Journal of Enzyme Inhibition and Medicinal Chemistry, 2010; 25(5): 608–614

informa
healthcare

ORIGINAL ARTICLE

Antioxidant properties of benzylchroman derivatives from *Caesalpinia sappan* L. against oxidative stress evaluated *in vitro*

Min-Ja Lee[1], Hye-Sook Lee[1], Hyuck Kim[1], Hyo-Seung Yi[2], Sun-Dong Park[2], Hyung-In Moon[1], and Won-Hwan Park[1]

[1]*Cardiovascular Medical Research Center and Department of Diagnostics, and* [2]*Department of Prescriptionology, College of Oriental Medicine, Dongguk University, Gyeong-Ju, Gyeongbuk, Republic of Korea*

Abstract

The antioxidant activity of extracts from *Caesalpinia sappan* L. (CSL) was studied *in vitro* by evaluating the total phenolics, measuring the antioxidant activity by TEAC, measuring the scavenging effects on reactive oxygen species (ROS) and on reactive nitrogen species (RNS), and measuring the inhibitory effect on Cu^{2+}-induced human low-density lipoprotein (LDL) oxidation. The CSL extracts were found to have a potent scavenging activity against all of the reactive species tested, as well as an inhibitory effect on LDL oxidation, especially in the ethyl acetate (EA) fraction. Therefore, we isolated and identified benzylchroman derivatives sappanchalcone (**1**) and 3′-deoxy-4-*O*-methylepisappanol (**2**) from the EA fraction of CSL and their antioxidant activities were evaluated. The studied CSL extracts and the compounds **1** and **2** were revealed to be very effective against the evaluated pro-oxidant species, including ROS and RNS.

Keywords: Caesalpinia sappan *L.; reactive oxygen species; reactive nitrogen species; antioxidant activity; LDL oxidation*

Introduction

Oxidative stress, which is defined as an imbalance between the production of reactive oxygen species (ROS; superoxide anion, hydroxyl radical) or reactive nitrogen species (RNS; nitric oxide and peroxynitrite) and antioxidant defense, is considered to be an important pathogenic factor in degenerative diseases such as cardiovascular dysfunction, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury, and neurodegenerative diseases[1]. ROS and RNS inactivate and destroy macromolecules (proteins, lipids, deoxyribonucleic acids, carbohydrates, and polyunsaturated fatty acids), thereby rapidly disrupting the cell architecture, ultimately leading to death. Ongoing research indicates that the abundance of ROS or RNS in the vasculature results in an increased oxidation of proteins that induce oxidized low-density lipoprotein (Ox-LDL), which then initiates an inflammatory process and causes damage to the arterial wall[2]. This oxidative and nitrosative damage can be retarded by endogenous defense systems such as enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Re), catalase, thiol enzymes) and non-enzymatic (glutathione (GSH), ascorbic acid (AA), tocopherols, phenolic compounds, phenylpropanoids, carotenoids, and flavonoids) antioxidative systems[3]. The antioxidative systems evolved not only to eliminate ROS and RNS, but also to adjust the cellular redox state and enable redox signal transduction; however, these systems are not completely efficient. Antioxidants also inhibit the oxidative modification of LDL. Ox-LDL is a primary constituent of atherosclerotic lesions; therefore, if antioxidant nutrients have the ability to inhibit its formation they may be useful for the prevention and treatment of atherosclerotic cardiovascular disease (CVD).

Polyphenols are present in most oriental medicines of plant origin. They are common constituents of the human diet that are considered to contribute to the prevention

Address for Correspondence: Hyung-In Moon, Won-Hwan Park, Cardiovascular Medical Research Center and Dept. of Diagnostics, College of Oriental Medicine, Dongguk University, Sukjang-Dong 707, Gyeong-Ju 780-714, Republic of Korea. Tel: +82-54-770-2373. Fax: +82-54-770-2376. E-mail: himun68@gmail.com; diapwh@dongguk.ac.kr

(Received 11 August 2009; revised 11 September 2009; accepted 26 September 2009)

ISSN 1475-6366 print/ISSN 1475-6374 online © 2010 Informa UK, Ltd.
DOI: 10.3109/14756360903373376

of various degenerative diseases. Recently, polyphenols, including catechin and its derivatives, resveratrol, and curcumin, have attracted attention as functional foods that have various bioactivities including anticancer, antimutagenic, antimicrobial, and antiviral activities. In addition, several studies have shown that plant polyphenol compounds such as flavonoids[4], tannins[5], catechins, proanthocyanidins, and polyphenolic acids[4] exert antioxidant effects.

Caesalpinia sappan L. (CSL) is a Chinese traditional folk medicine that has been used as an analgesic and anti-inflammatory agent to cure emmeniopathy, sprains, and convulsions[6]. It has been reported that extracts of CSL have pharmacological activities such as antihypercholesteremic, sedative, and depressant effects on the central nervous system, anti-hepatitis B surface antigen (HBsAg) capability, anti-complementary activity on the complement system, and an antimotility effect on human sperm. In addition, CSL is used for the treatment of diabetic complications and to promote blood circulation[6]. However, no studies concerning the antioxidant properties of CSL and its compounds have been conducted.

The antioxidant activity of a plant is significantly influenced by its qualitative and quantitative composition, which can be reversible depending on the method of evaluation, and whether the results reveal a positive or a negative correlation. In recent years, different methods have been proposed for evaluation of the antioxidant capacity of plants. The chemical principles of the methods used are based either on biological oxidants (peroxyl radical, superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid, singlet oxygen, nitric oxide radical, and peroxynitrite) or on non-biological oxidants (scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation (Trolox equivalent antioxidant capacity or TEAC assay), scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH assay), ferric reducing antioxidant power (FRAP assay), Folin–Ciocalteu reducing capacity (FC assay), electrochemical total reducing capacity). Each method has advantages and shortcomings within the scope of application[7]. Therefore, this study was conducted to evaluate the *in vitro* scavenging activity and inhibitory effect of LDL oxidation of pro-oxidant reactive species in response to treatment with CSL and identified compounds from CSL using various screening methods, including biological and non-biological oxidants.

Materials and methods

Chemicals

Dihydrorhodamine 123 (DHR 123) and 6-carboxy-2′, 7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA). Agarose and Coomassie brilliant blue R-250 were purchase from Promega (Madison, WI, USA). Peroxynitrite was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Trolox, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), potassium chloride (KCl), Folin–Ciocalteu phenol reagent, human low-density lipoprotein (LDL), ethanol (E), hexane (H), dichloromethane (DCM), ethyl acetate (EA), and butanol (B) were purchased from Merck (Merck KGaA, Darmstadt, Germany). All other chemicals were purchased from Sigma Chemical Co. as analytical grade (St. Louis, MO, USA). Thin layer chromatography (TLC) was performed on precoated silica gel G and GP uniplates from Analtech (IL, USA) and visualized with 254-nm ultraviolet (UV) light. Vacuum liquid chromatography was carried out on silica gel 60 (Scientific Adsorbents Incorporated, MO, USA).

Instruments for compound structural analysis

^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded on a Bruker DPX 400 at 400 MHz and 100 MHz (Germany). The chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane, and *J* values are in Hz. Mass spectra were recorded with a Waters Micromass ZQ LC-mass system, and high resolution mass spectra (HRMS) were measured with a Bruker BioApex FTMS system by direct injection using an electrospray interface (ESI) (Germany).

Total and organic solvent fractionation for bioassay

The heartwood of CSL (500 g, purchased from Dongguk University Gyeongju Oriental Hospital, Gyeongju, Gyeongbuk) was ground (maximum particle size 0.4 mm) and refluxed three times (12 h, 6 h, 3h) with 70% ethanol (ethanol/water, 70:30, E) solution (20-fold) and then filtered through a glass filter funnel (G4). The extract was then gathered and the ethanol was evaporated under reduced pressure at 45°C in a rotary vacuum evaporator (Buchi RII, Switzerland), followed by lyophilization. The dried extract was then suspended in 50 mL of distilled water and the aqueous suspension was partitioned sequentially with hexane (H), dichloromethane (DCM), ethyl acetate (EA), *n*-butanol (B), and water (aqueous, A) in a 1:1 ratio (v/v) at room temperature. The resulting extracts were then evaporated in a rotary vacuum evaporator to dryness to give H, DCM, EA, and B fractions. They were then quantitatively re-dissolved in 30% ethanol solution. The stock solutions were kept at 4°C in the dark until further analysis. Prior to analysis, solutions were filtered through a 1.0 μm syringe filter.

Isolation of active compounds from CSL

The air-dried and chipped CSL (5 kg) was extracted with 70% ethanol by refluxing for 4 h (three times × 5 L) on a sonication bath at 35°C. The extract was filtered through a Buchner funnel using Whatman No. 1 filter paper. The combined 70% ethanol extract was evaporated under reduced pressure to yield a red residue. Vacuum liquid chromatography (150 g, 6 × 30 cm) of the 70% ethanol extract (50 g), using *n*-hexane/CH_2Cl_2 (1:0–0:1) and CH_2Cl_2/MeOH (1:0–0:1) step gradients, produced 19 fractions, with the exception of the red colored fraction. These were pooled by TLC profile into four fractions (SL1–SL4), from which fraction SL3 (4.3 g) was eluted with petroleum ether–acetone (1:0 (500 mL), 2:1 (500 mL), 1:1 (500 mL), 1:2 (500 mL), 1:5 (500 mL), and

0:1 (500 mL)). The major compound was purified by preparative high-performance liquid chromatography (Econosil C-18, 10×250 mm; 1.0 mL/min) with MeCN/MeOH (1:1) to afford compounds **1** (8.8 mg) and **2** (7.3 mg). The compounds were then quantitatively re-dissolved in 1% dimethylsulfoxide (DMSO) as stock solution.

*Compound **1** (sappanchalcone)* Yellow needles; EI-MS *m/z* (%) 286 (M^+, 100); ^{1}H-NMR (CD_3OD, 400 MHz) δ 7.46 (1H, d, *J*=8.5 Hz, H-6'), 7.40 (1H, d, *J*=15.8 Hz, H-β), 7.25 (1H, d, *J*=15.9 Hz, H-α), 7.00 (1 H, d, *J*=2.0 Hz, H-2), 6.88 (1H, dd, *J*=2.0, 8.3 Hz, H-6), 6.70 (1H, d, *J*=8.3 Hz, H-5), 6.42 (1H, d, *J*=2.2 Hz, H-3'), 6.33 (1 H, dd, *J*=2.3, 8.5 Hz, H-5'), 3.79 (3H, s, -OCH_3). ^{13}C-NMR (CD_3OD, 100 MHz) δ 193.10 (ketone), 164.4 (C-4'), 162.42 (C-2'), 149.46 (C-4), 146.68 (C-3), 144.59 (C-β), 133.71 (C-α), 128.63 (C-1), 125.07 (C-6'), 123.35 (C-6), 121.71 (C-1'), 116.56 (C-5), 115.25 (C-2), 108.92 (C-5'), 100.13 (C-3'), 56.12 (-OCH_3).

*Compound **2** (3'-deoxy-4-O-methylepisappanol)* Colorless powder; mp 98–99°C, $[\alpha]^{25}_D$=–21.0 (*c* 0.15, MeOH), EI-MS *m/z* (rel. int.): $[M]^+$ 302 (3), 272 (4), 153 (100), 123 (40), 107 (47), 77 (18); HR-EI-MS *m/z*: 302.1147 $[M]^+$ (calcd for $C_{17}H_{18}O_5$, 302.1149); ^{1}H-NMR (CD_3OD, 400 MHz) δ 7.15 (1H, d, *J*=8.5 Hz, H-2',6), 6.96 (1H, d, *J*=8.0 Hz, H-5), 6.76 (2H, d, *J*=8.5 Hz, H-3',5'), 6.33 (1 H, dd, *J*=8.0, 2.0 Hz, H-6), 6.28 (1 H, dd, *J*=2.0 Hz, H-8), 4.10 (1H, d, *J*=11.0 Hz, H-2), 3.81 (1H, d, *J*=11.0 Hz, H-2), 3.62 (1H, s, H-4), 3.31(3H, s, -OCH_3), 2.90 (1H, d, *J*=13.5 Hz, H-9), 2.70 (1H, d, *J*=13.5 Hz, H-9). ^{13}C-NMR (CD_3OD, 100 MHz) δ 160.7 (C-7), 158.1 (C-4'), 157.2 (C-8a), 134.3 (C-5), 133.8 (C-2',6'), 128.8 (C-1'), 116.7 (C-3',5'), 113.6 (C-4a), 108.9 (C-6), 104.5 (C-8), 78.8 (C-4), 71.7 (C-3), 71.1 (C-2), 56.8 (-OCH_3), 40.4 (C-9).

Determination of total phenolics

The total content of phenolic compounds was determined by the Folin–Ciocalteu reaction[8], using gallic acid (GA) as standard.

Antioxidant activity as determined by ABTS·$^+$ and DPPH assays

The total antioxidant activity of CSL extracts and compounds was measured by the ABTS radical cation (ABTS·$^+$) decolorization assay[9]. The DPPH radical scavenging activity of CSL extracts and compounds was determined by the method according to Gyamfi *et al*[10].

Superoxide anion and hydroxyl radical scavenging activity

In this assay, when O_2 is generated, nitroblue tetrazolium (NBT) is reduced, which produces a blue formazan color that is associated with an increase in the absorbance at 560 nm. When a scavenger compound is added, it competes with the NBT for oxidation of the generated superoxide anions, which leads to a decrease in the rate of NBT reduction and therefore a reduction in absorbance. More effective compounds, therefore, require lower concentrations to inhibit the NBT reduction by 50% (IC_{50}). The conditions of the NBT assay were adapted from Gotoh and Niki[11]. The hydroxyl radical (·OH) scavenging activity of CSL extracts and compounds was assessed using the method described by Halliwell and Gutteridge[12].

Nitric oxide radical and peroxynitrite scavenging activity

The 4,5-diaminofluorescein (DAF-2) assay[13] was used to measure the nitric oxide radical (NO) scavenging ability. The peroxynitrite ($ONOO^-$) scavenging activity of the CSL extracts and compounds was determined using the method described by Kooy *et al.*[14], with a slight modification. Briefly, 10 µL of CSL extracts of different concentrations was mixed with 175.8 µL of rhodamine buffer (50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, and 5 mM potassium chloride) containing 4 µL of 5 mM diethylenetriamine pentaacetic acid (DTPA) and 0.2 µL of 5 mM DHR 123. The reaction was then initiated by adding 10 µL of 10 µM peroxynitrite. After 10 min at room temperature, the fluorescent intensity of the mixture was monitored at excitation and emission wavelengths of 480 and 530 nm, respectively, using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA). The scavenging effect of the extract is expressed as the percent inhibition of DHR 123 oxidation. AA and BHT were used as positive controls.

Relative electrophoretic mobility assay

The relative electrophoretic mobility (REM) of human LDL was determined by agarose gel electrophoresis according to the method described by Yoon *et al.*[15].

Inhibitory effects of $CuSO_4$-induced human LDL oxidation

The inhibitory effects of CSL extracts and compounds on $CuSO_4$-induced human LDL oxidation were determined spectrophotometrically by measuring the amount of thiobarbituric acid reactive substances (TBARS) generated[16].

Statistical analysis

All experiments were performed at least three times by conducting each assay in triplicate. Data were analyzed using SPSS software (SPSS, Chicago, IL, USA) and are expressed as the mean ± standard deviation. Statistical analyses were conducted using analysis of variance (ANOVA–Tukey test) and a *p* level of 0.05 or less was considered significant.

Results and discussion

Yield of fractions, total phenolics content, and active compounds

The extract yields ranged from 0.23 g/500 g CSL (H extract) to 20 g/500 g CSL (E extract) (Table 1). The total phenolics content of the extracts, as estimated by the Folin–Ciocalteu reagent method, ranged from 18.08 µg GA eq/mg (H fraction) to 759.82 ± 11.15 µg GA eq/mg (EA fraction) (Table 1). Two compounds, sappanchalcone (**1**) and 3'-deoxy-4-*O*-methylepisappanol (**2**), were isolated from the CSL (Figure 1). All spectral data for these compounds were in good agreement with those previously reported in the literature[6,17]. Previous

pharmacological studies on sappanchalcone have demonstrated anticonvulsant[6] and anti-allergic activities[18].

Antioxidant activity as determined by ABTS·⁺ assay

Table 2 shows the antioxidant capacities of CSL extracts and compounds as determined by the TEAC assay. The extracts showed generally high antioxidant capacities that ranged from 0.023 to 1.149 mmol Trolox equivalents. In addition, the difference in the antioxidant capacities of the various extracts was also very large, being up to 50-fold. The ethyl acetate fraction of CSL possessed the highest antioxidant capacity (1.149 mmol Trolox equivalent), followed by the E fraction (0.736 mmol Trolox equivalent), B fraction (0.703 mmol Trolox equivalent), and the DCM fraction (0.591 mmol Trolox equivalent), with the A fraction (0.063 mmol Trolox equivalent) and the H fraction (0.023 mmol Trolox equivalent) showing the lowest antioxidant capacity. The antioxidant activities of **1** and **2**, which were isolated from the EA of CSL, showed a significant difference ($p < 0.05$), their TEAC values being 2.704 and 2.158 mmol Trolox equivalent units, respectively. In addition, the antioxidant activities of AA and BHT, which were used as the positive controls, were 0.985 and 0.071 mmol Trolox equivalent units, respectively. AA is a natural antioxidant and BHT is a synthetic antioxidant of common knowledge. AA is a relatively expensive antioxidant, while BHT is toxic to humans and therefore inappropriate for chronic human consumption. Therefore, the screening of inexpensive, non-toxic antioxidants from natural sources is demanded. Consequently, we evaluated the antioxidant activity of CSL objectively by comparing with natural and synthetic antioxidants. To our knowledge, there have been no prior reports regarding the antioxidant activity of this plant; therefore, the data generated by the present study provide valuable preliminary data.

Table 1. Extraction yields and contents of total phenolics in extracts of *Caesalpinia sappan* L.

Sample[1]	Yield (%)[2]	Total phenolics[3] (μg GA eq/mg)
E	4.000	723.67 ± 0.11[c]
H	0.046	18.08 ± 0.39[f]
DCM	0.788	742.28 ± 3.83[b]
EA	2.878	759.82 ± 6.44[a]
B	0.204	356.79 ± 4.10[d]
A	0.084	190.28 ± 1.27[e]

[1]E, 70% ethanol extract; H, hexane fraction; DCM, dichloromethane fraction; EA, ethyl acetate fraction; B, butanol fraction; A, aqueous layer.
[2]Extraction yield is expressed as the percentage dry weight of *Caesalpinia sappan* L.
[3]Each value represents the mean ± SE of triplicate measurements.
[a–f]Values with different superscripts in the same column are significantly different at $p < 0.05$ by Tukey test.

Free radical scavenging activity as determined by DPPH assay

The free radical scavenging effects of CSL extracts and compounds under investigation on DPPH are shown in Table 2. Among the extracts examined, the EA and DCM fractions exhibited the strongest efficiency and showed over 50% scavenging effect of DPPH at concentrations of 113.55 ± 0.13 and 127.16 ± 0.24 μg/mL, respectively, followed by the E extract (IC_{50} = 135.5 ± 0.17 μg/mL). These values were superior to that of the positive control, which was 144.15 ± 3.28 (AA), and **1** and **2**, which were isolated from the EA of CSL, showed effective scavenging activities, their IC_{50} values being 149.98 ± 1.15 and 175.55 ± 3.09 μg/mL, respectively. These data imply that CSL has a high hydrogen-donating capacity.

ROS (superoxide anion and hydroxyl radical) scavenging activity

The IC_{50} values for the superoxide anion scavenging activity of all of the test samples from CSL are shown in Table 3.

Table 2. Antioxidant activities of extracts and **1** and **2** from *Caesalpinia sappan* L. as determined by ABTS·⁺ assay.

Sample[1]	TEAC[2] (mM Trolox equivalent)	Free radical scavenging activity[2] (IC_{50} = μg/mL)
E	0.736 ± 0.024[e]	135.50 ± 0.17[f]
H	0.023 ± 0.009[g]	NA
DCM	0.591 ± 0.035[f]	127.16 ± 0.24[g]
EA	1.149 ± 0.042[c]	113.55 ± 0.13[h]
B	0.703 ± 0.029[e]	263.50 ± 0.35[a]
A	0.063 ± 0.018[g]	NA
1	2.704 ± 0.074[a]	149.98 ± 1.15[d]
2	2.158 ± 0.030[b]	175.55 ± 3.09[c]
AA	0.985 ± 0.004[d]	144.15 ± 3.28[e]
BHT	0.071 ± 0.015[g]	222.60 ± 3.91[b]

[1]E, 70% ethanol extract; H, hexane fraction; DCM, dichloromethane fraction; EA, ethyl acetate fraction; B, butanol fraction; A, aqueous layer; **1**, sappanchalcone; **2**, 3′-deoxy-4-*O*-methylepisappanol.
[2]Each value represents the mean ± SE of triplicate measurements.
[a–h]Values with different superscripts in the same column are significantly different at $p < 0.05$ by Tukey test.

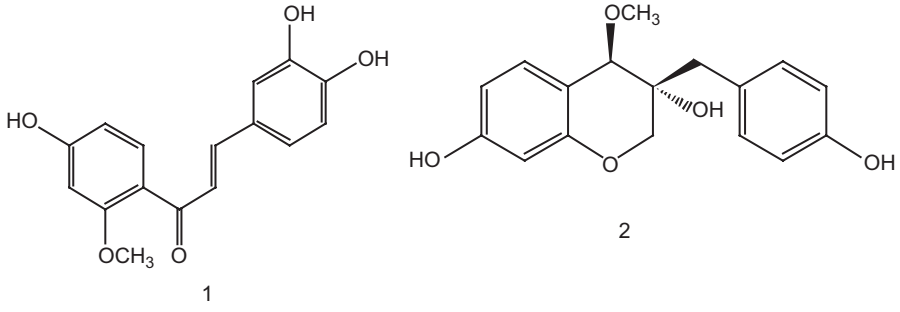

Figure 1. Structures of isolated compounds from *Caesalpinia sappan* L. **1**, sappanchalcone; **2**, 3′-deoxy-4-*O*-methylepisappanol.

Table 3. ROS (superoxide anion and hydroxyl radical) scavenging activities of extracts and **1** and **2** from *Caesalpinia sappan* L.

Sample[1]	Superoxide anion[2] (IC_{50} = μg/mL)	Hydroxyl radical[2] (IC_{50} = μg/mL)
E	121.72 ± 6.13[f]	127.46 ± 3.27[e]
H	NA	NA
DCM	172.24 ± 3.46[f]	106.95 ± 4.13[f]
EA	493.89 ± 12.24[b]	117.62 ± 3.56[ef]
B	173.16 ± 5.49[f]	284.94 ± 8.37[c]
A	584.64 ± 19.87[a]	1027.50 ± 15.45[a]
1	262.01 ± 15.67[d]	347.17 ± 6.11[b]
2	424.01 ± 17.30[c]	158.87 ± 6.79[d]
AA	NA	62.03 ± 2.12[g]
BHT	NA	NA

[1]E, 70% ethanol extract; H, hexane fraction; DCM, dichloromethane fraction; EA, ethyl acetate fraction; B, butanol fraction; A, aqueous layer; **1**, sappanchalcone; **2**, 3'-deoxy-4-*O*-methylepisappanol.
[2]Each value represents the mean ± SE of triplicate measurements.
[a–g]Values with different superscripts in the same column are significantly different at $p < 0.05$ by Tukey test. NA, not active.

Table 4. RNS (nitric oxide radical and peroxynitrite) scavenging activities of extracts and **1** and **2** from *Caesalpinia sappan* L.

Sample[1]	Nitric oxide radical[2] (IC_{50} = μg/mL)	Peroxynitrite[2] (IC_{50} = μg/mL)
E	1.75 ± 0.20[c]	7.32 ± 0.22[e]
H	669.09 ± 18.29[a]	288.76 ± 3.93[a]
DCM	1.81 ± 0.25[c]	4.76 ± 0.10[e]
EA	1.77 ± 0.22[c]	4.15 ± 0.09[e]
B	8.76 ± 1.42[c]	14.03 ± 0.29[d]
A	40.65 ± 1.59[b]	39.50 ± 0.19[c]
1	0.35 ± 0.00[c]	3.26 ± 0.16[e]
2	0.67 ± 0.01[c]	4.82 ± 0.42[e]
AA	9.13 ± 0.15[c]	3.45 ± 0.064[e]
BHT	NA	123.76 ± 2.34[b]

[1]E, 70% ethanol extract; H, hexane fraction; DCM, dichloromethane fraction; EA, ethyl acetate fraction; B, butanol fraction; A, aqueous layer; **1**, sappanchalcone; **2**, 3'-deoxy-4-*O*-methylepisappanol.
[2]Each value represents the mean ± SE of triplicate measurements.
[a–e]Values with different superscripts in the same column are significantly different at $p < 0.05$ by Tukey test. NA, not active.

CSL had a significant scavenging activity on the superoxide anion, and this effect occurred in a dose-dependent manner. In addition, the superoxide anion-scavenging activity of CSL extracts and compounds was significantly different from that of AA ($p < 0.05$). The E extract exerted the strongest scavenging activity (IC_{50} = 121.72 ± 6.13 μg/mL) ($p < 0.05$), showing 4.1-fold, 1.4-fold, 1.4-fold, and 4.8-fold greater activity when compared with EA (IC_{50} = 493.89 ± 12.24 μg/mL), DCM (IC_{50} = 172.24 ± 3.46 μg/mL), B (IC_{50} = 173.16 ± 5.49 μg/mL), and A (IC_{50} = 584.64 ± 19.87 μg/mL) extracts, respectively. The superoxide anion-scavenging activities of **1** and **2**, which were isolated from the EA of CSL, were lower than those of the E, DCM, and B extracts, their IC_{50} values being 262.01 ± 15.67 and 424.01 ± 17.30 μg/mL, respectively. In addition, AA and BHT exerted the lowest scavenging effect on the superoxide anion when all of the test samples were compared. Taken together, these results suggest that the CSL extracts exhibit a scavenging effect on superoxide anion generation that could help prevent or ameliorate oxidative damage.

The scavenging activities of CSL on the hydroxyl radical are shown in Table 3. CSL showed a high enough scavenging activity to be considered a potent hydroxyl radical-scavenger. The IC_{50} values of the E, EA, and DCM fractions of CSL were 127.46 ± 3.27, 117.62 ± 3.56, and 106.95 ± 4.13 μg/mL. The hydroxyl-radical activity of **2**, which was isolated from the EA of CSL, was lower than that of the B extract, its IC_{50} value being 158.87 ± 6.79 μg/mL.

RNS (nitric oxide radical and peroxynitrite) scavenging activity

The CSL extracts inhibited the ·NO-induced oxidation of DAF-2 to triazolofluorescein (Table 4), indicated by IC_{50} values of 1.75 ± 0.20, 1.81 ± 0.25, and 1.77 ± 0.22 μg/mL for the E, DCM, and EA fractions of CSL, respectively. These values were similar to that of the positive control, which was 9.13 ± 0.15 (AA), implying that CSL could act as a potent scavenger of ·NO. The scavenging activities of **1** and **2** on nitric oxide showed predominant scavenging effects, their IC_{50} values being 0.35 ± 0.00 and 0.67 ± 0.01 μg/mL, respectively; therefore, the above compounds may be useful as natural scavengers of ·NO. Peroxynitrite is a powerful biological oxidant that is produced by a diffusion-limited reaction of the superoxide anion with NO. Peroxynitrite induces the nitration of free L-tyrosine or tyrosine residues in protein, and affects normal protein structure. The rate of peroxynitrite formation depends on the concentrations of superoxide anion and NO, and even a relatively small increase in their concentrations may be responsible for a remarkable increase in the generation of peroxynitrite and its cytotoxic effects[19]. In addition, peroxynitrite serves as the injurious agent in cerebral injury and myocardial ischemia, and it may contribute to atherosclerosis through oxidation of LDL within the arterial walls[20]. For these reasons, the peroxynitrite scavenging activity of CSL was investigated, and the results compared with those of the reference antioxidants (Table 4). The need for a higher extract concentration to scavenge radicals indicates a lower antioxidant activity. The CSL extracts inhibited the peroxynitrite-induced oxidation of the DHR reaction mixture, with the peroxynitrite scavenging activity being the highest in the DCM fraction. The order of the peroxynitrite scavenging activity of the CSL extracts was as follows: EA (IC_{50} = 4.15 ± 0.09) > DCM(IC_{50} = 4.76 ± 0.10) > E (IC_{50} = 7.32 ± 0.22) > B (IC_{50} = 14.03 ± 0.29)> A (IC_{50} = 39.50 ± 0.19) > H (IC50 = 288.76 ± 3.93). In addition, the scavenging activities of **1** and **2** on peroxynitrite exhibited excellent effects, their IC_{50} values being 3.26 ± 0.16 and 4.82 ± 0.42 μg/mL, respectively. Taken together, these data imply that the CSL extracts and compounds may be effective scavengers of RNS.

Relative electrophoretic mobility assay

The oxidative modification of LDL appears to play a critical role in the pathogenesis of atherosclerosis. LDL is a heterogeneous molecule that is composed principally of phospholipids, cholesterol esters, cholesterol, and the

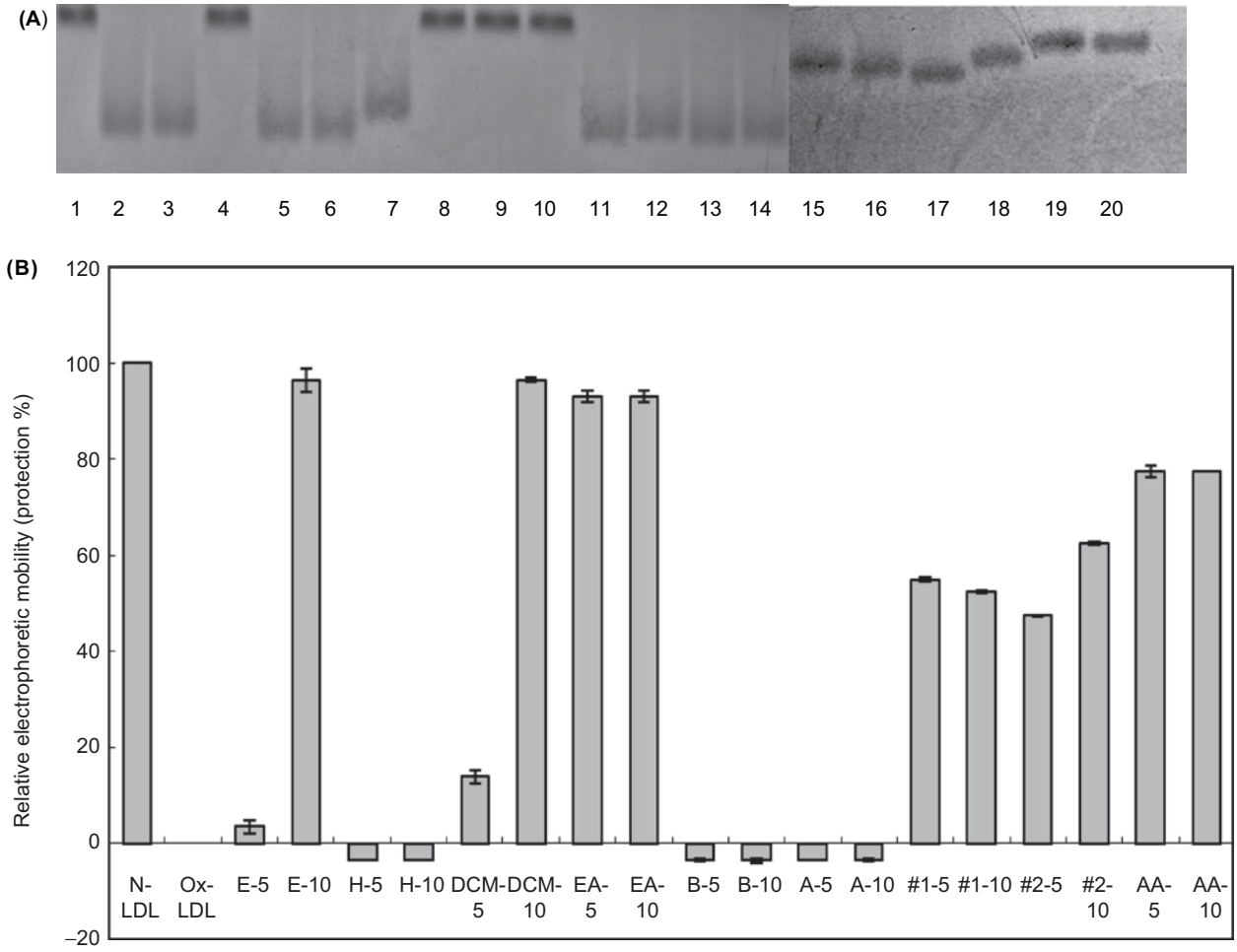

Figure 2. The relative electrophoretic mobility (REM) of human LDL incubated with Cu^{2+} and with or without extracts and **1** and **2** from *Caesalpinia sappan* L. (CSL). LDL (120 μg/mL) was oxidized with 10 μM $CuSO_4$ at 37°C in the presence of CSL extracts for 12 h. (A) Lane 1: native LDL; lane 2: LDL and Cu^{2+}; lanes 3, 4: LDL and Cu^{2+} and 5, 10 μg of E; lanes 5, 6: LDL and Cu^{2+} and 5, 10 μg of H; lanes 7, 8: LDL and Cu^{2+} and 5, 10 μg of DCM; lanes 9, 10: LDL and Cu^{2+} and 5, 10 μg of EA; lanes 11, 12: LDL and Cu^{2+} and 5, 10 μg of B; lanes13, 14: LDL and Cu^{2+} and 5, 10 μg of A; lanes 15, 16: LDL and Cu^{2+} and 5, 10 μg of sappanchalcone (#**1**); lanes 17, 18: LDL and Cu^{2+} and 5, 10 μg of 3′-deoxy-4-*O*-methylepisappanol (#**2**); lanes 19, 20: LDL and Cu^{2+} and 5, 10 μg of AA. (B) Protection rate (%); each value represents the mean ± SE of triplicate measurements.

apolipoprotein (apo) B-100. Some amino acid constituents of apo B-100 are susceptible to attack by reactive species, including those generated by Cu^{2+} in Fenton and Haber–Weiss reactions. Oxidative modification of apo B-100 generates LDL subfractions that are defined by their degree of electronegativity[21]. Consequently, dietary antioxidants that protect LDL from oxidation may help to reduce atherogenesis and prevent coronary heart disease. Numerous *in vitro* studies have shown that polyphenols from red and white wine, rapeseed, and pine bark phenols as well as raspberry, coffee, cocoa, and tea beverages are recognized as bioactive components with antioxidant properties. Figure 2 shows the effect of CSL on the REM of LDL peroxidation induced by Cu^{2+}. If the REM of native LDL is assumed to be 1, the REM increased to 6.8 in response to the addition of Cu^{2+}. In addition, the data showed that LDL peroxidation can be suppressed by the addition of extracts of CSL, as indicated by of the REM value being reduced to 1.2, 1.2, and 1.4 in response to treatment with a concentration of 10 μg/mL of the E, DCM, and EA fractions, respectively; these data were superior to those for **1** and **2**. In this study, the ability of CSL to scavenge free radicals was further confirmed by the inhibition of LDL peroxidation. These results revealed that CSL extracts could convert free radicals to more stable products and terminate the radical chain reaction, thereby supplying antioxidant action.

Inhibitory effects of $CuSO_4$-induced human LDL oxidation

In this study, the inhibition effects of CSL on LDL oxidative modification induced by Cu^{2+} were evaluated by means of a TBARS assay. Although the measurement of TBARS lacks specificity, it has been shown to be a very good indicator of LDL oxidation[22]. Table 5 shows the protective effect of CSL extracts on LDL oxidation induced by Cu^{2+}. The peroxidation of LDL was significantly inhibited in the presence of CSL, and the protective action of CSL on LDL oxidation occurred in a concentration-dependent manner. The IC_{50} values for the inhibition of LDL oxidation were 5.02 ± 0.25, 6.22 ± 0.51, 5.53 ± 0.17, 25.42 ± 0.42, and 32.97 ± 0.04 μg/mL

Table 5. Inhibitory effect on Cu^{2+}-induced LDL oxidation of extracts and **1** and **2** from *Caesalpinia sappan* L.

Sample[1]	Inhibitory effect on Cu^{2+}–induced LDL oxidation[2] (IC_{50} = μg/mL)
E	5.02 ± 0.25[c]
H	813.96 ± 14.10[a]
DCM	6.22 ± 0.51[c]
EA	5.53 ± 0.17[c]
B	25.42 ± 0.42[b]
A	32.97 ± 0.04[b]
1	4.37 ± 0.19[c]
2	4.80 ± 0.14[c]
AA	21.67 ± 0.64[b]
BHT	26.67 ± 0.44[b]

[1]E, 70% ethanol extract; H, hexane fraction; DCM, dichloromethane fraction; EA, ethyl acetate fraction; B, butanol fraction; A, aqueous layer; **1**, sappanchalcone; **2**, 3'-deoxy-4-*O*-methylepisappanol.

[2]Each value represents the mean ± SE of triplicate measurements.

[a–c]Values with different superscripts in the same column are significantly different at $p < 0.05$ by Tukey test.

for the E, DCM, EA, B, and A fractions, respectively, indicating that these extracts prevented oxidation of LDL. The inhibitory effects of **1** and **2** on LDL oxidation induced by Cu^{2+} were powerful, and their IC_{50} values were 4.37 ± 0.19 and 4.80 ± 0.14 μg/mL, respectively. Lipid peroxidation resulting in Ox-LDL production is a common occurrence in patients with systemic autoimmune diseases and in chronic inflammatory disorders. Moreover, Ox-LDL can stimulate endothelial cells and monocytes to produce tissue factor, which may contribute to thrombus formation in retyped plaques as well as enhance spontaneous fibrin deposition. These phenomena result in the gradual thickening of arteries, causing decreased elasticity, narrowing, and reduced blood supply, ultimately leading to atherosclerosis[23]. Based on the data shown in Table 5, CSL has the potential to prevent atherosclerosis via suppression of LDL oxidation. Collectively, these remarkable properties indicate that CSL has significant antioxidant activity.

Conclusion

These data imply that at least part of the observed antioxidant activity may be a result of the phenolic compounds of CSL, and show that CSL can be used as an easily accessible source of natural antioxidants.

Declaration of interest

This work was supported by the Dongguk University Research Fund and the MRC program of MOST/KOSEF (grant #: R13-2005-01001-0).

References

1. Vranova E, Inze D, Van Breusegem F. Signal transduction during oxidative stress. J Exp Bot 2002;53:1227–36.
2. Schmitz G, Grandl M. Role of redox regulation and lipid rafts in macrophages during Ox-LDL-mediated foam cell formation. Antioxid Redox Signal 2007;9:1499–518.
3. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 2003;91:179–94.
4. Rice-Evans CA, Miller NJ, Paganga J. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152–9.
5. Haslam E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. J Nat Prod 1996;59:205–15.
6. Baek NI, Jeon SG, Ahn EM, Hahn JT, Bahn JH, Jang JS, et al. Anticonvulsant compounds from the wood of Caesalpinia sappan L. Arch Pharm Res 2000;23:344–8.
7. Magalhães LM, Segundo MA, Reis S, Lima JLFC. Methodological aspects about in vitro evaluation of antioxidant properties. Anal Chim Acta 2008;613:1–19.
8. Kujala TS, Loponen JM, Klika KD, Pihlaja K. Phenolic and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effects of cold storage on the content of total phenolics and three individual compounds. J Agric Food Chem 2000;48:5338–42.
9. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231–7.
10. Gyamfi MA, Yonamine M, Aniya Y. Free-radical scavenging action of medicinal herbs from Ghana Thonningia sanguinea on experimentally-induced liver injuries. Gen Pharmacol 1999;32:661–7.
11. Gotoh N, Niki E. Rates of interactions of superoxide with vitamin E, vitamin C and related compounds as measured by chemiluminescence. Biochem Biophys Acta 1992;1115:201–7.
12. Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1–85.
13. Nagata N, Momose K, Ishida Y. Inhibitory effects of catecholamines and antioxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. J Biochem (Tokyo) 1999;125:658–61.
14. Kooy NW, Royall JA, Ischiropoulos H, Beckman JS. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic Biol Med 1994;16:149–56.
15. Yoon MA, Jeong TS, Park DS, Xu MZ, Oh HW, Song KB, et al. Antioxidant effect of quinoline alkaloid and 2,4-di-tert-butylphenol isolated from *Scolopendra subspinipes*. Biol Pharm Bull 2006;29:735–9.
16. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
17. Fu LC, Huang XA, Lai ZY, Hu YJ, Liu HJ, Cai XL. A new 3-benzylchroman derivative from Sappan Lignum (*Caesalpinia sappan*). Molecules 2008;13:1923–30.
18. Yodsaoue O, Cheenpracha S, Karalai C, Ponglimanont C, Tewtrakul S. Anti-allergic activity of principles from the roots and heartwood of *Caesalpinia sappan* on antigen-induced β-hexosaminidase release. Phytother Res 2009;23:1028–31.
19. Olas B, Nowak P, Kolodziejczyk J, Ponczek M, Wachowicz B. Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. J Nutr Biochem 2006;17:96–102.
20. Yen GC, Chen HY, Peng HH. Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants. Food Chem Toxicol 2000;11:1045–53.
21. Chen CY, Milbury PE, Chung SK, Blumberg J. Effect of almond skin polyphenolics and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation. J Nutr Biochem 2007;18:785–94.
22. Zhenbao J, Fei T, Ling G, Guanjun T, Xiaolin D. Antioxidant properties of extracts from juemingzi (Cassia tora L.) evaluated in vitro. Lebenson Wiss Technol Food Sci Technol 2007;40:1072–7.
23. Stoll G, Bendszus M. Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. Stroke 2006;37:1923–32.