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In Vitro Peroxynitrite Scavenging Activity of 6-Hydroxykynurenic Acid and Other Flavonoids from *Gingko biloba* Yellow Leaves

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As part of our research on phytochemicals that exert protective effects against diseases related to reactive nitrogen species, we have evaluated the scavenging activity of the yellow leaves of *Ginkgo biloba* on $ONOO^-$. The methanol extract and ethyl acetate fraction obtained from yellow leaves of *G. biloba* evidenced a marked scavenging activity on authentic $ONOO^-$. Repeated column chromatography of the active ethyl acetate soluble fraction on silica gel, Sephadex LH-20, and RP-18, resulted in the purification of 15 known compounds, including sciadopitysin (**1**), ginkgolide B (**2**), bilobalide (**3**), isoginkgetin (**4**), kaempferol (**5**), luteolin (**6**), protocatechuic acid (**7**), bilobetin (**8**), amentoflavone (**9**), β-sitosterol glucopyranoside (**10**), kaempferol 3-*O*-rhamnopyranoside (**11**), kaempferol 3-*O*-glucopyranoside (**12**), kaempferol 3-*O*-[6'''-*O*-*p*-coumaroyl-β-D-glucopyranosyl(**1**→**2**)-α-L-rhamnopyranoside] (**13**), kaempferol 3-*O*-rutinoside (**14**), and 6-hydroxykynurenic acid (**15**). Among the compounds isolated, flavonoids (**5**, **6** and **11-14**), protocatechuic acid (**7**), and 6-hydroxykynurenic acid (**15**) all exhibited marked scavenging activities on authentic $ONOO^-$. The IC_{50} values of **5-7**, **11-14** and 15 were as follows: 2.86 ± 0.70, 2.30 ± 0.04, 2.85 ± 0.10, 5.60 ± 0.47, 4.16 ± 1.65, 2.47 ± 0.15, 3.02 ± 0.48, and 6.24 ± 0.27 μM, respectively. DL-Penicillamine (IC_{50} = 4.98 ± 0.27 μM) was utilized as a positive control. However, the other compounds (**1-4**, **8-10**) exerted no effects against $ONOO^-$.

Key words: *Ginkgo biloba*, Peroxynitrite, Biflavonoids, Flavonol glycosides, Terpenoids, Protocatechuic acid, 6-Hydroxykynurenic acid

INTRODUCTION

Peroxynitrite ($ONOO^-$) is a highly toxic oxidizing and nitrating species, and can be generated *in vivo via* interactions between superoxide and nitric oxide (Beckman and Crow, 1993; Squadrito and Pryor, 1995; Beckman *et al.*, 1990). $ONOO^-$ is known to induce lipids peroxidation, the oxidation of sulfhydryl groups, and the nitration of tyrosine residues in proteins, processes that collectively result in DNA damage (Darley-Usmar and Halliwell, 1996; Spencer *et al.*, 1996). $ONOO^-$ toxicity has been associated with several diseases, including cancer, as well as several cardiovascular and neurological diseases (Deliconstantinos *et al.*, 1995; Beckman, 1996; Ronson *et al.*, 1999; Good *et al.*, 1998). Moreover, the importance of $ONOO^-$ regulation has been recognized, due to the absence of endogenous enzymes exerting specific effects against $ONOO^-$. Thus, the isolation of substances, from natural products, that exert protective effects against $ONOO^-$ derived diseases has become the focus of a great deal of recent research. Naturally occurring $ONOO^-$ scavengers, including ascorbic acid, γ-tocopherol, flavonoids, and polyhydroxyphenols, found in fruits, wine, tea, and green vegetables, have also been identified as effective antioxidants (Timmins *et al.*, 1997). Only a few reports concerning the $ONOO^-$ scavenging activities of herbs and their isolated compounds are currently available in relevant medical literature. In previous studies, leaf extracts of *Eriobotrya japonica* (Soung *et al.*, 1999), green tea (Van Dyke *et al.*, 2000; Chung *et al.*, 1998), *Artemisia iwayomogi* (Kim *et al.*, 2004) and *Curcuma longa* rhizomes (Kim *et al.*, 2003) have been demonstrated to be potent scavengers of $ONOO^-$.

Ginkgo biloba L. extracts have classically been used as a traditional herbal medicine in many regions of the world.

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A number of pharmacological and clinical studies have shown that the extracts of *G. biloba* possess antioxidant, anti-ischemic, neuro-protective, cardiovascular, and cerebrovascular properties, and have also been shown to exert beneficial effects against cognitive deficits, including Alzheimer's-type and multi-infarct dementia, as well as peripheral vascular disease (Pietri *et al.*, 1997; Mar and Bent, 1999; Diamond *et al.*, 2000; Ellnain-Wojtaszek *et al.*, 2003). Several previous reports have described the seasonal variations in flavonoid content and phytochemical constitution of *G. biloba* leaves (Kang *et al.*, 1993; Chang *et al.*, 1993; Kang *et al.*, 1990; 1995; Hyun *et al.*, 2005). The standardized EGb 761 from *G. biloba* extract has been reported to scavenge superoxide, hydroxy radicals, and peroxyl radicals, thereby exerting an inhibitory effect against the generation of reactive oxygen species (Pincemail *et al.*, 1989; Gardes-Albert M *et al.*, 1993; Maitra *et al.*, 1995). However, the scavenging effects of *G. biloba* yellow leaves against $ONOO^-$ remain to be clearly elucidated. Extracts of the yellow leaves of *G. biloba* exhibited profound activity in preliminary $ONOO^-$ assay. In a study, the antioxidant properties of the methanol extract of yellow leaves of *G. biloba*, as well as its organic solvent-soluble fractions, were evaluated *via* $ONOO^-$ assay. The results indicated that the EtOAc fraction was extremely active in this regard. Thus, the present study was undertaken in order to identify the active compounds in the EtOAc fraction of the MeOH extract of *G. biloba*. We isolated 15 known compounds (**1-15**) from the EtOAc-soluble fraction of the MeOH extract of *G. biloba* leaves, some of which exhibited profound scavenging activity.

MATERIALS AND METHODS

General experimental procedures

The ^1H- and ^{13}C-NMR spectra were measured using a JEOL JNM-ECP 400 (400 MHz for ^1H, 100 MHz for ^{13}C) spectrometer. The chemical shifts were referenced to the respective residual solvent peaks, and the values recorded in δ. Column chromatography was carried out using silica (Si) gel 60 (70-230 mesh, Merck, Germany) and Sephadex LH-20 (25-100 μ, Sigma, St. Louis, MO). TLC was carried out on precoated Merck Kieselgel 60 F_{254} plate (0.25 mm) and spots were detected under UV light using 50% H_2SO_4 reagent. All solvents for column chromatography were of reagent grade, and were acquired from commercial sources.

Chemicals

The DL-2-amino-3-methylbutanoic acid (DL-penicillamine) and kynurenic acid were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were purchased from Molecular Probes (Eugene, Oregon, U.S.A.) and Cayman Chemical Co.(Ann Arbor, MI, U.S.A.), respectively.

Plant materials

The yellow leaves were collected directly from a tree of *G. biloba* L. which was grown in the sunshine in Andong National University in November 2001.

Extraction and isolation

Dried *G. biloba* leaves (1.18 kg) were refluxed for three hours with MeOH. The total filtrate was then concentrated to dryness, *in vacuo* at 40°C, in order to render the MeOH extract (524 g). This extract was suspended in H_2O, then successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH, in order to yield the CH_2Cl_2 (111.7 g), EtOAc (48.3 g), and *n*-BuOH fractions (175.6 g), as well as the H_2O residue (146.0 g). The EtOAc fraction (48.3 g) was then chromatographed on a Si gel column, using CH_2Cl_2-MeOH (stepwise) to generate 20 subfractions. Fractions 3 and 10 were recrystallized from the MeOH to yield compounds **1** (1.44 g) and **8** (263 mg). Fraction 6 (1.70 g) was subjected to column chromatography over Sephadex LH-20 with CH_2Cl_2-MeOH (1:4) in order to purify compounds **2** (36 mg) and **3** (185 mg). Fraction 8 (3.66 g) was subjected to column chromatography on a Si gel column, coupled with gradient elution, using hexane:EtOAc eluent (3:2, gradient to MeOH), in order to generate compound **4** (218 mg). Fraction 9 (2.57 g) was then subjected to column chromatography over Sephadex LH-20 with MeOH, in order to generate compounds **5** (10 mg), **6** (5 mg), and **7** (41 mg). Fraction 11 (6.03 g) was subjected to Si gel (CH_2Cl_2-MeOH, 20:1), and Sephadex LH-20 (MeOH) chromatography, yielding compounds **9** (54 mg) and **10** (21 mg). Fraction 12 (0.84 g) was subjected to Si gel (CH_2Cl_2-MeOH-H_2O, 26:14:5), and Sephadex LH-20 (MeOH) chromatography, yielding compound **11** (15 mg). Separate fractions 14 (3.18 g) and 16 (2.85 g) were obtained by a separation method similar to that used to obtain fraction 12, and this method also yielded compounds **12** (116 mg), **13** (469 mg) and **14** (171 mg). Fraction 19 (0.99 g) was subjected to Si gel (CH_2Cl_2-MeOH-H_2O, 26:14:5), Sephadex LH-20 (MeOH), and RP-18 (10% MeOH) chromatography, yielding compound **15** (15 mg).

Measurement of $ONOO^-$ scavenging activity

Peroxynitrite scavenging was evaluated by monitoring the oxidation of DHR 123, using a slightly modified version of the method described by Kooy *et al.* (Kooy *et al.*, 1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored as a stock solution at -20°C. A working solution with 5 μM DHR 123 diluted from the stock solution was placed on ice in the dark

immediately prior to the experiment. A buffer (90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride at pH 7.4) was purged with nitrogen and placed on ice prior to use. Immediately before use, 5 mM diethylenetriaminepentaacetic acid (DTPA) was added. $ONOO^-$ scavenging ability, by the oxidation of DHR 123, was measured at room temperature on a microplate fluorescence reader (FL_X 800, Bio-Tek Instruments) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. The background and final fluorescent intensities were determined 5 minutes after treatment with and without authentic 10 μM $ONOO^-$ in 0.3N sodium hydroxide (NaOH). All compounds were dissolved in 10% EtOH. Authentic $ONOO^-$ easily oxidized DHR 123 with its final fluorescent intensity being stable over time. DL-Penicillamine was used as a positive control.

Statistical analysis

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

RESULTS AND DISCUSSION

$ONOO^-$, the formation of which occurs as the result of a reaction between superoxide and nitric oxide, is a cytotoxic species capable of oxidizing several cellular components, such as proteins, lipids, and DNA (Squadrito and Pryor, 1998). Moreover, the need for a strong $ONOO^-$ scavenger is quite clear, due to the absence of any enzymes that might exert protective effects against damage induced by $ONOO^-$. Thus, the isolation of substances, from natural products, which would exert protective effects against $ONOO^-$ derived diseases, would have clear beneficial effects.

As part of an ongoing series of studies, focused on the identification of active compounds from natural products, this particular study examined compounds inherent to the yellow leaves of *G. biloba*. As is shown in Fig. 1, the MeOH extract (IC_{50} = 3.22 ± 0.41 μg/mL) of *G. biloba* leaves exhibited clear scavenging activity, which manifested in a concentration-dependent manner. We also evaluated the levels of activity of the organic solvent-soluble fractions, including the CH_2Cl_2, EtOAc, and *n*-BuOH fractions, as well as the H_2O layer of the MeOH extract of the *G. biloba* yellow leaves. Among the five tested fractions, the EtOAc-soluble fraction (IC_{50} = 0.71 ± 0.10 μg/mL) exhibited the most profound $ONOO^-$ scavenging activity. The EtOAc-soluble fraction, which manifested potent scavenging activity, was purified further *via* repeated silica gel, Sephadex LH-20, and RP-18 column chromatography. Compounds **1**-**15** were isolated, and could be identified readily *via* comparisons of their spectral data with the values provided in the relevant literature: sciadopitysin (**1**), ginkgolide B (**2**),

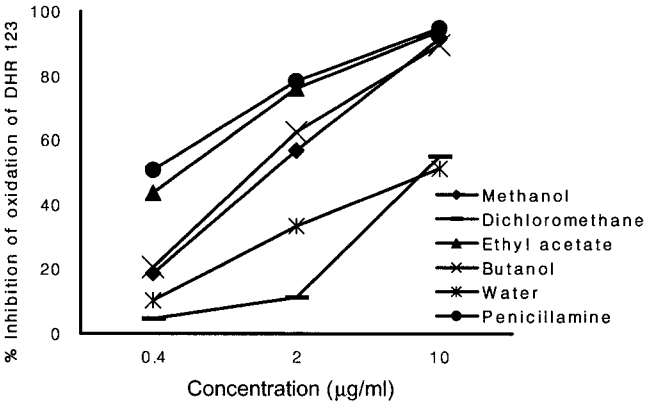

Fig. 1. Concentration-dependent scavenging activity of MeOH extract and its fractions from *G. biloba* on authentic $ONOO^-$

bilobalide (**3**), isoginkgetin (**4**), kaempferol (**5**), luteolin (**6**), protocatechuic acid (**7**), bilobetin (**8**), amentoflavone (**9**), β-sitosterol glucopyranoside (**10**), kaempferol 3-*O*-rhamnopyranoside (**11**), kaempferol 3-*O*-glucopyranoside (**12**), kaempferol 3-*O*-[6‴-*O*-*p*-coumaroyl-β-D-glucopyranosyl (**1**→**2**)-α-L-rhamnopyranoside] (**13**), kaempferol 3-*O*-rutinoside (**14**), and 6-hydroxykynurenic acid (**15**) (Weinges *et al.*, 1987; Kang *et al.*, 1990; 1995; Jung *et al.*, 2001; Jung *et al.*, 2003). The chemical structures of compounds **1**-**15** are provided in Fig. 2.

Compounds **5**-**7**, **11**-**14**, and **15** were found to exhibit profound $ONOO^-$ scavenging activities, at IC_{50} values of 2.86 ± 0.70, 2.30 ± 0.04, 2.85 ± 0.10, 5.60 ± 0.47, 4.16 ± 1.65, 2.47 ± 0.15, 3.02 ± 0.48, and 6.24 ± 0.27 μM, respectively. DL-Penicillamine, used as a positive control, exhibited an IC_{50} value of 4.98 ± 0.27 μM. Flavonoids (**5**, **6** and **11**-**14**), protocatechuic acid (**7**) and 6-hydroxykynurenic acid (**15**), exhibited profound scavenging effects on authentic $ONOO^-$. However, biflavonoids (**1**, **4**, **8** and **9**), terpenoids (**2** and **3**), and sterol glucoside (**10**), evidenced no activity against $ONOO^-$ at the highest tested concentrations (100 μM) (Table I). To the best of our knowledge, this is the first report of scavenging activity against $ONOO^-$ associated with the 6-hydroxykynurenic acid isolated from *G. biloba*. As is shown in Fig. 3, kynurenic acid, which is used commercially, and structurally similar to 6-hydroxykynurenic acid, exhibited no such activity, even at relatively high concentractions. A comparison of the inhibitory activities of the compounds with their structural features clearly indicated that the hydroxyl group located on the phenolic ring appears to play a pivotal role in the observed inhibitory activities.

The $ONOO^-$ scavenging effects evidenced by active flavonoids and protocatechuic acid are believed to be attributable to their electron-donating ability. As both flavonoids and phenolic acid are known to inhibit peroxynitrite-mediated tyrosine nitration, either *via* their functions

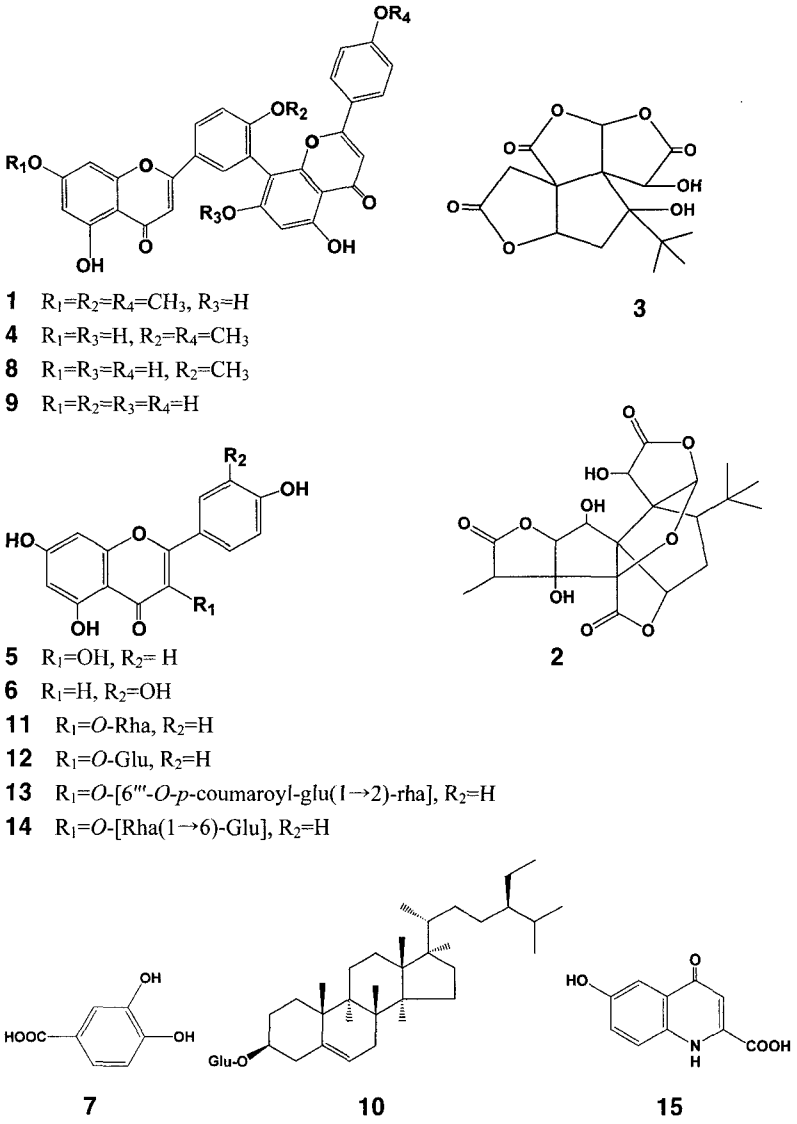

Fig. 2. Chemical structures of compounds **1-15** from the leaves of *G. biloba*

as alternative nitration substrates, as can be seen with monohydroxylated structures such as *p*-coumaric acid and ferulic acid, or *via* the reduction of reactive nitrogen species, as has been observed with catechol structures, including caffeic acid (Pannala *et al.*, 1997, 1998; Kerry and Rice-Evans, 1999). Although the inhibitory effects of terpenoids (ginkgolide A, B or bilobalide) on nitric oxide and superoxide production in macrophages (THP-1) have been previously reported (Maitra *et al.*, 1995; Scholtyssek *et al.*, 1997; Cheung *et al.*, 2001), these compounds exhibited no scavenging activities on authentic $ONOO^-$. With regard to the flavonoid constituents of *G. biloba*, including kaempferol and quercetin, their glycosides were found to harbor free radical scavengers, and also clearly exhibited antioxidative properties (Gardes-Albert *et al.*, 1993; Marcocci *et al.*, 1994; Maitra *et al.*, 1995; Pietta *et al.*, 2000; Ellnain-Wojtaszek *et al.*, 2003).

The present study demonstrated that isolated flavonoids, protocatechuic acid and 6-hydroxykynurenic acid in the extracts of *G. biloba* leaves are able to exert scavenging effects on authentic $ONOO^-$, which appear to be similar to those of the MeOH extract and its EtOAc fraction. Thus, $ONOO^-$ scavenging activities, as well as the antioxidant properties exhibited by these compounds, may play a vital role in the reported actions of *G. biloba*. The results of the present study indicate that the MeOH extract, as well as the EtOAc-soluble fraction of the MeOH extract of *G. biloba*, and its compounds, may serve to alleviate undesired accumulations of $ONOO^-$, which is generated in several conditions, including atherosclerosis, ischemic/reperfusion, and inflammation. A further evaluation of the $ONOO^-$ scavenging activities of these natural compounds with

Table I. Peroxynitrite scavenging activities of isolated compounds from the EtOAc fraction of the MeOH extract of *G. biloba*

Compounds	IC_{50} (μM)[a]
	Mean ± SE
5	2.86 ± 0.70
6	2.30 ± 0.04
7	2.85 ± 0.10
11	5.60 ± 0.47
12	4.16 ± 1.65
13	2.47 ± 0.15
14	3.02 ± 0.48
15	6.24 ± 0.27
Penicillamine	4.98 ± 0.27

[a]Inhibitory activity was expressed as the mean of 50% inhibitory concentrations of triplicate determines obtained by interpolation of concentration-inhibition curve.

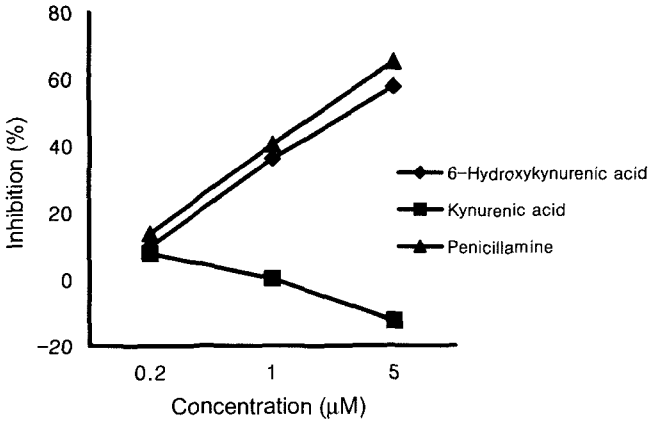

Fig. 3. Dose-dependent inhibitory effects on oxidation of DHR 123 by 6-hydroxykynurenic acid and kynurenic acid

regard to the *in vivo* prevention of a variety of peroxynitrite-mediated injuries in pathological situations might prove both interesting and instructive. Therefore, further investigation into such $ONOO^-$ scavenging principles is currently in progress.

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