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Antioxidative Activity of Phenolic Compounds in Roasted Safflower (*Carthamus tinctorius* L.) Seeds

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Abstract

Antioxidative compounds contained in roasted safflower seeds were investigated. Six phenolic compounds, N-feruloylserotonin, N-(*p*-coumaroyl) serotonin, matairesinol, 8′-hydroxyarctigenin, acacetin 7-*O*-β-D-glucoside (tilianine) and acacetin were isolated and identified from the extract of seeds. The inhibitory effects of six phenolic compounds on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and lipid peroxidation induced by H_2O_2/$FeSO_4$ in rat liver microsomes were determined. Two serotonins showed more potent DPPH radical scavenging activity, and a stronger inhibitory effect on the lipid peroxidation than that of α-tocopherol. In addition, acacetin and matairesinol also considerably inhibited lipid peroxidation, while 2-hydroxy- arctigenin and tilianine were inactive. These results suggest that phenolic compounds, including serotonins, lignans and flavonoids in the roasted safflower seeds can be used as potential dietary natural antioxidants.

Key words: safflower (*Carthamus tinctorius* L.) seed, antioxidative activity, phenolic compounds

INTRODUCTION

Antioxidants play an important role in protecting cell membranes against damages caused by active oxygen species, which are reportedly associated with several pathologic conditions, including carcinogenesis, mutagenesis, atherosclerosis, and aging (1,2). In particular, dietary natural antioxidants, such as L-ascorbic acid, α-tocopherol, β-carotene, and several flavonoids have been found to play considerable roles in preventing the above pathological diseases, as scavenger or quencher of active oxygen radicals (3,4), apart from synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), which are suspected to have carcinogenic and toxic effects (5,6). Many studies on the screening of more safe, novel natural antioxidants from dietary plants, therefore, are essential.

Recently, much attention has been devoted to the development of novel antioxidants from seed oil cakes, the waste product of the oil industry. In particular, safflower (*Carthamus tinctorius* L. Compositae) seeds, as major sources of dietary polyunsaturated fats, have been widely used for the treatment of cataclasis, osteoporosis and rheumatism in Korea. In addition, safflower seed powder was recently found to improve lipid metabolism in high fat and high cholesterol-fed rats (7). However, utilization of safflower meal as feed and food has been partially limited by some factors such as bitter taste (8,9) and cathartic effect (10). It was reported that the roasting process could mitigate considerably the above deleterious effects in fresh safflower seeds (11), and also enhance the antioxidative activity of the extracts of seeds (12). Until now, several serotonins and acacetin with antioxidative activity have been isolated from the roasted safflower seeds (12-14). However, few studies on the antioxidative activity of other phenolic constituents including serotonins, lignans and acacetin in biological systems, is available.

The objective of the present study is to isolate and identify polyphenolic compounds in roasted safflower seeds, and to investigate their antioxidative properties using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and rat liver microsome systems.

MATERIALS AND METHODS

Materials and reagents

Safflower seeds were harvested from a farm in Uisong, Kyungbuk, Korea. 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-tocopherol and H_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $FeSO_4 \cdot 7H_2O$, L-ascorbic acid and butylated hydroxyanisole (BHA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used for this study were analytical grade.

Extraction and isolation of phenolic compounds

Safflower seeds (500 g) roasted at 200°C for 5 min were ground and extracted twice with hexane to remove lipid under reflux. The defatted seeds were further extracted with MeOH (2.0 l) under reflux for 3 hr, and then evaporated under reduced pressure. The methanolic extract (45 g) was partitioned between n-hexane and 80% aqueous methanol. The concentrated methanolic extract was further partitioned between ethyl acetate and water. The ethyl acetate extract (8.1 g, Fig. 1) was subjected to Diaion HP 20 (Mitsubishi Chem. Co., Tokyo, Japan) column (4 cm × 50 cm), and then eluted with H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH and 100% MeOH, stepwise. The 60% MeOH fraction was

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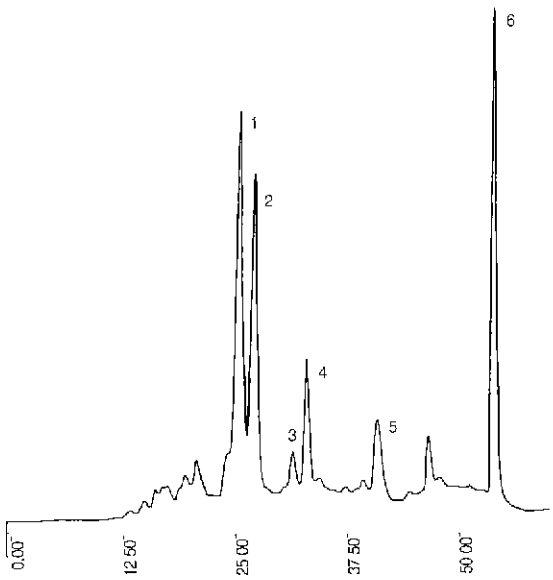

Fig. 1. HPLC chromatogram of the ethyl acetate fraction isolated from the defatted methanolic extracts of the roasted safflower seeds. **1**, N-feruoylserotonin; **2**, N-(*p*-coumaroyl)serotonin; **3**, matairesinol; **4**, 2-hydroxyarctigenin; **5**, tilianine; **6**, acacetin. HPLC condition: column, RCM Prep Nova-Pak C_{18} (2.5 cm × 10 cm); linear gradient elution from solvent A (0.1% TFA in 20% MeOH) to solvent B (80% MeOH) for 60 min; flow rate, 5 ml/min; detection, 280 nm.

chromatographed repeatedly on Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) with 90% MeOH as an eluent, and then separated into eight fractions. Fr. 4 and Fr. 5 were finally purified by preparative HPLC (Waters Delta Prep 4000, USA) using a Novapak C_{18} column (4 cm × 25 cm × 2 cartridge) at a flow rate of 5 mL/min, monitored at 300 nm, using MeOH-H_2O-TFA (20:80:0.1) as a mobile phase. Two serotonins (serotonin **1**, 280.3 mg; serotonin **2**, 245.3 mg) were repeatly isolated. Meanwhile, the 80% MeOH fraction was chromatographed repeatedly on Sephadex LH-20 with 90% MeOH as an eluent, and then separated into six fractions. Fr. 3 and Fr. 4 were finally purified by preparative HPLC using MeOH-H_2O-TFA (40:60:0.1) at 280 nm. Two lignans (lignan **1**, 13.5 mg; lignan **2**, 44.6 mg) were repeatly isolated. Finally, the 100% MeOH fraction was chromatographed repeatedly on Sephadex LH-20 with methanol as an eluent, and then separated into seven fractions. Fr. 6 and Fr. 7 were finally purified by preparative HPLC using MeOH-H_2O-TFA (70:30:0.1) at 330 nm. Two flavonoids (flavonoid **1**, 15.3 mg; flavonoid **2**, 130.3 mg) were repeatly isolated.

Instrumental analysis

The ultraviolet (UV) and infrared (IR) spectra were recorded with a Sinco 1030 spectrophotometer and an IFS 120 HR FT-IR spectrometer (Bruker, Germany), repectively. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained using a Unity Plus 500 spectrometer (Varian, USA). Chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. The electron impact-mass spectrometry (EI-MS) was determined with a Quattro II mass spectrometer (VG, U.K) at an ionization voltage of 70 eV.

DPPH radical scavenging activity

A test sample solution in methanol (200 µl) was added to 100 µM DPPH methanolic solution (4 ml). After vortexing, the mixture was incubated for 10 min at room temperature, and then the absorbance at 517 nm was measured (15). IC_{50} values were determined by regression analysis of the results at three different concentrations of the sample.

Antioxidative assay in rat liver microsome

Sprague-Dawley rats (8 weeks, 300±20 g) were sacrificed by decapitation after fasting for 24 hr, and their liver tissue was immediately removed. Microsomes were isolated from the liver tissue by differential centrifugation according to the method of Slater and Sawyer (16), and stored at -70°C until needed. The isolated microsomals were suspended in a 50 mM phosphate buffer (pH 7.4). The final protein concentration of the microsomal suspensions were adjusted to 10 mg protein/ml. The protein in the isolated microsomes was determined by the method of Lowry et al. (17) using bovine serum albumin as a standard.

The lipid peroxidation of rat liver microsomes was conducted according to the method of Laudicina and Marnett (18) with slight modifications. A mixture of microsomal suspension (0.5 ml), 50 mM phosphate buffer (pH 7.4, 0.8 ml), 30 mM H_2O_2 (0.3 ml) and 3.3 mM $FeSO_4$ (0.3 ml) solution and sample solution (0.1 ml, final concentration of 1.0 ~ 10.0 µM) was incubated at 37°C for 20 min, and then terminated by cooling in ice-water. The antioxidative activity was determined by using the thiobarbituric acid (TBA) method described by Ohkawa et al. (19). The solution of 20% acetic acid/HCl (1.5 ml) and 0.8% TBA/NaOH (1.5 ml) was added to the reacted solution (1.0 ml), and then heated at 100°C for 20 min. After cooling in ice-water for 5 min, centrifugation was carried out at 3,000 rpm for 10 min, and then the absorbance of supernatant was measured at 535 nm. The inhibition rate of lipid peroxidation of each sample was calculated as follows: [1-(A_{535} of reaction mixture with sample/A_{535} of control)] × 100. IC_{50} values were determined as described previously.

RESULTS AND DISCUSSION

Identification of six isolated phenolic compounds

Six phenolic compounds isolated from the roasted safflower seeds were identified by UV, IR, ^{1}H-/^{13}C-NMR and MS spectrometry.

Serotonin **1** (S1) and **2** (S2) showed UV maxima absorption at about 288 nm and 315 nm. Their IR spectra exhibited the presence of NH, OH (3400 cm^{-1}) and NHCO (1650 cm^{-1}) groups. The ^{1}H-NMR, ^{13}C-NMR and MS spectra were similar to those of published literature (13,14). Thus, **S1** and **S2** was readily identified as N-feruloylserotonin and N-(*p*-coumaroyl) serotonin, respectively.

Lignan **1** (L1) exhibited UV maximum absorption at 225 and 280 nm. The IR spectrum showed the presence of hydroxyl (3400 cm^{-1}), aliphatic (2970 cm^{-1}), cyclic esteric (1770, 1520 cm^{-1}) and aromatic (1600-1400 cm^{-1}) groups. The ^{1}H-NMR spectrum showed that the benzylic protons at C-7,

together with H-8 and H-8′, give a complex multiplet at near δ 2.5, while the other benzylic protons (H-7′) are more deshielded due to the proximity of the lactone carbonyl and appear as double doublets at δ 2.81 & 2.88. The two protons at C-9 characteristically appear as double doublets at δ 3.92 and 4.15. The rest of the spectrum revealed that the presence of six aromatic protons, two methoxyl groups and two hydroxyl groups. The ^{13}C-NMR spectrum is almost identical to the literature data for matairesinol (20). In particular, the resonances for C-7 and C-7′ at δ 38.90 and 35.35, respectively, correlate with the previously demonstrated *trans* configuration (20). The EI-MS of **L1** showed a molecular ion peak at *m/z* 358 with a base peak at *m/z* 137 which was deduced to hydroxymethoxybenzyl group. Thus, **L1** was elucidated as matairesinol.

Lignan **2** (L2) exhibited the similar UV, IR spectra as compared with those of **L1**. The ^{1}H-NMR spectrum of **L2** was very similar to that of **L1**, except for showing two doublets at δ 2.85 & 3.11 (H-7) and a double doublet at δ 2.80 (H-7′) caused by attaching the hydroxyl group at C-8′. The two protons at C-9 also were shifted upfield somewhat, as compared to **L1**. The rest of the spectrum revealed the presence of six aromatic protons, three methoxyl groups and an exchangeable hydroxyl group. Meanwhile, the ^{13}C-NMR spectrum indicated that the signal of C-5 was shifted upfield by 2.94 ppm, whereas the signal of C-4 was displaced downfield by 2.45 ppm, respectively, as compared to that of **L1**. However, the signal of C-4′ was not affected nearly. This indicated that the methoxyl group was attached to C-4 of the benzene moiety. In addition, the signal of C-8′ was shifted downfield greatly, whereas the signals of C-7′ and C-9′ were displaced upfield, respectively, indicating that the hydroxyl group was attached to C-8′. Other ^{13}C-NMR spectra of **L2** were consistent with that of **L1**, and complements the structure determined by ^{1}H-NMR. The EI-MS spectrum showed a molecular ion peak at *m/z* 388 with intense fragments at *m/z* 137 and 151, corresponding to benzyl fragments with hydroxymethoxy and dimethoxy substituents, respectively. Based on the above evidence, the structure of **L2** was shown to be 8′-hydroxyarctigenin. Complete assignments for the ^{13}C-NMR and MS spectra of **L2** were made for the first time.

Flavonoid **1** (F1) and **2** (F2) displayed the typical UV (λ_{max} 269 & 325 nm) and IR (ν_{max} 3397, 1648, 1612, 1074, 842 cm^{-1}) spectra of flavone (21). The ^{1}H-NMR, ^{13}C-NMR and MS spectra of **F1** and **F2** were consistent with those of previous reports (21-24). Thus, **F1** and **F2** were readily elucidated as acacetin 7-O-β-D-glucoside (tilianine) and acacetin. The detailed assignments of six phenolic compounds for ^{1}H-NMR and ^{13}C-NMR spectra, as well as their chemical structures, are shown in Table 1, 2 and Fig. 2, respectively. Matairesinol and 8′-hydroxyarctigenin and tilianine were found for the first time in the roasted safflower seeds, although six phenolic derivatives have already been reported in the fresh and roasted safflower seeds (9,13,14,22). In addition, we found that six phenolic glycosides in the fresh safflower seeds are easily converted into phenolic aglycones during the roasted process (11).

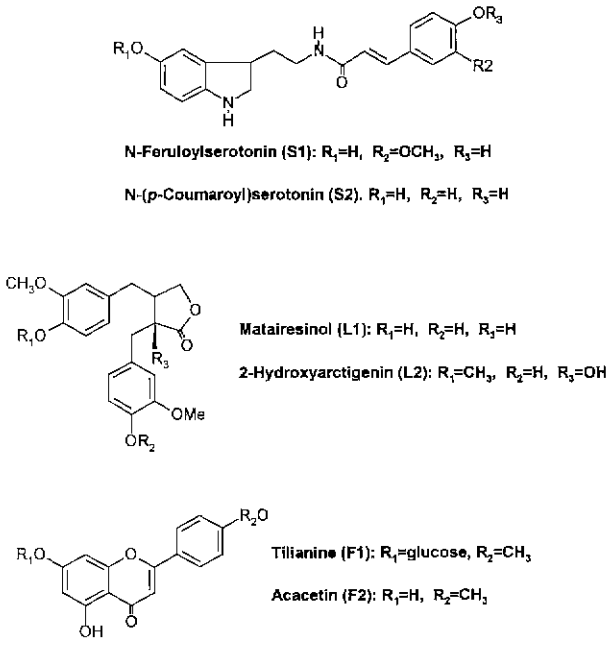

Fig. 2. Chemical structures of six phenolic compounds isolated from the roasted safflower seeds.

Antioxidative activity of isolated compounds in the DPPH radical system

The DPPH radical scavenging activity of six phenolic compounds isolated from the roasted safflower seeds is shown in Table 3. Among the isolated six compounds, two serotonins, the predominant components of the seeds, had more potent radical scavenging activity than that of α-tocopherol, BHA and L-ascorbic acid, well-known reference antioxidants. Two lignans also exhibited stronger activity, while two flavonoids showed less considerable activity. This observation supports previous reports that phenolic compounds containing NH and OH functional groups have a strong hydrogen donating or radical scavenging ability (25), and the methoxylation and glycosylation of the hydroxyl group in polyphenols causes a decrease in activity (26,27).

Antioxidative activity of isolated compounds in the rat liver microsome system

The antioxidative activity of six phenolic compounds in the rat liver microsome system is shown in Table 4. Two serotonins and acacetin exhibited much stronger antioxidative activity than that of α-tocopherol, a reference compound. Matairesinol also showed significant activity comparable to that of α-tocopherol. However, 8′-hydroxyarctigenin and tilianine showed weaker activity than their corresponding matairesinol and acacetin, indicating that addition of the hydroxyl and glucosyl groups to matairesinol and acacetin decreased antioxidative activity due to the low affinity of them for the lipid membranes (28). It is well known that hydroxyl radical is formed by the addition of H_2O_2 and Fe^{2+} in biological systems (29). Therefore, these findings suggest that the potent inhibitory effects of two serotonins, acacetin and matairesinol on lipid peroxidation induced by H_2O_2 and Fe^{2+} in micro-

Table 1. ^{1}H-NMR spectral data of six phenolic compounds isolated from the roasted safflower seeds (in CD_3OD)

	S1	S2	L1	L2	F1	F2
2	7.02 (s)	7.02 (s)	6.55 (d, *J*=2.0)	6.69 (d, *J*=3.0)		
3					6.67 (s)	6.54 (s)
4	6.95 (d, *J*=2.0)	6.95 (d, *J*=1.5)				
5			6.68 (d, *J*=8.0)	6.85 (d, *J*=7.5)		
6	6.66 (dd, *J*=2.5, 8.5)	6.65 (dd, *J*=2.5,7.5)	6.51 (dd, *J*=8.0, 2.0)	6.56 (dd, *J*=8.0, 2.0)	6.33 (d, *J*=2.0)	6.10 (d, *J*=2.0)
7	7.15 (dd, *J*=8.5)	7.15 (dd, *J*=2.0,6.5)	2.50 (m)	3.11 (d, *J*=14.0)		
				2.85 (d, *J*=14.0)		
8			2.66 (m)	2.48 (m)	6.65 (d, *J*=2.0)	6.32 (d, *J*=2.0)
9			3.92 (dd, *J*=8.3, 7.5)	3.84 (d, *J*=1.5)		
			4.15 (dd, *J*=8.8, 7.5)	3.97 (dd, *J*=8.0, 1.5)		
10	2.92 (t)	2.92 (t)				
11	3.57 (t)	3.56 (t)				
2′	7.11 (d, *J*=2.0)	7.38 (dd, *J*=2.5, 8.5)	6.66 (d, *J*=2.0)	6 85 (d, *J*=2.0)	7.92 (d, *J*=9.0)	7.91 (d, *J*=9.0)
3′		6.78 (dd, *J*=2.5, 8.5)			7.08 (d, *J*=9.0)	7.07 (d, *J*=9.0)
5′	6.78 (d, *J*=8.0)	6.78 (dd, *J*=2.5, 8.5)	6.70 (d, *J*=8.0)	6.85 (d, *J*=8.0)	7.08 (d, *J*=9.0)	7.07 (d, *J*=9.0)
6′	7.01 (d, *J*=2.0)	7 38 (dd, *J*=2 5, 8.5)	6.57 (dd, *J*=8.0, 2.0)	6.69 (dd, *J*=4.0, 1.5)	7.92 (d, *J*=9.0)	7.91 (d, *J*=9.0)
7′	7.43 (d, *J*=16.0)	7.44 (d, *J*=16)	2.81 (dd, *J*=14.0, 7.0)	2.80 (dd, *J*=14.0, 5.0)		
			2.88 (dd, *J*=14.0, 5.5)			
8′	6.41 (d, *J*=15.5)	6.39 (d, *J*=16)	2.66 (m)			
NH[1)]	9.75 (s)	9.78 (s)				
CONH[1)]	7.26 (t)	7.43 (t)				
OCH_3	3 87		3.78, 3.77	3.78, 3.77, 3.76	3.88	3.87
Glu-1					4.78 (d, *J*=7 34)	
Glu 2-6					3.18-3.77(m)	

Coupling constants (*J* in Hz) in parentheses. S, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.
[1)]Determined in DMSO-d_6

Table 2. ^{13}C-NMR spectral data of six phenolic compounds isolated from the roasted safflower seeds (in CD_3OD)

	S1	S2	L1	L2	F1	F2
1			131.45	133.40		
2	124.27	124.26	113.26	113.82	164.52	164.25
3	112.66	112.37	149.04	150.50	103.83	103.56
4	103.51	103 51	146.38	148.83	182.52	182.34
5	151.15	151.14	116.08	113.14	162.19	161.89
6	112.37	112.51	122.23	122.17	102.64	99.57
7	112.50	112.65	38.90	41.93	161.23	163.67
8	133.13	132.22	42.53	44.42	97.89	94.39
9	129.44	129.44	72.94	71.92	160.08	160.12
10	26.46	26.47			108.89	104.76
11	41.48	41.47				
1′	128.32	127.76	130.77	128.20	125.38	124.98
2′	111.52	130.55	113.89	114.92	129.45	129.11
3′	149.27	116.69	149.00	149.09	115.91	115.58
4′	149.80	160.09	146.19	146.69	162.87	162.40
5′	116.43	116.69	116.16	116.01	115.79	115.58
6′	123.21	130.55	123.04	124.06	129.41	129.12
7′	141.96	141.71	35.35	32.01		
8′	118.88	118.56	47.75	77.39		
9′			181.67	180.57		
CO	169.22	169.28				
OCH_3	56.37		56.33, 56.28	56.40, 56.52, 56 72	56.08	56.04
Glu-1					101.82	
Glu-2					73.12	
Glu-3					76.54	
Glu-4					72.43	
Glu-5					76.82	
Glu-6					61.13	

somes might be partly due to the action of hydroxyl radical scavengers. Recently, Roh et al. (12) reported that two serotonins in the roasted safflower seeds markedly inhibited the lipid peroxidation induced by ascorbic acid/Fe^{2-} and ADP/Fe^{3-}/NADPH in rat liver microsomes. Based on these results, the strong antioxidative activity of two serotonins, matairesinol and acacetin has been suggested to explain the claimed beneficial effects of safflower seeds on degenerative diseases such as osteoporosis and atherosclerosis. Futher study on the proliferative effects of six phenolic compounds on ROS 2.3W2

Table 3. DPPH radical scavenging activity of six phenolic compounds isolated from the roasted safflower seeds

Compound	IC_{50} (μM)
N-Feruoylserotonin	6.4±0.8
N-(*p*-Coumaroyl)serotonin	9.2±1.5
Matairesinol	52.6±4.3
8′-Hydroxyarctigenin	71.4±6.7
Tilianine	102.1±9.8
Acacetin	95.8±7.6
L-Ascorbic acid	34.3±4.3
α-Tocopherol	27.5±2.7
BHA	38.4±4.7

IC_{50} represents the sample concentration at which the absorbance shows 50% of control.
Values are shown as the mean±SD (n=3).
L-Ascorbic acid, α-tocopherol and BHA were used as positive references

Table 4. Inhibitory effects of six phenolic compounds isolated from the roasted safflower seeds on lipid peroxidation induced by H_2O_2 and $FeSO_4$ in rat liver microsome

Compound	IC_{50} (μM)
N-Feruoylserotonin	1.3±0.3
N-(*p*-Coumaroyl)serotonin	1.5±0.4
Matairesinol	2.3±0.7
8′-Hydroxyarctigenin	7.6±1.9
Tilianine	10.8±2.5
Acacetin	1.2±0.2
α-Tocopherol	4.4±1.3

IC_{50} represents the sample concentration required for 50% inhibition of lipid peroxidation.
Values are shown as the mean±SD (*n*=3).
α-Tocopherol was used as a positive reference.

osteoblast-like cells is now in progress.

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