Identification and Characterization of Antioxidants from *Sophora flavescens*

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The objectives of this study were to investigate the radical-scavenging activity and protective potential of *Sophora flavescens* from oxidative damage by the radical generator 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in renal epithelial LLC-PK$_1$ cells and to identify the active components using the bioassay-linked fractionation method. The MeOH extract and fractions of CH_2Cl_2, BuOH, and H_2O from *S. flavescens* showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effects in a dose-dependent manner ($p<0.01$),whereas only the BuOH and CH_2Cl_2 fractions showed protective effects against LLC-PK$_1$ cellular damage induced by AAPH in a dose-dependent manner ($p<0.01$). In particular, the BuOH fraction had the most effective ($p<0.05$) antioxidative capacity. Employing a bioassay-linked HPLC/MS method, the active constituents from the BuOH fraction of *S. flavescens* were isolated and characterized as sophoraflavanone G and kurarinone with potent antioxidant effects against the DPPH radical, with IC_{50} values of 5.26 and 7.73 μg/ml, respectively. Moreover, the compounds dose dependently recovered cell viability decreased by AAPH treatment ($p<0.01$), suggesting their protective roles against cellular oxidative damage. The results of this study suggest that *S. flavescens* has excellent antioxidative and kidney-protective potential and that flavonoids from *S. flavescens*, *i.e.*, sophoraflavanone G and kurarinone, are the active constituents.

Key words *Sophora flavescens*; antioxidative activity; HPLC/MS; sophoraflavanone G; kurarinone

Free radicals including active oxygen species are produced inside and outside the living body, affecting various biological functions. Their relation with aging, various inflammatory diseases, carcinogenesis, and intractable diseases has attracted much attention.[1—3] Living organisms have effective defense mechanisms to protect themselves from active oxygen or other free radicals and the resultant disorders and diseases.[4—6] However, if there is excessive oxidative stress, it may be beyond the capability of the defense mechanisms, and then the role of antioxidants assumes greater importance. For these reasons, antioxidants are of great interest, with particular attention focused on the search for natural antioxidants.

The medicinal properties of some herbal plants have been documented in ancient literature, and the preparations have been found to be effective in the treatment of various diseases, especially as sources of antioxidants.[7,8] Recent reports have indicated that increased dietary intake of antioxidant-rich natural products reduces the incidence of human disease.[9,10] Thus searching for new sources of synthetic and natural antioxidants is vitally important. During our continual search for new antioxidants,[11,12] the butanol fraction from the methanol extract of *Sophora flavescens* (Leguminosae) has been found to have potent antioxidative activity. The dried root of *S. flavescens* is an important herbal medicine traditionally used as antipyretic, analgesic, anthelmintic, and stomachic.[13,14] *S. flavescens* is known to contain numerous flavonoids, *e.g.*, formononetin, kushenol E, kushenol B, shphoraflavanone G, kushenol L, kushenol M, kuraridin, kurarinone, kushenol N, and kushenol F.[13—15] Thus, this study evaluated the radical-scavenging activity of *S. flavescens* in *in vitro* and cellular systems, attempted to identify the active components of *S. flavescens* using the bioassay-linked HPLC/MS method, and confirmed their antioxidative activity.

MATERIALS AND METHODS

Chemicals and Materials 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was purchased from Amresco (Solon, Ohio, U.S.A.). LLC-PK$_1$ cells (porcine renal epithelial cells) were provided by the ATCC (Manassas, VA, U.S.A.). Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, U.S.A.). The root of *S. flavescens* was purchased from an herbal market in Korea. Sophoraflavanone G (**1**) and kurarinone (**2**) were isolated from *S. flavescens*. HPLC-grade acetonitrile and water were purchased from Duksan Pure Chemical Co., Ltd. (Ansan, Kyungki, Korea). All other reagents were of analytical reagent grade and used without further purification.

Apparatus and Chromatographic Conditions The structural identification was performed on a Bruker Avance 500-NMR spectrometer (Bruker, Ettlingen, Germany, 500 MHz for ^{1}H) and a Jeol JMS-700 mass spectrometer (Jeol, Tokyo, Japan). HPLC/MS chromatograms and spectra for dereplication were measured on a Finnigan LCQ^{TM} mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) coupled with a Hewlett Packard HPLC HP 1100 system. LC/MS conditions were: capillary temperature, 250 °C; damping gas he-

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lium, sheath gas (N_2) flow, 80; auxiliary gas flow, 20 arbitrary units; source voltage, +4.5 kV; capillary voltage, +13 V; tube lens offset, +20 V. A phenomenex C_{18} column (150×4.6 mm i.d., 5.0-μm particles, Phenomenex, Torance, CA, U.S.A.) was used for LC/MS. The samples were separated by gradient elution of the mobile phase consisting of 0.01% acetic acid acetonitrile–0.01% acetic acid solution (30 : 70 to 80 : 20, v/v, for 30 min) at the flow rate of 0.3 ml/min at room temperature. Xcalibor software (Finnigan, U.S.A.) was used for data acquisition. Analytical and semipreparative HPLC were run on a Gilson chromatograph equipped with a Gilson pump 321 and a Gilson UV/VIS-155 detector set at 230 nm. The fraction collector was purchased from Gilson (U.S.A.). Analytical (RP-18, 5 μm, 250×4.6 mm) and semipreparative (RP-18, 5 μm, 250×10 mm) columns (Phenomenex, U.S.A.) were used throughout this study.

Extract Preparation and Fractions from *S. flavescens* The dried root pieces of *S. flavescens* (100 g) were refluxed with methanol for 3 h. The organic solvent was removed *in vacuo* to give 20 g of methanol extract. The methanol extract was suspended in 500 ml of water and further partitioned in succession with water, methylene chloride, and butanol, affording 6.5, 7.7, and 5.6 g of the respective fractions.

Bioactivity-Guided Fractionation and Isolation Twenty microliters of the butanol fraction (20 mg/ml) was injected onto an analytical ODS column (250×4.6 mm, 5 μm). A gradient elution system of CH_3CN and H_2O from 30% CH_3CN (0 min) to 80% CH_3CN (30 min) was applied at the flow rate of 1 ml/min. The column eluate was collected using a fraction collector at 300 μl/well in 96-well plates. The solvent in each well was removed in a vacuum oven, and the residue in each well was tested for antioxidative activity. Bioassay-linked HPLC/MS indicated that the peaks appearing at 19 and 24 min were responsible for the observed activities. These compounds were isolated using semipreparative HPLC with CH_3CN–H_2O (40 : 60). The chemical structures of the compounds were identified as sophoraflavanone G (**1**) and kurarinone (**2**) based on the spectral results (Fig. 1).[14]

DPPH Radical-Scavenging Activity The level of oxidation was spectrophotometrically determined as described previously with minor modifications.[16] One hundred microliters of an ethanol solution of the sample from *S. flavescens* or 100 μl of ethanol as a control was added to microwells, followed by the addition of 100 μl of DPPH 120 μM in ethanol. After gentle mixing and 30 min of standing at room temperature, the DPPH radical level was measured with a microplate reader (SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA, U.S.A.). The antioxidant activity was expressed as the inhibition rate of DPPH radical or the IC_{50} (concentration in μg/ml required to inhibit DPPH radical formation by 50%) determined from the log-dose inhibition curve.

Protective Activity against Oxidative Damage LLC-PK_1 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 on culture plates with 5% FBS-supplemented DMEM/F-12 medium. The AAPH-induced cellular oxidative model was used to investigate the protective activity against oxidative damage.[17] After confluence had been reached, the cells were seeded into 96-well culture plates at 10^4 cells/well. Two hours later, AAPH 1 mM was added to all of the wells, and then samples of extract, fractions, or compounds from *S. flavescens* were treated in the test wells. The plates were then incubated under routine conditions for 24 h. The appropriate concentration of AAPH and the incubation time were determined in a preliminary experiment. MTT 50 μl (1 mg/ml) solution was added to each well. After incubation at 37 °C for 4 h, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μl of dimethylsulfoxide. The absorbance in each well was then read at 540 nm using a microplate reader.

Statistical Analysis The results of each group are expressed as mean±S.D. Data were analyzed using one-way ANOVA between control and sample-treated groups with SAS 8.1 (SAS Institute Inc., Cary, NC, U.S.A.). A p of <0.05 was considered to represent a statistically significant difference among groups.

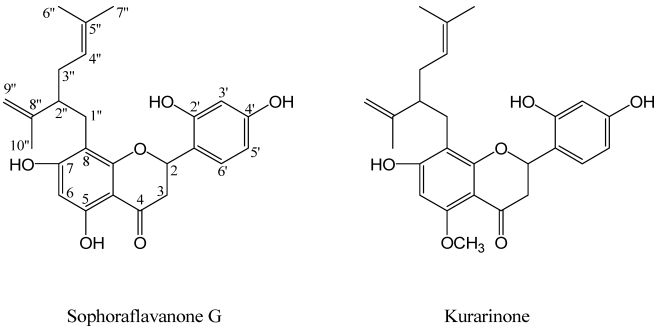

Fig. 1. Chemical Structures of Active Constituents Isolated from *Sophora flavescens*

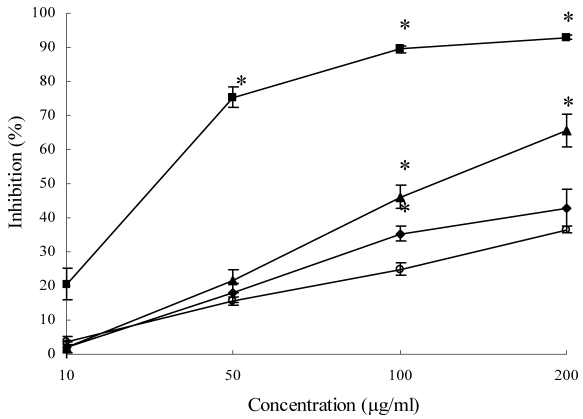

Fig. 2. Dose-Dependent Effects of MeOH Extract (◆) and CH_2Cl_2 (▲), BuOH (■), and H_2O (○) Fractions from *Sophora flavescens* on Inhibition of DPPH Radical Generation

The inhibition rate (%) increased quadratically ($p<0.01$) as the dose levels of MeOH extract and fractions of BuOH and CH_2Cl_2 increased whereas it increased linearly ($p<0.01$) for the H_2O fraction. * Significant difference ($p<0.05$) among the treatments within the same dose level.

RESULTS

DPPH Radical-Scavenging Activity As shown in Fig. 2, the MeOH extract and CH_2Cl_2, BuOH, and H_2O fractions from *S. flavescens* showed DPPH radical-scavenging activity ($p<0.05$) in a dose-dependent manner. In particular, the BuOH fraction exerted the strongest ($p<0.05$) inhibitory effect on DPPH radical generation, with inhibition of greater than 70% at 50 μg/ml and almost 90% at concentrations

greater than 100 μg/ml.

Protective Effects against AAPH-Induced Cellular Damage in LLC-PK$_1$ Cells Figure 3 shows the protective activity of the extract and fractions of *S. flavescens* against AAPH-induced cellular damage. Treatment with AAPH resulted in a decrease ($p<0.05$) in LLC-PK$_1$ cell viability to 60.2% compared with untreated normal cells. On the other hand, the CH_2Cl_2 and BuOH fractions increased ($p<0.01$) cell viability dose dependently. In particular, the BuOH fraction recovered cell viability to 70.1% even at the low concentration of 5 μg/ml, and to greater than 90% at 50 μg/ml, and thus the BuOH fraction revealed the greatest ($p<0.05$) protective activity.

HPLC and HPLC/MS Analysis of Antioxidant Compounds in the Butanol Fraction of *S. flavescens* The active components in the BuOH fraction of the root of *S. flavescens* were investigated using a bioassay-linked HPLC/MS dereplication process. The BuOH fraction was selected because it showed the most effective antioxidant activity on DPPH and LLC-PK$_1$ cells. The major antioxidant compounds in the BuOH fraction of *S. flavescens* were identified and measured with HPLC and positive ESI-MS. A phenomenax ODS column was selected and used in this analysis. Various mobile phase systems were evaluated to achieve satisfactory separation of all of these compounds, and a water (0.01% acetic acid) and acetonitrile (0.01% acetic acid) gradient was selected. No interfering peaks were noted for *S. flavescens* extract samples, and good resolution was achieved among all compounds. Two antioxidant constituents were detected. The retention times for the two active compounds were 18.2 and 22.8 min, respectively, detected by UV monitoring at 230 nm (Fig. 4A) and DPPH radical-scavenging activitygrams (Fig. 4B). Figure 4C shows the positive total ion chromatogram of the butanol fraction of *S. flavescens*. In addition, the two antioxidant constituents of the BuOH fraction appeared at 19.8 min at *m/z* 439 and 24.5 min at *m/z* 425, respectively.

Isolation and Identification of the Antioxidant Compounds from *S. flavescens* Extract The active components in the BuOH fraction were further purified using semipreparative HPLC, and their structures were elucidated to be sophoraflavanone G and kurarinone based on their mass spectral, ^{1}H-, and ^{13}C-NMR data.[14]

Compound **1** was found to have the molecular formula $C_{25}H_{28}O_6$ and showed a positive ESI-MS molecular ion peak at *m/z* 425 $[M+H]^+$. Combined with the analysis of its ^{1}H- and ^{13}C-NMR spectra, compound **1** was identified as sophoraflavanone G.

Compound **2** was found to have the molecular formula $C_{26}H_{30}O_6$ and showed a positive ESI-MS molecular ion peak at *m/z* 439 $[M+H]^+$. Combined with the analysis of its ^{1}H- and ^{13}C-NMR spectra, compound **2** was identified as kurarinone.

Antioxidative Effects of Sophoraflavanone G and Kurarinone The DPPH radical-scavenging effects of the active components of *S. flavescens* are shown in Table 1. The IC_{50} vales were 5.26 and 7.73 μg/ml of sophoraflavanone G and kurarinone, respectively. In addition, as shown in Table 2, sophoraflavanone G and kurarinone dose dependently recovered the cell viability decreased ($p<0.01$) by treatment with AAPH. Sophoraflavanone G and kurarinone increased cell viability to 96.12% and 89.29%, respectively, at 5.0 μg/ml from 66.7% in the AAPH-treated control cells. Moreover, treatment with sophoraflavanone G at the concentration of 10 μg/ml increased the viability of LLC-PK$_1$ cells to nearly normal values.

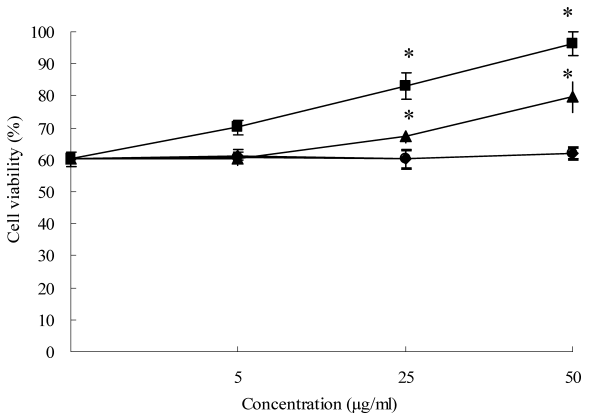

Fig. 3. Dose-Dependent Effects of MeOH Extract (◆) and CH_2Cl_2 (▲), BuOH (■), and H_2O (○) Fractions from *Sophora flavescens* on Viability of LLC-PK$_1$ Cells Treated with AAPH

Cell viabilities improved linearly ($p<0.01$) as the dose levels of the BuOH and CH_2Cl_2 fractions increased, whereas the dose of the MeOH extract and H_2O fraction did not affect ($p>0.05$) cell viability. * Significant difference ($p<0.05$) among the treatments within the same dose level.

Table 1. DPPH Radical-Scavenging Activity of Active Components from *S. flavescens*

Compound	IC_{50} (μg/ml)
Sophoraflavanone G	5.26±0.97
Kurarinone	7.73±1.90

Table 2. Effects of Flavanoids on Viability of Cells Treated with AAPH

Treatment (μg/ml)	Cell viability (%)	
	Sophoraflavanone G[a)]	Kurarinone[a)]
0.0	66.70±2.45[b)]	66.70±2.45[b)]
0.5	85.64±2.37[c)]	84.49±1.92[c)]
1.0	86.98±2.30[c)]	85.14±2.15[c)]
2.5	89.49±1.43[c)]	85.74±3.19[c)]
5.0	96.12±4.44[d)]	89.29±3.96[c)]
10.0	100.10±2.82[d)]	97.07±2.97[d)]
Normal	100±2.84	

a) Cell viability increased linearly ($p<0.01$) as dose levels increased. *b*,*c*,*d*) Means within the same column lacking common superscripts differ ($p<0.05$).

DISCUSSION

Free radicals and other reactive species are considered to be important causative factors in the development of various diseases and in the aging process.[18—21] This relationship has led to considerable interest in search for antioxidants to scavenge free radicals and boost defense systems. Although several synthetic antioxidants have been suggested to prevent and treat diseases, their various side effects and toxicities have become an issue. Therefore natural antioxidants have attracted much attention and great effort has been made to

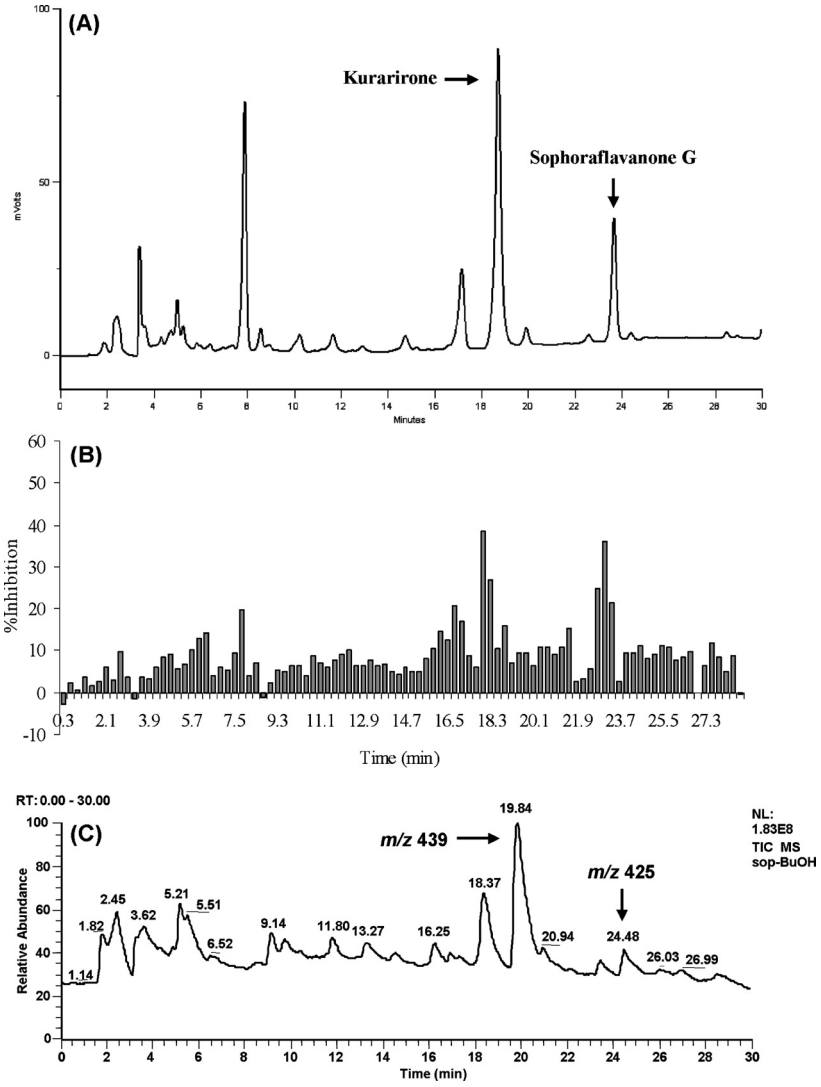

Fig. 4. HPLC Chromatogram (A) and Antioxidative Activity Profiles of DPPH Radical (B) and Positive-Ion Chromatogram (C) of the BuOH Fraction of *S. flavescens*

search for safe, effective therapeutic agents for the treatment of oxidative stress-related diseases.

In this study, we investigated the radical-scavenging effect and antioxidative potential of the extract and fractions of *S. flavescens* and identified its active components. The *in vitro* antioxidative effect was evaluated as the scavenging activity of DDPH radical and the cellular system of oxidative stress caused by AAPH using LLC-PK_1 renal tubular epithelial cells that are susceptible to oxidative stress.

To study the reactions of free radicals in biological systems, a well-designed *in vitro* model system is required. Numerous factors induce oxidative stress, such as irradiation, redox decomposition by metal ions of hydroperoxides or hydrogen peroxide, and thermal decomposition of free radical initiators including peroxides, hyponitrites, and azo compounds. To generate free radicals at a known, constant, and well-defined rate, the thermal decomposition of free radical initiators is preferred. It has been suggested that this can be achieved by the use of AAPH, a hydrophilic azo compound. AAPH generates free radicals at a constant and measurable rate during its thermal decomposition without biotransformation. The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals then attack other lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules and induces physiochemical alterations and cellular damage.[22] Finally, AAPH causes a diverse array of pathologic changes. Therefore an AAPH-intoxication experiment may be a promising assay system for the biological activities of antioxidants. In an appropriate model system, hydrophilic antioxidants can be used to scavenge radicals and suppress the oxidation initiated by AAPH within cells.[23,24] In addition, AAPH administration results in the *in vivo* damage of biological tissues such as lymphocytes, kidney, and liver, leading to pathologic conditions including atherosclerosis, ischemia-reoxygenation injury, and inflammatory disease.

AAPH leads to the decreased viability of LLC-PK_1 renal epithelial cells. Consistent with this result, several reports documented that AAPH decreased the viability of hepatic cells, neurons, and aortic endothelial cells and that treatment with AAPH induced apoptosis in the cells, causing loss of viability.[25—27] On the other hand, this study demonstrated that the extract and fractions of *S. flavescens* exerted DPPH radical-scavenging activity. In addition, the oxidative damage to

LLC-PK_1 cells by AAPH was recovered after treatment with *S. flavescens*, resulting in a significant, dose-dependent increase in cell viability. In particular, the *in vitro* and cellular oxidative damage model system showed that the BuOH fraction has the greatest activity against free radical generation and oxidative damage. The treatment with *S. flavescens* extract or fractions of LLC-PK_1 cells damaged by AAPH recovered cellular loss by peroxyl radical from AAPH, suggesting that the compounds in *S. flavescens*, especially those from the BuOH fraction, may have a vital role in the protection from oxidative damage due to AAPH.

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