

## *Houttuynia cordata* Thunberg exhibits anti-tumorigenic activity in human gastric cancer cells

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### ABSTRACT

**Objectives** : Gastric cancer is a leading cause of cancer-related deaths, worldwide. *Houttuynia cordata* Thunberg (*H. cordata*) has been used as a medicinal plants and it has an anti-cancer activity in human colorectal cancer and leukemic cancer. However, the potential anti-cancer activity and mechanisms of *H. cordata* for human gastric cancer cells have not been tested so far. Thus, this study examined the biological effects of *H. cordata* on the human gastric cancer cell line SNU-1 and AGS.

**Methods** : Inhibition of cell proliferation and cell cycle by *H. cordata* was carried out by MTT assay and Muse cell cycle analysis and the expressions of protein associated with apoptosis and cell cycle regulation were investigated with Western blot analysis.

**Results** : In MTT assay, the proliferation of SNU-1 and AGS cells was significantly inhibited by *H. cordata* in a time and dose dependent manner. Inhibition of cell proliferation by *H. cordata* was in part associated with apoptotic cell death, as shown by changes in the expression ratio of Bax to Bcl-2 by *H. cordata*. Also, *H. cordata* regulated the expression of cell cycle regulatory proteins such as pRb, cyclin D1, cyclin E, CDK4, CDK2, p21 and p15.

**Conclusion** : The antiproliferative effect of *H. cordata* on SNU-1 and AGS gastric cancer cells revealed in this study suggests that *H. cordata* has intriguing potential as a chemopreventive or chemotherapeutic agent.

**Key words** : Anti-cancer activity; Apoptosis; Human gastric cancer; *Houttuynia cordata* Thunberg

### Introduction

Gastric cancer is one of the leading causes of cancer incidence and mortality around the world<sup>1)</sup>. Although surgery is the most effective therapy for gastric cancer, chemoprevention has been regarded as a promising strategy to reduce the incidence of gastric cancer. Thus, nowadays, many researchers have investigated the promising anti-cancer agents and found that natural compounds are important sources for cancer chemopreventive and chemotherapeutic agents.

*Houttuynia cordata* Thunberg (*H. cordata*) has been used as a vegetable and herbal plant and it is

estimated that *H. cordata* has anti-inflammatory<sup>2)</sup>, anti-cestodal<sup>3)</sup>, anti-viral<sup>4)</sup> and anti-obesity effects<sup>5)</sup>, and induces apoptosis in human colorectal cancer cells<sup>6,7)</sup> and leukemic cancer cells<sup>8)</sup>. However, the potential anti-cancer activity and mechanisms of *H. cordata* for human gastric cancer cells have not been tested so far. Thus, in light of the therapeutic potential of *H. cordata* in human gastric cancer, this study was performed to elucidate the potential mechanism by which *H. cordata* induces the cell growth arrest and apoptosis in human gastric cancer cells. Here, for the first time, we report that *H. cordata* leads to cell growth arrest and apoptosis

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which may be associated with the regulating cell cycle-regulatory proteins and Bax/Bcl-2 ratio in human gastric cancer cells.

## Materials and Methods

### 1. Chemicals

Cell culture media, RPMI 1640 was purchased from Life Technologies (Grand Island, NY, USA). Antibodies against Bax, Bcl-2 and p21 were purchased from Santa Cruz Inc. (Santa Cruz, CA, USA) and other antibodies against cyclin D1, cyclin E, CDK2, CDK4, p15, phospho-Rb (ser780) and  $\beta$ -actin were purchased from Cell Signaling (Danvers, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

### 2. Sample preparation

*H. cordata* was kindly provided by Bonghwa Alpine Medicinal Plant Experiment Station, Korea. One kilogram of *H. cordata* was extracted with 2L of 80% methanol with shaking for 24 hours. After 24 hours, the extract with 80% methanol was filtered, concentrated to approximately 400 ml volume using by a vacuum evaporator, and subsequently fractioned with ethyl acetate in a separating funnel. The ethyl acetate fraction (42 g, yield rate: 4.2%) was separated from the mixture and evaporated by a vacuum evaporator. Ethyl acetate fraction was kept at  $-80\text{ }^{\circ}\text{C}$ .

### 3. Cull culture and treatment

Human gastric cancer cell lines (SUN-1 and AGS) and human normal gastric epithelial cells were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. The cells were maintained at  $37\text{ }^{\circ}\text{C}$  under a humidified atmosphere of 5%  $\text{CO}_2$ . Ethyl acetate fraction from *H. cordata* was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration was not exceeded 0.1% (v/v).

### 4. MTT assay

SNU-1, AGS and human normal gastric epithelial cells ( $1 \times 10^5$  cells) were plated onto 96-well plate and grown overnight. The cells were treated with 0, 5, 25 50

$\mu\text{g}/\text{ml}$  of extracts in media for 24 and 48 hours at  $37\text{ }^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . Then, the cells were incubated with 50  $\mu\text{l}$  of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/ml) for an additional 4 hours and then the formazan crystal produced was measured by an enzyme-linked immunosorbent assay reader at 570 nm. Cell growth inhibition was estimated as the reduction in values from a vehicle.

### 5. Cell cycle analysis

Cell cycle analysis was carried out using Muse<sup>TM</sup> Cell Cycle Kit (Millipore, Billerica, MA, USA) according to the manufacturer protocol. Briefly, the cells treated with ethyl acetate fraction from *H. cordata* were harvested, centrifuged at  $300 \times g$  for 5 min, and then washed once with 1 x PBS. One milliliter of ice cold 70% ethanol was added to cells and then cells were incubated for 3 hours at  $-20\text{ }^{\circ}\text{C}$ . After 3 hours, 200  $\mu\text{l}$  of fixed cells were transferred into new tubes, centrifuged at  $300 \times g$  for 5 min, and then washed once with 1 x PBS. After washing, cells were stained with 200  $\mu\text{l}$  of Muse<sup>TM</sup> Cell Cycle reagent at the room temperature in the dark for 30 min, and subsequently cell cycle was analyzed using Muse<sup>TM</sup> Cell Analyzer.

### 6. SDS-PAGE and Western blot

The cells were washed with 1 x phosphated-buffered saline (PBS), and lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA), and centrifuged at  $15,000 \times g$  at  $4\text{ }^{\circ}\text{C}$  for 20 min. After protein concentration was determined by Bradford protein assay. Equal amounts of proteins were subjected to SDS-PAGE and then transferred onto PVDF membrane. The membranes were blocked for non-specific binding with 5% non-fat dry milk in tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 hour at room temperature and then probed with the primary antibodies overnight at  $4\text{ }^{\circ}\text{C}$ , followed by incubation with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 hour at room temperature. Chemiluminescence was detected with ECL Western blotting substrate (GE Healthcare, Pittsburgh, PA, USA) and visualized in polaroid film.

### 7. Statistical analysis

Statistical analysis was performed with the Students unpaired t-test, with statistical significance set at \*,  $P < 0.05$ .

## Results

### 1. Effect of the extracts from *H. cordata* on cell proliferation and cell cycle progression in human gastric cancer cells and normal gastric epithelial cells

We investigated whether the extracts from *H. cordata* affects the proliferation of human gastric cancer cells. SNU-1 and AGS cells were treated with 0, 5, 25, 50 µg/ml of the extracts for 24 and 48 hours. As shown in Fig. 1A, the extracts resulted in a significant reduction of cell growth by 53% and 71% at 25 and 50 µg/ml of the extracts at 24 hours, and 12%, 80% and 82% at 5, 25 and 50 µg/ml of the extracts at 48 hours in AGS cells, respectively. We also observed that the extracts reduced the cell growth of SNU-1 by 13% and 31% at 25 and 50 µg/ml of the extracts at 24 hours, and 15%, 52% and 70% at 5, 25 and 50 µg/ml at 48 hours, respectively (Fig.1B). However, the extracts induced the minimal cell growth arrest in normal gastric epithelial cells (Fig. 1C). In addition, we analyzed the effect of the extracts from *H. cordata* on cell cycle progression to determine if inhibition of cell proliferation by the extracts from *H. cordata* was resulted from cell cycle arrest. As shown in Fig. 2A and 2B, the extracts from *H. cordata* significantly inhibited G1/S transition in SNU-1 and AGS cells. These data indicate that *H. cordata* may have an anti-cancer activity in human gastric cancer cells and this activity may be cancer-specific.

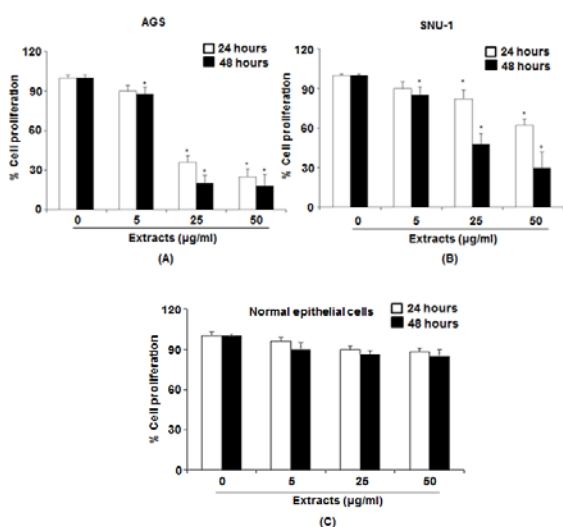


Fig. 1. Effect of *H. cordata* on cell proliferation in human gastric cancer cells, AGS and SNU-1. The effects of *H. cordata* on cell proliferation were measured by MTT assay. The values of absorbance were calculated as % cell proliferation. The cells without *H. cordata* were treated with DMSO. Statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at \*,  $P < 0.05$ .

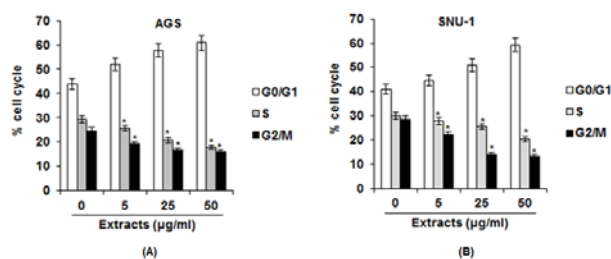


Fig. 2. Effect of *H. cordata* on cell cycle progression in human gastric cancer cells, AGS (A) and SNU-1 (B). Cell cycle progression was measured by Muse cell cycle analysis. The values of absorbance were calculated as % cell cycle. The cells without *H. cordata* were treated with DMSO. Statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at \*,  $P < 0.05$ .

### 2. Effects of the extracts from *H. cordata* on the expression of Bax and Bcl-2

To determine whether the extracts from *H. cordata* induce apoptosis in SNU-1 and AGS cells, the expression levels of Bax and Bcl-2 were evaluated by Western blot. Overexpression of Bcl-2, one of the anti-apoptotic proteins and down-regulation of Bax, one of the pro-apoptotic proteins mediates resistance to apoptosis in cancer cells<sup>9</sup>. As shown in Fig. 3A and 3B, the extracts from *H. cordata* induced overexpression of Bax in both SNU-1 and AGS cells, while it did not affect the expression levels of Bcl-2.

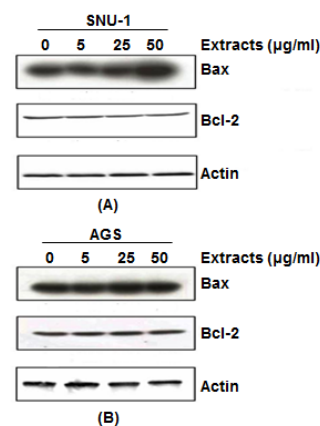


Fig. 3. Effect of *H. cordata* on the expression of Bcl-2 and Bax. SNU-1 (A) and AGS (B) cells were cultured in 6-well plate in presence of *H. cordata* for 48 h and equal amount of protein (40 µg) from cell lysates was subjected to SDS-PAGE as described in Materials and methods. Relative ratio was calculated using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.). The cells without *H. cordata* were treated with DMSO.

### 3. Inhibitory effect of the extracts from *H. cordata* on the expression of cell cycle-regulatory proteins

One of the major cell cycle regulators is retinoblastoma protein (pRb) which is considered to be a potent

tumor suppressor. It inhibits the E2F family transcription factors for transition to S phase, resulting in control of the cell cycle at the G<sub>1</sub>-to-Sphasecheckpoint<sup>10,11</sup>.

Hypophosphorylated pRb inhibits cell cycle progression by associating with E2F, while sequential pRb phosphorylation results in G<sub>1</sub>/S transition<sup>12-14</sup>. Based on this literature, we tested the regulation of pRb phosphorylation by *H. cordata* to determine whether the cell growth inhibition by *H. cordata* is mediated from cell cycle arrest. As shown in Fig. 3A and 3B, *H. cordata* down-regulated pRb phosphorylation at Ser780 in both SNU-1 and AGS cells. There are growing evidence that pRb phosphorylation is induced by the complex between cyclin D and cyclin dependent kinase (CDK) 4/6, and cyclin E and CDK2<sup>12-14</sup>. Thus we also investigated the expression levels of cyclin D1, cyclin E, CDK4 and CDK2 by Western blot. As results, expressions of cyclin D1, cyclin E, CDK4 and CDK2 were suppressed by the treatment of *H. cordata* at the dose-dependent manner in SNU-1 and AGS cells (Fig. 4A and 4B). Tumor suppressor proteins, p21 and p15 regulate cell cycle progression by inhibiting the activity of cyclin E/CDK2 and cyclin D1/CDK4 complex<sup>15,16</sup>. As shown in Fig. 4A and 4B, p21 and p15 were down-regulated in SNU-1 and AGS cells without *H. cordata*, but *H. cordata* dose-dependently up-regulated the expression of p21 and p15 in AGS and SNU-1 cells.

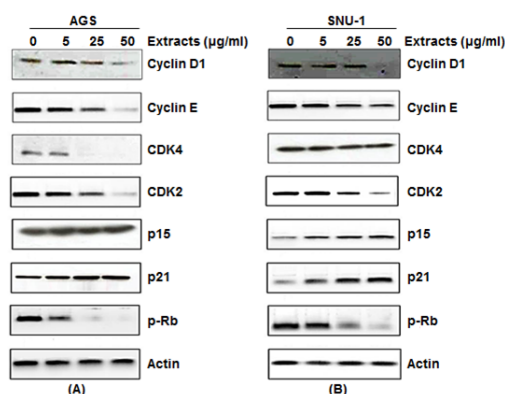


Fig. 4. Effect of *H. cordata* on the expression of cell cycle regulatory proteins. AGS (A) and SNU-1 (B) were treated with *H. cordata* for 48 h and equal amount of protein (40  $\mu$ g) from cell lysates was subjected to SDS-PAGE as described in Materials and methods. The cells without *H. cordata* were treated with DMSO. Relative ratio was calculated using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.).

## Discussion

Among cancers, gastric cancer is a leading cause of cancer-related deaths worldwide<sup>17</sup>. Recently, chemoprevention has received a great attention, and herbal medicinal plants have been recognized as an

effective anti-cancer agent<sup>18</sup>. There are some reports that *H. cordata* inhibits cell growth and induces apoptosis in human colorectal cancer cells<sup>6,7</sup> and leukemic cancer cells<sup>9</sup>. However, the potential anti-cancer activity and mechanisms of *H. cordata* for human gastric cancer cells have not been studied. Thus, this study was performed to elucidate the potential mechanism by which *H. cordata* induces the cell growth arrest and apoptosis in human gastric cancer cells.

Although apoptosis is a process that removes damaged cells, resulting in the maintenance of cell numbers, cancer cells avoid apoptosis in response to various physiologic stimuli<sup>19</sup>. Therefore, the induction of apoptosis has been regarded as a major target for cancer chemoprevention. There is a growing advance that the process of apoptosis is associated with increase of Bax expression, a proapoptotic protein and decrease of Bcl-2 expression, anti-apoptotic protein<sup>20</sup>. Thus, the ratio of Bax to Bcl-2 is important in determining susceptibility to apoptosis<sup>21</sup>. Although *H. cordata* did not affect Bcl-2 expression, it up-regulated Bax expression. Taken together, *H. cordata* induced the increase of the ratio of Bax to Bcl-2. Therefore, apoptosis was induced in *H. cordata*-treated SNU-1 and AGS.

More and more investigations indicated that cell cycle arrest and apoptosis are closely associated with cell proliferation<sup>22</sup>. Also, the sensitivity to apoptosis often depends on the cell cycle<sup>23</sup>. Many studies have shown that inhibition of the cell cycle has been considered as a target for cancer chemoprevention<sup>24</sup>. Retinoblastoma protein (pRb) controls G<sub>1</sub>/S phase transition and is a major target of the cyclin-CDK complexes. The pathway controlled by pRb may be aberrant in most human cancers<sup>25</sup>. In normal cells, Rb is functionally inactivated by multiple phosphorylations mediated sequentially by a series of cyclin-CDK complexes<sup>25</sup>. Phosphorylation of Rb disrupts complexes with E2Fs, allowing for cell cycle progression into S phase<sup>25</sup>. G<sub>1</sub>-phase progression is mediated by the combined activity of the cyclinD1/Cdk4 and cyclin E/Cdk2 complexes<sup>23</sup>. CyclinD1-associated kinase activity increases in mid-G<sub>1</sub>, while cyclin E/Cdk2 activity increases in late G<sub>1</sub> and peaks in early S phase<sup>23</sup>. We examined the effects of *H. cordata* on the expression of cell cycle-regulatory proteins in the G<sub>1</sub> phase of the cell cycle. *H. cordata* suppressed the expression of cyclin D1, cyclin E, CDK4 and CDK2. In addition, *H. cordata* increased the expression of tumor suppressor proteins, p15 and p21. It has been reported that p15 inhibits CDK4 activity and p21 acts as broad specific inhibitors of cyclin D and E<sup>26</sup>. These findings indicate that the inhibition of SNU-1 and AGS proliferation by

*H. cordata* may be mediated from the cell cycle distribution.

## Conclusion

In summary, the results of the present investigation indicate the below.

1. *H. cordata* inhibited cell proliferation in human gastric cancer cells, AGS and SNU-1.
2. *H. cordata* induced increase in Bax protein, while it did not affect Bcl-2 expression in AGS and SNU-1 cells
3. *H. cordata* down-regulated cell cycle regulatory proteins such as cyclin D1, CDK4, cyclin E and CDK2, while it activated the expression levels of tumor suppressor proteins, p21 and p15, which resulted in inhibition of pRb hyper-phosphorylation

In conclusion, this study demonstrates that human gastric cancer cells can be killed by *H. cordata* and these antiproliferative and apoptotic effects of *H. cordata* may account for some chemopreventive or chemotherapeutic effects on gastric cancer cells.

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