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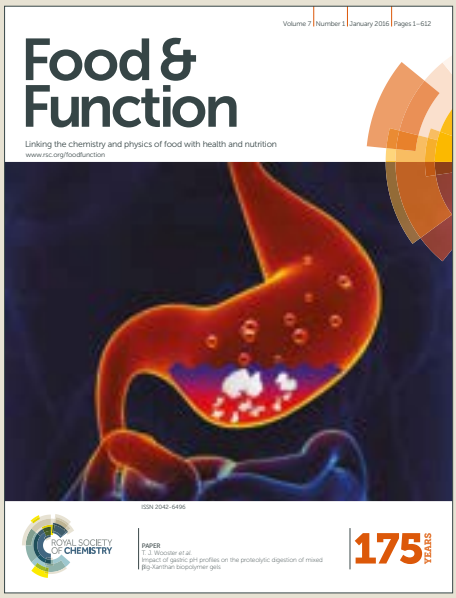

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Exploring the chemical and bioactive properties of *Hibiscus sabdariffa* L. calyces from Guinea-Bissau (West Africa)

Inès Jabeur[a,#], Eliana Pereira[a,#], Cristina Caleja[a], Ricardo C. Calhelha[a], Marina Soković[b], Luís Catarino[c], Lillian Barros[a,*], Isabel C.F.R. Ferreira[a,*]

[a]*Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal*

[b]*Institute for Biological Research "Siniša Stankovic", Department of Plant Physiology, University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia.*

[c]*Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal*

[#]Both authors contributed equally

*Corresponding authors. I.C.F.R. Ferreira: Tel.+351 273 303219; fax +351 273 325405. *E-mail address*: iferreira@ipb.pt; L. Barros: Tel.+351 273 303285; *E-mail address*: lillian@ipb.pt

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ABSTRACT

Different external factors influence the chemical characteristics of natural products, varying according to the geographic origin. The present study determined the nutritional and chemical composition of *Hibiscus sabdariffa* L. dried calyces (Guinea-Bissauan origin), as well as the phenolic composition, antioxidant, anti-inflammatory, cytotoxicity and antimicrobial activity of its infusion and hydroethanolic extracts. Among the chemical composition, glucose and quinic acid showed the major concentration for sugars and organic acids, respectively. Palmitic acid and α-tocopherol were the most abundant lipophilic compounds. The individual phenolic compounds were analysed through HPLC-DAD-ESI/MS. Thirteen compounds were identified in the hydroethanolic extract, while twelve were identified in the infusion, being 3-*O*-caffeoylquinic acid the major non-anthocyanin compound. Three anthocyanins were identified, being delphinidin-3-*O*-sambubioside the most abundant. Both extracts showed promising results in all the bioactive assays. This study exhibited beneficial properties of *H. sabdariffa* and also emphasized the potential applications of this plant in different industrial sectors.

Keywords: *Hibiscus sabdariffa* L.; antioxidant, antimicrobial activity; nutritional composition; phenolic compounds.

1. Introduction

Flowers, leaves and fruits of many plants have been used all over the world since ancient times and, in addition to the food properties, their consumption seems to be associated with different beneficial properties for consumers' health. Thus, several scientific studies have been carried out with the objective of confirming these properties in addition to assessing their nutritional quality[1]. The bioactive properties exhibited by plants have been associated with the presence of several classes of phytochemical compounds, such as phenolic

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compounds, vitamins, alkaloids, terpenoids, and natural pigments[2,3]. However, climate, geographical area, solar radiation, temperature and humidity, as well as the state of maturation, influence the profile and amount of bioactive compounds in plants[4].

Hibiscus sabdariffa L. (commonly known as roselle), is an annual or perennial plant with red stems and calyces, belonging to Malvaceae family. It is cultivated mainly in the tropical and subtropical areas of both hemispheres[5]. In Africa roselle has two main uses: as a vegetable and for preparation of a beverage[6]. In Guinea-Bissau it is known as ondjo and both leaves and calyces are widely consumed and marketed[7].

The use of this plant comes from ancestral times and is mainly used in the preparation of hot and cold beverages mainly due to its proven antioxidant function in humans, especially against chronic diseases[8,9]. These effects have been associated to the presence of some phenolic compounds namely, anthocyanins (cyanidin derivatives), flavonols (quercetin and kaempferol derivatives), phenolic acids (chlorogenic acid) and also due to the presence of a specific organic acid from *H. sabdariffa* (hibiscus acid)[10].

Several studies have been scientifically proving these bioactive properties of roselle from different geographic areas. A study by Rasheed et al.[11] revealed that roselle from Egypt had higher amounts of sugars and anthocyanins compared to the same plant of Sudan origin. The hydromethanolic extract of *H. sabdariffa* L. from Saudi Arabia demonstrated an excellent antimicrobial performance[12]. Similarly, the aqueous extract of California roselle revealed an excellent antimicrobial activity, in which Higginbotham, Burris, Zivanovic, Davidson, & Stewart[13] suggested this plant as a natural preservative, preventing the growth of pathogens in food products. Moreover, a methanol extract prepared from red calyces of roselle from Nigerian was also studied by Tolulope[14], highlighting its cytotoxic and antimicrobial capacities. Both aqueous and hydroethanolic extracts of *H. sabdariffa* with Portuguese origin showed excellent antimicrobial activity, and the hydroethanolic extract presented

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bactericidal/fungicidal inhibition ability for all bacteria and fungi tested[15]. The aim of this study was to characterize the calyces of *Hibiscus sabdariffa* L. from Guinea-Bissau, regarding its nutritional (proteins, fat, ash, carbohydrates and energy) and chemical (sugars, organic acids, tocopherols, fatty acids and phenolic acids) composition, as also its bioactive properties (antioxidant, anti-inflammatory and cytotoxicity) using aqueous and hydroethanolic extracts, and compare the results with previous studies from different geographic origins.

2. Materials and methods

2.1. Samples

The plant material used in the study, dried calyces of *H. sabdariffa* was acquired at Bandim market, Bissau, in February 2018 and transported to the laboratory CIMO, Bragança, in Portugal (**Figure 1**).

2.2. Nutritional composition

Proteins, fat, carbohydrates and ash were estimated using AOAC[16] procedures and following a procedure previously reported by Jabeur et al.[15]. Energy was estimated by applying the following equation: Energy (kcal) =4 × (g protein + g carbohydrates) + 9× (g fat).

2.3. Chemical composition

Free sugars. The free sugars were prepared using 1 g of lyophilized sample and melezitose as the internal standard (IS, 5 mg/mL). Extraction was performed with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged at 15,000*g* for 10 min (Centurion K24OR refrigerated centrifuge, West Sussex, UK) and then concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL

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and filtered through 0.2 μm Whatman nylon filters. The free sugars were analysed by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany) using the internal standard (IS, melezitose) method, according to a procedure previously described by Barros et al.[17]. The results were expressed in g per 100 g of dry weight

Organic acids. 2 g of dry sample was extracted with 25 mL of meta-phosphoric acid (25 °C, 150 rpm, 45 min) and subsequently centrifuged (10,000*g* for 5 min) and then filtered through 0.2 μm Whatman nylon filters. The organic acids were determined, according to a procedure previously described by Barros et al.[17], using an Ultra-Fast Liquid Chromatography (UFLC, Shimadzu 20A series, Kyoto, Japan) and a photodiode array detector (215 nm and 245 nm (for ascorbic acid) as preferred wavelengths). The results were expressed in g per 100 g of dry weight.

Fatty acids. After extraction by Soxhlet, the obtained fat extract was subjected to a methylation process with 5 mL of 2:1:1 (v/v/v) methanol/sulphuric acid/toluene in a water bath at 50 ºC and 160 rpm, for 12h; after this process and to obtain the phase separation, 3 mL of deionized water were added. The FAME was recovered by adding 3 mL of diethyl ether, stirring on a Vortex stirrer. The sample was collected in a Teflon flask and filtered with 0.2 μm Whatman nylon filter prior to injection. The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC-FID, DANI model GC 1000, Contone, Switzerland), following a procedure previously described by Barros et al.[17]. The results were expressed as relative percentage of each fatty acid.

Tocopherols. Tocopherols were extracted by adding to the sample (500 mg), 100 μL of the BHT solution in hexane (10 mg/mL) and 400 μL of the internal standard solution in hexane (tocol; 50 μg/mL). Afterword's, the samples were homogenized with 4 mL of methanol by mixing in a vortex (1 min). Subsequently, 4 mL of hexane was added and vortexed again (1

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min). Then, 2 mL of saturated aqueous NaCl solution was added and the mixture was homogenized (1 min), centrifuged (5 min, 4000 g) and the clear top layer was carefully transferred to a flask. Finally, the sample was re-extracted twice with hexane and the extracts were brought to dryness under a stream of nitrogen. They were then re-dissolved in 2 mL of hexane, dehydrated with anhydrous sodium sulphate and finally filtered through a 0.2 μm Whatman nylon filter. Tocopherols were determined by HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, USA) programmed for excitation at 290 nm and emission at 330 nm, using the IS (tocol) method for quantification, following a method previously described by Barros et al.[17]. The results were expressed in mg per 100 g of dry weight.

2.4. Extracts preparation

The hydroethanolic extract was prepared by macerating with agitation (150 rpm) the dried sample (1 g) with 30 mL of ethanol/water (80:20 v/v, for the anthocyanin identification 0.5% of TFA was added to the extracting solvent), at 25 °C for 1 h. After filtration (Whatman No. 4 paper) the residue was extracted with an additional 30 mL of the same solution (25 °C at 150 rpm) for 1 h. The combined extracts were evaporated (rotary evaporator Büchi R-210, Germany) at 40 °C, frozen, and further lyophilized.

The infusions were prepared by adding boiling distilled water (100 mL, at 100 °C) to 1 g of the dry sample, following the procedure described by Jabeur et al.[15]. Afterwards, the mixtures were left to stand for 5 min at room temperature, filtered, frozen and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

The hydroethanolic and infusion extracts were respectively, re-dissolved in ethanol/water (80:20 v/v) and water. For the phenolic compounds characterization, a concentration of 10 mg/mL was used, thus for the anthocyanin identification 0.1% of TFA was added to the

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ethanol/water mixture and water. For the antioxidant and antimicrobial assays, a concentration of 10 mg/mL and 100 mg/mL, respectively, were the stock solution, while 8 mg/mL was used for the cytotoxicity and anti-inflammatory evaluation. The stock solutions were diluted to different concentrations to be submitted to different *in vitro* bioactivity evaluation assays.

2.5. Identification and quantification of phenolic compounds

The phenolic compounds (non-anthocyanin and anthocyanin compounds) were separated, identified, and quantified using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA), following a previously described procedure[15]. The detection was performed with a DAD (280, 330, 370, and 520 nm, as preference wavelengths) and in a mass spectrometer (LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) working in negative mode for non-anthocyanin compounds and positive mode for anthocyanin compounds. Analytical curves (200-5 μg/mL) of the available phenolic standards were constructed based on the UV-Vis signal: chlorogenic acid (y = 168823x - 161172; R^2 = 0.9999; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL); quercetin 3-*O*-glucoside (y = 34843x – 160173; R^2 = 0.9998; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL); and cyanidin-3-*O*-glucoside (y = 134578x –3E+06; R^2 = 0.9986; LOD = 0.25 μg/mL; LOQ = 0.83 μg/mL). The results were expressed as mg/g of extract.

2.6. Evaluation of the antioxidant activity

Four colorimetric assays: DPPH radical-scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition, were performed following the procedures previously described by Barros et al.[17]. Trolox was used as a positive control and

the results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay).

2.7. Evaluation of the anti-inflammatory activity

The anti-inflammatory activity was accessed following a protocol previously described by Correa et al.[18]. Dexamethasone (50 μM) was used as a positive control and the results were expressed as EC_{50} values (μg/mL), corresponding to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production).

2.8. Evaluation of cytotoxicity

The cytotoxic methodology was accessed following a procedure described by Barros et al. [17]. HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung cancer) were the human tumor cell lines used to evaluate the cytotoxic properties of the extracts. To evaluate the hepatotoxicity of the extracts a porcine liver primary cell culture established in our laboratory (PLP2) was applied. The cell growth inhibition was measured using sulforhodamine B assay. Ellipticine was used as positive control and the results were calculated as GI_{50} values, corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.

2.9. Evaluation of the antimicrobial activity

The antibacterial and antifungal was evaluation following a methodology previously described by Carocho et al.[19] and Jabeur et al.[15], using four Gram-negative and Gram-positive bacteria (*Bacillus cereus, Micrococcus flavus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Enterobacter cloacae, Pseudomonas aeruginosa,*

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Salmonella typhimirium), and eight microfungi (*Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Trichoderma viride, Penicillium funiculosum, Penicillium ochrochloron, Penicillium verrucosum var. cyclopium*). The minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MBF) concentrations were determined using the microdilution method.

2.10. Statistical analysis

Three samples were used, and all the assays were carried out in triplicate, being the results expressed as mean values and standard deviation (SD). In order to determine the significant difference among the different extracts, with $\alpha = 0.05$, the results were analysed using a Student´s t-test (SPSS v. 23.0 program).

3. Results and discussion

3.1. Nutritional and chemical characterization

Plants are daily subject to several external factors that can cause changes in their composition and bioactive activities. Different studies regarding the effects of drought and osmotic stress on the polyphenols composition in distinct species have demonstrated that these compounds could increase or decrease (depending on the species), with the type and intensity of stress[20]. Some studies have revealed that some environmental factors such as UV radiation, blue light, high light intensity, wounding, pathogen attack, drought, and nutrients deficiency have an influence on the accumulation of anthocyanins in plants[21,22]. In addition to all these external factors, all the parameters applied during the extraction (temperature, time and percentage of solvent) also have a great influence on the amount of extracted compounds[23]. Therefore, it is of utmost importance to study different species of plants submitted to different conditions of

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cultivation and growth, and therefore different stress conditions, in order to understand how these differences may influence the chemical and nutritional composition.

Table 1 presents the results of the nutritional composition of *H. sabdariffa* calyces, showing that carbohydrates were the most abundant macronutrient. This would be expected, considering other studies found in literature of *H. sabdariffa*, which also mention carbohydrates as the main macronutrients, although some variations in the concentrations were observed[15,24]. These small differences found between the various works studying the same plant species, but with different origins, may be related as previously mentioned with environmental factors specific to each region, as well as harvesting, drying and storage conditions.

Fructose, glucose, sucrose, and threalose were the free sugars found in the sample, and glucose was the most abundant followed by fructose (**Table 1**). In a previous work[15] of Portuguese *H. sabdariffa*, glucose was also the most abundant sugar, despite being found in a slightly lower amount. Thus, the amount of total sugars present in *H. sabdariffa* from Guinea-Bissau (7.80±0.01 g/100 g dw) was lower than the quantity presented in the Portuguese *H. sabdariffa* sample (11.1±0.9 g/100 g dw). According to some authors, exposure to water stress causes an increase in sugar content in some fruit and vegetables. Specifically, a study with *H. sabdariffa* calyces proved the effect of seasonal variation in organic acids and sugars, demonstrating that in general the content of sugars and organic acids were higher during the dry season[20]. Therefore, these considerations could be taken into account to justify the observed differences found between the samples.

Table 1 presents the organic acids identified and quantified in *H. sabdariffa* calyces. The most representative organic acids were quinic acid followed by malic acid. Portuguese *H. sabdariffa* did not reveal the presence of quinic acid and presented malic acid as the most abundant organic acid. The total amount of organic acids was much higher in the Guinea-

Bissauan sample in comparison with the Portuguese origin[15]. Also, a review study of *H. sabdariffa* revealed citric acid, hydroxycitric acid, hibiscus acid, malic acid, and tartaric acid as the main organic acids found in aqueous extracts of *H. sabdariffa* (from Egypt, Senegal, India, Thailand and Central America), whereas oxalic acid and ascorbic acid were shown as minor components[24]. These observed differences might be due to different response to environment conditions, genetics, ecology, as also harvest conditions[25].

For tocopherols identification, only α and β isoforms were identified in the *H. sabdariffa* samples and the most abundant isoform was α-tocopherol (**Table 1)**. Tocopherols are important compounds that have been highly studied due to their association with their antioxidant activity. Some studies report the direct relationship between the consumption of tocopherols and the reduction of oxidative stress and the prevention of certain diseases. The identification of tocopherols in the tested parts (seed, leaf, stem and sepals) of *H. sabdariffa* cultivated at laboratory scale was previously performed by Mohamed et al.[26], revealing the absence of β-tocopherol in all the plant parts. These authors also concluded that the seeds presented the highest amount of total tocopherols, revealing the presence of α, γ, and δ-tocopherols, and the sepals were the parts that presented the lowest concentration. Nevertheless, in the present study, as also in a previous study performed by Jabeur et al.[15], α- and β- tocopherol were the only isoforms identified. Given the importance of this vitamin it is interesting to note that the various parts of roselle have good amounts of the different isoforms.

Finally, the last part of **Table 1** presents the eighteen fatty acids identified and their respective percentages in the sample under study. The major fatty acids found were palmitic acid (C16:0), followed by linoleic (C18:2n6) and oleic (C18:1n9) acids. The sample revealed a greater predominance of saturated fatty acids (SFA), followed by polyunsaturated fatty acids (PUFA), and monounsaturated fatty acids (MUFA). Other studies in literature also refer to the

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predominant presence of these three fatty acids in *H. sabdariffa*[24, 27]. The study developed by Jabeur et al.[15] revealed a higher percentage of linoleic acid than the one obtained in the present study, which may be justified by the predominant levels of PUFA, nevertheless, a similar fatty acid profile was observed. Once again it is possible to verify that the origin of the samples has a great influence in the chemical composition of *H. sabdariffa*.

3.2. Phenolic compounds quantification

Table 2 presents the peak characteristics, tentative identities and quantification of the phenolic compounds in *H. sabdariffa* hydroethanolic and infusion extracts. Identification of the phenolic compounds was carried out and crossed checked through their chromatographic characteristics, such as retention time, mass spectrum, and UV-Vis absorption. A total of thirteen different phenolic compounds were detected, five phenolic acids and flavonols, and three anthocyanins. The hydromethanolic extract presented all the identified compounds, while the infusion revealed the presence of twelve molecules, revealing the absence of caffeic acid. In general, we can observe that the hydroethanolic extraction seems to have favored the extraction of phenolic compounds, both flavonoids and non-anthocyanic compounds. In contrast, it was verified that the infusion was the ideal methodology for the anthocyanin's extraction, having presented an amount of 36.7±0.4 mg/g extract in comparison to the hydroethanolic extract that showed a lower amount of 26.6±0.3 mg/g extract. The phenolic compounds were identified considering the previous findings by Jabeur et al.[15], as also those described in previous studies of *H. sabdariffa* [28,29]. Phenolic acids were present in higher amounts, mostly due to the presence of *trans* 3-*O*-caffeoylquinic acid, and the hydroethanolic extract revealed the highest concentration (44.8±0.9 mg/g extract). Flavonols were found in lower amounts, being all the detected compounds present in similar quantities, thus hydroethanolic extracts revealed a slightly higher amount (5.01±0.01 mg/g extract) compared

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to the infusion (4.70±0.01 mg/g extract). Both extracts, hydroethanolic and infusion, presented *trans* 3-*O*-caffeoylquinic acid as the major compound, being present in a higher amount in the hydroethanolic extract (32±1 mg/g extract) than in the infusion (20.3±0.3 mg/g extract).

Ifie et al.[20] compared the phenolic compounds content in *H. sabdariffa* grown in two different seasons in Ibadan, concluding that growing season had a great effect on the amounts of anthocyanins and other constituents. These authors revealed that the anthocyanins content was significantly higher during the dry season, while the concentrations of 3-*O*-caffeoylquinic acid and myricetin 3-*O*-arabinogalactoside were higher in the humid season. The ethanolic extract of different varieties of Mexican roselle were chemically characterized by Borrás-Linares et al.[10] and they reported the presence of a great variety of phenolic compounds (including phenolic acids and quercetin glycosides as the major flavonols).

Anthocyanins are characteristic compounds of *H. sabdariffa* and as reported in **Table 2**, both tested extracts revealed the presence of three anthocyanins, delphinidin-3-*O*-sambubioside, cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-glucoside, in which the first molecule was the major anthocyanin in both studied extracts. Contrary to the previously described phenolic compounds, all anthocyanins appeared in higher amounts in the infusion extract (36.7±0.4 mg/g extract), in comparison to the hydroethanolic extract (26.6±0.3 mg/g extract), thus leading to statistically significant differences between both preparations. This could be due to the fact that anthocyanins are more soluble in water than ethanol, and in addition, it is interesting to note that despite the application of a higher temperature in the infusion extraction, this did not contribute to the degradation of anthocyanins relatively to hydroethanolic extraction. Moreover, a previous study which aimed to optimize the extraction of phenolic compounds from *H. sabdariffa* calyces by microwave-assisted extraction, proved that different factors, such as temperature, time, and solvents proportion, can cause changes in

the quantitative composition of bioactive compounds[23]. Thus, these factors could highly influence the concentration of these molecules.

Delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside have been described as predominant anthocyanin compounds in the aqueous extracts of dried calyces of *H. sabdariffa* from different origins[30,31]. Sindi, Marshall, & Morgan[32] identified delphinidin-3-*O*-sambubioside, delphinidin3-*O*-glucoside, cyaniding-3-*O*-sambubioside, and cyanidin-3-*O*-glucoside in water and methanol extracts of *H. sabdariffa*. The presence of these compounds in the calyx of *H. sabdariffa* been also described as being responsible for the red color of this plant[10].

3.3. Bioactive properties

3.3.1 Antioxidant activity

Regarding the antioxidant activity evaluation of the hydroethanolic and the infusion extracts, four different *in vitro* tests (DPPH radical-scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition) were performed and the results obtained are shown in **Table 3**. As it can be observed, the extract obtained by infusion presents lower EC_{50} values in the four *in vitro* tests, than the hydroethanolic extract, thus revealing a higher antioxidant activity, which may be related to its higher content in anthocyanins[33]. There are several studies (*in vitro*, *in vivo* and human) that widely describe anthocyanins and classify them as excellent antioxidants[34,35]. Cácedas et al.[36] classified cyanidin-3-*O*-glucoside as one of the most important anthocyanins in nature with a very strong antioxidant activity. Anthocyanins have been associated with the antioxidant action, due to their deficiency in electrons and consequent reactivity to oxygen species (ROS). Some studies have shown that anthocyanins have greater antioxidant power in comparioson to several antioxidant compounds, such as catechol and Trolox[37].

Excellent antioxidant activity has been reported in several *in vitro*[38-40] and *in vivo*[38,41,42] studies using different *H. sabdariffa* extracts. The bioactive potential of this plant has also been reported in the different plant parts, such as flowers, seeds, and leaves[43]. A study using twenty-five samples of roselle samples from different origins (Cuba, Georgia, Ghana, India, Jamaica, Malaysia, Poland, Senegal, South Africa, Sudan, Taiwan, Thailand, and Zambia) showed a variation in the antioxidant activity between 101.5 ± 17.5 to 152.5 ± 18.8 μmol Trolox/g[44], thus these results cannot be compared to the herein study. The study developed by Jabeur et al.[15] showed higher EC_{50} values than those obtained in the present study, for all the tested assays. In this way, we can verify that the origin also influences the antioxidant activity demonstrated by the plant.

3.3.2 Anti-inflammatory activity and cytotoxicity

Table 3 also presents the results regarding the anti-inflammatory activity and cytotoxicity in tumor and non-tumor cell lines. Nevertheless, only the hydroethanolic extract presented anti-inflammatory activity. In relation to the cytotoxic potential of these extracts, the infusion only revealed activity only against the HeLa line; nevertheless, the hydroethanolic extract presented a lower GI_{50} value, which therefore reveals a greater activity. In addition, the hydroethanolic extract also showed activity against the HepG2 cell line. None of the extracts revealed toxicity against the non-tumor cell line (PLP2, porcine liver cells), up to the maximal tested concentration (400 μg/mL).

The greater anti-inflammatory and cytotoxic potential of the hydroethanolic extract could be related to its higher concentration in phenolic acids, especially *trans* 3-*O*-caffeoylquinic acid. Moreover, the excellent anti-inflammatory and cytotoxic activity of *H. sabdariffa* extracts has been previously reported, being associated with the presence of various phenolic compounds[24,45]. Although these studies could point to a possible relationship between the

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presence of phenolic compounds and the display of bioativities, we consider that it would be pertinent to carry out further studies to prove the direct relationship between the presence of some of these phenolic compounds, namely *trans* 3-*O*-caffeoylquinic acid, with the displayed activities, such as anti-inflammatory and cytotoxic potential.

3.3.3. Antibacterial and antifungal properties

The antibacterial and antifungal activities of *H. sabdariffa* hydroethanolic and infusion extracts were tested against a panel of eight bacteria and fungi strains selected on the basis of their relevance to public health, and the results are presented in **Table 4**. The two extracts revealed bactericidal (MBC) and fungicidal (MFC) effects for all the tested bacteria and fungi. *Bacillus cereus*, *Trichoderma viride*, *and Aspergillus ochraceus* presented the lowest MIC values for both extracts. In general, both extracts revealed a good antibacterial and antifungal activity in comparison with the control standards. The infusion extracts were also studied in a previous work performed by Jabeur et al.[15], thus these extracts did not show inhibitory capacity for the several studied strains. The authors attributed the differences found to their phenolic compounds, since there was a great difference between the studied extracts. In the present study the highest MICs were presented against *Micrococcus flavus, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa*, and *Salmonella typhimirium* in both extracts, although these values were lower for infusion, highlighting that this extract had a higher antibacterial potential.

4. Conclusions

H. sabdariffa is consumed worldwide because it is associated with various beneficial properties for the human health, related with the presence of different nutritional and bioactive compounds. The antioxidant, cytotoxic, antimicrobial properties were demonstrated

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in this study for both of the studied extracts (infusion and hydroethanolic). These properties maybe related to the presence of some very interesting compounds such as α-tocopherol, organic acids (quinic acid as the major), and phenolic compounds, such as 3-*O*-caffeoylquinic and anthocyanins (such as delphinidin-3-*O*-sambubioside the most abundant). Comparing the different studies with roselle, we can conclude that the different plant parts and/or its origin have a great influence on the nutritional, chemical and bioactive properties. This study demonstrates the high potential of the species highlighting its usage as a functional beverage and source of nutraceuticals and natural pigments, giving an add value for future applications in food, pharmaceutical and textile industries, for example.

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		Hibiscus sabdariffa L. calyces	Fatty acids (%)	*Hibiscus sabdariffa* L. calyces
Nutritional value (g/100 g dw)	Proteins	9.14 ± 0.06	C6:0	0.41 ± 0.01
	Ash	12.3 ± 0.5	C8:0	0.14 ± 0.04
	Fat	0.77 ± 0.07	C10:0	0.22 ± 0.01
	Carbohydrates	77.8 ± 0.5	C11:0	0.641 ± 0.004
	Energy (Kcal/100 g dw)	355 ± 2	C12:0	0.304 ± 0.005
Sugars (g/100 g dw)	Fructose	4.4 ± 0.2	C13:0	0.035 ± 0.003
	Glucose	7.80 ± 0.01	C14:0	1.70 ± 0.01
	Sucrose	1.81 ± 0.05	C15:0	0.9 ± 0.01
	Threalose	1.53 ± 0.03	C16:0	34.03 ± 0.04
	Total soluble sugars	15.6 ± 0.1	C17:0	1.36 ± 0.03
Organic acids (g/100 g dw)	Oxalic	0.58 ± 0.02	C18:0	7.1 ± 0.4
	Quinic	13.65 ± 0.01	C18:1n9	14.3 ± 0.2
	Malic	6.29 ± 0.04	C18:2n6	23.7 ± 0.4
	Citric	0.40 ± 0.01	C18:3n3	7.5 ± 0.2
	Succinic	0.87 ± 0.05	C20:0	1.1 ± 0.2
	Fumaric	0.0081 ± 0.0002	C22:0	5.1 ± 0.1
	Total organic acids	21.80 ± 0.06	C23:0	0.75 ± 0.01
Tocopherols (mg/100 g dw)	α-tocopherol	0.200 ± 0.004	C24:0	0.73 ± 0.04
	β-tocopherol	0.170 ± 0.003	**SFA**	54.5 ± 0.4
	Total tocopherols	0.37 ± 0.01	**MUFA**	14.3 ± 0.2
			PUFA	31.2 ± 0.2

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Table 1. Nutritional and chemical composition of *Hibiscus sabdariffa* L. calyces.

Values expressed in mean values ± standard deviation (n=9); C6:0 caproic acid; C8:0 caprylic acid; C10:0 Capric acid; C11:0 undecylic acid; C12:0 lauric acid; C13:0 tridecanoic acid; C14:0 myristic acid; C15:0 pentadecanoic acid; C16:0 palmitic acid; C17:0 decanoic acid; C18:0 stearic acid; C18:1n9 oleic acid; C18:2n6 linoleic acid; C18:3n3 linolenic acid; C20:0 arachidic acid; C22:0 behenic acid; C23:0 tricosanoic acid; C24:0 lignoceric acid; SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the UV–Vis region (λmax), mass spectral data, identification and quantification of phenolic compounds in *Hibiscus sabdariffa* L. lyophilized hydroethanolic and infusion extracts (mean ± SD).

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Hydroethanolic (mg/g extract)	Infusion (mg/g extract)	*t*-Students test *p*-value
Non-anthocyanins								
1	4.6	324	353	191(100),179(45),173(5),161(3),135(12)	*cis* 3-*O*-Caffeoylquinic acid[1]	8.9±0.1	2.01±0.07	<0.0001
2	4.94	325	353	191(100),179(43),173(5),161(3),135(8)	*trans* 3-*O*-Caffeoylquinic acid[1]	32±1	20.3±0.3	<0.0001
3	6.4	322	353	191(12),179(48),173(100),155(3),135(8)	4-*O*-Caffeoylquinic acid[1]	0.98±0.01	0.88±0.03	0.002
4	6.9	325	353	191(100),179(12),173(8),161(3),155(3),135(3)	5-*O*-Caffeoylquinic acid[1]	2.8±0.1	1.53±0.04	<0.0001
5	9.43	320	179	135(100)	Caffeic acid[1]	0.32±0.01	nd	-
6	13.45	350	611	449(52),371(100)	Myricetin-*O*-sambubioside[2]	1.00±0.02	0.935±0.001	0.002
7	15.47	350	595	463(27),301(100)	Quercetin-*O*-sambubioside[2]	1.06±0.01	0.96±0.01	<0.0001
8	17.25	350	609	301(100)	Quercetin-3-*O*-rutinoside[2]	1.00±0.01	0.94±0.01	<0.0001
9	18.44	350	463	301(100)	Quercetin-3-*O*-glucoside[2]	0.98±0.01	0.9291±0.0001	<0.0001
10	20.47	340	593	285(100)	Kaempferol-3-*O*-rutinoside[2]	0.97±0.01	0.933±0.001	<0.0001
					Total phenolic acids	44.8±0.9	24.8±0.5	<0.0001
					Total flavonols	5.01±0.01	4.70±0.01	<0.0001
					Total non-anthocyanin phenolic compounds	49.8±0.9	29.5±0.5	<0.0001
Anthocyanins								
Peak	**Rt (min)**	**λmax (nm)**	**$[M+H]^+$ (*m/z*)**	**MS^2 (*m/z*)**	**Tentative identification**	**Hydroethanolic (mg/g extract)**	**Infusion (mg/g extract)**	***t*-Students test *p*-value**
11	16.42	524	597	303(100)	Delphinidin-3-*O*-sambubioside[3]	18.1±0.1	23.7±2.8	<0.0001
12	17.83	523	465	303(100)	Delphinidin-3-*O*-glucoside[3]	2.56±0.04	3.00±0.03	<0.0001
13	21.14	517	581	287(100)	Cyanidin-3-*O*-sambubioside[3]	6.0±0.2	7.8±0.8	<0.0001
					Total anthocyanins	26.6±0.3	36.7±0.4	<0.0001

nd - not detected; Values expressed in mean values ± standard deviation (n=9); Analytical curves used for quantification results: 1- chlorogenic acid; 2- quercetin 3-*O*-glucoside; 3- cyanidin-3-*O*-glucoside.

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Table 3. Bioactivity assays of *Hibiscus sabdariffa* L. calyces.

	Hibiscus sabdariffa L. calyces			Positive Control
	Hydroethanolic	Infusion	*t-Students test p-value*	
Antioxidant activity (EC_{50} values, mg/mL)				Trolox
DPPH scavenging activity	0.43 ± 0.01	0.37 ± 0.01	<0.0001	41 ± 1
Reducing power	0.54 ± 0.01	0.466 ± 0.002	<0.0001	18 ± 1
β-cartotene bleaching inibition	0.87 ± 0.08	0.50 ± 0.03	<0.0001	42 ± 1
TBARS inhibition	0.072 ± 0.001	0.068 ± 0.004	0.010	23 ± 1
Anti-inflammatory activity (EC_{50} values, µg/mL)				Dexamethasone
RAW 267.4	306 ± 10	>400	-	16 ± 1
Cytotoxicity (GI_{50} values, µg/mL)				Ellipticine
MCF-7 (breast carcinoma)	>400	>400	-	1.00 ± 0.04
NCI-H460 (non-small cell lung cancer)	>400	>400	-	1.0 ± 0.1
HeLa (cervical carcinoma)	250 ± 13	321 ± 9	<0.0001	2.0 ± 0.1
HepG2 (hepatocellular carcinoma)	334 ± 10	>400	-	1.0 ± 0.2
Hepatotoxicity (GI_{50} values, µg/mL)				
PLP2	>400	>400	-	3 ± 1

The antioxidant activity was expressed as EC_{50} values, which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Cytotoxicity results are expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. Anti-inflammatory activity is expressed as EC_{50} values corresponding to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). Trolox, dexamethasone and ellipticine, respectively, were used as positive controls in the assays. All values are expressed as means ± SD (n = 9).

Table 4. Antimicrobial activities of infusion and hydroethanolic extracts of *Hibiscus sabdariffa* L. calyces (mg/mL).

		Antibacterial activity							
		B.c.	***M.f.***	***S.a.***	***L.m.***	***E.c.***	***En.cl.***	***P.a.***	***S.t.***
Hydroethanolic	**MIC**	0.15	0.45	0.45	0.45	0.20	0.30	0.45	0.45
	MBC	0.30	0.60	0.60	0.60	0.90	0.60	0.60	0.60
Infusion	**MIC**	0.10	0.30	0.30	0.30	0.20	0.15	0.30	0.45
	MBC	0.30	0.60	0.60	0.60	0.300	0.30	0.60	0.60
Streptomycin	**MIC**	0.10	0.20	0.04	0.20	0.20	0.20	0.20	0.25
	MBC	0.20	0.30	0.10	0.30	0.30	0.30	0.30	0.50
Ampicillin	**MIC**	0.25	0.25	0.25	0.40	0.40	0.25	0.75	0.40
	MBC	0.40	0.40	0.45	0.50	0.50	0.50	1.20	0.75
		Antifungal activity							
		A.fum.	***A.v.***	***A.o.***	***A.n.***	***T.v.***	***P.f.***	***P.o.***	***P.v.c.***
Hydroethanolic	**MIC**	0.45	0.10	0.15	0.60	0.075	0.30	0.30	0.30
	MFC	0.90	0.15	0.30	0.90	0.15	0.60	0.60	0.60
Infusion	**MIC**	0.60	0.15	0.04	0.60	0.075	0.15	0.20	0.60

	MFC	1.20	0.30	0.075	1.20	0.15	0.30	0.60	1.20
Ketoconazole	**MIC**	0.25	0.20	1.50	0.20	1.00	0.20	2.50	0.20
	MFC	0.50	0.50	2.00	0.50	1.00	0.50	3.50	0.30
Bifonazole	**MIC**	0.15	0.10	0.15	0.15	0.15	0.20	0.20	0.10
	MFC	0.20	0.20	0.20	0.20	0.20	0.25	0.25	0.20

B.c.: *Bacillus cereus*; M.f.: *Micrococcus flavus*; S.a.: *Staphylococcus aureus*; L.m.: *Listeria monocytogenes*; E.c.: *Escherichia coli*; En.cl.: *Enterobacter cloacae*; P.a.: *Pseudomonas aeruginosa*; S.t.: *Salmonella typhimirium*; A.fum.: *Aspergillus fumigatus*; A.v.: *Aspergillus versicolor*; A.o.: *Aspergillus ochraceus*; A.n.: *Aspergillus niger*; T.v.: *Trichoderma viride*; P.f.: *Penicillium funiculosum*; P.o.: *Penicillium ochrochloron*; P.v.c.: *Penicillium verrucosum var. cyclopium*. MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration and MFC- minimum fungicidal concentrat

Figures

Figure 1 – *Hibiscus sabdariffa* from Guinea-Bissau. *A* – plant with mature edible flowers; *B* – calyces drying on the ground; *C* - sale of dried calyces (ondjo) in Bandim market, Bissau.

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Graphical abstract

Exploring the chemical and bioactive properties of *Hibiscus sabdariffa* L. calyces from Guinea-Bissau (West Africa)

Inès Jabeur, Eliana Pereira, Cristina Caleja, Ricardo C. Calhelha, Marina Soković, Luís Catarino, Lillian Barros, Isabel C.F.R. Ferreira

***Hibiscus sabdariffa* L. calyces:**

- Nutritional composition;
- Chemical (free sugars, organic acids, fatty acids and tocopherols) composition.

Infusion and hydroethanolic extracts:

- Phenolic composition;
- Antioxidant, anti-inflammatory, cytotoxicity and antimicrobial activity.