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Antioxidant Principles of *Nelumbo nucifera* Stamens

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In our ongoing study to identity antioxidants from natural sources, the antioxidant activity of *Nelumbo nucifera* stamens was evaluated for their potential to scavenge stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, inhibit total reactive oxygen species (ROS) generation, in kidney homogenates using 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and scavenge authentic peroxynitrites ($ONOO^-$). A methanol (MeOH) extract of the stamens of *N. nucifera* showed strong antioxidant activity in the $ONOO^-$ system, and marginal activity in the DPPH and total ROS systems, so were therefore fractionated with several organic solvents, such as dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The EtOAc soluble fraction, which exhibited strong antioxidant activity in all the model systems tested, was further purified by repeated silica gel and Sephadex LH-20 column chromatographies. Seven known flavonoids [kaempferol (**1**), kaempferol 3-*O*-β-D-glucuronopyranosyl methylester (**2**), kaempferol 3-*O*-β-D-glucopyranoside (**3**), kaempferol 3-*O*-β-D-galactopyranoside (**4**), myricetin 3′,5′-dimethylether 3-*O*-β-D-glucopyranoside (**5**), kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**6**) and kaempferol 3-*O*-β-D-glucuronopyranoside (**7**)], along with β-sitosterol glucopyranoside (**8**), were isolated. Compound **1** possessed good activities in all the model systems tested. Compounds **2** and **7** showed scavenging activities in the DPPH and $ONOO^-$ tests, while compounds **3** and **4** were only active in the $ONOO^-$ test. Conversely, compound **8** showed no activities in any of the model systems tested.

Key words: *Nelumbo nucifera*, Nymphaeceae, Flavonoids, Antioxidant, Free radical, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, Reactive oxygen species (ROS), Peroxynitrite ($ONOO^-$)

INTRODUCTION

Reactive oxygen and nitrogen species (ROS and RNS, respectively), such as superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), singlet oxygen ($^1O_2$), alkoxyl radical (RO·), peroxyl radical (ROO·) and peroxynitrite ($ONOO^-$), are generated under normal and/or pathological conditions. These ROS and RNS are likely to damage several cellular molecules, such as lipids, proteins, nucleic acids and DNA, due to oxidation and nitration, and cause inflammation, or lesions, of various organs (Beckman *et al.*, 1990). Also, these reactive species are associated with various degenerative diseases, such as cancer (Dreher *et al.*, 1996), aging (Sohal, 2002), and arteriosclerosis and rheumatism (Griffiths and Lunec, 1996; Squadrito and Pryor, 1998; Choi *et al.*, 2002).

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There has been much research on the powerful, nontoxic natural antioxidants from edible or medicinal plants to prevent not only these oxidative stress-induced disorders in humans, but also the oxidative deterioration of food by auto-oxidation and lipid peroxidation. Many naturally occurring compounds from plant sources have also been reported to retard the oxidation process, in their natural environment, by acting as free radical scavengers, reactive oxygen scavengers or as reducing agents *in vitro* (Pratt, 1994; Larson, 1988).

In our ongoing investigation to identify natural antioxidants, we have dedicated much time to the study of *Nelumbo nucifera* Gaertner, from the Nymphaeceae family. This edible, medicinal plant is an aquatic, perennial herb, which is distributed in all over the world, including Korea (Van Bergen *et al.*, 1997; Kim, 1996). Due to its various therapeutic effects, it has been used as a traditional folk medicine for the treatment of diarrhea, gastritis, insomnia, nervous prostration and as a haemostatic in Korea and other countries, such as China and India (Kim, 1997; La Cour *et al.*, 1995). The biological activities of *N. nucifera*,

such as anti-diarrheic (Mukjerjee *et al.,* 1995), psycho-pharmacological (Mukjerjee *et al.,* 1996), hypoglycemic (Mukjerjee *et al.,* 1997), hypolipemic (La Cour *et al.*, 1995), anti-pyretic (Sinha *et al.*, 2000) and antioxidant activities (Liou *et al.*, 1999; Guo *et al.*, 2000) have all previously been reported.

In the present study, the antioxidant activity of the methanol (MeOH) extract, and its organic solvent soluble fractions, such as those from dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and the water (H_2O) layer, from the stamens of *N. nucifera,* were evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, total ROS and $ONOO^-$ scavenging/inhibitory tests. The isolation and identification of seven known flavonoids (**1**-**7**), together with compound **8** from the active EtOAc fraction of *N. nucifera* stamens was also investigated. Furthermore, the antioxidant properties of these isolated flavonoids (**1**-**4** and **7**) were measured by three model systems, those being the DPPH radical, total ROS and $ONOO^-$ model systems.

MATERIALS AND METHODS

Plant materials

The stamens of *N. nucifera* were purchased in April 2000 from the herbal medicine co-operative association of Busan Province, Korea, and authenticated by Hae Young Chung, professor of the College of Pharmacy, Pusan National University. A voucher specimen has been deposited in the Herbarium of the Medicinal Plant Garden, the College of Pharmacy, Pusan National University.

Chemicals

The DPPH, L-ascorbic acid, Trolox and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The high quality DCFH-DA and DHR 123 (dihydrorhodamine 123), and $ONOO^-$ were purchased from Molecular Probes (Eugene, Oregon, USA) and Cayman (Ann Arbor, MI, USA), respectively.

General experimental procedures

The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian UNITY-400 spectrometer. Chemical shifts were referenced to the respective residual solvent peaks, and the values recorded in δ. The HMQC and HMBC spectra were recorded using pulsed field gradients, rather than phase cycling, for coherence pathway selection. The multiplicities of the ^{1}H- and ^{13}C-NMR signals are indicated as s (singlet), d (doublet) and m (multiplet). The column chromatography was performed with silica gel (Merck, 70-230 mesh), and the TLC on precoated Merck Kieselgel 60 F_{254} plate (0.25 mm), and spots detected using 50% H_2SO_4 reagent.

Isolation of compounds 1-8

The dried *Nelumbo nucifera* stamens (3.4 kg) were refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40°C, to render the MeOH extract (560 g). This extract was suspended in H_2O and then successively partitioned with CH_2Cl_2, EtOAc and *n*-BuOH, to afford the CH_2Cl_2 (330 g), EtOAc (20 g), and *n*-BuOH fractions (66 g), and the H_2O residue (140 g). The EtOAc extract (20 g) was fractionated with Sephadex LH-20, using MeOH, to obtain 5 fractions (Fr. 1-Fr. 5). Fractions 1 (3.5 g) and 2 (5.7 g) were separately purified by Sephadex LH-20, with MeOH, to obtain compounds **8** (100 mg) and **5** (25 mg). Fraction 3 (9.7 g) was subjected to column chromatography on a Si gel column, with gradient elution, using an EtOAc:MeOH eluent (10:1, gradient to MeOH), to give compounds **2** (20 mg), **3** (450 mg), **4** (180 mg) and **6** (45 mg). Fractions **4** (1.5 g) and **5** (2.0 g) were recrystallized to obtain compounds **7** (80 mg) and **1** (260 mg), respectively.

Kaempferol (1)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.19 (1H, d, J = 2.0 Hz, H-6), 6.44 (1H, d, J = 2.0 Hz, H-8), 6.92 (2H, d, J = 8.0 Hz, H-3′, 5′), 8.06 (2H, d, J = 8.0 Hz, H-2′, 6′), 12.47 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 175.9 (C-4), 163.9 (C-7), 160.7 (C-5), 159.2 (C-4′), 156.2 (C-9), 146.8 (C-2), 135.7 (C-3), 129.5 (C-2′, 6′), 121.7 (C-1′), 115.4 (C-3′, 5′), 103.0 (C-10), 98.2 (C-6), 93.5 (C-8).

Kaempferol 3-*O*-β-D-glucuronopyranosyl methyl ester (2)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.57 (1H, s, OMe), 5.47 (1H, d, J = 7.4 Hz, H-1″), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.43 (1H, d, J = 2.0 Hz, H-8), 6.89 (2H, d, J = 8.0 Hz, H-3′, 5′), 8.02 (2H, d, J = 8.0 Hz, H-2′, 6′), 12.51 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.2 (C-4), 169.0 (C-6″), 164.3 (C-7), 161.2 (C-5), 160.2 (C-4′), 156.6 (C-9), 156.4 (C-2), 133.0 (C-3), 130.9 (C-2′, 6′), 120.6 (C-1′), 115.1 (C-3′, 5′), 104.0 (C-10), 101.4 (C-1″), 98.9 (C-6), 93.8 (C-8), 75.6 (C-5″), 75.5 (C-3″), 73.9 (C-2″), 71.5 (C-4″), 51.9 (OMe).

Kaempferol 3-*O*-β-D-glucopyranoside (3)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.47 (1H, d, J = 7.3 Hz, H-1″), 6.21 (1H, J = 2.0 Hz, H-6), 6.44 (1H, d, J = 2.0 Hz, H-8), 6.88 (2H, d, J = 8.0 Hz, H-3′, 5′), 8.05 (2H, d, J = 8.0 Hz, H-2′, 6′), 12.62 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.6 (C-4), 164.2 (C-7), 161.3 (C-5), 160.0 (C-4′), 156.5 (C-9), 156.4 (C-2), 133.3 (C-3), 131.0 (C-2′, 6′), 121.0 (C-1′), 115.2 (C-3′, 5′), 104.1 (C-10), 100.9 (C-1″), 98.8 (C-6), 93.7 (C-8), 77.6 (C-5″), 76.5 (C-3″), 74.3 (C-2″), 70.0 (C-4″), 60.9 (C-6″).

Kaempferol 3-*O*-β-D-galactopyranoside (4)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.41 (1H, d, *J* = 7.7 Hz, H-1″), 6.21 (1H, *J* = 2.0 Hz, H-6), 6.43 (1H, d, *J* = 2.0 Hz, H-8), 6.86 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 8.08 (2H, d, *J* = 8.0 Hz, H-2′, 6′), 12.63 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.6 (C-4), 164.2 (C-7), 161.2 (C-5), 160.0 (C-4′), 156.4 (C-9), 156.4 (C-2), 133.2 (C-3), 131.0 (C-2′, 6′), 120.9 (C-1′), 115.1 (C-3′, 5′), 104.0 (C-10), 101.7 (C-1″), 98.7 (C-6), 93.7 (C-8), 75.8 (C-5″), 73.1 (C-3″), 71.2 (C-2″), 67.9 (C-4″), 60.2 (C-6″).

Myricetin 3′,5′-dimethylether 3-*O*-β-D-glucopyranoside (5)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.84 (6H, s, OCH_3), 5.59 (1H, d, *J* = 7.3 Hz, H-1″), 6.22 (1H, *J* = 2.1 Hz, H-6), 6.50 (1H, d, *J* = 2.1 Hz, H-8), 7.49 (2H, s, H-2′, 6′), 9.17 (1H, brs, OH), 10.87 (1H, brs, OH), 12.60 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-9), 156.3 (C-2), 147.4 (C-3′, 5′), 138.6 (C-4′), 133.1 (C-3), 119.8 (C-1′), 106.9 (C-2′, 6′), 104.0 (C-10), 100.7 (C-1″), 98.7 (C-6), 93.8 (C-8), 77.4 (C-5″), 76.4 (C-3″), 74.3 (C-2″), 69.8 (C-4″), 60.5 (C-6″), 56.2 (OCH_3).

Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (6)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.97 (3H, s, *J* = 6.2 Hz, CH_3), 4.38 (1H, s, H-1‴), 5.30 (1H, d, *J* = 7.4 Hz, H-1″), 6.23 (1H, *J* = 2.1 Hz, H-6), 6.45 (1H, d, *J* = 2.1 Hz, H-8), 6.89 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 7.97 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 10.22 (1H, brs, OH), 11.01 (1H, brs, OH), 12.55 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.4 (C-4), 164.2 (C-7), 161.1 (C-5), 160.0 (C-4′), 156.8 (C-9), 156.4 (C-2), 133.2 (C-3), 130.8 (C-2′, 6′), 120.8 (C-1′), 115.1 (C-3′, 5′), 103.9 (C-10), 101.3 (C-1″), 100.7 (C-1‴), 98.7 (C-6), 93.7 (C-8), 75.1 (C-5″), 76.4 (C-3″), 74.1 (C-2″), 71.8 (C-4‴), 70.6 (C-2‴), 70.3 (C-3‴), 69.9 (C-4″), 68.2 (C-5‴), 66.9 (C-6″), 17.7 (C-3‴).

Kaempferol 3-*O*-β-D-glucuronopyranoside (7)

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.43 (1H, d, *J* = 7.2 Hz, H-1″), 6.15 (1H, d, *J* = 2.0 Hz, H-6), 6.36 (1H, d, *J* = 2.0 Hz, H-8), 6.86 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 8.02 (2H, d, *J* = 8.0 Hz, H-2′, 6′), 12.48 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.4 (C-4), 171.4 (C-6″), 164.5 (C-7), 161.1 (C-5), 160.1 (C-4′), 156.4 (C-9), 156.4 (C-2), 133.2 (C-3), 131.0 (C-2′, 6′), 120.7 (C-1′), 115.1 (C-3′, 5′), 103.8 (C-10), 101.3 (C-1″), 98.8 (C-6), 93.8 (C-8), 76.1 (C-5″), 75.2 (C-3″), 73.9 (C-2″), 71.8 (C-4″).

Determination of the scavenging effect on DPPH radicals

The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (1958). A hundred and sixty microliters (μL) of a MeOH solution of various sample concentrations (10-320 μL/mL) was added to 40 μL of a DPPH methanol solution (1.5×10^{-4} M). After mixing gently, and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a microplate reader spectrophotometer VERSAmax (Molecular Devices, CA, USA). The antioxidant activity of each sample was expressed in terms of IC_{50} (μg/mL or μM required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

Measurement of the inhibition of the total ROS generation

Rat kidney homogenates, prepared from the kidneys of freshly killed male Wistar rats, weighing 150-200 g, were mixed with or without a suspension of extracts/or compounds, then incubated with 12.5 μM DCFH-DA, at 37°C for 30 min. Fifty mM of pH 7.4 phosphate buffer was used. DCFH-DA is a stable compound, which easily diffuses into cells, and is hydrolyzed by intracellular esterase to yield a reduced non-fluorescent compound, DCFH, which is trapped within cells. The ROS produced by cells oxidize the DCFH to the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively (Label and Bondy, 1990).

Measurement of the $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123, by modifying the method of Kooy *et al.* (1994). The DHR 123 (5 mM), in dimethylformamide, was purged with nitrogen, stored at -80°C and used as a stock solution. This solution was then placed on ice, and kept from exposure to light, prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which were prepared with high quality deionized water, and purged with nitrogen. The final concentration of the DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment, both with and without the addition of authentic $ONOO^-$. The DHR 123 was oxidized rapidly by authentic $ONOO^-$, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.), with excitation and emission wavelengths of 480 and 530 nm, respectively. The results were expressed as the mean±standard error (*n*=3) of the final fluorescence

intensity minus the background fluorescence. The effects were expressed as the percentage of inhibition of the DHR 123 oxidation.

Statistical analysis

All values were expressed as the mean±standard error of three or five replicate experiments.

RESULTS AND DISCUSSION

The formation of reactive species, such as H_2O_2, O_2^-, ·OH, NO· and $ONOO^-$, in the body, as a consequence of aerobic metabolism, play roles in the pathogenesis of human degenerative diseases (Pincemail, 1995; Beckman *et al.*, 1990), and are also likely to damage intracellular components, such as nucleic acids, proteins and lipids (Sagar *et al.*, 1992; Ames *et al.*, 1993).

In our ongoing investigations to identify powerful, nontoxic and natural antioxidants, we evaluated general antioxidants for their potential to scavenge authentic $ONOO^-$ and DPPH radicals as well as to inhibit the total ROS generation of the MeOH extract, along with its solvent soluble fractions, including the CH_2Cl_2, EtOAc and *n*-BuOH fractions, as well as the H_2O layer derived from *N. nucifera* stamens. As summarized in Table I, the EtOAc, *n*-BuOH and H_2O fractions possessed good scavenging activities for the DPPH radical, with 50% inhibition concentration (IC_{50}) values of 13.5, 34.5 and 37.5 μg/mL respectively, compared to the 6.2 μg/mL of the well-known antioxidant, L-ascorbic acid. In the total ROS system, the IC_{50} values of these three fractions were 7.81±0.91, 42.25±0.33 and 64.64± 3.35 μg/mL, respectively, with the EtOAc fraction especially exhibiting more potent inhibitory activity than the positive control Trolox (IC_{50} 28.42±1.21 μg/mL). The MeOH extract, and all the fractions from the stamens of *N. nucifera,* showed marked scavenging activity on the authentic $ONOO^-$ in the following order: EtOAc fraction (IC_{50} 0.11± 0.06 μg/mL) > *n*-BuOH fraction (IC_{50} 3.41±0.26 μg/mL) > MeOH extract (IC_{50} 18.90±2.14 μg/mL) > H_2O layer (IC_{50} 28.01±1.21 μg/mL) > CH_2Cl_2 fraction (IC_{50} 35.52±1.01 μg/ mL). The $ONOO^-$ scavenging activities of the EtOAc and *n*-BuOH fractions were about eight and two time as good, respectively, as that of the positive control (penicillamine), which has an IC_{50} value of 8.27±0.06 μg/mL. These results suggest that there are likely to be many antioxidants in the EtOAc soluble fraction, so much attention should be given to the isolation of the antioxidative compounds from this fraction.

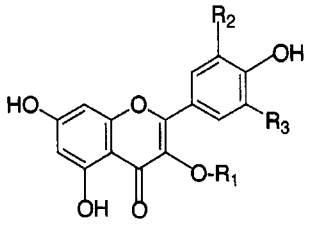
Table I. Antioxidant activities of the MeOH extract and its solvent soluble fractions of the stamens of *N. nucifera*

Samples	DPPH[a] IC_{50} (μg/mL)	Total ROS[b] IC_{50} (μg/mL) Mean±SE[d]	$ONOO^-$[c] IC_{50} (μg/mL) Mean±SE[d]
MeOH	273.5	> 100	18.90±2.14
CH_2Cl_2	279.0	> 100	35.52±1.01
EtOAc	13.5	7.81±0.91	0.11±0.06
n-BuOH	34.5	42.25±0.33	3.41±0.26
H_2O	37.5	64.64±3.35	28.01±1.21
L-Ascorbic acid	6.2		
Trolox		28.42±1.21	
Penicillamine			8.27±0.06

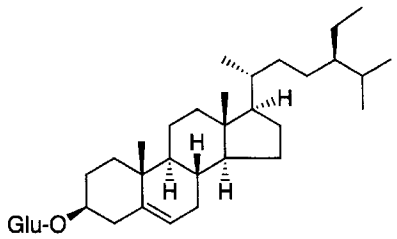
[a]DPPH is the free radical scavenging activity (IC_{50}: μg/mL). [b]Total ROS is the inhibitory activity of the total free radical generation in the kidney postmicrosomal fraction (IC_{50}: μg/mL). [c]$ONOO^-$ is the inhibitory activity of authentic peroxynitrite (IC_{50}: μg/mL). [d]Values of total ROS and $ONOO^-$ were expressed as the mean±standard error of three or five experiments.

The EtOAc soluble part of the MeOH extract, from the stamens of *N. nucifera,* repeatedly underwent chromatography on the Si gel and Sephadex LH-20 to yield compounds **1**-**8**. The structural identifications of these compounds were elucidated from the 1D (^{1}H- and ^{13}C-NMR) and 2D NMR (HMQC and HMBC) spectral data, and by comparison with published spectral data (Park *et al.*, 1991; Mpalantinos *et al.*, 1998; Agrawal, 1992; Pauli, 2000; Ismail and Alam, 2001; Sung *et al.*, 1997). The isolated compounds were readily elucidated as: kaempferol (**1**), kaempferol 3-*O*-β-D-glucuronopyranosyl methylester (**2**), kaempferol 3-*O*-β-D-glucopyranoside (**3**), kaempferol 3-*O*-β-D-galactopyranoside (**4**), myricetin 3′,5′-dimethylether 3-*O*-β-D-glucopyranoside (**5**), kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**6**), kaempferol 3-*O*-β-D-glucuronopyranoside (**7**) and β-sitosterol glucopyranoside (**8**). The chemical structures of compounds **1**-**8,** from the stamens of *N. nucifera, are* shown in Fig. 1. Each chemical shift, corresponding to the aglycone of seven compounds **1**-**4**, **6** and **7** were similar to those of the ^{1}H- and ^{13}C-NMR spectra of kaempferol. This indicated that the compounds **2**-**4**, **6** and **7** were kaempferol glycosides and their structures were further confirmed from the HMQC and HMBC spectroscopies, as well as by comparison with the published NMR spectral data, of several monoglycosides (Agrawal, 1992; Pauli, 2000).

The ^{1}H-NMR data for compound **5** suggested the presence of three hydroxyl signals (1H, brs), in the downfield region, at δ_H 9.17, δ_H 10.87, δ_H 12.60, along with two characteristic *meta*-coupled doublets (J = 2.1 Hz) at δ_H 6.22 (H-6) and δ_H 6.50 (H-8). Additionally, there was a two-proton singlet at δ_H 7.49 (H-2′, H-6′), a six-proton singlet at δ_H 3.84, corresponding to two methoxyl groups at the *meta*-position in the B ring, as well as one anomeric proton at δ_H 5.59 (1H, J = 7.3 Hz). The above ^{1}H-NMR data and values of the coupling constants of **5** led us to conclude it was the glycoside of myricetin dimethylether, which further supported by the ^{13}C-NMR data. In the



Kaempferol (**1**): R_1=H, R_2=H, R_3=H
Kaempferol 3-O-β-D-glucuronopyranosyl methyl ester (**2**): R_1=Gln-Me, R_2= R_3=H
Kaempferol 3-O-β-D-glucopyranoside (**3**): R_1=Glu, R_2= R_3=H
Kaempferol 3-O-β-D-galactopyranoside (**4**): R_1=Gal, R_2= R_3=H
Myricetin 3',5'-dimethylether 3-O-β-D-glucopyranoside (**5**): R_1=Glu, R_2=R_3=OCH_3
Kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**6**):
R_1=Rha-(1→6)-Glu, R_2 = R_3 = H
Kaempferol 3-O-β-D-glucuronopyranoside (**7**): R_1=Gln, R_2= R_3=H



β-sitosterol glucopyranoside (**8**)

Fig. 1. Chemical structures of compounds **1**-**8** from the stamens of *N. nucifera*

HMBC, the proton signal for two *meta*-methoxyl groups, at δ_H 3.84 (6H, s), was correlated with that of the myricetin C-2′ and 6′, at δ_C 115.4. Also, an anomeric glucose proton signal, at δ_H 5.59 (1H, *J* = 7.3 Hz), showed a cross peak with the myricetin C-3, at δ_C 133.1. These results confirmed that **5** was myricetin 3′,5′-dimethylether 3-*O*-β-D-glucopyranoside.

The ^{1}H-NMR data for compound **6** showed signals for two *meta*-coupled protons at δ_H 6.23 (1H, *J* = 2.1 Hz, H-6) and δ_H 6.45 (1H, d, *J* = 2.1 Hz, H-8), together with two *ortho*-coupled systems at δ_H 6.89 (2H, d, *J* = 8.8 Hz, H-3′, 5′) and δ_H 7.97 (2H, d, *J* = 8.8 Hz, H-2′, 6′), indicating a kaempferol derivative (Harborne, 1980; Agrawal, 1992). From the analysis of the ^{1}H-NMR and coupling constants of **6**, there were characteristic signals of rhamnopyranoside and glucopyranoside, at δ_H 4.38 (1H, s, H-1‴) and at δ_H 5.30 (1H, d, *J* = 7.4 Hz, H-1″), respectively (Pauli, 2000). These were further supported by the HMBC spectrum; the signals at δ_H 4.38 and δ_H 5.30 correlated with those of glucose C-6, at δ_C 66.9, and kaempferol C-3, at δ_C 133.2, respectively. These results suggested that the order of sugars was α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (Agrawal, 1992), which confirmed **6** to be kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

Table II shows the antioxidative activities of the flavonoids **1**-**4** and **7**, as kaempferol, and its derivatives isolated from the active EtOAc fraction of *N. nucifera* stamens, on the DPPH radical, total ROS and $ONOO^-$ systems. Flavonoids,

Table II. Antioxidant activities of isolated compounds **1**-**4** and **7** from the stamens of *N. nucifera*

Compounds	DPPH[a] IC_{50} (μM)	Total ROS[b] IC_{50} (μM) Mean±SE[d]	$ONOO^-$[c] IC_{50} (μM) Mean±SE[d]
1	13.8	26.84±2.05	3.00±0.07
2	61.4	> 100	10.51±0.77
3	> 100	> 100	6.98±0.37
4	> 100	> 100	16.59±3.99
7	20.1	> 100	3.18±0.39
L-Ascorbic acid	11.5		
Trolox		6.09±1.29	
Penicillamine			5.11±0.23

[a]DPPH is the free radical scavenging activity (IC_{50}: μM). [b]Total ROS is the inhibitory activity of the total free radical generation in the kidney postmicrosomal fraction (IC_{50}: μM). [c]$ONOO^-$ is the inhibitory activity of authentic peroxynitrite (IC_{50}: μM). [d]Values of total ROS and $ONOO^-$ were expressed as the mean±standard error of three or five experiments.

a family of diphenylpropanes (C_6-C_3-C_6), which are naturally occurring compounds that are widely distributed in the plant kingdom. In particular, flavonoids have been found to owe their antioxidant activity to both their excellent metal chelating and radical scavenging properties (Morel *et al.*, 1993; Salah *et al.*, 1995; Van Acker *et al.*, 1998; Rice-Evans *et al.*, 1996). The differences in flavonoids are dependent on their basic structure, as well as the number, and arrangement of the functional groups, such as hydroxyl groups, methoxyl groups and glycosidic units (Rice-Evans *et al.*, 1997). Of the five flavonoids tested, compound **1** possessed good activities in the three model systems tested. Compounds **2** and **7** showed scavenging activities in the DPPH and $ONOO^-$ tests, while compounds **3** and **4** were only active in the $ONOO^-$ test. Because compounds **5** and **6** were isolated as minor components, their antioxidant activities were not determined. Conversely, compound **8** exhibited no activity in any of the model systems tested, although the data is not shown. These results were in accordance with the other research on the structure-antioxidant activity relationship of flavonoids. As shown in Table II, the differences in the structures of flavonoids affected their antioxidant activity in the three model systems tested. When the hydroxyl groups at the C-3 positions are masked by a glycoside, and other factors, the IC_{50} values were dramatically decreased, although differences according to the diversity of the systems used were shown. The above results suggest that the hydroxyl groups at the C-3 positions are a structural requirement for the antioxidant activities of the flavonoids in the model systems tested (Van Acker, S.A.B.E. *et al.*, 1996; Cos *et al.*, 1998; Nagao *et al.*, 1999; Haenen *et al.*, 1997; 2001;

Choi *et al.*, 2002).

The present work found that the antioxidant capacities of the MeOH extract, its various fractions and active components, from the *N. nucifera* stamens, could be useful for the treatment of oxidative damage. Furthermore, it will be interesting to investigate the antioxidative activity of these natural compounds still further for the prevention of various radical-mediated injuries in pathological situations *in vivo*. The investigation of further antioxidant principles, and the biochemical mechanisms of the flavonoids remain to be elucidated in future studies.

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