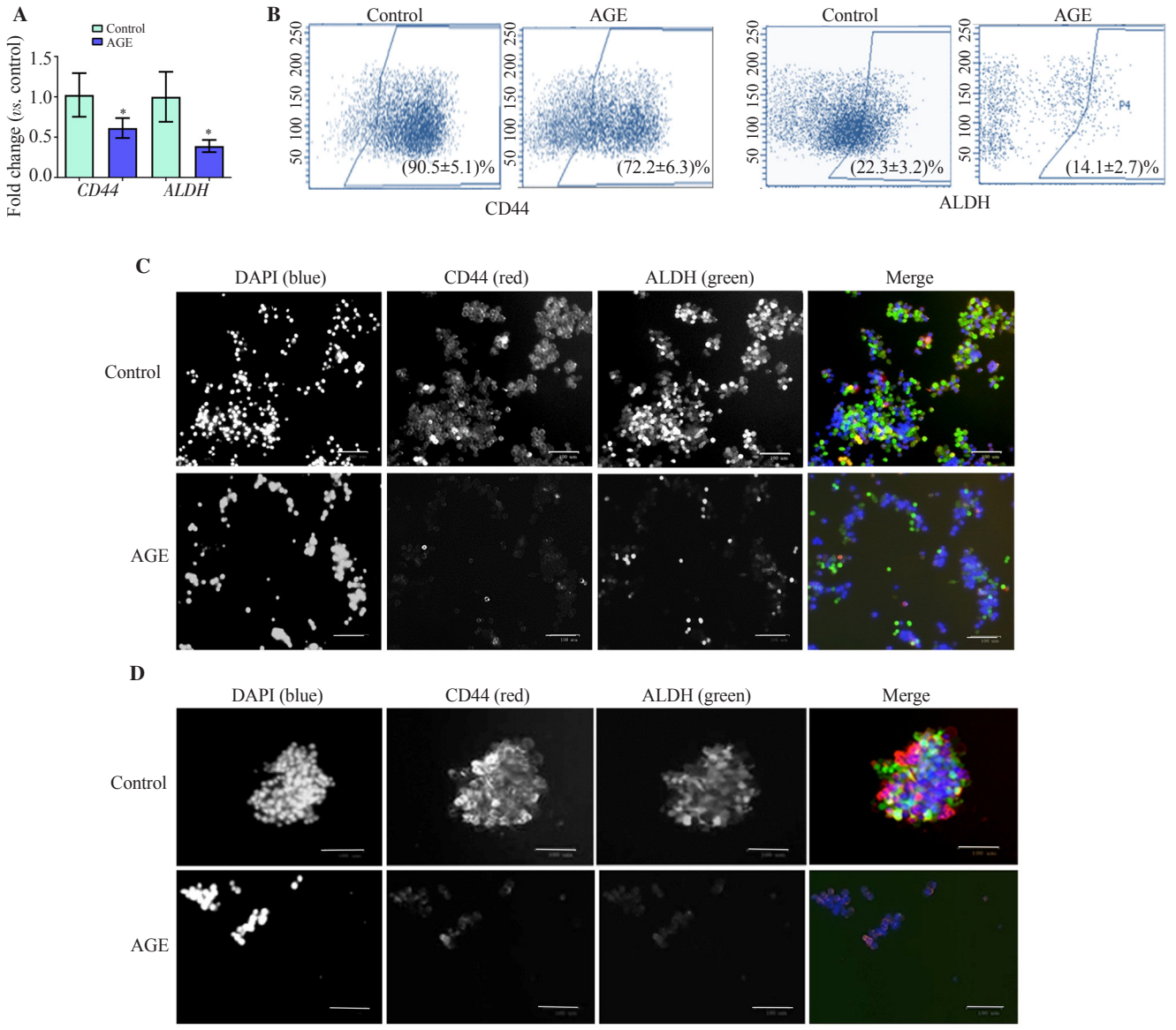

Figure 5. Effect of AGE on the expression of cancer stem cell marker genes and the number of gastric cancer stem cells in MKN45 cells. Cells were treated with AGE at concentrations of IC_{50} for 48 h. (A) The expression levels of *CD44* and *ALDH* genes in MKN45 cells. The results are expressed as mean ± SD and analyzed by one way ANOVA with Bonferroni's multiple comparison test, $^{*}P < 0.05$ compared with the control. (B) The percentage of cells expressing *CD44* and *ALDH* genes was measured by flow cytometry in a 2D culture model. (C) The immunofluorescence staining images of 2D cultured cells expressing CD44 (red) and ALDH (green) after 48 h of treatment with AGE or DMSO (control) (magnification: ×200; scale bar = 100 μm). (D) The expression of CD44 (red) and ALDH (green) by immunofluorescence staining in 3D tumorspheres treated or non-treated with AGE. DAPI (blue) was used for cell nucleus staining (magnification: ×200; scale bar = 100 μm).

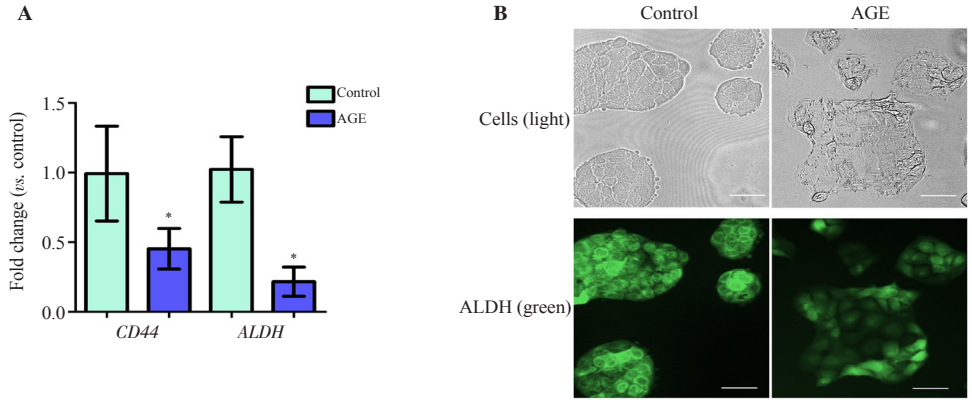

Figure 6. Effect of AGE on the expression of gastric cancer stem cell markers in MKN74 cells. (A) The expression level of *CD44* and *ALDH* genes in 2D culture cells. The results are expressed as mean ± SD and analyzed by one way ANOVA with Bonferroni's multiple comparison test. $^{*}P < 0.05$ compared with the control. (B) The immunofluorescence staining images of ALDH in 3D tumorspheres (magnification: ×200; scale bar = 50 μm).

extract arrested the cell cycle of MKN45 and MKN74 gastric cancer cells in the G_0/G_1 phase, thereby initially identifying the cause of the inhibition of cell proliferation. Data also showed that the expression level of several genes encoding the proteins that are responsible for controlling the cell cycle, such as p53 and p21, was altered when cells were treated with the *A. gigantifolia* extract in both 2D and 3D culture models.

We observed the elevated expression of *p21* gene at both mRNA and protein levels in the gastric cancer cell lines MKN45 and MKN74. p21 is known as a critical protein in the regulation of the cell cycle and cell proliferation. Induction of p21 protein expression leads to inhibition of the expression of cyclin kinases, which causes cell cycle arrest, as demonstrated in previous studies[25]. In mouse models of the *p21* gene knockout (*p21*-/*p21*-), there was markedly increased tumor formation in the cases of hepatocellular carcinoma[26], intestinal cancer[27], and prolactinoma[28]. Therefore, p21 protein has recently been proposed as a promising cancer target[26,29]. A previous study showed all-*trans*-retinoic acid as an agent targeting gastric CSC, and an enhancement of p21 expression was indicated in gastric cancer cells treated with all-*trans*-retinoic acid *in vitro* and *in vivo*[17]. In addition, p21 has also been involved in the differentiation and self-renewal of CSC. Increased expression of p21 is believed to inhibit mesenchymal-epithelial translocation and downregulate the expression of genes involved in breast CSC properties[29].

CSC is known as a small population in tumors but plays an important role in drug resistance and cancer recurrence[30,31]. Current studies have shown that killing these cells can reduce resistance and the recurrence of tumors[32], and tumorspheres formation and CSC marker expression are important features of CSC[33,34]. The 3D culture model has shown many advantages over the traditional culture model in current drug evaluation because it contains some similarities with *in vivo* evaluation[35-37]. Evaluating the effect of *A. gigantifolia* extract on the properties of gastric CSC is also an important goal of this study. Here, using 3D culture models to generate tumorspheres, we observed that *A. gigantifolia* extract inhibited the formation of tumorspheres, as well as reduced the size of the formed tumorspheres. Furthermore, using real-time PCR and flow cytometry analyses, we showed that the expression of CD44 and ALDH gastric CSC markers was decreased at both RNA and protein levels. Simultaneously, immunofluorescence staining analyses for 2D cells and 3D tumorspheres also indicated a significant reduction in the number of cells expressing these markers in both MKN45 and MKN74 cells. Previously, the effect of reducing tumorsphere production in 3D culture models has been used to evaluate the inhibitory effect of gastric CSC from a number of different drugs, such as all-*trans* retinoic acid[17] and metformin[38]. Zhu *et al.* showed that the extract of *Celastrus orbiculatus* reduced CSC properties in gastric cancer by targeting PDCD4 and EIF3H proteins[39]. An extract from *Aloysia polystachya* was also shown to reduce the number of CSC in colorectal cancer[40]. Thus, it can be seen that *A. gigantifolia* has the potential to inhibit cell growth as well as reduce the number of CSC in gastric cancer. This may be related to natural compounds present in this plant. However, the limitations of this study are that the chemical components related to the anti-gastric cancer activity of the extract have not been identified. In addition, in-depth analyses of the signaling pathways related to gastric CSC and evaluations of animal models with implanted tumors have not been performed. The combination of chemical composition analysis with screening of each potential compound is necessary to identify the important compounds that play a role in targeting gastric CSC.

Conflict of interest statement

The authors declare no competing interests.

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Authors' contributions

TTHL collected and analyzed data as well as prepared and revised the article. PHN supervised the work, designed and analyzed data, and revised the article. VPN and TNN reviewed and revised the article. All authors approved the final manuscript.

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