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ORIGINAL PAPER

African baobab (*Adansonia digitata*) fruit as promising source of procyanidins

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Abstract
Baobab is an endemic African plant rich in health-promoting compounds and still not extensively studied. Characterization of bioactive molecules in baobab fruit by means of LC system coupled with a photodiode array and a triple quadrupole mass spectrometer detector is here reported. This work provides a qualitative and quantitative profile of polyphenols in baobab pulp, shell, kernel and seeds. Baobab pulp is a rich source of ascorbic acid, (−)-epicatechin and procyanidin B2 (135.6, 104.8, and 100.1 mg/100 g). Moreover, a HPLC preparative method to isolate procyanidin fraction from pulp was developed, revealing how the antioxidant activity of baobab pulp not only depend on the ascorbic acid content but also on the presence of procyanidins. The use of baobab fruit pulp as promising healthy food or food supplement is also discussed.

Keywords Ascorbic acid · Procyanidin · Baobab · HPLC/PDA/MS–MS · Antioxidant activity · Bioactive molecules

Introduction

Bioactive molecules' content in foodstuffs has been extensively studied both for their characterization and to verify their positive effects on human health. In the last decade, from a quick search on the most common scientific databases, more than 1000 research articles have been published reporting composition in bioactive molecules of fruits and vegetables and their potential biological activities [1–3]. Knowledge of the biologically active molecules content in plant foods consumed daily by the most of the world population is therefore wide.

However, a huge number of novel plant species, in particular those consumed by local populations of developing countries and little used by western society are interesting for food and pharmaceutical industries due to their promising healthy properties, even if their chemical composition was not so extensively studied [4]. An example is the baobab (*Adansonia digitata*) fruit. Baobab tree is a large tree endemic of Africa [5], belonging to the family of Bombacaceae, a sub-family of Malvaceae [6, 7]. The African tree grows wild in Western Madagascar, sub-Saharan Africa, South Africa, Botswana, Namibia, Mazambique and in arid and semi-arid zones of America and Asia where 44 different species of *Adansonia* have been reported [7, 8]. Baobab fruits are usually globose or ovoid, irregular in shape, and covered by velvety yellowish or greenish hairs [9]. The resistant external irregular capsule of the fruit is named pericarp, and the baobab fruit pulp is contained inside this capsule. The pulp, with filaments (red fibers), constitute the internal part of baobab fruit, called endocarp [10], which contains several seeds. When the fruit is mature, pulp is a free-flowing coarsely milled powder characterized by a distinctive tart and acidic flavor, and color ranges from off-white to cream [11].

Edible parts of baobab trees are: leaves, seeds, and fruit pulp. These are generally consumed as foodstuffs and for medicinal purposes by indigenous people [10]. They are

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also believed to be useful for spiritual welfare [12]. Fresh leaves are cooked as vegetables, dried and crushed for later use, or generally employed to prepare sauces [5, 13, 14]. Seeds, which are enveloped by a layer of dehydrated pulp, are eaten fresh, dried or roasted; moreover, they are used for the production of vegetable oil [13, 15, 16]. They can also be ground into a powder and used as substitute for coffee, or as thickening agents, or as flavor enhancers when fermented [13, 14, 16]. The pulp is usually employed for juice, snacks, and sweets preparation, as well as a fermenting agent in local brews, porridge and in food recipes [14, 16].

Thanks to its therapeutic potential baobab is also named "chemist tree" or "the small pharmacy" [5, 10, 17]. Indeed pulp and leaves are used as insect repellent, analgesic, febrifuge, anti-diarrhea/anti-dysentery, anti-tuberculosis, antimicrobial infections, and for treatment of anemia, gingivitis, smallpox, cough, haemoptysis and measles [5, 10, 17, 18]. Seeds are employed as medicines in the treatment of fever, diarrhea, dysentery, muscle wounds, dandruff and other skin ailments [17]. At least to our knowledge, there are no reports in the literature concerning the amount of baobab fruit consumed by local people in the African areas where this plant spontaneously grown.

There are many literature data on the composition in macronutrients, micronutrients, amino acids, and fatty acids of baobab fruit [5, 7, 9, 11, 16]. Baobab pulp showed a high vitamin C content, 150–550 mg/100 g dw, and contains a high amount of both soluble and insoluble dietary fiber [8]. Leaves are rich in calcium (307–2640 mg/100 g dw). Seeds have a relatively high lipid content, 11.6–33.3 g/100 g dw, representing an excellent source of mono- and polyunsaturated fatty acids, such as linoleic and oleic acids [9].

In the last decade the western society has been interested in this fruit, in particular for producing dietary supplements and cosmetics [9]. In 2008, PhytoTrade Africa issued a GRAS (Generally Recognized As Safe) notification on baobab pulp to suggest its use in the western diet. In this notification, the use of the pulp is encouraged in several different formats: as a source of fiber (es. in cereal bars or snacks), as a source of fruit (es. in soft drinks or smoothies), and as a structural agent to replace the starches in sauces and desserts [11]. For these reasons baobab fruit pulp was approved as a novel food ingredient both in Europe and in the United States of America by the European Commission [19] and the Food and Drug Administration [11]. In the last 10 years, demand for baobab products has grown.

Several research articles described the total phenolic content and the antioxidant activity of fruit pulp [4, 5, 8, 9, 20]. Some articles are focalized on the isolation of five procyanidins from baobab pericarp [13], on identification of phenolic acids from red fiber [10] and stem bark [21]. Six iridoid glycosides, three phenylethanoid glycosides and four hydroxycinnamic acid glycosides have also been identified in the whole fruit [17]. Recently, an article by Tsetegho Sokeng and co-workers has reported metabolites identification in powdered fruits and leaves from *Adansonia digitata* [22]. Few articles in the literature concern the quantification of polyphenols in *Adansonia digitata* fruit pulp. Procyanidin B2, (−)-epicatechin and kaempferol isomers content were previously determined in baobab pulp [23, 24].

In this work, a systematic study on the qualitative and quantitative characterization of bioactive molecules (vitamin C and phenolic acids) in all the part (seed, pulp, shell and kernel) of baobab fruit by means of RP-HPLC/PDA/MS–MS is proposed. Moreover, the contribution of the different classes of molecules to the total antioxidant properties of the pulp has also been investigated.

Materials and methods

Materials and samples

Edible parts of baobab fruit (pulp and seeds) were collected during the first decade of August 2017 in a local market in Iringa (Tanzania, Africa). Pulp and seeds were stored at room temperature for a few days until use. According to traditional processing of baobab seeds [9, 14], these were boiled in distilled water for 1 h at 100 °C, to separate the shell from the kernel. Prior to the bioactive molecules' extraction, seeds, kernels and shells were finely ground with a laboratory grinder, and then stored at − 80 °C.

The standard compounds and reagents namely gallic acid, ascorbic acid (ASC), epicatechin, potassium persulfate, sodium carbonate, dithiothreitol (DTT), NEM (*N*-ethylmaleimide), iron (III) chloride ($FeCl_3$) and trichloroacetic acid (TCA), *ortho*-phosphoric acid, α,α′-dipyridyl, Folin–Ciocalteu reagent, ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic)-diammonium salt], trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and DPPH (2,2-diphenyl-2-picryl-hydrazyl) were purchased from Merck KGaA (Darmstadt, Germany). Procyanidin A2 and procyanidin B2 were obtained from Extrasynthese (Genay Cedex, France). EDTA (ethylenediaminetetraacetic acid) was purchased from J. T. Baker (Deventer, Holland).

Solvents employed for the extraction procedure and for HPLC–MS/MS analyses formic acid, methanol, ethanol, water and acetonitrile were obtained from Merck KGaA (Darmstadt, Germany).

Instrumentation

Sample extractions were carried out in an Elma ultrasonic bath S 30H (Elma Schmidbauer GmbH, Germany), and the extracts were centrifuged in an Eppendorf 5804 R centrifuge (Eppendorf AG, Germany). Spectrophotometric analyses

were carried out with a UV-1800 UV/Vis spectrophotometer (Shimadzu, Milan, Italy). Total phenolic content and antioxidant activity evaluation were determined with an Infinite® 200 PRO multimode reader (Tecan, Milan, Italy).

HPLC–PDA and HPLC preparative analyses were carried out using a Shimadzu system (Shimadzu, Milan, Italy), including a SCL-10A VP controller, two LC-10 AD VP pumps, a 7725I Rheodyne® 6-port 2-position valve and a CTO-10ASVP column oven. As detector, an SPD-M10Avp diode array was employed for quantification of ascorbic acid and isolation of procyanidins fraction from baobab pulp. Data acquisition was performed by the Shimadzu LCsolution software ver 1.24 SP1 (Shimadzu).

HPLC–PDA-MS/MS separations were conducted on a Nexera-e liquid chromatograph system (Shimadzu, Kyoto, Japan) equipped with a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a CTO-20AC column oven, a DGU-20A5R degasser, a SIL-30AC autosampler, and a SPD-M30 PDA detector (1.8 µL detector flow cell volume) connected in series to a LCMS-8050 triple quadrupole MS, interfaced through an electrospray ionisation (ESI) source. Data acquisition and processing were performed using Shimadzu LabSolution software ver. 5.65 (Shimadzu, Kyoto, Japan).

Ascorbic acid determination

Samples extraction

1 g of each part of Baobab fruit (pulp, whole seed, shell, and kernel) was finely homogenized by mortar and pestle with ten volumes of cold 0.5% (v/v) formic acid at 4 °C. The homogenates were centrifuged at 14,000×*g* for 15 min at 4 °C. The supernatant (8 mL) was collected for spectrophotometric and HPLC–PDA analyses of ascorbate. For HPLC analyses the pulp extract was diluted 1:10 in water/formic acid (99.5:0.5 v/v) before the injection. Whole seeds, kernel, and shell extracts were directly subjected to HPLC separation. Each sample was analyzed in triplicate.

HPLC–PDA analytical conditions

Ascorbic acid analyses were carried out on a Luna C18, 250 × 3 mm I.D. with particle size of 5 µm (Phenomenex, PA, USA). The injection volume was 2 µL, mobile phase consisted of water/formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile/formic acid (99.5:0.5, v/v) (solvent B) and the step-wise gradient profile was as follows: 0 min, 0% B, 5 min, 0% B, 15 min, 22% B, 20 min, 100% B, 25 min, 100% B. Flow rate was 0.3 mL/min, data were acquired using a photodiode array detector in the range 190–400 nm and the chromatograms were extracted at 245 nm. Time constant was 0.64 s and sample frequency 1.5625 Hz. Data acquisition was performed by Shimadzu LCsolution software ver 1.24 SP1.

Spectrophotometric determination of ascorbic acid content

The analysis of ascorbic acid was determined according to de Pinto et al. [25]. Briefly, total ascorbate was determined after reduction of DHA (dehydroascorbate) to ASC with DTT. 100 µL of supernatant (obtained as described in "Samples extraction") of each sample was diluted with water/formic acid (99.9:0.5, v/v) 4 and 2 times for pulp and whole seed, respectively. 250 µL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA was added to all the diluted extracts. These reaction mixtures were added of 50 µL of 10 mM DTT and then incubated for 10 min at room temperature. 50 µL of a solution of 0.05% NEM was then added to remove DTT excess. Colorimetric reaction was developed by adding to the mixture the following reagents: 0.15 mL of 10% TCA, 0.2 mL of 44% *ortho*-phosphoric acid, 0.2 mL of 4% α,α′-dipyridyl in 70% ethanol and 0.05 mL $FeCl_3$ (63 mg/mL) in 10% TCA. After vortexing, the mixture was incubated at 40 °C for 40 min. Ascorbic acid content was determined using a UV/Vis spectrophotometer, and the absorbance of the solution was read at 525 nm. A standard curve was developed based on ASC in the range 1–10 µg/mL, following a validated procedure previously adopted [26].

Determination of phenols

Samples' extraction

The extraction procedure was carried out on 1 g of each sample, extracted with three aliquots of 10 mL of water (0.1% of formic acid), water/acetonitrile/formic acid (5:4.9:0.1 v/v/v), and acetonitrile (0.1% of formic acid) in an ultrasonic bath at room temperature (for 10 min at 50 Hz). The extracts were centrifuged at room temperature (for 5 min at 4000 g), combined, filtered on a filter paper, and brought to dryness in a rotary evaporator. The extracts thus obtained were dissolved in 1 mL of a mixture of water/acetonitrile/formic acid (5:4.9:0.1 v/v/v), filtered on Acrodisc filter 0.45 µm Merck KGaA (Darmstadt, Germany), and injected into HPLC. Each sample was analyzed in triplicate.

HPLC–PDA-MS/MS analytical conditions

Bioactive molecules present in baobab samples were analyzed with an Ascentis® Express C18, 150 × 4.6 mm I.D. with particle size of 2.7 µm (Merck KGaA, Darmstadt, Germany) column. The injection volume was 2 µL, mobile phase consisted of water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile/formic acid (99.9:0.1, v/v) (solvent B)

and the step-wise gradient profile was as follows: 0 min, 0% B, 5 min, 0% B, 40 min, 40% B, 50 min, 100% B. Flow-rate was 1.0 mL/min, data were acquired using a photodiode array detector in the range 190–400 nm and the chromatograms were extracted at 280 and 325 nm. Time constant was 0.16 s and sample frequency 12.5 Hz. MS acquisition was performed using ESI, both in negative and positive mode. ESI conditions: mass spectral range, *m*/*z* 100–1200; interval 1.0 s; scan speed 2000 amu/s; nebulizing gas (N_2) flow 3.0 L/min; ESI temperature 300 °C; heat block 300 °C; DL (desolvation line) temperature, 250 °C; interface voltage of 3.5 kV and detector voltage of 1.8 kV.

Isolation of flavonoids fraction

2 g of baobab pulp was extracted with 50 mL of water/methanol mixture (1:1 v/v) and the extract was brought to dryness in a rotary evaporator. The extract thus obtained was dissolved in 1 mL of water/methanol mixture (1:1 v/v) and subjected to preparative HPLC separation using a Discovery® HS C18 (250 × 10 mm I.D. × 5 µm) (Merck KGaA, Darmstadt, Germany) preparative column. Mobile phases: water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile/formic acid (99.9:0.1, v/v) (solvent B); flow rate: 3 mL/min gradient: 0 min, 0% B, 20 min, 30% B, 55 min, 100% B, 60 min, 100% B. Injection volume was 200 µL. Data were acquired using a UV detector at 280 nm. Data acquisition was performed by Shimadzu LCsolution software ver 1.24 SP1.

After five consecutive injections three fractions were collected. Each fraction was analyzed at the same chromatographic conditions optimized for the samples. Only fraction 3 contained flavonoids, so was concentrated to 1 mL and subjected to antioxidant activity assays.

Sample extraction methods validation

Recovery of the bioactive components was determined by carrying out the extraction procedure on the pulp sample fortified of known amounts of ascorbic acid, (−)-epicatechin, procyanidin A2, and procyanidin B2.

Every extract thus obtained was analyzed in triplicate. Recovery was calculated using the following formula:

$$\text{Recovery } \% = \left[(\text{conc. sample fortified} - \text{conc. sample unfortified})/\text{fortification}\right] \times 100.$$

RP-HPLC methods validation and statistical analysis

To quantify bioactive molecules content in the various samples tested, external calibration curves have been constructed using each commercial standard. Five different concentrations of each component, in the range between 100 and 0.1 mg/L, prepared by diluting a stock solution of about 1000 mg/L, using water as a solvent, were analyzed for five consecutive times by HPLC under the same chromatographic conditions optimized for the samples. Concentration of the different compounds in baobab extracts was determined using the calibration curves of the compounds with the same chromophore. By means of the calibration curves described above, it was possible to quantify ascorbic acid and procyanidins' content in the analyzed samples.

Limit of detection (LOD) and limit of quantification (LOQ) values, following the EURACHEM guidelines [27], for reference compounds were also calculated.

The precision of the method was tested by performing intra- and inter-day multiple injections of standard compounds mixture and then checking the %RSD of retention times.

Antioxidant activity

Free radical scavenging activity of bioactive molecules extract obtained from baobab pulp, whole seed, kernel and shell samples and from fraction isolated using preparative HPLC was evaluated by colorimetric assay according to a previously described method with some modification [28]. The radical cation $ABTS^{\cdot+}$ was produced by reacting 7 mM ABTS aqueous phosphate buffer (5 mM NaH_2PO_4–H_2O and 5 mM Na_2HPO_4–$2H_2O$, pH 7.4) solution with 2.5 mM potassium persulfate (final concentration). This solution was allowed to react for 12 h at room temperature and in the dark. $ABTS^{\cdot+}$ working solution was obtained by the diluting in phosphate buffer of the stock solution to an absorbance of 0.70 ± 0.05 at 734 nm. 50 µL of supernatant (obtained with extraction method reported in "Samples extraction") of each sample and fraction isolated using preparative HPLC were diluted 50 times (pulp and fraction) and 2 times (whole seed, shell and kernel) with 5 mM aqueous phosphate buffer before performing procedure described below. 10 µL of each diluted sample was mixed with 190 µL of $ABTS^{\cdot+}$ working solution in a 96-multiwell plate (Greiner Bio-one, Germany). The absorbance was recorded at 734 nm after 20 min using a multifunctional microplate reader. A calibration curve was prepared with Trolox at a concentration 50–700 µM, following a validated procedure previously adopted [29]. All measurements were carried out in triplicate. Results were expressed as µmol Trolox equivalent (TE) per g of sample.

The DPPH assay was performed according to the method described by Padmanabhan and Jangle [30], with few modifications. 50 µL of supernatant (obtained with extraction method reported in "Samples extraction") of each sample and the fraction isolated using preparative HPLC were diluted 50 times (pulp and fraction) and two times (whole seed, shell and kernel) with methanol/water (50:50, v/v) before performing procedure described

below. 20 µL of each diluted sample was mixed with 180 µL of ethanolic solution of DPPH (0.1 mM) in 96-well plates. The mixture was shaken and kept in the dark for 20 min at room temperature. The absorbance was detected at 518 nm, against a blank of ethanol and a control with 20 µL of ethanol with 180 µL of DPPH 0.1 mM in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader. Experiments were performed in triplicate. The antioxidant capacity of sample was determined by a calibration curve, using Trolox as reference compound (20–200 µM), following a validated procedure previously adopted [29].

Total phenolic content

Total phenolic content (TPC) of baobab pulp, whole seed, kernel, and shell extract was determined by Folin–Ciocalteu phenol reagent method, according to the procedure reported by Singleton and co-workers [31]. A volume of 1.58 mL of a methanol/water (50:50, v/v) and 100 µL of Folin–Ciocalteu were added to 20 µL of supernatant (obtained with extraction method reported in "Samples extraction"). The supernatant of pulp sample was diluted 2 times with methanol/water (50:50, v/v) before performing procedure described previously. The mixture was incubated for 8 min at room temperature. Then 300 µL of sodium carbonate solution (20% w/v) was added. The solution was mixed and allowed to stand in a dark environment at room temperature for 2 h, and finally centrifuged at 20,800*g* for 5 min. The absorbance reading was 765 nm in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader. The results were expressed as mg of gallic acid equivalents (GAE)/g of sample. Total phenolic content was determined from the gallic acid calibration curve (0.05–1.6 mg/mL), following a validated procedure previously adopted [29].

Results and discussion

Our work aimed to achieve a qualitative and quantitative determination of the bioactive molecules content of a baobab fruit. To tackle this task, spectrophotometric essays, RP-HPLC/UV, and RP-HPLC/PDA/MS–MS methods were successfully employed. We focused our attention on vitamin c and phenolic composition of pulp and seeds of baobab fruit, since their nutritional and healthy values are well known.

Total phenolic and ascorbic acid contents, and the consequent effect on antioxidant activities, were first investigated. Table 1 shows total phenolic compounds, ascorbic acid concentration and the values of antioxidant activities obtained by spectrophotometric assays for each sample. Pulp samples were the richest in ascorbic acid and phenolic compounds. Vitamin C and total phenolic content found in *Adansonia digitata* fruit pulp (167.1 ± 6.72 mg/100 g and 24.3 ± 0.51 mg GAE/g, respectively) were totally in accordance with previously published data (150–500 mg/100 g and 21.8–40.6 mg GAE/g, respectively) [4, 5, 8–10, 32]. As expected, the baobab pulp showed the highest antioxidant activity between the analyzed samples (Table 1). The two methods used for measuring the total antioxidant activity gave similar trends, even if, in our conditions, TEAC methods seem to be more sensitive than DPPH (Table 1).

Ascorbic acid content was also evaluated using a liquid chromatographic method. According to literature data [33, 34], an RP-HPLC/UV method for the purpose of vitamin C quantification in baobab fruit samples was developed. The validation process provided the results shown in Table 2 for LOD and LOQ. Table 3 reports the ascorbic acid concentration in mg/100 g in all the samples analyzed. Quantitative data obtained with the RP-HPLC/UV method are totally in accordance with those obtained with the spectrophotometric assay.

These results confirm the high level of vitamin C and antioxidant activity of baobab fruit pulp already reported by other authors on baobab fruits [5, 9]. For elucidating the quali-quantitative profile in the bioactive molecules of phenolic nature, seeds, pulp, shell and kernel were extracted as reported in "Samples extraction". Extracts were subjected to RP-HPLC system coupled with a photodiode array and a triple quadrupole mass spectrometer detector, to quantify and

Table 1 Values represent total phenolic [TPC, expressed as mg gallic acid equivalent (GAE)/100 g], ascorbic acid contents (AAC, mg/100 g), and antioxidant activities [TEAC and DPPH, expressed as trolox equivalent (µmol TE/100 g)] of the analyzed samples

Sample	TPC	AAC	TEAC	DPPH
Whole seed	0.01 ± 0.001	–	0.05 ± 0.003	–
Pulp	0.24 ± 0.005	167.10 ± 6.720	0.90 ± 0.063	0.49 ± 0.013
Shell	0.01 ± 0.001	–	0.12 ± 0.017	0.01 ± 0.001
Kernel	0.01 ± 0.001	–	0.06 ± 0.001	0.01 ± 0.001

Table 2 Regression equations, correlation coefficients (R^2), LOD, LOQ, recoveries, retention times, λ_{max} and MS values for each bioactive molecule

Compound	λ_{max}	*m/z*	Rt	Rt (RSD %)	Area (RSD %)	Regression line	R^2	LOD (mg/kg)	LOQ (mg/kg)	Recovery (%)
Ascorbic acid	245	176	7.07	0.82	2.28	$y=46{,}650x-6696.7$	0.999	0.023	0.042	82.8
Procyanidin B2	279	578	19.06	0.75	3.12	$y=15{,}125x+236{,}133$	0.990	0.045	0.096	87.8
(−) Epicatechin	279	290	20.05	0.78	3.24	$y=16{,}383x+56{,}568$	0.994	0.037	0.083	90.3
Procyanidin A2	280	576	22.52	0.86	3.09	$y=14{,}755x-11{,}353$	0.990	0.028	0.050	85.2

Table 3 Bioactive molecules content (mg/100 g ± standard deviation) in four samples analyzed

Compound	*m/z*	MS/MS ions $[M-H]^-$	MS/MS ions $[M+H]^+$	Whole seed	Pulp	Shell	Kernel
Polyphenols							
Quercetin-3-*O*-β-D-glucoside[a]	463	Low abundance ions	Low abundance ions	1.9 ± 0.04	< LOD	< LOD	1.9 ± 0.03
Procyanidin B isomer 1[b]	578	577, 413, 407, 289	579, 386	< LOD	5.2 ± 0.20	< LOD	< LOD
Catechin glucoronide[a]	564	563, 517	536	1.0 ± 0.04	5.7 ± 0.02	< LOD	1.4 ± 0.06
Procyanidin B2	578	577, 413, 407, 289	579, 386	10.5 ± 0.28	100.1 ± 1.75	1.5 ± 0.01	23.5 ± 0.08
Procyanidin tetramer isomer 1[c]	1154	1153, 1001, 865, 863, 576, 289	–	< LOD	42.8 ± 0.69	< LOD	< LOD
Procyanidin tetramer isomer 2[c]	1154	1153, 865, 576, 427	400	0.9 ± 0.02	23.3 ± 1.10	< LOD	8.0 ± 0.14
Epicatechin	290	289	291	7.2 ± 0.03	104.8 ± 2.34	1.8 ± 0.02	< LOD
Procyanidin C isomer 1[c]	866	865, 576, 289	867, 579	2.5 ± 0.03	7.2 ± 0.16	1.1 ± 0.03	< LOD
Procyanidin tetramer isomer 3[c]	1154	1153, 1001, 865, 863, 576, 289	867, 579	< LOD	11.3 ± 0.28	< LOD	< LOD
Procyanidin C isomer 2[c]	866	865, 576, 289	867, 579	2.0 ± 0.01	4.6 ± 0.13	0.8 ± 0.02	< LOD
Procyanidin A2	576	475	448	1.0 ± 0.03	8.6 ± 0.12	0.7 ± 0.03	< LOD
Gallocatechin hexoside[a]	488	487	465	< LOD	2.2 ± 0.03	< LOD	< LOD
Procyanidin B isomer 2[b]	578	577, 413, 289	579, 386	< LOD	12.2 ± 0.67	< LOD	< LOD
All				27.0 ± 0.34	328.2 ± 7.26	5.9 ± 0.08	34.8 ± 0.08
Ascorbic acid				1.0 ± 0.03	135.6 ± 1.01	< LOD	< LOD
Total bioactive molecules				28.0	463.8	5.9	34.8

Bioactive molecules were quantitatively determined based on calibration curves obtained with the correspondent standard compound: [a]epicatechin, [b]procyanidin B2, [c]procyanidin A2

identify the bioactive molecules present. For the chromatographic separation a fused core C18 (150 × 4.6 mm, 2.7 µm d.p.) column was used. The use of a photodiode array as detector, for the untargeted analysis of polyphenols is not sufficient for a deep characterization of unknown samples. Instead, the use of a triple quadrupole mass spectrometer detector, due to its great sensitivity and selectivity, allows the identification and structural analysis of targeted and untargeted compounds.

For the first step both SIM and scan modes were employed for the targeted screening of six flavonoids, already isolated from baobab pericarp [13]. The six target flavonoids selected for the initial baobab fruit screening were: (−)-epicatechin (*m/z* 290), procyanidin B2 (*m/z* 578), procyanidin B5 (*m/z* 578), procyanidin A2 (*m/z* 576), procyanidin C1 (*m/z* 866) and quercetin-3-*O*-β-D-glucoside (*m/z* 463). Procyanidins B2 and B5 are isomers, so have the same molecular weight. The results of the initial screening, showed the presence of quercetin-3-*O*-β-D-glucoside (15.20 min), (−)-epicatechin (20.05 min), 3 procyanidin B isomers (16.36, 19.06 and 24.23 min), a procyanidin A (22.52 min), 3 procyanidin tetramer isomers (*m/z* 1154, 19.56, 19.71, and 21.70 min), and 2 procyanidin C isomers (21.08, and 21.94 min). Therefore, in our samples of fruit pulp, more procyanidins were found than those isolated from baobab pericarp [13], and most of them were isobaric molecules. Procyanidin B2, (−)-epicatechin and procyanidin A2 standards were used to confirm the identification and for quantitative analysis as reported in paragraph 2.7, while for other molecules tentatively identified further investigation was needed to exploit the

correct identification of the isobaric compounds by means of the triple quadrupole mass spectrometer detector.

Due to the lack of available standards of procyanidins and the presence of isobaric molecules, the use of SIM mode was not sufficient for the correct identification of the compounds. To tentatively identify the correct procyanidin, the product ion scan (PIS) mode was used to obtain a fragmentation pattern correlated to the characteristic structures of the selected $[M-H]^-$ and $[M+H]^+$. From interpretation of the PIS carried out injecting the samples with different collision energy values, three characteristic fragments at $[M-H]^-$ 289, 577, and 865 were found. As recently reported in a review on procyanidins structure elucidation via mass spectrometry [33], procyanidins can be categorized by their degree of polymerization (DP). A procyanidin monomer is epicatechin ($[M-H]^-$ 289), so the linkage between epicatechin molecules leads to oligomers, further forming polymers. The three characteristic fragments found for the procyanidins analyzed confirmed the presence of procyanidins with 4, 3 and 2 DP [35]. To have a further confirmation of the presence of procyanidins with different DP, the analyses were replicated using the triple quadrupole mass spectrometer in neutral loss mode. Each procyanidin tentatively identified showed a 289 *m/z* neutral loss, corresponding to the presence of procyanidin monomer.

Unfortunately the analysis by triple quadrupole mass spectrometer is not sufficient to discriminate isobaric molecules, but thanks to its great sensitivity and selectivity, it allowed the identification and structural analysis of the procyanidins contained in our samples.

Calibration curves of (−)-epicatechin, procyanidin A2, and procyanidin B2 were constructed under the same chromatographic conditions optimized for the samples to quantify the polyphenols content. The validation process provided the results shown in Table 2 for LOD and LOQ. Good linearity was obtained for all the analytes, as confirmed by the correlation coefficient R^2, ranging from 0.990 to 0.999. Concerning the intraday repeatability, coefficient of variation (CV) values of < 4% demonstrated good precision at the concentration level tested. Good recovery values for all the analytes, ranging from 83 to 90%, demonstrate the exhaustiveness of the extraction method employed for polyphenols.

Figure 1 shows the chromatogram of polyphenols in baobab pulp, whereas Table 3 reports the corresponding quantitative data.

The analytical method developed allowed for the determination of 13 different flavonoids, with epicatechin and procyanidin B2 being the most abundant. As reported in Table 3, pulp is the richest sample in bioactive molecules, while shell sample is the poorest. Qualitative profile of pulp sample was coherent with previously published data [23, 24]. In all the analyzed samples, polyphenols were more abundant than ascorbic acid (e.g., in baobab pulp 328.2 ± 7.26 mg/100 g versus 135.6 ± 1.01 mg/100 g). Ascorbic acid content in pulp sample represented a quarter of antioxidant total bioactive content; while this molecule was almost undetectable in the seeds. This latter result is not surprising, since ascorbic acid is very low in mature dry seeds [36, 37]. Moreover, it has to be taken into account that a thermic treatment was performed to separate the kernel from the shell (see "Materials and samples" for detail), and due to its thermos-lability, the ascorbic acid putatively present in the seeds could have been degraded. Polyphenols content in seed samples was approximately 12 times lower than the amount in the pulp (27.0 ± 0.34 vs 328.2 ± 7.26). From Table 3 it is possible

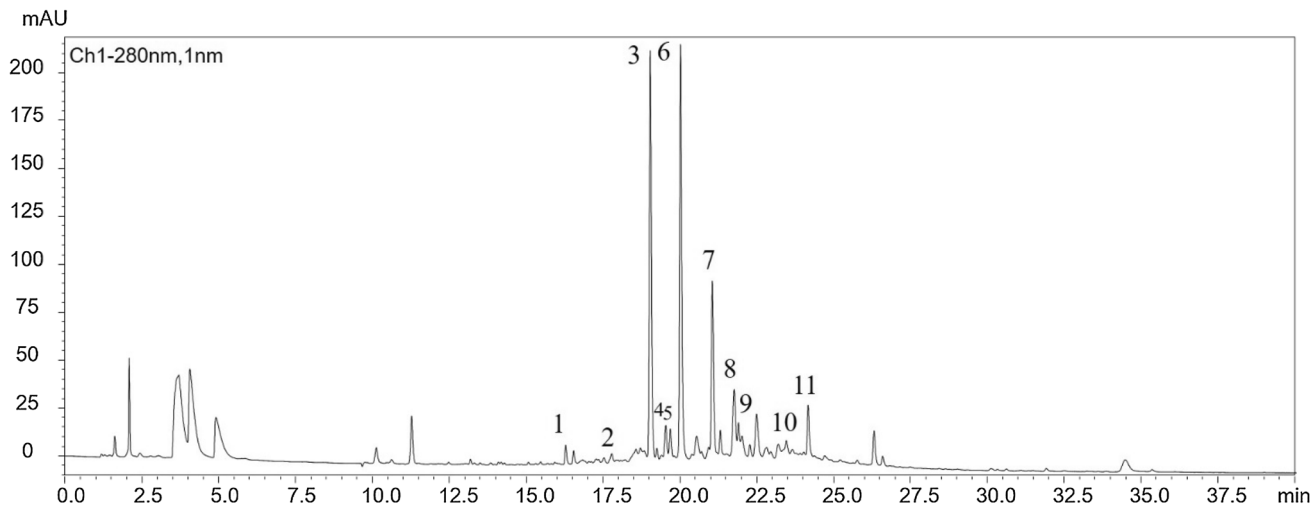

Fig. 1 RP-HPLC/PDA chromatogram of polyphenols extract in pulp sample. 1. procyanidin B isomer 1; 2. catechin glucoronide; 3. procyanidin b2; 4. procyanidin c tetramer isomer 1; 5. procyanidin tetramer isomer 2; 6. (−)-epicatechin; 7. procyanidin c isomer 1; 8. procyanidin tetramer isomer 3; 9. procyanidin c isomer 2; 10. gallocatechin hexoside; 11. procyanidin B isomer 3

to see how the polyphenols present in seed sample derived from those present in kernel sample, in spite of both thermal treatment having reduced the polyphenols present in the kernel sample and the different contribution of shell and kernel in the seed weight (7:3, w:w).

In relation to the thermic treatment, it has to be considered that the population using the kernel as a diet component usually performs this traditional processing [9, 14]; therefore, the characterized bioactive molecules in the kernel sample represent those that are of real nutritional interest.

As for ascorbic acid content, flavonoids total content in pulp sample showed how *Adansonia digitata* fruit is very rich in bioactive molecules even compared to more commonly consumed fruits and vegetables [38, 39].

The presence of procyanidins in baobab fruit increases its nutritional value, because these molecules have been reported to exhibit several biological activities such as anticancer, anti-inflammatory, antimicrobial and antiviral among others [35]. Procyanidins are also present in cosmetics and pharmaceuticals containing plant materials [35], so the use of baobab pulp and seeds could be encouraged also in cosmetic and pharmaceutical industries.

It has been reported that vitamin C represents the main molecule responsible for the high antioxidant activity of the baobab pulp [9]. On the other hand, flavonoids are also known to confer high antioxidant activity in other fruits [40]. To verify the flavonoids contribution to the antioxidant capability of baobab fruit pulp, 2 g of pulp was subjected to preparative HPLC analysis to isolate the whole flavonoids fraction. This fraction, containing procyanidins (the polyphenols that characterize baobab pulp) was subjected to TEAC and DPPH spectrophotometric essays, at the same conditions used for baobab samples. As expected, flavonoids fraction showed a good antioxidant activity but lower than that evaluated from the whole baobab pulp. From the obtained results, it was clear that the total antioxidant activity of baobab pulp not only depends on the ascorbic acid content, but is also influenced by polyphenols content, underlining the relevance of molecular biodiversity for improving food nutritional value.

In conclusion, from data presented here, baobab pulp is confirmed to be a rich source of ascorbic acid, with a high antioxidant activity. To the best of our knowledge, we can assert that this work provides for the first time a qualitative and quantitative profile of polyphenols in baobab pulp, shell, kernel, and seeds. Data here reported showed that baobab fruits, their pulp in particular, are rich in procyanidins. Moreover, a HPLC preparative method to isolate procyanidin fraction from pulp was developed, revealing how the antioxidant activity of baobab pulp not only depended on the ascorbic acid content but also on the presence of procyanidins. Bioactive molecules in baobab pulp could be useful as nutraceuticals or as healthful fortifiers in functional foods.

An EFSA scientific opinion [41] suggests a 100 mg/day intake of polyphenols, while the range of RDA for vitamin C is between 40 and 100 mg/day depending on the countries [42]. From the results presented here baobab pulp can represent a rich source of both polyphenols (328 mg/100 g) and vitamin C (136 mg/100 g). However, the high fiber content of the fruit has to be taken into consideration[8]. Despite the traditional use of baobab leaves and fruit as anti-diarrhea/anti-dysentery [5], a Food and Drug Administration (FDA) panel indicates that excessive consumption (no quantities reported) of baobab fruit pulp could have laxative effects [11] and suggests its use as ingredient in food supplements rather than as food. A way to use this promising healthy food could be to add baobab fruit pulp to smoothies and fruit bars (6–15% by weight) [11]. Then an addition of 6% of baobab pulp to 100 g of a food supplement allows the 20% (19 mg) of the polyphenols daily intake.

Moreover, a better knowledge of the pulp nutritional value as well as of the putative contraindications following its consumption, could be very useful for suggesting strategies aimed to improving the dietary intake of micronutrients in those areas of developing countries still affected by malnutrition, where baobab spontaneously grow.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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