

Original Article

## *Armeniaca* Semen Extract Induces Apoptosis in Mouse N2a Neuroblastoma Cells

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**Objectives :** In the present study, we investigated whether an aqueous extract of *Armeniaca semen* induces apoptotic neuronal cell death upon mouse N2a neuroblastoma cells.

**Methods :** 1. Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2. For in situ detection of apoptotic cells, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, 4,6-diamidino-2-phenylindole (DAPI) staining.

3. The fraction of cells was revealed by flow cytometric analysis used that .

4. For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed.

5. For detection of bax and bcl-2, Western blot analysis was performed.

6. Caspase enzyme activity was measured using caspase-3 assay.

**Results :** From the present results, N2a neuroblastoma cells treated with *Armeniaca semen* extract exhibited several characteristics of apoptosis. A treatment of *Armeniaca semen* extract was shown to increase the expression of Bax, a pro-apoptotic protein, and the treatment decreased the expression of Bcl2, an anti-apoptotic protein. In addition, *Armeniaca semen* extract increased the caspase-3 enzyme activity.

**Conclusions :** The present results show that *Armeniaca semen* extract induces apoptotic cell death in mouse N2a neuroblastoma cells.

**Key Words:** *Armeniaca semen*, N2a neuroblastoma cells, Apoptosis Caspase-3, Amygdalin

### Introduction

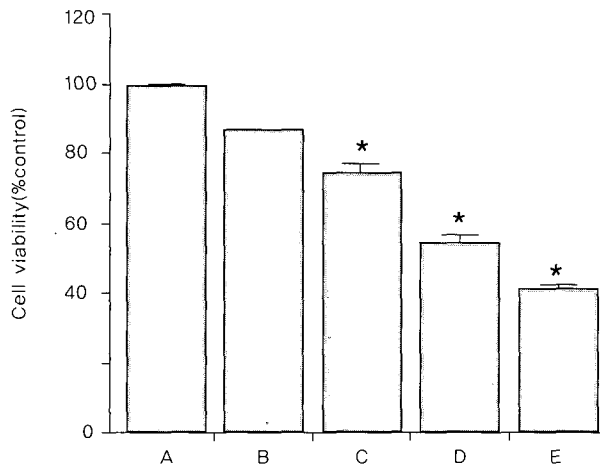
*Armeniaca semen* is the seed of *Prunus armeniaca* L. var *ansu* MAXIM, which is classified in Rosaceae. The *Prunus* species contains chemicals that are known to be able to relieve fever and quench thirst, and they have traditionally been used for the treatment of asthma,

bronchitis, emphysema, constipation, nausea, leprosy, and leucoderma<sup>1,2)</sup>. The major components of *Armeniaca semen* are emulin, amygdalin, and a-elaeostearic acid. Among them, amygdalin is abundant in the seeds of the *Prunus* genus such as almond, apricots, and other rosaceous plants. Amygdalin is also known as vitamin B17 and had been used as an anticancer drug named as laetril<sup>3)</sup>.

Apoptosis, also known as programmed cell death, is a form of cell death that occurs in several pathological situations in multicellular organisms, and it constitutes part of a common mechanism of cell replacement,

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**Fig. 1.** Cytotoxicity effect of *Armeniaca*e semen extract. (A) Control cells; (B) 1 mg/ml *Armeniaca*e semen-treated cells; (C) 3 mg/ml *Armeniaca*e semen-treated cells; (D) 5 mg/ml *Armeniaca*e semen-treated cells; (E) 10 mg/ml *Armeniaca*e semen-treated cells. The data are presented as the mean  $\pm$  standard error mean (S.E.M.). \*: represents  $p < 0.05$  compared to the control cells.

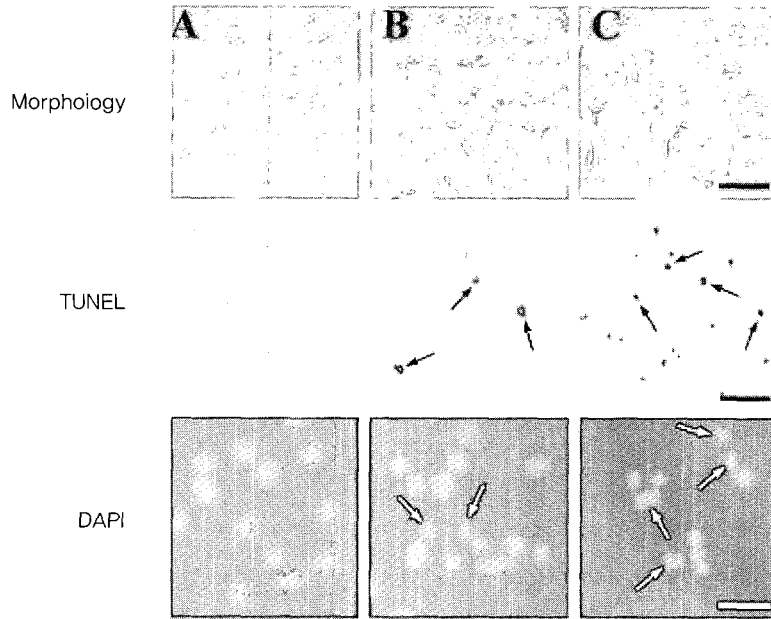
tissue remodeling and removal of damaged cells. Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation internucleosomal DNA fragmentation, and formation of apoptotic bodies<sup>9</sup>.

In numerous studies, it has been documented that the process of apoptosis is regulated by the expressions of several proteins. Two important groups of proteins involved in apoptotic cell death are members of the Bcl-2 family<sup>5</sup> and a class of cysteine proteases known as caspases<sup>6</sup>. The Bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, is known to regulate the apoptotic pathways and Bcl-2 protects against cell death, while Bax, a pro-apoptotic protein of the family, is expressed abundantly and selectively during apoptosis and Bax promotes cell death<sup>7</sup>. The caspase families are aspartate-specific cysteine proteases that have emerged as the central executioner of apoptosis. Caspases are known to mediate a crucial stage of the apoptotic process, and

they are expressed in many mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, and it is responsible either partially or wholly for the proteolytic cleavage of many proteins<sup>6,8</sup>.

Apoptosis is a very important mechanism in cancer treatment<sup>9</sup>). Tumor cells differ from normal cells in a number of traits. The process of carcinogenesis induces genetic changes, resulting in the alteration of the normal apoptotic response<sup>10</sup>. Cytotoxic drugs used in cancer therapy induce tumor cell death by apoptosis. Anticancer drugs induce the damage of DNA, inhibit DNA synthesis, deplete the intracellular nucleotide pool, or disrupt the mitotic apparatus<sup>11,12</sup>.

In the present study, we investigated whether an aqueous extract of *Armeniaca semen* induces apoptotic neuronal cell death on mouse N2a neuroblastoma cells. We observed morphological change by MTT assay, TUNEL assay and DAPI staining. Cell cycle distribution changes were observed



**Fig. 2.** Morphological changes induced by Armeniacaee semen extract. (A) Control cells; (B) 1 mg/ml Armeniacaee semen-treated cells; (C) 5 mg/ml Armeniacaee semen-treated cells. Above: Phase-contrast photomicrographs. Middle: N2a cells stained by TUNEL assay. Black arrows indicate where condensed and margined chromatin has been labeled. Below: N2a cells stained by DAPI. White arrows indicate the condensed nuclei. A scale bar represents 10  $\mu\text{m}$ .

by flow cytometric analysis. Reflecting the endonuclease activity, characteristic of apoptosis, was observed by DNA fragmentation assay. Expression of bax and bcl2 proteins was investigated by Western blot analysis. Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (pNA).

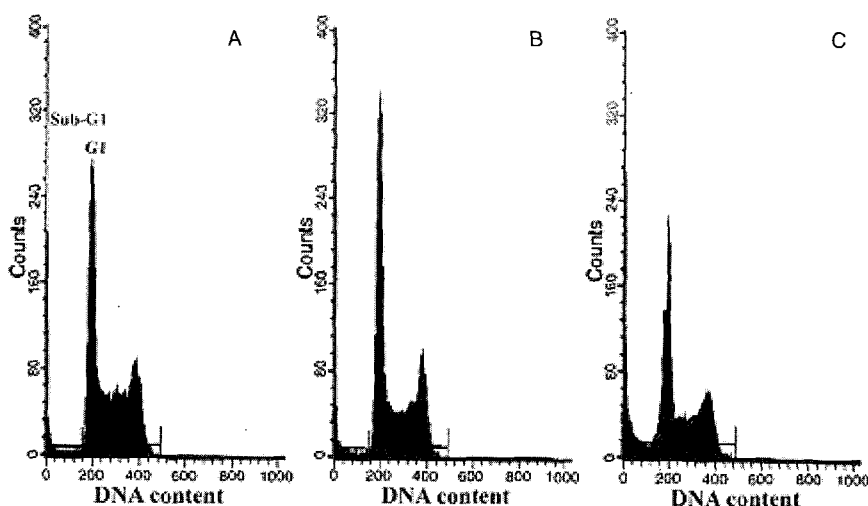
## Materials and Methods

### 1. Drugs and reagents

*Armeniacaee semen* used in this experiment was obtained from the Kyungdong market (Seoul, Korea). After immersion in 0.1 % citric acid for 1 min, it was rinsed and dried at room temperature for 24 hrs. Then, it was pulverized by a crusher (Hanil, Seoul, Korea), and the fine powder was sifted from the coarse particles using a mesh screen with a pore diameter of 2 mm. In order to obtain the aqueous extract of *Armeniacaee semen*, the

fine powder was subsequently heat-extracted by distilled water, pressure-filtered, and concentrated with a rotary evaporator (Eyela, Tokyo, Japan). The resulting 34.48 g powder (a yield of 6.88 %) was obtained from 500 g of *Armeniacaee semen* through lyophilization by a drying machine (Ilsin, Korea) for 24 hrs. The content of amygdalin in this powder, determined by high pressure liquid chromatography (HPLC), was 11.00 %. The aqueous extract of *Armeniacaee semen* used in this study contains 11% of amygdalin.

Propidium iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay were purchased from Boehringer Mannheim (Mannheim, Germany). The DNA fragmentation assay



**Fig. 3.** Flow cytometric analysis. (A) Control cells; (B) 1 mg/ml *Armeniacae semen*-treated cells; (C) 5 mg/ml *Armeniacae semen*-treated cells.

kit was obtained from TaKaRa (Shiga, Tokyo, Japan) and the caspase-3 assay kit was purchased from CLONTECH (Palo Alto, CA, USA). D-amygdalin and methanol were purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan).

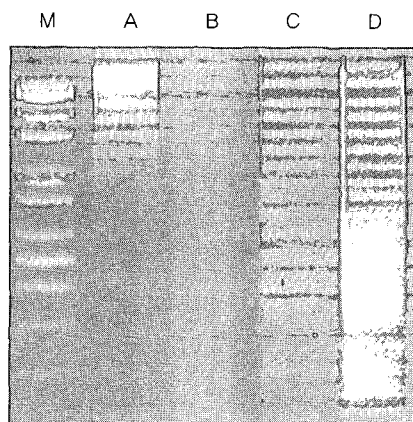
## 2. Cell culture

Mouse N2a neuroblastoma cells were cultured in

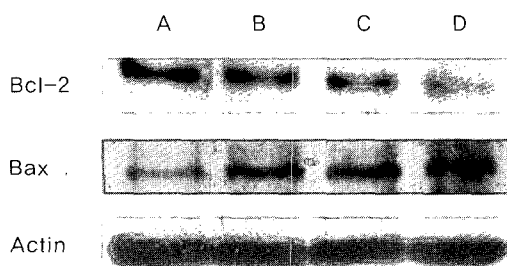
Dulbecco's Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco) at 37 °C in 5 % CO<sub>2</sub>, 95 % air in a humidified cell incubator, and the medium was changed every 2 days.

## 3. MTT cytotoxicity assay

Cell viability was determined using the MTT assay



**Fig. 4.** Electrophoretic examination of the genomic DNA of N2a cells. (M) Marker; (A) control cells; (B) 1 mg/ml *Armeniacae semen*-treated cells; (C) 3 mg/ml *Armeniacae semen*-treated cells; (D) 5 mg/ml *Armeniacae semen*-treated cells.



**Fig. 4.** Electrophoretic examination of the genomic DNA of N2a cells. (M) Marker; (A) control cells; (B) 1 mg/ml *Armeniaca semen*-treated cells; (C) 3 mg/ml *Armeniaca semen*-treated cells; (D) 5 mg/ml *Armeniaca semen*-treated cells.

kit as per the manufacturer's protocol. In order to determine the cytotoxicity of *Armeniaca semen*, the cells were treated with *Armeniaca semen* extract at concentrations of 1 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml for 24 hrs.  $10\mu\text{l}$  of the MTT labeling reagent was added to each well, and the plates were incubated for 4 hrs. Solubilization solution  $100\mu\text{l}$  was then added to each well and the cells were incubated for another 12 hrs. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. The percentage of cell viability was calculated as  $(\text{O.D. of drug-treated sample}/\text{control O.D.}) \times 100$ .

#### 4. TUNEL staining

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag<sup>®</sup> peroxidase in situ apoptosis detection kit. N2a cells were cultured on 3 chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of  $2 \times 10^4$  cells/chamber. After treatment with *Armeniaca semen* extract, the cells were washed with phosphate buffered saline (PBS) and fixed by incubating in 4 % paraformaldehyde (PFA) for

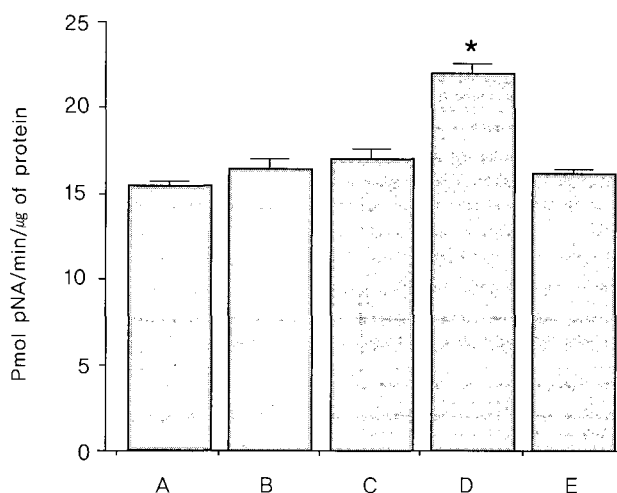
10 min at 4 °C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37 °C in a humidified atmosphere, and they were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using 3,3'-diaminobenzidine (DAB; Sigma Chemical Co, St. Louis, MO, USA) as the substrate for the peroxidase.

#### 5. DAPI staining

DAPI staining was performed according to the previously described protocol<sup>13</sup>). Cells were first cultured on 3-chamber slides (Nalge Nunc International). After treatment with *Armeniaca semen* extract, the cells were collected and fixed by incubation in 4 % PFA for 30 min. Following a washing in PBS, the cells were incubated in  $1\mu\text{g}/\text{ml}$  DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

#### 6. Flow cytometric analysis

For flow cytometric analysis, after treatment with *Armeniaca semen* extract, the cells were collected and fixed by incubation with 75 % ethanol in PBS at -20 °C



**Fig. 6.** Caspase-3 enzyme activity. The rate of DEVD-pNA cleavage was measured at a wavelength of 405 nm. (A) Control cells; (B) 1 mg/ml *Armeniaca* semen-treated cells; (C) 3 mg/ml *Armeniaca* semen-treated cells; (D) 5 mg/ml *Armeniaca* semen-treated cells; (E) cells treated with 1  $\mu$ g DEVD-fmk. DEVD-fmk is caspase-3 inhibitor. \*: represent  $p < 0.05$  compared to the control cells.

for 1 hrs. Afterwards, the cells were incubated with 100  $\mu$ g/ml RNase and 20  $\mu$ g/ml propidium iodide in PBS for 30 min at 37 oC, and the cells were analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

### 7. DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using an ApopLadder EXT<sup>M</sup> DNA fragmentation assay kit (TaKaRa, Shiga, Japan). The cells were treated with *Armeniaca semen* extract and then lysed with 100  $\mu$ l of lysis buffer. The lysate was incubated with 10  $\mu$ l of 10 % sodium dodecyl sulfate (SDS) solution containing 10  $\mu$ l of Enzyme A at 56 °C for 1 hrs, and this was followed by treatment with 10  $\mu$ l of Enzyme B at 37 °C for 1 hrs. After adding 70  $\mu$ l of precipitant and resuspending the resultant pellet in TE (Tris-EDTA) buffer, the genomic DNA was visualized by electrophoresis in a 2 % agarose gel containing ethidium bromide.

### 8. Western blot analysis

Cells were lysed in a ice-cold whole cell lysate buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 2  $\mu$ g/ml leupeptin, 1 mg/ml pepstatin, 1 mM sodium orthovanadate and 100 mM sodium fluoride. The protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein 30  $\mu$ g was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse bcl-2 antibody and mouse bax antibody (1:1000; Santa Cruz Biotech, CA, USA) were used as primary antibody. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibody (1:2000; Amersham Pharmacia Biothech GmbH, Freiburg, Germany) were used as secondary antibody for bax and bcl-2. B, and detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biothech GmbH).

### 9. Caspase enzyme activity assay

Caspase enzyme activity was measured using an ApoAlert® caspase-3 assay kit according to the manufacturer's protocol. In brief, after the treatment with *Armeniacae semen* extract, cells were lysed with 50  $\mu$ l of chilled cell lysis buffer. 50  $\mu$ l of 2  $\times$  reaction buffer (containing DTT) and 5  $\mu$ l of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37  $^{\circ}$ C for 1 hr, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

### 10. Statistical analysis

The results are expressed as the mean  $\pm$  standard error mean (S.E.M.). The data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc test. The differences were considered statistically significant at  $p < 0.05$ .

## Results

### 1. MTT cytotoxicity assay

The viabilities of cells incubated with *Armeniacae semen* extract at concentrations of 1 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml for durations of 24 hrs were 87.71  $\pm$  1.75 %, 75.10  $\pm$  2.57 %, 55.06  $\pm$  1.97 % and 42.06  $\pm$  1.01 % of the control value, respectively. A trend of decreasing viability with increasing *Armeniacae semen* concentration was observed. The results showed that *Armeniacae semen* exerts cytotoxicity on N2a neuroblastoma cells in a dose-dependent manner. In 3 mg/ml, 5 mg/ml and 10 mg/ml, cell vitality decreased significantly contrast to control cell (Fig. 1).

Under observation with the phase-contrast microscope, cells treated with *Armeniacae semen* extract of 1 mg/ml and 5 mg/ml for 24 hrs were noted as being detached from the dish, with cell rounding,

cytoplasmic blebbing and having irregularity in shape. Treatment with *Armeniacae semen* extract caused changes in the characteristic morphology of the N2a cells, that were indicative of apoptotic cell death.

DNA strand breaks occur during apoptosis, and it is known that nicks in the DNA molecules can be detected via TUNEL assay<sup>14)</sup>. TUNEL-positive cells were stained dark brown under the light microscope and nuclear condensation was observed in cultures treated with 1 mg/ml and 5 mg/ml *Armeniacae semen*. In the present study, TUNEL-positive cells that were indicative of the occurrence of apoptosis were observed among the *Armeniacae semen*-treated cells.

The DAPI assay revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon treatment with *Armeniacae semen* extract at concentrations of 1 mg/ml and 5 mg/ml for 24 hrs. Apoptotic bodies, one of the stringent morphological criteria of apoptosis, were characteristically present in *Armeniacae semen*-treated N2a cells stained with DAPI (Fig. 2).

### 3. Cell cycle distribution changes

Flow cytometric analysis revealed that the fraction of cells in the sub-G1 phase was increased from 5.85 % (control) to 10.13 % and 27.25 % following treatment with 1 mg/ml and 5 mg/ml *Armeniacae semen* extract for 24 hrs, respectively. The results of apoptotic cell analysis showed that number of cells in the sub-G1 phase was increased in a dose-dependent manner (Fig. 3).

### 4. DNA fragmentation analysis

In order to ascertain whether *Armeniacae semen* extract induces apoptotic cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As shown in Fig. 4, treatment with *Armeniacae semen* extract at concentrations of 1 mg/ml and 5 mg/ml for 24 hrs

resulted in the formation of definite fragments that could be seen via electrophoresis as a characteristic ladder pattern (Fig. 4).

#### 5. Western blot analysis of bax and bcl-2

After 24 hrs of exposure to *Armeniacae semen* extract at concentrations of 1 mg/ml, 3mg/ml and 5 mg/ml, the bax protein (26kDa) expression was increased, however bcl-2 protein (25kDa) was decreased in a dose-dependant manner (Fig. 5).

#### 6. Caspase-3 enzyme activity analysis.

Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (pNA). After 24 hrs of exposure to *Armeniacae semen* extract at concentrations of 1 mg/ml, 3 mg/ml and 5 mg/ml, the amount of DEVD-pNA cleavage product was increased from  $15.40 \pm 0.32$  pmol (control value) to  $16.55 \pm 0.48$  pmol,  $17.23 \pm 0.32$  pmol and  $22.05 \pm 0.56$  pmol, respectively (Fig. 6). On concentration of 5 mg/ml, caspase-3 enzyme activity significantly increased compare with control cells. ( $p < 0.05$ ).

### Discussion

The purpose of the present study is to find out whether *Armeniacae semen* extract induces apoptotic cell death in the mouse neuroblastoma cell line N2a.

Assessment of cell viability using MTT assay confirmed that high concentrations of *Armeniacae semen* extract exerts a cytotoxic effect on N2a cells. In addition, *Armeniacae semen* extract at concentration of 5 mg/ml was shown to induce characteristic apoptotic changes in the morphology of N2a cells. Apoptotic bodies, which are a stringent morphological criterion for apoptosis, were seen by DAPI staining in cells treated with *Armeniacae semen* extract. It has been reported that cells undergoing apoptosis exhibit cytoplasmic

blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and retraction of processes<sup>6</sup>. In addition, DNA strand breaks are known to occur during the process of apoptosis, and such breaks in the DNA molecules can be detected by TUNEL assay<sup>15</sup>. In the present study, TUNEL-positive cells, indicative of the occurrence of apoptosis, were observed in *Armeniacae semen*-treated cells.

Many anticancer drugs, such as Doxorubicin and Daunorubicin, induce apoptotic cell death in susceptible tumor cells<sup>16,17</sup>. It is also known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments that appear as a characteristic ladder pattern upon agarose gel electrophoresis<sup>18</sup>. To provide further evidence supporting the involvement of apoptosis in *Armeniacae semen*-induced cytotoxicity, DNA fragmentation assay was performed. The *Armeniacae semen*-treated cells presented with the distinctive ladder pattern characteristic of apoptosis. Flow cytometric analysis also showed the increase of apoptosis and the decrease of DNA synthesis that was caused by *Armeniacae semen* treatment. Flow cytometric analysis revealed that the fraction of cells in the sub-G1 phase was increased from 5.85 % (control) to 10.13 % and 27.25 % following treatment with 1mg/ml and 5 mg/ml *Armeniacae semen* extract for 24 hrs, respectively.

Members of the Bcl-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with Bax and homodimers with itself<sup>19</sup>. When Bax is overexpressed in cells, apoptotic death in response to a death signal is accelerated, resulting in its designation as a death agonist<sup>19</sup>. The present result demonstrated that Bax expression was increased by *Armeniacae semen* treatment in the N2a cells. When Bcl-2 is overexpressed, it heterodimerizes with Bax and cell



death is repressed. Presumably, the ratio of Bax to Bcl-2 serves to determine the susceptibility of cells to apoptosis<sup>9</sup>. In the present study, *Armeniacaese semen* extract decreased Bcl-2 expression. Down-regulation of the expression of Bcl-2 by ribozyme or antisense oligonucleotide resulted in apoptosis<sup>20,21</sup>.

Caspases, a family of cysteine proteases, are known to form integral parts of the apoptotic pathway. In particular, caspase-3, when activated, has many cellular targets and when these target proteins are severed and/or activated, this produces the morphologic features of apoptosis<sup>6</sup>. In the present study, caspase-3 enzyme activity was increased by *Armeniacaese semen* treatment in the N2a cells.

The present results showed that *Armeniacaese semen* extract induces apoptotic cell death in the N2a mouse neuroblastoma cells. Based on the present study, it is possible that the extract of *Armeniacaese semen* containing amygdalin may offer a valuable means for the treatment of tumors by inducing apoptotic cell death.

## Conclusion

N2a neuroblastoma cells treated with *Armeniacaese semen* extract exhibit several apoptotic features. Based on the present study, we get to the conclusion like this. The experimental results are as follow :

1. The viabilities of cells incubated with *Armeniacaese semen* extract at concentrations of 1 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml for durations of 24 hrs were 87.71  $\pm$  1.75 %, 75.10  $\pm$  2.57 %, 55.06  $\pm$  1.97 % and 42.06  $\pm$  1.01 % of the control value, respectively.

2. Under observation with the phase-contrast microscope, it was noted that cell rounding, cytoplasmic blebbing and having irregularity in shape. Nuclear condensation was observed in cultures by TUNEL

assay. The DAPI assay revealed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon treatment with *Armeniacaese semen* extract at concentrations of 1 mg/ml and 5 mg/ml for 24 hrs.

3. The fraction of cells in the sub-G1 phase was increased from 5.85 % (control) to 10.13 % and 27.25 % following treatment with 1mg/ml and 5 mg/ml *Armeniacaese semen* extract for 24 hrs, respectively.

4. Treatment with *Armeniacaese semen* extract at concentrations of 1 mg/ml and 5 mg/ml for 24 hrs resulted in the formation of definite fragments that could be seen via electrophoresis as a characteristic ladder pattern.

5. The bax protein (26kDa) expression was increased, however bcl-2 protein (25kDa) was decreased in a dose-dependant manner.

6. After 24 hrs of exposure to *Armeniacaese semen* extract at concentrations of 1 mg/ml, 3 mg/ml and 5 mg/ml the amount of DEVD-pNA cleavage product was increased from 15.40  $\pm$  0.32 pmol (control value) to 16.55  $\pm$  0.48 pmol, 17.23  $\pm$  0.32 pmol and 22.05  $\pm$  0.56 pmol, respectively.

It is possible that *Armeniacaese semen* may offer a valuable means for the treatment of cancer by induced apoptotic cell death.

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