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Ethanol extract of Kilkyung-baeksan, a traditional herbal formula, induces G0/G1 cell cycle arrest in human lung cancer cell lines

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ABSTRACT

Background: Despite current advances in diagnostics and medicines, the incidence of lung cancer is increasing and effective treatment is very challenging. Traditional herbal formulae as well as many herbal plant extracts have been recognized as attractive sources for novel multi-targeted therapy of cancer with minimal side effects.

Methods: The ethanol extract of Kilkyung-baeksan (EE-KKBS) and its component herbs were tested for their ability to inhibit cancer growth in several lung cancer cell lines. The effects of EE-KKBS and ethanol extract of *Croton tiglium* Linné seed (EE-CT) on cell cycle progression were measured by flow cytometric analysis using propidium iodide staining. Western blot analyses were performed to measure the expression profiles of proteins regulating cell cycle checkpoints.

Results: EE-KKBS inhibited the growth of lung cancer cells after 24–72 hours treatment. Lung cancer cells treated with either EE-KKBS or EE-CT showed strong G0/G1 cell cycle arrest. The expressions of p21 and p27, two key regulators of G1 cell cycle checkpoint, were significantly upregulated upon treatment with EE-KKBS and EE-CT.

Conclusion: EE-KKBS exerted its cytostatic activity through regulating G1 cell cycle checkpoint in lung cancer cells, and this activity is mainly mediated by one of its component herbs, seeds of *Croton tiglium*. Collectively, our data suggest that EE-KKBS could be a novel candidate for adjuvant therapy for lung cancer.

1. Introduction

Lung cancer is the most frequent malignancy and also one of the leading causes of cancer-related mortality in both men and women worldwide.[1] Based on the World Health Organization, there are four major types of lung cancer; squamous cell carcinoma, small-cell carcinoma, adenocarcinoma, and large cell carcinoma.[2] Non small cell lung cancer (NSCLC) is the most frequent type in all cases, accounting for 75–80% of lung cancer cases. Although common treatment for lung cancer include surgery, radiotherapy, and chemotherapy, the 5-year survival

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rate among lung patients remains very low[3] partly due to the late diagnosis and no optimal treatment option for lung cancer.

Using medicinal plants to fight against various human diseases including cancer have been widely practiced all around the world, especially in China, India, as well as in Korea. Compared with the conventional cancer chemotherapy, the mixture of phytochemicals in the medicinal plants may have synergistic effect that targets several pathways responsible for cancer pathogenesis, thus increasing the therapeutic efficacy along with reducing side effects.[4,5] The main issues of herbal extract for any therapeutic usage are hardship in quality control of plant materials, herbal standardization, and lack of scientific study supports its therapeutic value.

Kilkyung-Baeksan (KKBS) is three-herb containing traditional Korean Medicine (KM) formula: the root of *Platycodon grandiflorum* A.De Candolle (PG), the seed of *Croton tiglium* Linné (CT), and the bulb of *Fritillaria thunbergii* Miquel (FT). This formula first appeared in an old Chinese Medical book written in early 3rd century, Kumkye-yorak (Jin Gui Yau Lue in Chinese; Medical Treasures of the Golden Chamber) and repeatedly mentioned in various books on KM including Dongeui-bogam, a well-known Encyclopedia of Korean Medicine. It has been widely used for various lung diseases such as coughs, short breath, pneumonia, and lung cancer-like symptoms. Although there is no scientific study in regard to the pharmacological or biological effect of KKBS, one of its constituents, the root of *Platycodon grandiflorum* A.De Candolle, is known to have properties such as inducing apoptosis in various cancer cell lines including prostate[6] and colon cancer,[7] suppressing TGFβ1-induced epithelial-mesenchymal transition,[8] and hepatoprotective activity.[9]

In this study, we evaluated the effect of Kilkyung-Baeksan (KKBS) on various human lung cancer cell lines and found that ethanol extract of KKBS (EE-KKBS) and *Croton tiglium* seed (EE-CT), one of its constituents, possessed potent cell cycle arrest activities without high cytotoxicity which might confer on its anticancer ability.

2. Methods

2.1. Herbal formula and plant materials

KKBS is composed of three kinds of herbs. The names, parts, and proportions of each herb comprising KKBS are described in Table 1. These medicinal herbs were purchased from Kwangmyungdang Medicinal Herbs Co. (Ulsan, Republic of Korea) and voucher specimens for all herbs were deposited and coded (details are summarized in Table 1) at the Cancer Research Team, KM-Convergence Research Division, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea. The identification of each medicinal herb was confirmed by Dr. Go Ya Choi, K-Herb Research Center, Korea Institute of Oriental Medicine.

2.2. Preparation of ethanol extract of KKBS and its component herbs

The ethanol extracts of KKBS and each component were prepared as follows. The dried and ground plant materials were mixed accordingly and were extracted with 70% ethanol by sonication. After extraction, each extract was filtered using 3MM paper and freeze-dried to get a powered form. Each powdered herbal extract was first dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL concentration, then diluted to 2 mg/mL in phosphate-buffered saline (DMSO concentration in stock solution is 10%) and stored at −20 °C for further use.

2.3. Cell culture

All cell lines used in this study were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Human lung cancer cell lines including A549, NCI-H460, NCI-H23, and NCI-H226 were cultured in RPMI 1640 media, and normal lung fibroblast cell lines were cultured in Dulbecco's modified eagle medium (DMEM; Life Technologies, Grand Islands, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), and maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

2.4. Cell proliferation assay

Cells in the logarithmic growth phase were seeded into flat-bottom 96-well plates at a density of 5×10^3 cells per well and allowed to attach overnight prior to drug treatment. Cells were exposed to 100 ug/mL of KKBS and herbal extracts for 48 hours, and cell proliferative activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (Promega, Madison, WI, USA). The maximum concentration of vehicle (DMSO) in culture media was adjusted to 1% (v/v) and this concentration was used as control treatment. The relative proliferative index was calculated as percentage (%) of experimental optical density (OD) value divided by control (vehicle) OD value.

2.5. Cell growth and viability

Cells were seeded into six well plates at a density of 1×10^5 cells/well overnight and treated with 100 microgram/mL of herbal extracts. After 16 hours, 24 hours, 48 hours, and

Table 1 – Herbal constituents of KKBS

Voucher code	Latin name	Scientific name	Ratio (weight)
10032	*Platycodonis radix*	*Platycodon grandiflorum* A. De Candolle	3
10018	*Crotonis semen*	*Croton tiglium* Linné	1
10025	*Fritillariae bulbus*	*Fritillaria thunbergii* Miquel	3

KKBS, Kilkyung-Baeksan.

72 hours, all cells including detached cells were collected and the numbers of total (viable and dead) and dead cells were determined using the ADAM-MC automatic cell counter (Nanoentek, Seoul, Korea) as described previously.[10] The number of total cells and the cell viability were automatically calculated.

2.6. *Cell cycle analysis*

The ratio of cells in each phase of cell cycle was determined by cell's DNA content using propidium iodidie (PI) staining followed by flow cytometric analysis. Cells were plated onto six-well plates at the density of 2×10^5 cells/well were treated with indicated concentrations of KKBS and herbal extracts for indicated times as described in the figure legends. Cells were trypsinized, washed twice with PBS, and fixed with 70% ice-cold ethanol at −20 °C overnight. Fixed cells were washed with cold PBS and resuspended with PI staining solution containing 50 microgram/mL PI (Sigma-Aldrich, St. Louis, MO, USA), 100 microgram/mL RNase A, and 0.5% Triton X-100 (Sigma-Aldrich) and incubated in the dark for 30 minutes and analyzed using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle profiles were analyzed using ModFit LT 3.2 software (BD Biosciences).

2.7. *Western blot analysis*

Changes in intracellular protein levels by EE-KKBS and its component herbal extract treatments were determined by Western blot analysis. A549 human lung cancer cells were plated at 1×10^6 cells/dish in 60-mm culture dishes 16–24 hours before drug treatment. Cells were treated with 50 microgram/mL and 100 microgram/mL of each herbal extract for 24 hours. The total protein was extracted with ice-cold RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing Complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich), and the protein concentration was quantified by the BCA colorimetric method (Thermo Scientific). Twenty-five micrograms of proteins were separated on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to nitrocellulose membranes. The protein-blotted membrane was blocked with a 5% (w/v) skim milk solution in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 hour at room temperature (RT) and then probed with primary antibodies at 4 °C overnight. The primary antibodies were captured with species-specific secondary antibodies conjugated with horseradish peroxidase for 1 hour at RT. Membranes were visualized using SuperSignal West Femto Kit (Thermo Scientific) and the chemiluminescence was detected by FUSION SL (Vilber Lourmat, Marne La Vallée, France). Primary antibodies against p53, p21, and p27 were purchased from Cell signaling Technology (Danvers, MA, USA) and anti-Actin antibody was obtained from Santa Cruz Technology (Dallas, TX, USA).

3. Results

3.1. *Effect of ethanol extract of KKBS on the viability of lung cancer cell lines*

To investigate the effect of ethanol extract of KKBS (EE-KKBS) on lung cancer cells, four different human lung cancer cell lines were selected and treated with 100 microgram/mL EE-KKBS for 48 hours and the cell proliferative activities were measured by MTS assay. As shown in Table 2, the proliferation indices of cells treated with KKBS were not significantly different from those of control cells, ranging 73.66–94.09% of vehicle control. Since herbal formula contain many ingredients from individual herbs that might exert synergistic or antagonistic effects, each medicinal herb composing KKBS was investigated for its anti or proproliferative activity. It was shown that any ethanol extract of the herbal constituent in KKBS exerted neither cytotoxic effect nor proliferative effect on any lung cancer cell line tested (Table 2).

Next, effect of EE-KKBS on the growth and viability of lung cancer cells were assessed using NCI-H460 human lung cancer cell line. Cells plated and treated with 100 microgram/mL EE-KKBS were harvested at 16 hours, 24 hours, 48 hours, and 72 hours and total cell numbers and viabilities were calculated. The EE-KKBS exerted potent growth inhibitory effect starting at 24 hours post treatment in NCI-H460 human lung cancer cell line (Fig. 1A), however, there were little change in the viability of lung cancer cells treated with EE-KKBS even after 72 hours (Fig. 1B).

3.2. *Effect of EE-KKBS on cell cycle distribution of lung cancer cell lines*

Even though EE-KKBS did not show cytotoxicity on various lung cancer cell lines, it might exert cytostatic effect such

Table 2 – Cytotoxic activity of EE-KKBS and component herbs on lung cancer cell lines

Cell line	Cell viability (100 microgram/mL, 48 h, %)				
	Control	EE-KKBS	EE-CT	EE-PG	EE-FT
A549	100.05 ± 3.74	92.93 ± 3.96	93.85 ± 1.56	98.13 ± 4.57	97.13 ± 3.18
NCI-H23	100.07 ± 5.13	89.21 ± 5.19	87.42 ± 8.17	99.32 ± 1.50	95.14 ± 5.18
NCI-H226	100.22 ± 2.91	94.09 ± 2.02	100.31 ± 5.57	104.87 ± 7.71	112.73 ± 3.26
NCI-H460	100.00 ± 0.99	73.66 ± 8.77	83.73 ± 3.34	104.75 ± 8.43	96.67 ± 2.44

Cell viabilities after 48 hours drug treatment compared with control (0.5% DMSO) are expressed as mean ± SD (1 representative experiment done in triplicate/out of 3 independent experiments).
DMSO, dimethyl sulfoxide; EE-CT, ethanol extract of *Croton tiglium* Linné seed; EE-PG, ethanol extract of *Platycodon grandiflorum* A.De Candolle root; EE-FT, ethanol extract of *Fritillaria thunbergii* Miquel bulb; EE-KKBS, ethanol extract of Kilkyung-baeksan; SD, standard deviation.

Table 3 – Cell cycle distribution for NCI-H460 human lung cancer cells treated with EE-KKBS

		% Cells on each cell cycle phase		
		G0/G1	G2/M	S
Control	16 h	58.31 ± 2.48	11.36 ± 0.50	30.33 ± 1.98
	24 h	55.25 ± 3.72	15.77 ± 3.28	28.98 ± 0.45
	48 h	69.43 ± 7.20	7.08 ± 1.26	23.49 ± 5.94
EE-KKBS	16 h	83.99 ± 1.29	5.81 ± 1.19	10.21 ± 0.10
	24 h	82.63 ± 0.59	6.54 ± 1.37	10.83 ± 0.79
	48 h	91.24 ± 0.90	3.24 ± 0.07	5.52 ± 0.83

Data are presented as the mean ± standard deviation for three independent experiments.
EE-KKBS, ethanol extract of Kilkyung-baeksan.

as cell cycle arrest. To test this possibility, cell cycle profile changes after EE-KKBS treatment were assessed in human lung cancer cell lines. As demonstrated in Fig. 2A and Table 3, EE-KKBS treated NCI-H460 cells showed strong G0/G1 arrest in cell cycle profiles compared with nontreated control cells. The percentage of cells in G0/G1 phase increased from 58.31% to 83.99%, 55.25% to 82.63%, and 69.43% to 91.24% at 16 hours, 24 hours, and 48 hours after 100 microgram/mL EE-KKBS treatment, respectively (Table 3). At the same time point, there was significant decrease in cells in S phase and slight decrease in cells in G2/M phase (Fig. 2A and Table 3). Another human lung cancer cell line, A549, also showed strong G0/G1 arrest by EE-KKBS treatment (data not shown).

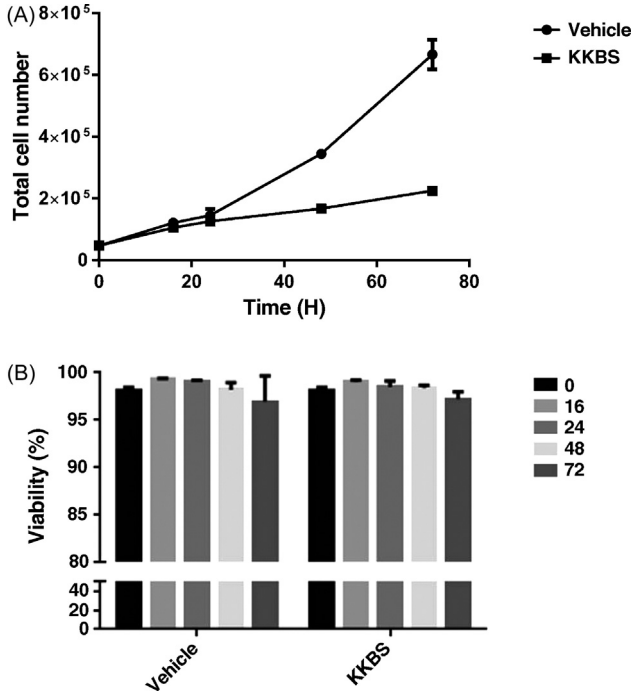

Fig. 1 – Effect of EE-KKBS on NCI-H460 human lung cancer cells. NCI-H460 cells were vehicle (0.1% DMSO) or EE-KKBS (100 microgram/mL) for the indicated time then collected for measuring total cell counts and cell viabilities. (One representative experiment done in triplicates). (A) Total cell numbers after 16 hours, 24 hours, 48 hours, and 72 hours of treatment. (B) Cell viabilities at each time point. DMSO, dimethyl sulfoxide; EE-KKBS, ethanol extract of Kilkyung-baeksan.

Table 4 – Cell cycle distribution for NCI-H460 human lung cancer cells treated with herbal extracts comprising EE-KKBS

	% Cells on each cell cycle phase at 24 h treatment		
	G0/G1	G2/M	S
Control	55.25 ± 3.72	15.77 ± 3.28	28.98 ± 0.45
EE-KKBS	82.63 ± 0.59	6.54 ± 1.37	10.83 ± 0.79
EE-CT	79.77 ± 1.85	6.25 ± 1.49	14.04 ± 0.45
EE-PG	54.71 ± 0.80	20.23 ± 0.96	25.07 ± 0.16
EE-FT	53.02 ± 0.65	19.95 ± 0.94	27.04 ± 1.59

Data are presented as the mean ± standard deviation for three independent experiments.
EE-CT, ethanol extract of *Croton tiglium* Linné seed; EE-PG, ethanol extract of *Platycodon grandiflorum* A.De Candolle root; EE-FT, ethanol extract of *Fritillaria thunbergii* Miquel bulb; EE-KKBS, ethanol extract of Kilkyung-baeksan.

3.3. *Effect of individual herbs comprising KKBS on cell cycle profile in lung cancer cell lines*

Since KKBS is a herbal formula comprised of three medicinal herbs, it is necessary to determine the cell cycle arrest induced by this formula comes from one of component herbs or the synergistic effect of multi-herbal extract. Among three component herbs, only ethanol extract of *Croton tiglium* Linné (EE-CT) showed potent G0/G1 cell cycle arrest which was comparable to EE-KKBS in both NCI-H460 and A549 human lung cancer cell lines (Fig. 2B, Table 4, and data not shown).

3.4. *Effect of EE-KKBS and EE-CT on cell cycle-related protein expressions*

The effects of KKBS and/or EE-CT on the expression of cell cycle-related proteins in NCI-H460 cells were evaluated by Western blot analysis. As presented in Fig. 3, EE-KKBS treatment caused upregulation of p21 and p27 proteins in NCI-H460 cells, two key proteins in G1/S cell cycle checkpoint, resulting in cell cycle arrest in G0/G1 phase. As expected, EE-CT treatment also increased the expression of both proteins (Fig. 3).

4. Discussion

Cancer has been the leading cause of death worldwide and has become a major public health problem. Lung cancer is the

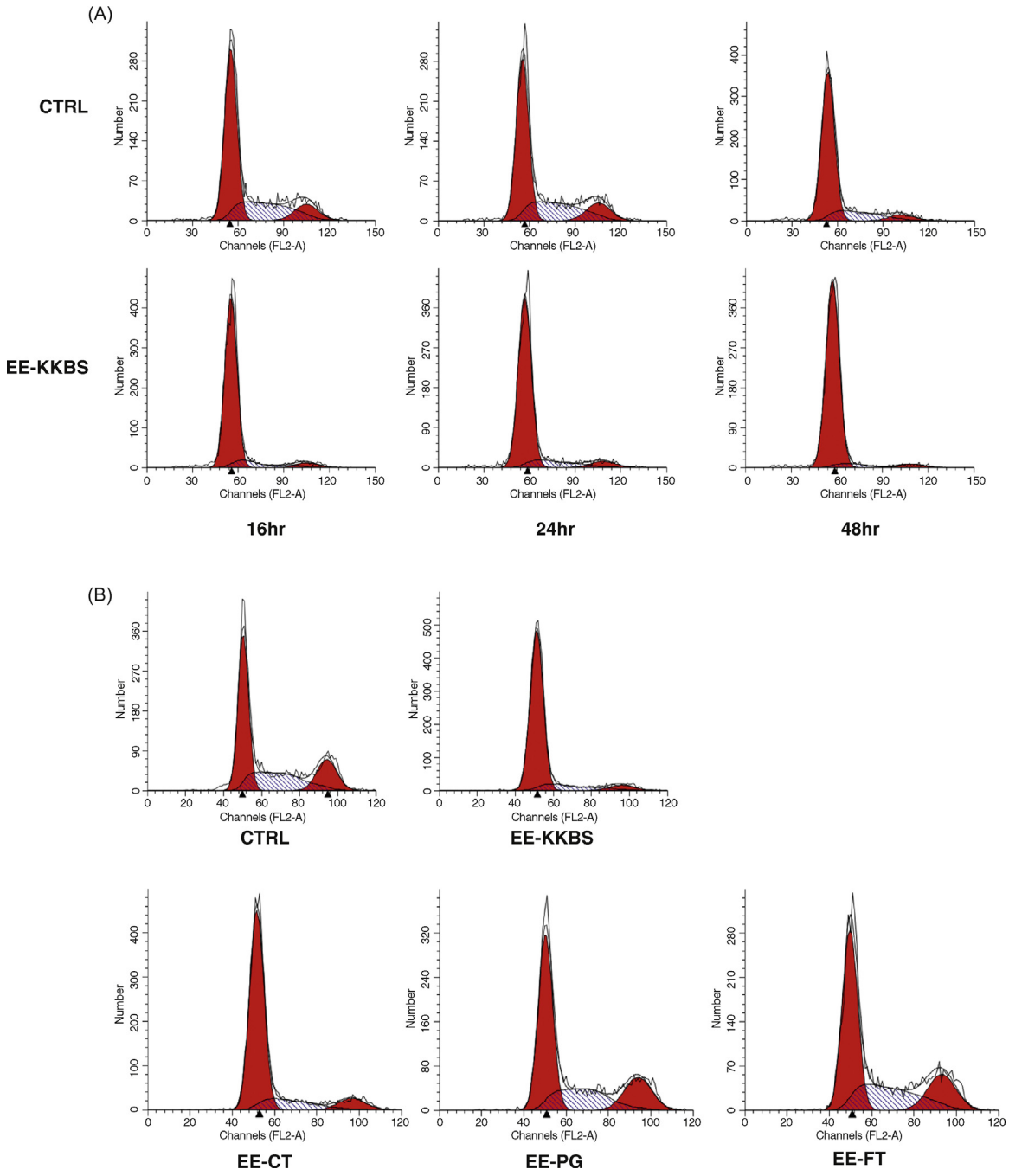

Fig. 2 – Effect of EE-KKBS (A) and component herbs (B) on NCI-H460 cell cycle progression. (A) Cells were treated with either vehicle (0.1% DMSO) or EE-KKBS (100 microgram/mL) for the indicated time, stained with PI and analyzed by flow cytometry. (B) Cells were treated with either vehicle (0.1% DMSO) or each herbal extracts (100 microgram/mL) for 24 hours, then stained with PI and analyzed by flow cytometry.
DMSO, dimethyl sulfoxide; EE-KKBS, ethanol extract of Kilkyung-baeksan; EE-CT, ethanol extract of *Croton tiglium* Linne seed; EE-PG, ethanol extract of *Platycodon grandiflorum* A.De Candolle root; EE-FT, ethanol extract of *Fritillaria thunbergii* Miquel bulb; PI, propidium iodide.

leading cause of cancer deaths, and in most cases, the cancer has already spread and/or metastasized at the time of diagnosis, therefore only limited therapeutic options are available. Currently platinum-based mixed chemotherapy is the standard treatment for advanced lung cancer, especially NSCLC, however, chemotherapy in patients with advanced NSCLC has response rate of only < 35% with severe toxicity.[11,12] Due to these limits of the current treatment, herbal medicines and phytochemicals draw a lot of attention for novel therapeutics development since these have been used traditionally with

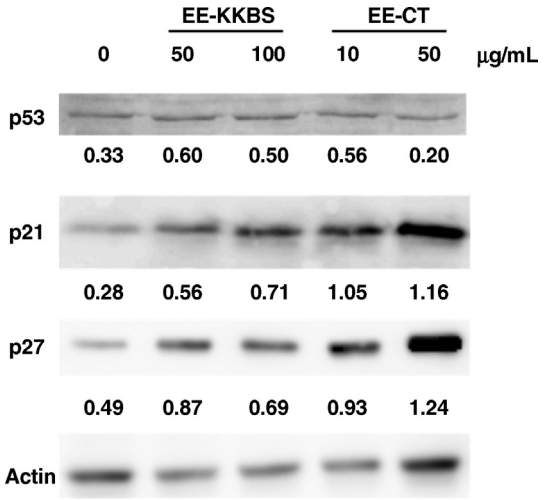

Fig. 3 – Expressions of cell cycle checkpoint proteins in NCI-H460 human lung cancer cell line treated with EE-KKBS (50 microgram/mL and 100 microgram/mL) or EE-CT (50 microgram/mL and 100 microgram/mL). Numbers below each lane represent normalized expressions of each protein over actin protein.
EE-CT, ethanol extract of *Croton tiglium*; EE-KKBS, ethanol extract of Kilkyung-baeksan.

little or no harmful effects. There are reports that suggest using herbal medicines and phytochemicals for lung cancer treatment and/or prevention.[13,14]

KKBS has a long history of traditional use in Korea and China for various symptoms of respiratory diseases. In the present study, we found that ethanol extract of KKBS (EE-KKBS) induced a significant growth arrest effect on various lung cancer cell lines in a time dependent manner (Table 2 and Fig. 1A). There was no change in the viability of EE-KKBS treated cells, we assumed that the effect of EE-KKBS on lung cancer cells was not cytotoxic (Fig. 1B). Furthermore, lung cancer cell lines treated with EE-KKBS displayed strong G0/G1 arrested cell cycle profiles without increased sub G1 population (Fig. 2A and Table 3) which strongly suggested cytostatic property of EE-KKBS.

One of the KKBS components, *Platycodon grandiflorum* Radix has been shown to elicit anticancer activity by inducing apoptosis in various cancer cell lines,[6,7] however, in our study, none of the component herbs comprising KKBS exhibited cytotoxic effect on lung cancer cell lines. Our effort to find the component herb with cell cycle arrest property revealed that the ethanol extract of *Croton tiglium* seed (EE-CT) possessed a potent G0/G1 cell cycle arrest activity (Fig. 2B and Table 4). We further analyzed which solvent fraction of EE-CT contained the substance(s) responsible for arrest of cell cycle. All subfractions except water fraction showed G0/G1 arrest between 24 hours and 48 hours treatment, although there were different magnitudes of cell cycle arrest (data not shown). Of these, the ethyl acetate subfraction displayed the most potent activity in G0/G1 arrest, up to 95% of cells in G0/G1 phase from 24 hours post treatment and this arrest continued to 48 hours (data not shown). Interestingly, our preliminary study indicated that this G0/G1 arrest effect of EE-CT and subsequent solvent fractions of EE-CT only limited to the cancer cells from lung origin and failed to induce cell cycle arrest in other types of cancer cell lines such as C33a cervical carcinoma cell or HT1080 fibrosarcoma cell lines (data not shown)

Many gene products such as p27, and p21 have been demonstrated to play important roles in regulating cell cycle checkpoints. Both proteins are members of the Cip/Kip family of cyclin-dependent kinase inhibitors and act as inhibitors of cell cycle progression. In association with CDK2 complexes, these proteins inhibit kinase activity of CDKs and block progression through G1/S.[15] To clarify the underlying molecular mechanism of G0/G1 arrest induced by EE-KKBS and EE-CT, the expression levels of these two proteins were examined. Our study indicated that p21 and p27, two key modulators of G1 checkpoint, were upregulated in lung cancer cells treated with EE-KKBS and EE-CT (Fig. 3).

In summary, our results suggested that EE-KKBS and its component herb, EE-CT, inhibited growth of lung cancer cells without cytotoxic effect. This antiproliferative property of EE-EE-KKBS was possibly mediated by enhancing the expressions of p21 and p27 proteins, regulating G1 checkpoint of cell cycle. Although the detailed mechanism of action, identification of active ingredients, and efficacy confirmation in animal models need to be verified, our data suggest that EE-KKBS could be a good candidate for lung cancer adjuvant therapy for regulating uncontrolled cancer growth.

Conflicts of interest

All authors have no conflicts of interest to declare.

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