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Metabolite characterization of powdered fruits and leaves from *Adansonia digitata* L. (baobab): A multi-methodological approach

Arold Jorel Tsetegho Sokeng[a,1], Anatoly P. Sobolev[b,1], Arianna Di Lorenzo[a], Jianbo Xiao[c], Luisa Mannina[b,d], Donatella Capitani[b,2], Maria Daglia[a,*,2]

[a] Department of Drug Sciences, Medicinal Chemistry and Pharmaceutical Technology Section, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy
[b] Istituto di Metodologie Chimiche, Laboratorio di Risonanza Magnetica "Annalaura Segre", CNR, 00015 Monterotondo (Rome), Italy
[c] Institute of Chinese Medical Sciences, State Key Laboratory of Quality Research in Chinese Medicine, University of Macau, Avenida da Universidade, Taipa, Macau, China
[d] Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, P.le Aldo Moro 5, 00185 Rome, Italy

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ABSTRACT

The metabolite profiling of extracts from *Adansonia digitata* L. (baobab) fruit pulp and leaf, and the quantification of their major components, was conducted by means of reverse-phase, high-performance liquid chromatography with photodiode array detection, coupled to electrospray ion-trap mass spectrometry (RP-HPLC-PDA-ESI-MS/MS) and high field nuclear magnetic resonance (NMR) spectroscopy. Water-soluble metabolites from chemical classes including sugars, amino acids, organic acids, and phenolic compounds, were identified, in addition to metabolites soluble in organic solvents such as triacylglycerides, sterols, and fatty acids, and most of these were quantified. The profiling of the primary and secondary metabolites of baobab fruit and leaves addresses the limited knowledge of the chemical composition of baobab, and helps support and explain the growing evidence on its nutritional and biological properties, and provide suggestions about the possible uses of baobab fruit and leaves by food, pharmaceutical and cosmetic industries.

1. Introduction

Adansonia digitata L. is a species of tree belonging to the Bombacaceae family, commonly known as baobab. It is widely distributed throughout sub-Saharan Africa and Western Madagascar and has many uses, including as an ingredient in food and beverages and in traditional medicine (Diop, Sakho, Domier, Cisse, & Reynes, 2006; Gebauer, El-Siddig, & Ebert, 2002). While in African tradition all parts of the baobab tree (fruits, seeds, flowers, roots and bark) are used as food, only the fruit pulp is consumed in Europe, where it has been authorized as a novel food since 2008 (Sugandha & Shashi, 2017), whereas in the United States of America the fruit pulp was approved as a food ingredient in 2009 (FDA, 2009). Over the last decade, the baobab tree has attracted significant attention due to the nutritional properties of its fruits, leaves and seeds and the beneficial effects of extracts from all of its parts on human health (Chadare, Linnemann, Hounhouigan, Nout, & Van Boekel, 2008; Rahul et al., 2015).

As far as the chemical composition of fresh baobab fruit is concerned, the literature is mainly focused on certain specific compounds. The ascorbic acid content has been found to be at least 3 times higher than that of other typical food sources of vitamin C (i.e. orange, kiwifruit, red fruits) (Vertuani, Braccioli, Buzzoni, & Manfredini, 2002). Furthermore, it has been reported that baobab fruit pulp is also rich in vitamins B_1, B_2, and B_6 (Diop et al., 2006) and in mineral salts (i.e. P, Mg, Ca, K, Na and Fe) (Glew et al., 1997). Baobab fruit pulp can contain as much as 56% pectin (Nour, Magboul, & Kheiri, 1980) and is rich in essential and nonessential fatty acids (i.e. linoleic acid, linolenic acid, oleic acid) (Glew et al., 1997). In 2017, Li et al. (2017) isolated and identified some polyphenolic compounds from dried baobab fruit pulp (i.e. four hydroxycinnamic acid glycosides, six iridoid glycosides, and three phenylethanoid glycosides).

Baobab leaves are traditionally used as a food in Africa, whereas they are not yet authorized as a novel food in Europe. Baobab leaves have been reported to be rich in palmitic acid, oleic acid and linoleic acid, and are an excellent source of Ca, K, Mg and Fe. Their vitamin content is also remarkable, being rich in Vitamins C, B_1, B_2, B_3, and β-

* Corresponding author.
E-mail address: maria.daglia@unipv.it (M. Daglia).
[1] These two authors share the first authorship.
[2] These two authors share the senior authorship.

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carotene, for young leaves. Baobab leaves contain mucilage (9–12%), which explains their traditional use as thickening agent in South-African culture. Phenolic acids (gallic and ellagic acids), hydroxycinnamic acids (chlorogenic and caffeic acid), flavonols (rutin, quercitrin, quercetin, kaempferol), flavan-3-ols (catechin, epicatechin), and a flavone (luteolin) were identified in fresh baobab leaves through reverse-phase high-performance liquid chromatography with diode-array detection (RP-HPLC-DAD) method (Irondi, Akintunde, Agboola, Boligon, & Athayde, 2017).

However, despite the growing importance of baobab fruit and leaves due to their nutritional and biological properties, data on the primary and especially the secondary metabolites are extremely sparse in literature and little is known about the concentration of nutrients and bioactive components to date.

To obtain a comprehensive picture of the composition of these foods, the most common analytical tools are nuclear magnetic resonance (NMR) and mass spectrometry (MS) based metabolomic approaches, allowing for "high-throughput" spectroscopic/structural information on a wide range of compounds with high analytical precision (Sobolev et al., 2015). Metabolomics is a technology-driven approach advanced by recent developments in analytical tools, software, and statistical data analysis.

Here, we report the identification and quantification of primary and secondary metabolites occurring in extracts obtained from baobab fruit pulp and dried leaf samples using a multi-methodological approach, which consists of the combination of untargeted NMR spectroscopy and targeted reverse-phase high-performance liquid chromatography with photodiode array detection coupled to electrospray ion-trap mass spectrometry (RP-HPLC-PDA-ESI-MS/MS). The combination of these methodologies has been optimized in our previous investigations of plant foods and beverages (Mannina et al., 2015; Marchese et al., 2014, Sobolev et al., 2018). The choice of dried samples instead of fresh samples was made in view of the most common forms of baobab consumption worldwide, both in the countries of origin and in importing countries.

2. Materials and methods

2.1. Materials

HPLC-grade water was obtained from a LC-Pak™ Millex system (Millipore Corporation, Billerica, MA, USA). Formic acid, MS grade methanol, citric acid, galacturonic acid, malic acid, glucose, sucrose, gallic acid, and caffeine were obtained from Sigma-Aldrich, St. Louis, MO, USA. Quinic acid, catechin, procyanidin B1, procyanidin C1, kaempferol glucoside, kaempferol, rutin, and quercetin rhamnoside were obtained from PhytoLab, Vestenbergsgreuth, Germany.

2.2. Food materials

In order to perform a more representative sampling, 5 samples of fresh baobab fruit pulp, baobab also known as *Adansonia digitata* var. congolensis (accepted name: *Adansonia digitata* L.), were collected, dried and mixed together, and 20 samples of dried leaves were collected and milled together. These samples were collected during the harvest season of January–February 2016, in a local market in Ngaoundéré, Cameroon, and transported by airplane in plastic bags inside a box to laboratories in Italy, where they were stored in vacuum plastic bags away from sunlight in a cool room.

2.3. Preparation of extracts from baobab fruit pulp and leaf

2.3.1. Extraction process for RP-HPLC-PDA-ESI-MS/MS analysis

Powdered baobab fruit pulp and dried leaves were ground into fine powders prior to extraction. Each extraction was carried out separately by weighing 10 g of each food powder into a conical flask and adding 100 mL of a 50:50 (*v/v*) mixture of methanol and Millipore grade water acidified with formic acid (0.1%). The conical flask, immersed in ice, was left under agitation for 24 h under nitrogen atmosphere. Then, the extracts were filtered under vacuum through paper filters, and methanol was evaporated under nitrogen flux. Finally, the obtained extracts were freeze-dried. The various extracts were dissolved in Millipore grade water prior to chromatographic analysis.

2.3.2. Extraction process for NMR untargeted analyses – Bligh-Dyer extraction

Powdered baobab fruit pulp and dried leaves were submitted to extraction according to a modified Bligh–Dyer methodology (Bligh & Dyer, 1959). A mixture of methanol/chloroform (2:1 *v/v*) was used to extract the greatest number of metabolites. The mixture (3 mL) was added to the sample (0.5 g) and agitated, followed by the addition of 1 mL of chloroform and 1.2 mL of Millipore grade water and the emulsion was stored at 4 °C for 40 min after stirring. The sample was then centrifuged (800*g* for 15 min at 4 °C). The upper (hydroalcoholic) and lower (organic) phases were carefully separated. The pellets were re-extracted using half of the solvent volumes (in the same conditions described above) and the separated fractions were pooled. Both fractions were dried under a nitrogen flow at room temperature until the solvent was completely evaporated. The dried phases were stored at −20 °C pending NMR analysis.

2.4. RP-HPLC-PDA-ESI-MS/MS analysis of the food extracts

Chromatographic analyses were performed using a Thermo Finnigan Surveyor Plus HPLC apparatus equipped with a quaternary pump, a Surveyor UV–Vis photodiode array detector (PDA), and a LCQ Advantage max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA, USA), coupled through an ESI source. RP-HPLC-PDA-ESI-MS/MS data were acquired under positive and negative ionization modes, using Xcalibur software. The ion trap operated in full scan (100–2000 *m/z*), data dependent scan and MSn modes; when greater discrimination was required, additional targeted MS^2 and MSn experiments were performed on selected pseudomolecular ions. To optimize the MS operating conditions, a preliminary experiment was performed: 10 μg/mL caffeine (H_2O/MeOH: 50/50 with 0.1% formic acid) and 10 μg/mL gallic acid (H_2O/MeOH: 50/50 with 0.1% formic acid) solutions were directly infused in the ESI interface at a flow rate of 25 μL/min into the mass spectrometer. Optimized conditions were as follows: sheath gas 60, capillary temperature 220 °C, auxiliary gas 25 and 20, spray voltage 4.5 and 5 kV, capillary voltage -26.13 V and 35 V respectively for negative and positive ionization modes.

2.5. HPLC separation of the baobab fruit pulp and leaves extracts

Separation was achieved on a Synergi Fusion RP18 80A (150 × 4.6 mm; 4 um) column operating at 25 °C, protected by its corresponding guard column, both from Phenomenex, California, USA. A gradient elution was executed with acidified water (0.1% formic acid) as mobile phase A and methanol as mobile phase B, at a flow rate of 0.3 mL/min. The elution gradient for the separation of the baobab fruit pulp extract involved moving from 10% B to 70% B in 104 min, 70% B to 80% B in 5 min, 80% B to 100% B in 5 min, a 5 min isocratic of 100% B, 100% B to 10% B in 5 min, and finally an isocratic run of 10% B for 6 min. The elution gradient for the separation of the baobab leaf extract involved moving from: 10% B to 70% B in 84 min, 70%B to 80% B in 5 min, 80% B to 100% B in 5 min, 100% B for 5 min, 100% B to 10% B in 5 min, and finally 10% B for 6 min. The temperature of the sample tray was set to 4 °C and the injection volume was 5 μL. Chromatograms for both extracts were recorded at λ 254, 280, 330 nm and spectral data were collected in the range of 200–800 nm for all peaks.

RP-HPLC-PDA-ESI-MS/MS analyses were performed in positive and negative ionization modes to obtain maximum information on the