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Isolation of Xanthine Oxidase Inhibitors from *Ginkgo biloba* Leaves-Derived Components

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Abstract

The extract of *Ginkgo biloba* leaves was measured for inhibitory activity against xanthine oxidase. Aceton extract of *G. biloba* leaves showed strong inhibitory activity. Inhibitory activities of the fractionated extract were in the order of water>ethyl acetate fractions. Two fractions exhibiting strong inhibitory activities were further purified via repeated silica gel, Amberlite IRN-78, Polyclar AT, and Sephadex LH-20 column chromatographies. Active components were isolated and identified through ^{1}H-NMR and ^{13}C-NMR. The compounds were characterized as kaempferol 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside), and quercertin 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside).

Key words: *Ginkgo biloba*, kaempferol 3-O- α -(6‴-p-coumaroylglucosyl-β-1,4-rhamnoside), quercertin 3-O- α -(6‴-p-coumaroylglucosyl-β-1,4-rhamnoside), xanthine oxidase

INTRODUCTION

Xanthine oxidase (XOD, EC 1.2.3.2) is a key enzyme that catalyses the oxidation of hypoxanthine into xanthine and of xanthine, in the presence of molecular oxygen, to yield uric acid and superoxide anions (1-3). The inhibition of xanthine oxidase is an effective therapeutic approach for treating hyperuricemia that causes gout, kidney stones, and myocardial ischemia (1-3). However, many of the naturally occurring XOD inhibitors have been limited in their practical usage due to their low effectiveness, even though they are considered to be active in controlling the toxic effects (4). These economic, healthy, and environmental concerns have highlighted the need to develop new types of XOD inhibitors.

Plants may be an alternative to currently used XOD inhibitors, because they are rich sources of bioactive organic chemicals and biodegradable into nontoxic products. Therefore, much effort has been focused on plant-derived materials for potentially useful products as commercial XOD inhibitors. A number of XOD inhibitors such as anthraquinones, flavonoids, xanthones, and caffeic esters have been found from plant sources (5-8). In particular, essential oils are known to be rich sources of XOD inhibitors, which are mainly composed of monoterpenes, sesquiterpenes, and diterpenes. Diterpenes from rosemary have been found to inhibit the generation of superoxide anion in the xanthine oxidase system and microsomal lipid peroxidation (9). The main diterpenes of rosemary are rosemarinic acid, carnosic acid, and carnosol, which have similar or higher inhibitory activity against XOD than those of the synthetic XOD inhibitors (9). However, much effort does not have been made to identify the relationship between the inhibitory activity against XOD and the components derived from *Ginkgo biloba* leaves.

We assessed the inhibitory activities of two components isolated from *G. biloba* leaves to search for plant-derived materials for potential XOD inhibitors.

MATERIALS AND METHODS

Chemicals

Bilobalide, ginkgolide (A and B), xanthine, xanthine oxidase grade I from buttermilk were provided from Sigma Chemical Co. (St. Louis, MO, USA). Kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)- α -L-rhamnopyranoside derived from *G. biloba* were kindly provided by Dr. Byeoung-Soo Park of Faculty of Biotechnology, College of Agriculture, Chonbuk National University, Chonju, Republic of Korea. All other chemicals were of reagent grade.

Isolation and identification

Leaves of *G. biloba* (10 kg) were dried in an oven at 60°C for 2 days, finely powdered, extracted twice with 60% aq. acetone at room temperature, and filtered (Toyo filter paper No. 2, Japan). The extract was concentrated via rotary evaporation at 35°C. The extract was isolated from the lyophilized methanol extract to yield about 13.4

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% (based on the weight of the leaves). The extract (20 g) was sequentially partitioned into hexane, ethyl acetate, butanol, and water (distilled) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 35°C, and water portion was freeze-dried. Compound I: Ethyl acetate fraction (51 g) was chromatographed on Amberlite IRN-78 column (Prolabo, 100 g, 200×2.5 cm i.d.) using water with increasing ratio of methanol and then methanol-0.05 N HCl. The active fraction (22.3 g) eluted with methanol was chromatographed on Silica gel 60 column (Merck, 70~230 mesh, 500 g, 70×5.5 cm i.d) packed with ethyl acetate and eluted with a mixture of ethyl acetate/methanol in an increasing ratio of methanol. The active subfraction (3 g) was further chromatographed on Polyclar AT column (Touzart and Matignon, 100 g, 200×2.5 cm i.d.) packed with chloroform/methanol (4:1) and eluted with the same solvent. The compound was finally purified successively on Sephadex LH-20 column (Pharmacia 25~100 mesh, 200×3.5 cm i.d.) and cellulose (Merck, 500 g, 100×2.0 cm i.d) eluted with methanol. Pure compound (470 mg) was obtained as an amorphous solid. Compound II: Water fraction (189 g) was defatted with C_6H_6 and chromatographed on Amberlite IRN-78 column (Prolabo, 100 g, 200×2.5 cm i.d.) using water in an increasing ratio of methanol and then methanol-0.05 M HCl. The active fractions (250 mL) were collected. The active fractions (42 g) eluted with methanol were chromatographed on Silica gel 60 column (Merck, 70-230 mesh, 500 g, 70×5.5 cm i.d) packed with chloroform and eluted with a mixture of chloroform/methanol in an increasing ratio of methanol. The fraction (4.5 g) was eluted with methanol on Sephadex LH-20 column (Pharmacia, 25~100 mesh, 200×3.5 cm i.d.). The active subfraction (250 mg) was chromatographed on Polyclar AT column (Touzart and Matignon, 100 g, 200×2.5 cm i.d.) packed with chloroform/methanol (4:1) and eluted with an increasing ratio of methanol. The compound was finally purified on a Sephadex column (915 g) eluted with methanol. Pure compound (200 mg) was obtained.

Structural determination of the active isolate was based on the spectral analysis. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer, and chemical shifts were given in ppm. UV spectra was obtained on a Waters 490 spectrometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Xanthine oxidase assay

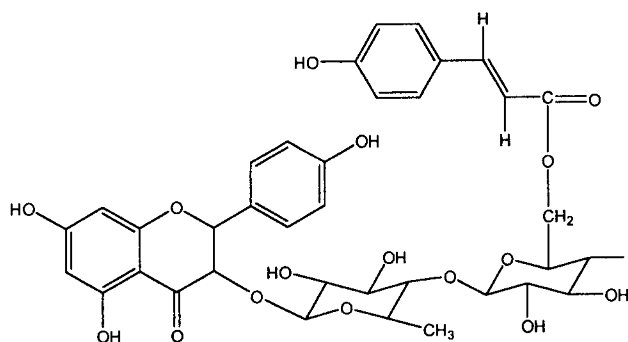
Xanthine oxidase inhibitory activities of the samples were tested spectrophotometrically with xanthine as the substrate through the method reported by Noro et al. (6) and was expressed as $(1-B/A)\times 100$, where A and B are the activities of the enzyme with and without the test material, respectively.

RESULTS

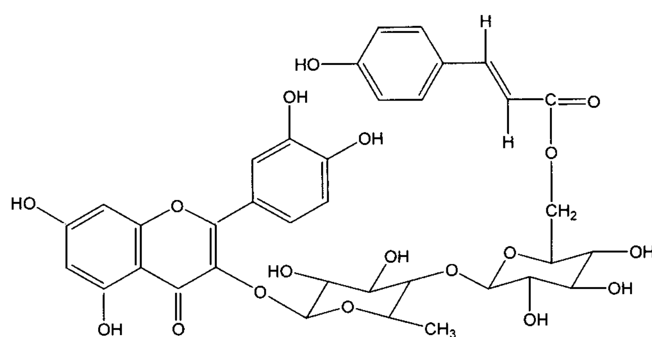
Acetone extract of *G. biloba* leaves was measured for the inhibitory activity against xanthine oxidase at 2 and 1 mg, and the significant inhibition of aceton extract (inhibition rate: 100%) was further tested to determine the inhibitory activity (Table 1). Two fractions obtained from aceton extracts showed the strong activities against xanthine oxidase, whereas hexane and butanol extracts had no inhibitory activities. Purification of the biologically active compounds from the fractions were chromatographed via repeated silica gel, Amberlite IRN-78, Polyclar AT, and Sephadex LH-20 column chromatography, and the isolates were bioassayed. Two active isolates showed the strong activity. Structural determination of the isolates were made via spectral techniques, and the compounds were characterized as kaempferol 3-*O*-α-(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside) (Compound I) and quercertin 3-*O*-α-(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside) (Compound II) (Fig. 1). The compounds were identified based on the following evidences: Compound I; Mp 335~339°C; $[\alpha]^{20}_{D}$-60° (EtOH; *c* 1). UV λ^{EtOH}_{max} nm: 355sh, 310, 263; +NaOAc: 370sh, 304, 267; +NaOAc-H_3BO_3: 355sh, 310, 263: +$AlCl_3$: 396sh, 307sh, 302, 275, 225;+$AlCl_3$-HCl: 397sh, 307sh, 303, 275, 225; ^1H NMR (200 MHz, DMSO-d_6): δ 0.92 (3H, *d*, *J*=6.0 Hz Me rhamnose), 3.06~4.24 (*m*, sugars protons), 4.35 (1H, *d*, *J*=8 Hz, H-1 glc), 5.61 (1H, *d*, *J*=2.0 Hz, H-1 rha), 6.11 (1H, *d*, *J*=2.5 Hz, H-6), 6.10 (1H, *d*, *J*=16.0 Hz, H-8 coum), 6.25 (1H, *d*, *J*=2.5 Hz, H-8), 6.72 (2H, *d*, *J*=9.0 Hz, H-3 coum and H-5 coum), 6.91 (2H, *d*, *J*=9.0 Hz, H-3′, and H-6′), 7.37 (2H, *d*, *J*=9.0 Hz, H-2 coum and H-6 coum), 7.45 (1H, *d*, *J*=16.0 Hz, H-7 coum), 7.72 (2H, *d*, *J*=9.0 Hz, H-2′ and H-6′), 8.49 (s, OH phenolic), CIMS 70 eV, m/z (rel. int.): 741 $[M+H]^+$ (2), 595 (10), 472 (14), 433 (100), 287 (1). ^{13}C NMR (50 MHz, DMSO-d_6): δ 17.4 (C-6 rha), 63.1 (C-6 glc), 69.8 (C-5 rha, 70.2 (C-4

Table 1. Inhibitory activities of *Ginkgo biloba* leaves-derived extracts and various fractions against xanthine oxidase

Fraction	Test concentration (mg)	Xanthine oxidase inhibition (%)
Control	1	-
Aceton extract	1	100
Hexane fraction	1	-
Ethyl acetate fraction	1	100
Butanol fraction	1	-
Water fraction	1	100



Kaempferol 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside)



Quercertin 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside)

Fig. 1. Structure of two compounds isolated from *Ginkgo biloba* leaves.

glc), 70.4 (C-3 rha) 71.7 (C-2 rha), 73.6 (C-2 glc, C-5 glc), 76.0 (C-3 glc), 81.6 (C-4 rha), 93.9 (C-8), 99.2 (C-6), 100.6 (C-1 rha), 103.3 (C-1 glc), 106.0 (C-10), 113.7 (C-8 coum), 115.4 (C-3′, C-5′), 115.7 (C-3 coum, C-5 coum), 120.3 (C-1′), 124.8 (C-1 coum), 130.0 (C-2′, C-6′), 130.4 (C-2 coum, C-6 coum), 134.2 (C-3), 144.2 (C-7 coum), 156.2 (C-9), 156.5 (C-2), 160.0 (C-4′), 160.2 (C-4 coum), 161.2 (C-5), 165.9 (C-7), 166.4 (C-9 coum), 177.4 (C-4). Compound II; Mp 229~233°C; UV λ^{EtOH}_{max} nm: 360sh, 316, 300sh, 268, 258; +NaOAc: 370sh, 315, 300sh, 269; +NaOAc-H_3BO_3: 373sh, 315, 300sh, 263; +$AlCl_3$: 410sh, 360sh, 315, 300sh, 272; +AlC_3-HCl: 400sh, 360sh, 315, 300sh; 277. ^{1}H NMR (200 MHz, DMSO-d_6): δ 0.91 (3H, *d*, *J*=6.0 Hz Me rhamnose), 3.03 ~4.15 (*m*, sugars protons), 4.28 (1H, *d*, *J*=8.0 Hz, H-1 glc), 5.52 (1H, *d*, *J*=2.0 Hz, H-1 rha), 6.16 (1H, *d*, *J*=2.0 Hz, H-6), 6.24 (1H, *d*, *J*=16.0 Hz, H-8 coum), 6.31 (1H, *d*, *J*=2.0 Hz, H-8), 6.70 (2H, *d*, *J*= 8.6 Hz, H-3 coum and H-5 coum), 6.88 (1H, *d*, *J*=8.4 Hz, H-5′), 7.25 (1H, *dd*, *J*=2.0 Hz and 8.4 Hz, H-6′), 7.36 (1H, *d*, *J*=2.0 Hz, H-2′), 7.41 (2H, *d*, *J*=8.6 Hz, H-2 coum and H-6 coum), 7.45 (1H, *d*, *J*=16.0 Hz, H-7 coum); CIMS 70 eV m/z (rel. int.) 757 $[M+H]^+$ (0.3), 611 (1), 595 (0.5), 472 (6.6), 449 (66.7), 303 (100). ^{13}C NMR (50 MHz, DMSO-d_6): δ 18.3 (C-6 rha), 63.8 (C-6 glc), 70.5 (C-2 rha, C-5 rha), 71.1 (C-4 glc), 72.7 (C-3 rha), 74.7 (C-2 glc, C-5 glc), 76.9 (C-3 glc), 82.6 (C-4 rha), 94.5 (C-8), 99.6 (C-1 rha), 101.6 (C-1 glc), 104.9 (C-10), 107.1 (C-6), 114.8 (C-8 coum, C-2′), 116.5 (C-5′, C-3 coum and C-5 coum), 121.5 (C-1′), 121.8 (C-6′), 125.9 (C-1 coum), 130.9 (C-2 coum, C-6 coum), 135.3 (C-3), 145.5 (C-3′), 146.1 (C-4′), 149.5 (C-7 coum), 157.3 (C-9), 157.5 (C-2), 160.6 (C-4 coum), 162.2 (C-5), 165.0 (C-7), 167.3 (C-9 coum), 178.6 (C-4).

Two isolates and other components, bilobalide, ginkgolide (A and B), and kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)- α -L-rhamnopyranoside, derived from *G. biloba* leaves were examined by measuring the inhibitory activities against xanthine oxidase (Table 2). On the inhibitory effect against xanthine oxidase, IC_{50} values of compounds I and II were 24 and 12 ppm, respectively. However, bilobalide, ginkgolide (A and B), and kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)- α -L-rhamnopyranoside derived from *G. biloba* leaves did not show inhibitory activities against xanthine oxidase.

DISCUSSION

Inhibitory activities of *G. biloba* leaves-derived materials were investigated against xanthine oxidase, although the effects varied with the test model (xanthine oxidase inhibition). Active components were identified as kaempferol 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside), and quercertin 3-*O*- α -(6‴-*p*-coumaroylglucosyl-β-1,4-rhamnoside). However, no inhibitory activities were observed from bilobalide, ginkgolide A, ginkgolide B, and kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)- α -L-rhamnopyranoside derived from *G. biloba* leaves. This plant species belongs to the family Ginkgoaceae. Though a great number of plant extracts were investigated for the antioxidative properties (10-12) and materials derived from *G. biloba* have been extensively studied for pharmacological and pesticidal effects, only a few investigations for the antioxidative mechanism were successful

Table 2. Inhibitory activities of *Ginkgo biloba* leaves-derived compounds against xanthine oxidase

Compounds	Xanthine oxidase inhibition
Compound I	24 ppm
Compound II	12 ppm
Kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)- α -L-rhamnopyranoside	-[1]
Bilobalide	-
Ginkgolide A	-
Ginkgolide B	-

[1]Not detected.

in obtaining free radical action and protective effect of *G. biloba* extracts against peroxidation (4,10). In this study, the strong inhibitory activities of the two isolates confirm their superiority and usefulness as potent inhibitors against xanthine oxidase.

It has been well-acknowledged that many of plant-derived extracts and phytochemicals were potential alternatives to synthetic inhibitors against xanthine oxidase (4). Various compounds including phenolics, terpenoids, and alkaloids exist in plants and, jointly or independently, contribute to the inhibitory activities against xanthine oxidase. Many of them appear to exert no secondary hazards to animals, but only a few studies have been conducted in this branch. The relationship between antioxidatants antimutagenicity and oxidative DNA damage has been reported. Antioxidative activity of tea extracts has been observed to correlate with the antimutagenicity (13). Halliwell and Gutteridge reported that the oxidative DNA damage, mediated by active oxygen radical, induces carcinogenesis (14). It has also been reported that green tea solution reduced hepatic lipid peroxide levels and effectively blocked oxidative DNA damage in liver as well as hepatotoxicity of rats treated with 2-nitropropane as a hepatocarcinogen (15).

In conclusion, the inhibitory activities of two components isolated from *G. biloba* leaves against xanthine oxidase may be indications of at least one of the pharmacological actions of the two components. Based upon our data, the isolated components could be promising compounds in the prevention of several diseases through their inhibitory function.

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REFERENCES

1. Li H, Meng JC, Cheng CHK, Higa T, Tanaka J, Tan RX. 1999. New guaianolides and xanthine oxidase inhibitory flavonols from *Ajania fruticulosa. J Nat Prod* 62: 1053-1055.
2. Izumida H, Adachi K, Mihara A, Yasuzawa T, Sano H. 1997. Hydroxyakalone, a novel xanthine oxidase produced by a marine bacterium, *Agrobacterium aurantiacum. J Antibiotics* 50: 916-918.
3. Halliwell B, Gutteridge JMC, Cross CE. 1992. Free radicals, antioxidants and human disease: Where are we now? *J Lab Clin Med* 119: 598-620.
4. Larson RA. 1997. *Naturally Occurring Antioxidants.* Larson RA, ed. Lewis Publishers, USA.
5. Noro T, Noro K, Miyase T, Kuroyanagi M, Umehara K, Ueno A, Fukushima S. 1987. Inhibition of xanthine oxidase by anthraquinones. *Chem Pharm Bull* 35: 4314-4316.
6. Noro T, Oda Y, Miyase T, Ueno A, Fukushima S. 1983. Inhibitors of xanthine oxidase from the flowers and buds of *Dephne Genkwa Chem Pharm Bull* 31: 3984-3987.
7. Noro T, Ueno A, Mizutani M, Hashimoto T, Miyase T, Kuroyanagi M, Umehara K, Fukushima S. 1984. Inhibitors of xanthine oxidase from the flowers and buds of *Athyrium mesosorum. Chem Pharm Bull* 32: 4455-4459.
8. Nakanishi T, Nishi M, Inada A, Obata H, Tanabe N, Abe S, Wakashiro M. 1990. Two new potent inhibitors of xanthine oxidase from leaves of *perilla frutescens britton* var. *acuta kudo. Chem Pharm Bull* 38: 1772-1774.
9. Haraguchi H, Saito T, Okamura N, Yagi A. 1995. Inhibition of lipid peroxidation and superoxide generation by diterpenoids from *Rosmarinus officinalis. Planta Med* 61: 333-336.
10. Potterat O. 1997. Antioxidants and free radical scavengers of natural origin. *Current Organ Chem* 1: 415-440.
11. Choi U, Shin DH, Chang YS, Shin JI. 1992. Screening of natural antioxidant from plant and their antioxidative effect. *Korean J Food Sci Technol* 24: 142-148.
12. Kim HK, Kim YE, Do JR, Lee YC, Lee BY. 1995. Antioxidantive activity and physiological activity of some Korean medicinal plants. *Korean J Food Sci Technol* 27: 80-85.
13. Yen GC, Chen HY. 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem* 43: 27-32.
14. Halliwell B, Gutteridge JMC. 1990. Role of free radicals and catalytic metal ions in human disease. *Meth Enzymol* 186: 1-85.
15. Hasegawa R, Chujo T, Sai-kato K, Uemura T, Tanimura A, Kurokawa Y. 1995. Preventive effects of green tea against liver oxidative DNA damage and hepatotoxicity in rats treated with 2-nitropropane. *Food Chem Toxicol* 33: 961-970.

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