Effect of controlled differential sieving processing on micronutrient contents and in vivo antioxidant activities of *Hibiscus sabdariffa* L. calyces powder

Markusse Deli¹ · Richard Marcel Nguimbou¹ · Elie Njantou Baudelaire^{1,2} · Nicolas Njintang Yanou^{1,3} · Joël Scher² · Carl Moses Mbofung¹

Received: 9 January 2020 / Revised: 24 August 2020 / Accepted: 9 September 2020 / Published online: 6 October 2020
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Abstract Sun-dried calyces of *Hibiscus sabdariffa* were finely grinded and fractionated by controlled differential sieving processing (CDSp) into four granulometric classes. The obtained powder fractions were examined for their micronutrient contents and in vivo antioxidant properties in high-fat diet induced oxidation model rats. Unsieved powder and lyophilized ethanolic extract were used for comparison. Micronutrient contents and antioxidant properties were significantly influenced ($p < 0.05$) by particle sizes. The mineral, vitamin, carotenoid and anthocyanin contents were shown to be higher in smaller powder particles. Comparatively, the ethanolic extract powder had the higher carotenoid, vitamin and anthocyanin contents while

the superfine powders $< 180 \mu\text{m}$ and $180\text{--}212 \mu\text{m}$ had the higher mineral contents and antioxidant properties which was characterized by high superoxide dismutase, catalase activities and low malondialdehyde production. Plant grinding followed by CDSp may be an option to improve micronutrient content and antioxidant activity of plant powders.

Keywords *Hibiscus sabdariffa* calyx · Powder fraction · Ethanolic extract · Micronutrient · Antioxidant activity

Introduction

Hibiscus sabdariffa L. is an herbaceous plant of the family of Malvaceae widely distributed in tropical and subtropical areas (Sinela et al., 2017). It is extremely attractive in various parts of Africa such as Cameroon from a food usages and medicinal point of view for its calyces, leaves and seeds. Specifically, its calyces representing the most used part of hibiscus plant, which used as food coloring. It is also used in preparation of local soft drink known as “foléré” in the northern regions of Cameroon. Previous phytochemical researches on *H. sabdariffa* calyces revealed the existence of macronutrients such as proteins, fats, carbohydrates and fiber (Deli et al., 2019a; Deli et al., 2019b). They have also been reported to be a good source of micronutrients, such as minerals, vitamins, as well as a whole series of compounds called the nonnutritive phyto-constituents especially polyphenols and food grade pigments (anthocyanins and carotenoids). All of these compounds play a vital role in health enhancement or an important role in health by functioning as natural antioxidants (Deli et al., 2019a; Jabeur et al., 2017). Plant antioxidant compounds have various mechanisms such as

✉ Richard Marcel Nguimbou
rm.nguimbou@univ-ndere.cm

Markusse Deli
markusdeli@yahoo.fr

Elie Njantou Baudelaire
elie.baudelaire@agritech-france.fr

Nicolas Njintang Yanou
njintang@yahoo.fr

Joël Scher
joel.scher@univ-lorraine.fr

Carl Moses Mbofung
cmbofung@yahoo.com

¹ Department of Food Sciences and Nutrition, ENSAI, University of Ngaoundere, PO Box 455, Ngaoundere, Cameroon

² Laboratoire d'Ingénierie des Biomolécules (LIBio), Université de Lorraine, 2, Avenue de la Forêt de Haye, B.P. 172, 54500 Vandœuvre-lès-Nancy, France

³ Department of Biological Sciences, Faculty of Sciences, University of Ngaoundere, PO Box 454, Ngaoundere, Cameroon

prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and a free radical scavenging capacity, which, among other biological effects, increases antioxidant activity and prevents cellular oxidation (Rao et al., 2004).

Generally, extraction is the most important and starting step in isolating different types of bioactive compounds from plant materials. However, an effective extraction and proper assessment of antioxidants from food and plant waste are crucial to explore the potential antioxidant sources and promote the application in functional foods, pharmaceuticals and food additives. To date, the development of a single standard method for efficient and rapid extraction of bioactive compounds from plant matrices has remained a challenge due to the inherent limitations of conventional extraction methods using solvents. Lately, many research studies concentrated on innovative technologies that promise to overcome such issues as time consuming, thermal decomposition of thermolabile compounds, high consumption of harmful chemicals, inefficiency, and low yield, which exist in most conventional techniques. Therefore, alternative drying and grinding (ADG), and controlled differential sieving process (CDSp) has been acknowledged as the most promising ones which have attracted much attention in food industries, and become the focus of research in many laboratories. Scientific communities and various publications have documented on ADG followed by CDSp and in a bid to validate claims of their interest or efficiency to concentrate a great variety of valuable compounds, including phenolic bioactive compounds and also improves the bioactivities of various experimental plant matrix (Baudelaire 2013; Becker et al., 2016; Becker et al., 2017; Deli et al., 2019a; Deli et al., 2019b; Zaiter et al., 2016). Due to the reduction in particle size of plant matrix to an appropriate, significantly enhance the physico-chemical and functional characteristics of food materials, resulting in the improvement of food powder quality (Chen et al., 2015; Jiang et al., 2017). In addition, ADG followed by CDSp as new and advanced technology deliver much higher process efficiency and extraction yield.

The present study aims at evaluating antioxidant activity related to the powder particle size generated by fine grinding of *H. sabdariffa* L. calyxes, followed by sieving into four granulometric classes. In this purpose, micronutrient contents, as well as in vivo antioxidant properties by measurement the levels of malondialdehyde, superoxide dismutase activity and catalase activity were determined. The contents in micronutrients compounds and in vivo antioxidant properties of all powder fractions were compared to those of lyophilized ethanolic extract to assess the

efficiency of the successive plant grinding follow by controlled differential sieving process.

Materials and methods

Procurement of reagents, standards and plant material

All chemicals and solvents were of the highest analytical grade and they were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). The dried calyxes of *H. sabdariffa* were used as plant material investigated in the present study. This plant material was purchased from the local market in the Adamaoua region of Cameroon. They were hand cleaned of foreign bodies (inorganic materials, dirt and dust particles) before been taken into grinding for powders production.

Grinding of dried calyxes and sieving of powder

CDSp procedures followed to obtain powder fractions was the same as reported in our previous studies (Deli et al., 2019a; Deli et al., 2019b). The CDSp is based on the separation of particles from a granular material by making them pass through several sieves of decreasing mesh size (315, 212, and 180 μm in the current study). Basically, 100 g ground plant sample is sieved in permanent vibratory mode at 0.5 mm amplitude for 10 min. The fraction of the powder retained on each sieve is recovered and weighed for the calculation of the mass fraction of each granulometric fraction. In this study, an electric Ultra-Centrifugal Mill ZM 200 (Haan, Germany) supplied with 24-tooth rotor of 99 mm and trapezoid holes a mesh sieve of 1 mm and operated at 12,000 rpm was used to make whole powder of dried calyxes. Then ground material was sieved with the Analysette 3 Spartan apparatus (Fritsch, Idar-Oberstein, Germany), operating by vertical vibration. The following powder fractions were obtained: < 180 μm , 180–212 μm , 212–315 μm , and > 315 μm , with a moisture content of about 10–12%. Non fractioned (unsieved) powder was taken as control, and the powdered samples were stored at 10 °C in polyethylene bags and placed at room temperature (25 ± 2 °C) until they were used.

Preparation of ethanolic extract powder

To prepare the ethanolic extract, hibiscus powder was mixed with ethanol in the ratio of 1/10 (w/v) and stand under stirring (Variomag Poly) for 24 h at 18 °C to improve the extraction. Thereafter, the homogenate obtained was then filtrated with Whatman No 1 filter paper (pore size 12–15 μm) and ethanol was removed using a

rotary evaporator (BUCHI - R210/215) at 40 °C and under reduced pressure of 17,500 Pa. Finally, the frozen extract (− 18 °C) was put into the freeze dryer chamber for 48 h and under pressure of 10 Pa. The temperature of freeze dryer was at − 60 °C. Collected powder extract was conditioned in polyethylene bags and stored at 10 °C until analysis.

Micronutrient analysis

Determination of mineral content

Dietary minerals (K, Ca, Mg, Na, Zn, Cu and Fe) concentrations in powder fractions and ethanolic extract of *H. sabdariffa* were analysed on atomic absorption spectrometer (Thermo Scientific, ICE 3000, version 2.0, Merckers Row, Cambridge, UK) (Oshodi, 1992). The powder samples were early incinerated in a muffle furnace at 550 °C, and obtained ashes were dissolved in 10% HCl solution prepare in deionized water. Calcium and magnesium extraction were done in 10 mL of lanthanum chloride solution. The solutions were filtrated on Whatman No 1 filter paper. Parameters of the instrument were chosen in accordance with the manufacturer's instructions. Then, standard solutions at various concentrations were injected to calibrate the atomic absorption spectrometer using acetylene gas. Finally, an aliquot of sample solutions was injected, and the mineral contents were calculated from standard curves and the results were expressed as mg/100 g dry weight.

Determination of vitamin content

Sample extracts were prepared and vitamins determination was performed using high-performance liquid chromatography (HPLC) method as previously described by Kucukkolbasi et al. (2013). 10 mL of mixed solvents of ratio 80/20 (v/v) (0.01% trichloroacetic acid prepared in ultrapure water and 100% methanol) were added to a 0.1 g of powder sample. Then, the mixture was stand under magnetic stirrer for 30 min and not exposed to bright light. Obtained solution was centrifuged at $603 \times g$ for 15 min at 4 °C and filtered through a nylon syringe filter (pore size 0.45 µm). Vitamin standards were dissolved in ultrapure water and methanol, respectively for water-soluble vitamins (thiamin, riboflavin, ascorbic acid, folic acid and p-aminobenzoic acid) and fat-soluble vitamins (retinol acetate and tocopherol). Then, mixed solutions of vitamin standards at varied concentrations were prepared in water-methanol mixture (50/50, v/v). For the quantification of vitamins, an HPLC equipped with a water pump, an automatic injector, thermostated column compartment at

30 °C, UV/photodiode array detector and a C18 column (150 mm × 2.1 mm, 5 µm; Alltima TM, Hichrom, serial number: 212120004) was used. Elution was performed using 0.01% trichloroacetic acid of pH 4 (mobile phase A) and 100% methanol of grade HPLC (mobile phase B), and 2 mL/min flow rate with a gradient run. Five microliters of sample solution or vitamin standard were injected into a column. The HPLC peaks of vitamins were identified by comparison with retention times with those of corresponding standards. The concentration of vitamins in the samples was determined from the calibration curves by plotting the peak areas of the vitamins identified against the concentration of the standards. The vitamin content was expressed as µg/100 g dry weight.

Determination of total carotenoid content

The total carotenoid content was determined according to the method of Rodriguez-Amaya (1999). For carotenoids extraction, 25 mL acetone and 75 mg of magnesium carbonate was added successively into the tube previously containing 2.5 g of hibiscus powder. Then, the mixture was stirred at 300 rpm (Variomag Poly) for 10 min at 18 ± 2 °C. The solution was then centrifuged (Thermo Scientific, Heraeus Megafuge 8R Centrifuge, Osterode am Harz, Germany) at $603 \times g$ for 10 min and at 10 °C. Organic phase was carefully removed by inverted tubes and extraction was repeated on retained residue with 5 mL of acetone and 5 mL of petroleum ether, followed by centrifugation. This procedure was repeated three times until the sample became colorless. The collected organic phases were combined and transferred into a 500 mL separation funnel containing 20 mL of petroleum ether. Acetone was removed through the moderate addition of 150 mL distilled water to prevent emulsion formation and the aqueous phase was discarded. The obtained extract was transferred through a funnel containing 15 g of anhydrous sodium sulfate and made up a volume of 25 mL with petroleum ether. The absorbance was measured at 450 nm using UV-spectrophotometer (Shimadzu UV-VIS 1605, Tokyo, Japan). The concentration of total carotenoid expressed in microgram per 100 g of dry weight were calculated using follow formula:

$$\text{Total carotenoid content}(\mu\text{g}/100 \text{ g DW}) = \frac{(\text{Abs} \times V \times 1000)}{A_{1\text{cm}}^{1\%} \times m} \times 100$$

where *Abs* absorbance at 450 nm, *V* sample's final volume (mL), *m* sample mass (g), and $A_{1\text{cm}}^{1\%} = 2592$ (β-carotene extinction coefficient in petroleum ether).

Determination of total anthocyanin content

Extraction of anthocyanins was performed according to the Tony et al. (1994) method. One gram of powder sample was added to 10 mL of solvent mixtures (0.1 M ethanol/HCl, 85/15, (v/v)). Then, mixture was stirred at 1.5 g (Variomag Poly Stirrer) for 2 h, and centrifuged (Thermo Scientific, Heraeus Megafuge 8R Centrifuge, Osterode am Harz, Germany) at $603 \times g$ for 10 min at 4°C . Supernatant was recovered for determination of total anthocyanin content using the pH-differential method (Giusti and Wrolstad, 2001). In this respect, an aliquot (100 μL) of appropriately diluted samples were introduced into the tubes containing respectively 4900 μL of 0.025 M potassium chloride buffer at pH 1.0 and 4900 μL of 0.4 M sodium acetate buffer at pH 4.5. The mixtures were stirred and incubated for 15 min at room temperature ($25 \pm 2^\circ\text{C}$). Absorbance was measured both at 520 and 700 nm using a UV–Vis spectrophotometer against a blank containing distilled water. Absorbance was calculated as the follow formula:

$$\Delta\text{Abs} = (\text{Abs}_{520\text{ nm}} - \text{Abs}_{700\text{ nm}})_{\text{pH } 1.0} - (\text{Abs}_{520\text{ nm}} - \text{Abs}_{700\text{ nm}})_{\text{pH } 4.5}$$

Total anthocyanin content was expressed as microgram of cyanidin 3-glucoside equivalents (CGE) per 100 g dry weight of sample was calculated as follow:

$$\text{Total anthocyanins}(\mu\text{g CGE}/100\text{ g DW}) = \frac{(\Delta\text{Abs} \times \text{MW} \times F \times 1000 \times 100)}{\varepsilon \times \lambda \times m \times \text{DW}}$$

where ΔAbs variation of absorbance, Abs absorbance, MW molecular weight for cyanidin 3-glucoside (449.2 g/mol), F dilution factor (–), ε molar extinction coefficient (29,600 L/mol/cm), λ path length (1 cm) and m mass of test powder sample (g), DW dry weight.

Protective effects of *H. sabdariffa* powders against oxidative damage

Animal experiments Three months old Wistar rats (*Rattus norvegicus*) weighting 200–300 g were used, and they were raised at the animal house of Laboratory of Biophysics, Food Biochemistry and Nutrition of the National School of Agro-Industrial Sciences, Ngaoundere University of Cameroon. The rats were maintained at room temperature ($25 \pm 2^\circ\text{C}$) under the lab conditions and were provided food and water ad libitum. Thus, there was free space for rats to get water, and formulated normal and high-fat diets (Hamlat et al., 2008; Ngatchic et al., 2013). The animals were randomly organised in group of 5 rats per cage. In total, 9 groups were formed that are 1 normal

control group, 1 negative control group, 1 positive control group, and 6 experimental groups (receiving powder fractions of $< 180\text{ }\mu\text{m}$, $180\text{--}212\text{ }\mu\text{m}$, $212\text{--}315\text{ }\mu\text{m}$, and $> 315\text{ }\mu\text{m}$, unsieved powder and ethanolic extract powder). The normal control group was submitted to normal food regime while the others received high-fat diet. Before foods being given to rats, they were administered per os 1 h before 10 mL of aqueous suspension of *H. sabdariffa* powder (for the experimental groups), vitamin C 1 mg/mL (for the positive control group), and distilled water (for the negative controlled group). *Hibiscus sabdariffa* powders were administered at the dose of 250 mg/kg body weight, while vitamin C was administered at the dose of 20 mg/kg body weight. The experiment last 28 days after which the rats were weighed before used for the antioxidant analyses.

Preparation of liver homogenate and blood plasma

At the end of the experience period (28 days), the rats were fasted overnight and ether anesthetized. After cardiac puncture, 1–2 mL of blood sample were collected and this blood was centrifuged at $603 \times g$ for 10 min and at 4°C to obtain the serum which was kept frozen at -4°C . After that animals had dissected, and liver was cutted from three rats in each group. Liver homogenate was prepared in phosphate buffer (0.1 M pH 7.4 having 0.15 M KCl) and kept frozen at -4°C until used to determination of oxidative stress markers. Based on the protein content determined according to the method of Lowry et al. (1951), malondialdehyde (MDA) content ($\mu\text{mol}/\text{mg}$ protein), catalase (CAT) activity (units/mg protein) and superoxide dismutase (SOD) activity (units/mg protein) were determined in the organ homogenates and blood plasma as recently reported (Deli et al., 2020). This study was carried out with approval from the Cameroonian National Ethics Committee Ref. No FWIRD00001954.

Lipid peroxidation assay

Lipid peroxidation was measured spectrophotometrically according to the method proposed by Yagi (1976) with modifications. The antioxidant capacity determined by this method is inversely correlated with the accumulation of lipid-derived conjugated dienes. This method depends on the formation of MDA as an end product of lipid peroxidation which reacts with thiobarbituric acid (TBA) producing thiobarbituric acid reactive substance, a pink chromogen, which can be measured spectrophotometrically at 532 nm. The assay mixture contained 100 μL of the sample, 400 μL of TBA reagent and 80 μL of HCl was thoroughly stirred and incubated in a boiling water bath for 15 min. After that, the mixture was cooled in a cold-water bath for 30 min, and mixture was centrifuged at $603 \times g$

for 15 min. The absorbance was measured on a UV/visible spectrophotometer. The results were expressed in terms of the MDA content in μmol per milligram of protein using molar extinction coefficient of MDA ($\epsilon = 1.56 \times 10^5$ - L/mol/cm).

Measurement of SOD activity

SOD activity was determined according to the method of Beauchamp and Fridovich (1971). Briefly, 2.5 mL of 0.1 M carbonate buffer solution at pH 10.2 was added to 0.2 mL of the sample. Then, 0.3 mL of aqueous solution of adrenalin ($5 \mu\text{g/mL}$) was added to the mixture to trigger the reaction and whole was vortexed. Absorbance was measured every 30 s until 150 s in order to follow an increase of absorbance at 480 nm. Three hundred microliters of distilled water were used in a reference tube. The SOD activity was thereafter expressed as units per milligram of protein.

Measurement of CAT activity

CAT activity was measured following the method proposed by Sinha (1972). This method is based on the direct measurement of the decrease in absorbance at 240 nm due to H_2O_2 consumption by CAT. One milliliter of 0.1 M phosphate buffer at pH 7.4 and 0.4 mL of 0.2 M H_2O_2 were added to 100 μL of the sample contained in tube. The reaction was stopped at 30, 60 and 90 s by adding 2 mL of dichromate/acetic acid mixture (5/95, v/v). Absorbance was measured at 620 nm, and CAT activity expressed in units per milligram of protein using molar extinction coefficient of CAT ($\epsilon = 0.036 \text{ L/mmol/cm}$).

Statistical analysis

Results were reported as means $\pm$ standard deviations and statistical analyses were done using statistical package of Statgraphics 11.1. The following statistical tests were performed: one-way analysis of variance and multiple comparison of Duncan's test with significance defined at $p < 0.05$. Principal component analysis (PCA) was performed for structuring correlation between studied samples, analysed micronutrients and their antioxidant properties (XLSTAT, version 2016, Addinsoft, New York, US).

Results and discussion

Mineral content

A total of seven mineral elements (Na, Ca, K, Mg, Fe, Cu and Zn) were determined in powder fractions (< 180 ,

180–212, 212–315 and $> 315 \mu\text{m}$), unsieved powder and lyophilized ethanolic extract from *H. sabdariffa* calyces (Table 1). In the mineral composition of unsieved powder, there was marked demarcation in the amount of potassium, calcium and magnesium (248, 975 and 338 $\text{mg}/100 \text{ g DW}$, respectively), while iron (10.22 $\text{mg}/100 \text{ g DW}$), zinc (4.01 $\text{mg}/100 \text{ g DW}$) and copper (0.31 $\text{mg}/100 \text{ g DW}$) contents were the lowest. In another study, the mineral contents in *H. sabdariffa* calyces were found different (Cissé et al., 2009a). These latest have reported potassium, calcium, magnesium, iron, zinc, sodium and copper contents of 2060, 1583, 316, 37.8, 6.5, 5.5 and 0.6 $\text{mg}/100 \text{ g DW}$, respectively. Tazoho et al. (2016) also reported the mineral contents of *H. sabdariffa* calyces of 770.6, 77.8, 52.0, 1.10, 3.25 and 0.56 $\text{mg}/100 \text{ g DW}$, respectively, for Ca, Mg, K, Na, Fe and Zn. The most likely reason for this difference in mineral contents was that the mineral composition of each plant organ is determined by a sequence of events. These events begin with bioavailability of mineral nutrients in soils and membrane transport in the roots to the final deposition of these nutrients into one or more cellular compartments (Grusak, 2002). Furthermore, changes in mineral nutrient homeostasis have been reported to influence multivariate changes in maturity of plants (Bennett et al., 2012). In addition, results show that, mineral contents varied significantly ($p < 0.05$) depending on the particle sizes. The mineral contents increased inversely to the particle sizes of the powders. Generally, a particle size of $< 180 \mu\text{m}$ showed the highest mineral contents. Obtained values ranged from 1248–2796, 975.5–1336, 338.9–480.1, 9.89–12.65, 10.22–24.01, 3.47–6.72 and 0.24–0.59 $\text{mg}/100 \text{ g}$, respectively, for potassium, calcium, magnesium, sodium, iron, zinc and copper. These results support the earlier reported hypothesis by Becker et al. (2016), Deli et al. (2019a; 2019b), Flávia et al. (2016) and Lucas-González et al. (2017). These authors reported the highest ash contents in the finest particle powders from grinded and sieved vegetable matrix as compared to that of larger particle size powders. Thus, higher total ash content of finer particle powders suppose that their mineral contents are higher. Comparing with traditional ethanolic extraction, CDSp has advantage to concentrate more minerals. The results suggest an interest of CDSp to enhance the mineral elements of plants matrix. It may therefore be hypothesized that finer powders may exhibit higher antioxidant activity as compared to powders with larger particle sizes and powder from ethanol extract. This need to be investigated.

Vitamin content

Vitamin contents in powder fractions obtained by CDSp and ethanolic extract of *H. sabdariffa* are represented in

Table 1 Changes in the mineral, vitamin, carotenoid and anthocyanin contents of powders (CDSp fractions, unsieved powder and freeze-dried ethanolic extract) from *H. sabdariffa* calyces

Constituents	Powder fractions obtained by CDSp				Unsieved powder	Ethanolic extract
	< 180 μm	180–212 μm	212–315 μm	> 315 μm		
Minerals (mg/100 g DW)						
Potassium	2 796 ± 196 ^c	2 309 ± 146 ^d	1 813 ± 77 ^c	1 366 ± 57 ^b	1 248 ± 92 ^b	511.9 ± 21.3 ^a
Calcium	1401 ± 87 ^c	1336 ± 53 ^c	1071 ± 58 ^b	927.4 ± 60.4 ^b	975.5 ± 42.0 ^{bc}	76.62 ± 0.98 ^a
Magnesium	580.1 ± 39.3 ^e	492.4 ± 34.9 ^d	403.9 ± 3.1 ^c	330.5 ± 8.1 ^b	338.9 ± 35.0 ^b	124.1 ± 0.3 ^a
Sodium	12.65 ± 0.83 ^c	11.60 ± 0.87 ^{bc}	11.40 ± 0.02 ^b	9.37 ± 0.08 ^a	9.89 ± 0.95 ^b	20.37 ± 0.25 ^d
Iron	24.00 ± 1.27 ^e	10.98 ± 0.67 ^d	4.61 ± 0.01 ^c	2.75 ± 0.01 ^a	10.22 ± 0.87 ^d	3.38 ± 0.02 ^b
Zinc	6.72 ± 0.38 ^d	6.60 ± 0.37 ^d	4.35 ± 0.04 ^c	3.47 ± 0.06 ^b	4.00 ± 0.40 ^c	0.75 ± 0.01 ^a
Copper	0.59 ± 0.04 ^e	0.48 ± 0.03 ^d	0.37 ± 0.01 ^b	0.27 ± 0.01 ^a	0.31 ± 0.03 ^a	0.41 ± 0.01 ^c
Vitamins (μg/100 g DW)						
*Ascorbic acid	56.35 ± 0.37 ^b	56.78 ± 0.80 ^b	56.48 ± 1.32 ^b	56.49 ± 1.25 ^b	54.22 ± 0.27 ^a	60.27 ± 0.43 ^c
Thiamin	10.32 ± 0.01 ^b	10.46 ± 0.09 ^b	10.30 ± 0.15 ^{ab}	10.40 ± 0.05 ^b	10.15 ± 0.08 ^a	11.16 ± 0.02 ^c
Riboflavin	248.2 ± 16.1 ^a	260.6 ± 15.7 ^{ab}	275.1 ± 14.8 ^a	244.6 ± 10.2 ^a	263.8 ± 13.8 ^{ab}	375.4 ± 9.27 ^c
Folic acid	939.0 ± 1.1 ^b	1 001 ± 3 ^c	1 011 ± 8 ^c	830.7 ± 16.9 ^a	919.3 ± 59.2 ^b	2 180 ± 6 ^d
PABA	4.51 ± 0.05 ^a	4.74 ± 0.03 ^c	4.62 ± 0.06 ^b	4.62 ± 0.02 ^b	4.52 ± 0.01 ^a	5.36 ± 0.05 ^d
Carotenoids (μg/100 g DW)						
	234.6 ± 5.1 ^f	169.6 ± 7.81 ^d	135.9 ± 5.3 ^c	101.8 ± 2.6 ^b	191.1 ± 10.9 ^e	626.1 ± 7.9 ^g
Anthocyanins (mg CGE/100 g DW)						
	604.6 ± 16.9 ^c	629.4 ± 7.8 ^c	673.3 ± 0.6 ^d	486.5 ± 24.6 ^a	572.4 ± 12.7 ^b	1561.9 ± 34.6 ^e

*Ascorbic acid (mg/100 g DW)

ND not determined, PABA *p*-aminobenzoic acid, CGE cyanidin 3-glucoside equivalents, DW dry weightMeans ± standard deviations with different superscripted letters within the same line differed significantly ($p < 0.05$) according to Duncan's multiple range test ($n = 3$)

Table 1. These results demonstrated that ascorbic acid was more concentrated in the *H. sabdariffa* unsieved powder (54.22 mg/100 g DW) than thiamin, riboflavin, folic acid, *p*-aminobenzoic acid (PABA) with 10.15, 263.8, 919.3 and 4.52 µg/100 g DW, respectively. The contents of all vitamins were found to be higher than those reported by Singh et al. (2017), with values of 17.0, 0.05, 0.07 and 0.06 mg/100 g DW, respectively, of vitamin C, thiamin, riboflavin and niacin. The differences observed between this study and the current work may be attributed to the treatments applied to the hibiscus calyces to produce powder. Such differences may also be affected by the maturity stage and the type of used sample. In accordance with previous studies (Singh et al., 2017), acetate retinol and α -tocopherol contents were investigated, this study confirms that these two vitamins were not also detected in all hibiscus powder samples. It could be explained that *H. sabdariffa* calyces are not a source of acetate retinol (vitamin A). In other hand, it must be recognized that production of hibiscus powder is accompanied by mechanisms of physicochemical alteration catalyzed by oxygen or heat (brought to plant during drying or grinding process) and can result in loss of the most sensitive vitamins. Vitamin A

is known to be sensitive to oxygen, heat and light (Loveday and Singh, 2008). It was reported that during the grinding operation, the particle temperature in the grinder surrounding can rise up to 90 °C. Consequently, alteration of acetate retinol and α -tocopherol may occur, thus inducing their loss. Otherwise, storage of powder affects the vitamin A stability. Hemery et al. (2010) reported a loss of 50% and 85% vitamin A in wheat powders stored at 25 °C for three months, respectively, in polyethylene and paper bags (permeable to oxygen and moisture).

Looking to powder fractions obtained by CDSp, there was difference of vitamin contents according to particle size, except for ascorbic acid. Generally, the finest particles or intermediate powder fractions obtained by CDSp exhibited significant ($p < 0.05$) and highest content of thiamin, riboflavin, folic acid and PABA. Indeed, in the plant cells, the vitamins are entrapped in chloroplasts, mitochondria and cytosol (Smith et al., 2007). This could be explained that grinding follow by sieving process altered or destroyed cell wall matrix, thus making the vitamin compounds released or expose with increase of specific surface area powder particles. Similarly, Yu and Kies (1993) noticed that the reduction of particle size was

correlated with an increase in the bioaccessibility of vitamin B (mainly niacin, pantothenic acid and thiamine). Nevertheless, there was often a loss of vitamins, testifying to deleterious impact of grinding process already discussed above. From a comparative point of view to powder fractions obtained by CDSp, higher contents of all analysed vitamins were recorded in ethanolic extract.

Total carotenoid content

Total carotenoid content among *H. sabdariffa* powders with different particle size were compared to those of lyophilized ethanolic extract (Table 1). It was found that the total carotenoid content was significantly affected by the particle size. With the decrease in particle size processed by superfine grinding followed by CDSp, the total carotenoid content rose. The highest total carotenoid content ($234.6 \pm 5.1 \mu\text{g}/100 \text{ g DW}$) was obtained using size particle of $< 180 \mu\text{m}$; when particle size of $212\text{--}315 \mu\text{m}$ was give the smaller total carotenoid content ($101.8 \pm 2.6 \mu\text{g}/100 \text{ g DW}$). Indeed, carotenoids are localized in chloroplasts and chromoplasts, which are surrounded by cell walls. Therefore, cell walls constitute a physico-chemical barrier which limits bioaccessibility of carotenoids. *Generally, the grinding of vegetables results in a breakdown of the plant cell structure, facilitating nutrients release and improvement of their bioavailability.* Release of carotenoids depends on the level of rupture of plant tissues or the disruptions in the cell wall fraction: the smallest particle size means that the highest specific surface area of particles, and more carotenoids are release on particle surface (Low et al., 2015). The results are consistent with other studies of the effect of particle size on carotenoid content. Gong et al. (2015) also showed for carrot powders an increase of about 50% in total carotenoid content when the particle size decrease from 240 to $40 \mu\text{m}$. Similarly, Van Het Hof et al. (2000) showed that bioavailability of carotenoids was increased by cell wall disruption. However, the total carotenoid content of ethanolic extract was $626.1 \pm 7.9 \mu\text{g}/100 \text{ g DW}$, which was much higher than those of all CDSp powder fractions and unsieved powder. Ethanolic extract concentrated the carotenoids about 3 times than unsieved hibiscus powder ($191.1 \pm 10.9 \mu\text{g}/100 \text{ g DW}$). Thus, carotenoids which are classified as non-polar compounds were solubilized in ethanol (Monroy et al., 2016). *Carotenoids have antioxidant activity, but few of them are converted into retinol, the active form of vitamin A* (Dini et al., 2013).

Total anthocyanin content

Hibiscus calyces have a characteristic deep red color, which is mainly due to the presence of anthocyanins.

Anthocyanins are a group of phenolic compounds found in a wide variety of flowers and fruits presenting a reddish-purple colour and have been used as natural alternatives for the replacement of synthetic coloring additives in the food industry. The relationship between anthocyanins and antioxidant activity of *H. sabdariffa* was reported previously; the author concluded that anthocyanin is the major source of antioxidant capacity in hibiscus (Tsai et al., 2002). According to the results, the anthocyanin content for different particle sizes increased from $486.51 \mu\text{g CGE}/100 \text{ g DW}$ (powder fraction $> 315 \mu\text{m}$) to $673.34 \mu\text{g CGE}/100 \text{ g DW}$ (powder fraction $212\text{--}315 \mu\text{m}$) (Table 1). Meanwhile, the anthocyanin values of powder fractions of $< 180 \mu\text{m}$, $180\text{--}212 \mu\text{m}$ and that of unsieved were: 604.66 , 626.42 and $572.45 \mu\text{g CGE}/100 \text{ g DW}$, respectively. Similar results in terms of the effect of particle size on anthocyanins were observed by Cid-Ortega and Guerrero-Beltrán (2014). In addition, results highlight that the ethanolic extract had higher anthocyanin content ($1561.92 \mu\text{g CGE}/100 \text{ g DW}$) when compared to all powder fractions obtained by CDSp. The above results show that CDSp has less advantageous than ethanol extraction of the anthocyanins from *H. sabdariffa* calyces. However, *the anthocyanin contents of dried roselle calyces were influenced by grinding processes. This may be due to the facts that anthocyanins are very unstable when exposed to high temperature, which generally cause a loss of chemical antioxidant compounds.* Indeed, an increase in the temperature over 70°C causes the thermal degradation of anthocyanins (Cissé et al., 2009b). Yet, it was reported the extreme rise in temperature (up to 90°C) experienced by fine particles during the grinding operation (Hu et al., 2012). Anthocyanins are more sensitive to heat than phenolic compounds (Karaaslan et al., 2014).

In vivo antioxidant activities of *H. sabdariffa* powders

Changes in body weight

In the beginning of the experiment, all groups had body weight between 200 and 300 g and received similar amount of calories for the entire period. The composition of the feed (by varying the amount of lipids and proteins) will have to lead high-fat groups to increase their weight and normal regime rats to regulate their body weight. The powder samples from *H. sabdariffa* exhibited an effect on body weight in high-fat diet rats through the reduction of body weight gain and this property depend on particle size of *H. sabdariffa* powders (Table 2). At the end of 28 days treatment, the body weight in negative control group gained more than those in normal group and others rat groups ($p < 0.05$). Meanwhile, the $< 180 \mu\text{m}$ and

Table 2 Effect of *H. sabdariffa* powders (250 mg/kg) on body weight in high-fat diet rats

Groups	Final body weight (g)	Body weight gain (g)
< 180 μm	399.6 + 23.3 ^a	190.5 + 15.9 ^a
180–212 μm	420.9 + 22.7 ^b	210.1 + 18.2 ^{ab}
212–315 μm	429.2 + 21.5 ^b	230.1 + 20.1 ^b
> 315 μm	430.9 + 27.9 ^b	232.1 + 22.2 ^b
Unsieved powder	440.9 + 20.8 ^b	235.1 + 20.2 ^b
Ethanollic extract powder	398.9 + 32.4 ^a	201.1 + 20.9 ^{ab}
Normal control	400.5 + 20.2 ^a	185.1 + 23.8 ^a
Positive control	395.9 + 23.1 ^a	195.1 + 21.5 ^a
Negative control	465.1 + 30.5 ^b	250.1 + 25.8 ^b

Means $\pm$ standard deviations with different superscripted letters within the same column differed significantly ($p < 0.05$) according to Duncan's multiple range test ($n = 5$)

ethanolic extract treated groups (250 mg/kg) same to the positive control group (vitamin C 20 mg/kg) were exhibited a remarkably reduced weight gain compared to negative control group.

Lipid peroxidation

Lipid peroxidation is a metabolic process under normal aerobic conditions and is one highly investigated consequence of free radical action on membrane structure and function. According to the results presented on Fig. 1, a significant production of liver and plasma MDA was observed in untreated rat group received a high-fat diet (negative control) compared to the normal control rat groups, indicated that the high-fat diet is associated with oxidative stress due to free radical production. MDA is produced by membrane lipid oxidation caused by a strong oxidizing agent (Thura et al., 2018). Thus, a high MDA

levels is an indicator of failure of antioxidant defense mechanism, which is supposed to inhibit the high MDA production. Generally, polyunsaturated fatty acids (PUFA), the main components of membrane lipids, are susceptible to peroxidation. Indeed, OH can react with the methylene groups of PUFA forming conjugated dienes, lipid peroxy-radicals (LOOP), and hydroperoxydes (LOOH) (Kumar et al., 2013). The lipid hydroperoxides produced can undergo reductive cleavage by reduced metals such as Fe^{2+} and produce lipid alkoxyl radical which can initiate additional chain reactions. The final stable products of peroxidation are aldehydes (including MDA) which react with thiobarbituric acid (TBA) to form TBA-malonaldehyde adduct with an absorbance maximum at 532 nm. Therefore, an increased MDA compared to normal is an indicator of reactive oxygen species generation in the tissue or organs (Deli et al., 2020). In addition, the MDA levels significantly decreased in treated rat groups with ascorbic

Fig. 1 Effect of *H. sabdariffa* powders (CDSp fractions, unsieved powder and freeze-dried ethanolic extract) on liver and plasma malondialdehyde contents in rats subjected to oxidative stress by high-fat diet. Error bars represent standard deviation. For liver or plasma, bars with different superscripted letters differed significantly ($p < 0.05$) according to Duncan's multiple range test ($n = 3$)

acid, ethanolic extract and CDSp powder fractions and having received concomitantly a high-fat diet. A reduction in liver and plasma MDA contents or decrease in lipid peroxidation indicates that the treatments reduce the effects of free radicals, lipid peroxidation and have antioxidant effects. The powder samples from *H. sabdariffa* inhibited lipid peroxidation through the reduction of MDA production and this property depend on particle size of *H. sabdariffa* powders. Thus, powder fraction of $< 180\ \mu\text{m}$ same to ethanolic extract powder were exhibited a significant lipid peroxidation inhibitory action. Indeed, their liver and plasma MDA contents were similar or lower to those of rat groups receiving ascorbic acid (positive control). The others powder fractions of 180–212 μm , 212–315 μm , $> 315\ \mu\text{m}$ and unsieved powder also inhibit lipid peroxidation, but to a lesser extent than that of ascorbic acid, except for 180–212 μm powder where liver MDA content was similar to those of ascorbic acid. These results highlight the stronger inhibitory activity of the finest powder. Indeed, finer particle powders (i.e., particle powder of $< 180\ \mu\text{m}$) had been found to exhibited the highest DPPH antiradical activity (Deli et al., 2019a) and richer in antioxidant compounds, especially in carotenoids, antioxidants minerals (Cu and Zn) and vitamin C. In particular, carotenoids are known to be very efficient physical and chemical quenchers of singlet oxygen ($^1\text{O}_2$), as well as potent scavengers of other reactive oxygen species (ROS) (Cvetkovic et al., 2013). This is of special significance, because the uncontrolled generation and concomitant increase of ROS level in the body results in “oxidative stress”.

SOD and CAT activities

The animal body has evolved highly complex enzymatic antioxidant systems which protect the cells and organ against free radical damage. This system includes enzymatic antioxidants such as SOD and CAT. According to Fig. 2a, SOD activity in rat groups received a high-fat diet was significantly ($p < 0.05$) lower than that in normal rat groups (untreated rat groups fed a normal diet). This decrease in SOD activity could be explained as previously report by Amit and Priyadarsini (2011). Indeed, that expression of SOD activity (which catalyzes a conversion of peroxide anions to hydrogen peroxides (H_2O_2)) was not sufficient to protect cell organs against oxidative stress caused by a high-fat diet. Interestingly, SOD activity in rat groups treated with the different powder fractions, ascorbic acid and lyophilized ethanolic extract was highest as compared to negative rat group, and suggests that they may prevent intense production of free radicals. It was also noted a significant ($p < 0.05$) effect of powder particle sizes on SOD activity. Liver SOD activity was greater in rat groups treated with powder fractions of $< 180\ \mu\text{m}$,

212–315 μm and ethanolic extract, while plasma SOD activity was high for rat group treated with powder fraction of $< 180\ \mu\text{m}$. An increase in the activity of SOD in the presence of *H. sabdariffa* powders may be link to the fact that plant powerful antioxidants can strongly scavenge most of the free radicals that accumulate in the body, thereby compensating the endogenous antioxidant potential in rats. Thus, observed difference according to the particle size of *H. sabdariffa* powders could resulted from a difference in their composition in antioxidant compounds. Indeed, the powder with particle sizes of $< 180\ \mu\text{m}$ was demonstrated higher in antioxidant minerals such as Zn and Cu which act as the cofactors to maintain catalytic activity of antioxidant enzymes (SOD and CAT).

From results in Fig. 2b, a significant ($p < 0.05$) decrease of CAT activity in rat groups received a high-fat diet but no treatment (negative control group) was noted, as compared to that received normal diet (untreated rat groups fed a normal diet). Similar to SOD activity, a decrease of liver and plasma CAT activities corroborates the previous results of MDA contents. Indeed, a high-fat diet is associated with oxidative stress due to free radical over production. Thus, the organs MDA contents increase, while SOD and CAT activities decrease. Otherwise, SOD and CAT activities supposed to maintain the pro-oxidant/antioxidant balance was not sufficient to control production of free radicals induced by high-fat diet, and consequently oxidative reactions take place, and an increased in MDA levels constitute a probably indicator. It can be also noted that rats have been treated with ascorbic acid, powder fractions, unsieved powder and ethanolic extracts of *H. sabdariffa*. A significant increase of CAT activity was observed as compared to that of negative control group. An administration of *H. sabdariffa* powders or vitamin C in rat groups (subjected to oxidative stress), probably entails an inhibition of free radical production, particularly H_2O_2 decomposition to water molecules, and thus restoration of CAT activity. Looking to granulometric powder fractions obtained by CDSp, CAT activity was significantly ($p < 0.05$) affected by powder particle size. The highest liver CAT activity was observed in rat groups treated with finer powder fractions (i.e., powders particle size of $< 180\ \mu\text{m}$ and 180–212 μm). From a comparative point of view to freeze-dried ethanolic extract, liver CAT activity of powder fractions of $< 180\ \mu\text{m}$ and 180–212 μm was equal, and interestingly greater to that obtained with vitamin C (positive control). Plasma CAT activity was equal for all *H. sabdariffa* powder samples, except for plasma CAT activity of powder fraction of 180–212 μm which has the low.

The results could be explained that the contained antioxidant compounds in *H. sabdariffa* powders such as phenolic compounds, carotenoids, vitamin C and minerals were contributed to their high antioxidant activity.

Fig. 2 Effect of *H. sabdariffa* powders (CDSp fractions, unsieved powder and freeze-dried ethanolic extract) on liver and plasma SOD (a) and CAT (b) activities in rats subjected to oxidative stress by high-fat diet. Error bars represent standard deviation. For liver or plasma, bars with different superscripted letters differed significantly ($p < 0.05$) according to Duncan's multiple range test ($n = 3$)

Phenolic antioxidants act by chelating metal ions, adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides, and improving the antioxidant endogenous system, while minerals such as Zn and Cu act as cofactors of SOD and CAT activities. In addition, carotenoids are known to be very efficient physical and chemical quenchers of singlet oxygen, as well as potent scavengers of other reactive oxygen species (Cvetkovic et al., 2013). The molecular mechanisms underlying these reactions are still not fully understood, especially in the context of the anti and pro-oxidant activities of carotenoids, which, although not synthesized by humans and animals, are also present in their blood and tissues, contributing to a number of biochemical processes (Fiedo and Burda, 2014). The antioxidant potential of carotenoids is of

particular significance to human health, due to the fact that losing antioxidant-ROS balance results in “oxidative stress”, a critical factor of the pathogenic processes of various chronic disorders. Thus, plant products contain a mixture of different chemical compounds endowed with antioxidant activity, and it would be difficult to attribute antioxidant effect to one or some active ingredients. Also, various plant bioactive compounds demonstrate their protective effects against free radicals or cooperate simultaneously with the activity of endogenous antioxidant enzymes (Kurutas, 2016). This would explain observed difference between different powder fractions obtained by CDSp and that for ethanolic extract.

Fig. 3 Principal components analysis of micronutrient contents and in vivo antioxidant properties of investigated *H. sabdariffa* powders (CDSp fractions, unsieved powders, and freeze-dried ethanolic extract). *MDA* malondialdehyde, *SOD* superoxide dismutase, *CAT* catalase, *PABA* *p*-aminobenzoic acid

Correlation and PCA

PCA of the antioxidant tests and micronutrients contents of powder fractions and lyophilized ethanolic extract from *H. sabdariffa* calyces are shown in Fig. 3. The powder samples, micronutrients contents and resulted antioxidant properties have been projected in the single system. The PCA explains 85.34% of the total variation which exists in this system with a contribution of 55.56% for the axis F1 and 29.79% for the axis F2. It is observed that the minerals, carotenoids, anthocyanins and vitamin C are highly positively correlated with the enzymatic activities of CAT and SOD, while negatively correlation was observed with MDA levels. As such, the results showed significant positive correlation between copper (Cu), vitamin C and carotenoids with the values of plasma CAT activity. As shown in Fig. 3, minerals and vitamins jointly contributed to the increase in CAT and SOD activities and decrease MDA in finer powder fractions ($< 180 \mu\text{m}$ and $180\text{--}212 \mu\text{m}$). The coarser powder fractions ($212\text{--}315 \mu\text{m}$ and $> 315 \mu\text{m}$) and unsieved powder are definitely lower in vitamins, while the antioxidant power of lyophilized ethanol extract derived particularly from its high level in anthocyanins, carotenoids and such vitamins (B1, B2, B9 and PABA).

The grinding followed by controlled differential sieving process (CDSp) was developed in this study and compared

to ethanolic extraction from *H. sabdariffa* calyces. It is concluded that the size reduction of *H. sabdariffa* calyces had successfully to improve the mineral, vitamin C and anthocyanin contents of powders. The smaller the particle size of the powder, the higher the proportions of minerals, anthocyanins and vitamin C, and also to exhibit higher preventive antioxidant activity as compared to coarser particles. However, ethanolic extract was essentially rich in total carotenoids contents. There is considerable evidence to suggest that the grinding followed by CDSp is helpful for improving the mineral, total carotenoid, anthocyanin and vitamin contents alongside the antioxidant properties of *H. sabdariffa* powder.

Acknowledgements The work was supported by the Francophone University Association (AUF) and its grant for Scientific Research, No. S0020ADM10607L. The work was carried out within the frame of the Extrapole project funded by the former Lorraine region (France). The authors are grateful to the staff of LABBAN (Laboratory of Biophysics, Food Biochemistry and Nutrition of Ngaoundere University, Cameroon) as well as at the LIBio (Biomolecular Engineering Laboratory, University of Lorraine, France) for their technical support.

Compliance with ethical standards

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

Ethical standard Research approval was obtained from the Cameroonian National Ethics Committee Ref. No. FWIRD00001954.

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