

***Orostachys japonicus* induces apoptosis and cell cycle arrest through the mitochondria-dependent apoptotic pathway in AGS human gastric cancer cells**

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Abstract. We investigated the anticancer mechanisms of the ethylacetate (EtOAc) fraction from *Orostachys japonicus* in human gastric cancer (AGS) cells. Flow cytometric analysis revealed that the number of total apoptotic cells following treatment with the EtOAc fraction increased in a dose-dependent manner. In the cell cycle analyses, the EtOAc fraction increased the peak in the sub-G1, indicating apoptosis, and in the G₂/M phases in a dose-dependent manner. In the RT-PCR analysis, the expression of cyclin-dependent kinase 1 (CDK 1) and cyclin B1 decreased in a dose- and time-dependent manner. The results of western blotting revealed that the protein levels of p53, cytochrome c, and cleaved caspase-3, -8 and -9 proteins increased and those of B cell lymphoma-2 (bcl-2) and pro-caspase-3, -8 and -9 proteins decreased in a dose- and time-dependent manner, whereas the levels of bcl-2-associated x protein (bax) remained unchanged. Furthermore, the changes in the levels of pro-caspase-3, -8 and -9 and cleaved caspase-3, -8 and -9 were abolished by the pan-caspase inhibitor Z-VAD-FMK. In addition, phosphorylation of p38 and JNK increased in a time-dependent manner. These results, for the first time, provide an understanding of the potential anticancer activity of the *O. japonicus*, which functions through the induction of apoptosis and cell cycle arrest.

Introduction

The incidence of cancer is increasing dramatically, and currently, there is a strong emphasis on identifying biologically active substances from traditional herbs with anticancer properties, because these are believed to have fewer side

effects than conventional chemotherapeutic agents (1,2). *Orostachys japonicus*, a perennial herbaceous plant belonging to the family Crassulaceae and referred to as *Wa-song* in Korea, has been used traditionally as an anti-inflammatory, anti-febrile, hemostatic, antidote and anticancer agent (3-7). In a previous study (8), dried powder of *O. japonicus* was extracted and fractionated using a series of organic solvents, including n-hexane (hexane), dichloromethane (DCM), ethylacetate (EtOAc), n-butanol (BuOH), and water (H₂O). These extracts were examined for their anticancer activities on various cancer cell lines, including human AGS gastric, A549 lung, HepG2 liver, and HT-29 colon cancer cells. Among the different *O. japonicus* fractions tested, the EtOAc-soluble fraction showed the highest anticancer activity against 4 cancer cell lines, among which, AGS showed the strongest effect. Furthermore, our previous study identified kaempferol, quercetin, and gallic acid in the EtOAc fraction (8). However, further research on *O. japonicus* is required considering the lack of fundamental data on the signaling pathways related to its physiological activity.

Apoptosis, the process of programmed cell death, is essential to maintain the normal development of organs and for the persistence of tissues in multi-cellular organisms. Failure of the apoptosis regulatory machinery is known to induce cancer or other degenerative diseases (4). Therefore, the rate of apoptosis in cancer cells is considered a principal indicator of anticancer activity. In cancer, cells continue to proliferate in an uncontrolled manner owing to their inability to sustain normal cellular activity (9). The level of cell cycle arrest has also been used as an indicator of anticancer activity. The p53 gene is a well-known transcription factor and a tumor suppressor gene that participates in several cellular processes, including apoptosis, cell signaling pathway, and cell cycle control. If cell DNA is damaged by extracellular stress, p53 is activated and moves to the nucleus, where it activates target genes. p53 activates genes involved in apoptosis and genes encoding cell division suppressors, thereby inducing apoptosis and suppressing cell division (10,11). During apoptosis, phosphatidylserine (PS) is exposed to the outer membrane. This results in impeding anti-apoptosis factors such as B cell lymphoma-2 (bcl-2), regulation of pro-apoptosis factors such as bcl-2 associated x protein (bax), release of mitochondria-mediated apoptosis factors such

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as cytochrome c, and activation of apoptosis-induced proteins such as caspase (12,13). Many therapeutic substances with anticancer activity arrest the cell cycle and simultaneously induce apoptotic cell death (14). Defects in the regulation of the cell cycle are common causes of abnormal proliferation of cancer cells; therefore, studies of the cell cycle and cancer have become closely interconnected (15). The eukaryotic cell cycle is traditionally divided into 4 sequential phases: G₁, S, G₂ and M. The cell cycle is regulated by cyclin/cyclin-dependent kinase (CDK) complexes (16). Mitogen activated protein kinases (MAPKs) are important mediators involved in the intracellular network of interacting proteins that transduce extracellular signals to intracellular responses. When MAPKs such as p38, c-Jun N-terminal kinase (JNK), and extracellular regulated protein kinase 1/2 (ERK1/2) are activated, their signals lead to the activation of diverse molecules that regulate proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis (17,18).

In this study, the effect of the EtOAc fraction derived from *O. japonicus* on the activation of signaling pathways involving p53 and its downstream and upstream effectors was investigated.

Materials and methods

Cell line and reagents. The EtOAc fraction was prepared from *O. japonicus* in our laboratory using methods described previously (5,8,19,20). After analyzing the EtOAc fraction by GC-MS system, 12 peaks were identified. Nine peaks were unknown, and 3 peaks were identified as gallic acid (4.24%), kaempferol (6.81%) and quercetin (5.08%) (8). AGS cells were obtained from the Korean cell line bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Thermo Scientific, Logan, UT, USA). Monoclonal antibodies against p53; bcl-2; bax; cytochrome c; caspase-3, -8 and -9; cleaved caspase-3, -8 and -9; phospho-p38 (p-p38), -JNK (p-JNK), -ERK1/2 (p-ERK1/2); p38; JNK; ERK1/2; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Z-VAD-FMK (a pan-caspase inhibitor) was purchased from BD Pharmingen™ (BD Biosciences, Franklin Lakes, NJ, USA). All other reagents used were of the highest available grade.

Cell culture. The AGS cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin and incubated at 37°C in a 5% CO₂ incubator. The cells were subcultured every 5-7 days at 1:5 split ratios, and the culture medium was changed every 2 days. The cells at ~80-90% confluency were used in the experiments. Cells were treated either with 0.1% dimethyl sulfoxide (DMSO) or with various concentrations of the EtOAc fraction for 12 h.

Flow cytometric analysis of apoptosis. Apoptosis in the AGS cells was evaluated by Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining by using the Annexin V-FITC apoptosis detection kit (BD Biosciences),

according to the manufacturer's instructions. AGS cells (4x10⁵ cells/ml in a 24-well plate) were added to different concentrations of the EtOAc fraction for 12 h and then harvested by centrifugation at 300 x g. After centrifugation, the pellets were washed twice with cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, pH 7.4) and suspended in 100 µl of 1X binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). The cells were incubated with 5 µl of Annexin V-FITC and 5 µl of PI at the room temperature for 15 min in the dark. After incubation, 400 µl of 1X binding buffer was added to each tube and the cells were analyzed immediately by FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Flow cytometric analysis of the cell cycle. The cell cycle phase was assayed by DNA fragment staining with PI using the Cell Cycle Phase determination kit (Cayman Chemical, MI, USA), according to the manufacturer's instructions. AGS cells (4x10⁵ cells/ml in a 24-well plate) were added to different concentrations of the EtOAc fraction for 12 h and then harvested. After centrifugation, the pellets were washed and suspended in cell-based assay buffer. The cells were fixed and permeabilized by adding 1 ml of a fixative to each tube for more than 2 h. After centrifugation, the fixatives were decanted and the cell pellets were suspended in 500 µl of a staining solution (200 µl of RNase and 200 µl of PI), followed by incubation for 30 min at room temperature in the dark. Then, the cells were analyzed immediately by FACSCalibur flow cytometry.

RT-PCR analysis. Total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). The cells were lysed using the TRI reagent, followed by the addition of chloroform (Sigma-Aldrich). RNA precipitated by 2-propanol (Sigma-Aldrich). The purity and concentration of the total RNA was checked by Optizen 2120 UV plus spectrophotometer (Mecasys Co., Ltd., Daejeon, Korea). Equal amounts of RNA were reverse-transcribed into cDNA using the DiaStar™ RT kit (Solgent Co., Ltd., Daejeon, Korea) by incubation at 65°C for 5 min, 50°C for 60 min, and 95°C for 5 min. PCR was performed using a 50 µl reaction mixture containing cDNA, 10X h-Taq buffer, 10 mM dNTP mix, 10 pM of each primer, h-Taq polymerase (Solgent Co., Ltd.), and RNase-free water. Primers used for amplification were as follows: i) CDK1: forward 5'-TTTTCAGAGCTTTGGGCACT-3' and reverse 5'-AAACATGGCAGTGACACCAA-3', ii) cyclin B1: forward 5'-CGGGAAGTCACTGGAAACAT-3' and reverse 5'-AAACA TGGCAGTGACACCAA-3', iii) GAPDH: forward 5'-GAGTC ACGGATTTCGGTCGT-3' and reverse 5'-TTGATTTTGG AGGGAGCTCG-3'. The samples were subjected to 25 cycles of denaturation for 20 sec at 95°C, annealing for 40 sec at 56°C (cyclin B1 and GAPDH) and 54°C (CDK1), extension for 1 min at 72°C, and a final extension step at 72°C for 5 min. The housekeeping gene GAPDH served as the control. The PCR products were electrophoresed in 2% agarose gel, followed by staining with 0.5 µg/ml ethidium bromide (EtBr) at 100 V for 30 min and visualization under a UV transilluminator.

Western blotting. The AGS cells were treated with the EtOAc fraction, washed twice with ice-cold PBS, and harvested using

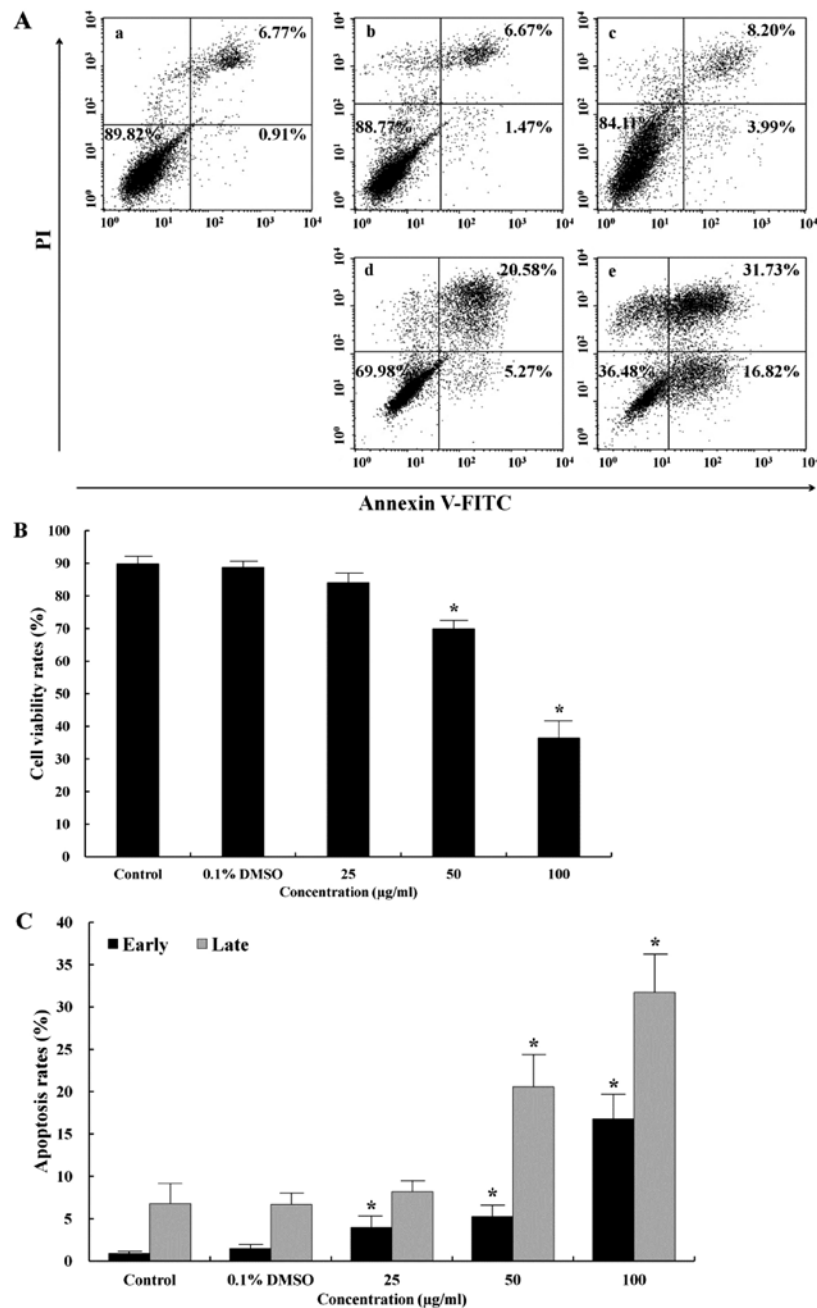
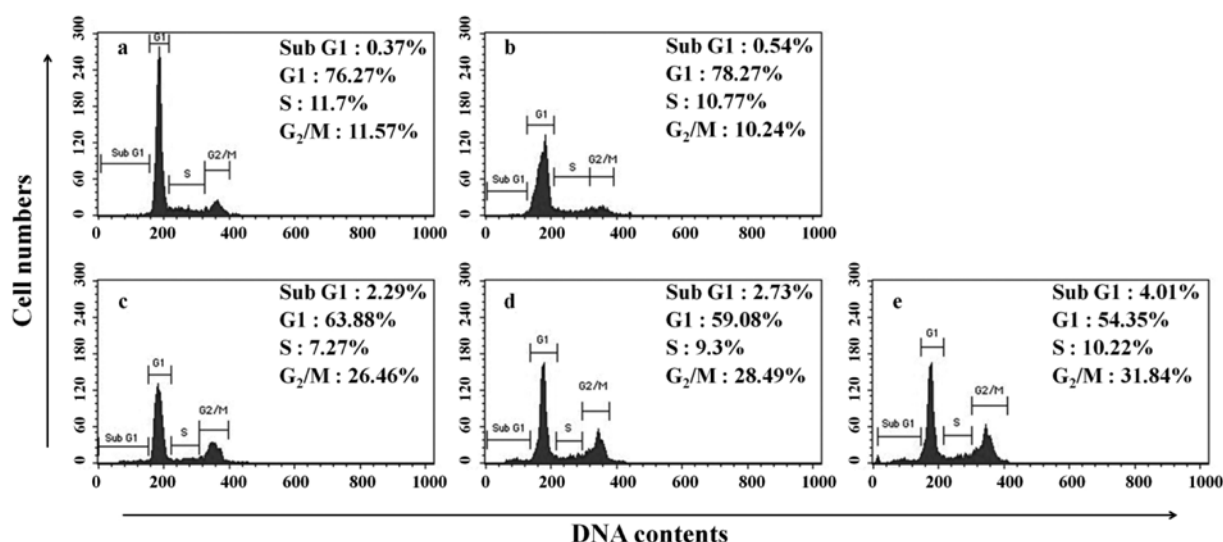


Figure 1. Flow cytometry analysis of the apoptotic death of AGS cells. (A) Dot plots display the apoptotic death of AGS cells treated without EtOAc fraction (a) or with 0.1% DMSO, 25, 50 and 100 μ g/ml EtOAc fraction (b-e) for 12 h. The cells were stained with monoclonal antibodies against Annexin V-FITC and PI. Percentages of (B) viable cells and (C) apoptotic cells are shown. The values are expressed as the mean \pm SD. Significantly different from control at * p <0.05.

a cell scraper. The cells were then pelleted by centrifugation, the pellets were resuspended in lysis buffer on ice for 1 h, and the cell debris was removed by centrifugation at 10,000 \times g for 10 min. Protein concentrations were determined using the Bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were mixed with 2X Laemmli loading buffer and preheated at 95°C for 5 min. The samples were electrophoresed on 10-15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a polyvinylidene fluoride (PVDF) membrane for 1 h by using a semi-dry transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 (PBST) for 2 h at 4°C and then incubated overnight

with primary antibodies. After hybridization with primary antibodies, the membranes were washed for 5 min with PBST, 3 times. The membranes were subsequently incubated with HRP-secondary antibody for 2 h at 4°C and washed for 5 min with PBST, 3 times. The signal of the membranes was developed by using a western blotting luminal reagent (Santa Cruz Biotechnology).

Statistical analysis. All experiments were performed at least 3 times. The data are expressed as the mean \pm standard deviation (SD). The statistical differences were calculated using the Student's t-test using the SigmaStat software (SigmaStat for Windows version 3.0.). A p-value of <0.05 was considered statistically significant.



Control (µg/ml)	Sub G ₁	G ₁	S	G ₂ /M
Control	0.37±0.14	76.08±1.35	11.63±1.35	11.39±2.88
0.1% DMSO	0.39±0.17	76.83±2.27	10.46±0.44	12.02±2.35
25	2.25±0.19*	64.14±3.05*	8.26±1.02*	25.08±4.28*
50	2.88±0.14*	59.17±1.13*	9.11±0.62	28.47±2.01*
100	4.57±0.51*	53.46±1.20*	10.05±1.56	31.83±0.47*

Figure 2. Flow cytometry analysis of cell cycle distribution of AGS cells. Histograms display sub G₁, G₁, S and G₂/M phase of AGS cells without EtOAc fraction (a) or with 0.1% DMSO, 25, 50 and 100 µg/ml EtOAc fraction (b-e) for 12 h. The results are presented as percentage of total treated cells. Data values are expressed as mean ± SD. (n=3). Significantly different from control at *p<0.05.

Results

Induction of apoptosis. At the initial stages of apoptosis, the PS is exposed, which is considered an early marker of apoptosis. In addition, Annexin V, a Ca²⁺-dependent phospholipid-binding protein, can now bind to the exposed PS (21). The exposed PS could be detected based on this characteristic. At the same time, the damaged DNA is stained using PI. Thus, from the amount of Annexin V binding and the intensity of PI staining, apoptosis can be detected. As shown in Fig. 1A, stained cell populations were defined as follows: lower left (LL), viable cells (Annexin V⁻/PI⁻); lower right (LR), cells undergoing early apoptosis (Annexin V⁺/PI⁻); upper right (UR), and late apoptotic or necrotic cells (Annexin V⁺/PI⁺). Apoptosis induction in AGS cells treated with the EtOAc fraction was evaluated by flow cytometry. The EtOAc fraction increased the number of early (LR) and late (UR) apoptotic cells in a dose-dependent manner (Fig. 1C). In addition, the total apoptosis rate was more than 6-fold higher than that in the control (48.55 vs. 7.68%) (Fig. 1A). Under control conditions, 89.82% of cells were viable (LL). In contrast, the survival rate induced by the 100 µg/ml EtOAc fraction was 36.48% (Fig. 1B).

Induction of cell cycle arrest. The DNA content of the PI-stained AGS cells was determined by flow cytometry. The fragmented DNA that serves as evidence of apoptosis is manifested on the left side rather than at the G₁ peak of

a cell cycle (22). Apoptosis can be observed by detecting the presence of this sub-G₁ peak. To probe the relationship between the inhibitory effect of the EtOAc fraction on AGS cell growth and the apoptotic effect, we investigated the alteration of the cell cycle using flow cytometry. In the control cells, this sub-G₁ peak was negligible (0.37%), but accounted for 4.57% of the cells treated with the 100 µg/ml EtOAc fraction. The sub-G₁ phase cell population of the EtOAc fraction-treated cells increased in a dose-dependent manner (Fig. 2). Further, apart from the sub-G₁ peak as evidence of apoptosis, the G₂/M peak was 31.83%, simultaneously being measured at a significantly higher rate than that of the control (11.39%). The G₂/M phase cell population of the EtOAc fraction-treated cells increased in a dose-dependent manner. Depending on the control period of DNA synthesis, the cell cycle is divided into four phases: G₁, S, G₂ and M. The cell cycle has a checkpoint that determines whether the general progress of cell division is implemented from one phase to another without disorder. The G₂/M transition is a point at which the completion of DNA replication is confirmed prior to cell division (23). An increase in G₂/M peak refers to the G₂/M cell cycle arrest shows inhibited cell division, incapacitation by further cell division due to the damage in the DNA of the AGS cells.

Effect of the EtOAc fraction on the expression level of the cell cycle-related genes. As the EtOAc fraction arrested the

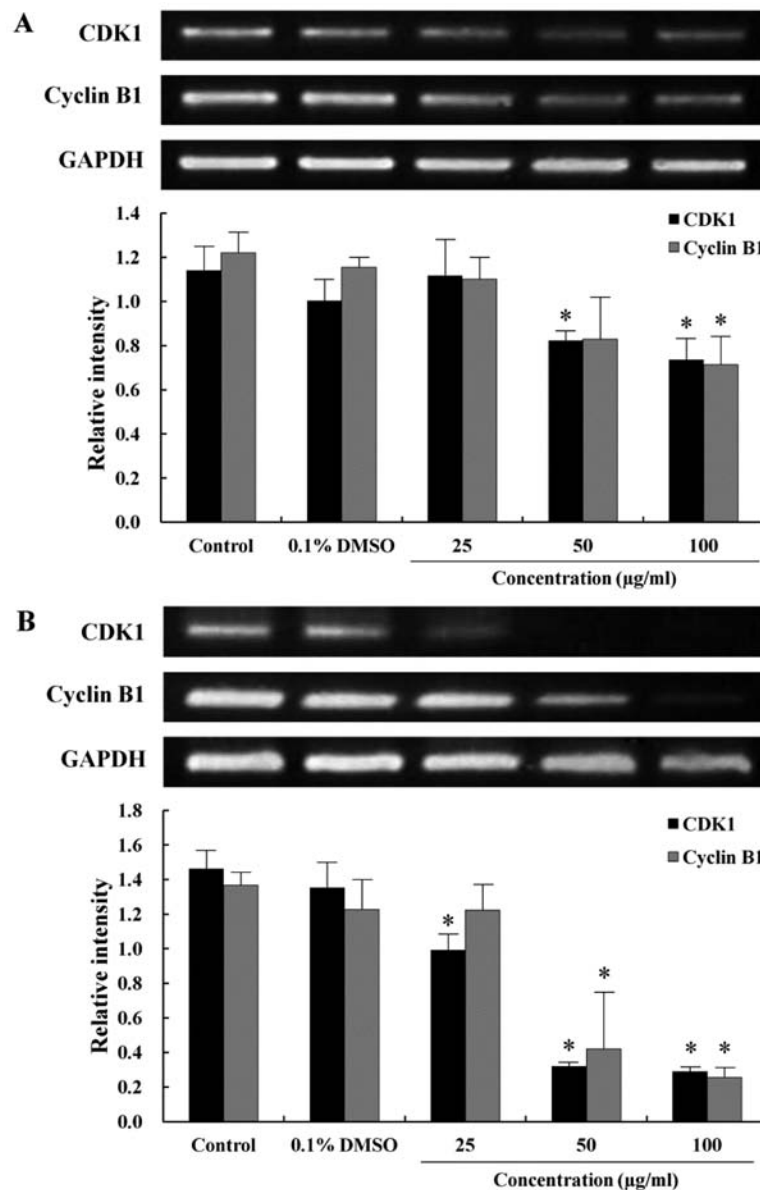


Figure 3. Effect of EtOAc fraction on mRNA expressions of CDK1 and cyclin B1 in AGS cells. The cells were treated with different concentration of EtOAc fraction for 6 or 12 h (A and B). Total RNAs were isolated from the cells and RT-PCR was performed. Band density was quantitated and plotted on the bar graph. GAPDH was used as a housekeeping control gene. The intensities of bands were determined by densitometry in three separate experiments with similar results. The values are expressed as the mean \pm SD. Significantly different from control at * $p < 0.05$.

AGS cells in the G₂/M phase of the cell cycle, RT-PCR was performed to study the correlation between G₂/M arrest and the transcription of the cell cycle-related genes. The CDK1 is primarily activated in association with cyclin B1 during the progression of the G₂/M phase (24). To examine the expression of mRNA levels regulating the cell cycle progression at the G₂/M phase, which was remarkably arrested as shown in Fig. 2, CDK1 and cyclin B1 expression were measured by RT-PCR. The expression of CDK1 decreased when the cells were treated with the EtOAc fraction in a time- and dose-dependent manner (Fig. 3). Cyclin B1 was also expressed at a lower level in the EtOAc fraction-treated cells than in the control cells. The decrease in the expression of CDK1 and cyclin B1 correlated to the increase in distribution of the G₂/M peak, which was consistent with the G₂/M arrest observed from the cell cycle analysis.

Induction of mitochondrial-mediated apoptosis. To further provide insight into the apoptotic effect of the EtOAc fraction, this study measured the expression level of p53, bcl-2, bax and cytochrome c proteins, which are relevant to the understanding of apoptotic signaling pathways after exposure to the EtOAc fraction for 12 h in the AGS cells. p53 is a tumor suppressor gene and transcription factor that induces either apoptosis or cell cycle arrest in response to extracellular stress signals and DNA damage (25). In addition, the imbalance of bcl-2 and bax protein expression could be influenced by p53 (26). The level of p53 protein expression increased in a dose-dependent manner (Fig. 4A). Major proteins involved in apoptosis are bcl-2 and bax. The bcl-2 protein inhibits the emission of cytochrome c from the mitochondria to defend the cell from apoptosis signals. In contrast, bax protein induces apoptosis by inducing stress in the mitochondria (27). The release of cytochrome c

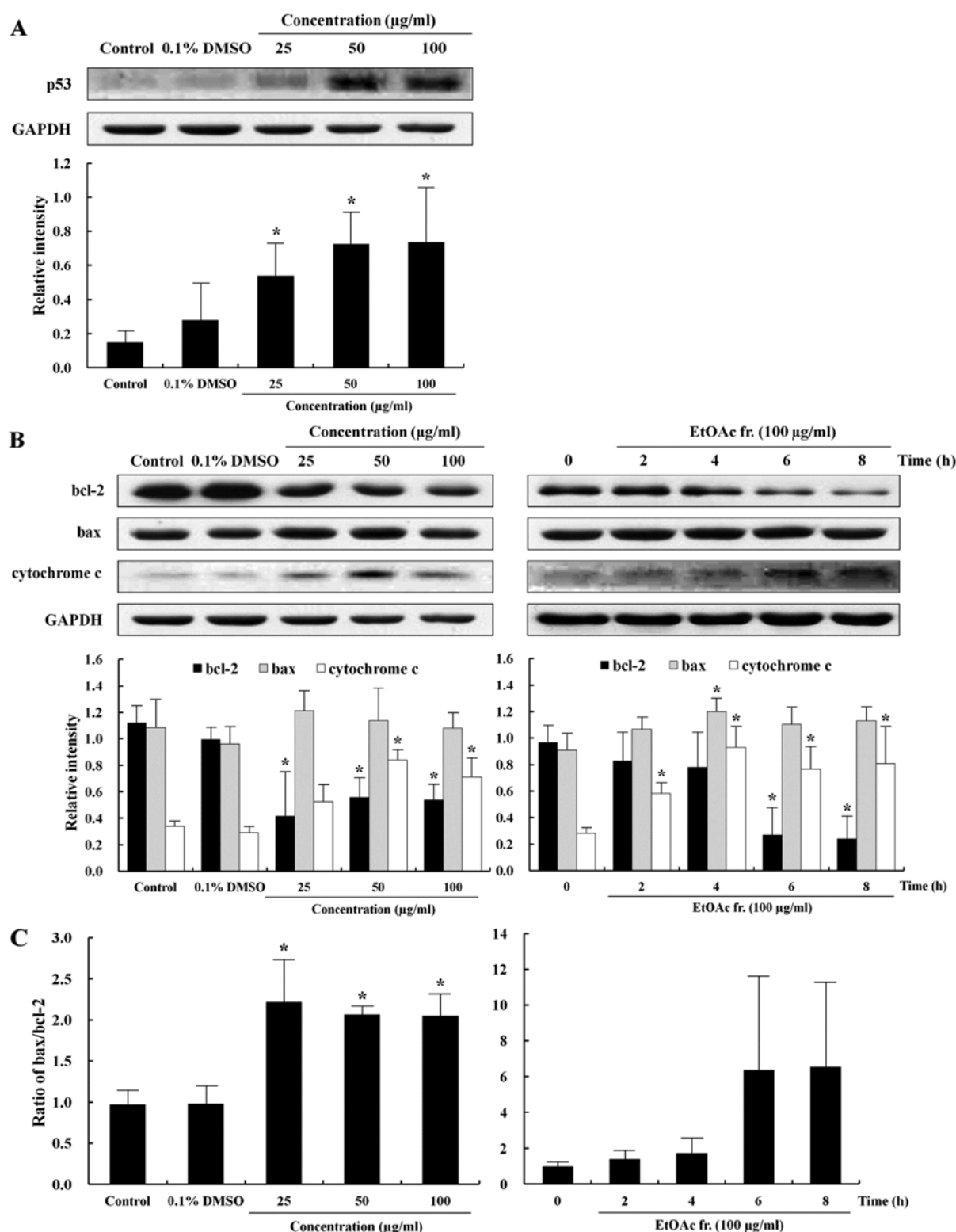


Figure 4. Effect of EtOAc fraction on protein expressions of p53, bcl-2, bax and cytochrome c in AGS cells. The cells were treated with different concentration of EtOAc fraction for 12 h or 100 $\mu\text{g/ml}$ EtOAc fraction for indicated times. The expressions of (A) p53, (B) bcl-2, bax, and cytochrome c were analyzed by western blotting. Band density was quantitated and plotted on the bar graph. (C) Ratio of bax/bcl-2 level was determined by ImageJ analysis. p53, 53 kDa; bcl-2, 26 kDa; bax, 23 kDa; cytochrome c, 14 kDa. GAPDH was used as an internal control. The values are expressed as the mean \pm SD. Significantly different from control at * $p < 0.05$.

from the mitochondria is promoted by bax and blocked by bcl-2 (28). The levels of anti-apoptotic and apoptotic proteins were examined by western blotting to determine whether the EtOAc fraction induced the apoptosis of AGS cells through the

regulation of bcl-2, bax and cytochrome c. The expression of bcl-2, bax and cytochrome c proteins in the AGS cells with the EtOAc fraction is shown in Fig. 4B. The level of bcl-2 protein expression decreased and that of cytochrome c increased in a

time- and dose-dependent manner, while that of bax was not significantly different between the EtOAc fraction-treated cells and the control cells. However, the bax/bcl-2 ratios increased significantly in a dose-dependent manner (Fig. 4C). These results suggest that the EtOAc fraction induced apoptosis of the AGS cells via the mitochondrial-mediated pathway.

Induction of caspase-mediated apoptosis. The apoptotic pathway is regulated by caspases that are responsible, either directly or indirectly, for the cleavage of other protein substrates within the cell, which is a characteristic of apoptosis (29). Caspase-3 is an effector caspase that can trigger the apoptotic process by activation of initiator caspases such as caspase-8 and -9 (30). In order to understand the mechanisms involved in the caspase-mediated apoptosis, we measured the expression levels of pro-caspase-3, -8, -9 and cleaved caspase-3, -8, -9 proteins. The treatment of the AGS cell with the EtOAc fraction caused decreases in the levels of pro-caspase-3, -8 and -9, inactive forms of caspase, in a time- and dose-dependent manner (Fig. 5A). The cleavage of caspases is directly related to the activation of the apoptotic process. Cleaved caspase-3, -8 and -9, the active forms of caspase, increased substantially in the EtOAc fraction-treated AGS cells in a time- and dose-dependent manner (Fig. 5A). To investigate whether apoptosis inductions was caspase-dependent, we used the general caspase inhibitor Z-VAD-FMK. As shown in Fig. 5B, the addition of 20 μ M Z-VAD-FMK significantly reduced the induction of apoptosis in response to the EtOAc fraction in a dose-dependent manner. These results show that caspase-3, -8 and -9 act as fundamental factors in the apoptotic effect of the EtOAc fraction in the AGS cells.

Involvement of MAPKs in the induction of apoptosis. MAPKs, such as p38, JNK and ERK1/2, activated by extracellular signals are involved in pro-survival and pro-apoptotic activity (31). To understand the role of pro-survival- and pro-apoptotic-signaling pathways in the induction of apoptosis, this study assessed 100 μ g/ml of the EtOAc fraction-treated AGS cells by western blotting using antibodies against the phosphorylated and total forms of MAPKs. Western blotting showed that the phosphorylation of p38 and JNK increased in the EtOAc fraction-treated AGS cells in a time-dependent manner. Activation of p38 and JNK was increased at 15 min and 30 min, respectively, and continued to increase until 120 min. On the other hand, activation of ERK1/2 increased markedly at 15 min, but thereafter, it decreased until 120 min in a time-dependent manner (Fig. 6). The expression of the total forms of p38, JNK and ERK1/2 did not change.

Discussion

Our results showed that the EtOAc fraction induced apoptosis and cell cycle arrest in the AGS cells. The apoptosis of the AGS cells with the EtOAc fraction was induced by p53 and mitochondrial-mediated apoptotic proteins through the MAPK signaling pathway. In addition, the EtOAc fraction arrested the AGS cells in the G₂/M phase of the cell cycle by regulating CDK1 and cyclin B1. In our previous study (8), we demonstrated that the EtOAc fraction, from among the 5 other fractions tested, possessed the highest anticancer

activity in the AGS cells, as evidenced by 4'-6-diamidino-2-phenylindole (DAPI) staining, DNA fragmentation assay and flow cytometry analysis. The present study is the first to investigate the signaling pathway at the molecular biological level in the context of exposure time and dose.

Apoptosis is the process of programmed cell death that is characterized by cell changes, including exposure of PS from the cell membrane and DNA fragmentation (32). To detect the characteristics of apoptosis in the AGS cells treated with the EtOAc fraction, flow cytometry analysis was performed following Annexin V/PI staining for detection of early or late apoptosis, and PI labeling assay was performed to detect DNA damage. As shown in Fig. 1, the early apoptosis rate (16.82%) in the AGS cells treated with the 100 μ g/ml EtOAc fraction was higher than that of the control (0.91%), and the late apoptosis rate (31.73%) was higher than that of the control (6.77%). The EtOAc fraction increased the total apoptosis rate and decreased the survival rate in a dose-dependent manner. The fragmented DNA that indicated the induction of apoptosis observed in the sub-G1 peak of the cell cycle. The EtOAc fraction increased the sub-G1 phase cell population in a dose-dependent manner (Fig. 2). These results suggest that the EtOAc fraction may not only induce apoptosis, but also inhibit the growth of AGS cells.

The normal cell growth is regulated through cell cycle progression, and uncontrolled cell growth is a characteristic of cancer. Several anticancer agents have been reported to arrest the cell cycle and simultaneously induce apoptosis. Cell cycle arrest has been used as an indicator of anticancer activity (33). The eukaryotic cell cycle is divided into the G1 phase for DNA duplication, the synthetic S phase for effective DNA duplication, the G₂ phase for cell growth ready for mitosis, and the M phase for mitosis (34). There are 3 checkpoints that check the general progress for problem-free cell division with regular transition between the phases, i.e., in the late G1 phase, G₂/M transition, and metaphase-to-anaphase transition (24). The CDKs are protein kinases involved in regulating the cell cycle and binding a regulatory protein called cyclin. The cyclin-CDK complexes regulate the general progress for a cell cycle. The cyclin D-CDK4 (and/or 6) complex is necessary for initiating the G1 phase, while the cyclin E-CDK2 complex is needed to progress to the S phase. The cyclin A-CDK2 complex is involved from S to G₂ phase, and the cyclin B1-CDK1 complex is required for progression to the M phase (22,35). The effect of the EtOAc fraction on the cell cycle progression of the AGS cells is shown in Figs. 2 and 3. The G₂/M phase cell population of the EtOAc fraction-treated cells increased in a dose-dependent manner. In the present study, we investigated the correlation between G₂/M arrest and the regulating genes of the cell cycle. Expression of CDK1 and cyclin B1 decreased comparison with the control in a time- and dose-dependent manner (Fig. 3). These results revealed that the EtOAc fraction act on the G₂/M transition checkpoint of the cell cycle.

p53 is a tumor suppressor gene, a transcription factor, which becomes activated in the cytoplasm and accumulated in the nucleus when the DNA is damaged by extracellular stress. p53 activates the target genes, including those encoding apoptotic proteins and cell division suppressor genes to induce apoptosis and arrest the cell cycle (36). The basal level of p53 was low in the AGS cells, and the EtOAc fraction upregulated

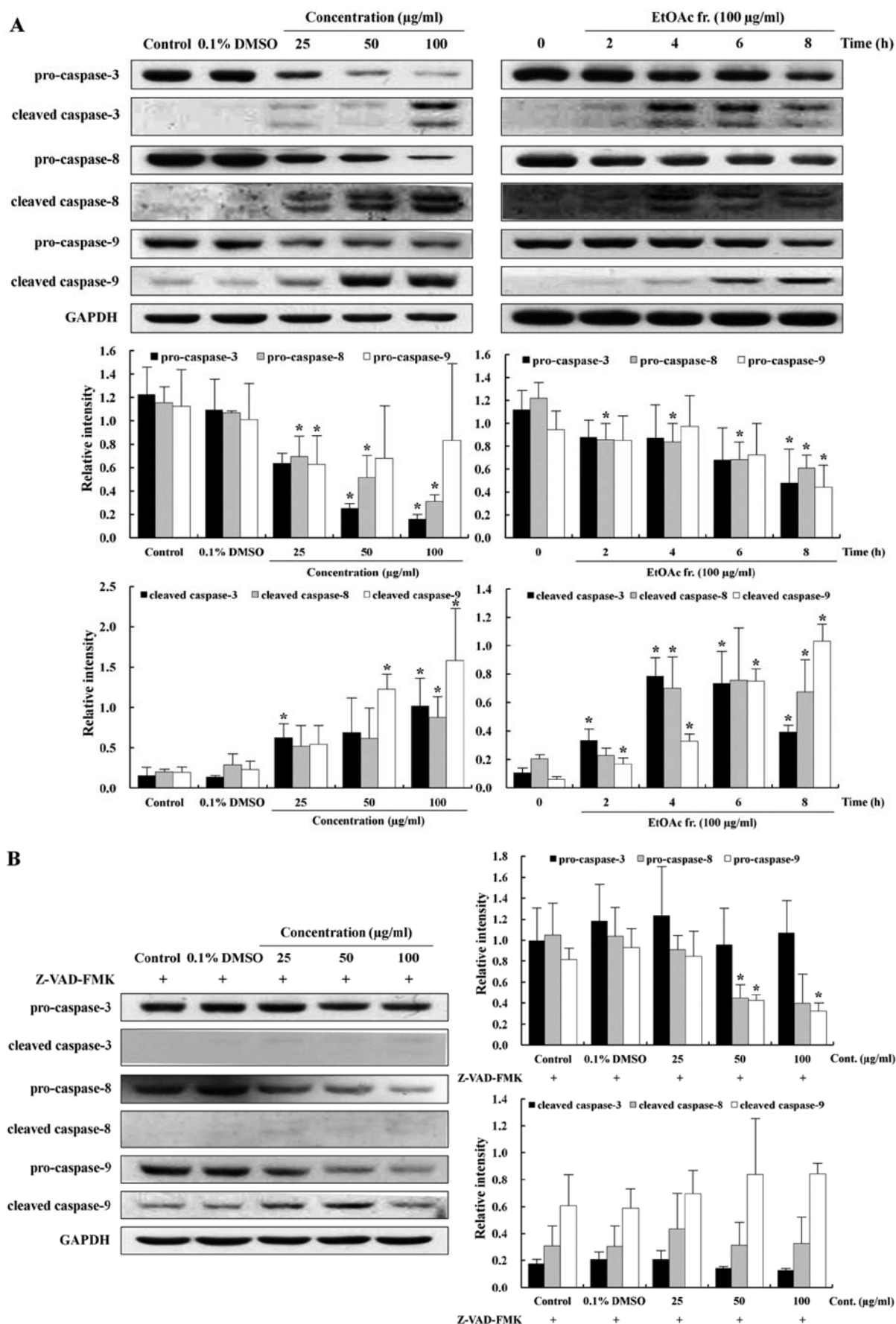


Figure 5. Effect of EtOAc fraction on protein expressions of caspase-3, -8 and -9 in AGS cells. (A) The cells were treated with different concentration of EtOAc fraction for 12 h or 100 $\mu\text{g/ml}$ EtOAc fraction for indicated times. (B) The cells were pretreated with the pan-caspase inhibitor Z-VAD-FMK (20 μM). Band density was quantitated and plotted on the bar graph. pro-caspase-3, 32 kDa; cleaved caspase-3, 17/19 kDa; pro-caspase-8, 57 kDa; cleaved caspase-8, 41/48 kDa; pro-caspase-9, 47 kDa; cleaved caspase-9, 35/37 kDa. GAPDH was used as an internal control. The values are expressed as the mean \pm SD. Significantly different from control at $*p < 0.05$.

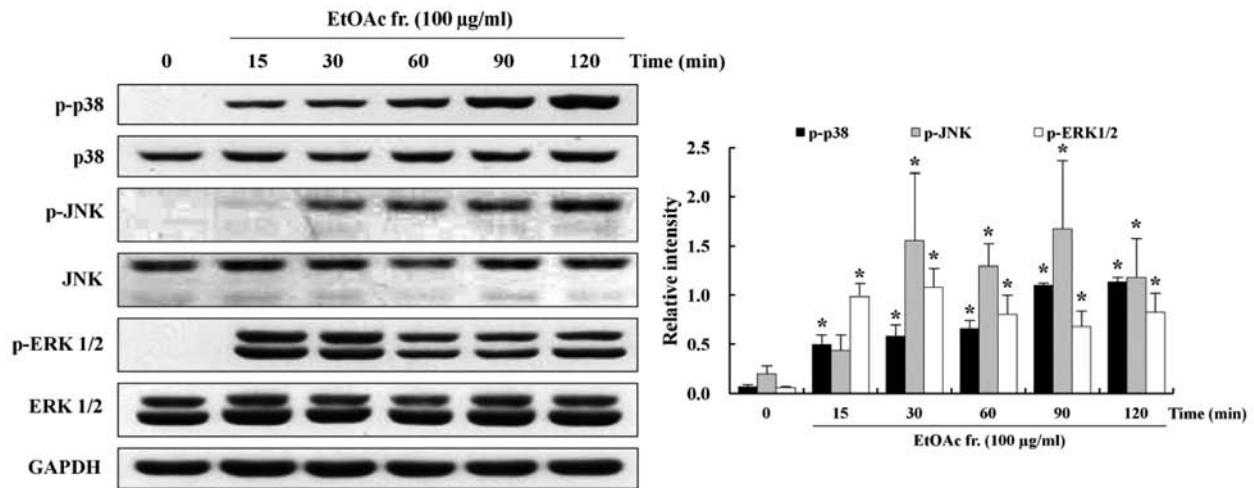


Figure 6. Effect of EtOAc fraction on the phosphorylation of p38, JNK and ERK1/2 in AGS cells. The cells were treated with EtOAc fraction (100 μ g/ml) for the indicated times. Band density was quantitated and plotted on the bar graph. phospho-p38, p38, 43 kDa; phospho-JNK, JNK, 46/54 kDa; phospho-ERK1/2, ERK1/2, 42/44 kDa. GAPDH was used as an internal control. The values are expressed as the mean \pm SD. Significantly different from control at * p <0.05.

its expression in a time- and dose-dependent manner (Fig. 4A). Previous studies investigating the involvement of p53 in the cellular response to active substances from natural plants have shown that it induces apoptosis and upregulates p53 in various cancer cells (37). The p53 activation causes induction of apoptotic cell death, which leads to the release of cytochrome c from the mitochondria (38). There are 2 major apoptotic pathways: the intrinsic mitochondrial pathway and the extrinsic death receptor pathway (39,40). Both these pathways are regulated by caspases for the cleavage of cellular proteins. The mitochondrial pathway induces apoptosis by releasing pro-apoptotic protein such as cytochrome c. The mitochondrial-mediated apoptosis is regulated by apoptotic proteins that suppress the anti-apoptotic protein bcl-2 and promote the pro-apoptotic protein bax (41). We showed that the EtOAc fraction decreased the expression of bcl-2 and increased the expression levels of cytochrome c and bax/bcl-2 ratio in a time- and dose-dependent manner (Fig. 4B and C). Cytochrome c is released from the mitochondria into the cytosol during apoptosis. The released cytochrome c forms an apoptosome composed of apoptotic protease activating factor-1 (Apaf1), cytochrome c, and caspase-9, which subsequently activates caspase-3 (29,42). As one of the primary pathways of apoptosis, the mitochondrial pathway is regulated by bcl-2 family proteins and is finalized by DNA fragmentation, and the apoptotic bodies are regulated through the activation of caspase-3, a final executing factor. The death receptor pathway is initiated by Fas receptor that transduces apoptotic signals into caspase-8-dependent cascade (43). During this cascade, caspase-8 induces the release of cytochrome c and activates caspase-3.

Several studies have provided evidence that caspase plays a crucial role in the induction of apoptosis (44,45). Caspases are grouped into initiator caspases such as caspase-8 and -9 and effector caspases such as caspase-3. Caspase-3 is an executioner caspase that is activated by a mitochondrial-mediated apoptosis involving caspase-9 or a death receptor-mediated apoptosis involving caspase-8 (46). In the present study, pro-caspase-3, -8 and -9, inactive forms of caspase, decreased in the cells treated with the EtOAc fraction in a time- and dose-

dependent manner (Fig. 5A). In contrast, cleaved caspase-3, -8 and -9, active forms of caspase, increased substantially in the cells treated with the EtOAc fraction in a time- and dose-dependent manner (Fig. 5A). In addition, pretreatment with the general caspase inhibitor Z-VAD-FMK significantly inhibited the EtOAc fraction-induced apoptosis, indicating that the EtOAc fraction induced caspase-dependent apoptosis in the AGS cells. The expression levels of caspase-3 and -8 were significantly blocked in the presence of Z-VAD-FMK, but the inhibition of caspase-9 had a mild inhibitory effect (Fig. 5B). Caspase-9 was activated at ~6 h, while caspase-3 was activated at 4 h. These findings indicate that caspase-3 has a feedback action on caspase-9. These results suggested that p53 is a key regulator of apoptosis and cell cycle arrest in the EtOAc fraction-treated AGS cells. Furthermore, bcl-2 and anti-apoptotic protein was impeded by the release of cytochrome c from the mitochondria, and caspases were sequentially activated to induce apoptosis of the EtOAc fraction-treated AGS cells.

The MAPK signaling pathways regulate intracellular functions such as apoptosis and cell proliferation (47). Major members in the MAPK signaling pathway belong to 3 subgroups of the MAPK family: p38, JNK and ERK1/2 (48). Several studies have reported that the activation of MAPKs is linked to cell growth and induction of apoptosis in various cancer cells (49-51). It has been shown that activation of p38 and JNK are involved in mitochondrial-mediated apoptosis and caspase activation and that ERK1/2 is associated with cell proliferation and survival. This study hypothesized that the anticancer activity of the EtOAc fraction was related to the activation of the MAPK signaling pathway. To screen the MAPK-signaling pathway underlying the anticancer effects of the EtOAc fraction, the phosphorylation of p38, JNK and ERK 1/2 was determined. We observed that the EtOAc fraction increased phosphorylation of p38 and JNK in a time-dependent manner (Fig. 6).

In conclusion, we showed that the EtOAc fraction could effectively suppress the growth of AGS cells by the induction of apoptosis and cell cycle arrest. The expression levels

of p53, cytochrome c, cleaved caspase-3, -8 and -9 were upregulated, and the expression levels of CDK1, cyclin B1, bcl-2, pro-caspase-3, -8 and -9 were downregulated through phosphorylation of p38 and JNK in the AGS cells treated with the EtOAc fraction. These results provide, for the first time, a useful foundation for understanding the anticancer effects of *O. japonicus* at the molecular level and show that *O. japonicus* could be used for the development of novel anticancer agents for the treatment of gastric cancers.

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