Original research article

Induction of caspase-mediated apoptosis using *Alnus japonica* extracts in AGS human gastric carcinoma cells

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ABSTRACT

Alnus japonica has been used as a traditional oriental medicine for many diseases such as fever, haemorrhage and alcoholism. In this study, *A. japonica* extracts were evaluated for their *in vitro* antioxidant potentials and anticancer effects in AGS human gastric carcinoma cell line. The antioxidant properties of *A. japonica* extracts were evaluated using several biochemical assays, including FRAP (ferric reducing antioxidant power) assay, ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), alkyl and hydroxyl radical scavenging activity assay. Our study showed that ethanol extract of *A. japonica* (AJE) has a more potent antioxidant activity than its water extract. In addition, AJE extract inhibited the cell growth and induced the cell death by increasing reactive oxygen species (ROS) production in AGS cells. Moreover, AJE extract specifically triggered the apoptosis mediated through the activation of caspase-8, 7, 3, and poly-ADP ribose polymerase (PARP). Thus, these results suggest that AJE extract could be potentially useful as a new promising strategy in the therapy for gastric carcinoma cancer.

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Introduction

There are many reports on the positive correlation between antioxidant activity and cytotoxicity in cancer cells of plant extracts, including *Plinia edulis, Mutellina purpurea* L., *Polygonum tinctorium* Lour., and *Sygygium fruticosum* Roxb (Heo et al., 2014; Sieniawska et al., 2013). Some plant extracts found to exhibit significant cytotoxicity in cancer cell lines are considered as potential anticancer agents. Such activity is ideally specific to the cancer cells but not to the normal cells. Gastric cancer is the second most common cause of cancer-related mortality worldwide and the fourth most prevalent cancer (Jemal et al., 2011). At present, surgery, radiation therapy, conventional chemotherapy, molecular targeted therapy, and biological therapy are used to manage gastric cancer; however, due to numerous side-effects, such as myelosuppression, hepatotoxicity, and immunosuppression, the curative effects of existing chemotherapeutic drugs are unsatisfactory (Ali et al., 2013; Leite de Oliveira et al., 2012). Therefore, it is getting important to search for new drugs from natural sources that have the capability of preventing and treating gastric cancer without or with minimum harmful side-effects to the patients. Several researchers have proposed to use traditional medicines, especially those derived from medicinal plants which could be used to increase the efficacy of anticancer drugs and decrease their toxicity or side effects (Zhang et al., 2013). Chemoprevention refers to administration of synthetic or naturally occurring agents to prevent the initiation and/or promotion of events associated with carcinogenesis and it has been increasingly appreciated as an effective approach for the management of neoplasia (Ahmad et al., 2001).

Apoptosis, or programmed cell death, is a mechanism by which cells die in response to DNA damage or to control cell proliferation. There are two major mechanisms of cell death such as necrosis and apoptosis. Cells that are damaged by external injury undergo necrosis; whereas, cells which are induced to commit programmed

death by internal or external stimuli undergo apoptosis (Ghobrial et al., 2005).

A. japonica, an indigenous *Alnus* species in Korea, North China, and Japan, is a deciduous, ovate elliptic-leaved tree that grows in wet lowlands and mountainous areas. The *Alnus* species has been recognised as a traditional Asian herbal medicine and it has been extensively used as a remedy for fever, haemorrhage, diarrhoea, and alcoholism (Lee, 1966). Previous studies on *A. japonica* extracts have led to the identification of numerous clinically relevant characteristics, including reduced atopic dermatitis (Joo et al., 2009), hepatoprotective effect and antioxidant effect (Kim et al., 2004). However, despite these protective properties, *A. japonica*'s ability to induce apoptosis in gastric carcinoma has not been studied. Therefore, the purpose of this study was to elucidate the molecular mechanism underlying the apoptosis induced by ethanol extract of *A. japonica* (AJE) in AGS human gastric carcinoma cells.

Materials and methods

Reagents

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as diammonium salt (ABTS), 2,4,6-tripyridyl triazine (TPTZ), 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH), (4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2′,7′-dichlorofluorescein-diacetate (DCFH$_2$-DA), and Hoechst 33342 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 were purchased from Hyclone (Thermo scientific, Logan, UT). Antibodies for Bax and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved caspase-8, 3, 7, and poly-ADP ribose polymerase (PARP) anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were obtained from Cell Signalling (Cell Signalling Technology, MA, USA). *A. japonica* was purchased from local herbal medicine market (Jecheon, Korea). All other reagents were of the highest grade available commercially.

Preparation of A. japonica extracts

Water and ethanol extracts of *A. japonica* bark were prepared as described below. Briefly, the water extract (AJW) was obtained by decocting *A. japonica* dried bark powder (100 g) with distilled water (1000 ml) for 2 h using an electric brewing pot (JS research Inc., Gongju, Korea) and ethanol extract (AJE) was prepared by shaking *A. japonica* dried bark powder (100 g) with 70% ethanol (1000 ml) for 1 day at 25 °C in a shaking incubator (Daewongbio, Goesan, Korea) for two times. Then, extracts were filtered through Whatman No. 41 filter paper under suction at room temperature. The filtered extracts were evaporated under reduced pressure using a rotary vacuum evaporator (EYELA, Tokyo, Japan) at 40 °C. After evaporation, the extracts were lyophilized in a freeze-dryer (Samwon, Busan, Korea) and stored at −20 °C until use.

ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation (ABTS+) scavenging

The ABTS assay was performed as described previously (Re et al., 1999) with slight modifications. The working solution was prepared by mixing the two stock solutions (7.4 mM ABTS solution and 2.6 mM potassium persulfate solution) in equal quantities and allowing them to react in the dark for 15 h at room temperature (R.T.). ABTS reagent (900 μl) was mixed with 100 μl of extract, and the absorbance was measured at 414 nm after 30 min of reaction time at R.T., using ethanol as the blank sample. Antioxidant activity

was expressed by Trolox equivalent antioxidant capacity (TEAC), as millimoles Trolox equivalents (mM Trolox eq/mg extract).

FRAP (ferric reducing antioxidant power) assay

FRAP assay was carried out by the method reported by Benzie & Strain (Benzie and Strain, 1996) with slight modifications. One milliliter of FRAP reagent (1 ml; mixture of 0.3 M acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl$_3$ (10:1:1 v/v/v) was mixed with 50 μl of extract and allowing them to react at 37 °C for 5 min. The absorbance was determined at 593 nm against distilled water as the blank. A calibration curve was obtained using an aqueous solution of ferrous sulphate (FeSO$_4$). The FRAP values were expressed as mM FeSO$_4$ equivalent per milligram of extract (mM FeSO$_4$ eq/mg extract).

Radical scavenging activity by ESR (Electron spin resonance) spectrometry

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured using an ESR spectrometer (JES-FA machine; JOEL, Tokyo, Japan) according to the technique described by Nanjo et al. (1996). Sample (30 μl) was added to 30 μl of DPPH (60 μM) in methanol. After 10 s of vigorous mixing, solutions were transferred to Teflon capillary tubes and inserted into the cavity of the ESR spectrometer. The spin adducts were determined by ESR spectrometer exactly after 2 min.

In addition, the alkyl radicals were generated by AAPH. The phosphate-buffered saline (PBS) reaction mixture containing 40 mM AAPH and 40 mM 4-POBN was incubated with different concentrations of samples at 37 °C in a water bath for 30 min and then they were transferred to 100 μl Teflon capillary tubes and spin adducts were analysed.

The hydroxyl radicals were generated via the Fenton reaction and reacted rapidly with nitrone spin trap DMPO. The resulted DMPO-OH adducts were detected using an ESR spectrometer. The reaction mixture containing 100 μl of 0.3 M DMPO, 100 μl of 10 mM FeSO$_4$, and 100 μl of 10 mM hydrogen peroxide (H$_2$O$_2$) was mixed with the samples and then transferred to Teflon capillary tubes. The spin adducts were measured by an ESR spectrometer exactly after 2.5 min.

Determination of total polyphenol and total flavonoid contents

Total polyphenol content (TPC) was determined using Folin–Ciocalteu assay (Singleton et al., 1999). Total flavonoid content (TFC) was determined by aluminum colorimetric method (Zhishen et al., 1999) with slight modifications. Briefly, the samples were individually dissolved in distilled water (100 μg/ml). Then, 50 μl of these solutions were mixed with 250 μl of distilled water and 15 μl of 5% NaNO$_2$ and allowed to react for 5 min. Following this, 30 μl of 10% AlCl$_3$ was added, and the mixture was allowed to stand for 6 min. Finally, 100 μl of 1 M NaOH and 55 μl of distilled water were added to the reaction mixture. After 10 min incubation at ambient temperature, the absorbance of the supernatant was measured at 510 nm against the blank in the spectrophotometer (Tecan, Mannedorf, Switzerland). The TFC was calculated based on the calibration curve of catechin. Total flavonoid contents were expressed as milligram catechin equivalents per micro gram of dry extract (mg CE/μg extract).

Cell culture

Human normal hepatic (Chang), human gastric epithelial carcinoma (AGS), human gastric polygonal carcinoma (SNU-1967), and human gastric epithelial carcinoma (SNU-601) cell

lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Korean Cell Line Bank (KCLB, Seoul, Korea). Chang cells were maintained in DMEM with 10% inactivated FBS and 1% penicillin-streptomycin-neomycin at 37 °C in a humidified incubator containing 5% CO_2. AGS, SNU-1967, and SNU-601 cells were grown in RPMI-1640 medium supplemented with 10% inactivated FBS, 2.05 mM L-glutamine, and 1% penicillin-streptomycin-neomycin at 37 °C in a humidified incubator containing 5% CO_2. Cultures were harvested, and cell numbers were counted using a hemocytometer. Experiments were determined using trypan blue exclusion and examined using phase contrast microscopy.

Cell viability analysis

The cytotoxicity assay was performed using Thiazolyl Blue Tetrazolium Bromide method as described with minor modifications. Briefly, Chang cells (1.5×10^4 cells/well), AGS cells (2.0×10^4 cells/well), SNU-1967 cells (2.0×10^4 cells/well), and SNU-601 cells (2.0×10^4 cells/well) were seeded in 48-well microtiter plates and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) for 12, 24, and 36 h, respectively. Doxorubicin was employed as a positive control. Following 12, 24, and 36 h of cell treatment, treatment medium was completely removed from all wells and 200 μl of fresh media supplemented with 8 μl of MTT stock solution (50 mg/ml) was added to each well and incubated for 3 h at 37 °C. Subsequently, the resulted formazan crystal products were dissolved by the addition of 200 μl of DMSO. The absorbance at 540 nm was measured using a microplate reader (SpectraMax M2/M2e, CA, USA).

Observation of morphological changes

AGS cells (4×10^5 cells/well) were seeded in 60 mm dishes, incubated for 12 h, and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) as a positive control for 24 h. Cellular morphology was observed using a phase contrast microscope (Nikon, Japan).

Cell cycle analysis by flow cytometry

Cellular DNA levels were determined using flow cytometry analyses of propidium iodide (PI)-labeled cells (Kim et al., 2012). In brief, AGS cells (4×10^5 cells/well) were seeded into 60 mm dishes, incubated for 12 h, and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) for 24 h. After incubation, cells were washed twice with PBS and re-suspended in PBS containing 1% PI and 0.1% ribonuclease A (RNase A) at 37 °C for 30 min. FACSCalibur flow cytometer (Becton & Dickinson Co., USA) was used for the flow cytometry analyses. The cell cycle analysis was performed using CellQuest software (BD Biosciences).

Observation of nuclear morphologic changes using Hoechst 33342 staining

Nuclear Hoechst 33342 staining was used to investigate changes in the nuclear morphology of apoptotic cells. AGS cells (1×10^4 cells/well) were seeded on an 8 well chamber slide, incubated for 12 h, and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) for 24 h. The cells were washed twice with PBS and then fixed in PBS containing 4% paraformaldehyde for 30 min at R.T. The fixed cells were washed with PBS containing 0.02% tween 20. Cells were stained with Hoechst 33342 (1 μg/ml) in the dark for 20 min at R.T. Cells were then washed thrice with PBS and the Hoechst 33342-stained nuclei were visualized under a fluorescence microscope (Carl Zeiss, UY, USA).

Annexin V-FITC/PI apoptosis assay

Cellular DNA levels were determined using flow cytometry analyses of PI-labeled cells using the annexin V-FITC apoptosis detection kit (BD Bioscience, CA, USA). AGS cells (4×10^5 cells/well) were seeded in 60 mm dishes, incubated for 12 h, and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) for 24 h. Cells were harvested, washed twice with PBS, and resuspended in 500 μl of binding buffer at a concentration of 1×10^6 cells/ml. The Cells were then incubated with 5 μl of annexin V-FITC and 5 μl of PI in the dark for 15 min at ambient temperature and analysed by FACSCalibur flow cytometer (Becton & Dickinson Co., USA). Events were recorded statistically (10,000 events/sample) using CellQuest software (BD Biosciences).

Measurement of intracellular ROS by flow cytometry

Intracellular ROS accumulation was detected *via* flow cytometry using the cell-permeable fluorogenic probe $DCFH_2$-DA, which is a stable nonpolar dye which can easily diffuse into the cell and it can be hydrolyzed by intracellular esterase yielding 2′,7′-dichlorodihydrofluorescin (DCFH) within the cell. $DCFH_2$-DA is non-fluorescence, but when this reagent is oxidised by ROS, it becomes green fluorescence. Briefly, AGS cells (4×10^5 cells/well) were seeded in 60 mm dishes, incubated for 12 h, and then exposed to different concentrations of AJE extract and doxorubicin (2 μM) for 24 h. After treatment, the cells were incubated with medium containing 10 μM $DCFH_2$-DA at 37 °C for 30 min. Then, the cells were harvested and re-suspended in 1 ml of PBS. Samples were analysed by FACSCalibur flow cytometer (Becton & Dickinson Co., USA). Data were analysed using CellQuest software (BD Biosciences).

Western blot analysis

After treatment, cells were washed once with PBS and then lysed using ice-cold RIPA buffer. Cell lysates were centrifuged at 13,000g for 30 min at 4 °C. Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% (v/v) nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) at R.T. for 1 h. This was followed by an overnight incubation of membrane at 4 °C with 1:1000 dilution of protein-specific primary antibodies (Bax, Bcl-2, cleaved caspase-8, 7, 3, PARP and cleaved PARP). The membrane was washed thrice with TBST for 5 min each. The membrane was incubated again with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at R.T. for 1 h and washed again three times with TBST buffer. The membrane was then incubated with ECL substrate solution for 5 min and visualised with autoradiography film. The imaging program Luminescent image analyser (LAS-3000; Fujifilm, Tokyo, Japan) was used for quantification.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Experimental data were analysed statistically using one-way analysis of variance (ANOVA) for multiple group comparisons. A probability value of $^*p < .05$, $^{**}p < .01$, or $^{***}p < .001$ were considered statistically significant. And data were analyzed by two-tailed unpaired t-test. Values of $p < .05$ were considered statistically significant. Each experiment was performed in triplicates (GraphPad-Prism Software Inc., San Diego, CA).

Results and discussion

The antioxidant properties of A. japonica extracts

Many concrete pieces of evidence have demonstrated that natural products have the capacity to be an important source of antioxidants which prevent many diseases including carcinogenesis (Milaeva, 2011).

Secondary plant metabolites, especially phenolic compounds have been found to be strong antioxidants. Antioxidants could scavenge or suppress the reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation and enhance the antioxidant defence systems in the body; consequently, it could lead to preventing carcinogenesis (Halliwell, 2007).

The antioxidant capacities of *A. japonica* water and ethanol extracts are shown in Table 1. Trolox equivalent antioxidant capacity (TEAC) is measured through hydrogen donors, which can terminate oxidation process by converting free radicals into stable forms (Re et al., 1999). According to our data, there is no significant different between ABTS radical cation scavenging activities of water and ethanol extracts of *A. japonica* (Table 1). The FRAP assay is commonly used to evaluate antioxidant capacity due to its rapid, simple, and sensitive nature. The antioxidant potentials of processed and unprocessed extracts are estimated from their ability to reduce the TPTZ-Fe (III) complex to a TPTZ-Fe (II) complex. In this study, the ethanol extract showed higher FRAP antioxidant activity than the water extract. Moreover, various radical scavenging activities against DPPH, alkyl and hydroxyl radicals were estimated using an ESR spectrometer. DPPH has been widely used to evaluate various antioxidant substances' free radical scavenging abilities (Kim et al., 2014). As shown in Table 1, DPPH assay half maximal inhibitory concentrations (IC_{50}) of water and ethanol extracts were 5.0 ± 2.0 and 4.0 ± 1.0 μg/ml, respectively. Thus, the both water and ethanol extracts displayed greater antioxidant activity. The alkyl radical spin adducts of 4-POBN/free radicals were generated from AAPH, and a decrease in ESR signals

Table 1
Extraction yields, FRAP antioxidant activity, various radical scavenging activity (ABTS, DPPH, alkyl, and hydroxyl), and total polyphenol and flavonoid content of *A. japonica* extracts.

Solvent	Extraction yields (% W/W)	TEAC (mM Trolox eq./mg extract)	FRAP (mM $FeSO_4$ eq./mg extract)	DPPH radical scavenging activity IC_{50} (mg/ml)	Alkyl radical scavenging activity IC_{50} (mg/ml)	Hydroxyl radical scavenging activity IC_{50} (mg/ml)	Total polyphenol content (mg GAE/100 μg extract)	Total flavonoid content (mg CE/100 μg extract)
Water	4.69	0.936 ± 0.00	2.042 ± 0.03	0.005 ± 0.002	0.020 ± 0.004	0.441 ± 0.001	97.651 ± 0.364	0.019 ± 0.001
Ethanol	3.77	0.924 ± 0.01^{ns}	$5.626 \pm 0.01^{***}$	0.004 ± 0.001^{ns}	0.016 ± 0.002^{ns}	$0.459 \pm 0.002^{***}$	$122.492 \pm 0.765^{***}$	$0.036 \pm 0.000^{***}$

GAE: Gallic acid equivalents, CE: Catechin equivalents, TEAC: Trolox equivalent antioxidant capacity, FRAP: ferric reducing antioxidant power.
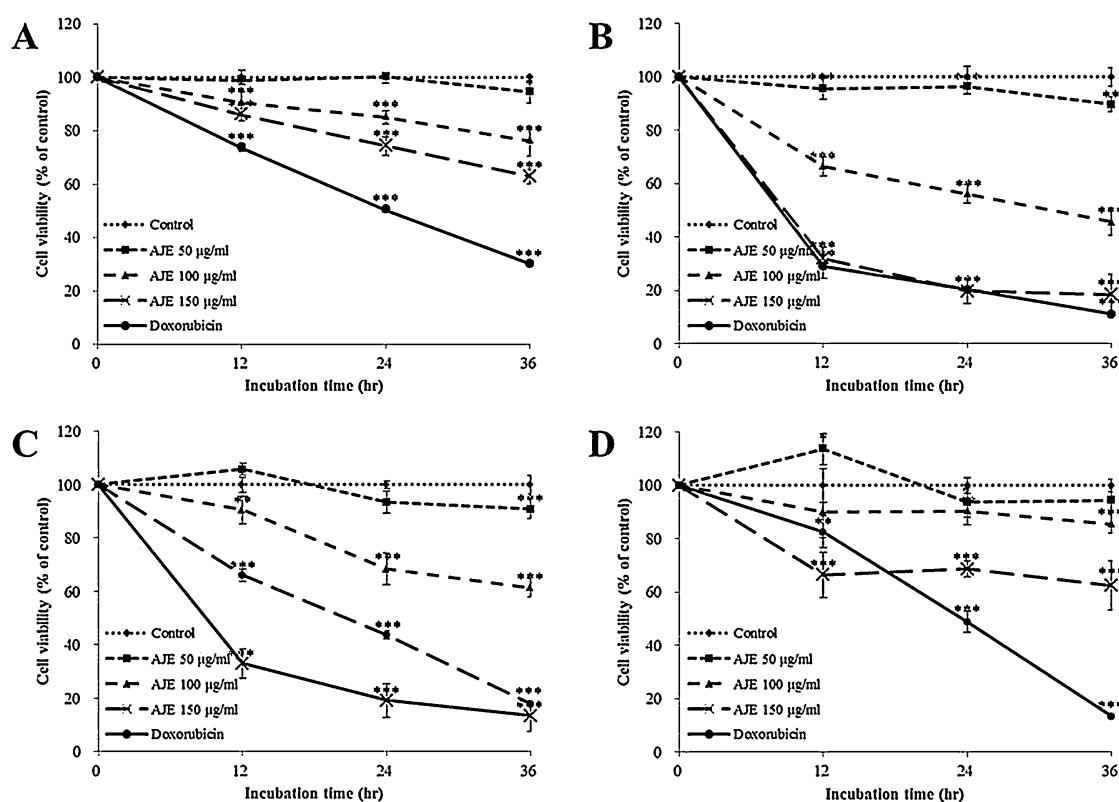Each value is the mean $\pm$ SD of three determinations (***$p < .001$ vs. water extract).

Fig. 1. Dose-dependent and time-dependent effects of *Alnus japonica* ethanol (AJE) extract on the cell viability of various cell lines. (A) Chang human normal hepatic cells; (B) AGS human gastric carcinoma cells; (C) SNU-1967 human gastric carcinoma cells; (D) SNU-601 human gastric carcinoma cells. Results are expressed as the percentage of the cell viability relative to the viability of control. Each value is the mean $\pm$ SD of three determinations (*$p < .05$, **$p < .01$ and ***$p < .001$ vs. control).

was observed with the dose-dependent increases in both water and ethanol extracts. Ethanol extract exhibited stronger alkyl radical scavenging activity than water extract. The hydroxyl radicals generated in a Fe^{2+}/H_2O_2 system were trapped by a DMPO forming spin adduct, which could be detected by an ESR spectrometer, and the typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed. The IC_{50} values of hydroxyl radical scavenging activity of water and ethanol extracts were 441.0 ± 1.0 and 459.0 ± 2.0 μg/ml respectively.

These results indicate that ethanol extract from *A. japonica* effectively scavenged various reactive radicals.

Total polyphenol and flavonoid contents

A. japonica water and ethanol extraction yields were 4.69 and 3.77 (% w/w) respectively (Table 1). The total polyphenol contents of water and ethanol extracts were 97.651 ± 0.364 mg GAE/100 μg and 122.492 ± 0.765 mg GAE/100 μg respectively. Similarly, the total flavonoid content of ethanol extract was markedly higher than that of water extract (Table 1). Our results showed that ethanol extract with higher total polyphenol and flavonoid contents than water extract. Recent evidence demonstrated that phenols, namely flavonoids, appear to be beneficial compounds in various stages of carcinogenesis (Clere et al., 2011).

Effects of AJE extract on the cell viability

The effects of AJE extract on the cell viability of human tumor and non-tumour cells were determined as shown in Fig. 1. AJE extract reduced the cell viability in time and dose-dependent manners.The IC_{50} values of 24 h treatment were >150 μg/ml in Chang cells (Fig. 1A), 116.99 μg/ml in AGS cells (Fig. 1B), 137.38 μg/ml in SNU-1967 cells (Fig. 1C), and >150 μg/ml in SNU-601 cells (Fig. 1D). More precisely, AJE extract profoundly reduced the cell viability in AGS cells compared to Chang, SNU-1967, and SNU-601 cells. When the cells were treated with

ascending concentration range of AJE extract from 50 to 150 μg/ml for 24 h, the cell viability (% of control) of Chang cells (non-tumorigenic) was decreased from 100.32 ± 0.95% to 74.42 ± 3.48%, whereas the cell viability of AGS cells (tumorigenic) was reduced from 96.40 ± 2.75% to 19.68 ± 4.57%. As a positive control, all four cell lines used in this study were treated with doxorubicin, a cancer chemotherapy drug. These results indicated that AJE extract might be less cytotoxic to non-tumorigenic cells. The present data also suggested that AJE extract has stronger and broader inhibitory effects on the growth of cancer cells than non-tumorigenic cells. Similar to our results, it had been reported that the aqueous extract of *Plinia edulis* leaves, which contain high amounts of polyphenols and flavonoids possess cytotoxic activity and inhibit cell growth of MCF-7 cells (Carvalho et al., 2012). Based on the cell viability analysis, we selected the doses and times for the following experiments.

Morphologic changes and cell death in AGS cells

In the present study, we examined the morphological changes and the cell death induced by AJE extract in AGS cells. Following the cell exposure to toxicants, the most readily observed effect is a morphological alteration in the cell-layer and/or cell-shape in monolayer cultures (Ekwall et al., 1990). The effect of AJE extract on the cell morphology of AGS cell line was markedly induced in a dose-dependent manner (Fig. 2). The AJE extract induced significant morphological abnormalities in AGS cells, which were characteristic features of cell death including cell shrinkage, detachment from neighbouring cells and reduced cell density (Fig. 2A). Cell membrane integrity assessment and counting of living and dead cells were performed using trypan blue dye exclusion technique. This dye does not enter living cells, but it passes through the membranes of dead cells. The AGS cell number (10^5) was significantly decreased by AJE extract treatment in a dose-dependent manner after exposing to 24 h (control; 15.5 ± 0.9, AJE 50 μg/ml; 14.6 ± 0.9, AJE 100 μg/ml; 7.4 ± 0.9,
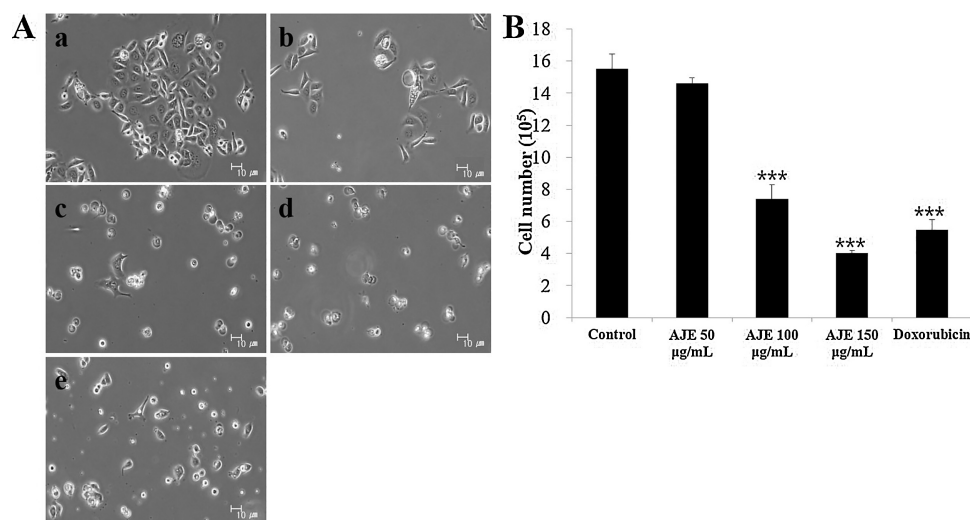

Fig. 2. Induction of morphologic changes and cell death by *Alnus japonica* ethanol (AJE) extract in AGS cells. (A) Observation of morphological changes. Cells were treated with (a) 0, (b) AJE 50 μg/ml, (c) AJE 100 μg/ml, (d) AJE 150 μg/ml, and (e) doxorubicin (2 μM), and morphological changes were monitored after 24 h. Morphological changes were observed under a phase-contrast microscope. Photographs were taken using a phase-contrast microscope at 100 × magnification. Scale bar: 10 μm. (B) Direct living cell counting was performed by trypan blue exclusion assay. Results are expressed as the cell number (10^5). Each value is the mean ± SD of three determinations (***$p < .001$ vs. control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
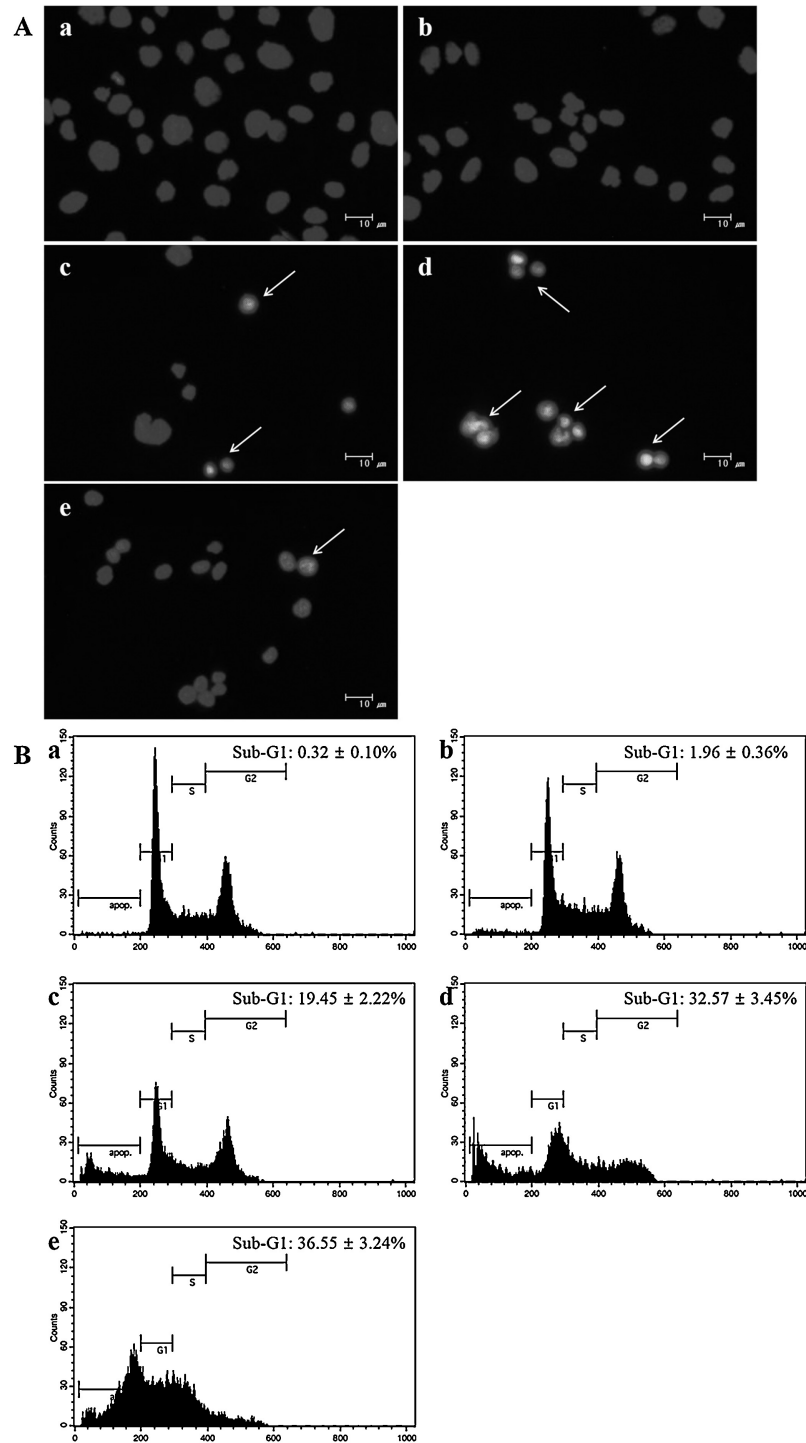
Fig. 3. *Alnus japonica* ethanol (AJE) extract causes AGS cell accumulation in the sub-G1 phase of the cell cycle. The Cells were treated with (a) 0, (b) AJE 50 μg/ml, (c) AJE 100 μg/ml, (d) AJE 150 μg/ml, and (e) doxorubicin (2 μM) for 24 h. (A) Morphological analysis of the nucleus was evaluated by Hoechst 33342 staining. Condensed and fragmented nuclei were observed under a fluorescent microscope. Photographs were taken using a fluorescence microscope at 200 × magnification. The arrows indicate chromatin condensation and nuclear fragmentation. Scale bar: 10 μm. (B) The effect of AJE extract on AGS cell cycle distribution. Histograms show the cell number per channel (vertical axis) vs. the DNA content (horizontal axis).
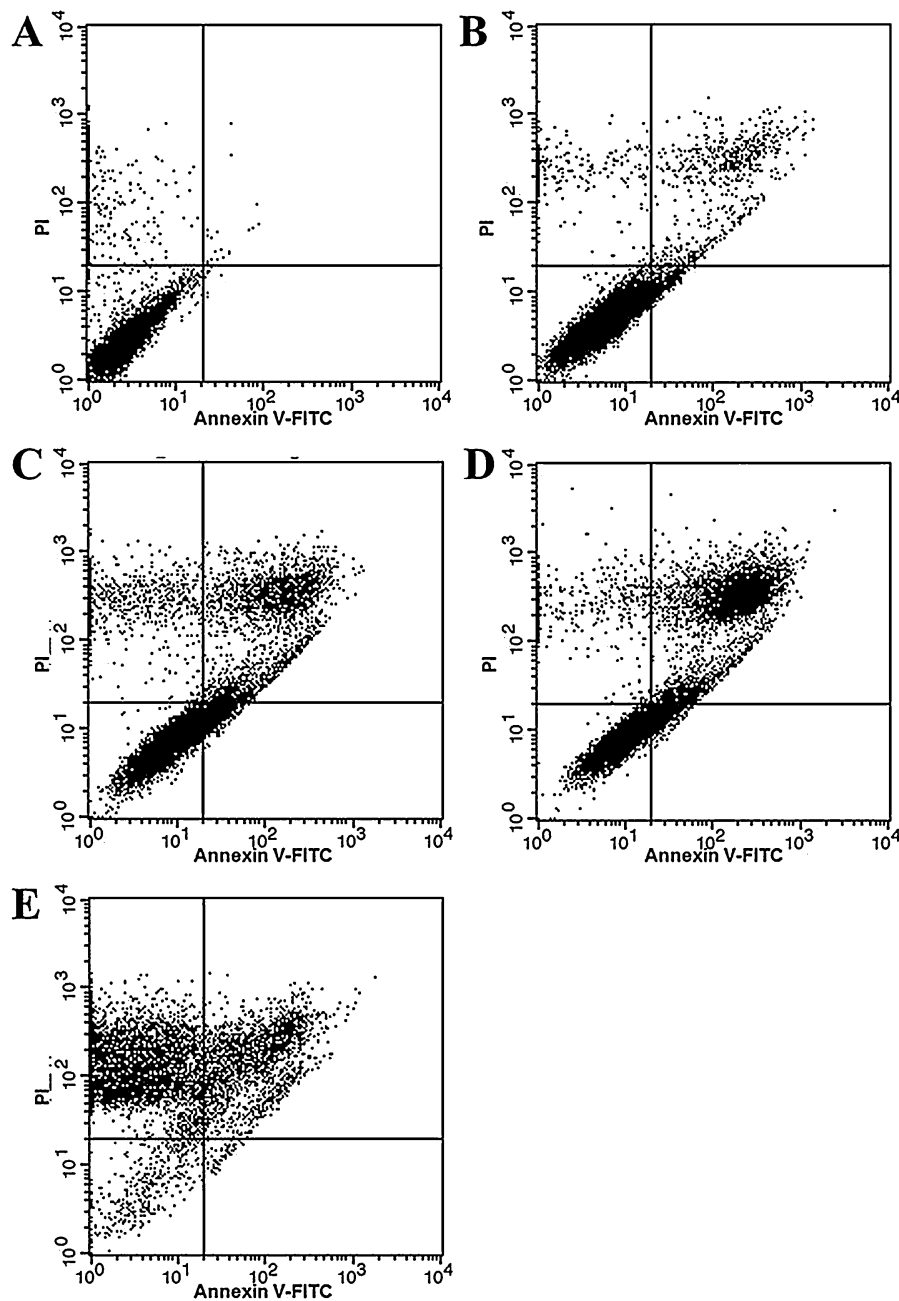
Fig. 4. The effect of *Alnus japonica* ethanol (AJE) extract on annexin V-FITC and propidium iodide (PI) counterstaining. The cells were treated with (A) 0, (B) AJE 50 μg/ml, (C) AJE 100 μg/ml, (D) AJE 150 μg/ml, and (E) doxorubicin (2 μM) for 24 h. Annexin V-FITC/PI double staining was analyzed by flow cytometry. Histograms show the cell number per PI fluorescence (vertical axis) vs. the cell number per annexin V-FITC fluorescence (horizontal axis).

AJE 150 μg/ml; 4.0 ± 0.2) (Fig. 2B). Doxorubicin is commercial anticancer compound. These results demonstrate that AJE extract induces morphological changes and cell death in AGS cells.

Effects of AJE extract on the cell cycle

Flow cytometric analysis and Hoechst 33342 staining assay were performed to analyse the AJE extract induced AGS cell death mediated through the induction of apoptosis. Our results revealed that untreated control cells displayed normal, round nuclei, while

cells treated with high concentration of AJE extract exhibited more condensed and fragmented chromatin and bright blue nuclei (Fig. 3A). All the above observations indicate that AJE extract could induce apoptosis or necrosis in AGS cells. In general, when the cells are exposed to toxicants, there should be a distinct increase in the percentage of cells with a sub-G1 DNA content, which is considered to be an indicator of cell damage (Carvalho et al., 2012). In this study, we examined the influence of AJE extract on cell cycle distribution using flow cytometry. Apoptotic nuclei were observed as a sub-diploid DNA peak (sub-G1) and they were

distinguished from cell debris based on PI fluorescence and forward light scatter (Fig. 3B). The percentage of cells in the sub-G1 phase was increased from 0.32% (control) to 1.96% (50 μg/ml), 19.45% (100 μg/ml) and 32.57% (150 μg/ml) following 24 h exposure of AJE extract. These results suggest that AJE extract causes cell accumulation in the sub-G1 phase of the cell cycle.

Effects of AJE extract on apoptosis in AGS cells

In the present study, we performed the annexin V-FITC/PI staining assay to further confirm the apoptosis induced by AJE extract. As shown in Fig. 4, AJE extract induced apoptosis in AGS cells in a dose-dependent manner. When the cells were exposed to AJE extract at different concentrations for 24 h, the proportion of AV+/PI- (early apoptosis) cells was increased from 0.09% to 9.09%, and the number of AV+/PI+ (late apoptosis or necrosis) cells was increased from 0.2% to 44.3% (Fig. 4). These results suggest that AJE extract effectively induces apoptosis in AGS cells.

Cellular ROS generation

Recently, several researchers have compared normal and cancer cells under increased oxidative stress. Oxidative stress is often associated with alterations in metabolic activities and increased ROS generation. In addition, elevated ROS in cancer cells have significant consequences, such as the promotion of mutations, stimulation of cellular proliferation, genetic instability and alterations in cellular sensitivity to anticancer agents and apoptosis (Behrend et al., 2003; Kang and Hamasaki, 2003). In the present study, we also examined the intracellular ROS generation induced by AJE extract treatment at various concentrations in AGS cells. The purpose of this assessment was to determine whether AJE extract-induced apoptosis is associated with elevated ROS levels. As shown in Fig. 5, AGS cells incubated with AJE extract for 24 h displayed a significant, does-dependent increase in DCF fluorescence. Therefore, these results strongly suggest that AJE extract promotes intracellular ROS generation which plays an essential role in the induction of apoptotic cell death in AGS cells.

Activation of caspases and PARP in AGS cells

We examined the expression levels of pro-apoptotic family protein Bax and pro-survival family protein Bcl-2 to investigate whether these proteins are involved in regulating AJE extract-induced apoptosis in AGS cells. The anti-apoptotic Bcl-2 protein family acts as a repressor of apoptosis by blocking the release of cytochrome-c, whereas the pro-apoptotic protein family acts as a promoter of the extrinsic and intrinsic apoptotic pathways. These effects are more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone (Reed, 1997). In the present study, however, AJE extract treatment could not significantly alter the
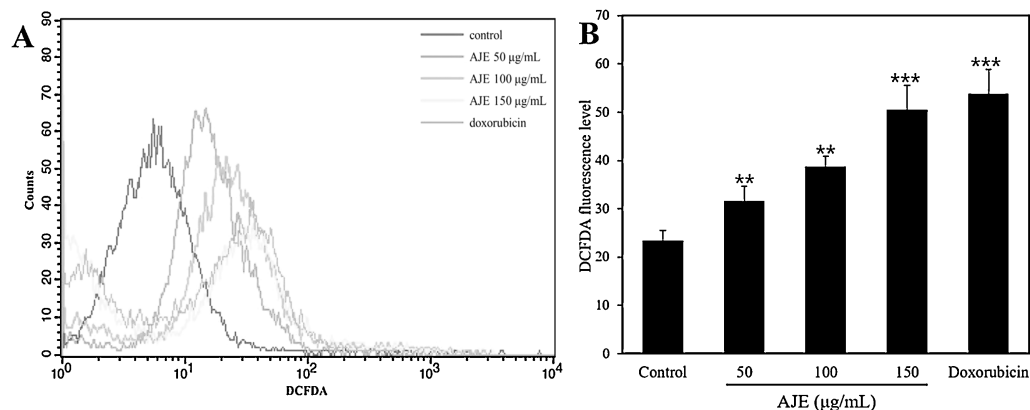

Fig. 5. The effect of *Alnus japonica* ethanol (AJE) extract on intracellular ROS levels in AGS cells. (A) Histograms show the cell number per channel (vertical axis) vs. the (2′,7′-dichlorofluorescein diacetate) DCFDA fluorescence (horizontal axis). (B) ROS levels expressed as the mean ± SD intensity of cell fluorescence. **$p < .01$, ***$p < .001$ vs. control.
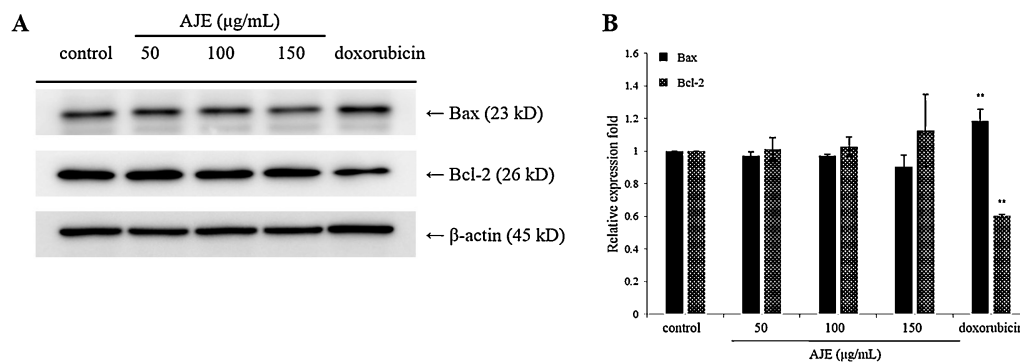

Fig. 6. The effect of *Alnus japonica* ethanol (AJE) extract on the expression levels of Bax and Bcl-2 proteins in AGS cells. The cells were treated with 0, 50, 100, or 150 μg/ml of AJE extracts and doxorubicin (2 μM) for 24 h. The Cell lysates were prepared and subjected to western blot analysis. Each value is the mean ± SD of three determinations (**$p < .01$ vs. control).
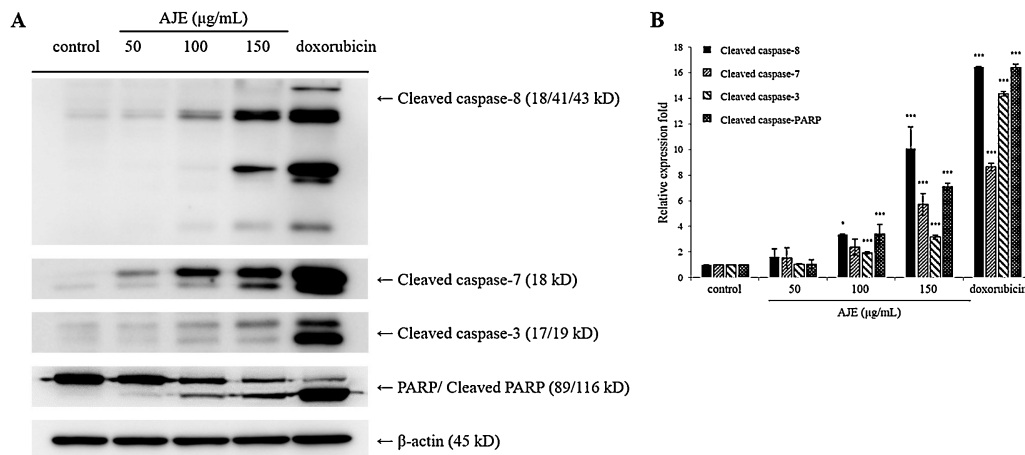
Fig. 7. The effect of *Alnus japonica* ethanol (AJE) extract on the expression levels of caspases and poly (ADP-ribose) polymerase (PARP) in AGS cells. The cell lysates were prepared and subjected to western blot analysis to detect apoptosis related cleaved caspase-8, 7, 3, and PARP/cleaved PARP. Each value is the mean ± SD of three determinations (***$p < .001$ vs. control).

protein expression levels of Bax and Bcl-2 proteins (Fig. 6). These results were closely similar to the results of the earlier study (Gross et al., 1999) and our results reveal that AJE extract-induced apoptosis is mitochondria-independent.

The activation of caspases-3, 6, 7, 8, and 9 is essential for both extrinsic and intrinsic apoptotic pathways (Fulda and Debatin, 2006). We also assessed the effects of AJE extract on the activation of various caspases and cleaved PARP. PARP seems to be involved in DNA repair in response to apoptotic signal stimulation, and it serves as a marker for cells undergoing apoptosis (Oliver et al., 1998). Our results showed that AJE extract could effectively induce the cleavage the caspase-8, 7, 3, and PARP/cleaved PARP in a dose-dependent manner (Fig. 7). Based on these results, we strongly suggest that AJE extracts induce the apoptotic cell death through the caspase-dependent pathway in AGS cells.

This activity might be attributed to the antioxidant and free radical scavenging properties of AJE extract. This is the first report on AJE extract cytotoxicity in AGS human gastric carcinoma cells, and these findings indicate that the extracts are potential for anticancer therapy. Further studies on the isolation, identification of bioactive compounds of *A. japonica* and their effects on *in vivo* model are needed for better understanding of their mechanisms of action.

Conclusion

In the present study, we evaluated the *in vitro* antioxidant and anticancer effects of *A. japonica* extracts in AGS human gastric carcinoma cells. The ethanol extract of *A. japonica* (AJE) showed strong antioxidant activities and induced cellular ROS generation. AJE extract inhibited cell viability and induced the cell death related morphological changes and cell death in AGS cells, in contrast, AJE extract had no effect on non-tumorigenic cells. Moreover, AJE extract induced apoptosis through the caspase-dependent pathway. Thus, these results suggest that AJE extract could be potentially useful as a new promising strategy in the therapy for gastric carcinoma cancer.

Conflict of interests

The authors declare no conflict of interests regarding this article.

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