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Palaeophytochemical Constituents of Cretaceous *Ginkgo coriacea* Florin Leaves

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Abstract

Chemical investigation of the organic solvent extract of Cretaceous *Ginkgo coriacea* Florin leaves by liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectrometry (GC-MS), analogous to those from extant leaves of *Ginkgo biloba* L., led to the detection of a group of natural flavonoids and other volatiles. The similarity of the chemical constituents in these two species of *Ginkgo* suggest that the secondary metabolism of extant *G. biloba* is close to that of the Cretaceous species. The remaining natural products may be one explanation why the leaves of the Cretaceous *G. coriacea* have been preserved morphologically in fossilization. The detection of flavonoids suggests that the leaves of *G. coriacea* experienced a mild post-depositional environment during their fossilization. This appears to be the oldest occurrence of flavonoids in plant fossils.

Key words*:* constituents; Cretaceous; fossil; *Ginkgo biloba*; *Ginkgo coriacea*; palaeophytochemistry.

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Chemical studies of ancient plants provide crucial information on fossil preservation. Characteristic natural products detected from fossils can be used as chemosystematic markers or biomarkers for their biological origin (Otto et al. 2002). Previous chemical investigation of plant fossils has indicated a high level of phytochemical fidelity between the fossils and their respective extant genera (Giannasi and Niklas 1977; Niklas and Giannasi 1977, 1978). Leaf fossils are used to reconstruct the paleoclimate and paleoenvironment (Wang et al. 2003; Sun et al. 2005). *Ginkgo*, as a "living fossil", has existed since the early Jurassic period, approximately 170 million years before present (BP)(Zhou and Zheng 2003). The following Cretaceous age (approximately 120 million years BP) is a crucial period during which the Jurassic-type *Ginkgo* species evolved the modern ovulate organs. Therefore, investigation of Cretaceous *Ginkgo*, bridging the gap between the Jurassic and present *Ginkgo* species, is very important in understanding the evolution of the morphology and secondary metabolism of *Ginkgo*. In the present study, we undertook a phytochemical investigation on Cretaceous *Ginkgo coriacea* (Florin 1963; Sun 1993) in order to determine the organic constituents of Cretaceous *Ginkgo* and the chemical reasons for its long-term preservation.

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Results

Volatiles from three samples of *Ginkgo* leaves

Twenty-six constituents, including four types of fatty acid (C_8 up to C_{18}) compounds, *n*-alkanes (C_{16} up to C_{29}), phthalates, and phenolic compounds were detected in the ether extract of Cretaceous *G. coriacea* leaves using GC-MS (Table 1). Similarly, 21 constituents were identified in *G. biloba* leaves preserved for 150 years and 12 constituents were found in extant *G. biloba*. Fatty acids and *n*-alkanes are the main constituents of the waxes in plant leaves (Gülz 1994); 3-alkanyl-phenols are

the major compounds in 150-year-preserved and extant *G. biloba* (Itokawa 1987). The volatiles detected from three samples of *Ginkgo* showed that four types of organic constituents from Cretaceous *G. coriacea* leaves were co-occurrence with difference on constituents compared with those extracted from the leaves of extant and 150-year-preserved *G. biloba*.

Analysis of flavonoids from Cretaceous *G. coriacea*

Flavonoids from methanol extracts of the three *Ginkgo* species were detected using HPLC analysis with a photodiode array (PDA) detector at 310 nm, which is among the absorption band (300–360 nm) used for the detection of flavonoids. The HPLC spectrum (Figure 1) showed that the flavonoid peaks in the Cretaceous *G. coriacea* leaves were less than those from extant and 150-year-preserved of *G. biloba* leaves. These experiments indicated that the flavonoids in Cretaceous *G. coriacea* leaves are different to those in extant and 150-year-preserved *G. biloba*. In contrast, the flavonoids obtained from extracts of extant and 150-year-preserved leaves of *G. biloba* were very similar.

Further analysis of flavonoids from the methanol extract of Cretaceous *G. coriacea* leaves using LC-MS at 310 nm recorded 10 obvious peaks (Figure 2). The UV spectra of these peaks possessed typical absorption bands of flavonoids at

Table 1. Comparison of constituents from ether extract of *Ginkgo* genus leaves by gas chromatography-mass spectrometry

No.	Constituents	Molecular formulation	Cretaceous *G. coriacea*	150 year-preserved *G. biloba*	Extant *G. biloba*
1	Tetradecane	$C_{14}H_{30}$	–	+	–
2	Pentadecane	$C_{15}H_{32}$	–	+	+
3	Hexadecane	$C_{16}H_{34}$	+	+	+
4	Heptadecane	$C_{17}H_{36}$	+	+	+
5	Octadecane	$C_{18}H_{38}$	+	+	+
6	Nonadecane	$C_{19}H_{40}$	+	+	–
7	Eicosane	$C_{20}H_{42}$	+	+	–
8	Heneicosane	$C_{21}H_{44}$	+	+	–
9	Docosane	$C_{22}H_{46}$	+	–	–
10	Tricosane	$C_{23}H_{48}$	+	–	–
11	Tetracosane	$C_{24}H_{50}$	+	–	–
12	Pentacosane	$C_{25}H_{52}$	+	–	–
13	Hexacosane	$C_{26}H_{54}$	+	+	–
14	Heptacosane	$C_{27}H_{56}$	+	+	+
15	Octacosane	$C_{28}H_{58}$	+	–	–
16	Nonacosane	$C_{29}H_{60}$	+	–	–
17	Diethyl phthalate	$C_{16}H_{22}O_4$	+	+	+
18	Dibutyl phthalate	$C_{16}H_{22}O_4$	+	+	+
19	Diisobutyl phthalate	$C_{16}H_{22}O_4$	+	+	–
20	Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	+	+	–
21	2,5-Bis (1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$	+	–	–
22	3-Undecyl-phenol	$C_{17}H_{28}O$	–	+	–
23	3-Tridecyl-phenol	$C_{19}H_{32}O$	–	+	+
24	3-Pentadecyl-phenol	$C_{21}H_{36}O$	–	+	+
25	Octanoic acid	$C_8H_{16}O_2$	+	–	–
26	Nonanoic acid	$C_9H_{18}O_2$	+	–	–
27	Decanoic acid	$C_{10}H_{20}O_2$	+	–	–
28	Dodecanoic acid	$C_{12}H_{24}O_2$	+	–	–
29	Tetradecanoic acid	$C_{14}H_{28}O_2$	+	+	+
30	Hexadecanoic acid	$C_{16}H_{32}O_2$	+	+	+
31	Octadecanoic acid	$C_{18}H_{36}O_2$	+	+	–
32	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	–	+	+
33	9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	–	–	+

+ stands for existence; – stands for inexistence.

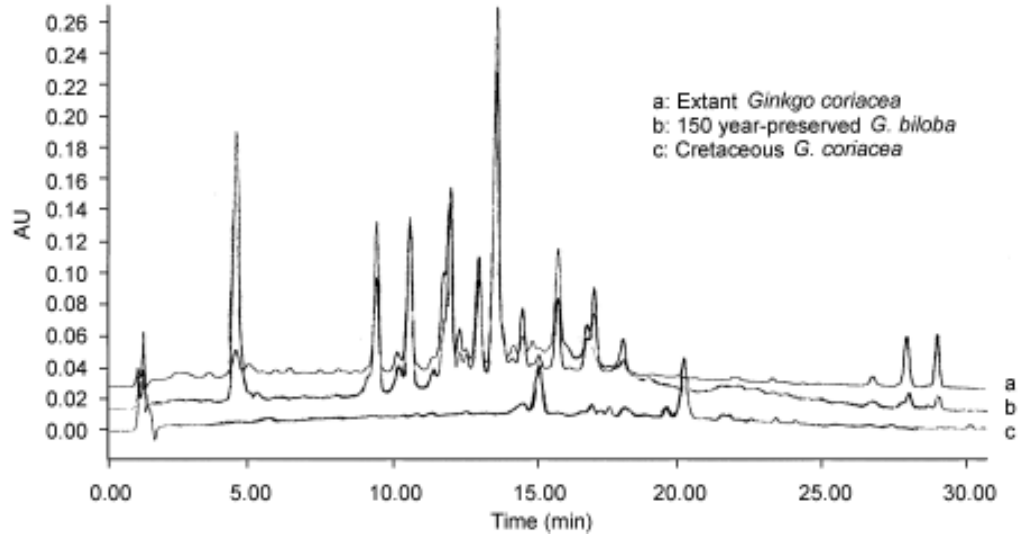


Figure 1. Chromatograms of flavonoids from three samples detected at UV absorption 310 nm by high-performance liquid chromatography (HPLC)

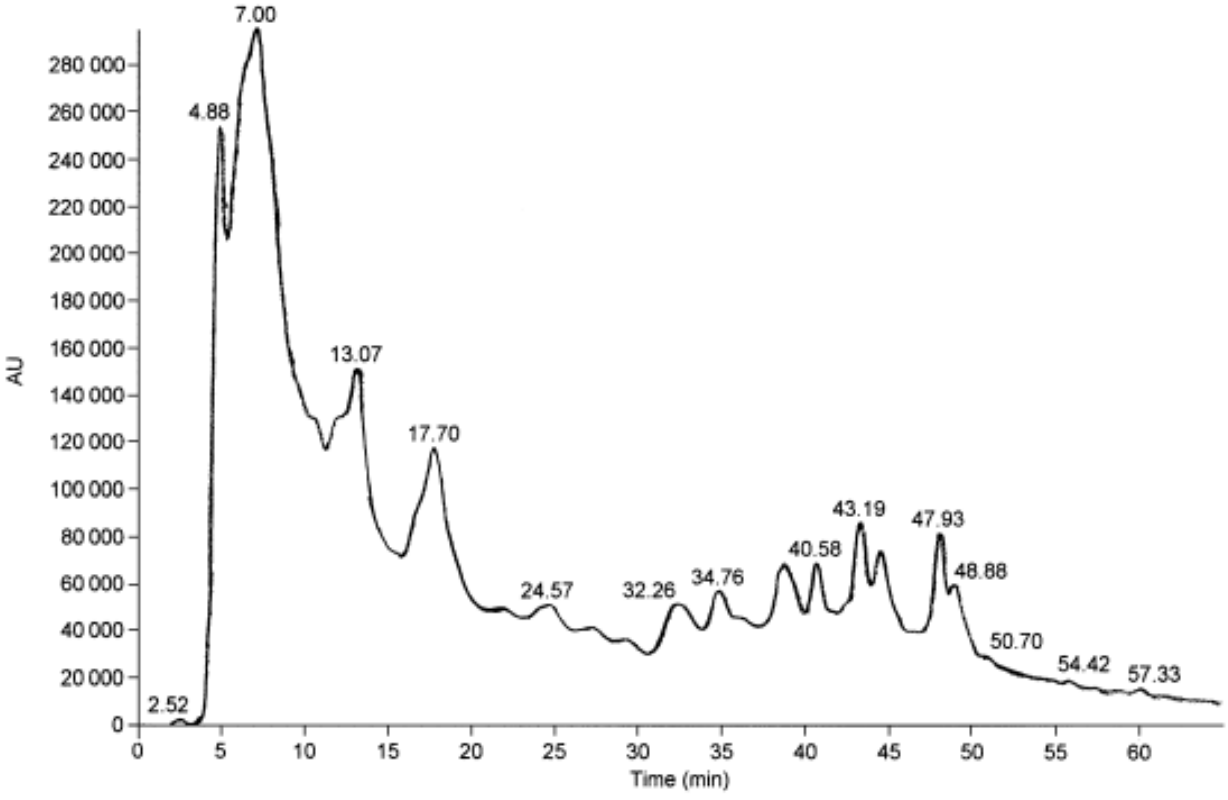
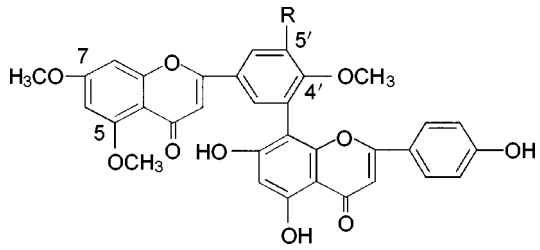


Figure 2. Spectrum of flavonoids from Cretaceous *Ginkgo coriacea* leaves by liquid chromatography-mass spectroscopy (LC-MS) analysis at 310 nm

300–400 and 240–280 nm. Two flavonoids from the Cretaceous *G. coriacea* corresponding to retention times of 47.93 and 40.58 min on LC-MS were deduced as 5-*O*-methylginkgetin (compound **1**) and 5'-methoxy-5,7-*O*-dimethylbilobetin (compound **2**; Figure 3) on the basis of their UV spectra, mass spectra, and using reasonable biopathway analysis.

Compound **1** showed a molecular ion peak at *m/z* 581 $[M+H]^+$ in the electronic spray ion/mass spectrometry (ESI/MS), suggesting its molecular formula as $C_{33}H_{24}O_{10}$. The molecular formula of compound **1** implies one more methene unit than that of ginkgetin (Markham 1984) obtained from extant *G. biloba* leaves. Compound **1** gave UV absorption bands at 315 and 250 nm (bands



1 5-*O*-methylginkgetin (R = H)

2 5'-methoxy-5,7-*O*-dimethylbilobetin (R = OCH_3)

Figure 3. Proposed structures of detected flavones (compounds **1** and **2)**

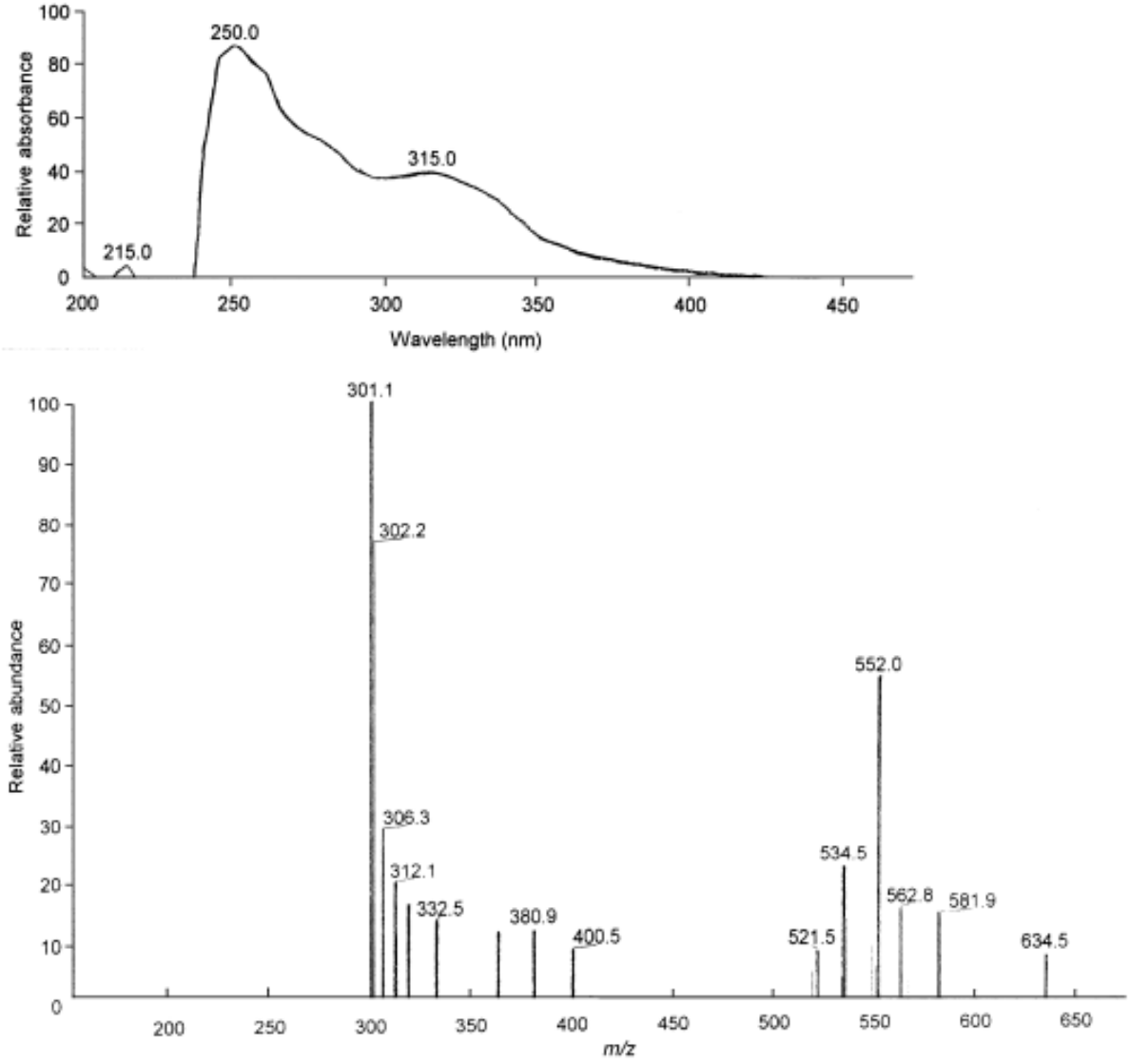


Figure 4. Ultraviolet and mass spectrum of compound **1**, corresponding to retentive time at 47.93 min in liquid chromatography-mass spectroscopy (LC-MS).

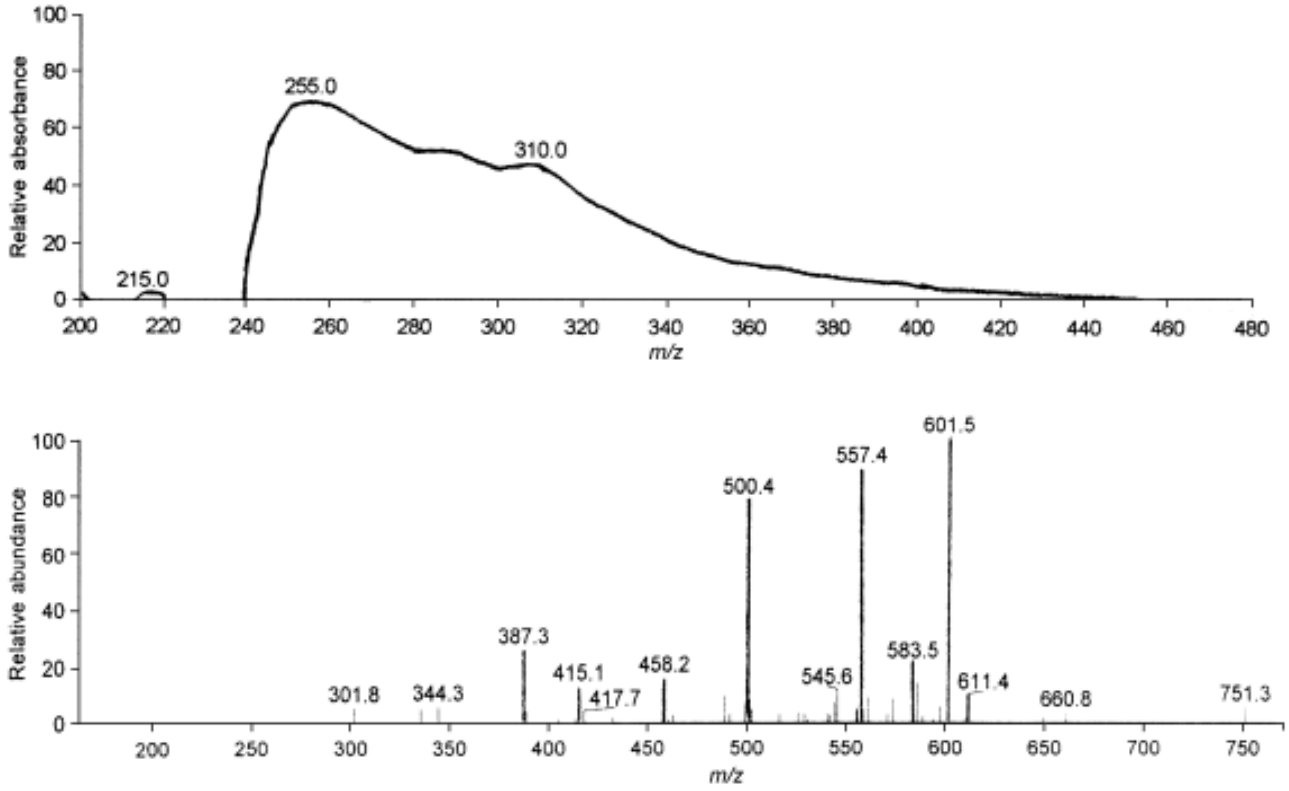


Figure 5. Ultraviolet and mass spectrum of compound **2**, corresponding to retentive time at 40.58 min in liquid chromatography-mass spectroscopy (LC-MS).

I and II; Figure 4), instead of the 330 and 268 nm bands of 7,4', 7''-tri-*O*-methyl amentoflavone (Parmar et al. 1993) and the 330 and 270 nm bands of ginkgetin (Markham 1984). The blue shift of the UV absorption bands may be due to methylation at 5-OH because the UV absorption bands of 5-methoxyflavone are approximately 15 nm lower than those of 5-hydroxyflavone (Xiao 1997). This assumption could be further supported by the fragment ion-peaks with reasonable MS cleavage (Figure 4). Based on the above analysis, compound **1** was deduced as 5-*O*-methylginkgetin.

Compound **2** showed *m/z* 611 [M+H$^+$] in its ESI/MS, which is in accordance with the formula $C_{34}H_{26}O_{11}$. Based on its UV absorption, indicating two typical bands at 310 nm (300–360 nm) and 255 nm (240–280 nm; Figure 5), compound **2** was assigned as a biflavonoid possessing two more methene units than that of 5'-methoxy-bilobetin (Joly et al. 1980), a biflavonoid obtained from extant *G. biloba* leaves. Methylation may have occurred at both the 5-hydroxy and 7-hydroxy according to a comparison of the UV absorption bands of the two compounds **1** and **2** and detailed analysis of MS fragment ion peaks (Figure 5). Based on the above information, compound **2** was deduced to be 5'-methoxy-5,7-*O*-dimethylbilobetin.

Results of anti-oxidation activity experiments for flavonoids from Cretaceous *G. coriacea* leaves indicated that some of the flavonoids had anti-oxidation activity on the basis of comparisons of the chromatography of flavonoids from Cretaceous *G. coriacea* leaves (Figure 6) and those of anti-oxidative flavonoids obtained using the on-line HPLC-DPPH system (Figure 6).

Discussion

Flavonoids are distributed widely throughout the leaves of extant *Ginkgo* species (Hasler and Sticher 1992; Sticher 1993). Ultraviolet (UV) spectra have been used to provide information for the identification of flavonoids because flavonoids show characteristic UV absorptions (Voirin 1983) at two bands, namely 300–400 and 240–280 nm. The two flavonoids, compounds **1** and **2**, detected from Cretaceous *G. coriacea* leaves were deduced to be 5-*O*-methylginkgetin (**1**) and 5'-methoxy-5, 7-*O*-dimethylbilobetin (**2**) on the basis of their UV spectra characteristics, mass spectra and comparisons with known biflavonoids. The two flavonoids detected both had a biflavonoid skeleton derived from ginkgetin and 5'-methoxybilobetin (Sticher

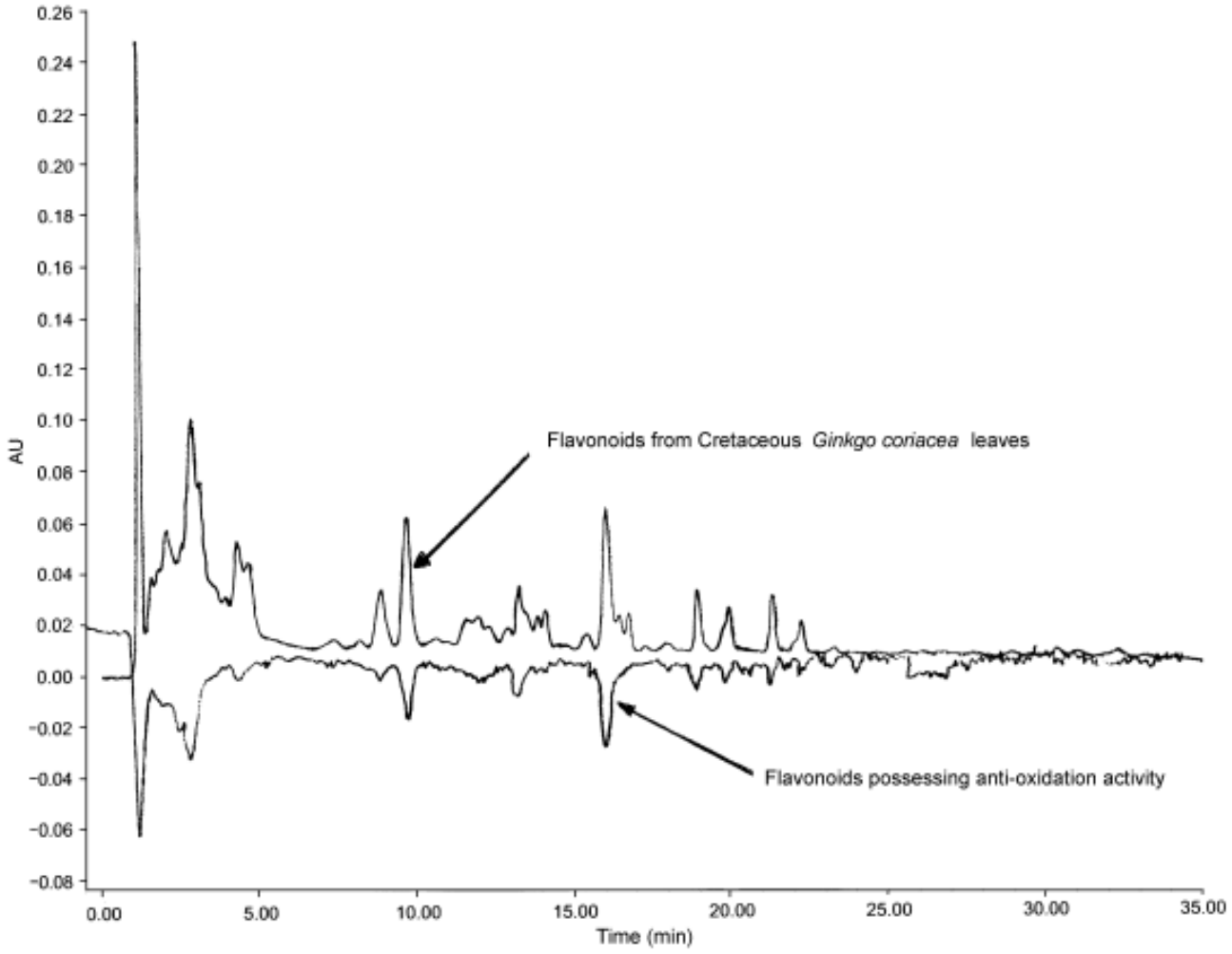


Figure 6. Online HPLC-DPPH photography for evaluation of the radical scavenging flavonoids.

1993; Xiao 1997) of extant *G. biloba* leaves. The methylation of the existing hydroxy groups at position 5 and/or 7 of two flavonoids may have resulted from either secondary metabolism or fossilization.

The fossilized Cretaceous plants may have experienced various physical, biological, and chemical changes over the long years, but never extreme digenetic conditions (temperatures higher than 80 °C and/or pH shifts beyond the range 6.3–7.2) because, under such conditions, the flavonoids would have been degraded during fossilization (Niklas and Giannasi 1977). The discovery of flavonoids in the present study from Cretaceous *G. coriacea* appears to document the oldest occurrence of flavonoids in ancient plants, older than those detected from Miocene fossils, and shows a high level of phytochemical fidelity between the fossils and their respective extant genera (Niklas and Giannasi 1977, 1978; Giannasi and Niklas 1977). The detection of flavonoids from Cretaceous *G. coriacea* revealed that the natural products were found in the fossil rather than the thermolytic fragments created by pyrolysis GC/MS analysis (Mösle et al. 1998).

Phytochemical investigation of the samples from the three *Ginkgo* species indicated that the leaves from all *Ginkgo* samples contained five types of natural products, specifically fatty acids, alkanes, phthalates, phenolic compounds, and flavonoids. These results suggest that little evolution of *Ginkgo* in terms of secondary metabolism has occurred over the past 120 million years. This assumption is consistent with the observation that the morphology of Cretaceous *Ginkgo* leaves is like that of present-day *G. biloba*, indicating little change for over 100 million years (Zhou and Zheng 2003). Well-known insect repellants (such as phthalates), anti-oxidants (including phenolic compounds and flavonoids), and wax (series of

alkanes and fatty acids) in the fossilized leaves have acted as a "great wall" for the preservation of Cretaceous *Ginkgo* cuticles in the fossil.

Material and Methods

Material preparation

The limited leaf material (547 mg) of *Ginkgo coriacea* Florin (voucher specimen HLH-28) was collected from an open coalmine of Huolinhe in eastern Inner Mongolia, China. The coal-bearing sediments were assigned to be Huolinhe Formation and dated as early Cretaceous, approximately 120 million years ago. The leaves of 150-year-preserved *G. biloba* (voucher specimen RS-1), which were collected from Beijing in 1850 and preserved in the Herbarium at V. L. Komorov Botanical Institute, Russian Academy of Sciences, St Petersburg, Russia, were kindly provided for the studies by Herbarium. Extant leaves of *G. biloba* L. (voucher specimen KUN-0040041) were collected in Kunming Institute of Botany, the Chinese Academy of Sciences, China.

Experimental procedures

The volatiles from the three *Ginkgo* samples were determined on a GC-MS apparatus (HP 6890GC/S973MS). Flavonoids from the three *Ginkgo* samples were analyzed on a Waters (Milford, MA, USA) 2695-2996 HPLC system. Further structures of flavonoids from Cretaceous *G. coriacea* were developed using LC-MS with chromatography on a Waters 2695 system and mass spectroscopy on a Thermo Finnigan LCQ Advantage. The experiment for anti-oxidation activities of flavonoids from Cretaceous *G. coriacea* leaves was performed on a Waters 2695-2996 HPLC system according to the on-line HPLC-DPPH method for the detection of radical-scavenging compounds (Koleva et al. 2000).

Analysis of volatiles from *Ginkgo* leaves

Powdered samples from the three sets of *Ginkgo* leaves (Cretaceous, 150-year-preserved and extant) were all extracted for 24 h with methanol at room temperature. The three extracts were concentrated in vacuum at room temperature to give three residues, which were partitioned between ether and methanol (approximately 95%) to give the ether layer and methanol layer, respectively. The three ether extracts were subjected to GC-MS analysis. The GC was operated as follows: analysis column, HP-5MS (30 m × 0.25 mm × 0.25 μm); column temperature, held from 80 °C and ramped at 5 °C/min to 280 °C/min; elution, pure He; concentration of ether extract, 2 mg/mL; and injection volume, 0.2 μL. The MS was operated in full scan mode (35–450, 1 scan/s, 70 eV ionization energy). Peaks were identified by comparison of their relative retention times, and mass spectral characteristics, with NBS mass spectral library software.

Analysis of flavonoids from Cretaceous *G. coriacea*

The methanol extract of the three samples of *Ginkgo* leaves were subjected to HPLC analysis for the detection of flavonoids. A Waters 2695-2996 HPLC system was used for the analysis of flavonoids in the three samples of *Ginkgo* leaves at 310 nm. A gradient elution from 30% solvent A (methanol) and 70% solvent B (0.2% formic acid : 0.2 g formic acid dissolved in 100 g water) to 100% of methanol at a flow rate of 1.0 mL/min in 30 min was chosen in the separation procedure.

Further analysis of flavonoids from Cretaceous *G. coriacea* was performed using LC-MS. The following procedure was chosen for LC: analysis column, XTTRA C_{18} (3.5 μm, 3.0 mm × 50.0 mm); flow rate, 0.1 mL/min; column temperature, 40 °C; concentration of methanol extract, 5 mg/mL; injection volume, 50 μL; and detection λ, 310 nm. A gradient elution procedure was chosen as follows: held at 50% solvent A (methanol) and 50% solvent B (0.2% formic acid) for 5 min, then graduated from 50% solvent A to 100% solvent A in 55 min, and kept at 100% solvent A for 10 min.

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