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Antioxidant Properties and Quantification of Phenolic Compounds from Safflower (*Carthamus tinctorius* L.) Seeds

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Abstract The antioxidant properties of twelve phenolic compounds, including matairesinol 4'-*O*-β-D-glucoside, 8'-hydroxyarctigenin 4'-*O*-β-D-glucoside, matairesinol, 8'-hydroxyarctigenin, *N*-feruloylserotonin 5-*O*-β-D-glucoside, *N*-(*p*-coumaroyl)-serotonin-5-*O*-β-D-glucoside, *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, luteolin 7-*O*-β-D-glucoside, luteolin, acacetin 7-*O*-β-glucuronide, and acacetin, isolated from defatted safflower (*Carthamus tinctorius* L.) seeds were evaluated with regard to the DPPH, superoxide and hydroxyl radicals. Additionally, levels of phenolic compounds were determined by HPLC in two cultivars of safflower seeds. Among them, four serotonin derivatives showed potent DPPH (IC_{50}=10.83-21.75 μM) and hydroxyl (IC_{50}=75.93-374.63 μM) radical scavenging activities, and their activities were significantly stronger than that of α-tocopherol. Four flavonoids (IC_{50}=170.65-275.83 μM) and four lignans (IC_{50}=114.22-406.10 μM) exhibited significant superoxide and hydroxyl radical scavenging activities, respectively, whereas these compounds contained less activity toward the DPPH and hydroxyl radicals than serotonin derivatives. The levels of serotonin derivatives, lignans and flavonoids in safflower seeds of two cultivars ranged from 49.30 to 260.40, 3.72 to 158.90, and 11.72 to 214.97 mg% (dry base), respectively. Of the two cultivars, 'Cheongsu' had somewthat higher concentrations of phenolic compounds than 'Uisan'. These results suggest that phenolic compounds in safflower seeds may play a role as protective phytochemical antioxidants against reactive oxygen-mediated pathological diseases.

Keywords: safflower (*Carthamus tinctorius* L.) seed, phenolic compound, antioxidant activity, quantification

Introduction

Oxidative stress arising from an imbalance in the human antioxidant status, reactive oxygen species vs. defense and repair mechanisms, is mainly responsible for the development of pathological diseases such as cancer, atherosclerosis, diabetes, brain dysfunction, immune-system decline, and aging (1, 2). In living systems, several natural antioxidants, including L-ascorbic acid, α-tocopherol, β-carotene, and polyphenolics are known to play important physiological roles in the inhibition of various oxidative stress-mediated degenerative diseases (3, 4). Naturally occurring polyphenolic compounds such as flavonoids, anthocyanins, cinnamic acids, phenolic acids, and tannins, etc. have recently gained much attention as dietary supplements for preventing many pathological diseases and improving health conditions (5, 6). For these reasons, an extensive search for effective antioxidants from natural sources has been undertaken.

Safflower (*Carthamus tinctorius* L.) seed, which is rich in linoleic acid, protein, and dietary fiber, is clinically used for the treatment of cataclasis, osteoporosis, and rheumatism in Korea. However, these effects have not been scientifically proven. Our recent studies revealed that phenolic compounds in safflower seeds, such as conjugated serotonins, lignans, and flavones, stimulated bone formation, reduced bone loss, and increased plasma HDL cholesterol level in estrogen deficient rats (7-9). Additionally, these phenolic compounds have been reported to have antioxidation (10-12), anti-inflammation (13), anticancer (14), and anti-aging (15, 16) properties. Thus, phenolic compounds in safflower seeds are receiving much attention as potential therapeutic agents against several pathological diseases. However, a systematic study involving the isolation and identification of phenolic compounds from safflower seeds, and the evaluation of their antioxidant properties as reactive oxygen radical scavengers is lacking, although some studies on the antioxidant activities of major phenolic aglycones isolated from roasted safflower seeds have been reported (10-12).

In addition, the levels of phenolic compounds in safflower seeds may vary depending on maturity, cultivar and processing. It is necessary to set up HPLC methods to quantify phenolic compounds for assessing high quality safflower seed powders. However, no information on the quantification of phytochemical phenolic compounds in different safflower cultivars is available, although quantitative analysis of the chemical compositions of safflower seeds has already been performed (17).

The objective of the present study was to isolate and identify the phenolic compounds in roasted safflower seeds, and further investigate their antioxidant properties as radical scavengers toward the 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and hydroxyl radicals using enzymatic and non-enzymatic *in vitro* systems. Additionally, the levels of phenolic compounds in two different safflower cultivars were also determined by HPLC.

Materials and Methods

Chemicals 1,1-Diphenyl-2-picrylhydrazyl (DPPH), xanthine oxidase (EC 1.2.3.2), xanthine, nitrobluetetrazolium chloride (NBT), thiobarbituric acid (TBA), H_2O_2, 2-deoxyribose, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO),

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ethylene-diaminetetraacetic acid (EDTA), and NMR solvents were obtained from Sigma Chemical Co. (St. Louis, MO, USA.). α-Tocopherol, L-ascorbic acid, and $FeCl_3 \cdot 6H_2O$ were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Sodium phosphate dibasic twelve hydrate and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents used in this study were of analytical grade.

Plant materials Safflower (*Carthamus tinctorius* L.) seeds of two different cultivars ('Uisan' & 'Cheongsu') were directly harvested in early August 2005 at an Experiment Station farm of the New Natural Products Institute, Uiseong, Gyeongbuk, Korea. The harvested seeds were dried at 50°C in a dry oven and stored at 4°C in a cooling room until use.

Extraction and isolation Dried 'Uisan' safflower seeds were roasted at 250°C for 5 min and milled to a 20 mesh size with a coffee maker. Roasted ground safflower seeds were refluxed twice with *n*-hexane and filtered to obtain the defatted safflower seed residue. The residue (600 g) was refluxed twice with methanol (MeOH, 10 L) for 2 hr, filtered and evaporated under reduced pressure. The MeOH extract was again solubilized in 80% MeOH and washed with *n*-hexane to remove lipids and pigments. The lower layer was evaporated to a small volume under reduced pressure, and further suspended in 40% aq. MeOH (500 mL). The MeOH souble extract was loaded onto a Diaion HP-20 column (6.5×50 cm), eluted successively with 40% (2 L), 60% (4 L), 80% (4 L), and 100% MeOH (2 L), and each fraction was then concentrated to yield 40% MeOH fr. (4.23 g), 60% MeOH fr. (3.48 g), 80% MeOH fr. (3.96 g), and 100% MeOH fr. (2.08 g). The 60% MeOH fr. (3.48 g) was repeatedly chromatographed on an ODS-A column (2.0×30 cm; YMC Inc., Milford, MA, USA) with 60% aq. MeOH and yielded five fractions; Fr. 1 (0.23 g), Fr. 2 (0.51 g), Fr. 3 (0.43 g), Fr. 4 (0.31 g), and Fr. 5 (0.12 g). Fr. 1 was rechromatographed on a Sephadex LH-20 column, to give pure compound **1** (Comp. **1**, 15.6 mg) and compound **2** (Comp. **2**, 68.4 mg). Fr. 2 and 3 were chromatographed separately on a precoated C_{18} TLC plate with 0.1% CH_3COOH in 60% MeOH to yield pure compound **3** (Comp. **3**, 18.3 mg) and compound **4** (Comp. **4**, 14.8 mg). Fr. 4 and 5 were rechromatographed separately on a Sephadex LH-20 column with 90% MeOH and yielded three subfractions; Fr. 1 (0.11 g), Fr. 2 (0.35 g), and Fr. 3 (0.19 g). Fr. 2 was further purified by using a preparative HPLC on YMC-Packed C_{18} column (20 mm, i.d. × 250 mm, YMC Inc.) with an isocratic elution using 0.1% trifluoroacetic acid (TFA) in 40% aq. MeOH, and a flow rate of 5 mL/min at 310 nm to yield pure compound **5** (Comp. **5**, 11.2 mg) and compound **6** (Comp. **6**, 23.3 mg). The 80% MeOH fr. (3.96 g) was chromatographed on a Sephadex LH-20 column (2.5×80 cm) with 90% MeOH and yielded five fractions; Fr. 1 (0.68 g), Fr. 2 (0.32 g), Fr. 3 (0.38 g), Fr. 4 (0.37 g), and Fr. 5 (0.10 g). Fr. 1 was further purified by a preparative isocratic HPLC using 0.1% TFA in 60% aq. MeOH to yield pure compound **7** (Comp. **7**, 101.4 mg) and compound **8** (Comp. **8**, 66.3 mg). Fr. 2 was rechromatographed on a Sephadex LH-20 column with 90% MeOH and yielded pure compound **9** (Comp. **9**, 15 mg). Fr. 3 and 4 were further chromatographed with ODS-A column with 60% aq. MeOH and yielded pure compound **10** (Comp. **10**, 25.6 mg) and compound **11** (Comp. **11**, 52.1 mg). Finally, the 100% MeOH fr. (2.08 g) was chromatographed on a Sephadex LH-20 column (2.5×80 cm) with 100% MeOH and yielded a pure compound **12** (Comp. **12**, 19.5 mg).

Identification of phenolic compounds UV and IR spectra were obtained using a Jasco 3334 UV-vis spectrophotometer (Osaka, Japan) and a FT-IR spectrometer (IFS 120 HR, Bruker, Germany), respectively. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured in pyridine-d_6 (lignans) and DMSO-d_6 (serotonin derivatives and flavones) on a Unity Plus 500 spectrometer (Varian, Palo Alto, CA, USA) and chemical shifts were expressed as δ values with tetramethylsilane (TMS) as an internal standard. Fast-atom bombardment mass spectrometry (FAB-MS) was recorded on a JMS-700 mass spectrometer (ion source, Xe atom beam; accelerating voltage, 10 kV; Jeol, Tokyo, Japan), using glycerol and *m*-butylalcohol as a mounting matrix. Column chromatography was performed with Diaion HP-20 (Mitsubishi Chem. Co., Tokyo, Japan), ODS-A (12 nm, 150 μm; YMC, Inc.), and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) columns. Preparative TLC (PTLC) and HPLC (PHPLC) were carried out on precoated silica C_{18} plates (0.25 mm, Merck, Damstadt, Germany) and Delta Prep 4000 (Waters, Milford, MA, USA), respectively. All solvents used for this study were of analytical and HPLC grades.

HPLC analysis Dried 'Uisan' safflower seeds were roasted at 250°C for 5 min and milled with a coffee maker. The ground roasted seed (10 g) was refluxed twice with 150 mL of *n*-hexane and filtered. The defatted seed residue was extracted twice with 80% aqueous EtOH under reflux, filtered and evaporated under reduced pressure. The EtOH extract was redissolved in 8 mL of 80 % aq. EtOH and left to stand overnight. The upper layer was then filled up to 10 mL with the same solvent. The soluble extract (2 mL) was diluted 10 times and passed through a 0.45 μm PVDF syringe filter (Whatman, Maidstone, England) and then subjected to HPLC for quantification of phenolic compounds. HPLC was performed on a Gilson 506B HPLC System coupled with a Gilson 170 UV-vis detector, and Gilson 231 XL autosampler with a 10 μL loop. HPLC analysis was carried out using a YMC-Pack Pro C_{18} column (46 mm i.d. × 250 mm, YMC Inc.) with a Guard-Pak C_{18} precolumn insert. The separation was conducted using a linear gradient from 0.05%v/v H_3PO_4 in 20% MeOH (solvent A) to 80% MeOH (solvent B) for 60 min at a flow rate of 0.8 mL/min with UV detection at 270 nm. The elution profile was as follows: 0-2 min, 100% A, 0% B; 5-10 min, 80% A, 20% B; 15-20 min, 60% A, 40% B; 25-30 min, 40% A, 60% B; 35-40 min, 0% A, 100% B. The column was returned to initial conditions for 10 min before the next injection. Individual phenolics were identified by a comparison of their retention time with those of the twelve standard phenolics isolated previously as described in the Materials and Methods. Peaks were identified by co-chromatography with authentic samples

isolated previously. Duplicate analyses were conducted on duplicate samples. Linear correlation coefficients were greater than 0.999 for each phenolic. The concentration of each phenolic was determined by the calibration curves of twelve standard phenolics, and expressed as mg% of dry weight. Recovery rates of serotonin derivatives, lignans, and flavonoids were 92, 90, and 80%, respectively.

DPPH, superoxide, and hydroxyl radical scavenging activities The DPPH, superoxide, and hydroxyl radical scavenging activities of phenolic compounds from safflower seeds were determined as reported previously (18).

In the assay for DPPH radical scavenging, each phenolic compound was added to 100 μM DPPH in methanol, followed by incubation at 25°C. Reactivity with DPPH was determined spectrophotometrically at 516 nm.

To assess superoxide radical scavenging, the reaction mixture (3.0 mL) comprising 0.05 M Na_2CO_3 buffer (pH 10.2) containing 3 mM xanthine, 3 mM EDTA, BSA (1.5 mg protein/mL), 0.75 mM NBT, test solution (1-300 mM in DMSO), and 5.0 U/mL xanthine oxidase was incubated at 25°C for 20 min. The reaction was then terminated by the addition of 6 mM $CuCl_2$. The production of formazan was measured at 560 nm.

To assess hydroxyl radical scavenging, the reaction mixture (1.0 mL) comprising 30 mM KH_2PO_4-KOH buffer (pH 7.4), 2 mM 2-deoxyribose, 0.1 mM $FeCl_3 6 \cdot H_2O$, 104 μM EDTA, test solution [100-1,000 μM in above buffer], 1.0 mM H_2O_2, and 0.1 mM L-ascorbic acid, was incubated at 37°C for 1 hr. The thiobarbituric acid reactive substance (TBARS) level was determined after terminating the reaction by the addition of TBA reagent. IC_{50} values of radical scavenging activity were determined by regression analysis of the results obtained at three different concentrations of the sample.

Results and Discussion

Identification of phenolic compounds in safflower seed Defatted MeOH extract from roasted safflower seeds was successively loaded onto a Diaion HP-20 and Sephadex LH-20 columns, PTLC silica C_{18} plates, ODS-A, and PHPLC, to give twelve phenolic compounds in a pure state. Among them, two lignans [Comp. **3**: matairesinol (MR), Comp. **4**: 8'-hydroxyarctigenin (HAG)], four serotonin derivatives [Comp. **5**: *N*-feruloylserotonin-5-*O*-β-D-glucoside (FSG), Comp. **6**: *N*-feruloylserotonin (FS), Comp. **7**: *N*-(*p*-coumaroyl)serotonin-5-*O*-β-D-glucoside (CSG), Comp. **8**: *N*-(*p*-coumaroyl)serotonin (CS)], and two flavonoids [Comp. **10**: luteolin (LT), Comp. **12**: acacetin (AT)] have already been isolated and identified from safflower seed (10, 19, 20). Additionally, we previously isolated and identified two flavonoids [Comp. **9**: luteolin-7-*O*-β-D-glucoside (LTG), Comp. **11**: acacetin 7-*O*-β-D-glucuronide (ATG)] from safflower leaf (21). Herein, we identify the complete structures of two lignan glycosides [Comp. **1**: matairesinol 4'-*O*-β-D-glucoside (MRG), Comp. **2**: 8'-hydroxyarctigenin 4'-*O*-β-D-glucoside (HAGG) through FAB-MS and 1D- & 2D-NMR analysis, although the 2 compounds were already identified in safflower seeds (19, 20).

Compound **1** showed a protonated molecule $[M+H]^+$ at *m/z* 521 in the positive FAB-MS spectrum, together with one significant fragment ion peak at *m/z* 359 [M^+-162 (hexose)] indicating the presence of matairesinol and one hexose moiety. ^{1}H-NMR (in pyridine) spectra of Comp. **1** exhibited two aromatic ABX-type proton signals [δ6.66 (1H, dd, *J*=2.0, 8.0 Hz, H-6), 6.78 (1H, d, *J*=2.0 Hz, H-2), 6.74 (1H, d, *J*=8.0 Hz, H-5), 6.94 (1H, dd, *J*=2.0, 8.0 Hz, H-6'), 7.02 (1H, d, *J*=2.0 Hz, H-2'), 6.88 (1H, d, *J*=8.0 Hz, H-5')], butylolactone signals [2.58 (2H, m, H-7), 2.58 (1H, m, H-8), 2.58 (1H, m, H-8'), 2.74 (1H, dd, *J*=7.0, 14.0 Hz, H-7'), 2.82 (1H, dd, *J*=5.5, 14.0 Hz, H-7'), 3.86 (2H, m, H-9)], two methoxyl signals (δ 3.78, 3.81), and glucose moiety signals [5.64 (anomeric proton, 1H, d, *J*=7.0 Hz, H-1), 4.56 & 4.58 (2H, brd, *J*=2.0, 2.5 Hz, H-6), 4.37 (1H, m, H-3), 4.37 (1H, m, H-5), 4.34 (1H, m, H-2), 4.22 (1H, m, H-4)]. ^{13}C-NMR spectral data [δ179.07 (CO, C-9'), 148.77 (C-3), 148.75 (C-3'), 146.91 (C-4), 146.88 (C-4'), 132.63 (C-1'), 129.85 (C-1), 122.32 (C-6'), 122.32 (C-6), 116.31 (C-5'), 115.11 (C-2'), 113.11 (C-2), 116.31 (C-5), 46.62 (C-8'), 71.45 (C-9), 41.72 (C-8), 34.54 (C-7'), 37.99 (C-7), two methoxyl carbons (55.98, 55.99), glucose carbons [102.43 (C-1), 78.80 (C-5), 78.51 (C-3), 74.89 (C-2), 71.25 (C-4), 62.34 (C-6)] of Comp. **1** coincided well with those of matairesinol binded with one glucose moiety. Interestingly, when compared to matairesinol, compound 1 has one glucose moiety in matairesinol through splitting patterns of proton (δ4.22-5.64) and carbon (δ62.34-102.43) signals. Additionally, the heteronuclear multi-quantum connectivity (HMQC) spectrum revealed the presence of two methoxyl residues, in which two OCH_3 proton signals (δ3.81, 3.78) were correlated with their carbon signals (δ55.98, 55.99). Finally, the heteronuclear multi-bond correlation (HMBC) spectrum showed a correlation between H-1 (δ5.64) of the glucose moiety and C-4' (δ146.88), indicating one glucose moiety should be attached to C-4' of the matairesinol skeleton. From 1D- & 2D-NMR spectral analysis, the structure of compound **1** was characterized as MRG.

Compound **2** showed a protonated molecule $[M+H]^+$ at *m/z* 551 in the positive FAB-MS spectrum, together with one significant fragment ion peak at *m/z* 389 [M^+-162 (hexose)], indicating the presence of 8'-hydroxyarctigenin and one hexose moiety. ^{1}H-NMR (in pyridine) spectra of Comp. **2** showed two aromatic ABX-type proton signals [δ6.88 (1H, dd, *J*=2.0, 8.0 Hz, H-6), 6.92 (1H, brs, H-2), 6.93 (1H, d, *J*=6.0 Hz, H-5), 7.06 (1H, dd, *J*=2.0, 8.0 Hz, H-6'), 7.18 (1H, d, *J*=1.5 Hz, H-2'), 7.61 (1H, d, *J*=8.5 Hz, H-5')], butylolactone signals [2.76 (1H, m, H-8), 2.96 (1H, dd, *J*=9.5, 14.0 Hz, H-7), 3.26 (1H, dd, *J*=4.5, 13.5 Hz, H-7), 3.33 (1H, d, *J*=14.0 Hz, H-7'), 3.65 (1H, d, *J*=13.5 Hz, H-7'), 4.18 (2H, t, *J*=8.0 Hz, H-9)], three methoxyl signals (δ 3.76, 3.77 & 3.81), and glucose moiety signals [5.66 (anomeric proton, 1H, d, *J*=7.0 Hz, H-1), 4.52 & 4.50 (2H, brd, *J*=2.0, 2.5 Hz, H-6), 4.38 (1H, m, H-3), 4.36 (1H, m, H-5), 4.33 (1H, m, H-2), 4.30 (1H, m, H-4)]. ^{13}C-NMR spectral data [δ179.36 (CO, C-9'), 149.88 (C-3), 149.67 (C-3'), 148.65 (C-4), 147.22 (C-4'), 132.54 (C-1'), 130.42 (C-1), 123.35 (C-6'), 121.33 (C-6), 116.19 (C-5'), 115.34 (C-2'), 113.45 (C-2), 112.83 (C-5), 78.90 (C-8'), 71.23 (C-9), 44.15 (C-8), 41.64 (C-7'), 31.82 (C-7), three methoxyl carbons (56.05, 56.05, 55.98), glucose carbons [102.43 (C-1), 78.57 (C-5), 76.70 (C-3), 74.89 (C-2), 70.96 (C-4),

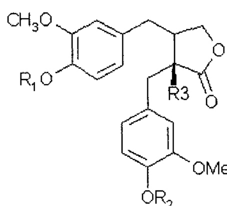
62.34 (C-6)] of Comp. **2** coincided well with those of 8'-hydroxyarctigenin conjugated with one glucose moiety. In particular, when compared to arctigenin, compound **2** exhibited a lower field shift of the C-8' (δ78.90), suggesting the substitution of a hydroxyl group at C-8' position of arctigenin (22). This fact was reinforced by the HMBC cross peak between H-7' (δ41.64) and C-8' (δ78.90). Additionally, the HMQC spectrum revealed the presence of three methoxyl residues, in which three OCH_3 proton signals (δ3.81, 3.77, 3.76) were correlated with their carbon signals (δ56.05, 56.05, 55.98). Finally, the HMBC spectrum showed a correlation between H-1 (δ5.66) of the glucose moiety and C-4' (δ147.22), indicating one glucose moiety should be attached to C-4' of the arctigenin skeleton. From the spectral analysis including 2D-NMR, the structure of compound **2** was elucidated as HAGG. The detailed UV, IR, and NMR spectral data of compounds **1** and **2** are shown in Table 1. This is the first report of the isolation and purification of twelve phenolic compounds from defatted safflower seeds. The chemical structures of the twelve compounds are shown in Fig. 1.

Antioxidant properties of phenolic compounds from safflower seeds Twelve phenolic compounds, including serotonin derivatives, lignans, and flavonoids, and their glycosides, were examined for their abilities to scavenge

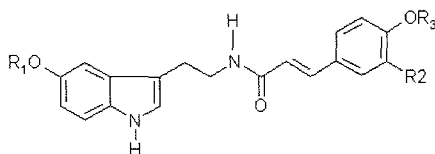
Table 1. NMR spectral data of MRG[1] and HAGG[2]

	MRG		HAGG	
UV (λ_{max})	230, 279		228, 278	
IR (ν_{max})	3400, 1765, 1450		3400, 1775, 1600, 1520, 1460	
NMR	δ_H	δ_C	δ_H	δ_C
1		129.85		130.42
2	6.78 (d, J=2.0 Hz)	113.11	6.92 (brs)	113.45
3		148.77		149.88
4		146.91		148.65
5	6.74 (d, J=8.0 Hz)	116.31	6.93 (d, J=6.0 Hz)	112.83
6	6.66 (dd, J=2.0, 8.0 Hz)	122.32	6.88 (dd, J=2.0, 8.0 Hz)	121.33
7	2.58 (m)	37.99	2.96 (dd, J=9.5, 14.0 Hz) 3.26 (dd, J=4.5, 13.5 Hz)	31.82
8	2.58 (m)	41.72	2.76 (m)	44.15
9	3.86 (m)	71.45	4.18 (t, J=8.0 Hz)	71.23
1'		132.63		132.54
2'	7.02 (d, J=2.0 Hz)	115.11	7.18 (d, J=1.5 Hz)	115.34
3'		148.75		149.67
4'		146.88		147.22
5'	6.88 (d, J=8.0 Hz)	116.31	7.61 (d, J=8.5 Hz)	116.19
6'	6.94 (dd, J=2.0, 8.0 Hz)	122.32	7.06 (dd, J=2.0, 8.0 Hz)	123.35
7'	2.74 (dd, J=7.0, 14.0 Hz) 2.82 (dd, J=5.5, 14.0 Hz)	34.54	3.33 (d, J=14.0 Hz) 3.65 (d, J=13.5 Hz)	41.64
8'	2.58 (m)	46.62		78.90
9'		179.07		179.36
OCH_3	3.81, 3.78	55.99, 55.98	3.81, 3.77, 3.76	56.05, 56.05, 55.98
Glucose				
1"	5.64 (d, J=7.0 Hz)	102.43	5.66 (d, J=7.0 Hz)	102.43
2"	4.34 (m)	74.89	4.33 (m)	74.89
3"	4.37 (m)	78.51	4.38 (m)	76.70
4"	4.22 (m)	71.25	4.30 (m)	70.96
5"	4.37 (m)	78.80	4.36 (m)	78.57
6"	4.56 (brd, J=2.5 Hz) 4.58 (brd, J=2.0 Hz)	62.34	4.50 (brd, J=2.5 Hz) 4.52 (brd, J=2.0 Hz)	62.34
FAB-MS $[M+H]^+$	537		551	

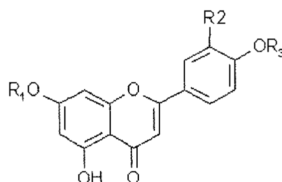
[1]Matairesinol-4'-O-β-D-glucoside. [2]8'-Hydroxyarctigenin-4'-O-β-D-glucoside.
Chemical shifts in ppm from TMS. Coupling constants in Hz, spectra recorded in DMSO-d_6 at 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR).



Matairesinol: R_1=H, R_2=H, R_3=H
Matairesinol-4'-*o*-β-D-glucoside: R_1=H, R_2=glucose, R_3=H
8'-Hydroxyarctigenin: R_1=CH_3, R_2=H, R_3=OH
8'-Hydroxyarctigenin-4'-*o*-β-D-glucoside: R_1=CH_3, R_2=glucose, R_3=OH



N-Feruloylserotonin: R_1=H, R_2=OCH_3, R_3=H
N-Feruloylserotonin-5-*o*-β-D-glucoside: R_1=glucose, R_2=OCH_3, R_3=H
N-(*p*-Coumaroyl)serotonin: R_1=H, R_2=H, R_3=H
N-(*p*-Coumaroyl)serotonin-5-*o*-β-D-glucoside: R_1=glucose, R_2=H, R_3=H



Acacetin-7-*o*-β-D-glucuronide: R_1=glucuronide, R_2=H, R_3=CH_3
Acacetin: R_1=H, R_2=H, R_3=CH_3
Luteolin 7-*o*-β-D-glucoside: R_1=glucose, R_2=OH, R_3=H
Luteolin: R_1=H, R_2=OH, R_3=H

Fig. 1. Chemical structures of twelve different phenolic compounds from safflower seeds.

the DPPH radical, and superoxide, radical generated enzymatically in a xanthine/xanthine oxidase system, and the hydroxyl radical generated via the Fenton reaction. As shown in Table 2, most of the phenolic compounds showed considerable DPPH, superoxide, and hydroxyl radical scavenging activities in a dose-dependent manner. Among them, four serotonin derivatives (IC_{50}=10.83-21.75 μM) were much more efficient at scavenging the DPPH radical than lignans (IC_{50}=70.56-86.85 μM) and flavonoids (IC_{50}=74.03-102.62 μM), and their activity was significantly stronger than that of α-tocopherol (IC_{50}=28.45 μM). The lignans and flavonoids also exhibited considerable DPPH radical scavenging activity, although their activity was less than that of α-tocopherol. Meanwhile, the flavonoids (IC_{50} =170.65-275.83 μM) exerted higher superoxide anion radical scavenging activity than the serotonin derivatives (IC_{50}=304.92-524.34 μM) and lignans (IC_{50}=641.82-1,256.93 μM), and their activities were stronger than that of α-tocopherol. Four serotonin derivatives also exhibited moderate inhibitory activity, but the lignans contained less activity. Finally, the lignan (IC_{50}=114.22-406.10 μM) and serotonin derivatives (IC_{50}=75.93-374.63 μM) had stronger hydroxyl radical scavenging activity than the flavonoids,

Table 2. DPPH, superoxide and hydroxyl radical scavenging activities of 12 phenolic compounds isolated from safflower seeds

Phenolic compound	IC_{50} (μM)[14]		
	DPPH radical	Superoxide radical	Hydroxyl radical
MRG[1]	71.54±5.45	878.36±32.43	114.22±1.54
MR[2]	70.56±3.65	641.82±22.54	279.66±2.59
HAGG[3]	86.85±5.76	1,256.93±73.44	134.85±2.92
HAG[4]	84.83±4.65	978.06±45.69	406.10±11.65
FSG[5]	12.14±0.34	372.84±21.64	126.72±4.23
FS[6]	10.83±0.12	304.92±12.64	374.63±12.01
CSG[7]	21.75±1.82	524.34±19.43	75.93±1.02
CS[8]	20.94±1.02	419.65±16.47	102.53±3.12
ATG[9]	102.62±5.34	275.83±7.45	832.65±18.65
AT[10]	99.63±5.23	220.34±5.67	1,274.27±43.65
LTG[11]	76.03±4.23	198.56±2.87	596.76±12.49
LT[12]	74.76±3.54	170.65±2.36	1,034.83±52.57
α-Toc[13]	28.45±1.20	>5,000	154.34±14.24

[1]Matairesinol 4'-*O*-β-D-glucoside. [2]Matairesinol. [3]8'-Hydroxyarctigenin 4'-*O*-β-D-glucoside. [4]8'-Hydroxyarctigenin. [5]*N*-Feruoylserotonin 5-*O*-β-D-glucoside. [6]*N*-Feruloyl- serotonin. [7]*N*-(*p*-Coumaroyl)serotonin 5-*O*-β-D-glucoside. [8]*N*-(*p*-Coumaroyl)serotonin. [9]Acacetin 7-*O*-β-D-glucuronide. [10]Acacetin. [11]Luteolin 7-*O*-β-D-glucoside. [12]Luteolin. [13]α-Tocopherol. [14]IC_{50} represents the concentration of compound required for 50% scavenging of DPPH, superoxide, and hydroxyl radicals. Values are mean±SD of duplicate analyses. α-Tocopherol was used as a positive reference.

whereas the flavonoids showed moderate hydroxyl radical scavenging activity comparable to that of α-tocopherol (IC_{50}=154.34 μM). Thus, these results suggest that the phenolic compounds in safflower seeds, specifically the serotonin derivatives, lignans and flavonoids, can function as potentially powerful oxygen radical scavengers, even though some phenolic compounds showed weak radical scavenging activity. It is also very interesting to note that serotonin derivatives, the predominant constituents in safflower seeds, have strong hydroxyl radical scavenging activity along with their potent free radical scavenging activity (10). For these reasons, phenolic compounds in safflower seeds are receiving renewed interest as promising sources of natural antioxidants acting as radical scavengers.

The structure-activity relationship of phenolic compounds as scavengers of the DPPH, superoxide, and hydroxyl radicals was also investigated. Of the phenolic compounds, the serotonin derivatives exhibited high DPPH radical scavenging activity, whereas the lignans and flavonoids showed less activity than the serotonin derivatives. Additionally, there were no significant differences in the activities of the lignans and flavonoids, as well as phenolic aglycones and glycosides. These facts support earlier reports that the DPPH radical scavenging activity of phenolic compounds is directly correlated to the number of hydroxyl groups in the aromatic ring (23, 24), and to the presence of phenolic amides with hydroxycinnamic acid and indole nuclei (10), regardless of the glycosylation of hydroxyl groups in phenolic compounds.

The flavonoids showed the strongest superoxide radical scavenging activity, followed by the serotonin derivatives and the lignans, in decreasing order. Of the flavonoids, LT, with a catechol group on the B ring, showed greater activity than AT, with a 4-methoxyl group on the B ring. With regard to the serotonins derivatives, FS was higher than CS, and of the lignans, MR had higher activity than HAG. These results support previous reports that the superoxide radical scavenging activity of phenolic compounds increased with increasing the numbers of hydroxyl groups in phenolic compounds, but decreased with the presence of a methoxyl group adjacent to the 4-hydroxyl group *para*-substituted on the aromatic ring (25, 26). Additionally, phenolic aglycones were more efficient at scavenging the superoxide radical than their corresponding glycosides, confirming that glycosylation of the hydroxyl group in phenolic compounds decreased superoxide radical scavenging activity with regard to the bio-assay system used (26).

The serotonin derivatives and lignans exhibited strong hydroxyl radical scavenging activity, although some differences in activity were found according to the nature of substituent in the aromatic ring. The flavonoids, with high DPPH and superoxide radical scavenging activities, showed less activity than the serotonin derivatives and lignans. This observation supports an earlier report that methoxylation of the hydroxyl group at the *o*-position, as in HAG and FS, resulted in a drastic decrease of the hydroxyl radical scavenging activity, and polar substituents at the *p*-position, as in CS, were correlated with higher hydroxyl quenching activity (27). Additionally, LT had greater hydroxyl radical scavenging activity than AT, supporting the idea that flavonoids with *ortho*-dihydroxyl groups on the B ring enhance the metal chelating ability of flavonoids (28, 29). In particular, the hydroxyl radical scavenging activity of the flavonoids, which are well-known as hydroxyl radical scavengers (28, 29), was less than that of the serotonin derivatives and lignans, suggesting that the presence of a hydroxyl group in the indole nuclei of the serotonin derivatives, and of a carbonyl function in the butylolactone group of the lignans, may exert beneficial effects on scavenging hydroxyl radicals.

On the basis of these results, the differences in three radical scavenging activities toward three different radicals observed among the different phenolic compounds in safflower seeds can be explained in terms of the number and position of hydroxyl and methoxyl groups in each phenolic compound, as well as the functional side groups, such as indole nuclei in the serotonin derivatives, and butylolactone in the lignans. Thus, the mechanism of antioxidant and other physiological actions of the phenolic compounds in safflower seeds seem to be due to their free and reactive oxygen radical scavenging properties. Additionally, it has previously been reported that some phenolic compounds in safflower seeds exhibited strong DPPH and hydroxyl radical scavenging activities (10, 11), and greatly inhibited non-enzymatic lipid peroxidation in rat liver microsomes (11, 12). Overall, these results suggest that the phenolic compounds in safflower seeds may play important roles in scavenging free radicals related to the intiation and propagation steps of lipid peroxidation, and quenching superoxide and hydroxyl radicals involved in the generation of several pathological conditions, including cancer, atherosclerosis, inflammation, and aging. Thus, defatted safflower seed residue containing antioxidant phenolic compounds may be as promising source of natural antioxidants having antiosteoporotic, anti-lipidemic, and anticarcinogenic activities without toxic effects (8, 9, 14). This is the first report on the antioxidant properties of phenolic compounds in safflower seeds functioning as superoxide and hydroxyl radical scavengers.

Quantification of phenolic compounds Levels of the twelve phenolic compounds in two different cultivars of safflower seeds were determined by HPLC (Table 3), and a typical HPLC chromatogram is shown in Fig. 2. Four lignans (MRG, HAGG, MR, and HAG), four serotonin derivatives (FSG, CSG, FS, and CS), and four flavonoids (LTG, LT, ATG, and AT) were detected in the defatted MeOH extract from roasted safflower seeds based on the retention time of each standard phenolic compound isolated previously. Levels of the serotonin derivatives, lignans and flavones in the two cultivars ranged from 49.30 to 260.40, 3.72 to 158.90, and 11.72 to 214.97 mg% (dry base), respectively. Of the two cultivars, the 'Cheongsu' cultivar had higher concentrations of phenolic compounds than the 'Uisan' cultivar. Thus, the phenolic content of safflower seeds varies with two cultivars, supporting earlier reports that the types and contents of phenolics in plants vary with cultivar, maturation and processing (4, 30).

In conclusion, twelve phenolic compounds were isolated from defatted MeOH extract of safflower seeds through a combination of Diaion HP-20, Sephadex LH-20,

Table 3. Levels of phenolic compounds in two different cultivars of safflower seeds

Phenolic compound	Content (mg%, dry weight)[13]	
	Uisan	Cheongsu
MRG[1]	20.32±1.48	32.43±1.92
MR[2]	3.72±0.17	6.62±0.92
HAGG[3]	123.08±3.73	158.90±2.93
HAG[4]	14.36±0.72	21.72±1.01
FSG[5]	49.30±1.76	58.21±6.91
FS[6]	234.13±4.47	260.40±18.11
CSG[7]	84.08±4.98	95.92±6.51
CS[8]	152.76±10.92	191.95±13.57
LTG[9]	81.60±4.81	101.06±3.20
LT[10]	118.40±2.84	165.94±10.39
ATG[11]	181.77±7.15	214.97±10.06
AT[12]	11.72±1.03	13.81±1.27

[1]Matairesinol 4'-*O*-β-D-glucoside. [2]Matairesinol. [3]8'-Hydroxyarctigenin 4'-*O*-β-D-glucoside. [4]8'-Hydroxyarctigenin. [5]*N*-Feruoylserotonin 5-*O*-β-D-glucoside. [6]*N*-Feruloyl- serotonin. [7] *N*-(*p*-Coumaroyl)serotonin 5-*O*-β-D-glucoside. [8] *N*-(*p*-Coumaroyl)serotonin. [9]Luteolin 7-*O*-β-D-glucoside. [10]Luteolin. [11]Acacetin 7-*O*-β-D-glucuronide. [12]Acacetin.
[13]Values are mean±SD of duplicate analyses.

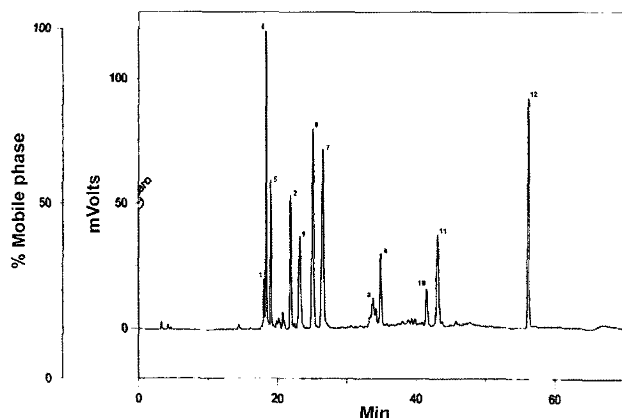


Fig. 2. HPLC chromatogram of twelve phenolic compounds from defatted MeOH extract of safflower seeds. 1, Matairesinol 4'-*O*-β-D-glucoside; 2, 8'-hydroxyarctigenin 4'-*O*-β-D-glucoside; 3, matairesinol; 4, 8'-hydroxyarctigenin; 5, *N*-feruoylserotonin 5-*O*-β-D-glucoside; 6, *N*-(*p*-coumaroyl)serotonin 5-*O*-β-D-glucoside; 7, *N*-feruloylserotonin; 8, *N*-(*p*-coumaroyl)serotonin; 9, luteolin 7-*O*-β-D-glucoside; 10, luteolin; 11, acacetin 7-*O*-β-D-glucuronide; 12, acacetin.

ODS-A column chromatographies, and PTLC and PHPLC techniques, and their structures have been elucidated using spectral analysis and previously published data. Among these compounds, the serotonin derivatives exhibited potent DPPH and hydroxyl radical scavenging activities, whereas the flavonoids showed strong superoxide radical scavenging activity. The lignans also exhibited moderate radical scavenging activity toward all three radicals. Additionally, the phenolic compound content in safflower seeds varied with their cultivars. These results suggest that the phenolic compounds in safflower seeds may function as strong radical scavengers of reactive oxygen species, thereby inhibit several oxygen radical-mediated pathological diseases. Further study is required to investigate the antioxidant activities of safflower seed phenolic compounds *in vivo*.

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