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Adulteration of *Ginkgo biloba* products and a simple method to improve its detection

Hans Wohlmuth[a,b,*], Kate Savage[c], Ashley Dowell[c], Peter Mouatt[a,c]

[a] Medicinal Plant Herbarium, Southern Cross University, PO Box 157, Lismore, NSW 2480, Australia
[b] Integria Healthcare, Gallans Road, Ballina, NSW 2478, Australia
[c] Southern Cross Plant Science, Southern Cross University, PO Box 157, Lismore, NSW 2480, Australia

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ABSTRACT

Extracts of ginkgo (*Ginkgo biloba*) leaf are widely available worldwide in herbal medicinal products, dietary supplements, botanicals and complementary medicines, and several pharmacopoeias contain monographs for ginkgo leaf, leaf extract and finished products. Being a high-value botanical commodity, ginkgo extracts may be the subject of economically motivated adulteration. We analysed eight ginkgo leaf retail products purchased in Australia and Denmark and found compelling evidence of adulteration with flavonol aglycones in three of these. The same three products also contained genistein, an isoflavone that does not occur in ginkgo leaf.

Although the United States Pharmacopeia – National Formulary (USP-NF) and the British and European Pharmacopoeias stipulate a required range for flavonol *glycosides* in ginkgo extract, the prescribed assays quantify flavonol *aglycones*. This means that these pharmacopoeial methods are not capable of detecting adulteration of ginkgo extract with free flavonol aglycones.

We propose a simple modification of the USP-NF method that addresses this problem: by assaying for flavonol aglycones pre and post hydrolysis the content of flavonol glycosides can be accurately estimated via a simple calculation. We also recommend a maximum limit be set for free flavonol aglycones in ginkgo extract.

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Introduction

Extracts of ginkgo (*Ginkgo biloba* L.) leaf are sold worldwide as the active ingredient of numerous dietary supplements, botanicals, herbal medicinal products and complementary medicines. Indeed, ginkgo is currently one of the most widely sold medicinal plants, and the global market for ginkgo has been estimated at more than US$700 million (Euromonitor International Ltd., 2009). In the United States, the most recent (2012) data show the retail market for ginkgo products to be worth US$30 million (Lindstrom et al., 2013).

Ginkgo is also one of the most intensely studied medicinal plants, with more than 3000 scientific papers published on the topic between 2001 and 2009 alone (van Beek and Montoro, 2009). Ginkgo leaf extracts are recommended for a range of conditions, including cerebral insufficiency, vertigo and tinnitus of vascular origin, and peripheral arterial disease (Blumenthal, 2003; Bone and Mills, 2013).

The pharmacologically active compounds in ginkgo leaf are considered to be flavonol glycosides (quercetin, kaempferol and isorhamnetin being the principal aglycones) and terpene lactones (bilobalide and ginkgolides). Most ginkgo leaf extracts on the market are produced by selective, multi-step extraction processes involving organic solvents and carry quantitative claims concerning their content of flavonol glycosides and terpene lactones. Accordingly, most ginkgo leaf extracts are more high-tech and high-cost than typical botanical extracts.

Botanical raw materials including extracts present special challenges in terms of quality control and assurance due to their chemical complexity and inherent natural variability. The most fundamental aspects of quality assurance for such materials are to ensure the correct morphological part(s) from the right botanical taxon is used, and that the material is not adulterated with other botanical or extraneous material. Adulteration, either accidental or intentional and economically motivated, is a well-known issue for botanicals, and one that potentially can jeopardise not only the quality but also the safety of the finished product (Khan, 2006; Walker and Applequist, 2012). The potential safety issues associated with adulterated or sub-standard ginkgo extracts have been

* Corresponding author at: Integria Healthcare, Gallans Road, Ballina, NSW 2478, Australia. Tel.: +61 2 6620 5180; fax: +61 2 6622 3459.
E-mail addresses: hans.wohlmuth@scu.edu.au, hans.wohlmuth@integria.com (H. Wohlmuth).

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highlighted by recent toxicology and carcinogenicity studies using such inferior materials, while other studies using pharmaceutical quality ginkgo extract have found it to be safe (Koch et al., 2013; Krenn et al., 2013).

Pharmacopoeial monographs play an important role in the quality assurance of botanicals and herbal medicinal products (Vlietinck et al., 2009). Monographs for ginkgo raw materials (leaf and extract) can be found in various pharmacopoeias, including the United States Pharmacopoeia-National Formulary (USP-NF) (United States Pharmacopeial Convention, 2013), the European Pharmacopoeia (EP) and the British Pharmacopoeia (BP) (British Pharmacopoeia Commission, 2012). The USP-NF monograph for Powdered Ginkgo Extract and the BP/EP monograph for Refined and Quantified Ginkgo Dry Extract specify a flavonoid content of 22–27%, calculated as flavonol/flavone glycosides and a maximum content of 5 ppm for ginkgolic acids (putative allergens). In addition, specifications are provided for the terpene lactones, bilobalide and ginkgolide A, B and C, but the ranges for these differ between the USP-NF and the BP/EP. The USP-NF also provides monographs for Ginkgo Tablets and Ginkgo Capsules.

Here we report on ginkgo retail products found to be adulterated with free flavonol aglycones and also containing the isoflavone genistein, which is not native to ginkgo. We demonstrate that current pharmacopoeial methods are inadequate for the detection of this type of adulteration, and we propose a simple modification of the USP-NF method that addresses this problem.

Materials and methods

Plant materials and botanicals

Five samples of dried *Ginkgo biloba* leaf were obtained from commercial suppliers. These leaf samples came from ginkgo cultivated in China (3), New Zealand (1) and Australia (1). They were authenticated by an experienced pharmacognosist (H.W.), and voucher materials were deposited in the Medicinal Plant Herbarium (PHARM) at Southern Cross University.

Eight retail products containing *Ginkgo biloba* as the sole active ingredient were purchased in Australia (6) and Denmark (2). Four of these were tablets and four were capsules.

Chemicals and reagents

Water was obtained from an in-house Milli-Q system (Millipore, Billerica, MA, USA). Methanol (HPLC grade) was obtained from Merck (Kilsyth, VIC, Australia), ethanol (AR grade) from Chem-Supply (Gillman, SA, Australia), acetonitrile from Scharlau (Sentmenat, Spain), phosphoric acid from Ajax Finechem (Sydney, Australia), and hydrochloric acid (AR grade), trifluoroacetic acid, dimethyl sulfoxide (DMSO), kaempferol (97.8%) and genistein (>98%) from Sigma-Aldrich (Sydney, Australia). Quercetin dihydrate (98.5%) was obtained from Chromadex (Irvine, CA, USA) and isorhamnetin (>98%) from Chengdu Biopurify Phytochemicals (Chengdu, Sichuan, China).

Standard preparation

Reference standards (quercetin, kaempferol and isorhamnetin) were dissolved in DMSO at a concentration of 2.0 mg/mL, then serially diluted in methanol using a Hamilton Microlab 500 Diluter (Reno, NV, USA). Genistein was dissolved in methanol.

Sample preparation

Ginkgo leaf samples were ground to a fine powder in a Retsch MM301 Mixer Mill (Haan, Germany). Approximately 5.5 g of powdered leaf material was placed in a 250-ml round bottom flask with 50 ml ethanol and 20 ml Milli-Q water and sonicated for 15 min (Soniclean Ultrasonic bath, Thebarton, SA, Australia). To achieve hydrolysis of flavonol glycosides, 8 ml of 37% hydrochloric acid was added (with boiling chips) and the mixture refluxed at moderate temperature in a fume-hood for 2 h 15 min. Once cooled, the solution was transferred quantitatively to a 100-ml volumetric flask and diluted to volume with Milli-Q water. Samples not hydrolysed were treated identically, except for the addition of hydrochloric acid.

Tablets were extracted by combining a quantity of 20 and grinding them to a fine powder in a Retsch MM301 Mixer Mill. Depending on the label claim for flavonol glycoside content, between 300 and 1000 mg of the powder was extracted by sonication, as detailed for the leaf samples. Capsules were opened and their content extracted in the same way as the tablets. All samples were extracted in triplicate.

HPLC

Reverse-phase HPLC analysis was performed on an Agilent (Palo Alto, CA, USA) 1100 HPLC system fitted with a Phenomenex (Torrance, CA, USA) Synergi C18 4 μM (250 mm × 4.6 mm i.d.) column, using an in-house validated method based on the USP34-NF29. Mobile phase A consisted of 0.5% aqueous phosphoric acid; mobile phase B consisted of methanol. The gradient eluting mobile phase was A/B (60:40, v/v) to A/B (50:50, v/v) over 40 min. This was followed by a 5 min column wash with A/B (5:95) and a 5 min equilibration period with A/B (60:40) prior to the next injection. Mobile phase was pumped at 1.2 ml/min, the column temperature 40 °C, and the injection volume was 10 μl. Data were collected using a UV/visible light diode array detector collecting absorption spectra from 200 to 400 nm with quantification performed at 270 nm. System control and data evaluation were achieved using ChemStation for HPLC software. Limits of detection for quercetin, kaempferol and isorhamnetin were 0.625 μg/ml, 0.25 μg/ml and 0.07 μg/ml, respectively. The range for precision, accuracy and linearity was 0.0025–0.5 mg/ml for quercetin, 0.000625–0.5 mg/ml for kaempferol and 0.00028–0.14 mg/ml for isorhamnetin.

LC–MS

LC–MS analysis was performed on an Agilent (Palo Alto, CA, USA) 1100 LC/MSD system equipped with an atmospheric pressure chemical ionisation (APCI) source and fitted with a Phenomenex (Torrance, CA, USA) Luna C18 3 μM (100 mm × 4.6 mm i.d.) column. Mobile phase A consisted of water and mobile phase B of acetonitrile, both with 0.005% trifluoroacetic acid added. The gradient eluting mobile phase was A/B (90:10, v/v) to A/B (5:95, v/v) over 18 min followed by a 3 min column wash with A/B (5:95), to A/B (90:10) over 3 min and a 5 min equilibration period with A/B (90:10). Rate of mobile phase flow was 0.750 ml/min, the column temperature was 40 °C, and the injection volume was 5 μl.

MS parameters in the positive ionisation mode were: Vcap 3000 V, nebuliser 60 psig, drying gas flow rate 5.0 l/min, gas temperature 350 °C, corona 4.0 μA, vaporiser 350 °C, scan range 100–1500 m/z, step size 0.15 m/z, peak width 0.1 min, time filter enabled, fragmentor 150 V. System control and data evaluation were performed with ChemStation for LC/MS software.

Calculation of flavonol glycoside content

Flavonol glycoside content was calculated according to the method provided in the Ginkgo Tablet and Ginkgo Capsule

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