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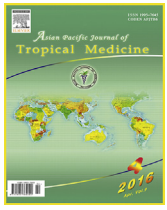

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Antioxidant potential, tannin and polyphenol contents of seed and pericarp of three *Coffea* species

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ABSTRACT

Objective: To investigate the antioxidant activity, total phenolic and total tannin content of the pericarp and the seed of *Coffea benghalensis* (*C. benghalensis)* and *Coffea liberica* compared to *Coffea arabica* (*C. arabica*).

Methods: The antioxidant potential, total tannin and polyphenol contents of the immature and mature seed and pericarp of *C. benghalensis* and *Coffea liberica* were quantified and compared to *C. arabica*. Enhanced chemiluminescence (ECL), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity, Folin-Ciocalteau method and total tannin content assays were used.

Results: Trolox equivalent (TE/g plant material) values obtained by ECL and DPPH methods showed loose correlation (r^2 = 0.587) while those measured by oxygen radical absorbance capacity assay were higher without correlation in each plant. A closer correlation was detected between the ECL method and the percentage antioxidant activity of the DPPH technique (r^2 = 0.610 7) in each species, however the immature pericarp of *C. benghalensis* showed much higher DPPH scavenging potential than was seen in the ECL assay. The immature pericarp of *C. benghalensis* expressed the highest tannin and polyphenol content, and a high polyphenol level was also detected in the immature seed of *C. arabica*. The immature pericarp of Bengal and Liberian coffees showed the largest amount of phenolic contents.

Conclusions: The obtained data highlight the potential role of *C. benghalensis* as a new source of natural antioxidants and polyphenols compared to *C. arabica*.

1. Introduction

Coffee (*Coffea*) species are evergreen shrubs or small trees which are native to the Ethiopian mountains [1,2]. They belong to the Rubiaceae family, which is the largest plant family in the world involving 450 genus and 6500 species [3,4]. Nowadays, more than 120 *Coffea* species and their varieties are mentioned in scientific reports [5,6]. They grow in the tropical and subtropical areas, especially in the Equatorial region at an altitude of (200–1 200) m and between (18–22) °C [7]. *Coffea arabica* (*C. arabica*) L., *Coffea robusta* (*C. robusta*) L. Linden, and *Coffea liberica* (*C. liberica*) Hiern have significant commercial value, occupying the second place after petrol on the international market [1,2]. Coffee is one of the most widely consumed beverages worldwide, with an annual consumption rate of approximately 7 million tons according to FAO [8].

C. arabica (Arabic coffee) originated from Ethiopia is the most widespread coffee species. It provides 80% of the coffee production of the world [9]. The wild species *Coffea benghalensis* (*C. benghalensis)* Roxb. (Bengal coffee), which has been reclassified into the *Psilanthus* genus *(Psilanthus benghalensis* Roxb. Ex Schult.), is a small shrub in South and

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Southeast Asia. Although, Bengal coffee is rarely used in the industry, the cafesterol and bengalensol content, as well as the antimicrobial and antioxidant effects of the fruit have been determined [10–12]. *C. liberica* (Liberian coffee) is also native to Africa, and it provides 2% of the total coffee production of the world. Despite its lower demand, the volatile extract of its immature beans possessed higher antioxidant capacity than that of *C. arabica* and *C. robusta* [13].

In oxidative stress, reactive oxygen species have been suggested to participate in the initiation and propagation of chronic diseases such as cardiovascular and inflammatory diseases, cancer, and diabetes [14]. Antioxidants which are found naturally in many foods and beverages provide health benefits in preventing heart disease and cancer by fighting against cellular damage caused by free radicals in the body [15]. Coffee species are rich in biologically active substances and polyphenols such as chlorogenic acid, ferulic acid, caffeic acid, sinapic acid, kaempherol, quercetin, nicotinic acid, trigonelline, quinolic acid, tannic acid, pyrogallic acid and caffeine which possess antioxidant, hepatoprotective, antibacterial, antiviral, anti-inflammatory and hypolipidemic effects [16–24]. These compounds play an important role against pathogens and abiotic stress such as changes in temperature, water content, exposure to UV light levels and deficiency in mineral nutrients [25]. In coffee species, chlorogenic acid content and its *cis*-isomers have been determined to be higher in the leaves than the seeds which prove the effect of UV radiation on the geometric isomerisation of chlorogenic acid in the leaves [26]. The local use of coffee extracts could prevent various dermatological disorders, in addition, they also could have UV protection for skin. During a clinical study 30 patients having dermatological face problems were locally treated with coffee seed extract. In comparison with the standard creams with placebo effect, coffee extract reduced wrinkles and pigmentation, as well as it improved the appearance of patients' skin [27]. The silverskin of *C. arabica* and *C. robusta* have antioxidant activity [28], while the extract of the green seed has an anti-inflammatory effect [29]. The regular consumption of coffee reduce the kidney, liver, premenopausal breast and colon cancer [30].

Although more than 100 *Coffea* species are known nowadays, only a few taxa have been extensively analyzed at present. Based on the widely used enhanced chemiluminescence (ECL), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) assays, total phenolic, and tannin methods [31], the aim of this study was to investigate the antioxidant activity, total phenolic and total tannin content of the pericarp and seed of *C. benghalensis* and *C. liberica* compared to the thoroughly studied *C. arabica*. The analyses were carried out to find new sources of natural antioxidants for nutraceuticals, and a new utilization of wasted residues of coffee products.

2. Material and methods

2.1. Plant materials

The mature and immature fruits of *C. benghalensis*, *C. liberica*, and *C. arabica* were collected in the Botanical Garden of the University of Pécs in the spring of 2014. The samples were air-dried at room temperature in the shade. Voucher specimens were deposited and labeled with unique codes at the Institute of Pharmacognosy, University of Pécs. For the antioxidant assays, samples were ground (0.25 g each) and extracted with 5 mL 50% ethanol (Merck). The extracts were shaken for 20 min (Edmund Bühler, Labortechnik-Materialtech, Johanno Otto GmbH), then filtered and stored at 4 °C in the dark until analyses (less than 7 d).

2.2. Chemicals and reagents

All chemicals, used for antioxidant assays, were of analytical or spectroscopic grade purity and highly purified water (<1 μS) was used in our experiments. Horseradish peroxidase (POD from Sigma-Aldrich), 1 mg/mL bovine serum albumin (BSA, Serva) in 50 mM phosphate buffer pH 7.4, H_2O_2 (Molar Chemicals) diluted with citric acid (Ph. Hg. Eur), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), luminol, para-iodophenol, diphenyl-2,2-picryl-hydrazyl (DPPH stable free radical), fluorescein-Na_2 salt, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH, all from Sigma-Aldrich), methanol and ethanol (Reanal, Hungary) were used as received. In the ORAC assay 75 mM phosphate buffer of pH 7.4 was applied.

Chemicals used for Folin-Ciocalteau methodology and for measurement of total tannin were the followings: $AlCl_3$ (Alfa Aesar), acetone, 25% HCl, ethyl acetate, 5% methanol-acetic acid (Molar Chemicals), distilled water, solution of sodium carbonate (Lach-Ner), phosphor-molybdo-tungstic reagent (Sigma-Aldrich), holystone (Reanal), hide powder, hexamethylene tetramine, and pyrogallol (VWR).

2.3. DPPH assay

Four mg DPPH in 100 mL methanol (0.1 mmol/L) was prepared and kept in the fridge being stable for at least 1 wk. For absorbance measurements standard 96-well microplates (Sarstedt) were applied. Twenty μL Trolox/blank/sample and 180 μL DPPH solution were pipetted into the wells (using a multichannel pipette), mixed and the absorbance was read at 517 nm after 30 min incubation in the dark at 25 °C [32–34].

2.4. ECL

We adapted and modified the method of Muller *et al* [35] as follows.

Reagents: Before the analysis 15 μU/mL POD working solution was freshly prepared from 1.5 U/mL POD stock stored at −20 °C in phosphate buffered saline (PBS, pH 7.4) by dilution with the BSA containing phosphate buffer and was kept on ice. A working reagent of 1360 μM H_2O_2 was also freshly diluted with 0.1% citric acid from 10 M concentrated stock solution and was also kept on ice, protected from light. During the whole period of measurements these reagents were stored in melting ice. Both working solutions were stable for at least several hours.

The chemiluminescence detection reagent was prepared separately by dissolving luminol and p-iodophenol in 0.2 M boric acid/NaOH buffer, pH 9.6 and was refrigerated at 4 °C in brown bottles with a shelf life of several weeks. Trolox was used as standard in both assays. Trolox at 1 mM concentration was dissolved freshly in 50% ethanol weekly and kept at 4 °C.

Depending on the assay, Trolox dilutions in the range of (0–100) μM were prepared on the day of the experiments with the same diluents that were applied for the samples.

ECL antioxidant method: The chemiluminescence reaction was performed in 96-well white optical plates (Perkin-Elmer). The enzyme working solution and the ECL reagent was pre-mixed (200 μL POD+70 μL ECL reagent) and kept on ice. The wells were filled with 20 μL Trolox/blank/sample and 270 μL of POD-ECL reagent was pipetted into each well with an 8-channel micropipette. The reaction was initiated by automated injection of 20 μL ice-cold H_2O_2 in citric acid (final concentrations of the components in the wells: 0.97 μU/mL POD, 101.6 μM luminol, 406.4 μM p-iodophenol, 88 μM H_2O_2). The chemiluminescence signal was followed for 20 min.

2.5. ORAC assay

Four μM fluorescein (FL) stock was prepared in 75 mM phosphate buffer of pH 7.4 (stable for 1 wk in the fridge). The working FL solution was made freshly diluting the stock with phosphate buffer at a 1:99 ratio (40 nmol/L). AAPH was also prepared just before the measurements in the phosphate buffer (400 mM). Trolox standard was used as described above. Into each well of black optical plates (Perkin Elmer) 25 μL of blank/standard/sample and 150 μL of diluted FL were pipetted and the plates were preheated to 37 °C for 20 min. The outer wells of the plates were filled with 200 μL phosphate buffer, and only the inner 6×10 matrix was used for the assay. The reaction was initiated by automated injection of 25 μL AAPH solution into each well and fluorescence intensities were immediately monitored for 80 min (490/520 nm) at 150 s intervals. The final concentrations of the components in the wells were as follows: FL 30 nM, AAPH 50 mM, Trolox (0–33.3) μM [36,37].

2.6. Folin-Ciocalteau methodology

The total phenolic concentration of the pericarp and seed was measured using the Folin-Ciocalteu method in each plant. 0.5 g powdered samples were mixed with 1 mL of 0.5% hexamethylene tetramine, 20 mL acetone, 2 mL of 25% HCl, and holystone. The mixtures were stored on reflow refrigerator for 30 min, and shaken with distilled water and ethyl acetate in shaking funnel. The extracts were used in two solutions, separately. The first part of the extracts were mixed with 1 mL $AlCl_3$ and 5% (v/v) methanol-acetic acid for further measurement, while the second parts were mixed with 5% (v/v) methanol – acetic acid producing the standard solution. After 30 min incubation, the absorbance of both samples was measured at 425 nm (A). The total phenolic concentration was calculated with the following formula: (1.25 × A)/m, where m = mass of the sample in grams. Each analysis was performed in duplicate following the procedure described in the 7th European Pharmacopoeia [38].

2.7. Total tannin content

Powdered immature and mature pericarp of 0.5 g and seed of the selected 3 species were mixed with 150 mL distilled water, and then heated on water-bath for 30 min at 70 °C. The cooled extracts were transferred quantitatively to a 250 mL volumetric flask, then filtrated and used for the reactions.

2.7.1. Total polyphenols

The filtrate with 5.0 mL was diluted to 25.0 mL with distilled water. The solution with 2.0 mL was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL of distilled water, and then it was diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (A_1) against distilled water.

2.7.2. Polyphenols not adsorbed by hide powder

The filtrate with 10.0 mL was mixed with 0.10 g of hide powder, and then shaken for 60 min. The filtrate with 5.0 mL was diluted to 25.0 mL with distilled water, then 2.0 mL of this solution was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL of distilled water. Then the mixture was diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (A_2) against distilled water.

2.7.3. Pyrogallol standard solution for polyphenol content

Pyrogallol with 50.0 mg was dissolved in distilled water and diluted to 100.0 mL with the same solvent. The solution with 5.0 mL was diluted to 100.0 mL with distilled water, and then 2.0 mL of this solution was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL water. This mixture was diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (A_3) against distilled water.

Each analysis was performed in duplicate. Polyphenol contents were calculated with the following formulas [38]:

Polyphenols not adsorbed by hide powder :

$$[62.5\times(A_1-A_2)\times m_2]/A_3\times m_1$$

m_1 = mass of the sample to be examined in grams
m_2 = mass of pyrogallol in grams

Total polyphenols :

$$[62.5\times A_1\times m_2]/A_3\times m_1$$

m_1 = mass of the sample to be examined in grams
m_2 = mass of pyrogallol in grams

2.8. Instrumentation and interpretation of data

For the ECL based measurements a Biotek Synergy HT plate reader equipped with programmable injectors was used. After initiation of the reaction by injection of H_2O_2, light detection was immediately begun with 0.2 s measuring time/well for 20 min at 64 s measuring intervals. Trolox standards in 50% ethanol were applied in the range of (0–15) μM final concentrations in the wells and a 32-fold dilution with 50% ethanol of the plant extracts were used for the measurements (n = 12 replicates for each sample). The total antioxidant capacity (TAC) of the extracts was calculated from the regression equation obtained for the standards, multiplied by the dilution factor and expressed as μM Trolox equivalent (TE). TE for each plant extract was referred to 1 g of initial dry material.

For the DPPH assay a Perkin Elmer EnSpire Multimode reader was used in absorbance mode, equipped with monochromators. Standardization of the assay was done by application of (0–25) μM Trolox/well final concentrations in 50% ethanol and absorbance values were read at 517 nm after 30 min of incubation at 25 °C (with 5 s shaking before the measurement). Antioxidant capacities were calculated either by using the equation of the calibration line or by expressing the antioxidant activity of the extracts in % of the blank using the formula: $(A_{blank} - A_{sample}/A_{blank}) \times 100$ [39]. TAC values were also referred to 1 g of dried plant and were given as TE/g or % TAC/g.

For the ORAC assay the Biotek Synergy HT plate reader was used in fluorescence mode at 37 °C with 490 nm excitation and 520 nm emission filter settings. After 20 min incubation of the plate containing blanks/standards/samples and FL at 37 °C the AAPH start reagent was automatically injected into the wells and readings were taken in every 150 s for 80 min with 100 intensity readings/well at each measuring point. TE was calculated by subtraction of the fluorescence intensities of the corresponding blank values from those of the Trolox standards (net area under curve) and in this way a calibration line was obtained based on net area under curve *vs.* Trolox concentrations. TE data for the examined plants were obtained from the regression equation of the standards and were also referred to 1 g of dry plant.

3. Results

3.1. Antioxidant activity tests (DPPH, ECL and ORAC)

We could quantify the antioxidant activity by both three methods of all tested plant extracts. The ECL and DPPH TE/g values showed loose correlation (r^2 = 0.587, P = 0.083 by Student's t-probe) while those obtained for the ORAC assay were considerably higher with a more uniform pattern and without correlation with the other two assays' data (Table 1).

The imprecision of the three assays was acceptable (ECL: ≤5%, DPPH: ≤10%, ORAC: ≤2%). The DPPH data were also calculated as % TAC using the equation described in 2.8. Our results showed closer correlation between the ECL method and the percentage antioxidant capacity obtained by the DPPH technique (r^2 = 0.610 7, P = 0.161 by Student's t-probe). The biggest difference was seen for the immature pericarp of *C. benghalensis* and for the mature pericarp of *C. liberica* where the DPPH method showed much higher antioxidant capacity than the ECL assay (Figure 1 and Figure 2). In our experiments, the ORAC technique showed the highest values which did not

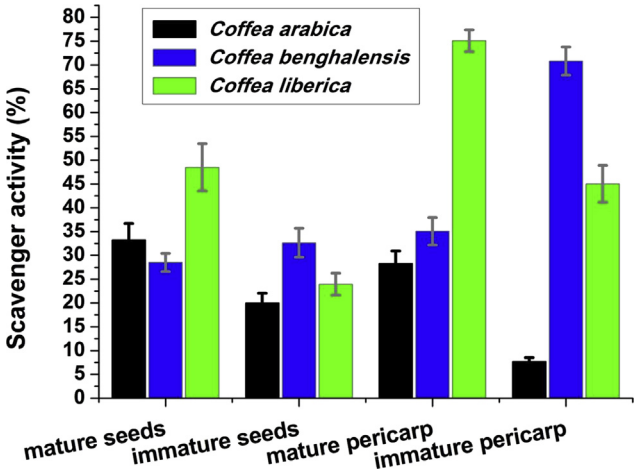

Figure 1. Scavenger activity of the studied *Coffea* species measured by DPPH method.

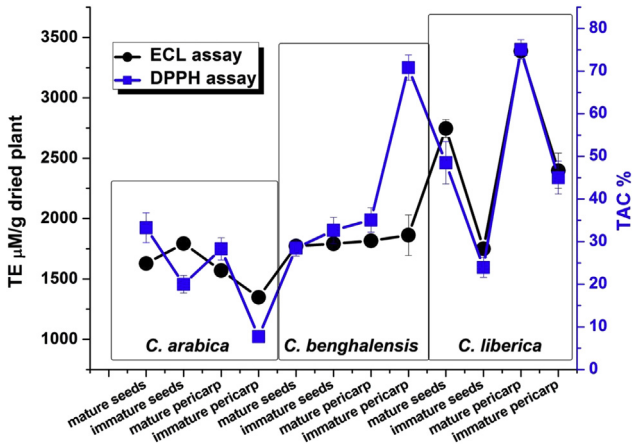

Figure 2. Comparison of the ECL assay with the DPPH method. Data obtained from the coffee plant extracts are expressed in TE/g dried plant (ECL method) and in TAC % (DPPH method).

Table 1
Total antioxidant capacity of *Coffea* species measured by three different spectroscopic methods (mean ± SD).

Investigated species		DPPH assay	Chemiluminescence assay	ORAC assay
		TE (μmol/g dried plant)	TE (μmol/g dried plant)	TE (μmol/g dried plant)
C. arabica	Mature seed	1627.089 ± 158.675	1628.254 ± 20.700	5139.01 ± 16.01
	Immature seed	1019.291 ± 94.423	1793.257 ± 1.332	5914.72 ± 78.76
	Mature pericarp	1429.857 ± 119.541	1570.281 ± 28.951	4816.47 ± 66.83
	Immature pericarp	1205.598 ± 73.270	1347.583 ± 35.621	4327.14 ± 39.41
C. benghalensis	Mature seed	1691.492 ± 153.326	1773.039 ± 34.323	5640.41 ± 68.91
	Immature seed	1598.913 ± 139.436	1791.784 ± 1.662	5608.26 ± 23.26
	Mature pericarp	1702.417 ± 146.458	1815.860 ± 28.173	5501.75 ± 51.16
	Immature pericarp	3132.134 ± 121.553	1862.025 ± 166.608	5558.02 ± 35.86
C. liberica	Mature seed	2212.817 ± 204.156	2745.598 ± 74.097	5872.52 ± 52.23
	Immature seed	1200.423 ± 106.076	1750.142 ± 9.326	5415.33 ± 15.04
	Mature pericarp	3307.812 ± 93.589	3386.733 ± 40.773	2740.56 ± 38.68
	Immature pericarp	2070.049 ± 159.152	2396.324 ± 145.552	5721.80 ± 8.53

TE: Trolox equivalent in μM, data are referred to 1 g of dried plant material. n = 12 for the ECL and DPPH methods, respectively while in the ORAC assay measurements were performed in triplicates.

Table 2
Total tannin, polyphenol and phenolic content of *Coffea* species measured by different spectroscopic methods.

Investigated plants and their parts		Total tannin content (%)	Total polyphenol content (%)	Total phenolic content (%)
C. arabica	Mature seed	0.558	2.123	0.005
	Immature seed	1.084	4.146	0.012
	Mature pericarp	0.793	1.687	0.022
	Immature pericarp	0.838	1.634	0.022
C. benghalensis	Mature seed	0.447	3.285	0.009
	Immature seed	0.581	2.503	0.002
	Mature pericarp	0.745	2.235	0.014
	Immature pericarp	1.464	3.677	0.148
C. liberica	Mature seed	0.402	3.129	0.003
	Immature seed	1.164	3.120	0.015
	Mature pericarp	1.134	0.938	0.010
	Immature pericarp	0.344	1.863	0.142

correlate with the results of the other two assays. In contrast to the data of Kiran, Baruah, Ojha, Lalitha & Raveesha, 2011 the antioxidant activity of mature fruit extracts of *C. benghalensis* (DPPH method) we found lower than theirs however, that of immature pericarp was higher in our study.

Although *C. liberica* is less used commercially, the antioxidant effect of its green seeds is comparable to that of *C. arabica* and *C. robusta* [40].

The ORAC and the ECL assays belong to the hydrogen atom transfer (HAT) mechanism group while the DPPH technique is considered to be based on single electron transfer [41]. Both techniques are considered to characterize the non-enzymatic total antioxidant capacity of the plant extracts. The ECL method applying phenolic compound as enhancer proved to be more sensitive than the ORAC assay however, the measuring range and precision of the ORAC method were more favorable. In the ECL technique the phenolic enhancer compound itself is also involved in the reaction with POD intermediates accelerating the reaction by increasing the turnover rate of the enzyme. Apart from POD-phenolic interaction the resulting phenoxyl radicals can directly oxidize luminal [42]. It is uncertain yet why the TE/g values obtained by the ORAC HAT assay were considerably higher than those seen for the other two methods. It might be postulated that in the ORAC microenvironment more antioxidant compounds could react with the AAPH oxidant than in the DPPH (single electron transfer) and ECL (HAT) type assays. In order to explain the differences between the various methods we plan to separate active compound of the *Coffea* species and measure their antioxidant capacity separately by both techniques.

3.2. Folin-Ciocalteau method and total tannin content

Total tannin and polyphenol content of the selected *Coffea* species were measured by different spectroscopic methods (Table 2). The highest tannin content was found in the immature pericarp of Bengal coffee followed by the immature seed of Liberian and Arabic coffee. The least tannin content was detected in the mature seed extract of each species. The highest polyphenol content was measured in the immature seed of *C. arabica* and in the immature pericarp of *C. benghalensis* while the least content was observed in the mature pericarp of *C. liberica*. In addition, a high polyphenol concentration value was detected in the mature seed of all three species. High phenolic content was measured in the immature pericarp extracts of *C. benghalensis* and *C. liberica*. The other plant extracts contained low phenolic concentrations.

In comparison with other studies the antioxidant activity and total polyphenol content of green coffee extracts of *C. arabica* were higher, when the extraction was made with isopropanol and water (60:40), than seen in our study. These differences could be explained by the different extraction methods used [20].

4. Discussion

Among the used antioxidant assays, the measured ECL and DPPH values indicated a loose correlation in contrast with the data of the ORAC assay, while a closer correlation was observed between the ECL technique and the expressed antioxidant potential studied by the DPPH method in each coffee species. The much higher antioxidant activities measured by the ORAC assay might reflect the differences in the reactive antioxidant compounds among the assays and/or the altered reactivity with the reporter molecules. The immature pericarp of Bengal and Liberian coffee produced a high phenolic content, and in comparison, the immature pericarp of Bengal coffee showed the most significant tannin and polyphenol content similarly to the high polyphenol content of the immature seed of Arabic coffee. These data highlight the potential role of Bengal coffee as a new source for natural antioxidants and polyphenols compared to the Arabic coffee.

Conflict of interest statement

The authors declare that there was no conflict of interest.

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