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Assessment of components of *Gingko biloba* leaves collected from different regions of China that contribute to its antioxidant effects for improved quality monitoring

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Abstract

Ginkgo biloba leaves are commonly used in traditional Chinese medicine. Owing to its wide distribution across China with varying geographical environments and climatic characteristics, *G. biloba* leaf products have different active ingredients and degrees of activities, which poses a challenge for quality control and standardization. We aimed to identify the key factors contributing to the quality of *G. biloba* based on assessment of anti-aging and antioxidative components. *G. biloba* leaf samples collected from 14 different regions in China were analyzed for antioxidant activities by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay, and the levels of terpene lactones and flavonoids were quantified by ultra-performance liquid chromatography-tandem mass spectroscopy and high performance liquid chromatography. Correlation analysis attributed the antioxidant activity of *G. biloba* leaf samples of different origins to isoginkgetin and ginkgetin, which also affected the matrix metalloproteinase-1 levels of fibroblasts, determined using enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction. Therefore, appropriate quality control of a collected *G. biloba* sample could be achieved by determining the levels of isoginkgetin and ginkgetin as anti-aging and antioxidant components. The results further provide a foundation for the application of *G. biloba* leaves as an antioxidant raw material.

Keywords: antioxidant; anti-aging activity; *Ginkgo biloba*; ginkgetin; isoginkgetin

Practical Application: *Ginkgo biloba* leaves can be used as antioxidant raw material.

1 Introduction

Ginkgo biloba L. is a large tree with an estimated age of at least 200 million years (Editorial Committee of Chinese Flora of the Chinese Academy of Sciences, 1978). *G. biloba* leaves have long been used in traditional Chinese medicine owing to its extensive pharmacological action such as scavenging free radicals (Shao et al., 2003; Wang & Yang, 2001) .*In vivo* assays of the anti-aging effect of *G. biloba* leaf extracts have shown a reduction in the development of skin wrinkles, abnormal hyperplasia of the epidermis, and degradation of elastic fibers during photoaging in BALB/c mice. Oxidative stress can change the expression of matrix metalloproteinases (MMPs) and affect the process of aging. Experiments with fibroblasts have shown that *G. biloba* leaf extracts inhibited induction of the pro-inflammatory factors interleukin (IL)-1, IL-1β, IL-6, and tumor necrosis factor-alpha by UVB exposure through the inhibition of extracellular signal-regulated kinase, c-Jun N-terminal kinase, p38 protein, and reactive oxygen species in the mitogen-activated protein kinase pathway, which ultimately inhibit the increase of MMPs (Chen et al, 2014). Other studies have shown that the main active ingredients of *G. biloba* leaves are flavonoids and terpene lactone compounds, which reduce oxidative stress and apoptosis to inhibit cell damage (Lin et al, 2008; Yao et al., 2014).

G. biloba leaves are considered a specialty plant material in China with an extensive cultivation area ranging from Shenyang in the northeast to Guangzhou in the south, and growing from altitudes of 40–1000 m in the east to below 2000 m (Gong et al., 2008; Guo et al., 2019). Moreover, the components of *G. biloba* leaves are complex and the most relevant components responsible for its well-established antioxidant activity remain unclear. To clarify these issues, in the present study, we collected *G. biloba* leaves from 14 different areas of China, which were quantitatively analyzed for five terpene lactones and eight flavonoids along with comparison of the antioxidant activity of the samples. These results can help to identify consistent markers of antioxidant activity despite geographical and environmental variation, which can be used for quality control of *G. biloba* and to potentially enhance the antioxidant activity of the leaves as a functional food.

2 Materials and methods

2.1 Materials

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Biodee Biotechnology Co., Ltd.

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(Beijing, China). The MMP-1 ELISA kit (No. I21032323) was purchased from CUSABIO (Wuhan, China). Reference standards of rutin (1), quercetin-3-*O*-*β*-glucoside (2), kaempferol-3-*O*-*β*-D-glucopyranoside (3), isorhamnetin-3-*O*-glucoside (4), myricetin (5), apigenin (6), isoginkgetin (7), ginkgetin (8), ginkgolide A (9), ginkgolide B (10), ginkgolide C (11), ginkgolide J (12), and bilobalide (13) were purchased from Beijing Simianti Tech. Co., Ltd. (Beijing, China). Human dermal fibroblasts (HDFs) were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The XGB-40-B laboratory water purifier was purchased from Shenyang Xinjie Technology Co., Ltd. (Shenyang, China). The Ultimate 3000 HPLC-UV system and the TSQ Altis mass spectrometer were purchased from Thermo Fisher Scientific (Shanghai, China). The TSK-GEL ODS column (4.6 mm ×25 cm) was purchased from TOSOH (Shanghai, China) and the ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) was purchased from Waters (Shanghai, China).

2.2 G. biloba leaves collection

Based on a fieldwork investigation of planted resources and collected samples, *G. biloba* leaves from 14 regions across China were selected for antioxidant analysis, and the 13 chemical constituents described above were quantified in each sample. Detailed sample location information is shown in Table 1, which is in the supplemental file, and the structures of the five terpene lactones and eight flavonoids are shown in Figure 1.

2.3 Extraction of G. biloba leaves

G. biloba leaves (10 g) were crushed and 200 mL 95% ethanol (w/v, 1:20) was added. The mixture was extracted for 1 h at 50 °C by ultrasonication three times, and the filtered supernatants were concentrated and dried. The *G. biloba* leaf extracts were further diluted to concentrations of 0.03, 0.06, 0.12, 0.25, 0.5, and 1.0 mg/mL with anhydrous ethanol to analyze the 13 compounds of interest and conduct the ABTS free radical scavenging assay.

2.4 Flavonoid quantification by high performance liquid chromatography/diode-array detection

The chromatographic analysis was conducted under the following conditions: mobile phase, water with 0.2% phosphoric acid (A) and acetonitrile (B); flow rate, 0.5 mL/min; column temperature, 30 °C; injection volume, 5.0 μL; and detection wavelength, 270 nm. The elution gradient was as follows: 85–75% (A) at 0-25 min, 75-70% (A) at 25-40 min, 70-50% (A) at 40-45 min, 50-40% (A) at 45-55 min, 40-30% (A) at 55-70 min, and 30-30% (A) at 70-75 min (Wang et al., 2020).

We accurately weighed an appropriate amount of the references rutin (1), quercetin-3-*O*-*β*-glucoside (2), kaempferol 3-*O*-*β*-D-glucopyranoside (3), isorhamnetin-3-*O*-glucoside (4), myricetin (5), apigenin (6), isoginkgetin (7), and ginkgetin (8), which were established as mix reference solutions with concentrations of 12, 6, 12, 12, 12, 6, 12, and 12 μg/mL, respectively. We then analyzed the prepared mixed standards according to the chromatographic conditions described above and constructed the calibration curve for each reference.

Terpene lactone quantification by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

The TSQ Altis mass spectrometer (Thermo Fisher) was used for mass spectrometric detection with the following conditions: ion source type, heated electrospray ionization; detection mode, selected reaction monitoring (SRM); ion, 3500 V; negative ion, 3500 V; sheath gas, 35 Arb; auxiliary gas, 10 Arb; vaporizer temperature, 350°C; ion transfer tube temperature, 300°C; CID gas, 2 mTorr. The information for the SRM mode of the reference compounds is provided in Table 2, which is in the supplemental file.

Table 1. The collection location *G. biloba* leaves resource.

No.	Origin	Latitude	Longitude
1	Anguo	N38°42′	E115°33’
2	Liaoning	N38°43′-43°26′	E118°53′-125°46′
3	Xuzhou	N33°43′-34°58′	E116°22′-118°40′
4	Jinan	N6°35′-36°40′	E116°54′-117°02′
5	Nanjing	N31°14′-32°37′	E118°22′-119°14′
6	Sichuan	N26°03′-34°19′	E97°21′-108°12′
7	Hubei	N29°01′-33°6′	E108°21′-116°07′
8	Tancheng	N34°22′-34°56′	E118°05′-118°31′
9	Shaoyang	N25°58′-27°40′	E109°49′-112°57′
10	Xinjiang	N34°22′-49°10′	E73°40′-96°23′
11	Lianyungang	N33°59′-35°07′	E118°24′~119°48′
12	Hebei	N36°05′-42°40′	E113°27′-119°50′
13	Guangdong	N20°13′-25°31′	E109°39′-117°19′
14	Zhengzhou	N34°16′-34°58′	E112°42′-114°14′

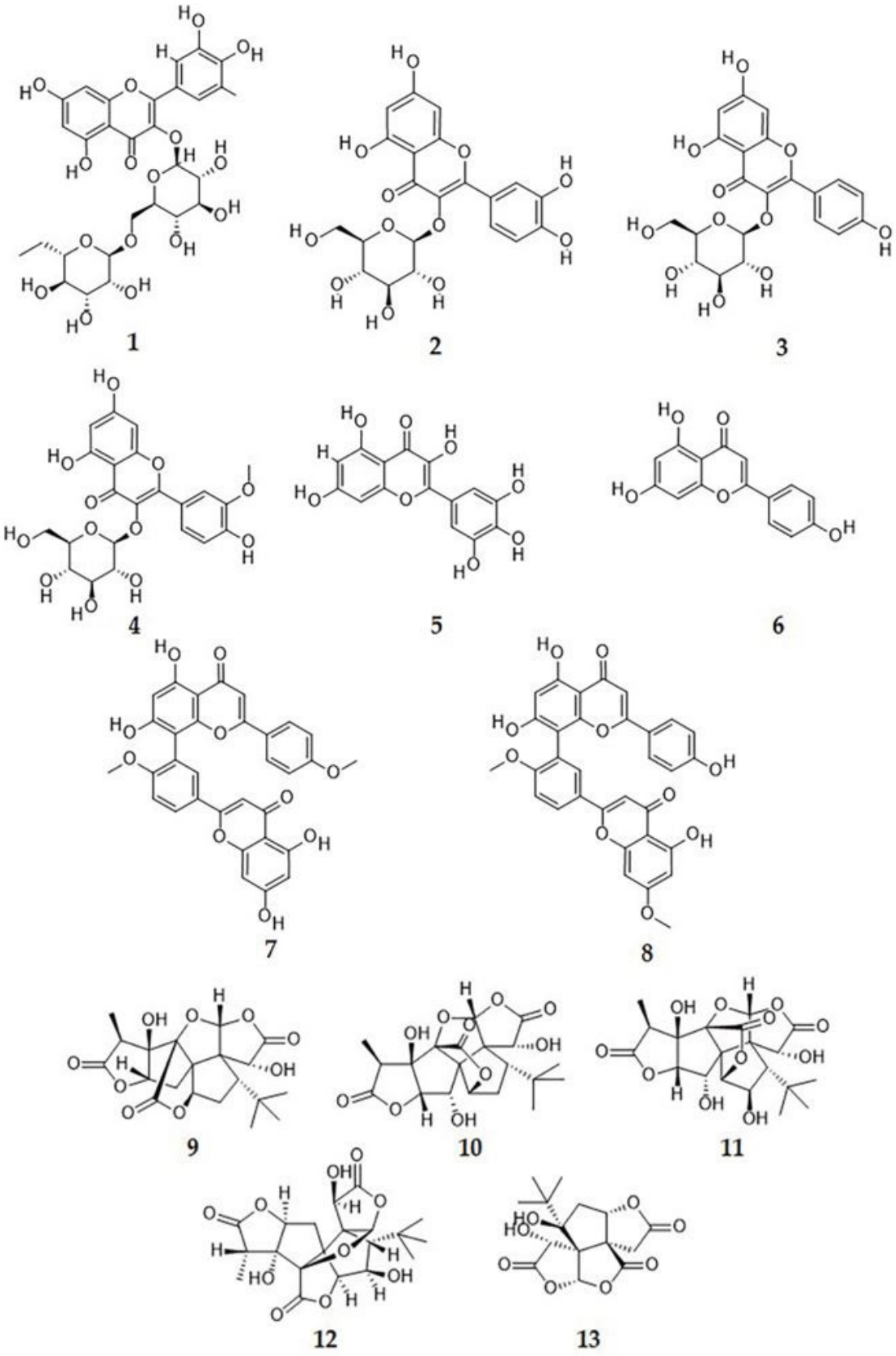

Figure 1. The structure of eight flavonoids in *G. biloba* leaves: rutin (1), quercetin-3-*O*-*β*-glucoside (2), kaempferol-3-*O*-*β*-D-glucopyranoside (3), isorhamnetin-3-*O*-glucoside (4), myricetin (5), apigenin (6), isoginkgetin (7) and ginkgetin(8); The structure of five lactones in G. biloba leaves: ginkgolide A (9), ginkgolide B (10), ginkgolide C (11), ginkgolide J (12), and bilobalide (13).

Table 2. Determination of eight flavonoids and five lactones in *G. biloba* leaf (n = 3, $\bar{x}$ ± s).

Origin	Rutin	Quercetin-3-*O*-*β*-glucoside	Kaempferol-3-*O*-*β*-D-glucopyranoside	Isorhamnetin-3-*O*-glucoside	Myricetin	Apigenin	Isoginkgetin	Ginkgetin	Ginkgolide A	Ginkgolide B	Ginkgolide C	Ginkgolide J	Bilobalide
1	5.28 ± 0.05	1.70±0.003	2.49±0.06	2.30±0.02	2.70±0.03	1.49±0.01	6.34±0.06	5.09±0.08	0.82±0.04	1.52±0.09	1.27±0.02	0.22±0.03	8.62±0.11
2	6.80 ± 0.01	1.93 ± 0.003	2.41 ± 0.00	2.61 ± 0.01	4.00 ± 0.02	1.45 ± 0.02	6.05 ± 0.04	5.29 ± 0.01	1.78 ± 0.04	1.76 ± 0.04	1.06 ± 0.01	0.43 ± 0.02	8.91 ± 0.42
3	8.68 ± 0.19	2.48 ± 0.03	2.53 ± 0.18	2.67 ± 0.01	6.03 ± 0.05	—	7.07 ± 0.14	6.63 ± 0.10	4.18 ± 0.04	2.77 ± 0.29	1.81 ± 0.53	0.92 ± 0.02	22.89 ± 0.25
4	6.44 ± 0.03	1.89 ± 0.01	2.57 ± 0.17	2.45 ± 0.06	3.72 ± 0.05	1.48 ± 0.03	6.63 ± 0.03	5.95 ± 0.01	1.18 ± 0.11	1.64 ± 0.23	1.05 ± 0.16	0.27 ± 0.09	7.85 ± 0.16
5	4.27 ± 0.08	1.36 ± 0.02	—	2.39 ± 0.03	4.01 ± 0.06	1.90 ± 0.01	6.33 ± 0.07	5.83 ± 0.08	1.93 ± 0.08	2.16 ± 0.19	2.05 ± 0.07	0.34 ± 0.13	11.43 ± 0.45
6	5.15 ± 0.01	1.60 ± 0.13	—	2.37 ± 0.01	3.40 ± 0.01	1.64 ± 0.01	6.09 ± 0.01	5.36 ± 0.01	1.61 ± 0.03	2.18 ± 0.13	1.82 ± 0.08	0.40 ± 0.13	10.75 ± 0.06
7	7.28 ± 0.01	2.14 ± 0.01	2.60 ± 0.01	3.29 ± 0.07	4.72 ± 0.01	1.31 ± 0.00	5.70 ± 0.02	6.37 ± 0.01	2.38 ± 0.02	2.98 ± 0.23	3.54 ± 0.01	0.66 ± 0.05	18.86 ± 0.13
8	4.20 ± 0.08	1.38 ± 0.01	—	—	3.30 ± 0.04	1.49 ± 0.01	5.76 ± 0.06	5.96 ± 0.07	2.37 ± 0.08	2.77 ± 0.15	3.12 ± 0.08	0.54 ± 0.15	11.83 ± 0.29
9	4.15 ± 0.03	1.77 ± 0.02	2.40 ± 0.02	2.43 ± 0.02	5.31 ± 0.04	—	2.78 ± 0.07	4.45 ± 0.06	2.53 ± 0.14	2.02 ± 0.16	2.17 ± 0.06	0.38 ± 0.03	21.92 ± 0.28
10	7.52 ± 0.28	2.51 ± 0.01	2.62 ± 0.01	2.75 ± 0.03	5.20 ± 0.01	1.56 ± 0.01	8.03 ± 0.01	6.36 ± 0.01	2.21 ± 0.19	2.33 ± 0.16	1.99 ± 0.04	0.52 ± 0.16	11.15 ± 0.19
11	4.94 ± 0.01	2.35 ± 0.01	2.78 ± 0.01	2.70 ± 0.02	3.63 ± 0.01	1.31 ± 0.01	7.56 ± 0.02	4.98 ± 0.02	1.37 ± 0.11	2.14 ± 0.28	1.41 ± 0.03	0.43 ± 0.03	10.62 ± 0.53
12	5.72 ± 0.02	2.01 ± 0.01	2.65 ± 0.01	2.75 ± 0.01	3.57 ± 0.02	—	7.16 ± 0.04	6.13 ± 0.03	1.01 ± 0.03	1.74 ± 0.08	1.82 ± 0.01	0.30 ± 0.12	11.04 ± 0.68
13	5.16 ± 0.01	1.65 ± 0.01	2.51 ± 0.01	2.34 ± 0.01	3.07 ± 0.01	1.51 ± 0.02	5.62 ± 0.04	5.83 ± 0.09	0.93 ± 0.10	1.69 ± 0.08	1.29 ± 0.07	0.27 ± 0.02	10.37 ± 0.25
14	6.94 ± 0.01	2.19 ± 0.002	—	2.63 ± 0.01	16.52 ± 0.01	1.15 ± 0.01	5.74 ± 0.04	4.81 ± 0.01	7.55 ± 0.19	3.60 ± 0.14	4.45 ± 0.75	1.42 ± 0.08	48.95 ± 1.45

"—"means no detection.

For chromatography, the references were separated with the ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm), using acetonitrile (A) and 0.1% formic acid solution (B) as the mobile phase with a gradient of 10% A at 0-10 min. The flow rate was 0.3 mL/min, the column temperature was set at 30°C, and the injection volume was 2.0 μL.

We accurately weighed an appropriate amount of the standard references ginkgolide A, B, C, and J, and bilobalide to prepare the mixed reference solutions at five concentrations of 8.6, 15.7, 3.8, 3.9, and 1.6 μg/mL. We then constructed each calibration curve using the mixed standard reference concentrations according to the UPLC-MS/MS conditions described above. The limit of detection and limit of quantitation were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively.

2.5 Determination of ABTS free radical scavenging activity

We thoroughly mixed 0.2 mL of the different concentrations of each sample with 0.8 mL ABTS solution and allowed the mixture to react in the dark for 30 min at 25 °C. The absorbance of the mixture was measured at 734 nm using 0.2 mL distilled water as the blank control, and the clearance rate was calculated according to the following formula (Equation 1):

$$Clearance\ rate\ (\%) = \left[(A_0 - A)/A_0\right] \times 100\% \quad (1)$$

where A_0 is absorbance of the control and A is absorbance of the sample

2.6 Correlation between antioxidant activity and components of G. biloba extracts

The Pearson correlation coefficient of the half-maximal inhibitory concentration (IC_{50}) value of the antioxidant index (clearance rate) measured using the ABTS assay and the 13 components of the extract was calculated using Statistical Package for the Social Sciences (SPSS) version 25. Student's *t*-test was used to determine the active components that were significantly related to the IC_{50} (Xu & Zhang, 2017).

2.7 Cell culture and cell viability assay

HDFs were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) and 1% penicillin-streptomycin (Gibco BRL, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. HDFs were seeded at a density of 1×10^4 cells/well in 96-well plates for 24 h. The medium was removed, the cells were washed with sterilized phosphate-buffered saline (PBS), and the buffer was replaced with the medium containing the samples diluted to varying concentrations (0.05, 0.1, 0.2, and 0.4 mg/mL). After 24-h incubation, the medium was removed, the cells were washed with sterilized PBS, and HDF proliferation was measured using the Cell Counting Kit (CCK)-8 assay after diluting the CCK-8 solution 1:10. The absorbance of the cell suspension was then read at 450 nm using a microplate reader. The cell viability is expressed as a percentage of that of the negative control, and each sample was assayed using three replicates (Lee et al., 2006).

2.8 Enzyme-linked immunosorbent assay (ELISA) of MMP-1 levels

HDFs were seeded in six-well plates (5×10^5 cells/mL) with DMEM containing 10% FBS for 24 h, and then the medium was replaced with serum-free medium containing the test samples. After incubation for 24 h, the supernatant was collected from each well and MMP-1 levels in the supernatant were quantified by ELISA according to the instructions of the kit (Du et al., 2017).

2.9 Reverse transcription-quantitative polymerase chain reaction (RT-PCR)

HDFs were seeded in six-well plates (1.8×10^5 cells/well) and incubated overnight at 37°C in a 5% CO_2 incubator. After treatment with the samples for 24 h, HDF cells were harvested, total RNA was extracted using TRIzol RNA extraction reagent, and then cDNA was synthesized from the total RNA. The *MMP1* primer sequences used to amplify the desired cDNA were forward, 5′-AAGGTGGACCAACAATTTCAGA-3′ and reverse, 5′-TGAAGGTGTAGCTAG GGTACATCAA-3′. Fluorescence quantitative PCR detection was then performed, and each sample was assayed in triplicate.

2.10 Statistical analysis

SPSS version 25.0 was used for all statistical analyses. Each experiment was repeated three times, and the data are expressed as the mean ± standard deviation. Data between groups were compared using analysis of variance, and $P < 0.01$ was considered statistically significant.

3 Results

3.1 Quantification of flavonoids and terpene lactones in G. biloba leaves

UPLC-MS/MS analysis of lactones in the 14 *G. biloba* leaf samples showed some differences in the levels of rutin (1), quercetin-3-*O*-*β*-glucoside (2), kaempferol-3-*O*-*β*-d-glucopyranoside (3), isorhamnetin-3-*O*-glucoside (4), myricetin (5), apigenin (6), isoginkgetin (7), ginkgetin (8), ginkgolide A (9), ginkgolide B (10), ginkgolide C (11), ginkgolide J (12), and bilobalide (13). The results of the HPLC analysis and UPLC-MS/MS analysis are shown in Table 2. Part of the HPLC analysis results and the standard diagram of UPLC-MS/MS analysis is shown in Figure 2.

3.2 Determination of antioxidant activity

There were also differences in the ABTS scavenging abilities of *G. biloba* leaf extracts collected from different areas (Table 3). Many oxidative stress-related diseases and physiological processes such as aging are caused by the accumulation of free radicals in the human body. Flavonoids and terpene lactones from *G. biloba* leaf extracts are reported as good antioxidants (Kingsley et al., 2019; Qiu et al., 2017). Therefore, we further determined the specific components contributing to the variation in antioxidant properties of *G. biloba* leaves of different regions.

3.3 Correlation between the IC_{50} value determined using ABTS and active components

Analysis of the 13 compounds in *G. biloba* leaf extracts from 14 different regions and their antioxidant activity (Table 4) demonstrated a significant correlation between the levels of isoginkgetin and ginkgetin and the IC_{50} value ($r = -0.761$ and -0.703, respectively; both $P < 0.01$). The lower the IC_{50}, the stronger the antioxidant activity, which explains the negative correlation. Therefore, these two flavonoids were considered to be mainly responsible for the antioxidant property of *G. biloba* leaves.

3.4 Effect of isoginkgetin and ginkgetin on HDF viability

Pretreatment of HDFs for 24 h with two different concentrations (0.1 and 0.2 mg/mL) of isoginkgetin and ginkgetin showed no significant differences in cell viability compared with that of the control group ($P > 0.05$). Furthermore, pretreatment of HDF cells with the two compounds at concentrations of 0.1 and 0.2 mg/mL caused no cytotoxicity: In 0.1 mg/mL, isoginkgetin and

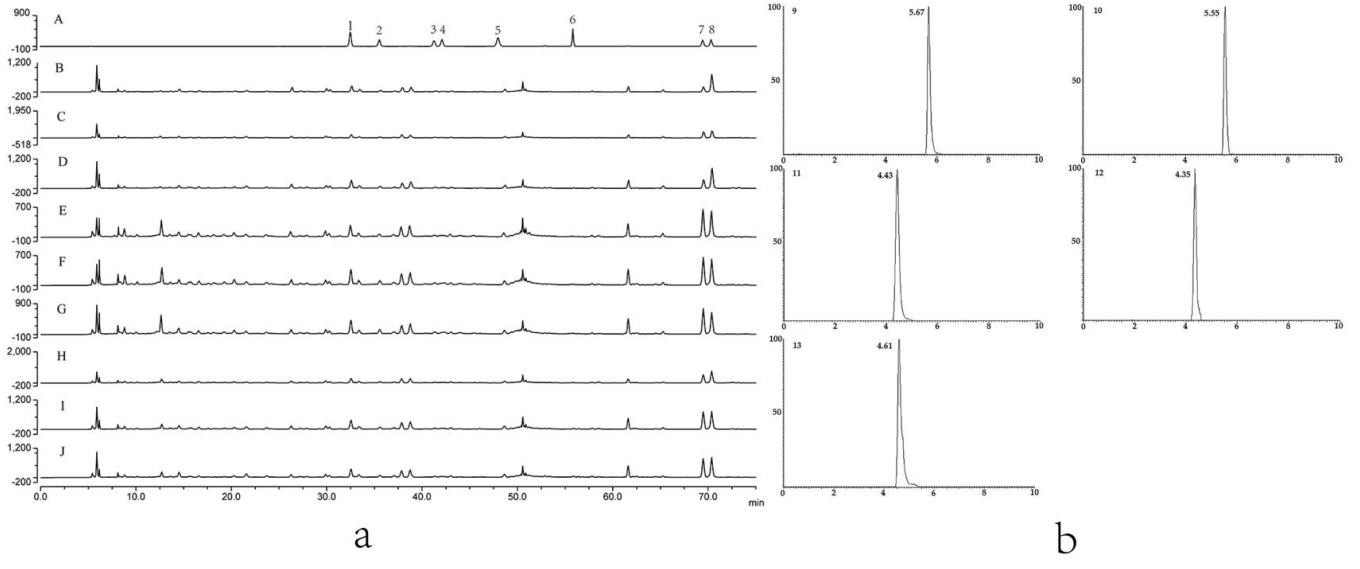

Figure 2. (a) is the result of HPLC analysis: rutin (1), quercetin-3-*O*-*β*-glucoside (2), kaempferol-3-*O*-*β*-D-glucopyranoside (3), isorhamnetin-3-*O*-glucoside (4), myricetin (5), apigenin (6), isoginkgetin (7), ginkgetin (8). A-J are the results of part of origin. (b) is the standard diagram of UPLC-MS/MS analysis: ginkgolide A (9), ginkgolide B (10), ginkgolide C (11), ginkgolide J (12), and bilobalide (13).

Table 3. ABTS free radical scavenging ability of *G. biloba* leaves from 14 different places (%) (n = 3, $\bar{x}$ ± s).

Origin	1 (mg/ mL)	0.5 (mg/mL)	0.25 (mg/mL)	0.125 (mg/mL)	0.06 (mg/mL)	0.03 (mg/mL)	IC_{50} (mg/mL)
1	90.31 ± 0.76	78.98 ± 1.28	48.48 ± 1.85	37.25 ± 1.78	26.30 ± 1.96	12.56 ± 2.11	0.296
2	82.44 ± 2.59	75.47 ± 0.29	46.81 ± 0.78	32.40 ± 1.31	19.98 ± 1.48	4.01 ± 0.05	0.379
3	93.50 ± 0.82	91.51 ± 0.87	66.89 ± 1.53	60.02 ± 1.75	37.48 ± 1.98	26.99 ± 1.29	0.131
4	96.22 ± 1.20	70.90 ± 2.66	60.20 ± 2.89	40.20 ± 1.57	33.74 ± 1.21	21.16 ± 1.78	0.237
5	98.33 ± 1.54	76.43 ± 2.25	58.01 ± 1.94	43.37 ± 1.78	30.54 ± 1.04	22.18 ± 1.27	0.221
6	85.07 ± 2.45	67.52 ± 2.11	51.11 ± 2.50	42.41 ± 1.70	30.59 ± 1.92	24.11 ± 1.06	0.301
7	90.47 ± 0.23	85.97 ± 2.91	55.01 ± 1.47	40.07 ± 1.26	31.98 ± 2.26	17.74 ± 0.85	0.239
8	86.06 ± 1.42	71.31 ± 1.51	53.40 ± 1.37	31.23 ± 1.30	33.58 ± 1.09	26.14 ± 0.90	0.254
9	76.22 ± 1.70	62.12 ± 1.61	35.33 ± 1.04	35.54 ± 2.02	24.40 ± 1.08	13.48 ± 1.86	0.461
10	96.66 ± 2.37	88.21 ± 2.50	62.48 ± 2.92	39.49 ± 2.34	37.54 ± 1.20	16.70 ± 5.08	0.196
11	86.35 ± 1.87	79.39 ± 1.67	69.71 ± 1.65	40.92 ± 2.02	25.70 ± 1.89	18.96 ± 0.19	0.262
12	97.47 ± 1.93	87.65 ± 1.40	62.40 ± 0.90	44.36 ± 1.33	28.43 ± 1.71	14.67 ± 1.90	0.203
13	80.66 ± 2.08	62.47 ± 1.84	48.19 ± 2.67	39.85 ± 2.25	29.86 ± 0.89	9.54 ± 1.01	0.386
14	95.81 ± 1.85	75.12 ± 1.18	53.08 ± 2.00	43.33 ± 0.46	30.12 ± 1.24	14.99 ± 2.21	0.254

Table 4. The correlation of the 13 chemical compounds and the activities in *G. biloba* leaves.

Compounds	Correlation	Compound	Correlation
Rutin	-0.518	Ginkgetin	-0.703**
Quercetin-3-*O*-β-glucoside	-0.426	Ginkgolide A	-0.215
Kaempferol-3-*O*-*β*-D-glucopyranosid	0.069	Ginkgolide B	-0.375
Isorhamnetin-3-*O*-glucoside	-0.079	Ginkgolide C	-0.20
Myricetin	-0.125	Ginkgolide J	-0.329
Apigenin	0.035	Bilobalide	-0.074
Isoginkgetin	-0.761**		

**$P < 0.01$.

ginkgetin are 98 ± 1.83 and 94 ± 1.73, respectively, in 0.2 mg/mL, isoginkgetin and ginkgetin are 93 ± 2.48 and 99 ± 1.59, respectively. Therefore, these concentrations were selected for further experiments.

3.5 Effect of isoginkgetin and ginkgetin on MMP-1 levels in HDFs

Isoginkgetin and ginkgetin at 0.1 and 0.2 mg/mL significantly reduced MMP-1 levels in HDFs ($P < 0.01$; Figure 3 A). PCR analysis showed that isoginkgetin and ginkgetin significantly ($P < 0.01$) inhibited *MMP1* expression (Figure 3 B).

4 Discussion

The widespread distribution of *G. biloba* and research focused on exploring the therapeutic value of natural medicine therapeutic agents are continuously developing, which have together promoted research on *G. biloba* leaves (Gong et al., 2008; Guo et al., 2019). Although many studies have been conducted on the antioxidant effects of *G. biloba* leaves in recent years, there is still a lack of research on the detailed antioxidant properties and mechanisms, especially with respect to identification of the most potent active ingredients. Further clarifying the compounds in *G. biloba* leaves that are most relevant for its antioxidant activity is of great significance for promoting the development of *G. biloba*-related industries. The purpose of the present study was therefore to confirm the active ingredients in *G. biloba* leaves with the strongest relation to its antioxidant activity, and to provide a scientific basis for the quality control of antioxidants in *G. biloba* leaves. Correlation analysis of the antioxidant activity of *G. biloba* leaves and active ingredients was used to identify the compounds significantly related to the antioxidant activity of *G. biloba* leaves collected across 14 disparate regions in China, and the activity of the relevant analysis results were verified by ELISA and PCR. These methods showed that isoginkgetin and ginkgetin have obvious antioxidant activity.

Quantitative analyses of eight flavonoids and five lactones in the 14 samples showed that the active ingredient and antioxidant properties of *G. biloba* leaves from different origins were significantly different, and flavonoids were not detected in samples from certain origins. To ensure that acceptable standards are met, we only collected the leaves of 4–7-year-old trees from August to October (Du et al., 2000). Differences in geographical environments and climatic conditions, including precipitation

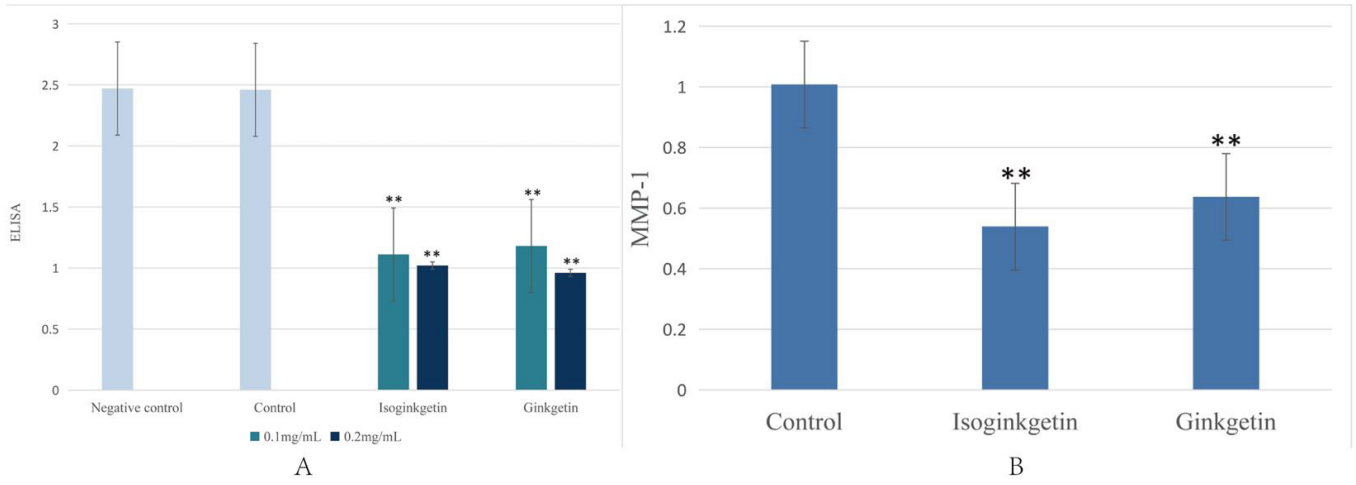

Figure 3. (A) is the results of ELISA on effects of two components on the secretion of MMP-1 from fibroblasts (n = 3, x,– ± s), $^{**}P < 0.01$; (B) is the results of PCR effects of two components on the secretion of MMP-1 from fibroblasts n = 3, x,– ± s), $^{**}P < 0.01$.

and altitude, in different cultivation regions of China lead to variations in the active components of *G. biloba* leaves, which ultimately affect the quality (Lin et al., 2019; Yao et al., 2017). This uncontrolled distribution creates a considerable challenge to conventional quality control strategies that currently depend on morphological characteristics, making the qualitative or quantitative determination of one or several bioactive marker components important (Xie et al., 2010).

Strong negative correlations between isoginkgetin or ginkgetin and antioxidant activity were identified, which was based on the IC_{50} value. This indicated that a higher content of these two flavonoids in *G. biloba* leaves results in stronger antioxidant activity. In general, peroxidation is one of the main factors leading to an increase in MMP levels. The ELISA results showed that isoginkgetin and ginkgetin at 0.1 and 0.2 mg/mL significantly reduced MMP-1 levels in HDFs at the protein and gene levels. Collectively, these results indicate that these two flavonoids are the two most active ingredients with strong relations to the antioxidant activity of *G. biloba* leaves. Therefore, when formulating the quality standards of *G. biloba* leaves, if the antioxidant effect is the main therapeutic effect, attention should be paid to the content of these two compounds.

Notably, the quantitative analysis showed that *G. biloba* leaves from Xinjiang province, Xuzhou (Jiangsu province), and Hebei province had higher levels of isoginkgetin and ginkgetin than leaves collected from other areas. Therefore, *G. biloba* growing in these locations should be prioritized as the raw materials of medicinal products when being applied for antioxidant effects.

Botanical medicines are a combination of multiple compounds, including those that play a pharmacological role (Wang et al., 2018); however, it is important to clarify the most relevant active ingredients contributing to the pharmacological effects. Although previous studies have reported the antioxidant activity of *G. biloba* leaves as well as the antioxidant activities of isoginkgetin and ginkgetin, the most relevant active ingredients contributing to the antioxidant activity of *G. biloba* leaves have not been scientifically studied to date. The present research can therefore provide a scientific basis for the development of the ginkgo leaf medicine industry. Moreover, these two compounds are also present in *Dioon edule* Lindl. leaves, and ginkgetin is present in *Taxus wallichiana* Zucc. and some *Selaginella* plants (Liu et al., 2002; Moawad & Amir, 2016; Qiu et al., 1989). Accordingly, our findings can also provide new directions for the pharmacological research of other plants.

5 Conclusions

Through correlation analysis of the antioxidant activity of *G. biloba* leaves and their active ingredients in different regions, and the use of ELISA and PCR to verify the correlation analysis results, we identified isoginkgetin and ginkgetin as the likely main active compounds in *G. biloba* with antioxidant and MMP-1 inhibitory activity. Therefore, monitoring the levels of these two compounds might improve estimating the quality of *G. biloba* leaf raw material. The results of this study provide a scientific basis and reference for the application of *G. biloba* leaf as an antioxidant raw material. The levels of these components were the highest in the materials collected from Xinjiang province, Xuzhou, and Hebei province, which indicates that these regions should be prioritized for obtaining *G. biloba* leaf materials with superior quality.

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