SUPPLEMENTARY MATERIAL

Comparative analysis of chemical constituents and bioactivities of the extracts from leaves, seed coats and embryoids of *Ginkgo biloba L.*

Xuanxuan Chen[a], Wu Zhong[a], Changqing Shu[b], Hong Yang[a, c], Erhu Li[a, c*]

[a] College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China

[b] College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, Hubei, China

[c] Key Laboratory of Environment Correlative Dietology (Huazhong Agricultural University), Ministry of Education, Wuhan 430070, Hubei, China

[*] Corresponding Author: Erhu Li

Email addresses：erhuli@mail.hzau.edu.cn (Erhu Li) ;

Abstract:

A total of 25 compounds including terpenoids, flavonoids, biflavonoids and ginkgolic acids were identified and quantified with a reliable, simple, and simultaneous method from Ginkgo leaves, seed coats and embryoids with different tree ages (approximately identified as 25, 500, 1000, and 2000 years). Leaves had the highest amount of total bioactive compounds. Seed coats had moderate contents of flavonoids, which was 15 time higher than embryoids. Furthermore, the effects of tree ages on bioactive compounds differ in 3 parts. The contents of bilobalide, ginkgolide J, ginkgolide C, ginkgolide B, ginkgolide A in embryoids and seed coats were highest from 500-year-old tree, while in leaves were highest from 25-year-old tree. This work first investigated the extensive bioactive compounds in ginkgo leaves, seed coats and embryoids from *Ginkgo* trees older than 500-year, it gives good reference for making better use of *Ginkgo* products.

Keywords: *Ginkgo biloba L.*; bioactive compounds; α-glucosidase; antioxidant; tree ages; UPLC-Q-Extractive Orbitrap mass spectrometer

Experimental

Chemicals and reagent

HPLC-MS grade acetonitrile and formic acid were purchased from Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Standards of flavonoids and terpenoids were purchased from Yuanye Biological Technology Company (Shanghai, China). All other chemicals were of analytical grade.

Plant materials and extracts

The *Ginkgo* parts (leaves and seeds) were collected from Suizhou Ginkgo Valley of Hubei Province, China and identified by Dr. Changqing Shu (Huazhong Agriculture University, college of Horticulture and Forestry Sciences). The ages of Ginkgo trees were tentatively identified mainly based on the site conditions, tree size, tree height and crown width. The voucher specimens (No.2018091303, No.2018091350, No.2018091362, No.2018091364) have been deposited in the college of Horticulture and Forestry Sciences, Huazhong Agriculture University (Wuhan, China).

The geographical coordinates address of the four plant samples are as follows: #50: E 113^{o}17’44.466”, N 31^{o}26’16.292”. #64: E 113^{o}17’45.571”, N 31^{o}26’05.903”. #62: E 113^{o}17’46.252”, N 31^{o}26’07.624”. #3: E113^{o}17’46.255”, N 31^{o}26’29.782”. As these plants are extremely close to each other, their site condition including soil, water and other environment factors can be regarded as identical.

Ginkgo leaves and seeds were harvested in September, 2018. A total of 12 samples, consisting of 3 different parts (leaf, seed coat and embryoid) with 4 different ages (approximately identified as 25, 500, 1000, and 2000 years) were collected. Each sample contains 100 seeds or 100 leaves randomly collected from four trees. Samples were recorded as L1: leaf of 25 years, L2: leaf of 500 years, L3: leaf of 1000 years, L4: leaf of 2000 years; S1: seed coat of 25 years, S2: seed coat of 500 years, S3: seed coat of 1000 years, S4: seed coat of 2000 years; E1: embryoid of 25 years, E2: embryoid of 500 years, E3: embryoid of 1000 years, E4: embryoid of 2000 years.

After collection, samples were air-dried outdoor for two days, then the seed coats were manually peeled, and the nutshell as well as mesosperms were removed and then the embryoids were obtained (Figure S1). Seed coats, embryoids and leaves were dried in a hot-air oven at 65 °C to constant weight. The extraction process was

performed referring to previous method with a slight change (Zhou G, Yao, et al. 2014). Each sample of 1.0 g of dry powder was mixed with 8 mL of 70% ethanol and extracted for 30 mins in the ultrasound device, and then in water bath at 60 °C for 1 hour. After 3 times of extraction, the filtrates were collected and centrifuged at 18900 g for 10 mins to collected the supernatant and filled with 70% ethanol to a final volume of 24 mL. The final extract was filtrated through 0.22 um reinforced nylon membrane filters prior to HPLC-MS analysis.

Total flavonoids and total phenolic contents

The total flavonoid content (TF) in *Ginkgo* samples was determined by the method described by (Gogna et al. 2015). 1 mL of leaf or embryoid or seed coat extract was added into 9 mL 70% (v/v) ethanol, 1 mL $NaNO_2$ (5%) was then added, the mixture was shaken well and then left in the dark for 6 min, followed by the addition of 4 mL NaOH (4%) and the distilled water was immediately added to reach the total volume of 25 mL, after shaken well, the mixture was put in dark again for 15 min. Absorbance was measured at 510 nm using a UV-spectrphotometer. The TF was expressed as mg rutin equivalent per gram of dry weight of *Ginkgo* samples.

The total phenolic (TP) content was evaluated by the Folin–Ciocalteu spectrophotometric method (Zhou KB et al. 2011). 1 mL of the sample extract was mixed with 1 mL of Folin–Ciocalteu phenol reagent, and then put in the dark for 3 min. Then 10 mL of 7% Na_2CO_3 solution were added, followed by addition of distilled water (25 mL) for dilution. The absorbance was measured at 750 nm after a 2 hours incubation period at room temperature. The standard curve was prepared using gallic acid. The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of dry *Ginkgo* sample. All measurements were performed three times and the results were averaged.

Determination of flavonoids, terpenoids, biflavonoids, and ginkgolic acids

UPLC-ESI-MS analysis

UPLC-ESI-MS analysis was carried out using an Q Exactive UPLC system connected to a thermo scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer via ESI interface (Thermo Fisher Scientific, CA, USA). Chromatographic separation was carried out on an Accucore UPLC a Q column (2.1 × 150 mm, 2.6 μm, Thermo Fisher Scientific, CA, USA). Gradient elution of acetonitrile (solvent A) and 0.1% formic acid solution (solvent B) was employed:

0-12 min, 5–20% B; 12–22 min, 20–35% B; 22–36 min, 35–60% B; 36–41 min, 60–95% B; 41–42 min, 95–5% B; 42–50 min, 5% B. The injection volume was 3 μL, with a flow rate of 0.2 mL/min. The constant column temperature was 30 °C. The tandem mass experiment was performed in negative ESI ionization modes with the data acquisition ranging of m/z 50-1200. The capillary voltage was optimized to 3.8 kV, and the capillary temperature was 320 °C. The Probe Heater temperature was 350 °C and the cone voltage was 40 V. The results were analysed by the Xcalibur software.

Quantitative analysis

The MS signal of each compound was collected in the selected ion-monitoring mode (SIM) according to their molecular ions. All quantification was based on a peak area ratio of the SIM signal of the analyte and the commercial standard. For compounds with no available standards, similar compounds of the same flavonoid and terpenoid subgroup were selected. The results were expressed as micrograms of commercial standard per gram of dry weight.

Assays of antioxidant activity in vitro

DPPH assay

The ability to scavenge the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated according to the method described by (Wan et al. 2012) with minor modifications. Ascorbic acid (vitamin C) was used as a positive control, and aqueous ethanol was used as a blank control. In this experiment, 1.0 mL of sample solution mixed with 3 mL of 2×10−4 mol/L DPPH radical solution in aqueous ethanol, after incubation in the dark at room temperature for 60 min. The absorbance of the solutions was measured at 517 nm. The analysis was performed in triplicate and the scavenging rate was calculated as equivalent ascorbic acid per gram dry weight samples (mg AAE/g DW).

ABTS assay

The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was conducted according to the slightly modified method of (Wang Q et al. 2018). $ABTS^{\cdot+}$ reagent was produced by reacting 7 mM ABTS solution with 2.45 mM/L potassium persulphate aqueous solution in the dark at room temperature for 16 hours before use. The ABTS·+ solution was diluted with 50% (v/v) ethanol to the appropriate absorbance (0.70±0.02) at 734 nm. Then 1 mL of sample solution was

mixed with 3 mL of ABTS solution, after incubated in the dark at room temperature for 1 hour, the absorbance of the solutions was measured at 734 nm. The analysis was performed in triplicate and the scavenging rate was calculated as equivalent ascorbic acid per gram dry weight samples (mg TE/g DW).

TRP assay

The total reducing power (TRP) of the 12 samples was evaluated by the previous method with a slightly modification (Wu et al. 2017). Extracts of Ginkgo leaves, seed coats and embryoids were used as reducers to convert Fe^{3+} to Fe^{2+} form in the assay system. In briefly, 2 mL of sample solution mixed with 2 mL of sodium phosphate buffer (0.2 mol/L, pH 6.6) and 2.0 mL of potassium ferricyanide (1%). Then, the mixtures were incubated in 50 ℃ water for 20 min, and cooled quickly by running cold water. 2 mL of trichloroacetic acid (10%) was added to end the reaction. After addition 2.5 mL distilled water and 0.5 mL ferric chloride (1%) solution, and reaction for 5 min, the absorbance was measured using a spectrophotometer at a wavelength of 700 nm. Each sample was repeated two times. Distilled water and vitamin C were used as blank and positive control, and the results were calculated as equivalent ascorbic acid per gram dry weight samples (mg AAE/g DW).

α-Glucosidase inhibition assay in vitro

For α-glucosidase assays, the methods described by (Mojica et al. 2015) were followed with a modification. In a 96-well plate, 40 μL of 100 times diluted sample extracts was added to 20 μL of 0.25 u/mL α-glucosidase solution in 0.1 M sodium phosphate buffer (pH 6.9) and incubated at 37 ℃ for 15 min. Then, 40 μL of 0.5 mM pNPG in PBS (pH 6.9) was added briefly to each well and incubated at 37 ℃ for 20 min. Before reading of the absorbance at 405 nm, the reaction was stopped with 100 μL of 0.1 M Na_2CO_3. Acarbose was used as positive control and PBS as blank control. The inhibition activity was expressed as micromole acarbose per gram dry weight sample (mg/g DW). The α-glucosidase inhibition percentage of samples and positive control was calculated using the following equation: Inhibition activity (%) = $[(A_{sample} - A_2) / (A_{control} - A_1)] \times 100\%$ where $A_{control}$ is the absorbance of PBS that replaces the samples; A_{sample} is the absorbance of the samples; A_1 is the absorbance of PBS that replaces the samples and the enzyme; and A_2 is the absorbance of PBS that replaces the enzyme.

Statistical analysis

All data was expressed as mean ± standard deviation (SD) and represented using GraphPad Prism 5.0. Statistical analysis of the data was performed with SPSS (version 24.0). One-way analysis of variance (ANOVA) Duncan's multiple range test was used to detect the statistical significance and differences were considered as significance, when $P < 0.05$.

Analysis of total flavonoid and phenolic content

The total flavonoid (TF) and total phenolic (TP) content of *Ginkgo* samples were shown in Figure S2. Considering the plant part, leaves exhibited the highest TF with an average of 1.19 mg/g DW, followed by sead coats with 0.46 mg/g DW, whereas embryoids showed much lower contents with 0.18 mg/g DW. Among leaf samples, 2000-year old trees showed the highest TF, while leaves from 1000-year old trees had the lowest TF value. Seed coats from 25-year old trees showed the highest TF, followed by 2000 and 1000-year samples, while the 500 year exhibited the lowest TF content. The total phenolic content (TP) of *Ginkgo* parts displayed similar trends with TF except for seed coats. The TP in seed coats ranged from 5.15 to 7.06 with an average of 6.07 mg/g DW, which was similar to leaf samples (6.45 mg/g DW). With regard to tree age, the TP in seed coats increased with the tree age and the highest TP was found in 2000-year old trees. As for leaves, the lowest TF was observed from 500-year-old tree, that may because the degradation rate of the flavonoids decreased when the tree age become older than 500 years.

Identification of the terpenoids, flavonoids, biflavonoids, and ginkgolic acids

A total of 25 compounds, including 5 terpenoids (bilobalide, ginkgolides A, B, J and C), 17 flavonoids, 2 ginkgolic acids and 1 biflavonoid in *Ginkgo* leaves, seed coats, and embryoids were identified by comparing the retention time and MS/MS fragments with standards or references (Table S1).

As for terpenoids, compound 4, 7, 8, 16, 17 were identified as bilobalide, ginkgolide J, ginkgolide C, ginkgolide A and ginkgolide B. The loss of 56 amu $[M-H-2CO]^-$, was characterized as the most common fragment pathway. Compound **4** showed $[M-H]^-$ precursor ion at *m*/*z* 325.093, with the major fragments of *m*/*z* 163, 251, 281, was identified as bilobalide. Compound **16** with the molecular ion at *m*/*z* 407.135 and fragment of *m*/*z* 351.15 was identified as ginkgolide A. Compound **8** with molecular weight at *m*/*z* 440.398, and product ion at *m*/*z* 383, was identified as ginkgolide C. Compound **7** and compound **17** had the same $[M-H]^-$ precursor ion at

m/z 423.127 and the same product ion at *m/z* 367, but compound **7** occurred at a shorter retention time, so it can be tentatively determined that compound **7** was ginkgolide J and compound **17** was ginkgolide B (Zhou G, Pang, et al. 2014).

17 flavonoid constituents were identified. Compound **1**, **3**, **6**, **10**, **18** were tentatively identified as coniferin, caffeic acid, 4-coumaric acid, rutin, and quercetin according to the previous studies (Wang L-J et al. 2015) or comparing the $[M-H]^-$ ions and MS^2 fragmentations to standards.

Kaempferol (compound **19)** displayed the MS^2 fragmentation of the ion at m/z 285.041 gave fragment ions at m/z 213, 257, 133. These fragments were consistent with those found for its standard solution. Despite the fact that compound **2** gave the same $[M-H]^-$ ion as compound **5**, it occurred at a much shorter retention time. For the m/z 289.072 and the MS^2 fragments of 109,123, they can be identified as epicatechin (compound **2**) and catechin (compound **5**), which are isomers. Compound **11** owed a $[M-H]^-$ ion at m/ 463. 376 and the fragment of m/z 300, 271, corresponding to the loss of a hexoside residue (163 amu) and was identified as isoquercetin (Wang L-J et al. 2015).

Compound **12** (m/z 593.151) showed the fragment of m/ z 447, with the loss of 146 amu corresponding to the fraction of raminoside, and the fragment of m/z 285 was kaempferol, so it was tentatively identified as Kaempferol-3-O-rutinoside. Compound **13** (m/z 447.093) showed the m/z 285 fragment as a consequence of the loss of 162 amu (a hexose unit), so it was tentatively identified as Kaempferol-3-O-glucoside (Gouveia and Castilho 2011).

Compound **21** showed a $[M-H]^-$ ion at m/z 269.046, with the main MS^2 fragment ions at m/z 251, 225, 151 and was identified as apigenin [20]. Compound **9** gave a $[M-H]^-$ ion at m/z 431.099 and subsequent fragmentation showed the loss of 162 amu, which corresponds to a hexose. The fragmentation at m/z 269 is typical of apigenin, and the most common substitution position for flavonoids is the 7-OH, so compound **9** was tentatively identified as apigenin-7-O-hexoside (Gouveia and Castilho 2011).

Compound **20** yielded the characteristic ions at *m/z* 299.056 in negative ion mode and produced MS/MS fragment at *m/z* 284, by the loss of CH_3 fragment, suggesting it was diosmetin. Compound **15** generated $[M-H]^-$ ion at *m/z* 461.109 with 162 amu higher than that of diosmetin, speculating that it was diosmetin glycoside, and it

produced a key negative MS^2 ion at *m/z* 285 may correspond to the neutral loss of glucose, so it was tentatively identified as diosmetin-7-O-glucoside (Chen et al. 2019).

Compound **22** and **14** were tentatively identified as isorhamnetin and isorhamnetin-7-O-glucoside. Because compound **22** presented a $[M-H]^-$ ion at m/z 315.051 and had a major MS/MS fragmention of m/z 269, the characteristic ion of isorhamnetin. Compound **14** showed a$[M-H]^-$ ion at m/z 477.104 and a product ion at m/z 315, a typical ion of isorhamnetin with the loss of 162 amu, a single glucoside assumed to be at the position of 7-OH (Ding et al. 2008).

Isoginkgetin (compound **23)** presented the $[M-H]^-$ ion at m/z 565.114 and produced the ion at m/z 533 in the MS/MS spectrum with a loss of CH_3OH, suggesting a methoxyl group could be linked to C4 or C7, it was preliminarily identified as isoginkgetin (Yao et al. 2017).

Ginkgolic acid (C15:1) and ginkgolic acid (C13:0) (compound **24** and **25)** showed deprotonated molecule of $[M-H]^-$ at *m/z* 345.244 and *m/z* 319.228, and ginkgolic acid (C15:1) has two more CH_2 than ginkgolic acid (C13:0) (Zhou G, Yao, et al. 2014).

The identification analysis indicated that kaempferol, quercetin and isorhamnetin were the major flavonoid aglycone in *Ginkgo* parts. The terpenoids can increase the activities of the antioxidant enzymes (SOD and catalase) and improve cell viability (Mahadevan and Park 2008). Except for ginkgolide A, ginkgolide B and ginkgolide C, ginkgolide J was found as a dominant terpenoids in *Ginkgo* in this work. Ginkgolic acid has been demonstrated to be a potent multi-target inhibitor of key enzymes in the biosynthesis of pro-inflammatory lipid mediators and was also abundant in *Ginkgo* leaves and seed coats.

Antioxidant activity and α-Glucosidase inhibitory activity

The DPPH radical scavenging capacities ranged from 0.16 to 9.05 mg AAE/g DW (Figure S3, A). Leaves and seed coats showed great DPPH radical scavenging capacities with average values of 6.89 and 5.79 mg AAE/g DW, while embryoids presented the lowest value with an average of 0.17 mg AAE/g DW. Leaves had the highest value from 2000-year-old tree (9.054±0.02 mg AAE/g DW), and the lowest from 500-year-old tree (4.861±0.04 mg AAE/g DW). As for seed coats, the highest DPPH value was from 25-year-old tree (7.65±0.06 mg AAE/g DW) and the weakest

value was from 500-year-old tree (4.58±0.10 mg AAE/g DW). The DPPH radical scavenging capacities of embryoids varied without significant differences ($p<0.05$).

The ABTS antioxidant activities of the 12 samples presented the similar trends with DPPH (Figure S3, B). The highest value was found in leaves of 2000-year-old tree (27.71±0.07 mg TE/g DW) and the lowest value was observed in embryoids of 500-year-old tree (1.44±0.09 mg TE/g DW). The ABTS antioxidant activities in leaves and seed coats were significantly higher than embryoids, with an average of 22.27 mg TE/g DW in leaves and 16.50 mg TE/g DW in seed coats and only 1.66 mg TE/g DW in embryoids.

The TRP-based antioxidant activities ranged from 2.38 to 21.25 mg AAE/g DW (Figure S3, C). The leaves and seed coats showed much greater TRP values than embryoids. As for seed coats, the TRP-based antioxidant activities presented the highest value in 2000-year old (21.25±0.27 mg AAE/g DW) and the lowest in 500-year-old tree (9.167±0.080 mg AAE/g DW). Similarly, the 2000-year old leaves had the highest TRP-based antioxidant activity of 20.45±0.07 mg AAE/g DW.

As the inhibition rate of embryoids was too low and the data was not presented. Seed coats and leaves exhibited strong α-glucosidase inhibition values. The results showed that the inhibitions of seed coats were significantly stronger, with an average of 7.04 mg acarbose/g DW, about 3 times of leaves (2.25 mg acarbose/g DW). The 25-year-old seed coats presented the highest α-glucosidase inhibition capacity, whereas the 500-year-old seed coats showed the lowest. The α-glucosidase inhibition capacity of leaves from different tree ages had slightly differences, with the highest in 2000-year-old tree (2.48 mg acarbose/g DW) and the lowest in 25-year-old tree (2.05 mg acarbose/g DW).

Tables

Table S1. Compounds tentatively identified in extracts of *Ginkgo* samples by UPLC-ESI-MS in negative ion mode.

NO.	Rt(min) [a]	Tentative identification	Mw	[M-H] -	MS/MS fragments	Molecular formula	Error (ppm)	References
1	2.89	Coniferin	342.341	341.109	179.06	$C_{16}H_{22}O_8$	0.93	(Zhou G, Yao, et al. 2014)
2	7.69	Epicatechin	290.268	289.072	109.03，123.04	$C_{15}H_{14}O_6$	-0.16	Standard [b]
3	8.74	Caffeic acid	180.157	179.034	135.04	$C_9H_8O_4$	0.15	standard
4	8.82	Bilobalide	326.299	325.093	281,251,237,163	$C_{15}H_{18}O_8$	0.59	(Zhou G, Yao, et al. 2014)
5	9.22	Catechin	290.268	289.072	109.03，123.04	$C_{15}H_{14}O_6$	0.27	standard
6	10.97	4-coumaric acid	164.158	163.039	119.05	$C_9H_8O_3$	-0.93	standard
7	11.37	Ginkgolide J	424.399	423.127	367.14	$C_{20}H_{24}O_{10}$	1.51	(Ding et al. 2008)
8	11.68	Ginkgolide C	440.398	439.124	383.13	$C_{20}H_{23}O_{11}$	-1.14	(Ding et al. 2008)
9	11.81	Apigenin-7-O-hexoside	432.378	431.099	269.05,251.11	$C_{21}H_{20}O_{10}$	0.18	(Soares et al. 2019)
10	11.97	Rutin	610.518	609.147	300.03	$C_{27}H_{30}O_{16}$	0.51	standard
11	12.38	Isoquercetin	464.376	463.088	300.03,271.03	$C_{21}H_{20}O_{12}$	1.31	Standard
12	13.25	Kaempferol-3-rutinoside	594.518	593.151	285.04,255.03,227.04	$C_{27}H_{30}O_{15}$	1.52	(Kelebek 2016)
13	13.79	kaempferol 3-O-glucoside	448.377	447.093	255.03,227.03,284.03	$C_{21}H_{20}O_{11}$	1.20	(Soares et al. 2019)
14	14.10	Isorhamnetin-7-O-glucoside	478.403	477.104	243.03,271.03,314.04	$C_{22}H_{22}O_{12}$	2.61	(Ding et al. 2008)
15	15.91	Diosmetin 7-O -glucoside	462.044	461.109	299,243,227	$C_{22}H_{22}O_{11}$	0.50	(Chen et al. 2019)
16	16.30	Ginkgolide A	408.399	407.135	351.15	$C_{20}H_{24}O_9$	1.30	standard
17	16.31	Ginkgolide B	424.399	423.127	367.14	$C_{20}H_{24}O_{10}$	2.45	(Ding et al. 2008)
18	18.59	Quercetin	302.236	301.035	149.02,191.03	$C_{15}H_{10}O_7$	0.27	standard
19	22.18	Kaempferol	286.236	285.041	133.03	$C_{15}H_{10}O_6$	1.09	standard
20	24.78	Diosmetin	300.263	299.056	284,256	$C_{16}H_{12}O_6$	2.31	(Chen et al. 2019)
21	29.09	Apigenin	270.237	269.046	251.11,227.03	$C_{15}H_{10}O_5$	0.97	(Gouveia and Castilho 2011)

22	31.12	Isorhamnetin	316.262	315.051	300.03,269.12	$C_{16}H_{12}O_7$	0.53	(Ding et al. 2008)
23	31.60	Isoginkgetin	566.511	565.114	374.04,117.03,533.03	$C_{32}H_{22}O_{10}$	1.62	(Yao et al. 2017)
24	37.33	Ginkgolic acid (C15:1)	346.504	345.244	301.25	$C_{22}H_{34}O_3$	0.92	(Zhou G, Yao, et al. 2014)
25	41.25	Ginkgolic acid (C13:0)	320.466	319.228	275.24,121.03,106.04	$C_{20}H_{32}O_3$	0.02	(Zhou G, Yao, et al. 2014)

[a] Rt, retention time.

[b] The compounds marked with 'standard' mean that they were identified by coelution with their corresponding standards and MS data.

Table S2. Quantification of terpene lactones, flavonoids, biflavonoid, and ginkgolic acids in extracts of *Ginkgo* embryoids, seed coats and leaves (μg/g of dry weight).

Category	Compounds	E1	E2	E3	E4	S1	S2	S3	S4	L1	L2	L3	L4
Terpene lactones	Bilobalide	22.66±1.52a	45.8±5.53b	31.6±8.28a	22.02±0.95a	20.42±3.01a	28.86±27.78a	29.72±10.70a	39.26±10.22a	1021.34±141.18b	470.2±176.51a	181.56±33.03a	278.07±5.98a
	Ginkgolide J	34.35±3.86ab	47.26±1.10b	36.68±15.60ab	15.11±0.68a	85.7±6.87b	93.67±5.36b	79.46±14.15b	37.32±0.70a	234.27±16.55a	175.03±22.78ab	148.22±25.31b	141.88±20.00b
	Ginkgolide C	472.26±0.05c	599.82±57.95c	329.68±75.63b	151.99±14.45a	1334.69±16.93d	1142.15±21.4c	891.1±2.65b	464.84±40.52a	2894.96±203.48c	1179.75±35.81b	834.23±6.94a	796.7±47.54a
	Ginkgolide A	1.88±0.12b	3.38±0.21c	0.92±0.02a	3.47±0.46c	55.73±3.86a	93.13±7.55c	73.26±4.90b	54.27±3.69a	268.54±10.23c	171.61±22.75b	91.09±10.03a	170.93±11.36b
	Ginkgolide B	984.54±21.38b	1987.79±42.12d	1525.28±170.09c	575.22±69.37a	2419.33±128.6c	2754.52±91.88d	1773.66±6.15b	1261.13±72.13a	6281.60±397.10d	5240.01±396.83c	2642.28±113.67a	3524.04±66.10b
	sum	**1515.69**	**2684.05**	**1924.16**	**767.81**	**3915.87**	**4112.33**	**2847.20**	**1856.82**	**10880.71**	**7236.46**	**3897.38**	**4929.62**
Flavonoids	Coniferin	0.59±0.06b	0.6±0.06b	0.28±0.08a	1.05±0.04c	1.02±0.07a	0.79±0.42a	0.77±0.23a	0.99±0.1a	0.62±0.88a	0.89±0.49a	1.08±0.45a	0±0a
	Epicatechin	nd	nd	nd	nd	2.58±0.74a	3.66±3.41a	2.69±2.90a	12.39±0.93a	72.11±0.03c	64.67±0.70b	40.62±0.66a	153.76±3.85d
	Caffeic acid	1.72±1.49a	0.9±1.28a	1.58±0.11a	0.86±0.12a	15.98±2.52a	30.53±3.22c	19.36±0.85ab	23.79±2.38b	27.03±3.60b	28.21±3.60b	20.3±2.92b	3.54±0.59a
	Catechin	0.22±0.01ab	0.6±0.27b	0.14±0.19ab	nd	2.87±0.02c	1.54±0.17b	0.39±0.22a	1.23±0.07b	47.58±1.69b	43.40±1.55b	37±1.09a	62.85±3.52c
	4-coumaric acid	nd	0.31±0.44a	0.53±0.04a	0.41±0.01a	2.94±0.26b	3.1±0.12b	3.74±0.24c	1.98±0.17a	33.42±2.99a	91.83±12.44b	108.52±6.33b	39.01±3.04a
	Apigenin-7-O-hexoside	nd	nd	nd	nd	nd	nd	nd	nd	11.83±1.16b	13.8±2.05b	7.09±0.10a	11.85±0.29b

	Rutin	nd	nd	1.79±0.07a	1.90±0.08a	38.95±2.85d	31.15±0.79b	10.74±0.94c	24.29±1.82a	1443.77±99.36b	1090.62±48.37a	1202.89±96.26ab	1242.97±94.68ab
	Isoquercetin	nd	nd	nd	nd	7.00±3.04a	4.85±0.55a	2.45±1.71a	4.31±0.20a	433.37±33.69c	248.08±8.35a	233.24±1.85a	354.54±6.29b
	Kaempferol-3-rutinoside	0.4±0.02a	0.34±0.03a	0.35±0.08a	0.32±0.05a	8.67±0.40d	6.34±0.13c	2.82±0.14a	5.37±0.34b	1062.12±47.61b	721.11±2.43a	753.18±12.13a	757.80±26.53a
	kaempferol 3-O-glucoside	nd	0.06±0.01c	0.49±0.15b	0.29±0.02a	2.79±0.01b	1.63±0.16a	1.58±0.09a	1.68±0.02a	441.81±3.73c	289.01±8.48a	257.81±21.45a	358.24±7.15b
	Isorhamnetin-3-glucoside	nd	0.39±0.42c	0.12±0.05b	0.09±0.02a	7.24±0.23d	4.45±0.08c	2.65±0.70b	1.56±0.16a	405.00±8.06c	209.53±7.17a	176.81±14.73a	253.63±20.25b
	Diosmetin 7-O-glucoside	nd	nd	nd	nd	nd	nd	nd	nd	116.92±13.08b	75.07±1.62a	92.53±2.09a	79.17±3.07a
	Quercetin	nd	nd	nd	nd	0.06±0.02a	0.16±0.02b	0.24±0.02c	0.19±0.01b	1016.99±26.53a	2635.70±690.16b	1807.23±349.93ab	1356.41±118.21a
	Kaempferol	nd	nd	nd	nd	0.01±0.02a	0.69±0.09c	0.01±0.01a	0.35±0.01b	0.63±0.13a	2.65±0.32b	2.17±0.37b	1.31±0.09a
	Diosmetin	0.11±0.02ab	0.22±0.04c	0.15±0.02b	0.07±0.01a	0.51±0.02a	1.5±0.42b	0.1±0.01a	0.28±0.01a	1.53±0.27a	2.11±0.17b	2.17±0.10b	1.15±0.05a
	Apigenin	nd	nd	nd	nd	0.46±0.05ab	0.62±0.1a	0.37±0.07a	0.39±0.02b	6.85±0.37a	10.41±0.77b	14.21±0.51c	8.72±1.03ab
	Isorhamnetin	nd	3.51±0.20b	1.19±0.18a	0.87±0.10a	2.7±0.05b	1.38±0.12a	1.74±0.08a	1.9±0.58a	2.69±0.01a	6.02±2.76a	5.57±2.02a	3.00±0.34a
	sum	**3.04**	**6.93**	**6.62**	**4.86**	**93.77**	**92.39**	**49.65**	**80.70**	**5123.07**	**5533.06**	**4762.42**	**4687.95**
Biflavonoids	Isoginkgetin	nd	nd	nd	nd	252.68±137.87ab	424.42±7.76b	179.87±16.87a	353.21±9.20ab	1982.78±380.93b	648.26±242.05a	1142.85±705.47ab	1680.03±162.71ab
Ginkgolic acid	Ginkgolic Acid (C15:1)	0.31±0.02b	0.19±0.03a	0.17±0.03a	0.17±0.01a	39.76±3.61a	43.5±4.06a	49.77±7.80a	43.27±6.73a	14.12±2.32a	13.96±12.93a	6.16±0.55a	3.98±0.40a
	Ginkgolic Acid (C13:0)	143.19±9.94b	171.98±14.78b	166.58±23.05b	56.36±17.49a	387.7±31.16a	463.35±5.12ab	547.61±0.82b	445.4±73.89ab	1475.97±46.61bc	1276.81±51.58a	1610.8±99.67c	1364.23±48.27ab
	sum	**143.50**	**172.17**	**166.75**	**56.53**	**427.46**	**506.85**	**597.38**	**488.67**	**1490.07**	**1290.77**	**1616.96**	**1368.21**

Values are means ± sdandard deviation.

Mean values within a line with different letters are significantly different at $p < 0.05$, and the significant analysis was made among four samples in one part (E1, E2, E3, E4 form the embryoid part, S1, S2, S3, S4 form the seed coat part, L1, L2, L3, L4 form the leaf part).

298 **Table S3.** Correlation matrix between chemical compositions and antioxidant activity

299 and inhibitory activity of α-glucosidase

	DPPH	ABTS	TRP	α-glucosidas
TP	0.930**	0.937**	0.948**	0.632*
TF	0.803**	0.865**	0.772**	0.096
Ginkgolide C	0.477	0.474	0.425	0.226
Ginkgolide A	0.714**	0.751**	0.727**	0.131
Ginkgolide B	0.604*	0.616*	0.545	0.014
Ginkgolide J	0.653*	0.731**	0.625*	0.065
Bilobalide	0.393	0.421	0.464	-0.164
Ginkgolic Acid (C15:1)	0.468	0.393	0.43	0.932**
Quercetin	0.539	0.624*	0.501	-0.163
Rutin	0.576	0.691*	0.621*	-0.18
kaempferol 3-O-glucoside	0.571	0.663*	0.616*	-0.187
Ginkgolic Acid (C13:0)	0.683*	0.819**	0.736**	0.031
Kaempferol-3-rutinoside	0.547	0.656*	0.602*	-0.191

300 Note. ** mean the correlated with each other significantly at $p<0.01$ level.

301 * mean the correlated with each other significantly at $p<0.05$ level.

Figures

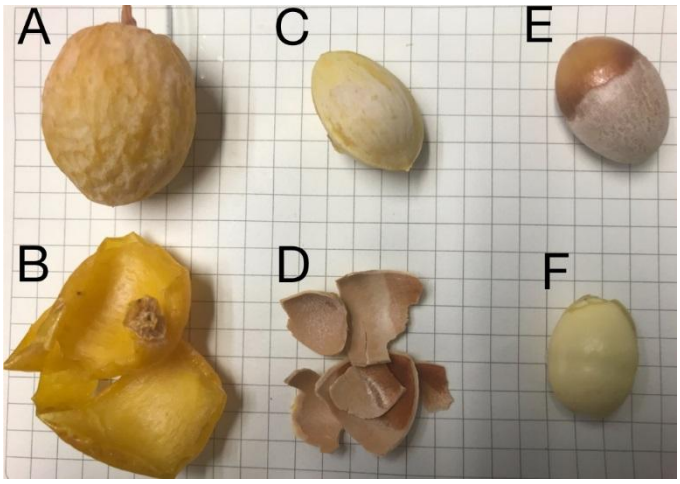

Figure S1. Dissection of *Ginkgo* seed. A: entire seed, B: seed coat, C: peeled seed, D: nutshell, E: seed nut with mesosperm, F: embryoid. Note: B and F were included in this assay.

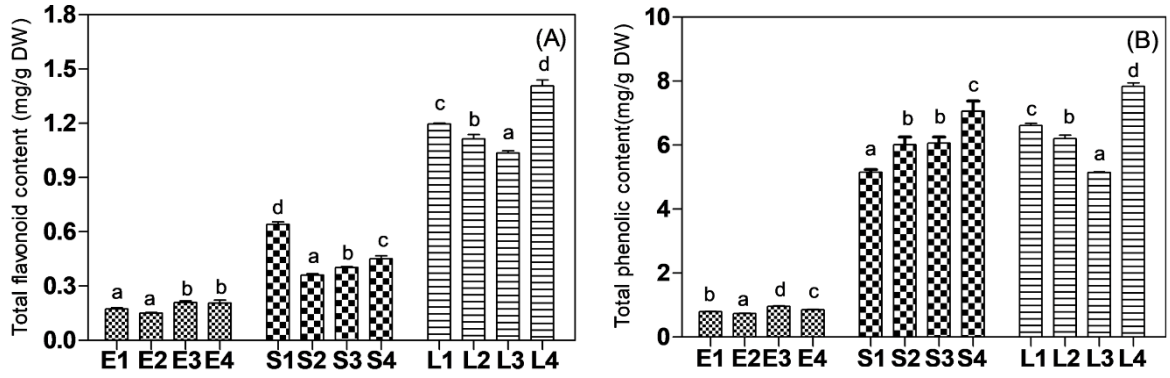

Figure S2. Contents of total flavonoids (A) and total phenolics (B) in *Ginkgo* embryoid, seed coat and leaf with different ages. (E1: embryoid of 25 years, E2: embryoid of 500 years, E3: embryoid of 1000 years, E4: embryoid of 2000 years; S1: seed coat of 25 years, S2: seed coat of 500 years, S3: seed coat of 1000 years, S4: seed coat of 2000 years; L1: leaf of 25 years, L2: leaf of 500 years, L3: leaf of 1000 years, L4: leaf of 2000 years). Data displays average of triplicate treatments ± SE. Different letters denoted the significant difference at $p < 0.05$ level within each part.

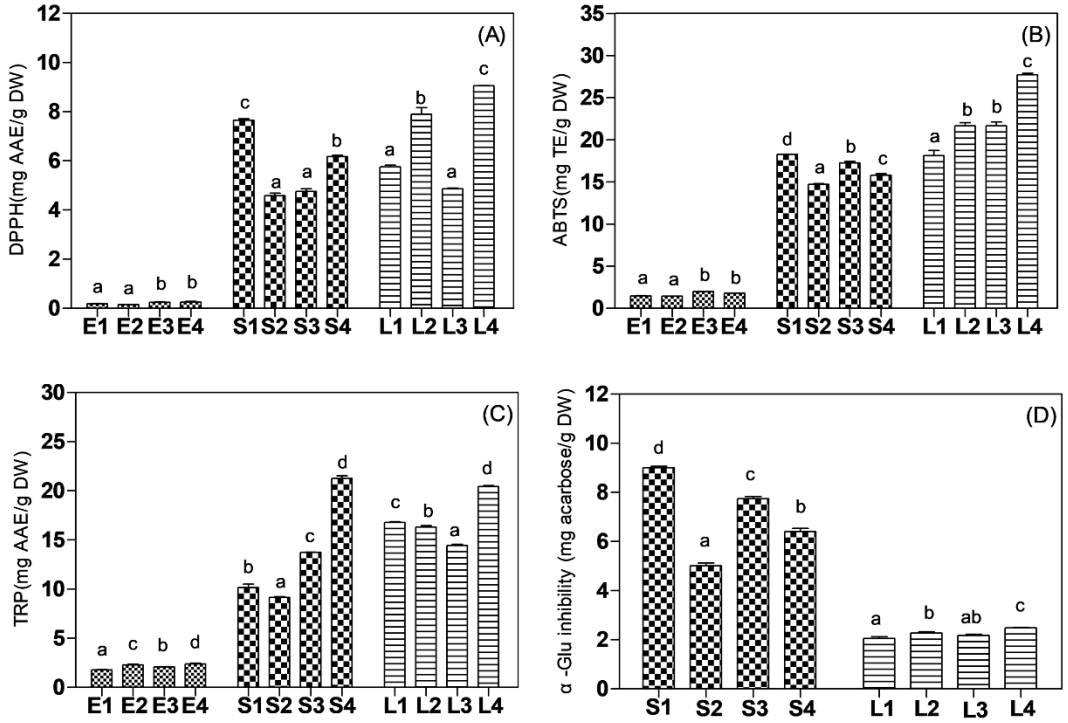

Figure S3.

Antioxidant activity and α-Glucosidase inhibitory activity of 12 Ginkgo samples. (E1: embryoid of 25 years, E2: embryoid of 500 years, E3: embryoid of 1000 years, E4: embryoid of 2000 years; S1: seed coat of 25 years, S2: seed coat of 500 years, S3: seed coat of 1000 years, S4: seed coat of 2000 years; L1: leaf of 25 years, L2: leaf of 500 years, L3: leaf of 1000 years, L4: leaf of 2000 years). Data displays average of triplicate treatments ± SE. Different letters denoted the significant difference at $p < 0.05$ level within each part.

References

Chen X, Xu L, Guo S, Wang Z, Jiang L, Wang F, Zhang J, Liu B. 2019. Profiling and comparison of the metabolites of diosmetin and diosmin in rat urine, plasma and feces using UHPLC-LTQ-Orbitrap MS(n). Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 1124:58-71.

Ding S, Dudley E, Plummer S, Tang J, Newton RP, Brenton AG. 2008. Fingerprint profile of Ginkgo biloba nutritional supplements by LC/ESI-MS/MS. Phytochemistry. 69(7):1555-1564.

Gogna N, Hamid N, Dorai K. 2015. Metabolomic profiling of the phytomedicinal constituents of Carica papaya L. leaves and seeds by 1H NMR spectroscopy and multivariate statistical analysis. Journal of pharmaceutical and biomedical analysis. 115:74-85.

Gouveia S, Castilho PC. 2011. Characterisation of phenolic acid derivatives and flavonoids from different morphological parts of Helichrysum obconicum by a RP-HPLC-DAD-(-)-ESI-MS(n) method. Food chemistry. 129(2):333-344.

Kelebek H. 2016. LC-DAD-ESI-MS/MS characterization of phenolic constituents in Turkish black tea: Effect of infusion time and temperature. Food chemistry. 204:227-238.

Mahadevan S, Park Y. 2008. Multifaceted therapeutic benefits of Ginkgo biloba L.: chemistry, efficacy, safety, and uses. Journal of food science. 73(1):R14-19.

Mojica L, Meyer A, Berhow MA, de Mejía EG. 2015. Bean cultivars (Phaseolus vulgaris L.) have similar high antioxidant capacity, in vitro inhibition of α-amylase and α-glucosidase while diverse phenolic composition and concentration. Food Research International. 69:38-48.

Soares JC, Rosalen PL, Lazarini JG, Massarioli AP, da Silva CF, Nani BD, Franchin M, de Alencar SM. 2019. Comprehensive characterization of bioactive phenols from new Brazilian superfruits by LC-ESI-QTOF-MS, and their ROS and RNS scavenging effects and anti-inflammatory activity. Food chemistry. 281:178-188.

Wan C, Yuan T, Cirello AL, Seeram NP. 2012. Antioxidant and alpha-glucosidase inhibitory phenolics isolated from highbush blueberry flowers. Food chemistry. 135(3):1929-1937.

Wang L-J, Wu J, Wang H-X, Li S-S, Zheng X-C, Du H, Xu Y-J, Wang L-S. 2015. Composition of phenolic compounds and antioxidant activity in the leaves of blueberry cultivars. Journal of Functional Foods. 16:295-304.

Wang Q, Rehman M, Peng D, Liu L. 2018. Antioxidant capacity and α-glucosidase inhibitory activity of leaf extracts from ten ramie cultivars. Industrial Crops and Products. 122:430-437.

Wu Y, Zhou Q, Chen XY, Li X, Wang Y, Zhang JL. 2017. Comparison and screening of bioactive phenolic compounds in different blueberry cultivars: Evaluation of anti-oxidation and alpha-glucosidase inhibition effect. Food Res Int. 100(Pt 1):312-324.

Yao H, Chen B, Zhang Y, Ou H, Li Y, Li S, Shi P, Lin X. 2017. Analysis of the Total Biflavonoids Extract from Selaginella doederleinii by HPLC-QTOF-MS and Its In Vitro and In Vivo Anticancer Effects. Molecules. 22(2).

Zhou G, Pang H, Tang Y, Yao X, Ding Y, Zhu S, Guo S, Qian D, Shen J, Qian Y et al. 2014. Hydrophilic interaction ultra-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (HILIC-UPLC-TQ-MS/MS) in multiple-reaction monitoring (MRM) for the determination of nucleobases and nucleosides in ginkgo seeds. Food chemistry. 150:260-266.

Zhou G, Yao X, Tang Y, Qian D, Su S, Zhang L, Jin C, Qin Y, Duan JA. 2014. An optimized ultrasound-assisted extraction and simultaneous quantification of 26 characteristic components with four structure types in functional foods from ginkgo seeds. Food chemistry. 158:177-185.

Zhou KB, Wang H, Mei WL, Li XN, Luo Y, Dai HF. 2011. Antioxidant Activity of Papaya Seed Extracts. Molecules. 16(8):6179-6192. English.