

Anti-Proliferative Effect of Polysaccharides from *Salicornia herbacea* on Induction of G2/M Arrest and Apoptosis in Human Colon Cancer Cells

Ryu, Deok-Seon¹, Seon-Hee Kim², and Dong-Seok Lee^{1,2*}

¹Department of Smart Foods and Drugs supported by the Second Stage BK21 Project of Korea Research Foundation, Inje University, Gimhae 621-749, Korea

²Department of Biomedical Laboratory Science, Inje University, Gimhae 621-749, Korea

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In this study, we investigated the anti-proliferative effect of polysaccharides from *Salicornia herbacea* on HT-29 human colon cancer cells. Crude polysaccharides from *S. herbacea* (CS) were prepared by extraction with hot steam water, and fine polysaccharides from *S. herbacea* (PS) were obtained through further size exclusion chromatography. The anti-proliferative effect of CS and PS were measured using the MTS assay, apoptosis analysis, cell cycle analysis, and RT-PCR. HT-29 cells were treated with CS or PS at different dosages (0.5, 1, 2, 4 mg ml⁻¹) for 24 or 48 h. CS and PS inhibited proliferation and stimulated apoptosis of cells in a dose-dependent manner. Flow cytometric analysis after Annexin V-FITC and PI staining revealed that treatment with CS or PS increased total apoptotic death of cells to 24.99% or 91.59%, respectively, in comparison with the control (13.51%). PS increased early apoptotic death substantially - up to 12 times more than the control. Treatment with CS or PS resulted in a concentration-dependent increase of the G2/M cell population of the cell cycle as determined by flow cytometry. G2/M arrest was induced significantly with the highest concentration (4 mg ml⁻¹) of PS. RT-PCR was performed to study the correlation between G2/M arrest and transcription of cell cycle control genes. The anti-proliferative activity of CS and PS was accompanied by inhibition of cyclin B1, and Cdc 2 mRNA. Moreover, both CS and PS induced expression of the p53 tumor suppressor gene and the Cdk inhibitor p21. These results suggest that polysaccharides from *S. herbacea* have anti-cancer activity in human colon cancer cells.

Keywords: *Salicornia herbacea*, anti-proliferation, apoptosis, cell cycle, G2/M arrest

*Corresponding author

Phone: +82-55-320-3262; Fax: +82-55-334-3426;
E-mail: mbdslee@inje.ac.kr

Colorectal cancer is the leading cause of death by cancer in the United States [13]. For the medical treatment of cancer, surgical operation, radiation-therapy, immunotherapy, and/or chemotherapy are often applied. Currently, most medicines used in chemotherapy are combinations of chemical substances with no selectivity toward cancer cells and usually show toxicity to normal cells as well. Anti-cancer drugs destroy the immune system of the host through suppression of lymphocytes and bone marrow cells, which play important roles in the immune system. Drug resistance and high rates of disease recurrence are associated with many of the currently-used chemotherapeutic drugs [2]. In recent years, many studies have therefore attempted to develop new anti-cancer drugs that are selective toward cancer cells to avoid damaging normal cells. Non-cytotoxic agents with high anti-cancer efficacy and less cellular toxicity to normal cells have been suggested as possible candidates to be investigated for their synergistic effects in combination with chemical substances [5, 17, 35]. In response to this situation, there is increasing interest in research on maritime plants in the search for novel agents for health promotion and the development of physiologically active materials [28]. This study focuses on the anti-proliferative activity of one of the maritime plants, *Salicornia herbacea*.

S. herbacea (Chenopodiaceae, Korean name: Hamcho) is an annual herb that grows in salt marshes and salt field along the seashores along the southern and western coasts of Korea [15]. It is used in Korea as a folk medication to treat a variety of diseases such as indigestion, gastroenteric disorders, hepatitis, and nephropathy [2, 14]. *S. herbacea* contains large amounts of salts and minerals, especially calcium, magnesium, and iodine [8, 30]. In addition, their high dietary fiber content (50–70%) makes them effective at preventing constipation. Indeed, some investigators have reported medicinal effects and synergistic effects in the prevention of tumors with *S. herbacea* [10, 11, 19, 22].

However, systematic research on its anti-proliferative activity is insufficient at this time. Because of its diverse medicinal properties, *S. herbacea* is expected to yield valuable therapeutic agents, but much scientific research will be required due to the lack of biological mechanisms regarding its physiological activity.

The eukaryotic cell growth is regulated through the cell cycle and apoptosis progression. Many cytotoxic agents or DNA-damaging agents arrest the cell cycle and subsequently induce apoptotic cell death [16, 26]. Cells pass through several checkpoints as they proceed through the cell cycle, and certain criteria must be met to pass each of these checkpoints [4]. Defects in the cell cycle regulation are a common cause of the abnormal proliferation of cancer cell, so studies of cell cycle and cancer have become closely interconnected. Therefore, the induction of cell cycle arrest and/or apoptotic cell death is an important mechanism that is involved in the anti-cancer agents.

In this study, we investigated the mechanism of suppression of proliferation, induction of apoptosis, and expression of genes related to cell cycle control by *S. herbacea* polysaccharides in human colon cancer cells.

MATERIALS AND METHODS

Materials

S. herbacea was harvested in the south and west reclaimed land along the shore in Korea and provided by DASARANG Ltd. (Shinna, Korea). *S. herbacea* were dried and sliced, and the polysaccharides were extracted by boiling in water for 3 h under high pressure. Crude polysaccharides of *S. herbacea* (CS) were collected by filtration through filter paper (Whatman[®] No. 1). Fine polysaccharides of *S. herbacea* (PS) were purified using the protocol described by Klarzynski *et al.* [20], with slight modification. The PS were fractionated by filtration using sephadex G-50 column (200×15 mm) at a flow rate of 3.5 ml min⁻¹. The average molecular mass of PS was measured by gel filtration chromatography with phenol/sulfuric acid monitoring [32]. The CS and PS were freeze-dried using a Freeze Dryer (Clean-Vac 24T, Biotron, Korea) to obtain powder and stored at -4°C.

Cell Culture

The HT-29 cell line (human colon cancer cells) was purchased from the Korean Cell Line Bank. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) with 5,000 units penicillin ml⁻¹ and 5,000 µg streptomycin ml⁻¹ in 0.85% saline (Gibco, Grand Island, NY, U.S.A.) in a humidified incubator at 37 with 5% CO₂.

Proliferation Assay

Inhibition of cell proliferation by CS or PS was measured by the MTS method using the Cell Titer 96[®] Aqueous One Solution Cell Proliferation Assay (Promega, Madison, MI, U.S.A.). MTS assays were performed as directed by the manufacturer. HT-29 cells were plated in 96-well microtiter plates (5×10⁵ cells ml⁻¹). After 24 h

incubation, the cells were treated with different concentration (0.5, 1, 2, 4 mg ml⁻¹) of CS or PS for 24 and 48 h. Culture medium was aspirated and 20 µl of MTS reagent in culture medium was added to each well. Cells were incubated for an additional 4 h at 37°C. Proliferation levels were detected by O.D. 490 nm using a Fluorescence Multi-Detection Reader (Synergy HT, Biotex, U.S.A.).

Apoptosis Assay

The apoptotic status of HT-29 cells was evaluated by measuring the exposure of phosphatidylserine on the cell membranes using Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining [7]. The BD Pharmingen Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used for the apoptosis assay. HT-29 cells were plated in a 24-well plate (1×10⁶ cells ml⁻¹), and after a 24 h incubation, the cells were treated with graded concentrations of CS or PS for 24 or 48 h and harvested. After centrifugation, the cell 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 1× binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). The cells were incubated with 5 µl Annexin V-FITC and 10 µl PI at room temperature for 15 min in the dark. After the incubation, 400 µl of 1× binding buffer was added to each tube. The cells were analyzed immediately by FACSCalibur flow cytometry (Becton Dickinson, U.S.A.) [18].

Cell Cycle Analysis

The cell cycle phase was assayed by measuring DNA fragment staining with PI. A Cell Cycle Phase Determination Kit (Cayman Chemical, Ann Arbor, Michigan, U.S.A.) was used as described by the manufacturer. 1×10⁶ cells were plated in each well of a 12-well plate for 24 h followed by treatment with different concentrations (0.5, 1, 2, 4 mg ml⁻¹) of CS or PS for 24 or 48 h. After treatment, the cells were harvested and centrifuged. Cell pellets were suspended to a density of 1×10⁶ cells ml⁻¹ in assay buffer after washing twice with assay buffer. The cells were fixed and permeabilized by adding 1 ml fixative (100% ethanol) to each tube for more than 2 h. After centrifugation, fixatives were decanted and cell pellets were suspended in 0.5 ml of staining solution containing 200 µl DNase-free RNase and 200 µl PI, and incubated for 30 min at room temperature in the dark. The cells were analyzed immediately by FACSCalibur flow cytometry.

RT-PCR

Cells were plated in a petri dish (1×10⁶ cells ml⁻¹) for 24 h and then treated with 4 mg ml⁻¹ CS or PS for 24 h. Total RNA was isolated using an Easy-spin [DNA-free] Total RNA Extraction Kit (iNtRON Biotechnology, Korea) as described by the manufacturer. The purity and concentration of the total RNA was checked by spectrophotometry. Equal amounts of RNA were reverse-transcribed into cDNA using the Maxime RT-PCR Premix Kit (iNtRON Biotechnology, Korea) by incubation at 45°C for 30 min and at 94°C for 5 min. PCR was performed on cDNA (primers shown in Table 1). Samples were subjected to 30 cycles of denaturation for 40 s at 94°C, annealing at different temperatures (shown in Table 1), and extension for 1 min at 72°C; this was followed by a final extension step at 72°C for 5 min. Amplified cDNA products were electrophoresed in a 1.5% agarose gel followed by staining with ethidium bromide.

Table 1. Sequences of primers used for RT-PCR and annealing temperatures.

Gene name	Annealing Temp. (°C)		Sequence
Cyclin B1	56.1	Sense	5'-AAG-AGC-TTT-AAA-CTT-TGG-TCT-GGG-3'
		Anti-sense	5'-CTT-TGT-AAG-TCC-TTG-ATT-TAC-CAT-G-3'
Cdc 2	51	Sense	5'-GGG-GAT-TCA-GAA-ATT-GAT-CA-3'
		Anti-sense	5'-TGT-CAG-AAA-GCT-ACA-TCT-TC-3'
p53	53.1	Sense	5'-GCT-CTG-ACT-GTA-CCA-CCA-TCC-3'
		Anti-sense	5'-CTC-TCG-GAA-CAT-CTC-GAA-GCG-3'
p21	56.2	Sense	5'-CTC-AGA-GGA-GGC-GCC-ATG-3'
		Anti-sense	5'-GGG-CGG-ATT-AGG-GCT-TCC-3'
GAPDH	61.8	Sense	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
		Anti-sense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

Statistical Analysis

All experiments were performed in triplicate. Data are expressed as means±standard error. Statistical comparisons of results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of control and CS or PS treated cells were analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

Extraction and Purification of CS and PS from *S. herbacea*

Crude polysaccharides (CS) were extracted from *S. herbacea* and were approximately 10% of the whole mass. Fine polysaccharides (PS) were serially purified from CS by gel filtration chromatography (Fig. 1). The average molecular weights of PS were determined to be between 1 and 5 kDa. Although composition analysis of the polysaccharides has not been conducted yet, it has been reported that *S. herbacea* contained approximately 46% polysaccharides

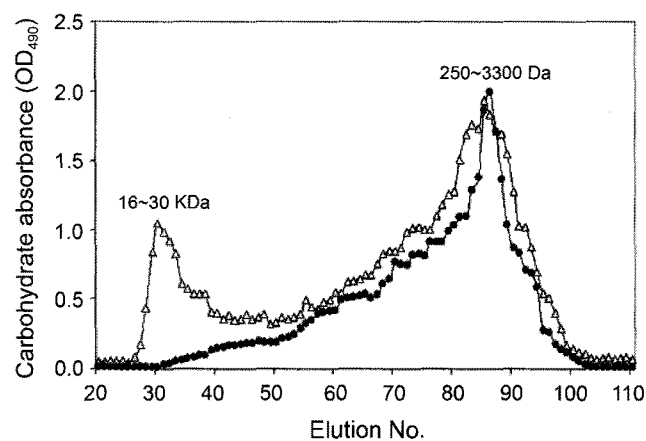


Fig. 1. Gel filtration chromatography of *S. herbacea* polysaccharide (PS).

Aliquots of polysaccharides (CS and PS) dissolved in distilled water were applied to Sephadex G-50 column chromatography. Fractions were collected and monitored using the phenol- H_2SO_4 method. CS (Δ), crude extract of *S. herbacea*; PS (\bullet), polysaccharides extract of *S. herbacea*.

and the average molar ratio of galactose, glucose and mannose was 5:1.5:1 [10].

Inhibition of Cell Proliferation and Induction of Apoptosis by CS and PS

The effect of CS and PS treatment on cell proliferation of HT-29 was detected by MTS assay. CS and PS both had a

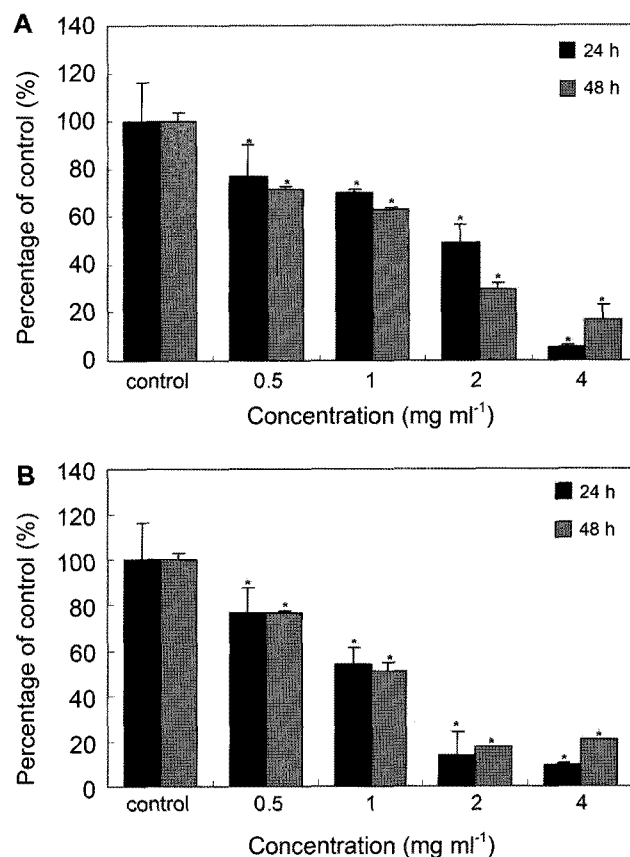


Fig. 2. Proliferative inhibition of HT-29 cells by CS (A) and PS (B). The cells (5×10^5 cells ml^{-1}) were treated with graded concentration of CS or PS for 24 or 48 h. Cell proliferation was determined by MTS assay. Results are represented as percentage of control. *Significantly different from control at $p < 0.05$.

dose-dependent anti-proliferative effect on the human colon cancer cell line HT-29 (Fig. 2). After treatment with CS for 24 h, the cells survival rate was more than 50% as compared to the control at a concentration of 0.5 mg ml⁻¹ and 1 mg ml⁻¹. However, the survival rate of cells treated with 4 mg ml⁻¹ CS decreased dramatically to 5.9% of the control. PS showed a similar anti-proliferative activity to CS. Treatment with PS concentrations of 0.5 mg ml⁻¹ and 1 mg ml⁻¹ inhibited proliferation of cells by approximately 50% compared to the control. When a high concentration of PS was used to treat HT-29 cells, growth was remarkably inhibited - the cell survival rate decreased to 9.5% of that of the control.

The ability of CS or PS treatment to induce apoptosis in HT-29 cells was determined using an Annexin V-FITC assay to establish the relationship between anti-proliferation and apoptosis. Treatment of HT-29 cells with CS or PS (0.5–4 mg ml⁻¹) for 24 h increased apoptotic death approximately 1.8- or 6.8- fold and suppressed cell survival rate in culture by 74.47% or 6.72%, respectively (Table 2 and Fig. 3). As shown in Fig. 3, the early apoptotic death rate (16.67%, lower right region) of HT-29 cells treated with CS was higher than that of the control (7.27%), and the late apoptotic or necrotic death rate (8.32%, upper right region) was higher than that of the control (6.24%). Similarly, the early apoptotic death rate (85.67%) of the HT-29 cells treated with PS was remarkably higher than the control. However, the late apoptotic or necrotic death rate (5.92%) was not significantly different from that of the control. After a 48 h treatment with CS or PS (Table 2), the amount of apoptosis induced by PS was the same as that induced by CS. Specifically, the apoptotic-inducing activities of CS and PS caused in an increase in the early apoptotic death rate that resulted in poor survival of cells after treatment.

Overall, these results exhibit that the polysaccharides of *S. herbacea* may not only inhibit growth of HT-29 cells but

also induce apoptosis of the cells. Especially, since many anti-cancer drugs suppress cancer by inducing apoptosis [1, 25], *S. herbacea* extract shows promise as a novel anti-cancer agent.

G2/M Arrest of the Cell Cycle by CS and PS

Uncontrolled cell proliferation is the characteristic of cancer, and CS and PS were shown to inhibit HT-29 cells proliferation. To probe the mechanism of CS and PS-mediated cell growth inhibition, we examined cell cycle alteration by flow cytometry. The effects of CS and PS on the cell cycle progression of HT-29 cells are shown in Table 3 and Fig. 4. After 24 h of growth, the G2/M phase cell population of control was 14.24%. Meanwhile, the G2/M phase cell population of CS- and PS-treated cells increased in a dose-dependent manner. A concentration of 4 mg ml⁻¹ PS had the highest percentage of cells (37.77%) in the G2/M phase of the cell cycle. As the G2/M phase population increased, the G1 phase cell population decreased, while the S phase cell population showed slight change within 24 h. This G2/M arrest was also found after 48 h of treatment. At the highest concentration (4 mg ml⁻¹) of CS and PS, the proportion of cells in the G2/M phase increased to 19.31% (2.5- fold) and 21.36% (2.7- fold), respectively, compared to the control (7.74%). These results indicate that the polysaccharides of *S. herbacea* act on the G2-M transition check-point of the cell cycle.

Effects of CS and PS on the Transcription of Cell Cycle Control Genes

Since CS and PS arrested HT-29 cells in the G2/M phase of the cell cycle, RT-PCR was performed to study the correlation between G2/M arrest and transcription of cell cycle control genes. A number of Cdks and Cdk inhibitors have been isolated, and have been shown to regulate the events of the cell cycle [29, 36]. Various cyclin-Cdk complexes composed of different kinds of cyclin and Cdk proteins

Table 2. Apoptotic death of HT-29 cells to CS or PS for 24 or 48 h.

		mg ml ⁻¹	Viable	Apoptotic death (early)	Apoptotic death (late)
24 h	Control	0	85.00±2.47	7.27±1.80	6.24±0.96
	CS	0.5	84.92±0.91	11.25±3.20	6.93±0.67
		4	74.47±2.95*	16.67±5.52*	8.32±2.45*
	PS	0.5	86.62±0.06	5.27±0.70	6.58±0.34
		4	6.72±0.91*	85.67±0.71*	5.92±0.24
	48 h	Control	0	79.36±0.60	16.27±0.91
CS		0.5	77.00±0.37	16.63±0.89	13.10±0.91
		4	53.00±7.54*	19.96±1.54	25.50±5.81*
PS		0.5	74.75±5.95	15.62±0.73	12.30±1.19
		4	6.87±0.37*	74.45±1.26*	18.26±1.49*

The results were represented as percentage of total treated cells.

Data values are expressed as mean±SE (n=3).

*Significantly different from control at $p < 0.05$.

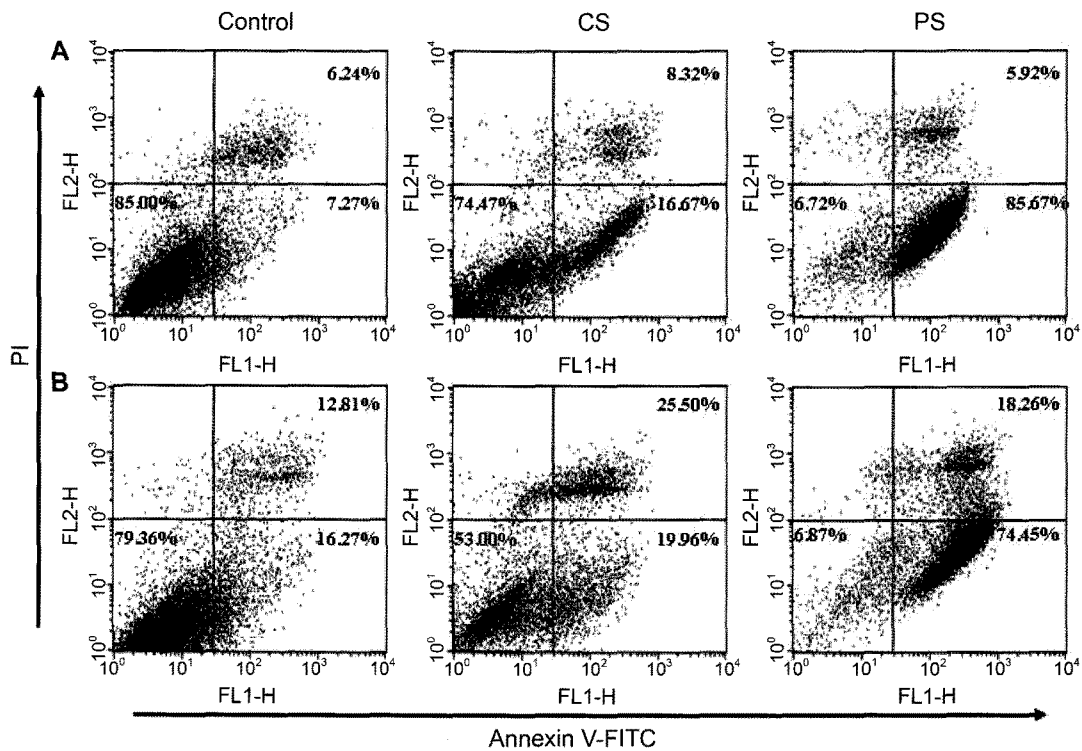


Fig. 3. Flow cytometry analysis of the apoptotic death of HT-29 cells. The cells were treated with different concentration of CS or PS for 24 (A) or 48 h (B). Dot plots display the apoptotic death of HT-29 cells treated with 4 mg ml⁻¹ of CS or PS. The cells were stained with monoclonal antibodies against Annexin V-FITC and PI. Annexin-/PI- (LL), viable cells; Annexin+/PI- (LR), cells undergoing apoptosis; Annexin+/PI+ (UR), cells that are in end-stage apoptosis or are already dead. LL, lower left; LR, lower right; UR, Upper right.

activate different cell cycle progression. Cyclin D and cyclin E are known to be essential to induce progression of the G1 phase. The cyclin D/Cdk 4 (and/or Cdk 6) complex is necessary for initiating the early G1 phase, while the cyclin E/Cdk 2 complex is needed to progress from the late G1 phase to early S phase [21, 24]. Expression of the cyclin A/Cdk 2 complex increases from the S to G2 phase, and the cyclin B1/Cdc 2 complex is required for progression

from the G2 phase to the M phase of the cell cycle [27]. Cdc2 kinase is primarily activated in association with cyclin B1 during the progression of G2/M phase [31, 37].

To examine the expression of mRNA levels regulating cell cycle progression at the G2/M phase which was remarkably arrested as shown in Fig. 4, cyclin B1 and Cdc2 expression were measured by RT-PCR (Fig. 5). Expression of cyclin B1 decreased when cells were treated

Table 3. Cell cycle distribution in HT-29 cells treated with CS or PS for 24 or 48 h.

		mg ml ⁻¹	Sub G1	G1	S	G2/M
24 h	Control	0	4.41±1.08	63.16±3.50	16.82±1.11	14.24±0.33
	CS	0.5	1.87±0.14*	54.57±1.52*	19.10±0.36*	20.40±1.20*
		4	3.01±0.93	52.17±0.18*	20.83±1.13*	23.05±0.41*
	PS	0.5	2.91±0.30	56.81±0.45*	17.76±0.20	18.32±0.80*
		4	1.66±0.33*	41.80±0.47*	15.71±0.45	37.77±0.35*
48 h	Control	0	13.01±2.90	69.14±3.04	6.50±0.71	7.74±1.49
	CS	0.5	13.02±0.36	71.01±1.33	6.25±0.01	11.00±1.44
		4	17.14±4.01	46.92±3.61*	14.08±1.43*	19.31±5.69*
	PS	0.5	12.07±1.65	71.24±1.59	5.19±0.30	9.57±0.14
		4	14.27±2.17	52.00±0.34*	11.13±0.67*	21.36±1.22*

The results were represented as percentage of total treated cells.

Data values are expressed as mean±SE (n=3).

*Significantly different from control at *p*<0.05.

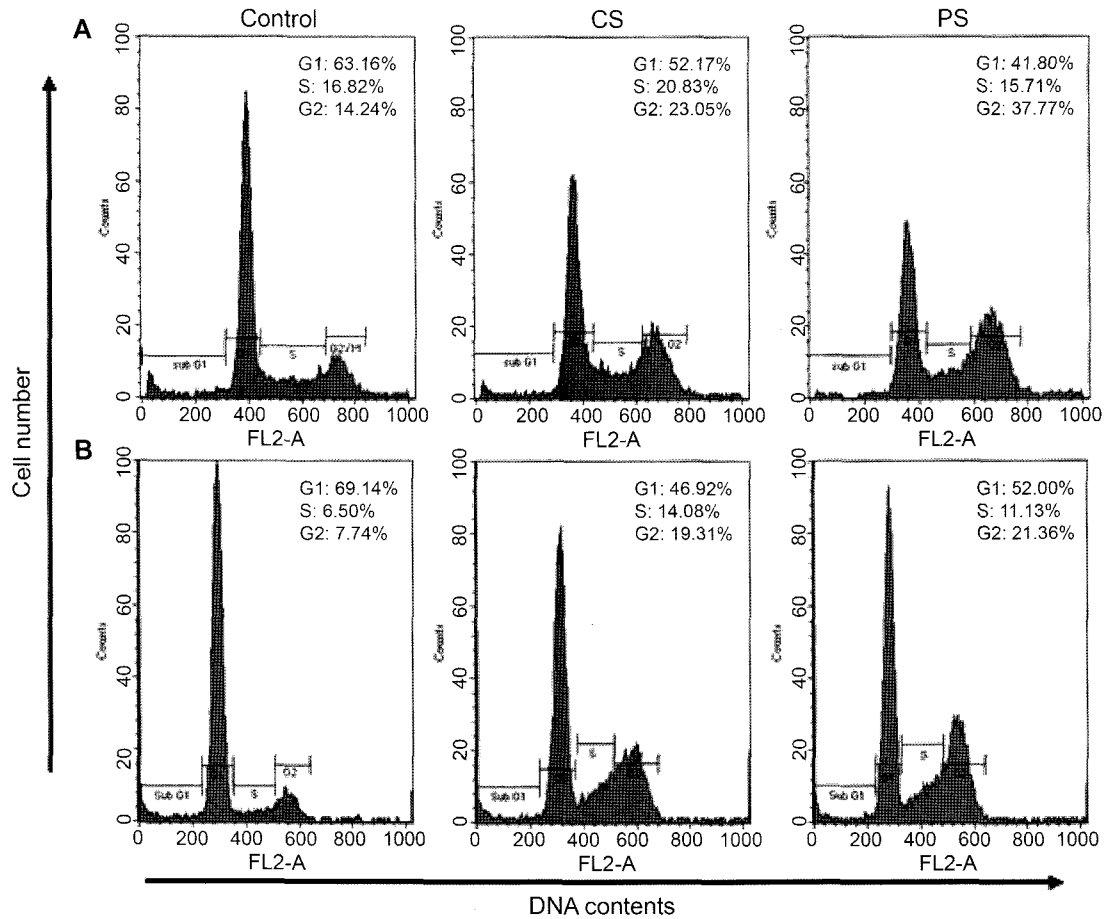


Fig. 4. Flow cytometry analysis of cell cycle distribution of HT-29 cells. The cells were treated with different concentration of CS or PS for 24 (A) or 48 h (B). Histograms display cell cycle distribution of HT-29 cells treated with 4 mg ml⁻¹ of CS or PS. The cells were stained with PI to measure the contents of DNAs. Histograms display sub G1, G1, S, and G2/M phase of HT-29 cells.

with CS or PS as compared with the control. Cdc 2 was also expressed at a lower level in CS- or PS-treated cells than control cells. The decrease in the expression of cyclin

B1 and Cdc 2 correlated to the increase in numbers of cells in the G2 phase, which is consistent with the G2/M arrest observed from the cell cycle analysis.

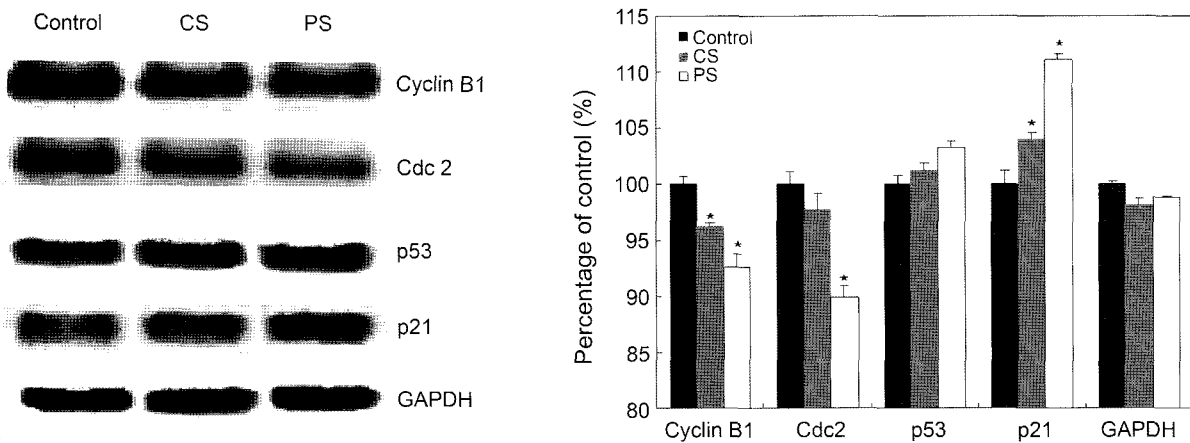


Fig. 5. Effect of CS or PS on the levels of cyclin B1, Cdc2, p53, and p21 in HT-29 cells. Total RNAs were isolated from the cells treated with CS or PS for 24 h and RT-PCR was performed. GAPDH was used as a house-keeping control gene.

Cdk inhibitors are known to bind and inactivate cyclin/Cdk complexes. Activation of p21, a Cdk inhibitor is controlled by p53. The concentration and activation of the p53 tumor suppressor gene increase when DNA is damaged [6, 23]. Activated p21 protein inactivates the cyclin-Cdk complex that helps the progression of the cell cycle to the S phase. Consequently, the cell cycle ceases at the G1 phase, so that repair of damaged DNA can occur before DNA replication [9]. The mechanism by which p53 mediates cell cycle arrest at the G1 check-point involves induction of the cyclin-dependent Cdk inhibitor p21. Recently, a novel form of p21 protein was shown to inhibit growth by acting not at G1, but at G2 arrest [3, 23, 34].

The expression of p53 and p21 mRNAs increased slightly when cells were treated with CS or PS as compared with the control (Fig. 5). Several investigators have noted that cells deficient in p21 escaped G2/M phase cell cycle arrest when exposed to DNA damaging agents [33] and p53 arrested the cell cycle at the G2/M phase by lowering cyclin B1 levels [12]. These results show that both CS and PS could influence the expression of p53 and p21 and be effective as an anti-proliferative agent for HT-29 cells in the maintenance of G2/M cell cycle arrest.

In conclusion, this study showed that both CS and PS extracts from *S. herbacea* have an anti-proliferative effect related to changes in the expression of cell cycle control genes as well as apoptotic effect on human colon cancer cells. Although further study will be needed to elucidate the detailed correlation between cell cycle arrest and apoptosis, this study provides a useful foundation for studying and developing new anti-proliferative substances based on these extracts for the treatment of colon cancer.

Acknowledgments

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