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Anti-Tumorigenic Activity of Sophoflavescenol against Lewis Lung Carcinoma *In Vitro* and *In Vivo*

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This study examined the *in vitro* cytotoxic activity and *in vivo* antitumor activity as well as intracellular apoptotic capacities of a prenylated flavonol, sophoflavescenol from *Sophora flavescens*, to evaluate prospective anti-tumorigenic drugs, and antitumor potential. In addition, the *in vitro* antioxidant and anti-inflammatory capacities were evaluated. Despite the small effect on human breast adenocarcinoma (MCF-7), sophoflavescenol showed cytotoxicity against human leukaemia (HL-60), Lewis lung carcinoma (LLC), and human lung adenocarcinoma epithelial (A549) cells. Interestingly, it also exerted potent *in vivo* antitumor activity by tumor growth inhibition in the LLC tumor model as well as apoptotic activity by caspase-3 activation in HL-60 cells. In addition, it exhibited potent antioxidant activities in 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radicals and lipid peroxidation assays. Sophoflavescenol exerted notable anti-inflammatory activity by inhibiting nitric oxide generation and *tert*-butylhydroperoxide-induced ROS generation rather than inhibiting nuclear factor kappa B activation in RAW 264.7 cells. The findings show that the antioxidant, anti-inflammatory, and apoptotic activities of sophoflavescenol might contribute to the antitumor activity without severe side effects, highlighting its potential for chemoprevention and/or anticancer drugs due to multi-effective targets in almost all stages of tumorigenesis, including initiation, promotion, and progression.

Key words: Sophoflavescenol, Anti-tumorigenesis, Cytotoxicity, Antioxidant, Anti-inflammation

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INTRODUCTION

Cancer is the second leading cause of death after heart disease throughout the world, and its growing risk is associated with many health problems (Huang et al., 2010). Since carcinogenesis is believed to consist of three stages, initiation, promotion, and progression, anticancer agents are expected to intervene and retard and/or inhibit each stage. As parts of carcinogenesis, tumorigenesis normally means tumor development and tumor progression, and in practice, cancer mortality depends on the latter. Therefore, anti-tumorigenic agents to reduce the tumor volume and delay tumor growth at the tumorigenic progression might be effective therapies in cancer patients (Okada and Fujii, 2005). In addition to the inhibition of tumor growth, the activation of caspases plays crucial roles in the suppression of carcinogenesis through the execution of an apoptotic cascade (Gerl and Vaux, 2005). Moreover, the multi-stages of carcinogenesis are believed to involve free radicals, cellular oxidative stress, such as reactive oxygen/nitrogen species (ROS/RNS), and inflammation. In particular, excessive and pro-

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longed exposure to free radicals and ROS/RNS might result in the extensive oxidative damage of biomolecules (e.g., lipids, proteins, DNA), such as lipid peroxidation (thiobarbituric acid reactive substances (TBARS)) and protein oxidation/nitration (3-nitrotyrosine), which can lead to the initiation of cancer, followed by promotion and progression (Weinberg and Chandel, 2009). Inflammation was also reported to involve in the promotion and progression of the tumorigenic process. The suppression of the transcription factor, nuclear factor kappa B (NF-κB) and proinflammatory genes, cyclooxygenase-2 (COX-2) also provide potential targets in cancer chemoprevention (Surh et al., 2005; Thangapazham et al., 2006). In addition, another proinflammatory mediator, nitric oxide (NO•), produced by inducible nitric oxide synthase (iNOS) in the prostaglandin synthesis pathway, is related to tumor promotion by the activation of COX-2 (Kensler, 1997; Mei et al., 2000). As part of an ongoing search for anticancer therapy to intervene in carcinogenesis, the alleviation of excessive oxidative stress and inflammation, as well as the scavenging/inhibition of carcinogen-derived free radicals have attracted increasing attention, and antioxidants have emerged as potent candidates (Poulson et al., 1998; Surh et al., 2005).

In particular, a range of phenolics and flavonoids as phytochemicals have been reported to possess antioxidant, anti-inflammatory, and chemopreventive activities, which are closely related (Choquenet et al., 2009; Huang et al., 2010). A range of prenylated flavonoids have attracted interest as possible anticancer and antiproliferative agents, as well as antioxidants due to their prenyl group and flavonoid-skeleton (Delmulle et al., 2006; Yazaki et al., 2009). Moreover, Wätjen et al. (2007) reported that the addition of a prenyl group at the C-8 position increased the cytotoxicity due to the easy permeability in the lipid bilayer of the intestine, thus increasing its cellular uptake, without altering its antioxidant capacity. Although the antitumor activities of some prenylated flavonoids isolated from *Sophora flavescens*, including kurarinone, 2'-methoxykurarinone, sophoraflavanone G, kuraridin, and kurarinol were investigated (Ryu et al., 1997; Sun et al., 2007), limited studies on sophoflavescenol, a prenylated flavonol from *Sophora flavescens*, was accomplished as an anticancer agent. Moreover, this compound was reported to possess a range of bioactivities, including antiproliferative, (Rickles et al., 2009) and inhibitory activities against tyrosinase (Lee et al., 2004) and cyclic guanosine monophosphate-specific phosphodiesterase type 5 (Shin et al., 2002), all of which are connected to the anticancer activity.

Therefore, the possibility of sophoflavescenol as an anticancer and chemopreventive agent was studied by the *in vitro* cytotoxicity against a range of cancer cell lines, such as human lung adenocarcinoma epithelial (A549), Lewis lung carcinoma (LLC), human leukaemia (HL-60), and human breast adenocarcinoma (MCF-7) using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay method. Its *in vitro* apoptotic activity was assessed using caspase-3 activation and inhibitory activity against the total ROS generation in HL-60 cells. The *in vivo* antitumor activity of sophoflavescenol was evaluated using the mouse LLC model, and its *in vitro* antioxidant activities and intracellular anti-inflammatory activities were investigated.

MATERIALS AND METHODS

Cells and animals

The mouse macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection. The cell culture medium and all the other associated materials were purchased from Gibco BRL Life Technologies. To obtain rat liver homogenates for lipid peroxidation, this lab followed the Guidelines for the Care and Use of Laboratory Animals as approved by Pukyong National University. All experiments were performed using male Institute of Cancer Research (ICR) mice and female BDF-1 mice weighing 28~30 g, which were purchased from the Orient Co., Ltd., a branch of Charles River Laboratories. The animal treatment and maintenance of anti-tumorigenic activity were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Catholic University of Daegu, Korea.

Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), xanthine oxidase (XOD) from bovine milk, diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), sodium nitroprusside dihydrate, xanthine (2,6-dihydroxypurine), 2-thiobarbituric acid (TBA), bovine serum albumin (BSA), 4,5-diaminofluorescein diacetate (DAF-2), L-2-amino-3-mercapto-3-methylbutanoic acid (L-penicillamine), L-ascorbic acid, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (Carboxy-PTIO) potassium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), allopurinol, 4-methylumbelliferyl phosphate (MUP), l-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), MTT, doxorubicin hydro-

chloride (adriamycin), and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma-Aldrich Co. Dihydrorhodamine 123 (DHR 123) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were of high quality and obtained from Molecular Probes, and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) and peroxynitrite ($ONOO^-$) from Cayman Chemicals Co. Anti-nitrotyrosine, clone 1A6 (mouse-monoclonal primary antibody, IgG2b) and anti-mouse IgG, HRP conjugate (goat, polyclonal secondary antibody) were purchased from Millipore Co., and the polyvinylidenefluoride (PVDF) membrane (Immobilon-P) was supplied by Millipore Co. The Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. Roswell Park Memorial Institute medium (PRMI) 1640, Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum (FBS), and antibiotics were purchased from Gibco BRL. Unless stated otherwise, all chemicals and solvents used were purchased from E. Merck, Fluka, and Sigma-Aldrich Co., unless stated otherwise.

Isolation of sophoflavescenol

The dried roots of *S. flavescens* (10 kg) were heated in methanol (MeOH) under reflux for 3 h (3 × 10 L). The total filtrate was concentrated to dryness *in vacuo* at 40°C, to render the MeOH extract (2.2 kg). The extract was suspended in distilled water (H_2O) and partitioned successively with methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), to yield CH_2Cl_2 (230 g), EtOAc (250 g), and *n*-BuOH (610 g) fractions, respectively, as well as a H_2O residue (1, 110 g). Repeated chromatography of the CH_2Cl_2 fraction over a Si gel column with CH_2Cl_2-MeOH (gradient) followed by Sephadex LH20 yielded sophoflavescenol (600 mg). This compound was characterized and identified by spectroscopic methods including ^{1}H- and ^{13}C-NMR as well as by a comparison with published data (Jung et al., 2004). The spectral data is as follows, and the structure is shown in Fig. 1.

^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.58 (1H, s, 7-OH), 9.99 (1H, s, 4'-OH), 7.98 (2H, d, J = 8.8 Hz, H-2'/H-6'), 6.91 (2H, d, J = 8.8 Hz, H-3'/H-5'), 6.44 (1H, s, H-6), 5.15 (1H, t-like, H-2''), 3.80 (3H, s, 5-OCH_3), 3.46 (2H, brd, J = 6.7 Hz, H-1''), 1.75 (3H, s, H-5''), 1.62 (3H, s, H-4''). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 171.2 (C-4), 159.5 (C-7), 158.5 (C-4'), 157.9 (C-5), 155.4 (C-9), 141.8 (C-2), 136.8 (C-3), 130.9 (C-3''), 128.6 (C-2'/ C-6'), 122.7 (C-2''), 122.3 (C-1'), 115.4 (C-3'/ C-5'), 106.8 (C-8), 105.2 (C-10), 95.4 (C-6), 55.7 (5-OCH_3), 25.4 (C-4''), 21.5 (C-1''), 17.8 (C-5'').

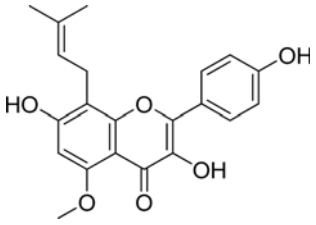

Fig. 1. Structure of sophoflavescenol

Assay for cell viability

The cytotoxicity of sophoflavescenol was evaluated using a MTT assay. The RAW 264.7 cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated at 37°C for 24 h. The cells were then treated with various sophoflavescenol concentrations. After additional 24 h incubation at 37°C, 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells and incubated for another 3 h. The resulting color was assayed at 540 nm using a microplate spectrophotometer (Molecular Devices).

Cytotoxicity assay

The cancer cell lines (A549, HL-60, LLC, MCF-7) were maintained in RPMI and/or IMDM, which included L-glutamine with 10% FBS and 2% penicillin-streptomycin. The cells were cultured at 37°C in a 5% CO_2 incubator. The cytotoxicity was measured using a modified MTT assay (Van le et al., 2009). The viable cells in the growth medium (180 μL) were seeded into 96-well microtiter plates (1×10^4 cells per well) and incubated at 37°C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to the final sample concentrations (f.c.) ranging from 5.0 to 100 μg/mL by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After leaving to stand for 4 h, 20 μL of the test sample was added to each well. The same volume of 0.1% DMSO in growth medium was added to the control wells. After 48 hours incubation, 20 μL of MTT (5 μg/mL) was also added to each well. Four hours later, the plate was centrifuged for 5 min at 1500 rpm, the medium was removed and the resulting formazan crystals were dissolved in DMSO (150 μL). The optical density (OD) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, ICN Flow). The IC_{50} value was defined as the sample concentration that reduced the absorbance by 50% relative to the vehicle-treated control.

In vivo antitumor activity

The LLC cells were purchased from the Korea Cells

Bank (ATCC No. CRL-1642). The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. The LLC cells (5×10^6 cells/mL) in PBS with 0.5% of Tween 20 were implanted carefully intradermally into the left axila of the BDF-1 mice (n = 5, 4 weeks old, female; Orient Inc.). The compound was dissolved in PBS with 2% (w/v) Tween 20 and 0.5% DMSO, and injected intraperitoneally in a volume of 0.1 mL per 20 g of body weight, with both 10 mg/kg and 1 mg/kg of sophoflavescenol daily for 14 days. Adriamycin (2 mg/kg) was used as a positive control. On the 21st day, the tumor volumes (length (mm) × width (mm) × height (mm)/2), and tumor weight were measured (Utsugi et al., 1996).

Assay for activation of caspase-3 by sophoflavescenol in HL-60 cells

To detect caspase-3 activation, the HL-60 cells were incubated with 15 and 30 μM of sophoflavescenol for various time periods, and then cells were washed with ice-cold PBS, and lysed in a cell lysis buffer (Cell Signaling Technology, Inc.) on ice for 30 min. The cell extracts were obtained by centrifugation at 14,000 × g at 4°C for 20 min. The protein concentrations were measured using a Bradford assay. The cytosolic proteins were separated electrophoretically on SDS-PAGE and transferred to PVDF membranes. The membranes were placed immediately in a blocking solution (5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) buffer) at room temperature for 1 h. The membranes were washed three times (10 min) in TBST buffer and incubated overnight with the primary antibodies at 4°C. After washing three times in TBST buffer (10 min), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. After washing three times in TBST buffer (10 min), the antibody labels were visualized with a supersignal west pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions, and exposed to X-ray film. Molecular weight markers were used for the molecular-weight determination.

Assay for effect of sophoflavescenol on ROS generation in HL-60 cells

To determine the intracellular ROS scavenging activity, HL-60 cells (2.0×10^4 cells/well) were seeded in a black 96-well plate. The cells were treated with sophoflavescenol for 2 h and DCFH-DA (20 μM) for 30 min. The fluorescence intensity of 2',7'-dichlorofluorescein was measured using a microplate fluorescence reader with excitation and emission wavelengths of 485 and 530 nm, respectively.

Assay for the effects of sophoflavescenol on the *tert*-butylhydroperoxide (*t*-BHP)-induced ROS generation in RAW 264.7 cells

The intracellular ROS scavenging activity of sophoflavescenol was measured using the oxidant-sensitive fluorescent probe, DCFH-DA. DCFH converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to dichlorofluorescein (DCF), a highly fluorescent compound. RAW 264.7 cells (2.0×10^4 cells/well) were seeded in a black 96-well plate. After 24 h, the cells were treated with sophoflavescenol for 2 h and *t*-BHP (200 μM) to induce ROS generation. After incubating the cells with DCFH-DA (20 μM) for 30 min, the fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation).

Assay for inhibition of cellular nitric oxide formation

The macrophage RAW 264.7 cell line was incubated in Dulbecco's modified Eagle's medium (DMEM) at 37°C under a humidified atmosphere containing 5% CO_2/air. The cells (1.0×10^5 cells/well in a 24-well plate with 500 μL of culture medium) were pretreated with sophoflavescenol for 2 h and incubated for 18 h with LPS (1.0 μg/mL). After incubation, the nitrite concentration of the supernatants (100 μL/well) was measured by adding 100 μL of Griess reagents (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). To quantify the nitrite concentration, standard nitrite solutions were prepared and the absorbance of the mixture was determined using a microplate spectrophotometer (Molecular Devices) at 540 nm. An inducible nitric oxide synthase inhibitor, AMT was used as a positive control.

Assay for the inhibition of NF-κB activation by a reporter gene assay

The RAW 264.7 cells harboring the pNF-κB-secretory alkaline phosphatase-neomycin phosphotransferase (SEAP-NPT) reporter construct were plated at a density of 1.0×10^5 cells/well in a 24-well plate with 500 μL of the culture medium and incubated for 24 h. The cells were then pre-treated with sophoflavescenol for 2 h before stimulation with LPS (1.0 μg/mL) for 16 h. The supernatants were heated at 65°C for 6 min and reacted in a SEAP assay buffer [2.0 M diethanol-

amine, 1.0 mM $MgCl_2$, 500 μM MUP] in darkness at 37°C for 1 h. The fluorescence from an adduct of the SEAP/MUP reaction was measured in relative fluorescence units (RFU) using a microplate spectrofluorometer (Molecular Devices) at excitation and emission wavelengths of 360 and 449 nm, respectively. A selective inhibitor of NF-κB, TPCK was used as a positive control.

Assay for DPPH radical scavenging activity

The DPPH radical scavenging activity was evaluated using a slight modification of the method reported by Blois (1958). Sophoflavescenol and DPPH were dissolved in MeOH. One hundred sixty microliters of sophoflavescenol at various concentrations (f.c. 100 μM) were added to 40 μL of a DPPH solution (1.5×10^{-4} M). After mixing gently and leaving stand at room temperature for 30 min, the optical density of the reactant was measured at 520 nm using a microplate spectrophotometer (Molecular Devices). The antioxidant activity of sophoflavescenol is expressed in term of the IC_{50} values (μM, needed to inhibit DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve. L-Ascorbic acid was used as a positive control.

Assay for trolox equivalent antioxidant capacity (TEAC)

This assay was based on the ability of different substances to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) compared to the positive control trolox: this method was developed by Re et al. (1999). To oxidize colorless ABTS to blue-green $ABTS^{\bullet+}$, a 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulfate (1:1, v/v) and left in the dark at room temperature for 12-16 h until the reaction was complete and the absorbance was stable. The blue/green $ABTS^{\bullet+}$ solution was diluted in ethanol (EtOH) to an absorbance of 0.70 ± 0.02 at 734 nm for the measurement. The photometric assay was carried out on 180 μL of the $ABTS^{\bullet+}$ solution and 20 μL of sophoflavescenol dissolved in EtOH solution (f.c. 100 μM), which was stirred for 30 sec. The optical density was measured at 734 nm after 2 min using a microplate spectrophotometer (Molecular Devices). The antioxidant activities of sophoflavescenol were calculated by determining the decrease in absorbance at different concentrations using the following equation: $E = [(A_c - A_t) / A_c] \times 100$, where A_t and A_c are the absorbance with and without sophoflavescenol, respectively. Trolox and L-ascorbic acid were used as the positive controls. The TEAC results are expressed as the IC_{50} values (μM), trolox equivalent (TE), and L-ascorbic acid equivalent (AE).

Assay for $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was assessed using the modified Kooy's method, which involved the monitoring of highly fluorescent rhodamine 123, which was produced rapidly from non-fluorescent DHR 123 in the presence of $ONOO^-$ (Kooy et al., 1994). Briefly, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5 μM. The assay buffer was prepared prior to use and placed on ice. Sophoflavescenol was dissolved in 10% DMSO (f.c. 100 μM). The background and final fluorescent intensities were measured for 5 min after treatment with and without the addition of authentic $ONOO^-$ (10 μM), dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate spectrofluorometer (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 530 nm, respectively. The $ONOO^-$ scavenging activity was calculated as the final fluorescence intensity minus the background fluorescence via the detection of DHR 123 oxidation. L-Penicillamine was used as a positive control.

Assay for nitric oxide scavenging activity

The $NO\bullet$ scavenging activity was determined using the modified method reported by Kim et al. (2004) with DAF-2 monitoring. DAF-2, as a specific $NO\bullet$ indicator, selectively traps $NO\bullet$ between the two amino groups within the molecule and yields triazolofluorescein, which emits green fluorescence when exited at 490–495 nm. Briefly, a stock solution of DAF-2 (4.5 mM) was diluted in 50 mM potassium phosphate buffer (pH 7.4, 1/45-fold). Sodium nitroprusside, as a $NO\bullet$ donor, was also dissolved (f.c. 2.0 mM). Sophoflavescenol was dissolved in 10% DMSO (f.c. 100 μM). The reaction was initiated by adding the diluted DAF-2 solution (f.c. 25 μM) to the mixed solution of sodium nitroprusside and sophoflavescenol in a 96-well black microplate. The fluorescence intensity caused by the reaction of DAF-2 with $NO\bullet$ was evaluated using a microplate spectrofluorometer (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 495 and 515 nm, respectively. Carboxy-PTIO was used as a positive control.

Assay for superoxide anion scavenging activity

The inhibition of XOD activity was evaluated by measuring the formation of uric acid from xanthine (Park et al., 2008). The assay mixture consisted of 50 μL of sophoflavescenol (f.c. 5.50 μg/mL) and 350 μL of xanthine (1.0 mM) as the substrate. Xanthine and

XOD were dissolved in 50 mM potassium phosphate buffer (pH 7.4) with 0.25 mM EDTA. The reaction mixtures were preincubated at room temperature for 2 min and the reaction initiated by adding 400 μL of XOD (50 mU/mL). The mixtures (800 μL) were incubated at 37°C for 30 min. The reaction was quenched by adding of HCl (100 μL, 5.0 M). The production of uric acid was determined with an UV/Visible spectrophotometer (Amersham Biosciences) at 295 nm. Allopurinol was used as a positive control.

Assay for *in vitro* lipid peroxidation

The rats were sacrificed and the livers removed. The liver was homogenized in 5.0 mL of 0.9% sodium chloride and diluted up to 10 mL with the same solution. A mixture of the liver homogenate (0.3 mL) was then mixed with 0.3 mL of aqueous 8.1% sodium dodecyl sulfate (SDS). The SDS/homogenate mixture was then split between two test tubes, one with and one without 0.1 mL of sophoflavescenol (f.c. 500 μg/mL). The mixtures were incubated at 37°C for 2 h. After adding 1.5 mL of 20% acetic acid and 1.0 mL of the 1.2% TBA solution, the test solutions were boiled at 100°C for 30 min and cooled to room temperature. The solutions were centrifuged at 2,500 rpm for 15 min and the absorbance of the upper layer was measured using a UV/Visible spectrophotometer (Amersham Biosciences) at 532 nm (Igarashi and Ohmuma, 1995). One TBA unit corresponded to an optical density of 0.1 at 532 nm, and was calculated to a TBA value per g liver weight. L-Ascorbic acid was used as a positive control.

Inhibition of $ONOO^-$-mediated tyrosine nitration

To examine the inhibition of $ONOO^-$-induced BSA nitration, 2.5 μL of sophoflavescenol (f.c. 6.25, 12.5, 25 μM) dissolved in 10% EtOH (v/v) was added to 95 μL of BSA (0.5 mg protein/mL) and mixed with 2.5 μL of $ONOO^-$ (200 μM). After incubation with shaking at 37 °C for 20 min, the mixed sample was added to a Bio-Rad gel buffer at a 1:1 ratio and boiled for 5 min to denature the proteins. The total protein equivalent of the reactant was separated on 10% SDS-polyacrylamide minigel at 80 V for 30 min, followed by 100 V for 1 h, and transferred to a PVDF membrane at 80 V for 110 min in a wet transfer system (Bio-Rad). The membrane was placed immediately in a blocking solution (5% non fat dry milk in TBS-Tween buffer (w/v), Bio-Rad TBS, and 0.1% Tween-20, pH 7.4) at room temperature for 1 h. The membrane was washed three times (10 min) in TBS-Tween buffer and incubated with the monoclonal anti-nitrotyrosine antibody (5% non fat dry milk), diluted 1:2000 in TBS-Tween buffer at 4°C overnight. After washing twice in TBS-Tween buffer (10 and 5 min), the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (5% w/v non fat dry milk), and diluted 1:2000 in TBS-Tween buffer at room temperature for 1 h. After washing twice in TBS-Tween buffer (10 and 5 min), the antibody labeling was visualized with a supersignal west pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions, and exposed to X-ray film (Kodak). Pre-stained blue protein markers were used for the molecular-weight determination.

Reaction of sophoflavescenol to $ONOO^-$

To explain the scavenging mechanism of sophoflavescenol, a mixture of sophoflavescenol and $ONOO^-$ were detected by spectrophotometric analysis (Kim et al., 2004). Briefly, tyrosine (f.c. 0.1 mM) was incubated without or with $ONOO^-$ (f.c. 0.5 mM in 0.3 N NaOH) in 50 mM phosphate buffer (pH 7.0) at room temperature for 10 min. Various concentrations of sophoflavescenol (f.c. 0.2, 0.1, 0.05, 0.025 mM) were incubated without or with $ONOO^-$ under the same conditions. The absorbance of each reactant was scanned from 200 to 600 nm using a UV/Visible spectrophotometer (Amersham Biosciences).

Statistics

The Kruskal-Wallis test and Mann-Whitney *U* test were used to determine the statistical significance of the differences between the values for various experimental and control groups. The results are expressed as the mean ± S.E.M.

RESULTS

In vitro cytotoxicity of sophoflavescenol

To assess the potential as anticancer drugs, sophoflavescenol isolated from *S. flavescens* was investigated via the *in vitro* cytotoxicity against various cancer cell lines, such as A549, LLC, HL-60, and MCF-7, using a MTT assay. Sophoflavescenol showed potential inhibitory effect on HL-60 cancer cells with an IC_{50} value of 12.5 μg/mL, as compared with a positive control, adriamycin (IC_{50} value = 0.23 μg/mL). In addition, sophoflavescenol exhibited moderate cytotoxicity against A549 and LLC cells with IC_{50} values as 69.9 and 38.1 μg/mL, respectively. Adriamycin displayed cytotoxicity against A549 and HL-60 with respective IC_{50} values of 0.41 and 0.18 μg/mL (Table I). On the other hand, sophoflavescenol did not show cytotoxicity against MCF-7 cells at concentrations up to 150 μg/mL. Sophoflavescenol had opposite effects among

Table I. Cytotoxicity of sophoflavescenol against cancer cell lines by MTT

Compounds	IC_{50} (μg/mL)			
	A549	LLC	HL-60	MCF-7
Sophoflavescenol	69.9	38.1	12.5	> 150
Adriamycin[a]	0.41	0.23	0.18	0.65

[a]Positive control

cancer cell lines, indicating this compound might possess specific anticancer activity for lung cancer and leukaemia.

In vivo antitumor activity of sophoflavescenol

Since sophoflavescenol showed moderate cytotoxicity, its effect against LLC cells was examined to assess its effectiveness on solid tumors. This compound was injected intraperitoneally into LLC-treated mice at doses of 10 mg/kg and 1 mg/kg. During experimental period after the injection (6 days), the growth of tumors was significantly inhibited by sophoflavescenol (Fig. 2A). As shown in Fig. 2B, the tumor volumes in the sophoflavescenol-treated groups were also decreased significantly in a dose-dependent manner as inhibition % of 74.6 and 16.8 at test concentrations of 10 mg/mL and 1 mg/mL, respectively, as compared to the positive control, adriamycin (72.7% at a concentration of 2 mg/mL). In addition, the decrease in tumor weight by sophoflavescenol was 87.3 and 41.7 % at 10 mg/kg and 1 mg/kg, respectively, compared to adriamycin (76.6% at 2 mg/kg, Fig. 2C).

Apoptotic activity of sophoflavescenol in HL-60 cells

In Western blot analysis, caspase-3 is present as an inactive 32 kDa precursor and its activation is indicated by the presence of a double protein band representing the proteolytic fragment p19 and active subunit p17 (Kondoh et al., 2005). As shown in Fig. 3, the cleavage of caspase-3 to 17 and 19 kDa fragments occurred 16 h after the sophoflavescenol treatment (15 and 30 μM). On the other hand, sophoflavescenol had negligible effects on ROS metabolism (Table II). This suggests indicate that the activation of caspase-3 in sophoflavescenol-treated HL-60 cells may be responsible for the induction of apoptosis and anticancer activity.

Antioxidant activities of sophoflavescenol

As shown in Table III, sophoflavescenol exhibited potent scavenging activity against DPPH and ABTS radicals with respective IC_{50} values of 9.01 ± 0.21 and 8.38 ± 0.28 μM, as compared to the positive controls,

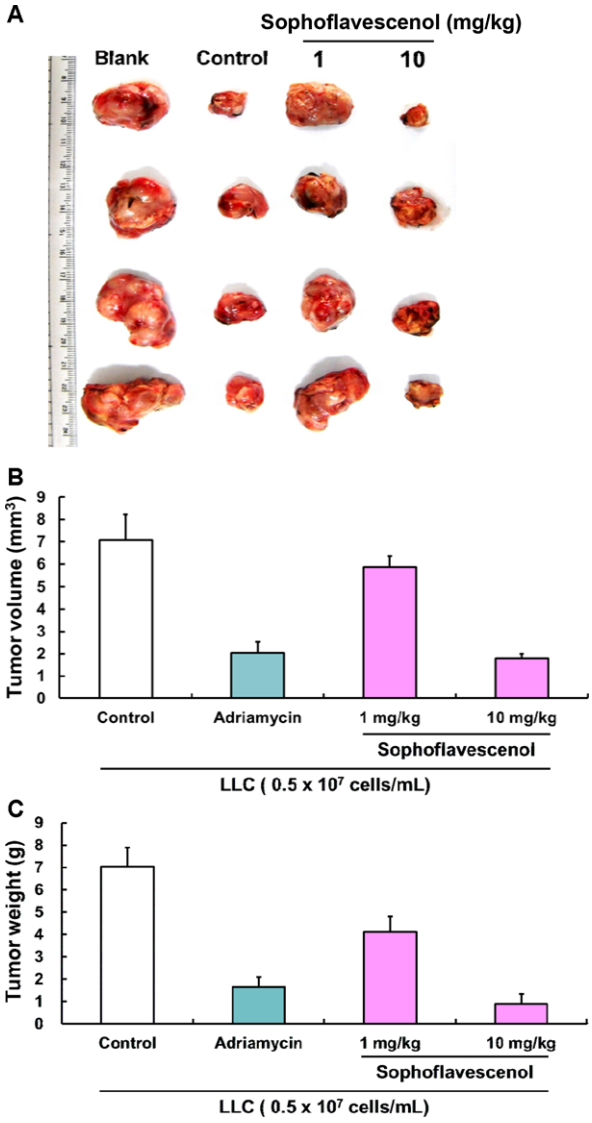

Fig. 2. Inhibitory effect of sophoflavescenol on tumor growth (**A**), volume (**B**), and weight (**C**) after 21 days. LLC cell concentration was 0.5 × 10^7 cells/mL. The mice in the negative control group (Blank) were injected LLC without administration of any inhibitors. The positive control group (Control) used was adriamycin at a concentration of 2.0 mg/kg.

L-ascorbic acid and trolox (IC_{50} values of 21.89 ± 0.41 and 7.78 ± 0.17 μM, respectively). This compound exhibited good scavenging activities against NO• with an IC_{50} value of 4.97 ± 0.76 μM. The positive control, carboxy-PTIO, had an IC_{50} value of 1.10 ± 0.25 μM. In addition, sophoflavescenol showed moderate scavenging activities against $ONOO^-$ and superoxide anion ($\cdot O_2^-$) with respective IC_{50} values of 14.50 ± 0.72 and 17.49 ± 1.56 μM, as compared with the positive controls, L-penicillamine and allopurinol (IC_{50} values of 0.62 ± 0.95 and 0.98 ± 0.28 μM, respectively). Since $ONOO^-$ is a well-known initiator in the carcinogenic

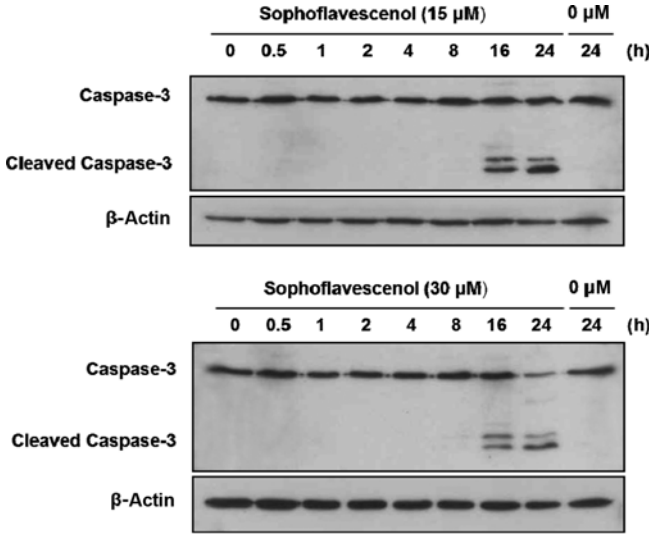

Fig. 3. Activation of caspase-3 by sophoflavescenol in HL-60 cell line. The HL-60 cells were incubated with 15 and 30 μM sophoflavescenol for 0.5-24 h. The protein concentrations were measured using a Bradford assay. The cytosolic proteins were electrophoretically separated on SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The antibody labelings were visualized with a supersignal west pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions, and exposed to X-ray film. The molecular weight markers were used for molecular-weight determination.

process, it is worth noting that sophoflavescenol possesses scavenging activities against this RNS.

As shown in Table III, sophoflavescenol inhibited TBARS formation with an IC_{50} value of 4.24 ± 0.00 μM, which is 10-fold better than the positive control, L-ascorbic acid (IC_{50} value = 50.48 ± 0.03 μM). In order to determine the inhibition against $ONOO^-$-induced tyrosine nitration as well as the scavenging mechanism of sophoflavescenol, the Western blot analysis using the 3-nitrotyrosine antibody and the spectrophotometric analysis detecting 3-nitrotyrosine were performed (Figs. 4 and 5). 3-Nitrotyrosine is a major product resulting from the reaction of proteins (BSA) and $ONOO^-$ and is detect at approximately 430 nm using

Table II. Effect of sophoflavescenol on ROS generation in the HL-60 cell line

	f.c. (μM)	Fluorescence		ROS (%)	
		Average	SD	Average	SD
Sophoflavescenol	1	15.82	0.42	96.48	2.58
	10	15.29	0.34	93.29	2.05
	15	16.05	0.91	97.92	5.57
	20	16.52	0.26	100.79	1.61
	30	18.12	1.05	110.52	6.41
	50	18.00	0.47	109.81	2.88
Control		16.39	0.21	100.00	1.27

Cells were treated with different concentrations (1, 10, 20, 30, and 50 μM) of sophoflavescenol and incubated for 2 h. Control values were obtained in the absence of sophoflavescenol.

a UV spectrophotometer. As demonstrated in Fig. 4, the sophoflavescenol pretreatment (f.c. 25, 50, 100 μM) inhibited the nitration of tyrosine significantly in a concentration-dependent manner. To estimate the $ONOO^-$ scavenging mechanism, the absorbance from 200 to 600 nm was measured after mixing sophoflavescenol (f.c. 25, 50, 100, 200 μM), tyrosine (f.c. 100 μM), and $ONOO^-$ (f.c. 500 μM) (Fig. 5). Sophoflavescenol decreased the absorbance intensity at 430 nm, corresponding to 3-nitrotyrosine in a concentration-dependent manner (98.5%, 84.3%, 60.5%, 21.0% at test concentrations of 200, 100, 50, 25 μM, respectively), suggesting that this compound acts as a $ONOO^-$ scavenger

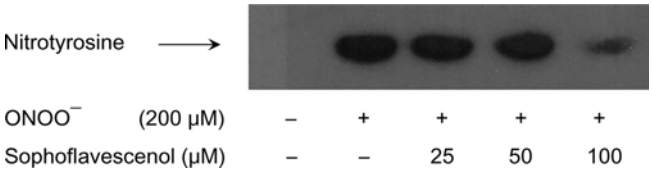

Fig. 4. Effect of sophoflavescenol on the protein nitration of BSA by $ONOO^-$. A mixture of sophoflavescenol, BSA, and $ONOO^-$ was incubated with shaking at 37°C for 30 min; this reactant was resolved by electrophoresis in 10% SDS-polyacrylamide gel.

Table III. *In vitro* antioxidant activities of sophoflavescenol

Compounds	DPPH[a]	TEAC (ABTS)[b]	TE[l]	AE[m]	TBARS[c]	$ONOO^-$[d]	NO•[e]	$•O_2^-$[f]
Sophoflavescenol	9.01 ± 0.21	8.38 ± 0.28	0.9	0.7	4.24 ± 0.00	14.50 ± 0.72	4.97 ± 0.76	17.49 ± 1.56
L-Ascorbic acid[g]	21.89 ± 0.41	5.82 ± 0.72		1.0	50.48 ± 0.03			
L-Penicillamine[h]						0.62 ± 0.00		
Carboxy-PTIO[i]							1.10 ± 0.25	
Allopurinol[j]								0.98 ± 0.28
Trolox[k]		7.78 ± 0.17	1.0					

[a-f]IC_{50} (μM); [g-k]Positive controls; [c] 50% inhibition concentration of lipid peroxidation; [l]Trolox equivalent (TE); [m]L-Ascorbic acid equivalent (AE) values.

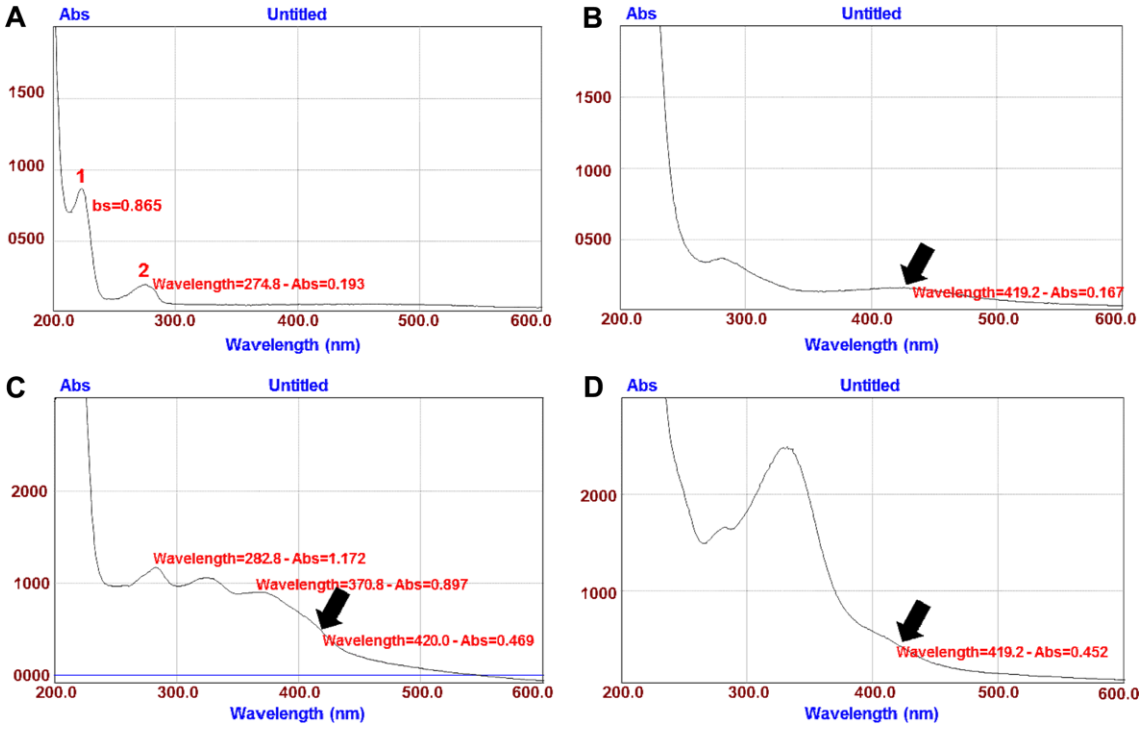

Fig. 5. Interaction of sophoflavescenol to $ONOO^-$. Tyrosine (f.c. 0.1 mM) was incubated without (**A**) or with ONOO (f.c. 0.5 mM in 0.3 N NaOH) (**B**) in 50 mM phosphate buffer (pH 7.0) at room temperature for 10 min. Sophoflavescenol (f.c. 0.2 mM) was incubated without (**C**) or with ONOO (**D**) at room temperature for 10 min. Spectrophotometric analysis was performed, as described in the Materials and Methods.

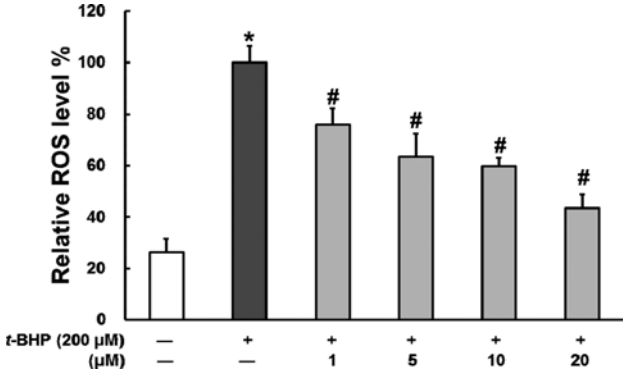

Fig. 6. Effect of sophoflavescenol on the *t*-BHP-induced ROS generation in RAW 264.7 cells. The cells were pre-treated with different concentrations (1, 5, 10 and 20 μM) of sophoflavescenol and *t*-BHP (200 μM) and incubated for 1 h. The control values were obtained in the absence of *t*-BHP and sophoflavescenol and after the addition of *t*-BHP (200 μM). $^*p < 0.05$ indicates significant differences from the control group. $^\#p < 0.05$ indicates significant differences from the *t*-BHP-treated group.

through nitration.

Anti-inflammatory activities of sophoflavescenol

Prior to the detailed inflammatory assays, the cytotoxicity of sophoflavescenol on RAW 264.7 cells were examined using *in vitro* cell-based assays. Although the data was not shown, this prenylated flavonol exhibited cytotoxicity at higher concentration of 20 μM (IC_{50} value = 21.31 μM). Therefore, sophoflavescenol was injected at a concentration range of 1 to 20 μM in subsequent experiments. To evaluate the effect of sophoflavescenol on *t*-BHP-induced ROS generation in RAW 264.7 cells, the cells were treated with *t*-BHP (200 μM) and a compound (0, 1.0, 5.0, 10, 20 μM) for 1 h. As shown in Fig. 6, sophoflavescenol had dose-dependent inhibitory effects on *t*-BHP-induced ROS levels in RAW 264.7 cells. To examine the effect of sophoflavescenol on LPS-induced NO• production in RAW 264.7 cells, the cells were treated with LPS for 18 h after treatment in the presence or absence of a compound (0, 1.0, 5, 10, 20 μM) for 2 h. The amount of nitrite, a stable metabolite of NO•, was used as an indicator of NO• production in the medium. As shown in Fig. 7A, NO• production in RAW 264.7 macrophages increased dramatically during 18 h incubation with LPS (1.0 μg/mL), and after the sophoflavescenol treatment, NO• production showed concentration-dependent inhibition with an IC_{50} value of 8.93 μM (3.4, 28.5, 55.8, and 89.4% inhibition at 1.0, 5, 10, 20 μM, respectively). As a positive control, AMT also exhibited a significant inhibitory effect with an inhibition % of

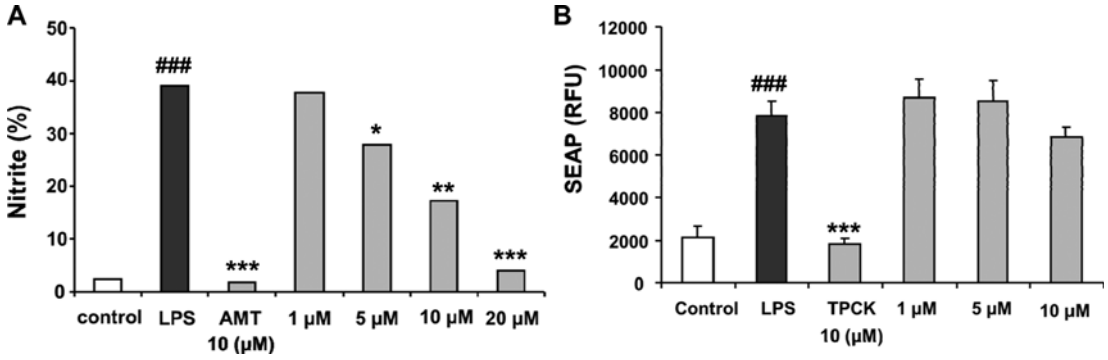

Fig. 7. Anti-inflammatory effect of sophoflavescenol in RAW 264.7 cells. (**A**) Effect of sophoflavescenol employed on LPS-induced nitrite formation in RAW 264.7 cells. The RAW 264.7 cells were pretreated with the indicated concentrations of sophoflavescenol for 2 h and LPS (1.0 μg/mL). After an 18 h incubation, the amount of nitrite in the culture supernatants was measured using a Griess reaction assay, as described in the Materials and Methods. The values are expressed as the mean ± S.E.M. of three individual experiments. $^{###}p < 0.001$ indicates significant differences from the unstimulated control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences from the LPS-treated group. (**B**) Effect of sophoflavescenol was performed on LPS-induced NF-B activation in RAW 264.7 cells. The RAW 264.7 cells harboring the NF-κB-SEAP-NPT reporter construct were pretreated with the indicated concentrations of sophoflavescenol for 2 h and treated with LPS (1.0 μg/mL). After 16 h incubation, the SEAP activity was measured in RFU using a microplate fluorometer. The values are expressed as the mean ± S.E.M. of three individual experiments. $^{###}p < 0.001$ indicates significant differences from the unstimulated control group. $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate significant differences from the LPS-treated group.

95.2 upon NO• production at a concentration of 10 μM. After 16 h, the LPS treated-transfected cells increased the level of SEAP expression by approximately 3.8-fold over basal levels (Fig. 7B). As shown in Fig. 7B, sophoflavescenol exhibited weak inhibition of SEAP expression by 12.8% at 10 μM. The positive control, TPCK, showed 76.7% inhibition at a concentration of 10 μM. Interestingly, sophoflavescenol did not show marked inhibition against LPS-induced SEAP expression despite the significant concentration-dependent inhibition of NO• production. This suggests that sophoflavescenol has an effect on inflammation-related carcinogenesis through the inhibition of ROS and NO• production rather than suppression of NF-κB activation.

DISCUSSION

Since cancer is the second leading cause of death, many researchers have focused on the development of preventive and therapeutic agents from natural products (Huang et al., 2010). Several studies have examined anticancer agents that inhibit the multi-stages progress of carcinogenesis, which are believed to involve free radicals, cellular oxidative stress, such as ROS/RNS, and inflammation, as well as suppress tumorigenesis. Considering the effective reduction of A549, LLC, and HL-60 cell, sophoflavescenol may be of benefit as an anticancer drug to inhibit/treat the pathogenesis of lung cancer and leukaemia rather than breast cancer (Table I). Since lung cancer is the most common cause of cancer mortality worldwide in adults due to the growing smoking population and severe air pollution, and leukaemia is the most mortal cause of cancer death in children, an in-depth study on this prenylated flavonol was accomplished using an *in vivo* anti-tumorigenic assay.

To examine the mechanism for potent cytotoxic activities of sophoflavescenol in HL-60 cells, its intracellular apoptotic capacity was also determined by activation of caspase-3 as well as *t*-BHP-induced ROS generation. Apoptosis is a well known programmed cell death due to the activation of a series of molecular events, including intrinsic and extrinsic apoptosis signaling, resulting in morphologic changes, chromatin condensation, and DNA fragmentation. In particular, the activation of proteases termed caspases, including caspases-8 and -9 as apoptotic initiators as well as the effector caspase-3, is important biochemical markers of apoptosis on the cell surface (Yu et al., 1998; Gerl and Vaux, 2005). Among the several caspases known, caspase-3 is believed to be the main executioner caspase and its activation has been shown to be essential for both intrinsic and extrinsic apoptotic cell death (Kim, 2005). As a result, the anticancer activity of sophoflavescenol might be due to the activation of caspase-3, followed by the induction of apoptosis in HL-60 cells (Fig. 3).

An *in vivo* LLC model was selected for the inhibition of tumor growth due to its relatively easy and rapid

tumor growth rate in the common mouse and its strong vulnerability against angiogenesis, which is an important process in the progression of carcinogenesis (Lee et al., 2006). In particular, the crucial step in cancer-derived death is due to tumor progression, including angiogenesis and migration (Okada and Fujii, 2005). Moreover, lung cancer is a major cause of cancer mortality throughout the world, as mentioned above (Lee et al., 2006). There was no significant difference in the body weight between the vehicle control group and sophoflavescenol-treated groups at any concentration during experimental periods (data not shown), indicating no remarkable side effects, such as lethargy and mortality were observed in the injection of this compound. Therefore, it is worthy of noting that the potent antitumor effect of sophoflavescenol is accompanied by non-toxicity *in vivo*, indicating minimal side effects in the human body.

Until now, there is compelling evidence that antioxidants, such as natural products, might play a pivotal role in the development of chemoprevention (Drisko et al., 2003; Nishino et al., 2004; Serafini et al., 2006). Since free radicals and cellular oxidative stress have been highly implicated in the initiation of carcinogenesis, the antioxidant capacity of sophoflavescenol was evaluated via the scavenging activities against free radicals and reactive oxygen/nitrogen species (ROS/RNS), including DPPH, ABTS radicals, $ONOO^-$, NO•, and $\cdot O_2^-$. Although sophoflavescenol exhibited moderate scavenging/inhibitory activities against NO•, $\cdot O_2^-$, $ONOO^-$, and $ONOO^-$-mediated tyrosine nitration, it exhibited potent antioxidant activities in the DPPH, ABTS, and lipid peroxidation assays. As a result, the antioxidant of sophoflavescenol might be responsible at least partly for the anticancer activity. Furthermore, these external radicals and cellular oxidative stress might damage the cellular components and subsequently accelerate the production of lipid peroxidation (TBARS) and protein oxidation/nitration (3-nitrotyrosine). Since TBARS and 3-nitrotyrosine might be important as biomarkers in cellular oxidative stress and play pivotal roles in cancer promotion, the inhibitory activities of sophoflavescenol against TBARS and 3-nitrotyrosine production were determined. Moreover, two possible mechanisms for phenolic compounds to scavenge $ONOO^-$ through nitration and electron donation have been proposed: $ONOO^-$-mediated nitration of tyrosine in the case of monohydroxylated structures, such as p-coumaric acid and ferulic acid; electron donation to reduce $ONOO^-$ for catechol structures, such as caffeic acid (Pannala et al., 1998; Kerry and Rice-Evans, 1999). Phenolic compounds can inhibit by either acting as alternative substrates for nitration, or as electron donors depending on their skeleton (Pannala et al., 1998).

In addition to the antioxidant capacity, anti-inflammation has emerged as an important target in anticancer remedies. In particular, two crucial factors, NF-κB and ROS/RNS are indispensible for tumorigenic promotion and progression (Surh et al., 2005). The effects of sophoflavescenol on inflammation-related carcinogenesis was determined in cell-based assays, including inhibitory activities of *t*-BHP-induced ROS generation as well as LPS-induced NO• generation and NF-κB activation in RAW 264.7 cells. Another important factor implicated in inflammation, is the activation of NF-κB as a transcription factor. The activated NF-κB transcription factor has been evidenced to result from LPS, followed by the extensive expression of pro-inflammatory genes, including iNOS, COX-2, and various cytokines (Xie and Nathan, 1994). Since these directly and indirectly lead to an increased inflammatory response, excessive ROS/RNS formation, and severe damage to biomolecules, a series of inflammation might take part in the promotion and progression of the carcinogenic process. The RAW 264.7 cells were transfected stably with the NF-κB-SEAP-NPT plasmid, containing four copies of the κB sequence fused to SEAP as the reporter (Moon et al., 2001). To examine the molecular inhibitory mechanism of inflammation and oxidative damage by sophoflavescenol, the NF-κB transcription activity was examined using a reporter gene assay system.

In conclusion, sophoflavescenol showed potent *in vivo* antitumor activity in the LLC tumor model and *in vitro* cytotoxicity against HL-60, LLC, and A549 cancer cells. In particular, sophoflavescenol showed cytotoxicity through caspase-3 dependent apoptosis in HL-60 cells. Despite the moderate *in vitro* antioxidant activities in NO•, $\cdot O_2^-$, $ONOO^-$, and $ONOO^-$-mediated tyrosine nitration, it exhibited potent scavenging/inhibitory activities against DPPH, ABTS, and lipid peroxidation. In addition, it exerted notable anti-inflammatory activity in RAW 264.7 cells through the inhibition of ROS and NO• generation rather than the suppression of NF-κB activation. As shown in Fig. 8, sophoflavescenol is proposed to act as an anticancer agent through (a) free radicals scavengers; (b) ROS/RNS scavengers; (c) inhibitors against protein nitration and lipid peroxidation; (d) inhibitors against *in vitro* and *in vivo* tumorigenesis; (e) inhibitors against NO•-mediated inflammation. These findings demonstrate that antioxidant and anti-inflammatory activities of sophoflavescenol might contribute to the antitumor activity without significant side effects, clearly suggesting its potential for chemoprevention and/or

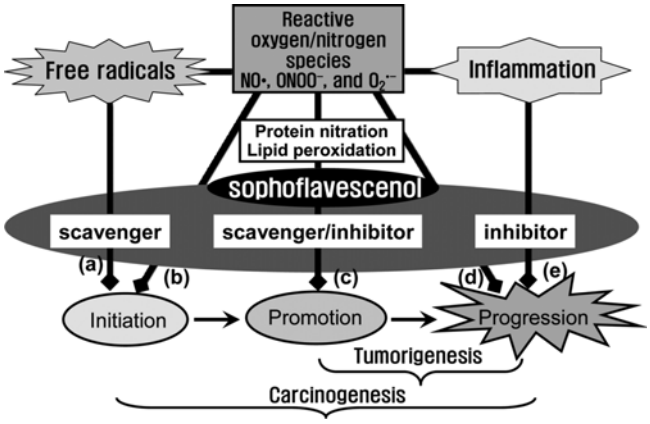

Fig. 8. Plausible multifunctional actions of sophoflavescenol in each stage of carcinogenesis. (**A**) Scavenging of free radicals, including DPPH and ABTS; (**B**) Scavenging of ROS/RNS, including NO•, $\cdot O_2^-$, and $ONOO^-$; (**C**) Inhibition of RNS-mediated protein nitration and ROS-mediated lipid peroxidation; (**D**) *In vitro* cytotoxicity and *in vivo* reduction of tumor growth and tumor volume; (**E**) Inhibition of NO•-mediated inflammation

anticancer drugs due to multi-effective targets in most stages of tumorigenesis, including initiation, promotion, and progression as well as the apoptotic cascades.

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