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Elucidation of micromolecular phenylpropanoid and lignan glycosides as the main antioxidants of Ginkgo seeds

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ABSTRACT

Ginkgo biloba L. is a widely used medicinal plant that has a high concentration of its bioactive components, flavonoids and ginkgolides, in its leaves. Gingko seeds are also consumed as a functional food and medicine throughout the world to slow the ageing process. However, there have been few phytochemical studies of the phenylpropanoids from Ginkgo seeds. To elucidate the active components, we studied the antioxidant activities and related main compounds in Ginkgo seeds. Four main phenylpropanoids and lignans were isolated and identified from the antioxidative fraction and their antioxidant activities were measured. An HPLC/DAD-Q-TOF-MS/MS method was developed to identify the other minor phenylpropanoid and lignan glycosides to elucidate the antioxidant profile of Gingko seeds. Four compounds were identified by NMR and mass data, three of which (**8**, **17**, and **28**) exhibited substantial antioxidant activities (IC_{50}: 67–100 μM). Sixteen minor compounds were tentatively identified based on their tandem mass spectrometry data and the fragmentation of four isolated standards from antioxidative *n*-butanol fraction. Fifteen compounds were reported in Ginkgo seeds for the first time, and compound **16** was tentatively identified as a new chemical structure. Thus, understanding their antioxidant profiles will lay the foundation for research and development of the health benefits of Ginkgo seeds.

1. Introduction

Ginkgo biloba L. is the sole surviving species of the Ginkgoaceae family and Ginkgoales order. Recently, extracts of *G. biloba* leaves have been used in commercial medical products and food supplements in China and many other countries. Terpene trilactones and flavonoid glycosides are the main bioactive constituents in ginkgo leaves and extracts, which are prescribed to improve the circulation and treat dementia. The highest annual expenditure on herbal medications is on *G. biloba* products. To meet this demand, 50 million *G. biloba* trees have been planted worldwide (Nakanishi, 2005; Singh et al., 2008; Van Beek, 2002). Much research has focused on *G. biloba* leaves (Strømgaard and Nakanishi, 2004; Van Beek and Monotoro, 2009). However, the seeds of *G. biloba*, produced by female trees, have a long history of being used as a traditional medicine for treating ailments, such as coughs, asthma, and leucorrhea, and are consumed as a functional food to slow the ageing process (Deng et al., 2011; Tredici, 1991). Studies have reported that commonplace nutritional components, such as proteins, carbohydrates, and amino acids, are the major constituents of Gingko seeds, and that flavonoid glycosides and terpene lactones are minor constituents (Huang et al., 2010; Singh et al., 2008; Zhou et al., 2014).

The main functions of Ginkgo seeds are to improve the skin and slow the ageing process, which are partly related to the antioxidant activity of the seeds. It has also been reported that the antioxidant activity is the most important effect of Ginkgo seeds (Goh and Barlow, 2002; Huang et al., 2010). However, the compounds that contribute to the antioxidant activity are unknown. Flavonoid glycosides and terpene trilactones are reported to be effective in treating cerebrovascular insufficiency and peripheral circulatory problems (Singh et al., 2008), which is far from the traditional effects. Thus, the antioxidants of this widely used functional product should be elucidated. During our previous research on the ginkgolides from Ginkgo seeds using LC–MS (Niu et al., 2017), we found the presence of micromolecular phenylpropanoid and lignin glycosides in the crude extract and we speculated whether these compounds are antioxidants.

In the present work, we elucidated the micromolecular antioxidants of Ginkgo seeds by isolating the main constituents and detecting the minor constituents of the antioxidant fraction by using HPLC/DAD-Q-

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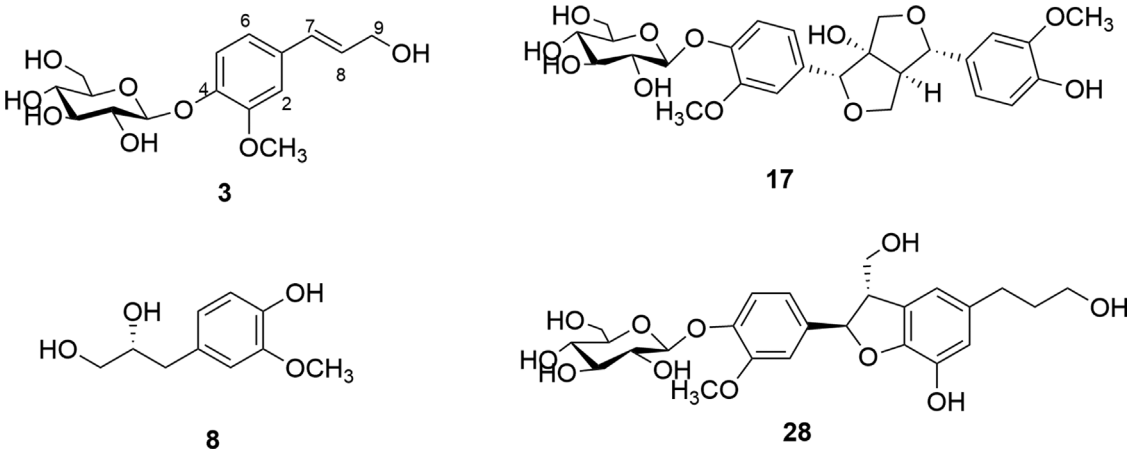

Fig. 1. Chemical structures of compounds isolated from Ginkgo seeds.

TOF-MS/MS. As a result, four major antioxidative phenylpropanoid and lignan glycosides were obtained and identified (IC_{50}: 67–100 μM), and the 16 minor compounds were tentatively identified based on the established HPLC/DAD-Q-TOF-MS/MS method. Elucidating the antioxidant profiles will lay the foundation for the research and development of the health benefits of Ginkgo seeds.

2. Materials and methods

2.1. Chemicals and reagents

Four reference compounds, coniferin (**3**), (2*R*)-3-(4-hydroxy-3-methoxyphenyl)-propan-1,2-diol (**8**), (+)-1-hydroxypinoresinol 4″-O-β-D-glucopyranoside (**17**), and (7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol-4-O-β-D-glucopyranoside (**28**) (Fig. 1), were isolated and purified from Ginkgo seeds. Their identities were confirmed by ^{1}H NMR, ^{13}C NMR, and MS analysis. The purity of each reference standard was > 95%, as determined by HPLC.

LC–MS grade acetonitrile, MeOH, and formic acid were purchased from Roe Scientific Inc. (New Castle, USA). Ultra-pure water was provided by Watsons (Hong Kong, China). The solid-phase extraction (SPE) cartridges (C18E; 6 mL, 500 mg) were purchased from Welch Materials, Inc. (Potomac, MD, USA). Other reagents used for preparation and purification of the extracts were of analytical purity.

2.2. Plant material

Ginkgo seeds were purchased from the Chinese medicinal market in Xuzhou, Jiangsu Province, China, and were identified by Prof. Mian Zhang, Department of Medicinal Plants, China Pharmaceutical University. A voucher specimen (No. 2014-GGL) was deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University. The seeds were pulverized and screened through a 100-mesh sieve before extraction.

2.3. Isolation of the main phenylpropanoid and lignan glycosides

Air-dried Ginkgo seeds (5.0 kg) were extracted with 95% ethanol three times (10 L each, 3, 2, and 1 h) to give a crude extract (200.0 g), which was suspended in water and then extracted with petroleum ether to remove the fatty components. The defatted crude extract was partitioned with *n*-butanol to afford an *n*-butanol-soluble fraction (84.0 g). The *n*-butanol fraction was applied to a D-101 macroporous resin column (700 g), and eluted with an EtOH-H_2O step gradient (0:100, 20:80, 40:60, 60:40, 80:20 v/v) to yield fractions Frs. A–E. Frs. A and B were combined (15 g) and applied to an MCI column eluted with H_2O to remove carbohydrates and obtain one sub-fraction (Fr. A-B-1). Fr. A-B-1 (5.0 g) was then applied to an MCI column and eluted with a MeOH-H_2O step gradient (10:90, 20:80, 30:70, 40:60 v/v) to obtain eight sub-fractions (Frs. A-B-1.1–1.8). Fr. A-B-1.4 (300 mg) was purified with a Sephadex LH-20 column and by preparative HPLC (MeOH-H_2O, 20:80, v/v) to give compounds **3** (20 mg) and **8** (5 mg). After the same procedures, Fr. A-B-1.5 afforded compounds **17** (2.2 mg) and **28** (5.1 mg) (Fig. 1). The pure isolates were characterized by NMR, UV, and MS analysis.

2.4. DPPH free radical scavenging activity

2,2-Diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activities were evaluated by a test similar to a previously described method (Luo et al., 2012). The test samples and DPPH were dissolved in ethanol. A series of concentrations of the test samples (100 μL) and 0.15 mmol/L DPPH (100 μL) were added to a 96-well plate, and the mixture was allowed to react at 25 °C in the dark for 30 min. The absorbance was determined at 517 nm. Ethanol was used instead of samples and Trolox was used as a positive control. The analysis was performed in triplicate. Free radical scavenging activity was expressed as the inhibition percentage and was calculated by

$$\text{DPPH radical scavenging (\%)} = [1 - (A_s - A_b)/A_o] \times 100$$

where A_s is the absorbance of the reaction between DPPH· and the sample or Trolox, A_b is the absorbance of the blank, and A_o is the absorbance of the control.

2.5. Sample preparation

The *n*-butanol extract was pretreated and enriched SPE to remove polar impurities and interference. Before SPE, the C18E cartridge was preconditioned with MeOH-H_2O (6 mL, 20:80 v/v). An *n*-butanol extract (80 mg) was dissolved in MeOH-H_2O (1 mL, 25:75 v/v), and then processed via the cartridge cleanup procedure. The cartridge was eluted successively with MeOH-H_2O (10 mL each, 25:75, 40:60, and 55:45, v/v) to afford three fractions. Finally, each fraction from the cartridge was evaporated to dryness under a gentle stream of nitrogen gas. The residue was reconstituted with MeOH, vortex mixed, and analyzed by HPLC/DAD-Q-TOF-MS/MS.

2.6. HPLC/DAD-Q-TOF-MS/MS analysis

Chromatographic analysis was performed on an HPLC system (1290, Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an online degasser, an auto plate-sampler, and a thermostatically controlled column compartment. The HPLC system was