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Phytochemicals of Black Bean Seed Coats: Isolation, Structure Elucidation, and Their Antiproliferative and Antioxidative Activities

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Bioactivity-guided fractionation of black bean (*Phaseolus vulgaris*) seed coats was used to determine the chemical identity of bioactive constituents, which showed potent antiproliferative and antioxidative activities. Twenty-four compounds including 12 triterpenoids, 7 flavonoids, and 5 other phytochemicals were isolated using gradient solvent fractionation, silica gel and ODS columns, and semipreparative and preparative HPLC. Their chemical structures were identified using MS, NMR, and X-ray diffraction analysis. Antiproliferative activities of isolated compounds against Caco-2 human colon cancer cells, HepG2 human liver cancer cells, and MCF-7 human breast cancer cells were evaluated. Among the compounds isolated, compounds **1**, **2**, **6**, **7**, **8**, **13**, **14**, **15**, **16**, **19**, and **20** showed potent inhibitory activities against the proliferation of HepG2 cells, with EC_{50} values of 238.8 ± 19.2, 120.6 ± 7.3, 94.4 ± 3.4, 98.9 ± 3.3, 32.1 ± 6.3, 306.4 ± 131.3, 156.9 ± 11.8, 410.3 ± 17.4, 435.9 ± 47.7, 202.3 ± 42.9, and 779.3 ± 37.4 μM, respectively. Compounds **1**, **2**, **3**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **14**, **15**, **19**, and **20** showed potent antiproliferative activities against Caco-2 cell growth, with EC_{50} values of 179.9 ± 16.9, 128.8 ± 11.6, 197.8 ± 4.2, 105.9 ± 4.7, 13.9 ± 2.8, 35.1 ± 2.9, 31.2 ± 0.5, 71.1 ± 11.9, 40.8 ± 4.1, 55.7 ± 8.1, 299.8 ± 17.3, 533.3 ± 126.0, 291.2 ± 1.0, and 717.2 ± 104.8 μM, respectively. Compounds **5**, **7**, **8**, **9**, **11**, **19**, **20** showed potent antiproliferative activities against MCF-7 cell growth in a dose-dependent manner, with EC_{50} values of 129.4 ± 9.0, 79.5 ± 1.0, 140.1 ± 31.8, 119.0 ± 7.2, 84.6 ± 1.7, 186.6 ± 21.1, and 1308 ± 69.9 μM, respectively. Six flavonoids (compounds **14**–**19**) showed potent antioxidant activity. These results showed the phytochemical extracts of black bean seed coats have potent antioxidant and antiproliferative activities.

KEYWORDS: Black bean; phytochemicals; triterpenoids; flavonoids; antioxidant; antiproliferative activities

INTRODUCTION

Food provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention (*1*). Epidemiological studies have consistently shown that regular consumption of fruits, vegetables, whole grains, and other plant foods is associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease (*2*–*4*). Phytochemicals, the bioactive nonnutrient plant compounds in fruits, vegetables, grains, and other plant foods, have been suggested to be responsible for their bioactivity linked to the reduced risk of major chronic diseases (*1*, *5*). It has been estimated that a healthy diet could prevent approximately 30% of all cancers (*6*, *7*).

Legumes, especially black beans (*Phaseolus vulgaris* L.), are widely consumed in the world, and are a staple in Central America as a major source for protein, energy, vitamins, and minerals. It was reported that black beans reduced azoxymethane-induced colon cancer in a rat model (*8*). The phytochemicals of black beans, including phenolic compounds (flavonoids and tannins), phytic acid, triterpenes, and phytosterols, may be responsible for their anticancer activity. Recently, black bean crude extracts were reported to inhibit HeLa cell proliferation in vitro (*9*).

The World Cancer Research Fund/American Institute for Cancer Research recognized the potential of legume consumption and reduced risk of cancer and recommended further research in this area (*10*). Recent work from our laboratory showed that the extracts of black beans had potent antiproliferative activity toward MCF-7 human breast cancer cells, HepG2 human liver cancer cells, and Caco-2 human colon cancer cells. However, the bioactive compounds that may be responsible for their anticancer activity are not clear. In a continuation of the efforts seeking bioactive components from fruits, vegetables, and other natural products (*11*, *12*), bioactivity-guided fractionation of black bean seed coats was used to determine the identity of bioactive compounds, which inhibit tumor cell growth. The

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objective of this research was to isolate and identify the bioactive compounds present in the seed coats of black beans with potent antiproliferative and antioxidant activities.

MATERIALS AND METHODS

Plant Material. Black beans (*P. vulgaris* L.) were purchased from a local grocery store (Ithaca, NY).

Reagents. All chemicals used in the study, such as methanol, acetone, hexane, ethyl acetate, dichloromethane, and *n*-butanol, were of analytical grade and were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). All deuteriated solvents, chloroform-*d*, dimethyl-d_6 sulfoxide, and pyridine-d_5, for NMR measurement were purchased from Sigma-Aldrich (St. Louis, MO). Randomly methylated β-cyclodextrin (RMCD) for antioxidant activity assay was obtained from Cyclodextrin Technologies (High Springs, FL). 2,2′-Azobis(amidinopropane) (ABAP) was purchased from Wako Chemicals (Richmond, VA). Dichlorofluorescein diacetate was purchased from Sigma-Aldrich (St. Louis, MO).

Chromatographic Materials. Silica gel for column chromatography, 230−400 mesh, and precoated silica gel 60 TLC plates were purchased from Merck KGaA (Darmstadt, Germany). Precoated RP-18 TLC plates were obtained from Macherey-Nagel (Düren, Germany). Diaion HP-20 was purchased from Supelco, Inc. (Bellefonte, PA). Octadecylsilane (ODS) (230−400 mesh) for open column chromatography was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). The 100 × 7.8 mm i.d., 5 μm, XTera MS C_{18} semipreparative HPLC column was purchased from Waters Corp. (Milford, MA). The 250 × 22 mm i.d., 5 μm, Alltima C_{18} preparative HPLC column was purchased from Alltech Associates, Inc. (Deerfield, IL).

Instrumentation. HPLC analysis and purification were performed on a Millennium HPLC system composed of two 515 pumps with a 2480 UV−visible detector (Waters Corp.). The detector wavelength was 205 nm when acetonitrile/water was used as mobile phase or 210 nm when methanol/water was used as mobile phase. All NMR spectra were obtained on a Varian INOVA 400 NMR spectrometer (Varian, Inc., Palo Alto, CA) operating at 400 MHz for proton and at 100 MHz for carbon (*12*). Mass spectra were obtained on an ESI-TOF II mass spectrometer (Micromass, Wythenshawe, U.K.) equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode (*12*). Optimal ESI conditions were as follows: capillary voltage, 3000 V; source temperature, 110 °C; and cone voltage, 55 V. The ESI gas was nitrogen. Data were acquired in continuum mode until acceptable averaged data were obtained (*12*). The X-ray diffraction analysis was conducted using the method previously described (*13*, *14*) as follows: data collection, APEX2 (Bruker, 2004); cell refinement, SAINTPlus (Bruker, 2003); data reduction, SAINT-Plus; program(s) used to solve structure, SHELXTL (Bruker, 1999); program(s) used to refine structure, SHELXTL; molecular graphics, SHELXTL; software used to prepare material for publication, SHELXTL.

Extraction, Isolation, and Purification Procedures of Bioactive Constituents from Black Bean Seed Coats. Black beans (44 kg) were washed with distilled water and were gently dried using paper towel. Black beans were soaked in chilled 80% acetone solution on ice for 1 h. The soaked black beans were blended in a chilled Waring blender for 5 min at lower speed to separate seed coats from the whole seeds. The phytochemicals of black bean seed coats were extracted using the method reported previously from our laboratory (*15*, *16*). Briefly, the black bean seed coats were homogenized for 3 min with chilled 80% acetone (1:20, w/v) using a chilled Waring blender. The sample was homogenized further using a Polytron homogenizer for an additional 3 min. The homogenates were filtered, and the filtrate was evaporated under vacuum at 45 °C until ∼90% of the filtrate had been evaporated. The residue was then resuspended in 10000 mL of water and extracted with the same volume of ethyl acetate and then extracted with water-saturated *n*-butanol five times, respectively (*12*).

The ethyl acetate fraction (110 g) was further purified by silica gel chromatography (230−400 mesh, 500 × 75 mm) and eluted with a CH_2Cl_2/MeOH gradient elution (the ratios of CH_2Cl_2/MeOH were from 100:0 to 0:100), and compound **2** (35.5 mg) was obtained from CH_2Cl_2/MeOH (50:1) elution. The CH_2Cl_2 eluant (1.3 g) was further subjected to silica gel column chromatography (240 × 22 mm) and eluted with hexane/ethyl acetate gradient elution (the ratios of hexane/ethyl acetate were from 100:0 to 0:100), and compound **3** (13 mg) was purified from the hexane/ethyl acetate (50:1) fraction. The fraction eluted with CH_2Cl_2/MeOH (100:1, 3.2 g) was further applied to a silica gel column (43 × 250 mm) using hexane/ethyl acetate (100:0 to 0:100), and compounds **23** (1.1 g) and **24** (45 mg) were obtained from hexane/ethyl acetate (25:1) elution. The fraction eluted with CH_2Cl_2/MeOH (50:1, 5.0 g) was further applied to a silica gel column (240 × 42 mm) using hexane/ethyl acetate (100:0 to 0:100), and compound **1** (318 mg) was obtained from hexane/ethyl acetate (25:1) elution. The fraction eluted with CH_2Cl_2/MeOH (20:1, 6.7 g) was further applied to a silica gel column (280 × 42 mm) eluted with CH_2Cl_2/MeOH (100:0 to 0:100), and compounds **21** (10 mg) and **22** (12 mg) were obtained from CH_2Cl_2/MeOH (20:1) elution. The CH_2Cl_2/MeOH (10:1, 5.6 g) eluant was chromatographed on a silica gel column (430 × 31 mm) using CH_2Cl_2/MeOH gradient elution (the ratios of CH_2Cl_2/MeOH were from 100:0 to 0:100) as solvent. Then the subfraction CH_2Cl_2/MeOH (10:1) was further purified on preparative HPLC using the 250 × 22 mm i.d., 5 μm, Alltima C_{18} preparative HPLC column, which was eluted isocratically with 20% acetonitrile in water at a flow rate of 8.0 mL/min. Compounds **15** (35 mg) and **16** (44 mg) were obtained at retention times of 11.3 and 12.9 min, respectively. The other fraction eluted with CH_2Cl_2/MeOH (10:1, 3.3 g) was further applied to a silica gel column (420 × 25 mm) using CH_2Cl_2/MeOH (100:0 to 0:100); compound **13** (85 mg) was obtained from CH_2Cl_2/MeOH (20:1) elution, and then the other subfraction CH_2Cl_2/MeOH (20:1) was further purified on semipreparative HPLC using the 100 × 7.8 mm i.d., 5 μm, XTera MS C_{18} semipreparative HPLC column, which was eluted isocratically with 1% (v/v) acetonitrile in water at a flow rate of 4.0 mL/min. Compound **19** (21 mg) was obtained at a retention time of 5.6 min. The subfraction of CH_2Cl_2/MeOH (10:1) was further purified on the C_{18} preparative column, which was eluted isocratically with 10% acetonitrile in water at a flow rate of 8.0 mL/min. Compounds **17** (21 mg), **18** (28 mg), and **14** (8 mg) were obtained at retention times of 5.5, 17.7, and 32.5 min, respectively.

The butanol fraction (328 g) of black bean seed coat extracts was subjected to a Diaion HP-20 column (550 × 75 mm) and eluted with water, 10% methanol (v/v), 30% methanol (v/v), 50% methanol (v/v), and 80% acetone, respectively. The 80% acetone eluant (43 g) was further purified by silica gel column (480 × 68 mm) and eluted with CH_2Cl_2/MeOH with a gradually increased ratio of methanol. Compound **20** (95 mg) was obtained from CH_2Cl_2/MeOH (50:1). The subfraction of CH_2Cl_2/MeOH (5:1, 13 g) was further purified on the C_{18} preparative column, which was eluted isocratically with 75% (v/v) methanol with 0.1% trifluoroacetic acid in water at a flow rate of 8.0 mL/min. Then four fractions were collected at retention times of 28.2, 35.7, 38.6, and 46.7 min, respectively. The first fraction (28.2 min, 202 mg) was further purified on the C_{18} semipreparative HPLC column, which was eluted isocratically with 65% (v/v) methanol with 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. Compounds **4** (103 mg) and **5** (54.4 mg), were obtained at retention times of 3.1 and 6.6 min, respectively. The second fraction (35.7 min, 134.8 mg) was further purified on the C_{18} semipreparative HPLC column, which was eluted isocratically with 75% methanol with 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. Compound **8** (22.5 mg) was obtained at a retention time of 10.8 min. The third fraction (38.6 min, 450 mg) was further purified on the C_{18} semipreparative HPLC column, which was eluted isocratically with 75% methanol with 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. One subfraction was collected at a retention time of 1.7 min. The subfraction (274.9 mg) was further purified on the C_{18} semipreparative HPLC column, which was eluted isocratically with 60% methanol and 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. Compounds **9** (69.1 mg) and **10** (120.2 mg) were obtained at retention times of 12.1 and 22.6 min, respectively. The last fraction (46.7 min, 128.7 mg) was further purified on the C_{18} semipreparative column, which was eluted isocratically with 75% methanol with 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. One subfraction (67.3 mg) was collected at retention time of 1.7 min. The subfraction was further eluted isocratically with 65% methanol with 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. Compounds **11** (9.6 mg) and **12** (1 mg)

were obtained at retention times of 7.1 and 13.2 min, respectively. The subfraction of CH_2Cl_2/MeOH (2:1) from 80% acetone eluate was further purified on the C_{18} preparative HPLC column, which was eluted isocratically with 75% methanol and 0.1% trifluoroacetic acid in water at a flow rate of 8.0 mL/min. One subfraction was collected at a retention time of 41.0 min. The subfraction was further purified on the C_{18} semipreparative HPLC column, which was eluted isocratically with 70% methanol and 0.1% trifluoroacetic acid in water at a flow rate of 4.0 mL/min. Compounds **6** (21.9 mg) and **7** (30 mg) were obtained at retention times of 22.0 and 38.8 min, respectively.

Measurement of Inhibition Activities against Tumor Cell Proliferation. Antiproliferative activities of the pure compounds isolated from black bean seed coats against HepG2 human liver cancer cells (*17*−*19*), MCF-7 human breast cancer cells (*20*), and Caco-2 human colon cancer cells (*21*) were measured by MTS assay as previously reported. HepG2 cells [The American Type Culture Collection (ATCC), Rockville, MD] were maintained in William medium E (WME), containing 10 mM Hepes, 5 μg/mL insulin, 2 μg/mL glucagon, 0.05 μg/mL hydrocortisone, 5% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY), 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin (*22*, *23*). HepG2 cells were maintained at 37 °C in 5% CO_2. A total of 2.5×10^4 HepG2 cells in growth media were added into each well of a 96-well flat-bottom plate. After 4 h of incubation at 37 °C in 5% CO_2, the growth medium was replaced by media containing different concentrations of purified compounds. Control cultures received the same solution minus the purified compounds, and blank wells contained 100 μL of growth medium with no cells. After 96 h of incubation, cell proliferation was determined by colorimetric MTS assay. Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490 nm) reading for each concentration of samples (*24*).

MCF-7 human breast cancer cells (ATCC) were maintained at 37 °C in 5% CO_2 in minimum essential medium (MEM) α medium containing 10 mM Hepes, 10 μg/mL insulin, 10% fetal bovine serum (Gibco, Life Technologies), 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin as described previously (*12*, *20*). MCF-7 cells in growth media were placed in each well of a 96-well flat-bottom plate at a concentration of 2.5×10^4 cells/well. Antiproliferative activities against MCF-7 breast cancer cells of pure compounds were measured using the same procedure described above.

Caco-2 human colon cancer cells (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies) supplemented with 5% fetal bovine serum (Gibco Life Technologies), 10 mM Hepes, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin and were maintained at 37 °C with 5% CO_2 as described previously (*25*). Caco-2 cells in growth media were placed in each well of a 96-well flat-bottom plate at a concentration of 2.5×10^4 cells/well. Antiproliferative activities against Caco-2 colon cancer cells of pure compounds were measured using the same procedure described above (*21*).

Measurement of Cytotoxicity. The cytotoxicity of isolated pure compounds was measured by methylene blue assay as described previously (*26*). Cells were plated in a 96-well plate at a density of 4×10^4 cells/well in 100 μL of growth medium and incubated for 24 h at 37 °C in 5% CO_2. The medium was removed, and the cells were treated with isolated pure compounds in 100 μL of treatment medium, and the plates were incubated at 37 °C for another 24 h. The treatment medium was removed, and the cells were washed with PBS. A volume of 50 μL/well methylene blue staining solution (98% HBSS, 1.25% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37 °C for 1 h. The dye was removed, and the plate was immersed in fresh deionized water three times, or until the water was clear. The water was tapped out of the wells, and the plate was allowed to air-dry briefly before 100 μL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a benchtop shaker for 20 min to allow uniform elution. The absorbance was read at 570 nm with blank subtraction using the MRX II DYNEX spectrophotometer (DYNEX Inc., Chantilly, VA).

Measurement of Antioxidant Activity Using Peroxyl Radical Scavenging Capacity (PSC) Assay. Antioxidant activities of isolated pure compounds were determined according to the PSC assay described previously (*27*). Just prior to use in the reaction, 107 μL of 2.48 mM dichlorofluorescein diacetate was hydrolyzed to dichlorofluorescein with 893 μL of 1.0 mM KOH for 5 min in a vial to remove the diacetate moiety and then diluted with 7 mL of 75 mM phosphate buffer (pH 7.4). ABAP (200 mM) was prepared fresh in the buffer and was kept at 4 °C between runs. In an assay, 100 μL of pure compounds was diluted in 75 mM phosphate buffer (pH 7.4) and then transferred into reaction cells on a 96-well plate, and 100 μL of dichlorofluorescein was added. The 96-well plate was loaded into the Fluoroskan Ascent fluorescence spectrophotometer (Thermo Labsystems, Franklin, MA), and the solution in each cell was mixed by shaking at 1200 rpm for 20 s. The reaction was then initiated by adding 50 μL of ABAP from the autodispenser on the equipment. Each set of dilutions for a replicate and control was analyzed three times in adjacent columns. The reaction was carried out at 37 °C, and fluorescence was monitored at 485 nm excitation and 538 nm emission with the fluorescence spectrophotometer. The buffer was used for control reactions. Data were acquired with Ascent software, version 2.6 (Thermo Labsystems). The areas under the average fluorescence-reaction time kinetic curve (AUC) for both control and samples (up to 36 min) were integrated and used as the basis for calculating antioxidant activity according to the equation PSC unit $= 1 - (SA/CA)$, where SA is the AUC for sample or standard dilution and CA is the AUC for the control reaction using only buffer. Compounds inhibiting the oxidation of dichlorofluorescein produced smaller SA and higher PSC units. The median effective concentration (EC_{50}) was defined as the dose required to cause a 50% inhibition (PSC unit 0.5) for each pure compound. Results were expressed as micromoles of vitamin C equivalents per micromole of pure compound ± standard deviation (SD) for triplicate analyses.

Solubility of lipophilic compounds was ensured by dissolving them in 12% RMCD prepared in 50% acetone in water. The fluorescent dye was prepared by hydrolyzing 11 μL of 2.48 mM dichlorofluorescein diacetate with 898 μL of 1.0 mM KOH and then diluted with 7 mL of 75 mM phosphate buffer (pH 7.4). The reaction mixture of the lipo-PSC assay contained 100 μL of appropriate dilution of pure compounds in 12% RMCD, 100 μL of dichlorofluorescein, and 50 μL of 200 mM ABAP. Control reactions used 12% RMCD. The reaction conditions, data acquisition, and processing were as described above for the hydro-PSC assay. The AUC was calculated up to 40 min as described previously (*27*).

RESULTS AND DISSCUSION

Structure Identification of the Purified Compounds. Twenty-four compounds, including 12 triterpenoids, 7 flavonoids, and 5 other phytochemicals, were isolated and identified from black bean seed coat extracts. The chemical structures of compounds **1**−**24** are shown in **Figure 1**.

Compound **1** was obtained as colorless cubic crystals from the ethyl acetate faction of black bean seed coat extracts. The ESI-MS of compound **1** gave the $[M - H]^-$ ion at *m*/*z* 441, and its molecular formula was inferred as $C_{30}H_{50}O_2$ from the analyses of ^1H, ^{13}C NMR, and DEPT spectra. In the ^1H NMR spectra, there were seven methyl groups in the molecule, showing characteristic peaks of triterpenoids at high field. The signals at 145.3 and 121.6 ppm in the ^{13}C NMR spectrum were two olefinic carbons, which implied there was a carbon−carbon double bond in the molecule. On the basis of the analyses of spectra, compound **1** was identified as olean-12-en-3β,24-diol, which was the same as reported previously (*28*). The stereochemical structure of compound **1** (**Figure 2**) was confirmed by X-ray diffraction analysis.

Compound **2** was obtained as a white amorphous powder from the ethyl acetate faction. The negative ESI-MS gave the $[M - H]^-$ ion at *m*/*z* 441, and its molecular formula was inferred as $C_{30}H_{50}O_2$ according to analyses of ^1H and ^{13}C NMR spectra. The ^1H NMR of compound **2** showed eight methyl groups in the high field. In the ^{13}C NMR, the olefinic carbon signals at

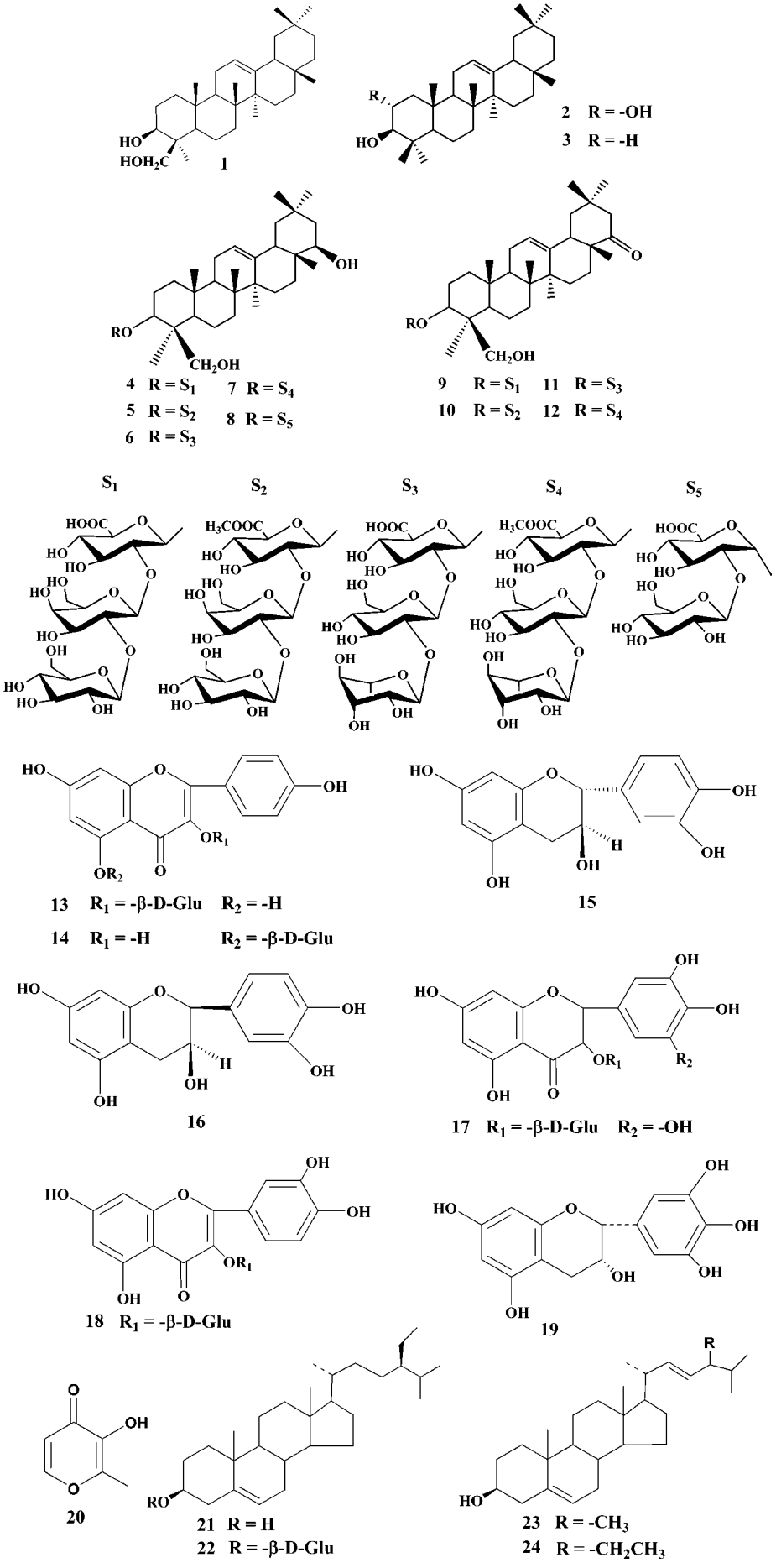

Figure 1. Chemical structures of compounds isolated and identified from black bean seed coats.

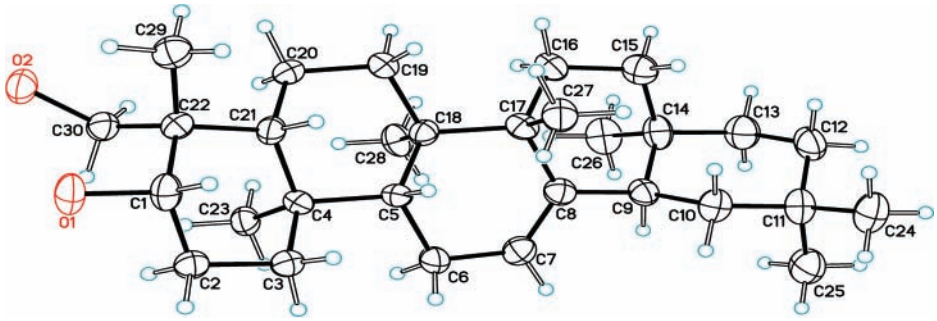

Figure 2. Single-crystal X-ray structure of compound **1**, olean-12-en-3β,24-diol.

145.6 ppm (C-13) and 122.7 ppm (C-12) were a carbon−carbon double bond at the position 12(13). There were two oxygen-linked carbons at 80.5 ppm (C-3) and 65.0 ppm (C-2), suggesting there were two hydroxyl groups in the molecule. Compound **2** was identified as olean-12-en-2α,3β-diol, which was consistent with the structure reported previously (*29*).

The molecular formula of compound **3** was inferred as $C_{30}H_{50}O$ on the basis of the analysis of ^{1}H and ^{13}C NMR spectra. Compared to that of compound **2**, compound **3** had only one hydroxyl group in the molecule, which was at 79.2 ppm (C-3). The ^{13}C NMR data of compound **3** were very similar to that of compound **2**, except for the carbon signals at the A-ring. Compound **3** was identified as olean-12-en-3β-ol, which was the same as the previously reported structure (*30*).

Compound **4** was obtained as a white amorphous powder from the butanol fraction of the extracts of black bean seed coats. The negative ESI-MS gave the $[M - H]^-$ ion at *m*/*z* 957, and the molecular formula was inferred as $C_{48}H_{78}O_{19}$ when combined with its ^{1}H and ^{13}C NMR spectra. The negative ESI-MS indicated the nature of the sugars present, showing ions at *m*/*z* 795 $(M - H - \text{hexose})^-$, 633 $(M - H - \text{hexose} - \text{hexose})^-$, and 457 $(M - H - \text{hexose} - \text{hexose} - \text{hexuronic acid})^-$. Combined with the ^{13}C NMR data, this suggested there were two hexoses and one hexuronic acid in the molecular structure. There were three anomeric carbon signals at 107.1, 105.0, and 103.1 ppm, showing there were three sugar units in the molecule. The three sugars were identified as D-glucuronic acid, D-galactose, and D-glucose, respectively. The β-configuration of the three sugars was inferred from the coupling constants of J_{H1-H2} ($J > 7$ Hz). Compound **4** was identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-3β,22β,24-triol, which was the same as reported previously (*31*).

The molecular formula of compound **5** was inferred as $C_{49}H_{80}O_{19}$ from its negative ESI-MS and ^{1}H and ^{13}C NMR data, which had an additional methyl group in the molecule compared with compound **4**. Compounds **4** and **5** had very similar ^{13}C NMR spectra except the data of glucuronic acid. Compound **5** was the methyl ester of compound **4**. Therefore, compound **5** was identified as 3-*O*-[(β-D-glucopyranosyl)(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-3β,-22β,24-triol methyl ester, which was the same as reported previously (*31*).

Compound **6** was obtained as a white powder. The negative ESI-MS gave the $[M - H]^-$ ion at *m*/*z* 941, and the molecular formula was inferred as $C_{48}H_{78}O_{18}$ on the basis of its ^{1}H and ^{13}C NMR spectra. The negative ESI-MS indicated the nature of the sugars present, showing ions at *m*/*z* 795 $(M - H - \text{rhamnose})^-$, 633 $(M - H - \text{rhamnose} - \text{hexose})^-$, and 457 $(M - H - \text{rhamnose} - \text{hexose} - \text{hexuronic acid})^-$. Combined with the ^{13}C NMR data, this suggested there were one rhamnose, one hexose, and one hexuronic acid in the molecule. Comparison of the ^{13}C NMR data of compound **6** with those of compound **4** showed that they had the same aglycone. There were three anomeric carbon signals at 105.7, 102.7, and 102.0 ppm, showing there were three sugar units in the molecule. The three sugars were identified as D-glucuronic acid, D-glucose, and L-rhamnose, respectively. The β-configuration of the D-glucuronic acid and D-glucose was inferred from the coupling constants of J_{H1-H2} ($J > 7$ Hz). The α-configuration of the anomeric carbon of rhamnopyranosyl units was confirmed by comparison of the chemical shift values of carbons 3 and 5 with those of the corresponding carbons of methyl α- and β-rhamnopyranoside. Compound **6** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-3β,22β,24-triol, which was the same as reported previously (*32*).

The molecular formula of compound **7** was inferred as $C_{49}H_{80}O_{18}$ from its negative ESI-MS and ^{1}H and ^{13}C NMR spectra, which had an additional methyl group in the molecule compared with compound **6**. Compounds **6** and **7** had very similar ^{13}C NMR spectra except the data of glucuronic acid. Compound **7** was the methyl ester of compound **6**. Therefore, compound **7** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-3β,22β,24-triol methyl ester as reported previously (*33*).

Compound **8** was obtained as a white powder and was another triterpenoid isolated from the butanol fraction. The negative ESI-MS gave the $[M - H]^-$ ion at *m*/*z* 795, and the molecular formula was inferred as $C_{42}H_{68}O_{14}$ when combined with its ^{1}H and ^{13}C NMR spectra. The negative ESI-MS indicated the nature of the sugars present, showing ions at *m*/*z* 633 $(M - H - \text{hexose})^-$ and 457 $(M - H - \text{hexose} - \text{hexuronic acid})^-$. Combined with the ^{13}C NMR data, this suggested there were a hexose and hexuronic acid in the molecule. The two sugar units were identified as D-glucose and D-glucuronic acid. Compound **8** had ^{13}C NMR data very similar to those of compound **6**, except compound **8** lacked a rhamnose unit in the molecule. Therefore, compound **8** was identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-3β,22β,24-triol, which was the same as reported previously (*34*).

Compounds **9** and **10** were a pair of triterpenoid derivatives and were obtained as a white amorphous powder from the butanol fraction. The negative ESI-MS gave the $[M - H]^-$ ion at *m*/*z* 955. The molecular formula was inferred as $C_{48}H_{76}O_{19}$ from its MS, ^{1}H, and ^{13}C NMR data. The negative ESI-MS indicated the nature of the sugars present, showing ions at *m*/*z* 793 $(M - H - \text{hexose})^-$, 631 $(M - H - \text{hexose} - \text{hexose})^-$, and 455 $(M - H - \text{hexose} - \text{hexose} - \text{hexuronic acid})^-$. There were seven methyl groups in the high field of ^{1}H NMR. In the lowest field of ^{13}C NMR, there was a carbonyl carbon signal at 215.9 ppm and a carboxyl carbon signal at 172.6 ppm. There

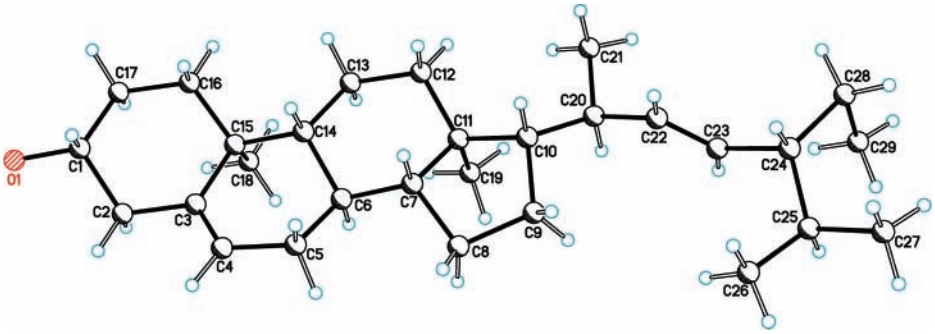

Figure 3. Single-crystal X-ray structure of compound **24**, stigmasta-5,22-dien-3β-ol.

were three anomeric carbons, which were at 107.1, 104.9, and 103.0 ppm. The three sugars were identified as D-glucuronic acid, D-galactose, and D-glucose, respectively. The β-configuration of the three sugars was determined by the coupling constants of J_{H1-H2} ($J > 7$ Hz). According to the literature (*35*), compound **9** was identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-22-oxo-3β,24-diol.

The molecular formula of compound **10** was inferred as $C_{49}H_{78}O_{19}$ from its negative ESI-MS and ^{1}H and ^{13}C NMR data, which had an additional methyl group in the molecule compared with compound **9**. Compounds **9** and **10** had very similar ^{13}C NMR spectra except the data of glucuronic acid. Therefore, compound **10** was identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-22-oxo-3β,24-diol methyl ester. Compound **10** is a new compound, which has not been reported in the literature.

Compounds **11** and **12** were another pair of triterpenoid derivatives isolated from the butanol fraction. They were obtained as a white powder. The negative ESI mass spectra of compounds **11** and **12** gave the $[M - H]^-$ ion at *m*/*z* 939 and 953, and their molecular formulas were inferred as $C_{48}H_{76}O_{18}$ and $C_{49}H_{78}O_{18}$, respectively, on the basis of the analyses of ^{1}H and ^{13}C NMR spectra. They had the same aglycone as that of compound **9** as shown by the ^{13}C NMR data. The ^{13}C NMR data of the sugar residue located at C-3 of compound **11** were identical with those of compound **6**. Thus, compound **11** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-22-oxo-3β,-24-diol, which was consistent with the literature reports (*33*). Accordingly, compound **12** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]olean-12-en-22-oxo-3β,24-diol methyl ester. Compound **11** is a new compound, which has not been reported in the literature. However, compound **12**, the methyl ester of compound **11**, was the same as reported previously (*33*).

Compounds **13**−**19** were seven flavonoids isolated from the ethyl acetate fraction of black bean seed coats. Compound **13** was obtained as a pale yellow powder. The ESI-MS showed the $[M - H]^-$ ion at *m*/*z* 447, and the molecular formula was inferred as $C_{21}H_{20}O_{11}$ on the basis of analyses of ^{1}H and ^{13}C NMR spectra. In the ^{1}H NMR spectra, an A_2B_2 spin-coupling system was ascribed to the four protons of the B-ring of a flavone, with signals at 8.04 ppm (H, br d, $J = 9.0$ Hz) and 6.89 ppm (H, br d, $J = 9.0$ Hz), respectively. The signals at 6.44 ppm (H, d, $J = 2.1$ Hz) and 6.21 ppm (H, d, $J = 2.1$ Hz) were meta-substituted protons of the A-ring. The signal at 12.62 ppm (H, s) was the characteristic signal of the hydroxyl at C-5 of a flavone. There was an anomeric proton at 5.47 ppm (H, d, $J = 7.5$ Hz). The signals in the ^{13}C NMR showed 21 carbons in the molecule. On the basis of the ^{1}H and ^{13}C NMR spectra, compound **13** was identified as kaempferol 3-*O*-β-D-glucopyranoside, which was identical to the structure reported previously (*36*).

Compound **14** had the same formula as compound **13**. It had the same aglycone and sugar unit as compound **13** as shown by the ^{13}C NMR data. The difference between them was the linkage position of the sugar unit. The D-glucose was linked at C-5 in compound **14**. Therefore, it was identified as kaempferol 5-*O*-β-D-glucopyranoside, which was consistent with that reported previously (*37*).

Compounds **15** and **16** were identified as (+)-catechin and (−)-epicatechin, respectively, on the basis of their ESI-MS and ^{1}H and ^{13}C NMR spectra (*38*).

Compound **17** was obtained as a pale yellow powder. The ESI-MS showed the $[M - H]^-$ ion at *m*/*z* 481, and the molecular formula was inferred as $C_{21}H_{22}O_{13}$ from ^{1}H and ^{13}C NMR spectra. The negative ESI-MS indicated the nature of the sugars present, showing ions at *m*/*z* 319 $(M - H - \text{hexose})^-$. In the ^{1}H NMR spectra, the signal at 6.56 ppm (2H, br s) was ascribed to the 2′,6′-protons of the B-ring. The signals at 5.85 ppm (H, d, $J = 2.1$ Hz) and 5.82 ppm (H, d, $J = 2.1$ Hz) were meta-substituted protons of the A-ring. There was an anomeric proton at 4.55 ppm (H, d, $J = 7.2$ Hz). The signals at 4.97 ppm (H, d, $J = 11.1$ Hz) and 4.47 ppm (H, d, $J = 11.1$ Hz) were ascribed to the protons at positions 2 and 3 of flavanone, which suggested these two protons were axile orientation. The signals in the ^{13}C NMR showed 21 carbons in the molecule. The sugar was identified as D-glucose according to its NMR data. The β-configuration of the glucose was derived from the coupling value of the anomeric proton. According to the data reported in the literature (*39*), the aglycone was identified as 3,5,7,3′,4′,5′-hexahydroxyflavanone. The sugar was linked at C-3 according to the HMBC and the chemical shifts at the A-ring. Therefore, compound **17** was identified as 3,5,7,3′,4′,5′-hexahydroxyflavanone-3-*O*-β-D-glucopyranoside. Compound **17** is a new compound, which has not been reported in the literature.

Compounds **18** and **19** were identified as 3,5,7,3′,4′-pentahydroxyflavone-3-*O*-β-D-glucopyranoside and (+)-gallocatechin, respectively, on the basis of their ^{1}H and ^{13}C NMR spectra, which were the same as reported in the literature (*38*).

Compound **20** was identified as 2-methyl-3-hydroxy-4-pyrone according to its ^{1}H and ^{13}C NMR spectra. This compound has a strong fragrant flavor and has been reported as a food flavor in the literature (*40*).

Compounds **21**−**24** were identified as β-sitosterol, β-sitosterol-3-*O*-β-D-glucoside, ergosta-5,22*Z*-dien-3β-ol, and stigmasta-5,22-dien-3β-ol, respectively, according to their MS and ^{1}H and ^{13}C NMR spectra. Compound **24** was obtained as a cubic crystal, and its structure was further confirmed by X-ray diffraction (**Figure 3**).

Table 1. Antiproliferative Activities (EC_{50}) and Cytotoxicities (CC_{50}) of Compounds Isolated from Black Bean Seed Coats against HepG2 Human Liver Cancer Cells, MCF-7 Human Breast Cancer Cells, and Caco-2 Human Colon Cancer Cells (Mean ± SD, $n = 3$)

	HepG2 cells			Caco-2 cells			MCF-7 cells		
compd	EC_{50} (μM)	CC_{50} (μM)	SI[a]	EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
1	238.8 ± 19.2	>226.1	~1.0	179.9 ± 16.9	>226.1	>1.3	>226.1	>226.1	
2	120.6 ± 7.3	>226.1	>1.9	128.8 ± 11.6	> 226.1	>1.8	>226.1	>226.1	
3	>234.5	>234.5		197.8 ± 4.2	>234.5	>1.2	>234.5	>234.5	
4	>208.7	>208.7		>208.7	>208.7		>208.7	>208.7	
5	>205.5	>205.5		105.9 ± 4.7	>205.5	>1.9	129.4 ± 9.0	274.1 ± 2.8	2.1
6	94.4 ± 3.4	>212.2	>2.3	13.9 ± 2.8	>212.2	>15.3	>212.2	>212.2	
7	98.9 ± 3.3	167.1 ± 5.0	1.7	35.1 ± 2.9	191.7 ± 7.7	5.5	79.5 ± 1.0	134.21 ± 8.4	1.7
8	32.1 ± 6.3	128.2 ± 8.0	4.0	31.2 ± 0.5	43.3 ± 2.1	1.4	140.1 ± 31.8	202.9 ± 3.24	1.5
9	>209.1	>209.1		71.1 ± 11.9	>209.1	>2.9	119.0 ± 7.2	>209.1	>1.8
10	>206.1	>206.1		40.8 ± 4.1	>206.1	>5.1	>206.1	>206.1	
11	> 212.7	>212.7		55.7 ± 8.1	135.8 ± 22.0	2.4	84.6 ± 1.7	>212.7	>2.5
13	306.4 ± 131.3	>446.1	>1.5	>446.1	>446.1		>446.1	>446.1	
14	156.9 ± 11.8	>446.1	>2.8	299.8 ± 17.3	>446.1	>1.5	>446.1	>446.1	
15	410.3 ± 17.4	>689.1	>1.7	533.3 ± 126.0	>689.0	>1.3	>689.0	>689.0	
16	435.9 ± 47.7	>689.1	>1.6	>689.0	>689.0		>689.0	>689.0	
17	>414.6	>414.6		>414.6	>414.6		>414.6	>414.6	
18	>430.7	>430.7		>430.7	>430.7		>430.7	>430.7	
19	202.3 ± 42.9	647.1 ± 69.2	3.2	291.2 ± 1.0	>653.0	>2.2	186.6 ± 21.1	2220.6 ± 76.4	11.9
20	779.3 ± 37.4	>1586	>2.0	717.2 ± 104.8	>1586	>2.2	1308 ± 69.9	>1586	>1.2

[a] SI, selective index = CC_{50}/EC_{50}.

Table 2. Antioxidant Activity of Selected Compounds Isolated from Black Bean Seed Coats (Mean ± SD, $n = 3$)

compd	EC_{50} (μM)	PSC value (μmol of vitamin C equiv/μmol of compd)
14	61.11 ± 8.97	0.22 ± 0.04
15	12.22 ± 0.48	1.08 ± 0.04
16	17.52 ± 0.81	0.76 ± 0.04
17	58.14 ± 6.75	0.23 ± 0.03
18	8.11 ± 1.34	1.67 ± 0.27
19	11.08 ± 0.62	1.2 ± 0.07

Antiproliferative Activities of the Pure Compounds Isolated from Black Bean Seed Coats. All compounds isolated were evaluated for their antiproliferative activities against HepG2 human liver cancer cells, Caco-2 human colon cancer cells, and MCF-7 human breast cancer cells. The antiproliferative activities and cytotoxicities are summarized in **Table 1**. Among the pure compounds isolated from black bean seed coat extracts, compounds **1**, **2**, **6**, **7**, **8**, **13**, **14**, **15**, **16**, **19**, and **20** showed potent inhibitory activities against the proliferation of HepG2 cells, with EC_{50} values of 238.8 ± 19.2, 120.6 ± 7.3, 94.4 ± 3.4, 98.9 ± 3.3, 32.1 ± 6.3, 306.4 ± 131.3, 156.9 ± 11.8, 410.3 ± 17.4, 435.9 ± 47.7, 202.3 ± 42.9, and 779.3 ± 37.4 μM, respectively. Compounds **1**, **2**, **3**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **14**, **15**, **19**, and **20** showed potent antiproliferative activities against Caco-2 cell growth, with EC_{50} values of 179.9 ± 16.9, 128.8 ± 11.6, 197.8 ± 4.2, 105.9 ± 4.7, 13.9 ± 2.8, 35.1 ± 2.9, 31.2 ± 0.5, 71.1 ± 11.9, 40.8 ± 4.1, 55.7 ± 8.1, 299.8 ± 17.3, 533.3 ± 126.0, 291.2 ± 1.0, and 717.2 ± 104.8 μM, respectively. Compounds **5**, **7**, **8**, **9**, **11**, **19**, and **20** showed potent antiproliferative activities against MCF-7 cell growth in a dose-dependent manner, with EC_{50} values of 129.4 ± 9.0, 79.5 ± 1.0, 140.1 ± 31.8, 119.0 ± 7.2, 84.6 ± 1.7, 186.6 ± 21.1, and 1308 ± 69.9 μM, respectively.

Antioxidant Activities of the Pure Compounds. The antioxidant activities of the 24 pure compounds were evaluated using the PSC assay (*27*). Six flavonoids (compounds **14**−**19**) showed potent antioxidant activities (**Table 2**). Compound **18** had the highest antioxidant activity with an EC_{50} of 8.11 ± 1.34 μM, followed by compound **19** (EC_{50} = 11.08 ± 0.62 μM), compound **15** (EC_{50} = 12.22 ± 0.48 μM), compound **16** (EC_{50} = 17.52 ± 0.81 μM), compound **17** (EC_{50} = 58.14 ± 6.75 μM), and compound **14** (EC_{50} = 61.11 ± 8.97 μM). The antioxidant activities of the six flavonoids were comparable to the activity of vitamin C with PSC values ranging from 0.22 to 1.67 μmol of vitamin C equiv/μmol.

In summary, 24 compounds, including 12 triterpenoids, 7 flavonoids, and 5 other phytochemicals, were isolated and identified from black bean seed coat extracts. Among the compounds isolated, compounds **1**, **2**, **6**−**8**, **13**−**16**, **19**, and **20** showed potent inhibitory activities against the proliferation of HepG2 cells; compounds **1**−**3**, **5**−**11**, **14**, **15**, **19**, and **20** showed potent antiproliferative activities against Caco-2 cell growth; and compounds **5**, **7**−**9**, **11**, **19**, and **20** had potent antiproliferative activities against MCF-7 cell growth in a dose-dependent manner. Six flavonoids (compounds **14**−**19**) showed potent antioxidant activities. These results showed the phytochemical extracts of black bean seed coats have potent antioxidant and antiproliferative activities. This further confirmed our hypothesis that the additive and synergistic effects of phytochemicals in whole foods are responsible for their potent antioxidant and anticancer activities and that the health benefits are attributed to the complex mixtures of phytochemicals present in whole foods (*5*, *11*, *41*).

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