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Research paper

Adulteration and poor quality of *Ginkgo biloba* supplements

Anthony Booker[a,d], Debora Frommenwiler[b], Eike Reich[b], Samuel Horsfield[a,c], Michael Heinrich[a,*]

[a] Research Cluster Biodiversity and Medicines/Centre for Pharmacognosy and Phytotherapy, UCL School of Pharmacy, London, UK
[b] CAMAG AG, 4132 Muttenz, Switzerland
[c] University of Birmingham School of Biosciences, Edgbaston, Birmingham, UK
[d] Division of Herbal and East Asian Medicine, Department of Life Sciences, University of Westminster, London W1W 6UW, UK

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ABSTRACT

Adulteration of Ginkgo products sold as unregistered supplements within the very large market of Ginkgo products (reputedly £650 million annually) through the post-extraction addition of cheaper (e.g. buckwheat derived) rutin is suspected to allow sub-standard products to appear satisfactory to third parties, e.g. secondary buyers along the value chain or any regulatory authorities. This study was therefore carried out to identify products that did not conform to their label specification and may have been actively adulterated to enable access to the global markets.

500 MHz Bruker NMR spectroscopy instrumentation combined with Topspin version 3.2 and a CAMAG HPTLC system (HPTLC Association for the analysis of *Ginkgo biloba* leaf) were used to generate NMR spectra (focusing on the 6–8 ppm region for analysis) and chromatograms, respectively.

Out of the 35 samples of Ginkgo biloba analysed, 33 were found to contain elevated levels of rutin and/or quercetin, or low levels of Ginkgo metabolites when compared with the reference samples. Samples with disproportional levels of rutin or quercetin compared with other gingko metabolites are likely to be adulterated, either by accident or intentionally, and those samples with low or non-existent gingko metabolite content may have been produced using poor extraction techniques. Only two of the investigated samples were found to match with the High-Performance Thin-Layer Chromatography (HPTLC) fingerprint of the selected reference material. All others deviated significantly. One product contained a 5-hydroxytryptophan derivative, which is not a natural constituent of *Ginkgo biloba*.

Overall, these examples either suggest a poor extraction technique or deliberate adulteration along the value chain. Investigating the ratio of different flavonoids e.g. quercetin and kaempferol using NMR spectroscopy and HPTLC will provide further evidence as to the degree and kind of adulteration of Gingko supplements. From a consumer perspective the equivalence in identity and overall quality of the products needs to be guaranteed for supplements too and not only for products produced according to a quality standard or pharmacopoeial monograph.

1. Introduction

Ginkgo biloba L. (Ginkgoaceae) is a popular phytomedicine with a high economic value. It is used principally for the treatment of problems associated with the peripheral circulation and to improve memory and cognitive function. It is a licensed drug in some European countries, e.g. Germany. In the UK, two products hold a Traditional Herbal Registration (THR) for the treatment of Raynaud's disease although the majority of products on the UK market are sold as unlicensed food supplements (now often referred to as botanicals).

According to 'Industry Experts', the global market for the sale of *Ginkgo biloba* supplements amounts to around £650 million annually (Daniells, 2013), typically ranging in price from 2.5 to 54 pence per individual daily dose. However, there have been frequent reports of poor quality and adulteration (Avula et al., 2015; Edwards et al., 2015; Wohlmuth et al., 2014).

Manufacturers of extracts complying with pharmacopoeial standards have to perform extensive testing. Besides HPTLC, for identification, the European Pharmacopeia (Ph. Eur.) requires three HPLC assays (flavonoids, terpene lactones and ginkgolic acids) and the United States Pharmacopoeia (USP) requires four HPLC assays

* Corresponding author.
E-mail address: m.heinrich@ucl.ac.uk (M. Heinrich).

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(content of free flavonol glycosides, content of terpene lactones, limit on rutin and quercetin, and limit of ginkgolic acids) (EP, 2014; USP, 2015). Supplement manufacturers may however choose not to claim compliance with a pharmacopeia and thus limit their analytical work to a minimum. Such simplified conventional testing is not always able to determine which products are of good quality, particularly as it has been reported that products are often 'spiked' with buckwheat-derived rutin in order to increase their total flavonoid content and to reduce the amount of authentic drug in the preparation (Ding et al., 2006). This has important implications for both the OTC market and the practitioner led market as middlemen involved in the early stages of the supply chain (cultivation and primary processing) may cross both markets.

NMR spectroscopy is an effective tool for the quality control of medicinal plants or HMPs (Shyur and Yang, 2008). The advantages of NMR spectroscopy over other techniques for metabolomics applications include the relative ease of sample preparation, the potential to identify a broad range of compounds and provision of structural information for unknown entities (Zulak et al., 2008).

NMR spectroscopy-metabolomics coupled with HPTLC can offer a better picture of the total metabolite profiles that can be obtained from plants and plant extracts and so may be a useful addition to other analytical methods (Liu et al.,, 2015; López-Gutiérrez et al., 2016), in the investigation of fake, adulterated and poor quality products (Booker et al., 2015).

2. Materials and methods

2.1. Test samples

35 Ginkgo products sold as food supplements were purposefully sampled from health food stores, supermarkets and pharmacies in the Central London area and from the internet. The internet search criteria used was 'Ginkgo, Ginkgo biloba, Ginkgo Products and Buy Ginkgo products'. The samples were marketed as either extracts of *Ginkgo biloba* formulated into tablets (22), hard capsules (11), or caplets (2). A detailed description of all investigated products is provided in the Supplementary data.

2.2. Solvents, reagents and chemicals

Deuterated dimethyl sulfoxide D6 lot no. 14F-145 and tetramethylsilane (for NMR spectroscopy) (99.9%) were purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. Methanol was purchased from Carl Roth GmbH, Karlsruhe, Germany, ethylacetate (99.5%) and formic acid (98+ %) pure purchased from Acros, New Jersey, USA. Acetic acid (99.5%), toluene (99 +%), acetone (pure), and dichloromethane (for HPTLC) were purchased from Sigma–Aldrich. Acetic anhydride (98.5%) was purchased from Merck. Polyethylene glycol, 2-aminoethyldiphenylborinate (derivatisation reagents) were purchased from Aldrich chemistry.

2.3. Standards

Reference standard

A quantified and licensed Ginkgo extract tablet EGb 761 (Tebofortin® intense) and *Ginkgo biloba* extract tablets (LI 1370 extract) were purchased through a pharmacy. Ginkgo tablet (S1312) and *Ginkgo biloba* leaf (S1310) were obtained from the National Institute of Standards and Technology (NIST) U.S. Department of Commerce; *Ginkgo biloba* leaf samples (S11311, S15564) and powdered *Ginkgo biloba* leaf extracts (S10925 and S15571) were obtained from the American Herbal Pharmacopeia (AHP). Quercetin, chlorogenic acid, rutin, and ginkgo terpene lactones (mixture) and *Ginkgo biloba* standard supplied by The European Pharmacopoeia (EP CRS for peak identification) were obtained from Sigma-Aldrich.

2.4. ^{1}H NMR spectroscopy

2.4.1. Preparations of standard solutions and samples

Approximately 50 mg of solid extracts were accurately weighed and transferred to a 1.5 ml Eppendorf reaction tube, 1 ml of deuterated DMSO containing 0.05% tetramethylsilane was added. The mixture was mixed on a rotary mixer for 60 s, sonicated for 10 min at room temperature and centrifuged for 10 min at room temperature (speed; 14,000 rpm). The reference standard solutions of rutin and *Ginkgo biloba* BRM were prepared at a concentration of 20 mg/ml in deuterated DMSO. 700 μl of supernatant was transferred to a 5 mm diameter NMR tube, and the samples were submitted on the same day for 1H NMR spectroscopy analysis.

2.4.2. Apparatus and instrumentation

The ^{1}H NMR spectra were acquired using 500 MHz NMR Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm cryoprobe head and operating at a proton frequency of 500.13 MHz. The acquisition parameters were: size of the spectra 64 k data points, line broadening factor = 0.16 Hz, pulse width (PW) = 30° and the relaxation delay d1 = 1 s. The acquisition temperature was 298 K.

Topspin software version 3.2 was used for spectra acquisition and processing. AMIX Bruker Biospin multivariate analysis software version 3.0 was used for converting spectra to an ASCII file. The numbers of scans chosen was 256 for optimum strength of signal, and locked at zero on the TMS peak.

2.5. Data reduction and multivariate statistics methods

The ^{1}H NMR spectra were phase-corrected, baseline-corrected, and zeroed to the TMS peak. The spectra were converted to an ASCII file using AMIX software for multivariate analysis.

AMIX was used to generate a number of integrated regions (buckets) of the data set in the region of 6.0–8.0 ppm. The size of buckets was 0.04 ppm. The data set was imported to Microsoft EXCEL, and the samples and standards were labelled 1–37. The Principal Component Analysis (PCA) was carried out using SIMCA software version 13.0.

2.6. High performance thin layer chromatography (HPTLC)

2.6.1. Preparations of standard solutions and samples

The extraction of plant samples was performed according to a method described by the HPTLC Association for the identification of dried *Ginkgo biloba* leaf (www.hptlc-association.org). Standard solutions of rutin, chlorgenic acid, and quercetin were prepared at a concentration of 0.2 mg/ml in methanol. The terpene lactones standard was prepared at concentration of 1 mg/ml. Approximately 100 mg of solid samples (extracts/products) were weighed individually into 10 ml centrifuge tubes and 10 ml of methanol were added. The mixture was sonicated for 10 min at room temperature and centrifuged for 5 min at 5000 rpm. The supernatant solution was transferred into individual vials, and then submitted for HPTLC analysis.

2.6.2. Chromatography

HPTLC analysis was performed on 20 × 10 cm HPTLC glass plates silica gel 60 F_{254} (Merck, Germany). Reference solutions and samples were applied onto the plate as bands 8.0 mm wide using a CAMAG Automatic TLC Sampler (ATS 4). Bands were applied at a distance of 8.0 mm from the lower edge of plate and 20 mm from