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# Cultivation and Evaluation of a High-Value *Ginkgo biloba* Variety "ZY 1"

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**Abstract**

*Ginkgo biloba* L., a native of China, enjoys unrivaled fame in the plant world, owing to its multipurpose characters. *Ginkgo* leave extract contains high levels of flavone glycosides and terpene lactones, which are widely used in orthodox or traditional medicine to treat diseases. However, there are few good *Ginkgo* varieties for leaf application to date. In this study, we collected fifteen core *Ginkgo* resources including the variety "Anlu 1" from different locations in China. Through $^{60}$Co-γ-ray irradiation of "Anlu 1", a variety named as "ZY 1" with good properties was cultivated. Field test assays showed that "ZY 1" has obvious advantages compared with "Anlu 1" (control). "ZY 1" grows faster and has no branches but higher leaf yield. More importantly, its leaf extracts contain higher flavonoids and terpenoids contents but lower undesired components such as ginkgolic acids. These results indicated that the variety "ZY 1" may have a high value of leaf application for clinical medicine, health care products and cosmetic.

**Keywords:** *Ginkgo biloba*, the variety "ZY 1", medical properties, leaves application

## 1. Introduction

*Ginkgo biloba* is one of the oldest living tree species in China. This tree is slow growing, reaching maturity at 20-30 years of age and bearing seeds at 30-40 years of age. It shows high resistance to environmental stresses, microbial diseases, pests and gaseous pollutants ozone and $SO_2$ (Hori et al., 1997). These distinguishing features make the *ginkgo* an ideal tree for afforestation, greening and ornament. More importantly, leaf and seed extracts of the ginkgo has been widely used for clinical medicine, health care products, foodstuff and cosmetic (Weinmann et al., 2010; Zuo et al., 2017). The global sale of *Ginkgo biloba* extract (GBE) is in the range of US$ 1 billion, of which US alone accounts for quarter of the amount (Van Beek & Montoro, 2009).

*Ginkgo* leaf extract attract contains many benefit components, including flavonol glycosides and terpene lactones, that have therapeutic actions in controlling cerebral blood flow, protecting against free radicals, and delaying the progress of dementia and diabetes (Dubber & Kanfer, 2004; Singh et al., 2008). In *Ginkgo* leaf extract, flavonol glycosides are present in their glycosidic forms of quercetin, kaempferol and isorhamnetin, and terpene lactones mainly comprise of ginkgolides A, B, C and bilobalides. In contrast, the ginkgolic acid derivatives from *Ginkgo* leaves extract are defined as the undesirable components. They provoke allergic reactions to humans (Liu & Zheng, 2009). The overall five individual common components in ginkgolic acids, namely 13:0 ginkgolic acid, 15:0 ginkgolic acid, 15:1 ginkgolic acid, 17:1 ginkgolic acid, and 17:2 ginkgolic acid are determined. Currently, a key issue in *Ginkgo* leaf application is how to control the contents of these medical properties effectively.

$^{60}$Co-γ radiation is a DNA damaging agent with high mutagenic potential in plants (van Harten, 1998). It not only causes DNA damage through strand breaks but also induces secondary DNA ionization through the induction of oxidation of by-products (Manova & Gruszka, 2015). This technology has been widely used for plant mutation breeding. For instance, a yellow-leaf mutant with chlorophyll-deficient is selected from the

progeny of an irradiated japonica rice variety "Jiahua 1" (Liu et al., 2012). Several wheat-alien translocations are produced from pollen irradiated by $^{60}$Co-γ (Cao et al., 2009). Recently, Pan et al. (2017) identified some $^{60}$Co-γ-induced differentially expressed genes that are responsible for regulation of chromatin states in rice. However, there are no reports about the *Ginkgo* varieties generated by $^{60}$Co-γ radiation.

Currently, there are few good *Ginkgo* varieties for leaf application, which severely hinders the development of *Ginkgo*-related industry. In this study, we cultivated a *Ginkgo* variety "ZY 1" via $^{60}$Co-γ radiation. This variety has obvious phenotypic advantages compared with the control ("Anlu 1", a core variety for leaf application in China). Particularly, "ZY 1" leaves extract contains higher levels of flavonoids and terpenoids but lower ginkgolic acids contents. Thus, the variety "ZY 1" presented here may be useful for leaf application in clinical medicine and other industries.

## 2. Material and Methods

### *2.1 Cultivation of the Ginkgo biloba Variety "ZY 1" by $^{60}$Co-γ Radiation*

Two-year-old "Anlu 1" plants were selected for $^{60}$Co-γ radiation at dose of 100 Gy in Shandong academy of agricultural sciences. The irradiated plants were grafted at appropriate sites of two-year-old rootstocks (the variety "Damaling") grown in different sites from Taian County (Shandong province, China). After two years of growth, a branch of an irradiated plant displayed phenotypic alterations and was then propagated by cuttings. Its phenotypes were consistent and stable in three-year field tests and named as "ZY 1".

### *2.2 Measurement of Growth Parameters*

The scions of the varieties "ZY 1" and "Anlu 1" (control) were grafted on three-year-old rootstocks in Jimo county (Shandong province, China) in March 2014. The parameters (area and weight) of leaves were measured after two years of growth. Ten grafted plants and twenty leaves at middle part of the main stem in each plant were selected for analysis.

### *2.3 Samples Collection and High Performance Liquid Chromatography (HPLC)*

Thirty green leaves of "ZY 1" and control ("Anlu 1") plants were sampled at middle part of main stems in October 2016. After collection, the samples were dried in oven at 60 °C for 48 h, grinded into fine powder with a grinder, and kept in a refrigerator until analysis.

Flavonoids, terpenoids and ginkgolic acids of *Ginkgo* dried leaves were extracted and detected with the HPLC method described in China Pharmacopoeia (Chin, 2015). The analysis was performed on a Waters 1525 Alliance HPLC system (Waters Corp., Milford, MA) equipped with binary pump (Waters 1525) and autosampler (Waters 2707). The raw data of flavonoids and ginkgolic acids were detected by an UV/Visible detector (Waters 2489), and terpenoids were detected by an Evaporative Light-Scattering Detector (ELSD, Waters 2424). Quercetin, kaempferol, isorhamnetin, ginkgolide A, ginkgolide B, ginkgolide C, bilobalide, and ginkgolic acids were used as reference compounds and purchased from Yuanye Bio-tech (Shanghai, China). HPLC grade methanol and acetonitrile were purchased from Kermel (Tianjin, China). Two independent control measurements were used for validating the HPLC method. At least three replicates were carried out to make sure the accuracy of the results.

### *2.4 Extraction and Measurement of Flavonol Aglycones Content*

The dried powder (1.0 g) was put in soxhlet extractors and refluxed with petroleum ether (60-90 °C) for 2 h. The residual was then added into methanol, refluxed for 4 h, and evaporated to dryness again with a rotary evaporation. A 25 mL mixed liquor of methanol and 25% (v/v) muriatic acid (4:1) was added and refluxed for 0.5 h. The acidic extract was diluted with methanol in a 50 mL volumetric flask and filtered through a 0.45 μm membrane filter. HPLC separation of flavonol aglycones was achieved using a ZORBAX SB C18 column (250 mm × 4.6 mm, 5 μm) (column temperature: 35 °C). The mobile phase was a mixture of methanol and 0.4% (v/v) phosphoric acid solution (52:48). UV/Visible detection wavelength was 360 nm and the flow rate of the mobile phase was 1 mL·min$^{-1}$. Standard solutions containing 30 μg quercetin, 30 μg kaempferol and 20 μg isorhamnetin were dissolved in 1 mL methanol. A 10 μL extract solution and reference solution were transferred into liquid chromatograph, respectively. The contents of quercetin, kaempferol and isorhamnetin were calculated according to the peak areas. Total flavonoid content (g/kg) = (quercetin content + kaempferol content + isorhamnetin content) × 2.51.

### *2.5 Extraction and Measurement of Terpenoids Content*

The dried powder (1.0 g) was put in soxhlet extractors and refluxed with petroleum ether (30-60 °C) for 2 h. The residual was then added into methanol, refluxed for 6 h, and evaporated to dryness. The extract was filtered through analytical filter paper, and the filtrate was evaporated *in vacuo* and dissolved in 10 mL methanol under

sonication. 5.0 mL of the solution was transferred to a solid-phase extraction column containing 3 g acidic $Al_2O_3$. HPLC separation of terpenoids was achieved using an Eclipse XDB C18 column. The mobile phase was a mixture of methanol and water (52:48), and the flow rate was 1 mL·min$^{-1}$. The drift tube temperature of the ELSD was set at 100 °C, and the nitrogen flow rate was 2.7 L·min$^{-1}$. A mixed standard solution containing 0.2 mg bilobalide, 0.18 mg ginkgolide A, 0.08 mg ginkgolide B, and 0.1 mg ginkgolide C was dissolved in 1 mL methanol. A 20 μL extract solution and reference solution were transferred into liquid chromatograph, respectively, for analysis. The units of terpenoids contents are g/kg fresh weight.

*2.6 Extraction and Measurement of Ginkgolic Acid Derivatives Content*

The dried powder (0.1 g) was dissolved in 10 mL methanol under sonication (40 kHz, 500 W) for 0.5 h. After centrifuging at 5 000 rpm min$^{-1}$ for 10 min, the supernatant was collected. The residues were added into 10 mL methanol for extraction again. All supernatant was concentrated up to dryness, and the residue was dissolved in 10 mL methanol. HPLC separation of ginkgolic acids was achieved using an Eclipse Plus C18 column (column temperature: 30 °C). The mobile phase was a mixture of acetonitrile and 0.4 % (v/v) phosphoric acid solution (90:10), and the flow rate was 1 mL·min$^{-1}$. The injection volume was set at 20 μL and the detection wavelength was 310 nm. 3.66 mg ginkgolic acids were dissolved in 10 mL methanol, and used as standard solutions. The statistical confidence values for the linearity of the calibration lines for C13:0, C15:1, C17:2, C15:0, and C17:1 are given in Figure 6. Total ginkgolic acids (TGA) were calculated as follows: TGA (%) = (C0 × A1 × V1)/(A0 × M1) × 100. C0 (mg mL$^{-1}$) is concentration of ginkgoneolic acid (control); A1 is the peak area of TGA in samples; V1 (mL) is the volume of extracted solution; A0 is the peak area of ginkgoneolic acid; M1 (g) is weight of samples.

*2.7 Statistical Analysis*

Statistical analyses were performed by one-way analysis of variance (ANOVA), and mean differences were compared by the lowest standard deviations (L.S.D.) test using SPSS 19.0. Data are presented as means±SD.

## 3. Results and Discussion

*3.1 Generation of the Variety "ZY 1"*

Fifteen core *Ginkgo* varieties were collected from 5 provinces in China, including Shandong, Jiangsu, Zhejiang, Hebei, and Guangxi. Of them, two-year-old "Anlu 1" plants were irradiated by $^{60}$Co-γ ray at dose of 100 Gy. This dose was relatively high, similar to those applied in Arabidopsis and wheat (Wi et al., 2007; Raut & Sainis, 2012), in order to produce more morphology-changed mutants. 220 irradiated plants were grown in the field for phenotypic observation. A branch of an irradiated plant was found to be different from others after two year of growth and was then propagated by cuttings. This variety was consistent and stable in three-year field tests and therefore named as "ZY 1".

*3.2 Physiological Characteristics of the Variety "ZY 1"*

The variety "ZY 1" had diverse phenotypic changes relative to "Anlu 1" (control, CK). It had no branches but 3.65-fold more buds in the main stem than CK (Figures 1A-C). This suggests that the variety "ZY 1" has potential advantages for high density plantings and mechanized harvest due to its only main stem. Actually, our field tests revealed that the suitable planting density of "ZY 1" may be up to 75 × 10$^3$ plants/hm$^2$, which was drastically higher than that of CK ("Anlu 1", 50 × 10$^3$ plants/hm$^2$).

"ZY 1" grew significantly faster than CK every year. Four-year-old "ZY 1" plants were 4 m in height and increased by 48.2% than CK (Figure 1D). It's known that the leaves of the ginkgo should be sampled for application in 4-7 years of growth, when both yield and major components (flavonoids and terpenoids) are optimal (Gu et al., 2011). Therefore, the fast-growing property of "ZY 1" is helpful for shortening the material period.

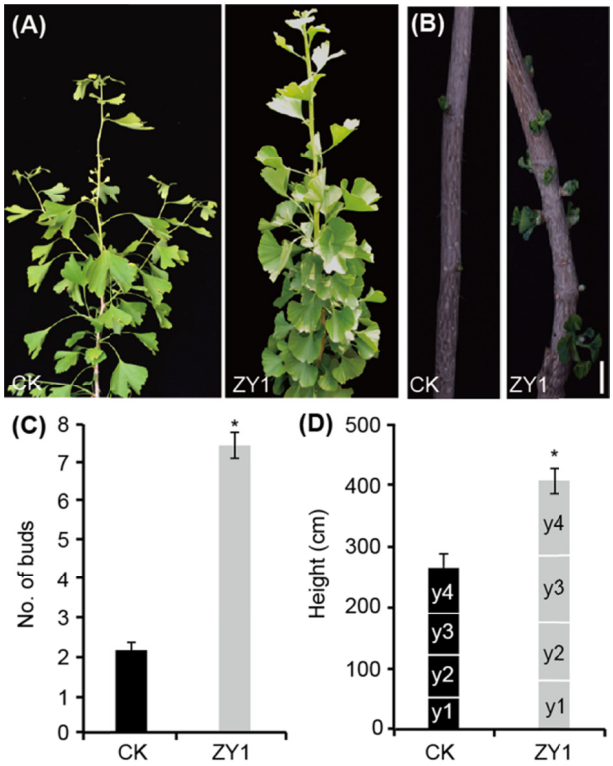


Figure 1. Phenotypes of the varieties "ZY 1" and CK (Control, "Anlu 1")

*Note*. (A) Two-year-old plants after grafting. (B, C) Numbers of buds in the main stems of two-year-old "ZY 1" and CK. (D) Height of "ZY 1" and CK plants after 1-4 year of growth. At least 20 plants for each variety are analyzed in (c-d). Data are means of three independent measurements and error bars indicate standard deviations. * $p < 0.05$ (*t* test). Bar = 2 cm.

Measurement of leaf parameters indicated that "ZY 1" had increased leaf length (155.6%), leaf width (133.1%) and leaf thickness (144.1%) compared with CK, which were correlated with the increases in leaf area and leaf fresh weight (Figures 2A-2C). Moreover, "ZY 1" had 7.9 whorled leaves, whose number was more than CK (4.8) (Figures 2D and 2E). Consistent with these results, leaf yield per plant in "ZY 1" was 938.5 g, which was higher by 54.9% than that in CK (605.6 g). Our data showed that leaf fresh weight and leaf yield per plant of "ZY 1" were separately higher by 35% and 5.26-fold than those of the recommended variety (Cao et al., 2011). Further prediction of leaf yield per unit based on leaf yield per plant and planting density indicated that "ZY 1" has the ability of producing 65.7 t leaves per $hm^2$, which is 2.17-fold higher than CK.

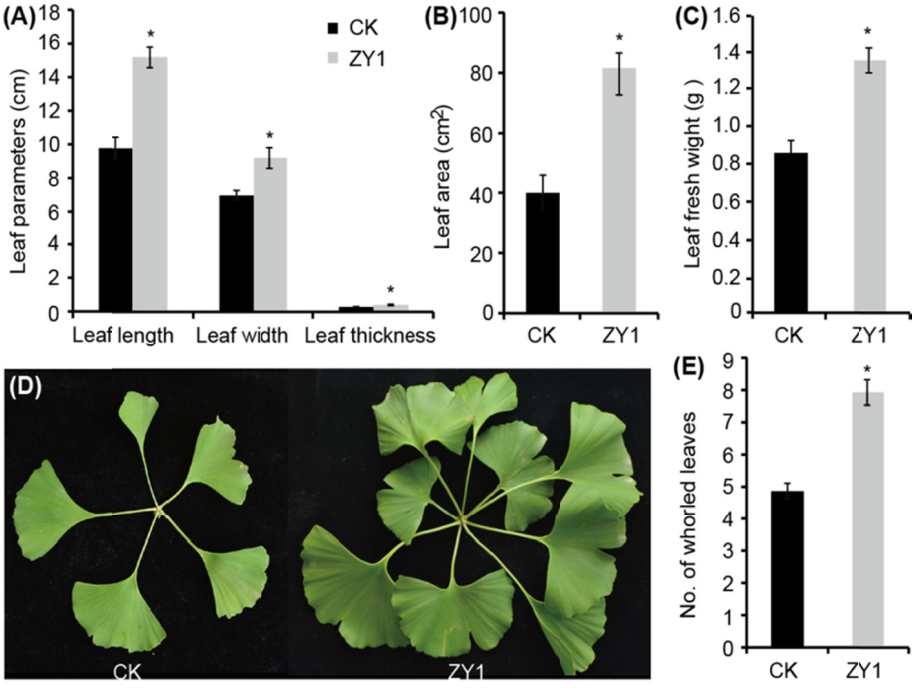


Figure 2. Morphology of "ZY 1" and CK leaves

*Note*. Leaf parameters (A-C) and whorled leaves numbers (D, E) of two-year-old "ZY 1" and CK plants. Ten plants and twenty leaves in each plant were analyzed. Data are means of three independent measurements and error bars indicate standard deviations. * $p < 0.05$.

### *3.3 Measurement of Flavonoids and Terpenoids Contents in "ZY 1" Leaves Extract*

Leaves of 2.5-year-old "ZY 1" and CK plants were sampled in October for detecting two major benefit components flavonoids and terpenoids contents following the method described in China Pharmacopoeia 2015 Edition. Total flavonoids content in "ZY 1" leave extract was 12.09±0.03 g/kg, which was higher by 28.3% than that in CK leave extract (9.44±0.45 g/kg) (Figure 3). Their glycosidic forms of quercetin (Q) and isorhamnetin (I) contents in "ZY 1" leave extract were separately 2.50±0.02 g/kg and 0.78±0.01 g/kg, and correspondingly higher by 48.2% and 36.7% than those in CK. However, there was no detectably difference for kaempferol (K) content (0.52±0.03 g/kg) between the two varieties. The Q/K/I ratio of CK was 1/0.87/0.33, which was consistent with the previous report that the Q/K/I ratio is in the range of 1/0.8-1.2/not less than 0.1 (Song et al., 2010). In contrast, the Q/K/I ratio in "ZY 1" was 1/0.63/0.31. The percentage of kaempferol content was obviously lower. This may be resulted from the differences in growing location of *Ginkgo*, harvest season or processing conditions (Song et al., 2010).

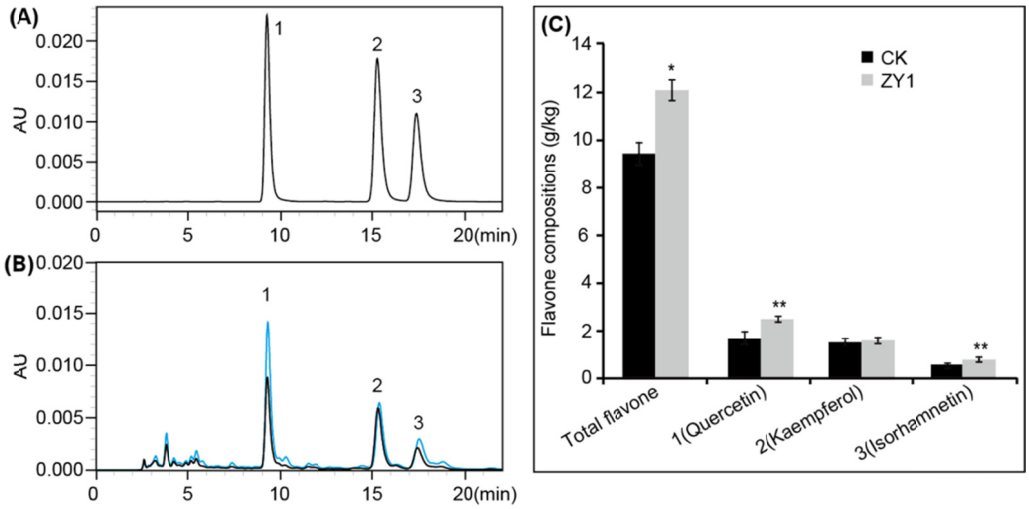


Figure 3. Typical HPLC-UV phytochemical fingerprint profiles of standards (A) and leaves extracts of 2.5-year-old "ZY1" (blue) and CK (black) plants (B, C) showing flavone glycosides in unhydrolyzed form

*Note*. Peak legend: 1: quercetin, 2: kaempferol, 3: isorhamnetin. All data are presented as means of three replicates. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

Total terpenoids content in "ZY 1" leave extract was 3.23±0.08 g/kg, and higher by 50% than CK (2.16±0.05 g/kg) (Figure 4). Bilobalides, ginkgolide A, ginkgolide B and ginkgolide C contents in "ZY 1" leaves extract were individually 0.36±0.02, 0.16±0.01, 0.20±0.01 and 0.26±0.07 g/kg, and correspondingly higher by 28.6%, 70.4%, 25% and 85.7% than those in CK. Together, these results showed that "ZY 1" had higher levels of flavone glycosides and terpenoids than "Anlu 1".

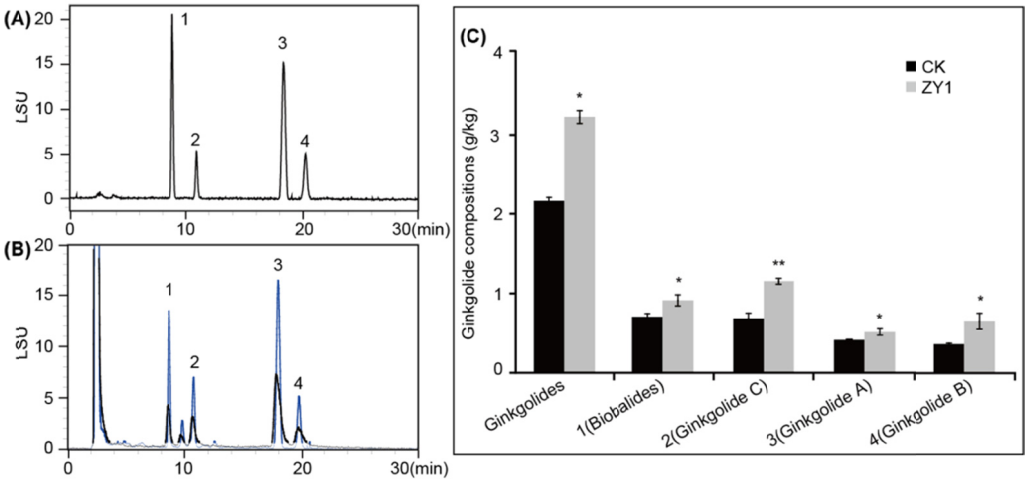


Figure 4. HPLC analysis of standards (A) and leaves extracts of 2.5-year-old "ZY1" (blue) and CK (black) plants (B, C) showing four terpenoid components

*Note*. Peak legend: 1: bilobalides, 2: ginkgolide C, 3: ginkgolide A, 4: ginkgolide B. All data are presented as means of three replicates. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

### *3.4 Measurement of Ginkgolic Acid Derivatives Content in "ZY 1" Leaves Extract*

The undesired compounds ginkgolic acid derivatives of *Ginkgo* leaves extract were further examined in the samples described above (Figure 5). Total ginkgolic acids content in "ZY 1" leaves extract was 4.41±0.20 g/kg, and lower by 3.05-fold than that in CK (13.34±0.13 g/kg). The contents of C13:0 ginkgolic acid, C15:0 ginkgolic acid, C15:1 ginkgolic acid, C17:1 ginkgolic acid and C17:2 ginkgolic acid were individually

1.25±0.08, 0.15±0.01, 1.31±0.06, 1.56±0.07 and 0.14±0.00 g/kg, which were correspondingly lower by 12.6%, 65.9%, 76.2%, 72.2% and 58.8% than those in CK. It's known that a small quantity of ginkgolic acids in *Ginkgo* leaves extract can provoke allergic reactions due to their similarity to the highly allergenic urushiol (van Beek & Montoro, 2016). Therefore, ginkgolic acids in *Ginkgo* leaves extract are defined as the marker for safety detection in leaf application. In the current European Pharmacopoeia (Ph. Eur.) 9.0 and United States Pharmacopoeia (USP) 39, the concentration of ginkgolic acids in standardized *Ginkgo* leaves extract is limited to below 5 μg/g. Our current data showed that "ZY 1" had significantly lower levels of ginkgolic acid derivatives than CK, which largely promotes its potential value of application.

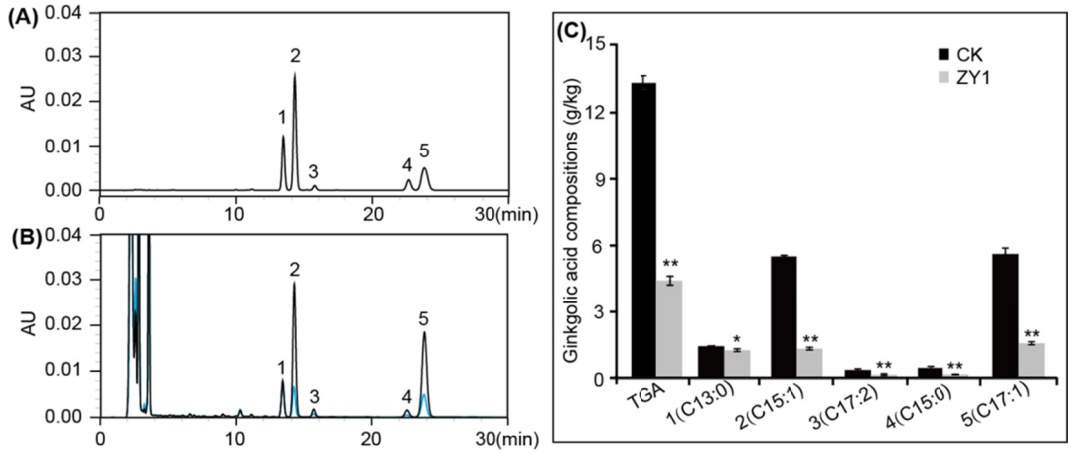


Figure 5. HPLC analysis of mixed standard (A) and leaves extracts of 2.5-year-old "ZY1" (blue) and CK (black) plants (B, C) showing five ginkgolic acid derivatives

*Note*. Peak legend: 1: C13:0 ginkgolic acid, 2: C15:1 ginkgolic acid, 3: C17:2 ginkgolic acid, 4: C15:0 ginkgolic acid, 5: C17:1 ginkgolic acid. All data are presented as means of three replicates. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$.

## 4. Conclusions

There is lack of good *Ginkgo* varieties for leaf application to date. In this study, we cultivated a *Ginkgo* variety "ZY 1" by $^{60}$Co-γ radiation. This variety grows faster and has higher leaf yield than the variety "Anlu 1". More importantly, "ZY 1" leaves extract contains higher levels of benefit components flavonoids and terpenoids but lower lever of undesired compounds ginkgolic acids. Therefore, the variety "ZY 1" has a high value of practical application.

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