

Adulteration Determining of Pharmaceutical Forms of *Ginkgo biloba* Extracts from Different International Manufacturers

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Abstract: In this study, *Ginkgo biloba* products used for the same purpose, but licensed to varying authorities were analyzed in point of similarity to each other. A group of these products were licensed from health authorities as herbal medicinal product (HMP), while the other groups of products were licensed as the food supplement (FS). The evaluation of their phytoequivalence was carried out comparing the chromatographic fingerprint profiles. Furthermore, ginkgolides (ginkgolides GA, GB, GC, and GJ) and flavonoid aglycones (quercetin, kaempferol, and isorhamnetin) were quantitatively analyzed by using liquid chromatography–mass spectrometry (LC-MS) and HPLC–diode Array detector (HPLC-DAD) assays. All six herbal medicinal products and two food supplements were found to be phytoequivalent to each other, but five of the seven food supplements did not possess similar content as herbal medicinal products, and the quantity of ginkgolides and flavonoid aglycones per tablet/capsule was found to be lower than declared on the labels. In addition, food supplements were found to be adulterated with rutin to reach expected total flavonoid glycosides amount.

Keywords: *Ginkgo biloba*; fingerprint analysis; flavonoids; diterpene lactone; LC-MS; HPLC-DAD. © 2014 ACG Publications. All rights reserved.

1. Introduction

Ginkgo biloba L. which is native to China, but also is cultivated in Australia, South Asia, Europe, Japan and the United States of America, the only surviving member of *Ginkgoaceae* family. EGb 761, the standardized extract prepared from the leaves of *Ginkgo biloba*, has been used for treatment of cerebrovascular insufficiency, cognitive disorders with demential syndromes, periferal arterial occlusive diseases, tinnitus, vertigo, and asthma [1].

Ginkgo biloba extracts contain two pharmacologically important chemical groups which are flavonoids and terpene trilactones, ginkgolides [2].

Plant extracts are complex mixtures of which the therapeutic effects are often attributed to cumulative or synergistic effects of many components. “HPLC fingerprint analysis” can give an overall view of all the components in the extract which is crucial to evaluate the quality of the plant product [3]. The fingerprint chromatographic technology was introduced and accepted by World

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Health Organization (WHO) as a strategy for identification and quality evaluation of herbal medicine [4]. Thus, chromatographic fingerprint analysis of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products.

The preparation process of the extracts from *G. biloba* leaves may differ for various manufacturers. *Ginkgo* extract has more than 30 different flavonoids derived from quercetin, kaempferol, and isorhamnetin which represent its chemical characterisation [5-6] (Figure 1). Fingerprint analysis is a qualitative method and cannot provide the quantity of individual flavonol aglycones [7-11].

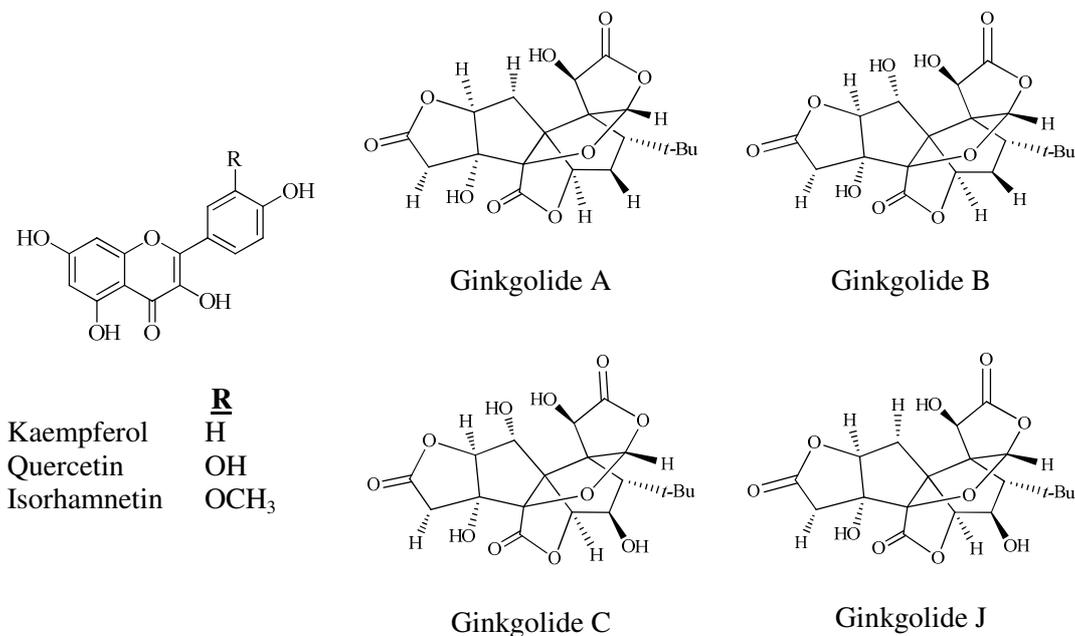


Figure 1. Flavonoid aglycones and ginkgolides in *G. biloba* leaf extracts

In this study, ginkgolides (ginkgolides GA, GB, GC, and GJ) and flavonoid aglycones (quercetin, kaempferol, and rutin) in 13 *Ginkgo biloba* extracts were quantitatively analyzed by LC-MS and HPLC-DAD, respectively. Six of them were licensed as HMP and seven of them used for the same purpose with HMP were licensed as FS. The evaluation of the phytoequivalence was carried out comparing the chromatographic fingerprint profiles.

2. Materials and Methods

2.1. Herbal Medicinal Products and Standards

Ginkgolides A, B, C, and J were purchased from Schwabe (Basel, Switzerland). Quercetin, kaempferol, and rutin were recently isolated by our research group. Isorhamnetin was prepared by acidic hydrolysis from kaempferol 3-*O*-gentiobioside. Isorhamnetin 3-*O*-gentiobioside was obtained in our previous studies. HPLC grade solvents were purchased from Riedel de Haen and J. T. Baker. Ethyl acetate, phosphoric acid, chloroform, hydrochloric acid, potassium hydroxide, sodium acetate and acetic anhydride were of analytical grade. The water used in the experiment was double distilled.

In the study, thirteen *Ginkgo biloba* extract products were analysed. Six of them were bought at a local pharmacy and seven of them were bought at different local markets.

2.2. Calibration standards

The standard stock solutions of ginkgolides (GA, GB, GC, and GJ) were prepared at the concentration of 200 ppm in methanol and diluted to reach working calibration standards of GA (2.5

ppm, 5 ppm, 10 ppm, 20 ppm); GB (1.25 ppm, 2.5 ppm, 5 ppm, 20 ppm); GC (1.25 ppm, 2.5 ppm, 5 ppm, 10 ppm, 20 ppm); GJ (0.31 ppm, 0.625 ppm, 1.25 ppm, 2.5 ppm, 5 ppm).

The standard stock solutions of quercetin (400 ppm), kaempferol and isorhamnetin were prepared at the concentration of 200 ppm in methanol and diluted to prepare quercetin (200 ppm, 100 ppm, 50 ppm, 10 ppm, 2 ppm), kaempferol and isorhamnetin (100 ppm, 50 ppm, 10 ppm, 5 ppm, 2 ppm) working calibration standards.

Calibration curves for each standard were constructed by plotting the peak area of flavonoid aglycones versus the concentration of flavonoid aglycones.

2.3. Preparation of Isorhamnetin by acidic hydrolysis

Acidic Hydrolysis: For preparing of isorhamnetin, kaempferol 3-*O*-gentiobioside and isorhamnetin 3-*O*-gentiobioside mixture was hydrolysed by 0.2 N HCl for 2 hours at 100 °C. Acidic solution was neutralised by 10 % KOH and then extracted with EtOAc for three times. Ethyl acetate extracts were combined and evaporated to dryness under vacuum. The yellow residue was dissolved in methanol and applied to silica column. Isorhamnetin is eluted with MeOH: H₂O (95:5).

2.4. Preparation of pharmaceutical formulations for chromatographic analysis for flavonoid aglycones

A portion of the powder equivalent to one tablet or capsule was weighed and hydrolysed with HCl as previously explained (Section 2.3.). The residue was dissolved in methanol and diluted to 50 mL. Sample solutions were filtered through 0.45 µm membran filter before injection.

For terpenoids and fingerprint analysis

10 tablets/capsules were powdered or mixed for each preparation, and a portion of the powder equivalent to one tablet or capsule was weighed. The sample was extracted with 7.5 mL volume of methanol for three times. The solution was centrifuged, combined and diluted to 25 mL with methanol. Sample solutions were filtered through 0.45 µm membrane filter prior to injection.

2.5. Instrumentation

HPLC-DAD apparatus was Agilent A 1100 series system on a Dionex C₁₈ column (5 µm, 250 x 4.6 mm i.d.) for fingerprint analysis of flavonoides. HPLC-MS apparatus was Waters 2695 Alliance HPLC system (Waters Corporation, Milford, MA, USA) equipped with Ace 5 C₁₈ (5 µm, 150 x 4.0 mm i.d.) column for the analysis of ginkgolides. The ESI-MS spectra were acquired with a Micromass ZQ 2000 electrospray mass spectrometer (Manchester, UK). The Masslynx v.4.1 software was used for data acquisition and processing. The electrospray ionization (ESI-MS) spectra were acquired in the positive ion mode (+ESI). Nitrogen was used as both desolvation gas at a flow rate of 500 L/h and cone gas at a flow rate of 50 L/h. The desolvation temperature was set at 350 °C. The ionization source was working at 120 °C. Capillary and cone voltages were 3.47 kV and 60V for +ESI. Mass spectra were acquired with scan mode from *m/z* 50-500 unit and SIR mode at *m/z* 431.30, 447.27, 447.28 and 463.26.

2.6. Chromatographic conditions Flavonoid analysis

The analytical experiments were performed using Dionex C18 column (5 µm, 250 x 4.6 mm) at a flow rate of 1 ml/min at ambient temperature. Samples were injected into the column at a constant volume of 20 µL and a UV detector at 408 nm was employed to obtain the response. For the analysis of flavonoid aglycones, mobile phase consisted of methanol (A) and deionized water adjusted to pH 4 using phosphoric acid (B). The gradient was run 40% A to 60 % A at 10 min, 60 % A for 11 min. 60 % A to 40 % A for 2 min [12].

Mobile phase used at the fingerprint analysis was B and acetonitrile (C). The gradient was run 14% C to 30% C at 40 min, 30% C to 14% C for 5 min [10].

Terpenoid analysis

The following analytical method for ginkgolides was developed by us. Mobile phase consisted of methanol (A) and deionized water (B). The gradient runs from 25% A to 62.5 % A over 15 minutes

linearly. Flow rate of the mobile phase was 0.8 mL/min and injection volume was 10 μ L. LC/ESI-MS was carried out in the positive ion scan mode from m/z 50-500 unit.

3. Results and Discussion

Standardized *Ginkgo biloba* extract called EGb 761 was reported to be specifically used as preventive against Alzheimer's disease. According to Commission E monographs, standardized *Ginkgo biloba* extract should contain 22-27% flavonol glycosides, calculated on the basis of kaempferol and isorhamnetin, and 5-7% diterpene trilactones (2.8-3.4% ginkgolides and 2.6-3.2% bilobalides) [13-15].

In this study, 13 samples were analyzed. Although they were being used for the same therapeutic purpose, six of them as HMP and seven of them as FS were licensed. LC-MS and HPLC-DAD method were used for fingerprint analysis and quantitative determination of ginkgolides (Table 1) and flavonoids (Table 2, Figures 2-4). Different mobile phase compositions (water-methanol, water-acetonitrile) were tried in order to get good resolution and acceptable peak shapes for the separation of flavonoid aglycones including quercetin, kaempferol and isorhamnetin. Finally, water-methanol and phosphoric acid were selected as an appropriate mobile phase with a step linear gradient. The mobile phase for flavonoid glycosides was acetonitrile-water (with phosphoric acid to pH=4) employing a gradient elution at a flow rate of 1.0 mL/min, and the detection wavelength was set at 370 nm. As seen in Figures 2-4, herbal medicinal products and food supplements were shown to have clearly different chromatograms from each other.

On the other hand, expected results for ginkgolides could not be obtained by HPLC-DAD. Ginkgolides were analyzed using by LC-MS. The method developed by our group was successfully used for analysis of different extracts. A water-methanol gradient method was developed for analysis of ginkgolides. As a mobile phase for ginkgolides, methanol-water was used at a flow rate of 1.0 mL/min, and the detection wavelength was set at 220 nm. ESI-MS was used in positive ion mode.

In order to quantify the flavonoid aglycones and ginkgolides, calibration curves for each standard were constructed by plotting the peak area of flavonoid aglycones versus the concentration of flavonoid aglycones. The calibration curves were found to be linear with the coefficient of determination (r^2) value of 0.998-0.999. The calculated and declared quantities of flavonoid glycosides and ginkgolides in HMP and FS were shown in Table 1 and 2. These results indicated that the developed assay methods could be considered as a suitable quality control method for flavonoids of *Ginkgo biloba*.

Extracts with identical contents licensed from different authorities cause confusion. In this paper, we want to find answers to the following questions; Are we sure enough in confidence while we use these different licensed products? How about their inconvenience? This paper clearly brings up this matter and search a solution proposal.

Although, all of the final extracts almost contain flavonol aglycones and diterpene trilactones which are considered the two pharmacologically most important groups, many different flavonol glycosides are present in *Ginkgo* leaves extracts, most of them derivatives of quercetin, kaempferol and isorhamnetin. The aglycones themselves occur only in relatively low concentrations. After acidic hydrolysis to the aglycones quercetin, kaempferol and isorhamnetin and separation by HPLC, quantitation is straightforward and yields an estimation of the original total flavonol glycoside content by recalculation.

As a result, we analyzed all herbal medicinal products and food supplements contain ginkgolides and flavonoids. However, the quantity of ginkgolides and flavonoid aglycones per tablet/capsule were found lower than declared on labels. When chromatographic fingerprint profiles of the flavonoid glycosides were compared, it was seen that six herbal medicinal products were phytoequivalent to each other.

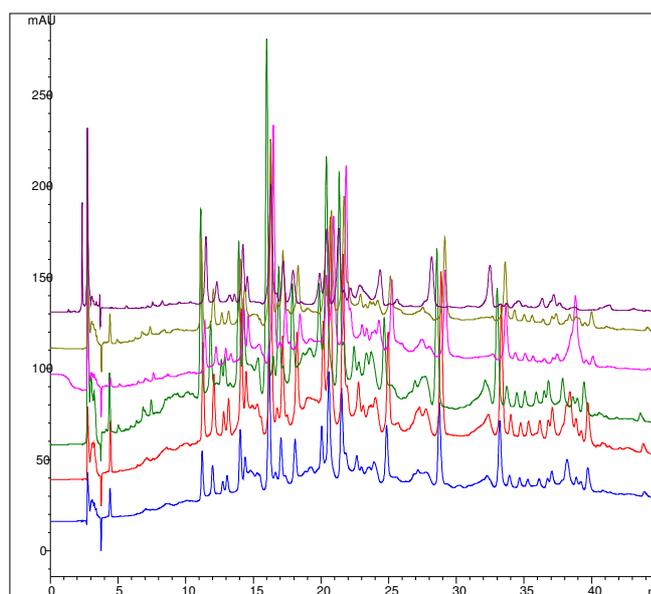


Figure 2. HPLC-DAD fingerprint chromatogram of flavonoid glycosides in HMP

But five of the seven food supplements not accepted as herbal medicinal product do not contain flavonoid glycosides. Also these products were adulterated with rutin to reach expected total flavonoid glycoside amount (Figures 3-4).

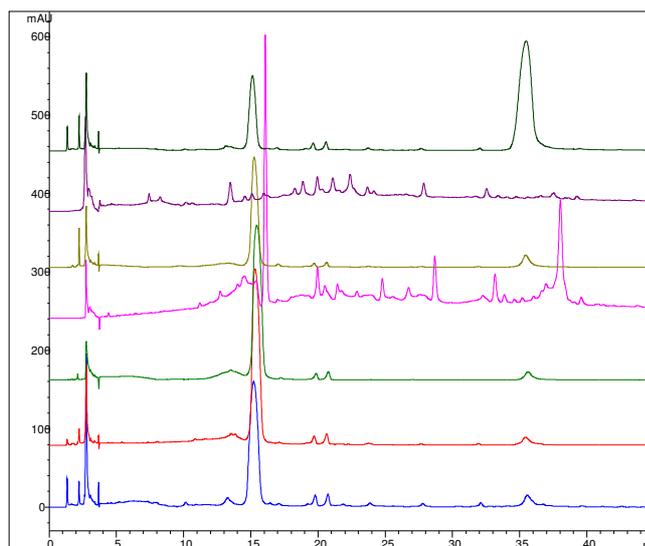


Figure 3. HPLC-DAD fingerprint chromatogram of flavonoid glycosides in FS

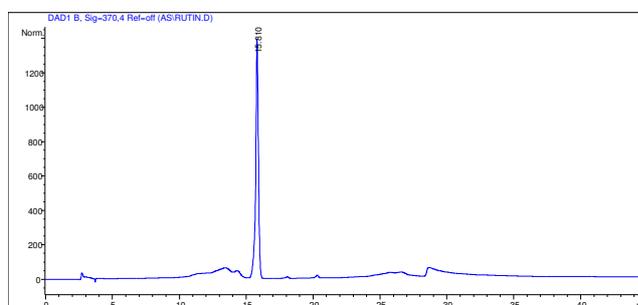


Figure 4. HPLC chromatogram of Rutin

Only two of them have shown similar profiles with that of herbal medicinal products, qualitatively. When these products were analysed to determine quercetin amount

spectrophotometrically expected correct results would be obtained, because rutin has quercetin aglycone. HPLC-DAD analysis makes rutin identification possible.

Table 1. Calculated and declared quantities of ginkgolides in HMP and FS (mg/tablet-capsule)

Products code	GA (mg/tb)	GB (mg/tb)	GC (mg/tb)	GJ (mg/tb)	Total (mg/tb)	Declared quantities on label (mg/tb)
HMP1	0.234 ± 0,01	0.080 ± 0,01	0.292 ± 0,01	0.113 ± 0,01	0.719 ± 0,02	1.12-1.36
HMP2	0.796 ± 0,01	0.329 ± 0,01	0.584 ± 0,01	0.153 ± 0,01	1.862 ± 0,02	2.24-2.72
HMP3	0.658 ± 0,02	0.277 ± 0,01	0.840 ± 0,02	0.215 ± 0,01	1.990 ± 0,03	2.24-2.72
HMP4	0.457 ± 0,01	0.063 ± 0,01	0.270 ± 0,01	0.086 ± 0,01	0.876 ± 0,02	1.12-1.36
HMP5	0.399 ± 0,01	0.157 ± 0,01	0.267 ± 0,01	0.084 ± 0,01	0.907 ± 0,02	1.12-1.36
HMP6	0.670 ± 0,01	0.305 ± 0,01	0.574 ± 0,02	0.144 ± 0,01	1.693 ± 0,03	2.24-2.72
FS1	0.092 ± 0,01	0.051 ± 0,01	0.286 ± 0,01	0.095 ± 0,01	0.524 ± 0,02	1.68-2.04
FS 2	0.028 ± 0,01	0.029 ± 0,01	0.140 ± 0,01	0.050 ± 0,01	0.247 ± 0,02	3.36-4.08
FS 3	-	0.016 ± 0,01	0.050 ± 0,01	0.021 ± 0,01	0.087 ± 0,02	3.36-4.08
FS 4	0.712 ± 0,01	0.293 ± 0,01	0.278 ± 0,01	0.090 ± 0,01	1.373 ± 0,02	1.40-1.70
FS 5	0.089 ± 0,01	0.067 ± 0,01	0.122 ± 0,01	0.047 ± 0,01	0.325 ± 0,02	3.36-4.08
FS 6	0.029 ± 0,01	0.102 ± 0,01	0.125 ± 0,01	0.047 ± 0,01	0.303 ± 0,02	0.28-0.34
FS 7	0.081 ± 0,01	0.053 ± 0,01	0.137 ± 0,01	0.048 ± 0,01	0.319 ± 0,02	8.40-10.20

Table 2. Calculated and declared quantities of flavonoid glycosides in HMP and FS (mg/tablet-capsule)

Products code	Quercetin (mg/ tb)	Kaempferol (mg/ tb)	Isorhamnetin (mg/ tb)	Total (mg/ tb)	Total flavonoid glycosides (mg/ tb)	Declared quantities on label (mg/tb)
HMP1	0.286 ± 0,01	0.951 ± 0,01	0.949 ± 0,02	2.186 ± 0,02	5.49	8.80-10.80
HMP2	0.536 ± 0,02	3.328 ± 0,02	2.664 ± 0,05	6.528 ± 0,06	16.39	17.60-21.6
HMP3	0.292 ± 0,01	2.052 ± 0,06	1.640 ± 0,02	3.984 ± 0,06	9.96	17.60-21.6
HMP4	0.414 ± 0,01	1.064 ± 0,01	2.522 ± 0,06	4.000 ± 0,06	10.00	8.80-10.80
HMP5	0.076 ± 0,01	1.248 ± 0,02	0.804 ± 0,01	2.128 ± 0,02	5.34	8.80-10.80
HMP6	0.944 ± 0,03	2.576 ± 0,06	3.672 ± 0,07	7.192 ± 0,10	18.00	17.6-21.6
FS1	2.072 ± 0,06	0.348 ± 0,01	0,666 ± 0,01	3.086 ± 0,06	7.75	13.20-16.20
FS2	1.554 ± 0,05	0.302 ± 0,01	0.654 ± 0,01	2.510 ± 0,05	6.1	26.4-32.4
FS3	6.412 ± 0,18	0.194 ± 0,01	1.286 ± 0,03	7.892 ± 0,18	19.81	26.4-32.4
FS4	0.956 ± 0,03	0.696 ± 0,01	1.388 ± 0,04	3.040 ± 0,05	7.6	11.0-13.5
FS5	3.186 ± 0,10	0.198 ± 0,01	0.664 ± 0,01	4.048 ± 0,10	10.1	26.4-32.4
FS6	0.660 ± 0,02	1.462 ± 0,04	1.108 ± 0,01	3.230 ± 0,04	8.08	2.20-2.7
FS7	7.804 ± 0,25	0.592 ± 0,02	2.492 ± 0,09	10.89 ± 0,27	27.2	66.0-81.0

Total flavonoids and ginkgolides are found to be higher in drug licensed formulations. Furthermore, there are no or very little flavonoids in the formulations of *Ginkgo biloba* extracts which are licensed as food supplements, as compared to drug, which is, therefore, an expected result in our study as well. The formulations licensed by health authority are recommended to use.

The correlation coefficients of the entire chromatographic patterns of samples were calculated by software and used for determining the similarity. The chemical information on the peaks in the chromatograms of samples was obtained by comparison of their exact mass data, UV spectra and literature values.

As a result, in this study 13 herbal medicinal products and food supplements contain ginkgolides and flavonoids per tablet/capsule lower than declared on the labels.

When the results were examined in point of ginkgolides calculated according to accepted standards by EMA, six herbal medicinal products were found to be phytoequivalent to each other. In addition, the two of food supplements showed similar profiles with that of herbal medicinal products. The results indicated that the chemical profiling of herbal medicinal products from pharmacies are quite different than samples obtained from different supplement manufacturer.

Supporting Information

Supporting Information accompanies with this paper on <http://www.acgpubs.org/RNP>

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