J Food Sci Nutr
Vol 11, p 198~203 (2006)

J Food Science and Nutrition

Phenolic Compounds with Antioxidant Activity on DPPH Free Radical Scavenging and Inhibition of Xanthine/Xanthine Oxidase from the Flowers of *Chrysanthemum morifolium*

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Abstract

The flowers of *Chrysanthemum morifolium* (Compositae) were investigated for their biofunctional components. Antioxidant compounds were identified from the flower of *Chrysanthemum morifolium*, through activity-guided fractionation and repeated column chromatographic separations. The structures of the isolated compounds were characterized as chlorogenic acid (1), luteolin (2) and quercitrin (3) by the analysis of physico-chemical and spectral data. Their antioxidant effects were evaluated by assaying for 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity and inhibition of xanthine/xanthine oxidase activity.

Key words: *Chrysanthemum morifolium*, Compositae, chlorogenic acid, luteolin, quercitrin, antioxidant potentials, DPPH assay, xanthine/xanthine oxidase assay

INTRODUCTION

Chrysanthemum morifolium R. (Compositae) is a perennial plant herb widely distributed in Korea. Flowers of the plant have been used for preparing healthy teas and alcohol beverages (1). In recent studies, acidic polysaccharides (2), essential oils (3), flavonoid glucuronide (4), and triterpenoids (5) have been reported to be the bioactive components of *Chrysanthemum morifolium.*

Scavenging or trapping agents are compounds that physically react with the activated (electrophilic) forms of carcinogens and oxygen free radicals. In a part of our study on the biological activity of Korean teas, an ethyl acetate (EtOAc) soluble fraction of *Chrysanthemum morifolium* flowers exhibited strong anti-oxidant activity based on scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (6).

In this study, we evaluated DPPH free radical scavenging and xanthine/xanthine oxidase inhibitory effects of the compounds. Three strong antioxidant phenolic compounds designated **1**, **2** and **3** were isolated from the flowers of *Chrysanthemum morifolium* using an activity monitoring assay. Identification of chemical structures was elucidated on the basis of physico-chemical and spectroscopic parameters with 1D-NMR and 2D NMR experiments. Our results suggest that the flowers of *Chrysanthemum morifolium* can be used as a bio-functional material with antioxidant properties, and that it might also contribute to the prevention and/or delay of degenerative diseases by interfering with pathologies associated with oxidative stress.

MATERIALS AND METHODS

Materials

The flowers of *Chrysanthemum morifolium* flowers were collected in Kyungki Province, Korea in 2005 and dried. The dried flowers were stored at ambient temperature and milled just prior to the present investigation. DPPH, xanthine and xanthine oxidase were obtained from Sigma Chemical Co (St. Louis, USA). The compounds were dissolved in dimethylsulfoxide (DMSO) and stored at -20°C. All other chemicals were purchased from commercial sources and were of the highest purity available.

Instrumental analyses

Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. Electron impact mass (EI-MS) and fast atom bombardment mass (FAB-MS) spectrometry spectra were obtained on a Hewlett Packard model 5985B gas chromatograph (GC)/MS and Kratos MS890/DS90 high resolution mass spectrometer system, respectively. The ultraviolet (UV) and infrared (IR) spectra were recorded on a Hitachi 3100 UV/Vis and JASCO Fourier transform

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(FT)-IR-5300 spectrophotometer, respectively. A Bruker AMX500 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS), and DMSO-d_6 as an internal standard and NMR solvents, respectively.

General experiment

Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel 60 F_{254} plates; 0.25 mm layer thickness; Merck, Darmstadt, Germany), with compounds visualized by spraying with 10% $FeCl_3$ in methanol (MeOH) and Molisch reagent after developing samples. Silica gel (Merck 60 A, 230~400 mesh ASTM) and Sephadex LH-20 (25~100 μm; Pharmacia Fine Chemicals, Piscataway, NJ, USA) were used for open column and vacuum column chromatographic separations.

Extraction and isolation of bioactive compounds

The dried flowers of *Chrysanthemum morifolium* (800 g) were refluxed with 80% ethyl alcohol (EtOH) three times for three hours in a hot water bath. The combined ethanol extract was partitioned with organic solvents of different polarities to afford chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol (BuOH) and aqueous fractions, respectively. The solvent fractions were assayed before separating additional chromatographic sub-fractions, then, sub-fractions with the desired activity were utilized for the isolation of bioactive components. The EtOAc fraction (3.2 g) was further chromatographed using Sephadex LH-20 and silica gel with MeOH and $CHCl_3$-MeOH gradient solvents, respectively. The elutes from the condensation yielded solid principles which were further purified by re-crystallization with highly purified MeOH to isolate the pure compounds **1**, **2** and **3**.

Antioxidant activity assay

Assay for DPPH free radical scavenging activity: Reaction mixtures containing test samples (5 mL, dissolved in DMSO) and DPPH ethanol solution (95 mL, final DPPH concentration was 300 mM) in 96-well micro filter plates were incubated at 37°C for 30 min, and absorbances were measured at 517 nm. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. IC_{50} values denote the concentration of samples which were required to scavenge 50% of DPPH free radicals (7). On the basis of reaction conditions, and in order to confirm the usefulness of the assay, commercial antioxidants including ascorbic acid and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) were also evaluated for their free radical scavenging activities.

Assay for inhibition of xanthine/xanthine oxidase activity: Xanthine solution (100 μM, 988 μL) in sodium phosphate buffer, pH 7.8, with 2 μL of xanthine oxidase solution (0.04 units), and 10 μL of DMSO were incubated for 3 min at room temperature, and uric acid was measured at 295 nm against a blank sample which did not contain the enzyme, but 2 μL of 0.1 M phosphate buffer solution (pH 7.8) instead. Optical density was recorded for 3 min and the tests were performed in duplicate. Various concentrations of each test sample (10 μL dissolved in DMSO) were added to xanthine buffer solution (988 μL) and phosphate buffer solution (2 μL) as blank tests. Enzyme solution (0.04 units) was added to each 10 μL of various concentrations of test samples in 988 μL of xanthine buffer solution and treated in the same manner as the control. Inhibitory effects on xanthine oxidase activity were measured by a decrease in uric acid generation. The IC_{50} values were calculated from percent inhibition of enzyme activity (8).

Spectral data of compounds 1~3

Chlorogenic acid (1): White powder from MeOH; mp 156~158°C; UV λ_{max} (MeOH) (log ε): 245 (4.20), 258 (4.18) nm; IR (KBr) ν_{max} 3360 (OH), 1720, 1709, 1640, 1529 (aromatic C=C) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity, %): 354 $[M]^+$ (100), 318 $[M-2H_2O]^+$ (23.1), 270 $[M-2H_2O-COOH]^+$ (40.1); 180 $[M-C_7H_{10}O_5]^+$ (32.7).

Luteolin (2): Pale yellow plate from MeOH; mp 320°C; UV λ_{max} (MeOH) (log ε): 254 (4.32), 267, 297 (sh, 4.25), 360 (4.21) nm; λ_{max} (MeOH+NaOH) (log ε): 268 (4.80), 313 (sh, 4.62), 402 (4.78) nm; λ_{max} ($AlCl_3$) (log ε): 271 (4.82), 303 (sh, 4.33), 356 (4.23), 424 (4.90) nm; λ_{max} ($AlCl_3$+HCl) (log ε): 272 (sh, 4.70), 270(4.76), 301 (sh, 4.40), 385 (4.49) nm, λ_{max} (NaOAc) (log ε): 275 (4.86), 372 (4.21) nm; λ_{max} (NaOAc+H_3BO_3) (log ε): 262 (4.50), 307 (sh, 4.35), 372 (4.37) nm; IR (KBr) ν_{max} 3410 (OH), 1656 (α,β-unsaturated C=O), 1607, 1512, 1460 (aromatic C=C) cm^{-1}; EI-MS *m/z* (relative intensity, %) 287 $[M+1]^+$ (12.1), 286 $[M]^+$ (100), 258 $[M-CO]^+$ (24.5), 229 $[M-CO-CHO]^+$ (21.1), 153 $[A_1+H]^+$ (49.3), 152 $[A_1]^+$ (10.1), 134 $[B_1]^+$ (18.5), 124 $[A_1-CO]^+$ (15.2), 123 $[A_1-CHO]^+$ (10.2).

Quercitrin (3): Yellow crystal from MeOH; mp 238~240°C; UV λ_{max} (MeOH) (log ε): 257 (433), 267 (sh, 4.26), 359 (4.20) nm; λ_{max} (MeOH+NaOH) (log ε): 280 (4.82), 315 (sh, 4.67), 420 (4.80) nm; λ_{max} ($AlCl_3$) (log ε): 272 (4.80), 303 (sh, 4.34), 350 (4.21), 430 (4.18) nm; λ_{max} ($AlCl_3$+HCl) (log ε): 272 (sh, 4.71), 270 (4.80), 304 (sh, 4.41), 424 (4.90) nm, λ_{max} (NaOAc) (log ε): 275 (4.86), 375 (4.49) nm; λ_{max} (NaOAc+H_3BO_3) (log ε): 265 (4.56), 305 (sh, 4.36), 368 (4.20) nm; IR (KBr) ν_{max} 3410 (OH), 1655 (α,β-unsaturated C=O), 1607,

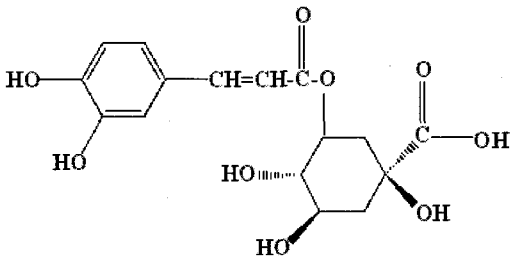
1510 (aromatic C=C), 1365, 1210, 1087 (glycosidic C-O) cm^{-1}; Positive FAB-MS *m/z* 541 $[M+glycerol+1]^+$, 449 $[M+1]^+$, 285 [M+1-Rhamnose]$^+$, 267 [M+1-Rhamnose-H_2O]$^+$; 249 [M+1-Rhamnose-$2H_2O$]$^+$, 221 [M+1-Rhamnose-$2H_2O$-CO]$^+$.

RESULTS AND DISCUSSION

Isolation and structure elucidation of compounds 1~3

The 80% ethanol extract of the flowers of *Chrysanthemum morifolium* was evaluated initially for antioxidant activity with 75.6% inhibition at a final concentration of 100 μg/mL using the DPPH free radical scavenging test system (6).

The extract was fractionated with $CHCl_3$, EtOAc, *n*-BuOH and aqueous soluble fractions. The EtOAc soluble fraction of *Chrysanthemum morifolium* flowers which had IC_{50} values of 30.1 μg/mL and 18.0 μg/mL in the DPPH free radical scavenging and xanthine/xanthine oxidase inhibition assay, respectively, were subjected to a series of activity-guided chromatographic separation steps to afford compounds **1**, **2** and **3** (Fig. 1). The pure isolates gave characteristic phenol color changes in $FeCl_3$ color reactions.



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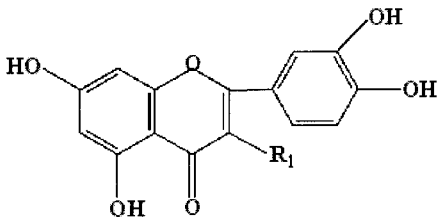

2 : R_1=H
3 : R_1=O-Rhamnose

Fig. 1. Chemical structure of compounds **1**~**3** from the flowers of *Chrysanthemum morifolium*.

Table 1. ^{1}H-NMR and ^{13}C-NMR spectral data of compounds **1**, **2** and **3**

Position	δ $^1H^{1)}$ (mult., *J* in Hz)			^{13}C		
	1	2	3		5	6
1				66.5		
2_α	1.90 (d, 13.4)			41.0	157.6	156.0
2_β	1.79 (d, 13.4)					
3	3.76	6.67		74.5	119.8	134.9
4	3.25			69.1	177.1	177.9
5					158.5	157.2
6_α	1.87 (d, 13.2)	6.22 (d, 1.8)	6.22 (d, 2.0)	37.4	99.0	98.6
6_β	1.72 (d, 13.2)					
7					166.0	165.1
8		6.58 (d, 1.8)	6.50 (d, 2.0)		94.7	94.6
9					161.5	161.0
10					105.6	105.1
1′				121.8	119.8	120.1
2′	7.01 (d, 1.8)	7.43 (dd, 1.8)	7.25 (d, 2.0)	116.7	121.1	121.0
3′				144.9	144.8	145.1
4′				150.1	135.2	134.5
5′	6.78 (d, 8.2)	6.92 (d, 8.4)	6.86 (d, 8.4)	115.3	115.8	116.0
6′	7.04 (dd, 1.8, 8.2)	7.41 (dd, 1.8, 8.4)	7.21 (dd, 2.0, 8.4)	122.3	120.2	120.7
5,7-OH		10.7, 12.0	10.9, 12.1			
CH_α	7.42 (d, 15.9)			40.5		
CH_β	6.19 (d, 15.9)			37.8		
CH_3			0.80 (d, 6.0)			17.9
COOH	12.4			175.4		
1″			5.26 (d, 2.0)			101.7
2″			4.01			70.5
3″			3.95			70.9
4″			3.16			71.0
5″			3.20			69.9

[1)]TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values in parenthesis.

Compound **1** was formulated as $C_{16}H_{18}O_9$ and obtained as a white powder, with a molecular weight of *m/z* 354 based on EI-MS data. The ^{1}H-NMR spectrum of **1** showed the presence of four methylenes δ 1.72 and 1.87 (J=13.2 Hz, H-6) and δ 1.79 and 1.90 (J=13.4 Hz, H-2), and one of benzene ring attached with quinic acid. The ^{13}C-NMR spectrum of **1** showed four methylenes, four oxygen bearing methines and two carboxyl groups. Assignments of each proton and carbon were confirmed by ^{1}H-^{1}H COSY and HMQC spectra. On the basis of these data and literature (9), the structure of compound **1** was identified as chlorogenic acid (Table 1). It has been reported that compound **1** is rapidly oxidized by peroxynitrite in a concentration and pH-dependent manner, and that it inhibits the formation of single strand breaks and nitration by peroxynitrite (10).

Compound **2** was formulated as $C_{15}H_{10}O_6$ and obtained as pale yellow plates with a molecular weight of *m/z* 286 based on EI-MS data. It contained a flavone structure by color reaction, Retro-Diels Alder (RDA) MS fragmentation pattern (Fig. 2) and ^{1}H-NMR spectral data. Compound **2** showed bathochromic shifts of band in the presence of NaOAc which indicated the presence of a C-7 free hydroxyl group. A bathochromic shift of NaOMe, without a decrease in intensity of band I, indicated the presence of C-4′ free hydroxyl groups, and a hypsochromic shift in band I of the $AlCl_3$ on addition of acid resulted the presence of *ortho*-dihydroxyl groups of B-ring (11). The ^{1}H-NMR spectrum showed that three aromatic proton signals at δ7.43, δ6.92 and δ7.41 were assigned to H-2′, H-5′ and H-6′ in B-ring on the basis of coupling constants and the data from the ^{1}H-^{1}H-COSY spectrum. The two *meta* coupled protons at δ6.22 and δ6.58 were assigned to H-6 and H-8 in A-ring. On the basis of these data and literature (12), compound **2** was identified as luteolin (Table 1). Compound **2** is widely distributed in many plants, and it has various biological activities, such as antioxidant, anti-inflammatory, cancer preventive and anti-arrhythmic activities (13).

Compounds **3** was formulated as $C_{21}H_{20}O_{11}$, and gave positive flavonoid color reactions according to purplish brown with $FeCl_3$, yellow with NaOH, yellowish orange with Mg-HCl, pink with Zn-HCl, and glycoside reaction with black green color in the Molisch test. In UV spectrum of compound **3**, 359 nm of Band I in MeOH spectra was very similar to those reported for a number of 3-hydroxy-substituted flavonols. Compound **3** exhibited bathochromic shifts of band in the presence of NaOAc which indicated the presence of C-7 free hydroxyl groups. A bathochromic shift of NaOMe, without a decrease in intensity of band I, indicated the presence of C-4′ free hydroxyl groups, and a hypsochromic shift in band I of

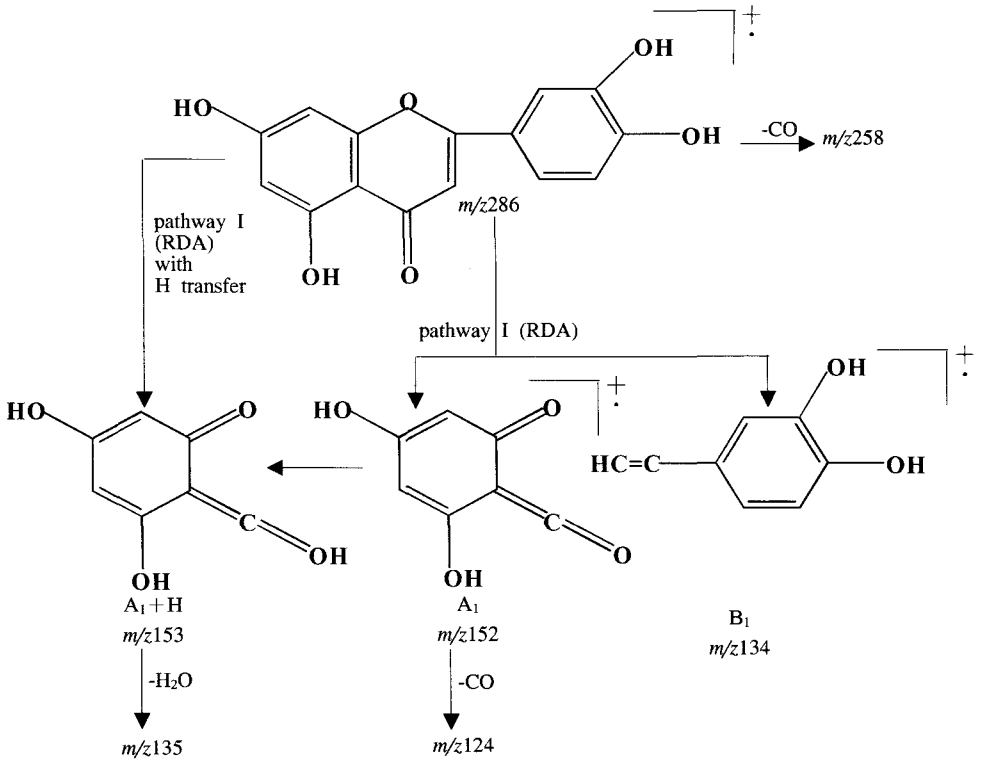

Fig. 2. Retro-Diels Alder MS fragmentation of compound **2**.

the $AlCl_3$ on addition of acid resulted the presence of *ortho*-dihydroxyl groups of B-ring (11). The ^{1}H-NMR spectra of compound **3** showed one anomeric proton signal at δ5.26 (J=2.0 Hz), two *meta*-coupled doublets ascribable to H-8 at δ6.50 and H-6 at δ6.22 of A-ring, and an *ortho*-coupled doublet, a *meta*-coupled doublet and a *ortho*, *meta*-coupled doublet-doublets attributable to H-2′ at δ7.25, H-3′ at δ6.86 and H-6′ at δ7.21 of B-ring. The sugar moiety of compound **3** was determined to be α-L-rhamnopyranose by the J values of the anomeric proton signal and ^{13}C-NMR data (Table 1). From these data and literature (14), compound **3** was characterized as quercitrin (quercetin-3-*O*-α-L-rhamnopyranoside).

Evaluation of the antioxidant activity of compounds

Assay for free radical scavenging activity: Bioassay-guided fractionation and separation of the 80% EtOH extract of the flowers of *Chrysanthemum morifolium* led to the isolation of three compounds isolated from EtOAc soluble fraction with moderate antioxidant activity for DPPH free radical scavenging and xanthine/xanthine oxidase inhibition. The antioxidant activity of compounds **1**, **2** and **3** was evaluated with the DPPH assay, using a free radical which shows a characteristic absorption at 517 nm. As shown in Table 2, compounds exhibited strong antioxidant activity with IC_{50} values of 30.1, 37.4 and 20.2 μg/mL, respectively, in the DPPH free radical scavenging assay. The DPPH test provides information on the reactivity of test samples with stable free radicals. Due to its odd electron, DPPH gives a strong absorption band at 517 nm (deep violet color). In assay systems, antioxidants can react with the stable free radical DPPH and convert it to 1,1-diphenyl-2-picrylhydrazine (15). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The change in absorbance produced in this reaction was assessed to evaluate the antioxidant activity of isolated compounds **1**, **2** and **3**.

Table 2. Antioxidant activity of compounds **1**~**3** by DPPH free radical scavenging and xanthine/xanthine oxidase assay

Compounds	DPPH[1)]	Xanthine/Xanthine oxidase[2)]
1	30.1	19.5
2	37.4	10.9
3	20.2	36.1
Ascorbic acid[3)]	22.4	71.1
BHA[3)]	20.6	>100

[1)]DPPH free radical scavenging activity (IC_{50}: μg/mL).
[2)]Xanthine/Xanthine oxidase activity (IC_{50}: μg/mL).
[3)]Control compounds.

Oxygen free radicals, or reactive oxygen species (ROS), are continuously generated by most cells and may be involved in several disease processes by damaging cellular molecules and structures (16). An important feature of free radical reactions with non-radicals is that they result in new radicals, which leads to chain reactions (17). In addition, reactive oxygen species are indeed a relevant class of carcinogens and can act at several stages in malignant transformation (18,19).

Assay for inhibition of xanthine/xanthine oxidase activity: Xanthine oxidase catalyses the hydroxylation of many purine substrates and converts hypoxanthine to xanthine, and then, uric acid in the presence of molecular oxygen to yield superoxide anion. The antioxidant activity of test samples can be measured by inhibition of reactive oxygen species formation generated through the xanthine/xanthine oxidase system. Uric acid formation in the reaction mixture was monitored for 3 min using a UV/VIS spectrophotometer. The superoxide anion (O_2^-)-scavenging capacity and inhibition of xanthine oxidase were studied by measuring the formation of uric acid at 295 nm using the xanthine/xanthine oxidase system.

As shown in Table 2, compounds **1**, **2** and **3** exhibited inhibitory effects on this enzyme-based antioxidant activity assay with IC_{50} values of 19.5, 10.9 and 36.1 μg/mL, respectively. A possible explanation for this is that the compounds **1**~**3** could form inter-molecular hydrogen bonding between the carboxylic acid and a hydroxyl group in the aromatic ring of compound **1** or between the hydroxyl groups in the compounds **2** and **3**. Phenolic compounds exhibit inhibitory effects on xanthine oxidase activity and inhibitory effects on TPA induced tumor formation in mouse skin, with an increase in the release of reactive oxygen species. It has also been reported that reactive oxygen species and other free radicals play an important role in tumor promotion (20,21). Thus, xanthine oxidase inhibitors should be considered to be potential antioxidants and thus tumor promotion inhibitors. Natural and/or synthetic antioxidants, by trapping electrophilic sites on activated carcinogens, scavenge free radicals, terminate lipid peroxidation, and enhance electrophile trapping potential by including Phase II metabolizing enzymes, have the potential to inhibit mutations in genes currently known to be associated with tumorigenensis.

From these results, we may conclude that the flowers of *Chrysanthemum morifolium* can be used as the biofunctional materials with antioxidant properties, and that they also might contribute to the prevention and/or delay of degenerative diseases by interfering with pathologies

associated with oxidative stress. In conclusion, phenolic acids such as chlorogenic acid, luteolin and quercitrin might be the active components responsible for the antioxidant activity of the flowers of *Chrysanthemum morifolium.*

ACKNOWLEDGEMENT

This research was supported by Institute of Natural Sciences, Duksung Women's University, Seoul, Korea (2005).

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(Received August 7, 2006; Accepted September 6, 2006)