***Glycine max* Merr. leaf extract possesses anti-oxidant properties, decreases inflammatory mediator production in murine macrophages, and inhibits growth, migration, and adhesion in human cancer cells**

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Abstract The present study aimed to investigate the *in vitro* anti-oxidant, anti-inflammatory, and anti-cancer properties of the ethanol extract of soybean (*Glycine max* Merr.) leaves (SLE). The total polyphenol and flavonoid levels were 142.0 ± 14.0 mg gallic acid equivalent/g and 104.9 ± 2.0 mg quercetin equivalent/g, respectively. The radical scavenging activity and ferric-reducing anti-oxidant power of SLE at the concentrations of 125–500 $\mu\text{g/mL}$ were 5–61%. In lipopolysaccharide-treated RAW 264.7 macrophages, treatment with SLE at concentrations of 62.5–500 $\mu\text{g/mL}$ dose-dependently decreased the production of nitric oxide and prostaglandin E_2 . In both HCT116 human colon cancer cells and H1299 human lung cancer cells, treatment with SLE inhibited the growth and anchorage-independent colony formation. SLE was also effective in inhibiting the migration of H1299 cells and the adhesion of both HCT116 and H1299 cells. These results suggest that SLE exerts anti-oxidant, anti-inflammatory, and anti-cancer activities *in vitro*. It needs to be determined whether similar effects are reproduced *in vivo*.

Keywords: soybean leaf, anti-oxidant activity, anti-inflammatory activity, anti-cancer activity, *in vitro*

Introduction

Soybean (*Glycine max* Merr.) has long been consumed in Asia, and its consumption in Western countries has increased significantly in the last decade (1). Soybean is a valuable dietary source containing high-quality proteins, minimal saturated fats, a large amount of fibers, and isoflavones, a group of compounds structurally similar to 17- β -estradiol and classified as phytoestrogens (2). Soybean and its constituents, mainly soy proteins and isoflavones, have been investigated extensively for their beneficial effects on human health (1–3). The leaves of soybean plant are consumed in several parts of Asian countries, including Korea (4). Soybean leaves contain isoflavones such as malonyl-genistin and genistin but not as the major phytochemicals (5). Rather, isoflavonols and pterocarpanes have been reported as the major constitutive phytochemicals in soybean leaves (5). Several biological activities of soybean leaves, including serum lipid profile-modulatory (6), carotid artery ring-relaxing (7), anti-diabetic (4,8–10), and anti-obesity (10–12) activities, have been reported. However, their anti-oxidant, anti-inflammatory, and anti-cancer potentials remain to be elucidated.

Reactive oxygen species (ROS) at physiological levels serve as

signaling messengers to mediate many essential biological responses (13). Unregulated and prolonged production of ROS, however, causes oxidative stress and is significantly implicated in the pathogenesis of many diseases, including chronic inflammation and cancers (13). Inflammation is a defense response of organisms to harmful stimuli, including infection, chemical exposure, and tissue damage (14). Inflammatory response is induced by inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) as well as by pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-6 (14). Chronic inflammation is induced by excessive and persistent production of inflammatory mediators and plays a critical role in the development of many diseases, including cancers (15). Cancer is a leading cause of mortality in humans worldwide (16). The colorectal and lung cancer burdens are among the highest in terms of their incidence and mortality (16). Prevention of these types of cancers, therefore, is a major issue in public health.

In the present study, we aimed to investigate the anti-oxidant content and activities of soybean leaves. Moreover, we evaluated their effects on the production of inflammatory mediators in murine macrophages and the important characteristics of cancer cells using human colon and lung cancer cell lines.

Materials and Methods

Preparation of soybean leaf extract Soybean leaves were purchased from a local retail store (Cheongju, Korea), washed, ground (MU-3000; TC Angel, Seoul, Korea), freeze-dried (PH1316; IshinBioBase, Yangju, Korea), and extracted using a 30-fold volume of 80% ethanol for 4 h at room temperature. The extracted solution was then centrifuged at 1,500 $\times$ g for 3 min (A320101; Gyrozen, Daejeon, Korea). Subsequently, the supernatant was collected, and the ethanol solvent in the supernatant was removed using a vacuum evaporator (NB-503CIR; N-biotech, Bucheon, Korea). The dried soybean leaf extract (SLE) was weighed and stored at -70°C for further analysis. The extraction yield was $22.3 \pm 1.3\%$ on a dry-weight basis.

Total polyphenol and flavonoid content The total polyphenol content was determined using the Folin–Ciocalteu method (17). Briefly, 35 μL of Folin–Ciocalteu's reagent (Sigma-Aldrich, St. Louis, MO, USA), 200 μL of 7.5% sodium carbonate (Sigma-Aldrich), 100 μL of distilled water, and 60 μL of SLE reconstituted in ethanol were mixed and allowed to stand for 30 min at room temperature. The reaction solution was centrifuged at 10,000 $\times$ g for 2 min (Gyrozen), the supernatant was collected, and the absorbance was measured at 650 nm using a plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The total polyphenol content was calculated using a standard curve of gallic acid (Sigma-Aldrich) and expressed as mg gallic acid equivalent/g dried leaves.

The total flavonoid content was determined using a method described previously (18). Briefly, 100 μL of diethylene glycol (Sigma-Aldrich), 50 μL of 1 N sodium hydroxide (Sigma-Aldrich), and 30 μL of SLE reconstituted in ethanol were mixed and allowed to stand for 1 h at room temperature. The absorbance was measured at 415 nm using a plate reader (Bio-Rad Laboratories). The total flavonoid content was calculated using a standard curve of quercetin (Sigma-Aldrich) and expressed as mg quercetin equivalent (QE)/g dried leaves.

Radical scavenging activities To determine the radical scavenging activities of SLE, two different assays were performed: one using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma-Aldrich) and the other using 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich). The ABTS radical scavenging activity was determined using a method described previously (19). Briefly, ABTS radicals were freshly prepared by reacting 7.4 mM ABTS with 2.6 mM potassium persulfate (Sigma-Aldrich) for 24 h in the dark at room temperature. Then, 1 mL of ABTS radical solution was mixed with 1 mL of SLE (at concentrations of 125, 250, and 500 $\mu\text{g}/\text{mL}$; reconstituted in ethanol) and allowed to stand for 20 min at room temperature. The ABTS radical solution mixed with ethanol in the absence of SLE was used as the control. The absorbance was measured at 750 nm using a plate reader (Bio-Rad Laboratories), and

percent decrease of absorbance was calculated.

The DPPH radical scavenging activity was determined using a method described previously (19). Briefly, a DPPH radical solution (1 mM) was freshly prepared in ethanol in the dark just before use and then reacted with SLE at concentrations of 125, 250, and 500 $\mu\text{g}/\text{mL}$ (reconstituted in ethanol) at a ratio of 1:1 (v/v) for 30 min in the dark at room temperature. The DPPH radical solution mixed with ethanol in the absence of SLE was used as the control. The absorbance was measured at 540 nm using a plate reader (Bio-Rad Laboratories), and percent decrease of absorbance was calculated.

Ferric reducing anti-oxidant power The ferric reducing anti-oxidant power (FRAP) was determined using a method described previously (20). Briefly, 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 5-tripyridyls-triazine in 40 mM hydrochloric acid, and 20 mM ferric chloride solution were mixed in a ratio of 10:1:1 (v/v) and used as a FRAP reagent. Then, 150 μL of the FRAP reagent and 100 μL of SLE (at concentrations of 125, 250, and 500 $\mu\text{g}/\text{mL}$; reconstituted in ethanol) were incubated at 37°C for 30 min. Ascorbic acid at the same concentration of SLE was used as the control. The absorbance was measured at 595 nm using a plate reader (Bio-Rad Laboratories). FRAP was expressed as a percentage of the absorbance of the sample in relation to the absorption of the control.

Cell culture Three types of cell lines, RAW 264.7 murine macrophages, HCT116 human colon cancer cells, and H1299 human lung cancer cells, were purchased from Korean Cell Line Bank (Seoul, Korea). The RAW 264.7 and H1299 cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Gibco Co., Rockville, MD, USA), and the HCT116 cells were maintained in McCoy's medium (Gibco Co.). Each medium contained 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 100 units/mL penicillin/0.1 mg/mL streptomycin (Welgene Inc., Daegu, Korea). Cells were cultured at 37°C in 95% humidity with 5% CO_2 .

Cell viability Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay (21). SLE was initially dissolved in dimethyl sulfoxide (DMSO; Biosesang Inc., Seongnam, Korea) to prepare a stock solution with a high concentration and then further diluted using the corresponding cell culture medium just before the cells were treated. The final concentration of DMSO in the culture medium was limited to less than 0.2% (v/v). RAW 264.7 (3×10^5 cells per well), HCT116 (5×10^3 cells per well), and H1299 cells (5×10^3 cells per well) were seeded in 96-well plates (Corning Inc., New York, NY, USA). After 24 h, macrophages and cancer cells were treated differently. RAW 264.7 cells were pretreated with SLE at concentrations of 0, 62.5, 125, 250, and 500 $\mu\text{g}/\text{mL}$ in a serum-free medium for 2 h and stimulated with 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS; Sigma-Aldrich) in a serum-free medium for 20 h. The medium was then replaced with a fresh medium containing 0.5 $\mu\text{g}/\text{mL}$ of MTT. The HCT116 and H1299 cells

were treated with SLE at concentrations of 0, 62.5, 125, 250, and 500 µg/mL in the corresponding serum-free media for 72 and 96 h. The media were aspirated and replaced with fresh media containing 0.5 mg/mL of MTT. After 4 h of incubation at 37°C, the MTT-containing media were removed. The reduced formazan dye at the bottom of the well was then dissolved by adding DMSO. After gentle mixing, the absorbance was measured at 540 nm using a plate reader (Bio-Rad Laboratories).

NO and PGE₂ levels RAW 264.7 (3×10^5 cells per well) were seeded in 96-well plates (Corning Inc.). After 24 h, RAW 264.7 cells were pretreated with 0, 62.5, 125, 250, and 500 µg/mL of SLE in a serum-free medium for 2 h and then stimulated with 1 µg/mL LPS in a serum-free medium for another 20 h. The medium was then collected for the determination of NO and PGE₂ levels.

The NO levels were determined by measuring nitrite, a primary stable and nonvolatile breakdown product of NO, using a method described previously (22). A mixture of 0.1% naphthylethylenediamine (Sigma-Aldrich) and 1% sulfanilamide (Sigma-Aldrich) prepared in 5% phosphoric acid (OCI Corporation, Seoul, Korea) was used as the Griess reagent. The culture medium was reacted with the Griess reagent for 15 min at room temperature. The absorbance was measured at 540 nm using a plate reader (Bio-Rad Laboratories). The NO concentration was calculated using a standard curve of sodium nitrite (Samchun Pure Chemical Co., Ltd, Pyeongtaek, Korea) and expressed as a percentage of the control.

The levels of PGE₂ in the culture media were measured using an enzyme-linked immunosorbent assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the procedure provided by the manufacturer. The absorbance at 450 nm was measured using a plate reader (Bio-Rad Laboratories). The PGE₂ concentration was calculated using a standard curve of PGE₂ (Cayman Chemical Company) and expressed as a percentage of the control.

Colony formation in soft agar Colony formation assay was performed in soft agar using a method described previously (23) with slight modification. The HCT116 (6×10^4 cells per well) and H1299 (8×10^3 cells per well) cells were mixed with 2 mL of culture media containing 0.3% agar (DC Chemical Co., Seoul, Korea) and then overlaid on 1 mL of 0.6% agar in 6-cm culture dishes (Corning Inc.). The cells were treated with 0 or 500 µg/mL of SLE in serum complete media for 21 days. After 3 weeks, colonies were visualized by staining the cells with 0.05% crystal violet for 1 h. The number of colonies was counted at 4 randomly selected points in each well under phase-contrast time-lapse microscope at a magnification of 100-fold (Primo Vert, Carl Zeiss, Oberkochen, Germany) and then expressed as a percentage of the control.

Staining of cell nucleus Apoptotic cells were observed morphologically by staining the DNA with the 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) (24). The

HCT116 (7×10^3 cells/well) and H1299 (6×10^3 cells/well) cells were seeded on an 8-well chamber slide (Corning Inc.). After 24 h, the cells were treated with 0 or 250 µg/mL of SLE in a serum-free medium for 48 h. The cells were fixed using 4% formaldehyde (Sigma-Aldrich) for 15 min at room temperature and then incubated with DAPI. The nuclear morphology was observed using a confocal laser scanning microscopy at a magnification of 200-fold (MRC-1024; Bio-Rad Laboratories). The apoptotic cells were identified from condensed nuclei (24). The DAPI staining intensity was determined using the Image-J software (NIH, Bethesda, MD, USA).

Cell cycle analysis The cell cycle was determined using a method described previously (25). Briefly, the H1299 cells (3×10^5 cells/well) were cultured in a serum complete medium containing 0 or 500 µg/mL of SLE for 24 h. The cells were then trypsinized, fixed in 70% ethanol overnight at 20°C, and incubated with ribonuclease A (1 µg/mL; Sigma-Aldrich) and propidium iodide (50 µg/mL; Sigma-Aldrich) for 20 min in the dark at room temperature. The cell population at the Sub-G1, G1/G0, S, and G2/M phases were identified using a flow cytometer (FACS Calibur-S System; BD Biosciences, Heidelberg, Germany) and the Cell Quest Pro software (BD Biosciences).

Cell migration Cell migration was determined using a scratch wound healing assay (25). The HCT116 and H1299 cells (5×10^3 cells/well) were seeded on 6-well plates (Corning Inc.). When the cells reached approximately 70% confluence, a wound was introduced by scratching the monolayer of cells with a pipette tip. The cells were then washed with phosphate buffer saline (PBS) and maintained in serum-free media containing SLE at concentrations of 0, 125, 250, and 500 µg/mL for 24 h. The images of the wound area were captured via the iSolution Lite software (IMT i-solution, Burnaby, Canada). The average widths of the wounds at 0 and 24 h time points were measured using the Image-J software (NIH). The wound closure during the time interval was calculated by subtracting the width of the wound at the 24 h time point from that at the 0 h time point.

Cell adhesion Cell adhesion was determined using a previously described method (25). Briefly, 96-well plates were pre-coated with fibronectin (1 µg/mL, Sigma-Aldrich) in Hank's buffer (Welgene Inc.) for 2 h at 37°C and then blocked with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at 37°C. The HCT116 and H1299 cells were suspended in serum complete media containing SLE at concentrations of 0, 125, 250, and 500 µg/mL and then plated into 96-well plates (1×10^3 cells/well). After 1 h, floating non-adherent cells in the media were aspirated. The adherent cells at the bottom of the plates were stained with 0.2% crystal violet for 10 min at room temperature, dissolved with 1% sodium dodecyl sulfate (Sigma-Aldrich), and then quantified by measuring the absorbance at 540 nm using a plate reader (Bio-Rad Laboratories).

Statistical analyses Each experiment was performed at least in

triplicate. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 12.0; SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) combined with Tukey's test was used for a comparison among multiple groups. Regression analysis was used for assessing a dose-response relationship. Student's *t*-test was used for a simple comparison between two groups. A probability value of $p < 0.05$ was considered as significant.

Results and Discussion

Anti-oxidant contents and activities of SLE Anti-oxidants have been of great interest in human health because they could play an important role in the prevention and treatment of many chronic diseases, including cancers (26). We first determined the anti-oxidant content in soybean leaves, such as total polyphenol and flavonoid levels. Polyphenols are a class of compounds characterized by the presence of multiple phenol units, and flavonoids are a class of polyphenols containing two phenyl rings and one heterocyclic ring (26). Both classes of compounds are found ubiquitously in plants and have been well-documented to possess a wide range of biological activities, including anti-oxidant, anti-inflammatory, and anti-cancer activities (26). As shown in Table 1, the total polyphenol and flavonoid levels in soybean leaves were 142.0 mg gallic acid equivalent/g and 104.9 mg quercetin equivalent/g, respectively. The polyphenol content in soybean leaves was higher than those in the leaves from nine different plants of the Leguminosae family (13–50 mg gallic acid equivalent/g) (27), which indicates the richness of polyphenols in soybean leaves. These results allowed us to further evaluate the biological activities of SLE, such as anti-oxidant, anti-inflammatory, and anti-cancer activities.

To determine anti-oxidant activities of SLE, three different assays were performed because any single assay does not perfectly reflect all the anti-oxidant activities. ATBS and DPPH assays measure the

Table 1. Anti-oxidant content of soybean leaves

Total polyphenols (mg gallic acid equivalent/g dried leaves)	Total flavonoids (mg quercetin equivalent/g dried leaves)
142.0±14.0 ¹⁾	104.9±2.0 ¹⁾

¹⁾Mean±SE of ≥3 determinations

activity of scavenging stable radicals (19), while FRAP measures the activity of reducing ferric ions to ferrous ions (20). As shown in Fig. 1, the ABTS radical scavenging activities of SLE at concentrations of 125, 250, and 500 µg/mL were 19–61%. The DPPH radical scavenging activities were lower than the ABTS radical scavenging activities, exhibiting a value of 5–17% at concentrations of 125, 250, and 500 µg/mL. The FRAP values of SLE at concentrations of 125, 250, and 500 µg/mL were 13–44%. Regression analysis showed a strong linear relationship between the concentrations and anti-oxidant activities of SLE ($R^2 > 0.9$, $p < 0.001$), thereby indicating dose-dependent anti-oxidant activities of SLE. Such anti-oxidant activities of SLE found in the current study are likely attributed to the high content of polyphenols and flavonoids.

Effect of SLE on the production of inflammatory mediators in RAW

264.7 macrophages Chronic inflammation is often linked to oxidative stress, and many anti-oxidants have been reported to act as anti-inflammatory agents (26). To evaluate the anti-inflammatory activities of SLE, the levels of two inflammatory mediators NO and PGE₂ were determined in LPS-stimulated RAW 264.7 macrophages, a widely used cell system for evaluating potential anti-inflammatory activities *in vitro* (28). NO is a ubiquitous mediator of a wide range of inflammatory conditions (29). PGE₂ is a key mediator that plays an important role in the pathogenesis of sepsis and inflammation (30). Their levels are known to reflect the degree of inflammation, thus providing a measure of the inflammatory response (14,29,30). As shown in Fig. 2A, treatment with SLE at concentrations of 62.5–500 µg/mL did not affect the viability of the LPS-stimulated RAW 264.7 cells. Therefore, a range of concentrations was selected to investigate

Fig. 1. Anti-oxidant activities of SLE. The ABTS radical scavenging activity (A), DPPH radical scavenging activity (B), and FRAP (C) of SLE at concentrations of 125, 250, and 500 µg/mL are presented as percentage values (mean±standard error of 3 determinations). Different letters (a–c) represent the statistical differences among different concentrations by Tukey's test ($p < 0.05$).

Fig. 2. Effects of SLE on NO and PGE₂ production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pre-treated with 0, 62.5, 125, 250, and 500 µg/mL of SLE for 2 h and cultured in the presence of 1 µg/mL LPS for another 20 h. Cell viability (A), NO levels (B), and PGE₂ (C) levels are presented as percentage values of the LPS-treated control (mean±standard error of 8 determinations for the cell viability or 4 determinations for the NO and PGE₂ levels). Different letters (a–e) represent the statistical differences among different treatments by Tukey's test ($p < 0.05$).

the effects of SLE on the production of NO and PGE₂. Treatment with SLE at concentrations of 62.5–500 µg/mL significantly decreased the levels of NO (to 24–69% of the control; $p < 0.05$; Fig 2B) and PGE₂ (to 19–68% of the control; $p < 0.05$; Fig 2C). There was a strong inverse linear relationship between the concentrations of SLE and levels of NO or PGE₂ ($R^2 \geq 0.9$, $p < 0.001$ by regression analysis), thereby indicating dose-dependent inhibitory activities of SLE against LPS-induced inflammatory response in RAW 264.7 cells. It needs to be further investigated how SLE decreases the production of NO and PGE₂.

Effect of SLE on the growth and colony formation of human cancer cells Carcinogenesis is a multistep process involving many pathogenic events, and oxidative stress and chronic inflammation are two of the most critical factors implicated in carcinogenesis (13,15). Since the anti-oxidant and anti-inflammatory properties of SLE were revealed in our study, we next evaluated the anti-cancer activities of SLE *in vitro*. The HCT116 colorectal carcinoma cells and H1299 non-small cell lung carcinoma cells have been utilized extensively for screening a variety of potential anti-proliferative and anti-metastatic compounds (25); therefore, these cells were used in the present study.

Uncontrolled growth is the most fundamental characteristic found in cancer cells (31). As shown in Fig. 3A, the treatment of the HCT116 cells with SLE at concentrations of 125, 250, and 500 µg/mL for 72 h significantly inhibited the growth (by 34–89%), with an estimated IC₅₀ value of 285 µg/mL. A longer treatment of the HCT116 cells with SLE (96 h) resulted in greater growth inhibition (by 62–87%), with an estimated IC₅₀ value of 121 µg/mL. The H1299 cancer cells were slightly more susceptible to SLE than the HCT116 cells; the treatment with SLE inhibited the growth by 45–85%, with estimated IC₅₀ values of 170 µg/mL at the 72 h time point and 95 µg/mL at the 96 h time point (Fig. 3A). In both the HCT116 and H1299 cells, the growth inhibitory effects of SLE increased with increasing the

concentrations of SLE ($R^2 \geq 0.8$, $p < 0.001$ by regression analysis), thereby showing a significant dose-response relationship.

The growth of cancer cells can be anchorage-dependent, similar to that of normal cells, or anchorage-independent (31). The effect of SLE on the anchorage-independent growth of human colon and lung cancer cells was investigated using a typical soft-agar assay. The control HCT116 cells produced a greater number but a smaller size of colonies than the control H1299 cells (data not shown), as previously reported (25). As shown in Fig. 3B, treatment with SLE at a concentration of 500 µg/mL resulted in a significantly decreased number of colonies (by 29–40%) in both the HCT116 and H1299 cells. Since the anchorage-independent growth assay is regarded as an *in vitro* test for tumorigenesis *in vivo* (32), it is speculated that SLE might reduce the tumorigenicity of these cells; further research on this is needed.

Effect of SLE on the apoptosis of human cancer cells The suppression of apoptosis, a process of programmed cell death, may lead to malignant cell growth. The restoration of apoptosis in cancer cells is regarded as an effective strategy for cancer prevention and treatment (31). To observe morphological evidence of apoptosis induced by the treatment with SLE, cell nuclei were visualized through DNA staining with a fluorescent dye, DAPI. The control HCT116 (data not shown) and H1299 cells (the upper panel of Fig. 4A) presented homogeneous and symmetric shape of the nucleus. The cells treated with SLE at a concentration of 250 µg/mL, however, exhibited heterogeneous and condensed nuclear chromatin. The DAPI staining intensity of apoptotic cells is known to increase because of their increased membrane permeability (33). Treatment with SLE at a concentration of 250 µg/mL resulted in an increase in the DAPI staining intensity to 2.1-fold of the control in the HCT116 cells and to 1.5-fold of the control in the H1299 cells (the lower panel of Fig. 4A), which indicates an apoptosis-inducing activity of SLE in

Fig. 3. Effect of SLE on the growth and colony formation of human cancer cells. The HCT116 colon cancer cells and H1299 lung cancer cells were treated with SLE at concentrations of 0, 125, 250, and 500 $\mu\text{g/mL}$ for 72 h and 96 h, respectively, and viable cells are shown as percentage values of the control (mean $\pm$ standard error of 8 determinations, A). The HCT116 and H1299 cells on agar were treated with SLE at concentrations of 0 or 500 $\mu\text{g/mL}$ for 21 days. The number of colonies was counted using phase-contrast time-lapse microscopy ($\times 100$) and shown as a percentage value of the control (mean $\pm$ standard error of 4 determinations, B). Asterisks indicate the statistical differences between the 72-h and 96-h time points or between the control and SLE-treated cells by the two-tailed Student's *t*-test (* $p < 0.05$, *** $p < 0.001$). Different letters (a–d) indicate the statistical differences between different concentrations of SLE at a specific time point by Tukey's test ($p < 0.05$).

both the HCT116 and H1299 cells. To confirm such an activity and further characterize the growth-inhibitory activity of SLE, cell cycle analysis was performed using the H1299 cells. Treatment with SLE at a concentration 500 $\mu\text{g/mL}$ resulted in an increase in the sub-G1 cell population to 1.7-fold of the control H1299 cells without causing any significant changes in the cell population at the G0-G1, S, and G2-M phases (Fig. 4B). These results suggest that the growth-inhibitory activities of SLE (Fig. 3) may be partially attributed to the apoptosis-inducing activities.

Effect of SLE on the migration and adhesion of human cancer cells

Metastasis is a critical process wherein cancer cells leave the site of the primary lesion, pass through the circulatory system, and establish a secondary tumor at a new distant organ site (31). The migration of cancer cells and the adhesion of the migrating cells to the secondary organ sites are prominent events in metastatic cascade (31). Treatment

of the H1299 cells with SLE at the highest concentration (500 $\mu\text{g/mL}$) significantly inhibited the migration in a wound-healing assay (by 50%, $p < 0.05$, Fig. 5A). The treatment of the HCT116 cells with SLE, however, did not affect the wound closure significantly (data not shown). The H1299 cells moved more rapidly than the HCT116 cells (data not shown), which might allow us to detect the obvious effects of SLE within a relatively limited time (24 h). Treatment of the HCT116 and H1299 cells with SLE also inhibited the cell adhesion to fibronectin by 21–31% (at 125–500 $\mu\text{g/mL}$) and 14% (at 500 $\mu\text{g/mL}$), respectively ($p < 0.05$, Fig. 5B). The inhibitory activities of SLE against the migration and adhesion were observed at much early time points (24 and 2 h, respectively) when cell growth was not significantly affected by SLE treatment (data not shown). Thus, the inhibitory activities of SLE against both migration and adhesion are likely independent of the growth-inhibitory activity. Further studies on the underlying mechanisms of the inhibition of migration and adhesion

Fig. 4. Effect of SLE on the apoptosis of human cancer cells. The nuclear morphology of the HCT116 colon cancer cells and H1299 lung cancer cells treated with 0 or 250 μg/mL of SLE for 48 h was observed by DAPI staining (A). The representative area of the HCT116 cells is shown in the upper panel, and the DAPI staining intensity is presented as a percentage value of the control (mean±standard error of 3 determinations) in the lower panel. The cell cycle distribution of the H1299 cells treated with 0 or 500 μg/ml of SLE for 24 h was analyzed, and the cell population (%) at the sub-G1 (M4) phase is shown (mean±standard error of 3 determinations, B). Asterisks indicate the statistical differences between the control and SLE-treated cells by the two-tailed Student's t-test ($p < 0.05$).

by SLE are needed in the future.

Several constituents of soybean leaves have been shown to possess different biological activities (4,7,9,10,34,35). Kaempferol glycosides have been shown to possess anti-diabetic, anti-obesity, and carotid artery ring-relaxing activities (4,7,10,34). Pterocarpans have been shown to possess anti-diabetic and α -glucosidase-inhibitory activities (9,35). However, further studies are needed to identify the major bioactive constituents responsible for the anti-oxidant, anti-inflammatory, and anti-cancer activities of SLE found in the current study and to clarify whether such activities are attributed to a single compound or different compounds in combination.

In summary, SLE possessed appreciable anti-oxidant activities most likely because of the high polyphenol and flavonoid contents. SLE decreased the production of NO and PGE₂ in LPS-stimulated RAW 264.7 macrophages, indicating the anti-inflammatory activities of SLE *in vitro*. SLE also inhibited the growth, anchorage-independent colony formation, and adhesion in both human colon and lung cancer cells as well as the migration of human lung cancer cells, suggesting the anti-cancer activities of SLE *in vitro*. To the best of our knowledge, this is the first report that systematically presents the anti-oxidant, anti-inflammatory, and anti-cancer activities of SLE in a

single study. If similar inhibitory effects of SLE are reproduced in relevant animal models and subsequently in humans and detailed mechanisms of its inhibitory actions are better understood, it would be a potent and readily available source for application to functional foods and medicinal products beneficial to human health.

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Fig. 5. Effect of SLE on the migration and adhesion of human cancer cells. The H1299 lung cancer cells were treated with 0, 125, 250, and 500 µg/mL of SLE for 24 h. The width of the wound was quantified using the Image-J software, and wound closure during the 24 h time interval was shown as a percentage value of the control (mean±standard error of 4–5 determinations, A). The HCT116 and H1299 cells were suspended with 0, 125, 250, and 500 µg/mL of SLE and then plated into a fibronectin-coated 96-well plate. After 2 h, the adherent cells were quantified and shown as percentage values of the control (mean±standard error of four determinations, B). Different letters (a, b) indicate the statistical differences among different concentrations by Tukey's test ($p < 0.05$).

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