Int. J. Plant Bas. Pharm. (2024) 4(1), 19-29

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

OPEN ACCESS

A North Cameroonian cultivar of *Hibiscus sabdariffa* (Malvaceae) with calyces enriched in anthocyanins

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ARTICLE INFO

Article History:

Received: 17 October 2023
Revised: 12 March 2024
Accepted: 13 March 2024
Available online: 16 March 2024

Edited by: B. Tepe

Keywords:

Taxonomy
Extraction
Chromatography
Pharmacopeia

ABSTRACT

Folere (*Hibiscus sabdariffa* L.) is a traditional plant cultivated in the north of Cameroon near the city of Yagoua. Calyces are used to prepare beverages while the leaves are incorporated into meals. This Cameroonian roselle is characterized by a higher intensity of red color than that found in conspecific samples from Egypt, and it was of interest to us to evaluate its beneficial activities in comparison with European pharmacopoeia standards. The samples were first subjected to phylogenetic analyses. Five samples including samples from Egypt and a white flower variety were subsequently examined according to the European pharmacopeia criteria i.e. *(i)* taxonomic identification, *(ii)* sample preparation and characterization (desiccation, TLC, coloring power, presence of foreign matter), and *(iii)* determination of the acidity index. Anthocyanins and anthocyanidins were quantified in three extracts from methanolic maceration, infusion, and boiling in water. Taken together, the results confirmed that the Cameroonian variety indeed belongs to *H. sabdariffa* and meets European Pharmacopoeia standards. All extracts contained common anthocyanins (delphinidin-3-sambioside, delphinidin-3-glucoside, cyanidin-3-sambioside, cyanidin-3-glucoside), and delphinidin and cyanidin aglycones were also found in the organic extracts. As a result of anthocyanin quantification by HPLC in Cameroon samples, it was determined that they showed a high concentration of delphinidin type anthocyanins and a concentration 10 times higher than the Egyptian variety. This variety appears to be a very promising candidate for inclusion in industrial preparations, thanks to its high anthocyanin qualitative and quantitative properties, and also maintains economic interest for local populations.

1. Introduction

Located in tropical West Central Africa, Cameroon has a wide range of subclimates and is considered to be home to 90% of diverse African ecosystems (e.g. Sahel, Sudan, humid tropical forest, coastal and mountain ecoregions called Afro mountains) (Fokunang et al., 2011). To date, approximately 7850 plant species have been recorded, of which 160 are endemic (Onana, 2011). *Hibiscus sabdariffa* L., belonging to the family Malvaceae, was recorded by Fongnzossie et al. (2017) and Jiofack et al. (2010) in Cameroon. It is a worldwide species found in Asia (India, China, Malaysia, Indonesia, Vietnam, Taiwan), in the Middle East (Egypt, Saudi Arabia), as well on Africa (Sudan, Nigeria, Cameroon) but also in America (Mexico) and its vernacular name varies according to the localization (Maganha et al., 2010) even in a same country: for example its name is Folere in extreme north of Cameroon and in the Sudano Sahelian region (Jiofack et al., 2010), Mbanga in the east (Fongnzossie et al., 2017) and Oseille in Douala (Emmanuel & Di-

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e-ISSN: 2791-7509
doi: https://doi.org/10.62313/ijpbp.2024.153

dier, 2011). According to its large geographical distribution, genetic polymorphism is considered important (Da-Costa-Rocha et al., 2014). All parts of *H. sabdariffa* are used for their nutritional value and medicinal properties (as well as for their economic value in the cosmetic and textile industries) (Cisse et al., 2009). The protein content in fresh calyces is approximately 1.9% (m/m), in leaves 3.3% (m/m) and in seeds approximately 27.8% (m/m) (Cisse et al., 2009; Riaz & Chopra, 2018). Calyces are also rich in vitamins (niacin, riboflavin, ascorbic acid) and minerals (calcium, iron, magnesium) (Riaz & Chopra, 2018) and are frequently used as beverage in the preparation of aqueous extracts to obtain cold or hot drinks and as food coloring (Maganha et al., 2010; Riaz & Chopra, 2018). As a medicinal plant, infusions of calyces are prepared to treat gastric and hepatic disorders in India but also fever and hypercholesterolemia. In Malaysia, oil obtained from seeds **(Figure 1C)** is incorporated in soaps (Agrawal, 2020; Riaz & Chopra, 2018). In Northern Cameroon, the decoction of the leaves is reportedly used in dental care (Fongnzossie et al., 2017), but the leaves are also mixed with meat to prepare typical meals. The decoction of the fruits and leaves is also used in the treatment of amoebiasis, anemia and sexually transmitted diseases, as well as as a regular drink. Another commercial outlet is the use of fibers which can be useful to obtain jute, or woven into fishing nets and ropes (Da-Costa-Rocha et al., 2014). A great number of preclinical studies and clinical trials have been conducted with *H. sabdariffa* extracts or powder (Najafpour Boushehri et al., 2020). The most relevant effects are antihyperlipidemic, antihypertensive, and antidiabetic activities (Da-Costa-Rocha et al., 2014; Maganha et al., 2010; Ojulari et al., 2019; Riaz & Chopra, 2018). While some antilithiatic and diuretic results have been more contradictory in human studies (Laskar & Mazumder, 2020), other pharmacological activities such as antimicrobial, anti-inflammatory, anti-anemic, and anticarcinogenic need to be confirmed in human studies (Maganha et al., 2010; Ojulari et al., 2019; Riaz & Chopra, 2018). Therefore, with global demand increasing by up to 15.000 tonnes per year, increasing cultivation of this species seems clearly economically beneficial (Raghu et al., 2019).

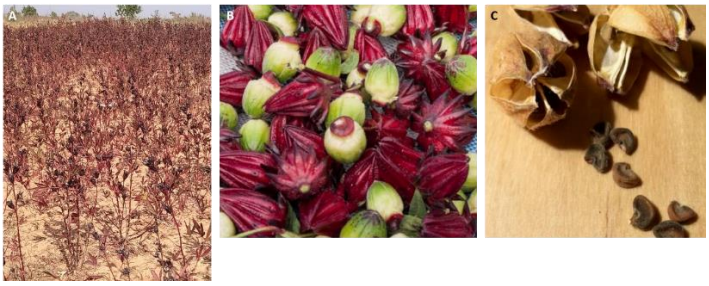

Figure 1. *H. sabdariffa*: cultivation of the red variety in Yagoua (Cameroon) (A), fruit and calyces (B), seeds from the fruits (C)

Various families of compounds, such as organic acids, phenolics, polysaccharides, and essential oils, have been isolated from *H. sabdariffa*, and the composition varies depending on the part of the plant and solvent extraction. For calyces, the organic acids give the species its sour taste and the most common acids are citric, malic, tartaric, hydroxycitric, succinic, and fumaric acids. Among polyphenols, flavonols (hibiscitrin, gossytrin, and their relative glycosides) are the major flavonoids while four anthocyanins have been reported (Da-Costa-Rocha et al., 2014). The major anthocyanins **(Figure 2)** are delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (gossypicyanin) and the others (delphinidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside) are less present (Cisse et al., 2009; Da-Costa-Rocha et al., 2014). Some phenolic acids (protocatechuic acid, chlorogenic acid), and tannins (catechin, ellagic acid, epigallocatechin) have also been described in aqueous extracts as well as polysaccharides and volatile terpenoids (Da-Costa-Rocha et al., 2014).

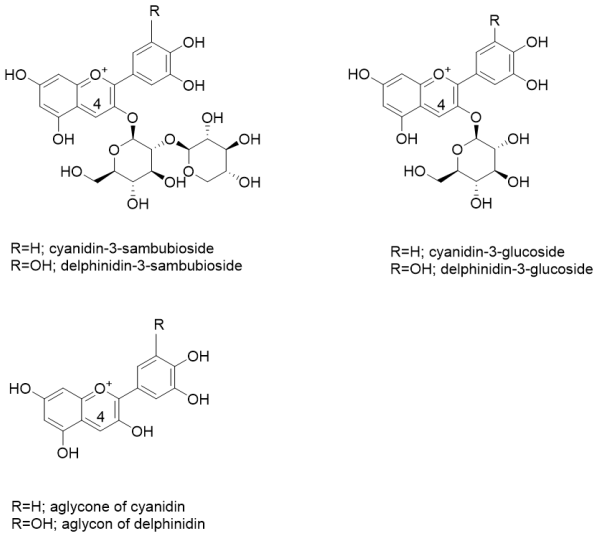

Figure 2. Anthocyanins reported in *H. sabdariffa*

In the extreme north of Cameroon near Yagoua, a cultivar of *H. sabdariffa* has been cultivated for a long time by women to ensure their subsistence. The calyces have a particularly intense red color; this is a sign of potential richness in anthocyanins. Given the great interest in the search for anthocyanins of economic importance (Veluru et al., 2022), the scope of this study was to describe this red

Cameroonian cultivar and to submit it to several assays of the European pharmacopeia (Karkade Monograph, 2017). Additionally, the dosage of anthocyanins was measured by comparing three different traditionally used extraction methods, respectively: decoction, infusion, and maceration. Phylogenetic analyses were also carried out independently. Three other varieties originating from Egypt and sold in pharmacies and a white variety from Cameroon were also compared with this traditional Cameroonian variety for interesting characteristics.

2. Materials and methods

2.1. Material

Thin layer chromatography (TLC) plates were 10 x 20 cm aluminum sheets coated with silica gel 60F254 (10-12 µm, Kieselgel 60 F254, MERCK 5554). Analytic grade solvents used for TLC, e.g. anhydrous formic acid (VWR Prolabo Chemicals, 13G220509), *n*-butanol (VWR Prolabo Chemicals, 16G084005), and sodium hydroxide were obtained from Sigma-Aldrich. Distilled water was obtained by using a 0.45µm filtration system (Vent Filter MPKO1, F7MA56578).

CAMAG system® including the Automatic TLC Sampler 4 (CAMAG, Switzerland) for depositing the samples on plates and the TLC Visualizer 2 (CAMAG, Switzerland) for photographing the plate before and after revelation were used in TLC analysis. All the system is controlled by the CAMAG® HPTLC software VisionCat.

Absorbances were recorded by using a spectrophotometer (Analytik Jena, Specord PC205) and the pH with a pH Meter (Fisher Scientific, Accumet AE150).

HPLC separation of the anthocyanins was accomplished on a Shimadzu Prominence chromatograph equipped with a quaternary pump LC-20AD 5P, an autosampler SIL-20A(HT), a photodiode array detection (SPD-M20A) with a degassing unity (DGU-20 A(5R). The gradient elution method was performed using a Phenomenex®, Kinetex C18 column (100 mm x 4.6 mm, 2.6 µm particle size, 100A), and the column oven CTO-20A was fixed at 30 °C. Mass spectrometric detection was performed using an Advion® expression cmS mass spectrometer (MS). The solvents used for HPLC analyses were of HPLC-grade (formic acid, acetonitrile).

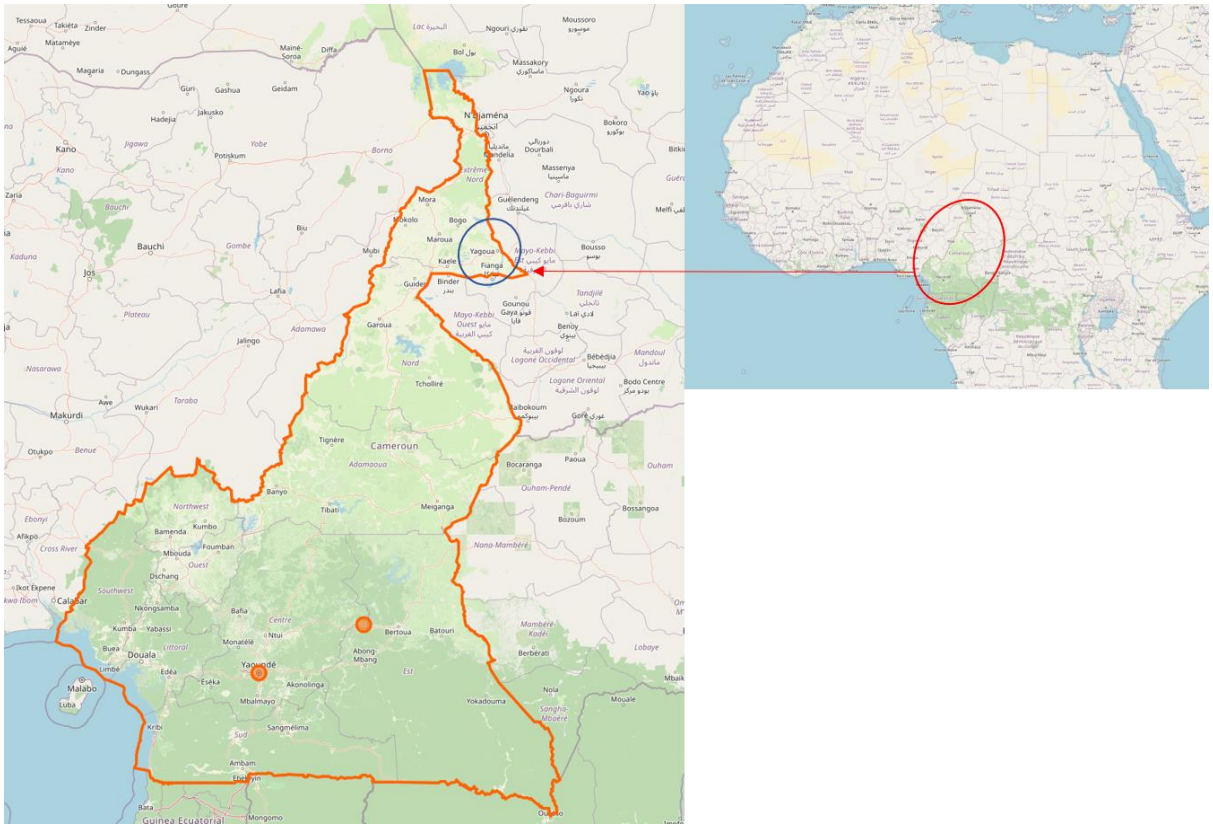

Figure 3. Localization (Yagoua district) of the red cultivar of sabdariffa in Cameroon.
(https://www.openstreetmap.org/#map=5/13.902/-2.900)

2.2. Description of the cultivation area and the crop

The red cultivar of *H. sabdariffa* is cultivated in the north of Cameroon in the region of Yagoua (capital of the department of Mayo-Danay), 115 km south-east of Maroua **(Figure 3)**. The cultivation fields are located around Yagoua city (10°20'34° N and 15°14'26° E, altitude 336 m) and are surrounded by Sahelian Savannah (Fongod et al., 2014). The Limani-Yagoua mega sand ridge itself has a warm and dry desert climate (Ngatcha et al., 2001). The calyces **(Figure 1A, B)** are collected twice a year: *(i)* a smaller crop during the rainy July-August season when cloudy and with rainfalls over 80 mm, and *(ii)* a large crop during the longer dry period (October-June) when sunny with temperatures exceeding 35 °C and with little rain (< 30 mm). The calyces are collected at maturity before fructification **(Figure 1C)** by women, with special attention to prohibiting children from working in the fields. Then calyces are dried in the shade away from the dust.

2.3. Plant material

Five samples of *Hibiscus* calyx (A-E) were selected: three samples originated from the north of Cameroon and two samples from Egypt but commercialized by different suppliers **(Table 1)**. Batch E was of officinal grade. The calyces from the north of Cameroon were

purchased from local producers in Yagoua after being dried traditionally under shade far away from the dust.

All dried-out samples were ground using a mortar and pestle and the resulting powder was sifted under a granulometry of 0.5 mm.

Table 1. Batches of *H. sabdariffa* and their acid percentage (mean of three assays, expressed in citric acid equivalent)

Code	Commercial batch number	Suppliers	Voucher number	Expressed in citric acid (%)
A	FDH90501	Cameroon (Yagoua)	REN_JB/A/21/01	13.27 ± 0.08
B	H1600239	Egypt (Cailleau)	REN_JB/A/21/02	17.66 ± 0.10
C	FDH803001	Cameroon (Yagoua)	REN_JB/A/21/03	15.90 ± 0.05
D	FDH803001	Cameroon (Yagoua)	REN_JB/A/21/04	13.93 ± 0.02
E	1974443	Pharmacist/Egypt	REN_JB/A/21/05	18.30 ± 0.01

2.4. Morphological analyses

Calyces from the five batches were examined macroscopically by using a Müller (HG550775) stereomicroscope (Gx6) and anatomical details of the ground powders were observed under an Olympus Cx41 light microscope. The identification and the microscopical examination of the specimens were done according to the European pharmacopeia protocols (Karkade Monograph, 2017).

2.5. Phylogenetic analyses

DNA was extracted from fresh seed samples according to the protocol of Werner et al. (2016), since DNA from the original plant material was degraded during and after the drying process. The nuclear ITS region and the chloroplast *rpl16* region were amplified and sequenced as explained by Werner et al. (2016).

The obtained sequences were then used to search via BLAST (Altschul et al., 1990) for the most similar sequences in GenBank. Sequences with more than 95% identity and ≥ 90% query coverage were selected. In the case of the ITS sequences, sequences of *Peltaea speciosa* (Kunth) Standl., *Taliparitihamabo* (Siebold & Zucc.) Fryxell, *Malvaviscus penduliflorus* DC., and *Pavonia spinifex* (L.) Cav. was added as the outgroups. Selected sequences were aligned with CLUSTALX (Larkin et al., 2007) using the default settings. Minor manual adjustments were made to correct evident errors using BioEdit (Hall, 1999), and the local alignment quality was checked with the TCS module of T-Coffee (Notredame et al., 2000).

Genetic distances were calculated by MEGAX counting the number of differences (Kumar et al. 2018). All ambiguous positions were removed for each sequence pairs (pairwise deletion option).

MrBayes v3.2 was used for the phylogenetic analyses (Ronquist et al., 2012). Trees were sampled across the substitution model space in the Bayesian MCMC analysis (Huelsenbeck et al., 2004) by using the option nst = mixed, removing the need for a priori model testing. Two runs with four chains (2 x 10^7 generations each) were run simultaneously. Chains were sampled every 10.000 generations and the respective trees were written into a tree file. Consensus trees and posterior probabilities of clades were calculated by combining the two runs and using the trees sampled after the chains converged. The inspection of the sump file created by MrBayes showed that (i) the chains had converged and that there was no tendency for the log-likelihood values to decrease or increase over time, (ii) the standard deviation of split frequencies was below 0.01 upon completion of the analyses, (iii) the potential scale reduction factor for each of the parameters was in the range of 0.999-1.001, and (iv) the effective sample size was above 500 for all parameters.

2.6. Determination of physicochemical properties

All assays were done according to the European Pharmacopeia protocols (Karkade Monograph, 2017).

2.6.1. Thin-layer chromatographic (TLC) analyses

The calyces (500 mg) were extracted with 5 ml of ethanol (60%) (Sigma-Aldrich, STBH9428) under stirring for 15 minutes at room temperature and then filtrated to obtain the TLC extracts.

Thin layer chromatography consisted of loading the extracts and the standard silica plates using the automatic TLC Sample IV. The volume for spotting was 5 µl with a band of 10 mm. Then, the plates were developed with anhydrous formic acid/water/*n*-butanol (10/12/40). All TLC plates were observed under visible light. The standard solution for TLC consisted of an equal mixture of quinaldine red (2.5 mg, Sigma Aldrich, MKCK2556) with sulfan blue (2.5 mg, Sigma Aldrich, MKCJ0273) dissolved in methanol (10 ml, Sigma Aldrich, STBJ4298).

2.6.2. Coloring intensities

Powder of each batch of *Hibiscus* calyces (500 mg) was extracted in hot water for 15 minutes and the temperature was stabilized at 30 °C. Then the volume of the filtrate was adjusted to 25 ml with distilled water to constitute the water solution. The absorbance of this solution was recorded at 520 nm. This assay was repeated three times. The threshold absorbance value required to comply with pharmacopeia criteria must be 0.350 for a whole plant and 0.250 for a fragmented plant.

2.6.3. Desiccation assay

The weight of calyces (1 g) of each batch number was determined before (m1) and after (m2) the incubation in an oven at 105 °C for 2 hours. The loss on drying was calculated according to the equation given below. This assay was done in triplicate. The threshold desiccation value required to comply with pharmacopeia criteria must be 11%.

$$Loss\ on\ drying\ (\%) = \frac{(m1 - m2)}{m1}\ x\ 100$$

2.6.4. Acid percentages

Powder of each batch of *Hibiscus* calyces (1 g) was mixed under agitation with 100 ml of water for 15 minutes. Then the filtrate (25 ml) was titrated by the addition of sodium hydroxide (0.1 M). The pH was recorded with a pH meter after each addition of NaOH (0.5 ml). The equivalent volume Veq (ml) is determined graphically, and the acid percentage is calculated according to the equation given below. The assays were repeated three times. The threshold acid

percentage value required to comply with pharmacopeia criteria must be 13.5% expressed in citric acid equivalent.

$$Acid\ (\%) = \frac{Veq\ x\ 6.4}{250}\ x\ 100$$

2.6.5. Foreign matters

Calyces of *Hibiscus* (100 – 500 mg) corresponding to a weight called m were examined for each batch under the stereomicroscope. Two types of foreign matter were identified and weighted: some fruit fragments (p1) and other diverse fragments (p2). The percentage of foreign matter was calculated according to the equation given below. The threshold for fruit fragments and diverse fragments values required to comply with pharmacopeia criteria must be respectively 2% maximum.

$$Foreign\ matters\ (\%) = \frac{weight\ (p1\ or\ p2)}{m}\ x\ 100$$

2.7. Determination of total anthocyanin content

2.7.1. Extraction

Protocols were followed according to ANSM guidelines for infusion, maceration, and preparation of herbal tincture (ANSM Monograph, 2013). Three extracts (organic, decocted, and infused) were realized to analyze their total anthocyanin contents. Extraction was done in triplicate for each sample. The organic extract (OE) consists of mixing 500 mg of *Hibiscus* powder with 40 ml of methanol and 0.1 ml HCl (1 M) for 30 minutes at room temperature. The decocted extract (DE) was obtained after boiling 500 mg of powder with 75 ml of water for 30 minutes. The infused extract (IE) was obtained after infusing 500 mg of powder in 25 ml of boiling water for 15 minutes. Then, all extracts were filtrated. They were evaporated, weighed, and stored in the dark at 4 °C under nitrogen.

An aliquot of each dried extract was dissolved either in water or in methanol (by the solvent of extraction) and filtrated (0.45 µm) to make an extract solution at 5 mg/ml. Then extract solutions from samples A and E were diluted to 1/3 and sample C to 1/5 before chromatographic analysis.

2.7.2. HPLC-DAD-MS analysis

An analytical method for identifying and measuring anthocyanins in *Hibiscus* was developed. The following gradient system was applied for separation: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) T 0 min: 5% B, T 0-10 min: 5% B, T 10-40 min: 40% B, T 40- 47 min: 100% B, T 47-51 min: 100% B, T 51-53 min: 5% B, T53-60 min: 5% B. The injection volume was 20 µl and the flow rate was 0.8 ml/min. UV detection was performed at 530 nm. Mass spectrometric detection was performed using Advion® expression cmS mass spectrometer (MS) and all analyses were recorded in the negative ESI mode in a mass range of 100 to 1200 Da, applying the following parameters: detector gain 1200, ESI voltage 3.5 kV, capillary voltage 180 V, source voltage 20 V, source voltage dynamic 20 V, nebulizer gas pressure 60 psi, desolvation flow gas rate 4 l/min, capillary temperature 250 °C and gas temperature 20 °C. Data processing and evaluation for MS measurement were performed with the Data and Mass Express 2.2.29.2 software (Advion).

2.7.3. Validation of the anthocyanins quantification method

All validation parameters were determined following the International Conference on Harmonization (ICH) Guidelines (Branch, 2005) using HPLC analyses, and the following characteristics were evaluated: linearity, limits of detection (LOD) and quantification (LOQ), and repeatability inter-day and intra-day.

A stock standard solution of 3-*O*-glucoside cyanidin (C3OG) in methanol was prepared at 600 µg/ml, this was done in triplicate. Then, five working standard solutions were prepared by appropriate dilutions of each stock solution with methanol to generate concentrations ranging from 1.9 µg/ml to 30 µg/ml for the external standard calibration curve and the determination of the regression line. Three calibration curves for C3OG were built after injection in HPLC-DAD at 530 nm. Hence, the concentrations of anthocyanins were calculated, based on peak areas in the equivalent of 3-*O*-cyanidin glucoside at 530 nm and the calibration curve Peak Area C3OG = f (CC3OG). The limits of detection and quantification were determined from the y-intercept standard deviation and the slope of the calibration curve. For the calculation of the intra-day repeatability, a diluted solution of 3-*O*-cyanidin glucoside at 7.5 µg/ml was injected six times on the same day. These assays were repeated on three different days for inter-day repeatability. The coefficient of variation and standard deviation were then calculated. Coefficients of variation of less than 5% for intra-day and inter-day were accepted.

2.7.3. Statistical analysis

Statistical analyses were carried out for loss on drying and acid percentage. Data were analyzed using the one-way analysis of variance (ANOVA) and Tukey-test to compare the mean according to the single variable *Hibiscus* variety. For the results concerning the quantification of anthocyanins, data were analyzed using the two-way ANOVA and Tukey test to compare the significance between the varieties of *Hibiscus* for each extraction method.

3. Results and discussion

3.1. Botanical analyses

3.1.1 Morphological studies

Two Cameroonian red calyce samples were compared macroscopically and microscopically with a white one also from Cameroon but also with a sample from Egypt and another sample of officinal grade **(Table 1)**. All calyces were concrescent and urceolated in the lower part while the upper part showed five acuminated and recurved tips **(Figure 1SA, 1SB, supplementary file)**. Except for the white sample **(Figure 1SB, supplementary file)**, the others were of red and purple color. The calyculus consisted of 8-12 narrow leaflets **(Figure 1SC, supplementary file)** which were lighter in the lower part **(Figure 1SD, supplementary file)**. A median midrib slightly in relief **(Figure 1SD, supplementary file)** was observed on the tips as well as the excretory gland **(Figure 1SE, 1SF, supplementary file)**.

The powder of all red samples showed the presence of polygonal cells from the epidermis **(Figure 1SG, supplementary file)** with a thick wall **(Figure 1SH, supplementary file)** and some of them contained some prisms of calcium oxalate **(Figure 1SG, 1SL, supplementary file)** and anisocytic stomates **(Figure 1SG, supplementary file)**. Some unicellular flexuous trichomes **(Figure 1SI, supplementary file)** were observed as well as some glandular trichomes **(Figure 1SJ, supplementary file)**. Numerous spiral vessels were observed in the parenchyma **(Figure 1SJ, supplementary file)**. Some scarce smooth bent trichomes and parenchyma with cells containing mucilage-filled cavities **(Figure 1SK, 1SH, supplementary file)** and calcium

oxalate crystals **(Figure 1SL, supplementary file)** were observed in the powder, but no pollen grains were found. The preliminary observation under the microscope was important to characterize a species (Raghu et al., 2019) and in our case, all these data were in accordance with the description of *H. sabdariffa* reported in the European monograph (Karkade Monograph, 2017).

3.1.2 Phylogenetic analyses

The two samples showed no differences in the *rpl16* sequence (GenBank accession numbers ON157056 and ON157057, respectively) in comparison with one sequence of another *H. sabdariffa* sample from Sudan and a sequence record of *H. mechowii* Garcke. In comparison with another GenBank record of *H. sabdariffa*, one difference was observed. All other sequences included in the *rpl16* alignment were separated by at least 6 mutational steps. The Bayesian tree **(Figure 4)** supported the observations made based on the distance values. All *H. sabdariffa* samples are placed on a clade with good support (posterior probability 1) together with a specimen of *H. mechowii*.

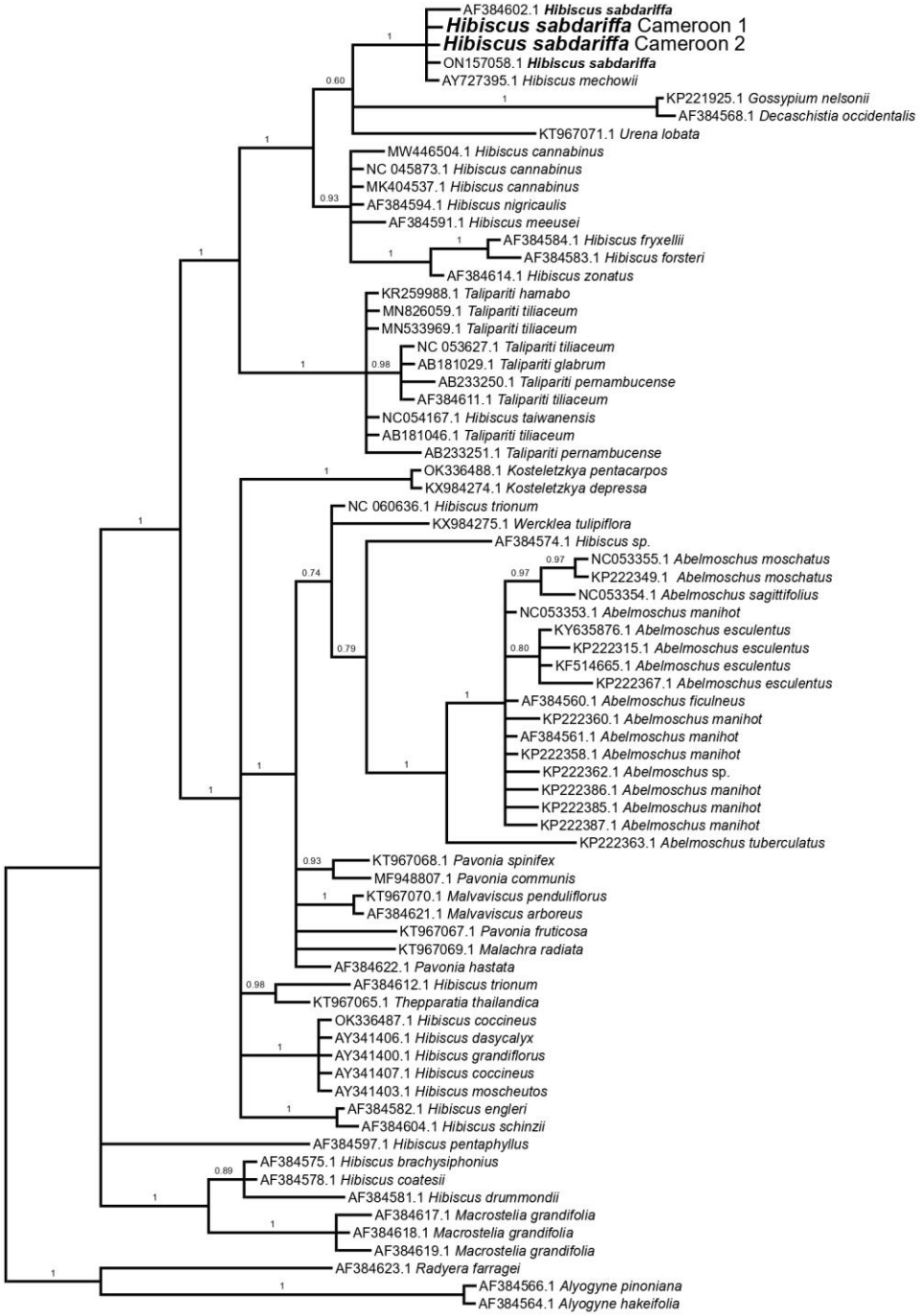

Figure 4. Baysian tree based on chloroplast *rpl16* sequences

GenBank accession numbers, species names, and support values for clades (posterior probability) are given. Sequences of *H. sabdariffa* are highlighted using boldface and the sequences from Cameroon by bigger font size. All samples of *H. sabdariffa* are situated on a clade with solid support together with one sequence of *H. mechowii*.

The ITS sequences for the two studied *H. sabdariffa* samples (GenBank accession numbers ON127554 and ON127555, respectively) were identical except for three ambiguous positions in the alignment, where sample 2 showed double peaks, indicating the

presence of different copies of the multicopy ITS region in the genome. In comparison with therpl16 sequences, a much more complicated pattern was observed. The available 14 *H. sabdariffa* sequences were separated into three groups. The more numerous group (11 samples) included specimens one and two from Cameroon, which were separated by 1-7 mutational steps from the other samples of this group. However, two identical samples of *H. sabdariffa* were separated by 21–25 differences but very close to *H. mechowii* (1 difference). The third group was represented by one individual only with a great distance from both other groups (25 and 35 differences).

The Bayesian tree (Figure 5) showed that the most numerous groups of *H. sabdariffa* are close to *H. asper* Hook.fil., *H. cannabinus* L., and *H. radiatus* Cav. The two-specimen group of *H. sabdariffa* was situated on a clade with a posterior probability of 1 together with the two sequences of *H. mechowii* and one sequence of *H. berberidifolius* A.Rich., while the isolated specimen was close to two *H. asper* sequences on a clade with solid support.

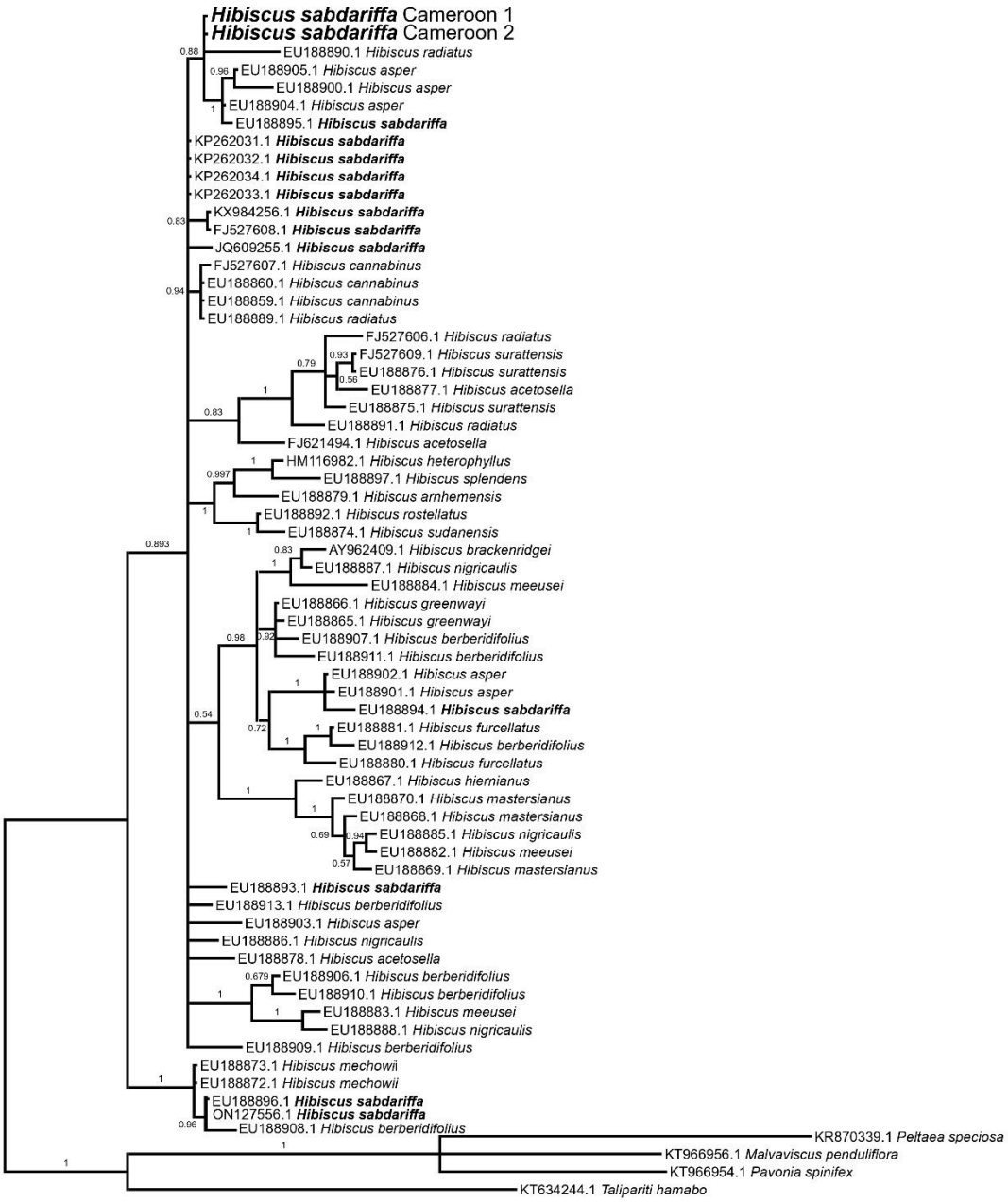

Figure 5. Baysian tree based on nuclear ITS sequences

GenBank accession numbers, species names, and support values for clades (posterior probabilities) are given. Sequences of *H. sabdariffa* are highlighted using boldface and the sequences from Cameroon by bigger font size. Most of the *H. sabdariffa* sequences from GenBank are close to the two samples from Cameroon, but several sequences are situated clearly apart. A similarly complex pattern can be observed for other species like *H. asper*.

Gene trees and species trees were not necessarily congruent; these results could be explained by incomplete lineage sorting, by which "ancestral polymorphisms persisted through several speciation events" (Maddison, 1997). Large effective population sizes and short phylogenetic branches (expressed in generations) favored incomplete lineage sorting (Pamilo & Nei, 1988). Another possible explanation for the incongruencies of gene trees and species trees was hybridization (Maddison, 1997).

In the case of *Hibiscus* section Furcaria, to which *H. sabdariffa* belongs, hybridization and alloploidy were well documented (Wilson, 2006) and the tetraploid *H. sabdariffa* (chromosome

number *n* = 36) was an allopolyploid (Wilson, 2006). This complex evolutionary pattern may explain the partial incongruence between morphological and sequence data, especially in the ITS tree that not only affected *H. sabdariffa*, but also species like *H. asper*, *H. berberidifolius*, *H. nigricaulis* Baker F., and *H radiatus*. In any case, the samples of *H. sabdariffa* from Cameroon were genetically very close to most of the *H. sabdariffa* sequences available at GenBank and taken together the available data support the correct identification of the samples with the present knowledge on the taxon.

3.2. Determination of physicochemical properties and analyses of foreign matters

Raw ethanol extracts of powder samples from each batch were deposited on a TLC plate. European Pharmacopeia monographs reported two bands (one blue and one purple) of high intensity and some other weaker bands were observable in visible light. As seen in **Figure 2S (supplementary file)**, *H. sabdariffa* extracts from batches A, B, C, and E also showed two bands with high intensity as well as weaker ones. The extract of *H. sabdariffa* from batch D exhibited no bands suggesting the absence or a very weak quantity of colored compounds. At this same concentration, batches A and C revealed two bands with higher intensity than batches B and E.

The coloring assay was based on the extraction of powder of *Hibiscus* with hot water. Then the absorbances of the five solutions were recorded at 520 nm **(Table 1S, supplementary file)**. Batches A, B, C, and E showed a saturation of the absorbances and agreed with the recommendation of the European Pharmacopeia (absorbance over 0.350 for powder). In contrast, the sample from batch D exhibited an absorbance under 0.350.

The loss on drying assay evaluated whether the sample was dried sufficiently; this is useful to prevent drug degradation. The loss on drying percentage for *H. sabdariffa* powders in batches A, B, and C was below 11%, while batches D and E were above this value recommended by the Pharmacopoeia **(Figure 3S, supplementary file)**. Statistical analysis indicated that loss on drying percentage varies significantly according to the variety studied. They are all different from each other (*p* value ranging from 0.0469 to < 0.0001), except for variety B, which is not different from either A or C **(Table 2S, supplementary file)**.

The acid percentage was determined for the five batches after the neutralization of the citric acid in the aqueous extracts with sodium hydroxide. The results are summarized in **Table 1** and show that batches B, C, D, and E exhibited an acid percentage of over 13.5%, which is recommended by the European guidelines. The percentage of batch A reached 13.27%, which is slightly lower. Statistical analysis indicated that there is also a significant difference between varieties, but this time they are all different from each other (*p* value ranging from 0.0067 to < 0.0001) **(Table 3S, supplementary file)**.

The analyses of foreign matters were realized in the five batches and two types of foreign matters were found in the samples: from the fruit and other sources **(Figure 4S, supplementary file)**. All samples respect the European Pharmacopeia (Karkade Monograph, 2017) criteria i.e. the samples must not contain more than 2% of fragments from the fruits (red funicles, grey or yellow fragments of the capsules or seeds) and less than 2% of others elements **(Figure 5S, supplementary file)**.

Three assays were performed on each of the five batches of *H. sabdariffa*. The white specimens of *H. sabdariffa* were used as negative control and the results showed that they did not meet the recommendations given by the European Pharmacopeia (for coloring and desiccation assays, for TLC analysis) in contrast to the red specimens from Cameroon and Egypt. Commercial samples stored in plastic bags and sold in the pharmacy did not respect the value of desiccation as condensation could be involved. Conservation for botanical drugs should be done in paper bags far from atmospheric humidity to guarantee better sample preservation and long-term quality of plants, akin to herbarium specimens (Fournier et al., 2011). The sour taste of karkade is specific to this drink and the relatively lower percentage of acidity for both samples retrieved for the red Cameroonian cultivar explained why it was less acidic (about 15% vs over 17%). Nevertheless, the values obtained here were in the range recommended by the pharmacopeias in contrast to some samples bought in a Turkish market (Özdogan et al., 2011). In the literature, the malic acid content in fresh calyces of *H. sabdariffa* was between 0.12 to 2.70% (m/m) (Cisse et al., 2009) and other authors reported a percentage of organic acids of about 15% whatever the acid (Da-Costa-Rocha et al., 2014).

3.3. Validation of the HPLC method for anthocyanins quantification

Three calibration curves for C3OG were built after injection in HPLC-DAD at 530 nm. Linearity was validated for the range of concentration (1.9–30 µg/ml). All results are available in **Table 4S (supplementary file)**. The method for the anthocyanins quantification in C3OG equivalent was validated for all parameters.

3.4. Extraction of H. sabdariffa, identification of major anthocyanins and their quantification in cyanidin 3-O-glucoside (C3OG) equivalent

Three types of extractions were done for the five batches of *H. sabdariffa*: (i) a maceration with the organic solvent methanol, (ii) a decoction, and (iii) an infusion process, the latter two used water. The choice of the solvents was an important step because it conditioned the chemical composition of the extract as reported by Laskar and Mazumder (2020), and thus the biological activity of the extract. Vasudeva and Sharma (2008) reported for example that methanol extracts of the calyx increased the hepatic enzymes (ASAT, ALAT), while aqueous extracts remained non-toxic even at high doses. In the same manner, water or ethanolic extracts from flowers showed different antioxidant activities (Da-Costa-Rocha et al., 2014). The higher yields for aqueous extractions could be explained by the extraction not only of anthocyanins and glycosylated flavonols but also by the extraction of mucopolysaccharides, pectin, and catechins the plant contains (Da-Costa-Rocha et al., 2014).

All extracts were analyzed by High-Performance Liquid Chromatography-Diode-Array Detector (HPLC-DAD-MS) and the analysis was focused on the anthocyanins easily detected at 530 nm as seen in **Figure 6**. Four peaks were detected for the methanolic macerated extracts A, C, and E named peak 1 (Rt = 16.6 min), peak 2 (Rt = 18.1 min), peak 3 (Rt = 20.9 min), peak 4 (Rt = 22.9 min), while two peaks (corresponding to peaks 1 and 2) were extracted with water whatever the method (infusion or decoction). The mass detection allowed us to determine two major ions for peak 1 with *m/z* $[M+H]^+$ = 597 and 465, peak 2 corresponded to two ions with *m/z* 581 and 449, and peaks 3 and 4 corresponded to respectively *m/z* 303 and 287. One could be noticed that the retention time for the anthocyanins differed along with the solvent and this was demonstrated by injecting the standard 3-*O*-cyanidin glucoside. No anthocyanins were detected in extract D.

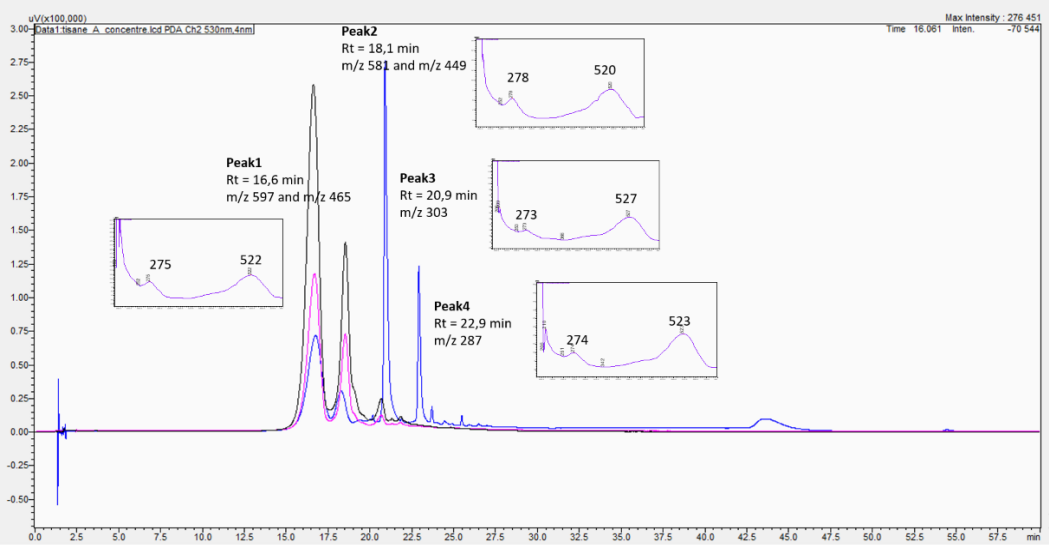

Figure 6. Chromatographic profiles of the three extracts of *H. sabdariffa* for batch A (in blue: maceration, in pink: decoction, in black: infusion)

The chromatographic data of each peak are the retention time (Rt), the mass spectra of the main fragment (*m/z*) and the UV spectra (in nm)

The mass detection allowed us to determine that peak 1 corresponded to delphinidin-3-sambubioside and delphinidin-3-glucoside, that peak 2 was cyanidin-3-sambubioside and cyanidin-3-glucoside. Peaks 3 and 4 were identified as the aglycones of delphinidin and cyanidin respectively (Schütz et al., 2006). These identifications agreed with the polarity of the solvents of extraction: the water extractions extracted the heterosides, while the organic extractions extracted the more apolar compounds, i.e. aglycones. Thanks to the mass spectrometry, delphinidin glycosides and cyanidin glycosides could be distinguished. Thus, the Cameroonian cultivar contained the major anthocyanins found in *H. sabdariffa*.

Quantification by High-Performance Liquid Chromatography–Diode Array Detector (HPLC-DAD) was calculated thanks to a calibration curve realized using the standard cyanidin-3-*O*-glucoside (C3OG). Each area under the peaks 1-4 of anthocyanins was determined and reported on the calibration curve to give a concentration in C3OG equivalent. As seen in Figure 7, the higher concentrations of anthocyanins were obtained for batch C > batch A > batch B and E whatever the mode of extraction. The extracted content was between 1.5 and 3.25 % m/m eq C3OG DM in extracts A and C and 0.16 and 0.55 % in extracts B and E (Table 2). Statistical analysis of the anthocyanin content indicated significant variations between Hibiscus varieties 2 by 2 for each given extraction method ($p < 0.0001$), except for varieties B and E for which $p < 0.05$ for decoctions and macerations, and which are not even significantly different for infusions ($p = 0.9556$). The anthocyanins content for the Cameroonian extracts was significantly higher whatever the mode of extraction (Table 5S, supplementary file).

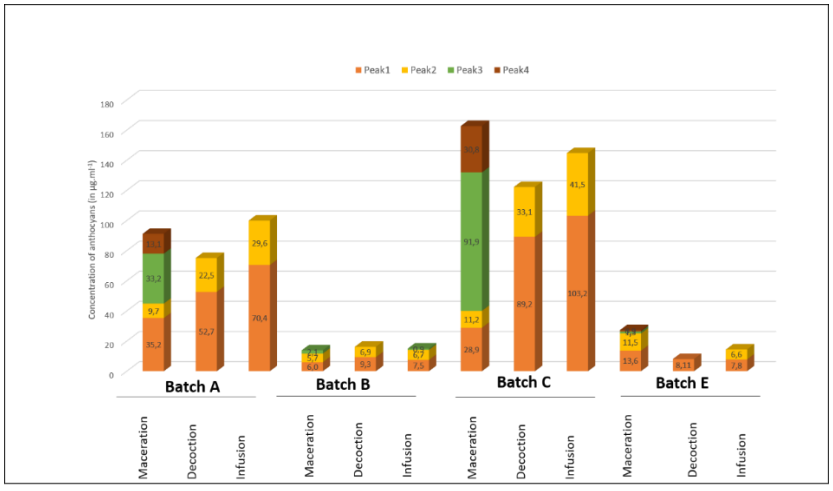

Figure 7. Concentration of the four anthocyanins in the three extracts of *H. sabdariffa* for the batches A, B, C, E (in µg/ml)

Peak 1: Delphinidin glycosides, Peak 2: Cyanidin glycosides, Peak 3: Aglycon of delphinidin, Peak 4: Aglycon of cyanidin

Table 2. Anthocyanin content in the five batches extract of *H. sabdariffa* along with the mode of extraction (mean of three assays, expressed in m/m eq C3OG (%)

	Maceration	Decoction	Infusion
Batch	Anthocyanin content (% m/m) ± δ	Anthocyanin content (% m/m) ± δ	Anthocyanin content (% m/m) ± δ
A	1.82 ± 0.06	1.50 ± 0.16	2.00 ± 0.08
B	0.35 ± 0.06	0.36 ± 0.06	0.32 ± 0.04
C	3.25 ± 0.08	2.45 ± 0.09	2.90 ± 0.05
E	0.55 ± 0.02	0.16 ± 0.01	0.29 ± 0.01

These results confirmed that the solvent of extraction was of great influence on the composition of the extracts: an alcoholic extraction allowed the extraction of more diversified anthocyanins with a great quantity of aglycons and fewer glycosides even if the yield was about the same whatever the solvent. For all batches, a slight decrease in anthocyanin content was observed for the extracts coming from decoction compared to infusion. It could come from the method of preparation because it is well-known that anthocyanins were unstable and were readily degraded after thermal or after long storage. Infusion was therefore recommended to obtain a better content of anthocyanins.

The anthocyanin quantification exhibited that the red cultivar from Cameroon (batches A and C) contained about ten-fold more content of anthocyanins than the Egyptian batches and overpassed the usual values found in the literature: 0.62% (m/m) (Riaz & Chopra, 2018) or about 0.15% (m/m) (Cisse et al., 2009). These extracts were about three-fold higher in delphinidin glycosides compared to cyanidin derivatives in agreement with the literature (Opletal et al., 2017; Riaz & Chopra, 2018). It was already described that upon drought stress, the plants could accumulate secondary metabolites and this high percentage of anthocyanins could be correlated to the place where the Folere is cultivated in semi-aridic places (Besharati et al., 2022). Jiofack et al. (2010) reported *H. sabdarrifa* cultivated in the Sudano-Sahelian region and it could be interesting to compare them.

As a result, *H. sabdariffa*, and especially this Cameroon cultivar is a huge source of anthocyanins, and greater than those fixed by the pharmacopeia for *Vitis vinifera* (0.2%) even if the advantage for the latter plant is the better stability of anthocyanins with the compounds having a hydroxyl group on C-4 position.

4. Conclusions

This *Hibiscus* cultivar cropped in Cameroon was taxonomically verified, chemically analyzed and scientifically characterized for its useful properties for the first time. Molecular analyses confirmed its identification as *H. sabdariffa* and the sequences were deposited at GenBank. All samples respected the identification tests, quantification of acidity, and several assays including desiccation, coloring, and foreign matters of the European Pharmacopeia. Moreover, the traditional extract realized as an infusion showed a very high content of delphinidin and cyanidin glycosides. This enriched source represents a great interest for companies because less herbal material is needed to realize standardized preparation for pharmacological purposes, cosmetic properties, and for coloring power. Until recently, the Arab Republic of Egypt was the only certified source country for commercial hibiscus flowers and in 2011 several producers and traders from Burkina Faso achieved certification through the FairTrade Labelling Organizations International FLO-CERT GmbH (Da-Costa-Rocha et al., 2014). We propose that Cameroon should also participate in this impulse. A political strategy supports the development of the local industry for producing drugs from medicinal plants (Fokunang et al., 2011) and this cultivar could also participate in local development communities.

Acknowledgments

The authors wish to acknowledge the Isabelle Rouaud for management of the vouchers and Solenn Ferron for assistance with chromatographic analyses.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Funding

None.

CRediT authorship contribution statement

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Supplementary File

The supplementary file accompanying this article is available at https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/19.

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