Hibiscus sabdariffa L. as a source of nutrients, bioactive compounds and colouring agents

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ABSTRACT

The nutritional and bioactive composition of plants have aroused much interest not only among scientists, but also in people's daily lives. Apart from the health benefits, plants are a source of pigments that can be used as natural food colorants. In this work, the nutritional composition of Hibiscus sabdariffa L. was analysed, as well as its bioactive compounds and natural pigments. Glucose (sugar), malic acid (organic acid), α-tocopherol (tocopherol) and linoleic acid (fatty acid) were the major constituents in the corresponding classes. 5-(Hydroxymethyl) furfural was the most abundant non-anthocyanin compound, while delphinidin-3-O-sambubioside was the major anthocyanin both in its hydroethanolic extract and infusion. H. sabdariffa extracts showed antioxidant and antimicrobial activities, highlighting that the hydroethanol extract presents not only lipid peroxidation inhibition capacity, but also bactericidal/fungicidal inhibition ability for all the bacteria and fungi tested. Furthermore, both extracts revealed the absence of toxicity using porcine primary liver cells. The studied plant species was thus not only interesting for nutritional purposes but also for bioactive and colouring applications in food, cosmetic and pharmaceutical industries.

1. Introduction

Plants are rich sources of several classes of bioactive compounds that have been reported to be responsible in the prevention and treatment of chronic health pathologies such as hypertension, cardiovascular diseases, inflammation and cancer (Barata, Rocha, Lopes, & Carvalho, 2016; Bresciani et al., 2015). Plants are also now being explored as sources of natural pigments to be used as food colorants, safer, with minimal toxic effects and displaying beneficial properties such as antioxidant and antimicrobial activities. Among the compounds present in plants with the potential to be used as colouring ingredients, anthocyanins emerge as promising molecules (Martins, Roriz, Morales, Barros, & Ferreira, 2016).

Hibiscus sabdariffa L., also known as roselle, is an annual, herbaceous medicinal plant that belongs to the Malvaceae family. Despite being native to Asia, it is also widely cultivated in many areas, including Central America and Africa. This species is usually cultivated for its fibers and calyces, and includes three different genotypes: green, red (which is the most common type used) and dark red (Barhé & Tchouya, 2015). The calyx is commercially important in the food industry for the production of beverages and foods such as tea, juices, jams, jellies and syrup (Borrás-Linares et al., 2015). The herbal tea of Hibiscus is commonly used around the world, as a sole ingredient in herbal infusions. In many countries, the leaves are also consumed as a leafy green vegetable (Zhen et al., 2016). Overall, there are several reports available on the consumption of the dried or fresh calyces, seeds and leaves of H. sabdariffa used in the preparation of herbal medicines, beverages and fermented drinks or even cooked raw (Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014). It is also rich in bioactive compounds such as anthocyanins and other flavonoids, organic acids and polysaccharides which are responsible for its antioxidant, antibacterial, anti-inflammatory, hepatoprotective and anticholesterol activities (Da-Costa-Rocha et al., 2014; Formagio et al., 2015). The composition of H. sabdariffa in terms of phenolic compounds has been previously analysed by some authors (Borrás-Linares et al., 2015; Christian & Jackson, 2009). Moreover, Borrás-Linares et al. (2015), chemically characterized the H. sabdariffa ethanolic extract, reporting the presence of a great variety of phenolic compounds and organic acids, such as citric acid, hibiscus acid, hydroxycitric acid and protocatechuic acid, being the main phenolic acid present the latest

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mentioned compound, otherwise, quercetin glycosides and gossypetin were the major flavonoids found.

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 colouring additives in the food industry. The calyx of *H. sabdariffa* has been reported to contain delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-sambubioside and cyanidin-3-sambubioside, being these compounds responsible for the red colour of this plant (Borrás-Linares et al., 2015). The intense red colour and some of the health promoting benefits of *H. sabdariffa* extracts, has triggered scientists to be more interested in exploiting this plant to obtain natural colouring ingredients for numerous applications in food industry (Frimpong, Adotey, Ofori-Kwakye, Lugrie-Kipo, & Dwomo-Fokuo, 2014). Aside from their well reported colouring potential, these compounds have displayed bioactive properties such as antioxidant, anti-inflammatory, antibacterial, antitumor and hepatoprotective potential and as such they can act as multifunctional ingredients; as natural colorants as well as health promoting ingredients (Liu, Chang, Liu, & Shen, 2016; Vagiri & Jensen, 2017). Thus, the multifunctional properties (colorant and bioactive properties) of *H. sabdariffa* can be explored in the food and pharmaceutical industry, as natural ingredients to be incorporated into the food products (e.g. as a multifunctional ingrediente) and pharmaceutical industries (e.g. as a natural colorante and for its bioactive properties).

To the best of our knowledge, there is no research article in literature that reports a complete study on the chemical composition and bioactive properties of *Hibiscus sabdariffa* L. calyces. Thus, the present study provides the nutritional composition, and chemical characterization, regarding sugars, tocopherols, fatty acids, organic acids and phenolic compounds. Furthermore, bioactive properties, regarding antioxidant and antimicrobial activity of *H. sabdariffa* calyces were further explored. These results will demonstrate the multiple functional applications of this plant species, namely as a source of bioactive compounds, with antioxidant, antimicrobial activity and as a natural source of natural colouring compounds.

2. Materials and methods

2.1. Samples

Dried aerial parts of *Hibiscus sabdariffa* L., namely flowers and inflorescences, were provided by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal), where the samples were air dried in order to obtain a constant weight measurement. The samples were reduced to a fine dried powder (~ 20 mesh), and mixed to obtain homogenous samples.

2.2. Standards and reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acid methyl ester (FAME) reference standard mixture (standard 47,885-U), L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(−)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/mL, was purchased from Matreya (Plesant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar. Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were supplied by Sigma Chemical Co. (Saint Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Nutritional composition

The protein, fat, carbohydrates and ash content were estimated using the AOAC (2016) procedures. The crude protein (N × 6.25) was determined by the Kjeldahl method (991.02); the ash content (935.42) was estimated by subjecting the sample to incineration at 600 ± 15 °C for 5 h, while the crude fat was determined using a Soxhlet apparatus with petroleum ether as recycling solvent (989.05) and total carbohydrate was estimated by difference. The total energy was calculated using the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

Free sugars were analysed by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), according to a procedure previously described by Pereira, Barros, and Ferreira (2015). Data were analysed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic) (fructose: LOD = 0.05 mg/mL; LOQ = 0.18 mg/mL; glucose: LOD = 0.08 mg/mL; LOQ = 0.25 mg/mL), and the results were expressed in g per 100 g of dry weight.

The organic acids were determined using the Ultra Fast Liquid Chromatography (UFLC, Shimadzu 20A series, Kyoto, Japan) and a photodiode array detector, according to a procedure previously described by Dias et al. (2013). The separation was achieved on a SphereClone reverse phase C18 column thermostatted at 35 °C (oxalic acid: LOD = 12.55 μg/mL; LOQ = 41.82 μg/mL; malic acid: LOD = 35.76 μg/mL; LOQ = 119.18 μg/mL; LOQ = μg/mL; fumaric acid: LOD = 0.08 μg/mL; LOQ = 0.26 μg/mL). The results were expressed in g per 100 g of dry weight.

The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC-FID/capillary column, DANI model GC 1000, Contone, Switzerland), a split/splitless injector and a Macherey–Nagel column. The methodology followed a procedure previously described by Pereira et al. (2015) and the fatty acids were identified by comparing the relative retention times of FAME peaks from samples with commercial standards. The results were treated using the chromatography station for Windows CSW (version 1.7) software from DataApex (Podohradska, Czech Republic) and expressed in relative percentages.

The tocopherols were determined according to a method previously described by Pereira et al. (2015). A HPLC coupled to a fluorescence detector and programmed for excitation at 290 nm and emission at 330 nm, was used. The quantification was carried out based on calibration curves obtained from commercial standards of each compound (α-tocopherol: LOD = 18.06 ng/mL; LOQ = 60.20 ng/mL; β-tocopherol: LOD = 25.82 ng/mL; LOQ = 86.07 ng/mL) and racemic tocol was used as the internal standard. The results were expressed in mg per 100 g of dry weight.

2.4. Non-anthocyanin and anthocyanin compounds

2.4.1. Extraction preparation

The hydroethanolic extract was prepared by using the dry sample (1 g) macerated with 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then additionally extracted with 30 mL of the same solution (25 °C at 150 rpm) for 1 h. The combined extracts were evaporated at 40 °C and further lyophilized.

An infusion was also prepared by adding boiling distilled water (100 mL, at 100 °C) to 500 mg of the dry sample. Afterwards, it was let to stand for 5 min at room temperature and subsequently, filtered and then lyophilized according to a procedure described by Pires, Dias, Barros, and Ferreira (2017).

The lyophilized hydroethanolic and infusion extracts were re-dissolved in ethanol/water (80:20 v/v) and water at 10 mg/mL,

respectively, for phenolic profile evaluation.

2.4.2. HPLC-DAD-ESI/MSn analysis

For both non-anthocyanin and anthocyanin compounds the chromatographic data was acquired from a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA). This system consists of a diode array detector coupled to an electrospray ionization mass detector (LC-DAD-ESI/MSn), a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostated column section (kept at 35 °C) and a Waters Spherisorb S3 ODS-2 C_{18} (3 μm, 4.6 × 150 mm, Waters, Milford, MA, USA) column provided chromatographic separations. For non-anthocyanin compounds, the solvents used were (A) 0.1% formic acid in water and (B) acetonitrile. The gradient elution applied was: 15% B (0–5 min), 15% B to 20% B (5–10 min), 20–25% B (10–20 min), 25–35% B (20–30 min), 35–50% B (30–40 min), and the column was then re-equilibrated, using a flow rate of 0.5 mL/min and with an injection volume of 10 μL.

For anthocyanin compounds, the solvents used were: (A) 0.1% trifluoroacetic acid in water and (B) acetonitrile. The gradient elution followed these parameters: 10% B for 3 min, from 10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min. Sixty minutes was the resulting total run time, followed by column reconditioning of 10 min, using a flow rate of 0.5 mL/min and with an injection volume of 10 μL. With a DAD, data were collected for both non anthocyanin compounds (280 nm and 370 nm) and anthocyanin compounds at 520 nm. In a mass spectrometer (MS) detection (Linear Ion Trap LTQ XL mass spectrometer, ThermoFinnigan, San Jose, CA, USA) negative mode was chosen for non-anthocyanin compounds whereas positive mode was selected for anthocyanin compounds. Retention times, UV-VIS and mass spectra were compared with available standards and with literature data, in order to identify the compounds. Calibration curves of available phenolic standards were constructed based on the UV signal to perform quantitative analysis. In the case of unavailable commercial standards, the compounds were quantified via calibration curve of the most similar standard available. The results were expressed as mg/g of dry extract.

2.5. Bioactivity evaluation

The hydroethanolic and infusion lyophilized extracts were, respectively, re-dissolved in ethanol/water (80:20 v/v) and water at 10 mg/mL for antioxidant and antimicrobial assays, and at 8 mg/mL for cytotoxicity evaluation. The stock solutions were diluted to different concentrations to be submitted to different in vitro bioactivity evaluation assays.

The antioxidant activity (DPPH radical-scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition) was evaluated by colorimetric assays, previously described by Jabeur et al. (2016). Trolox, a water-soluble analogue of vitamin E, was used as a positive control. 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).

The antibacterial activity was performed following a methodology previously described by Carocho et al. (2015). *Enterobacter cloacae* (ATCC 35030), *Salmonella typhimurium* (ATCC 13311), *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC (American type culture collection) 35210), were the tested Gram-negative bacteria, while *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973), *Micrococcus flavus* (ATCC 10240), *Bacillus cereus* (clinical isolate) and *Staphylococcus aureus* (ATCC 6538) were the tested Gram-positive bacteria. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by using the microdilution method.

For the antifungal activity, the procedure previously described by Carocho et al. (2015) was followed by using these microfungi: *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730),

Aspergillus niger (ATCC 6275), *Aspergillus fumigatus* (ATCC 1022), *Trichoderma viride* (IAM-Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *P. verrucosum* var. *cyclopium* (food isolate). Minimum inhibitory (MIC) and minimum fungicidal (MBF) concentrations were also determined by using the microdilution method.

A porcine liver primary cells culture, established in our laboratory (PLP2), was used to evaluate the hepatotoxicity of the extracts, following a previously described methodology (Rodrigues et al., 2012). The results were expressed in GI_{50} values, i.e. the extract concentration that inhibited 50% of the net cell growth. Ellipticine was used as positive control.

2.6. Statistical analysis

Three samples were used to obtain the extracts also in triplicate, and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD) and analysed using a Student's *t*-test, in order to determine the significant difference among the different extracts (hydroethanolic or infusion), with α = 0.05. This treatment was made using SPSS v. 23.0 program.

3. Results and discussion

3.1. Nutritional properties

The results of the nutritional composition of *Hibiscus sabdariffa* L. are shown in Table 1. Carbohydrates were the most abundant macronutrients, followed by ash, proteins and fat. The nutritional profile of *H. sabdariffa* was reviewed by Ismail, Ikram, and Nazri (2008); despite some slight differences in the concentration of several macronutrients, which can be attributed to environmental factors, carbohydrates were also the most abundant macronutrients, followed by proteins and fat.

Fructose and glucose were the two free sugars found in the sample, glucose being the most abundant molecule (6.5 g/100 g dw). According to other authors, these monosaccharides show benefits related with prolonged exercise performance (Rosset, Egli, & Lecoultre, 2017), due to the fact that these molecules induce a spectrum of effects on the muscle metabolism, possibly resulting in an improved performance (Rosset et al., 2017).

Oxalic, malic, shikimic and fumaric acids were the main organic acids identified and quantified (Table 1), malic acid (9.10 g/100 g dw) being the most abundant one. This acid has been reported to present some health benefits, namely its capacity to reduce the risk of metal poisoning, privilege fluidity, support the maintenance of oral health and increase immunity (Hossain, Akhtar, & Anwar, 2015). Therefore, this plant species could be industrially exploited for production of malic acid as well as its derivatives due to their medicinal benefits (Miglio, Veglia, & Fantozzi, 2015; Lin-Holderer, Li, Gruneberg, Marti, & Kunze, 2016). Nevertheless, other organic acids were previously described on this species, such as, citric, hydroxycitric, hibiscus, tartaric and ascorbic acids (Da-Costa-Rocha et al., 2014), which were not present in the herein studied sample. The observed differences could be related with the production of different metabolites in response to environment changes, so the same plant species grown in different geographic areas may produce different compounds (Santos, Manuela, & Saraiva, 2016).

α- and β-Tocopherols were the tocopherol isoforms found in *H. sabdariffa*, with α- isoform being the most abundant (39.19 mg/100 g dw; Table 1). Tocopherols have received increased attention because of their well-reported antioxidant activity, being consumed as an exogenous source of antioxidants to reduce oxidative stress and to prevent several chronic diseases (Shahidi & Camargo, 2016). Up to 18 fatty acids were also identified (Table 1), in which polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Linoleic acid (C18:2n6) was the

Table 1

Nutritional composition of *Hibiscus sabdariffa* L. (mean ± DS).

Nutritional value		Fatty acids (%)	
Ash (g/100 g dw)	7.4 ± 0.5	Caproic acid (C6:0)	0.40 ± 0.02
Proteins (g/100 g dw)	5.5 ± 0.4	Caprylic acid (C8:0)	0.14 ± 0.01
Fat (g/100 g dw)	0.47 ± 0.08	Capric acid (C10:0)	0.220 ± 0.001
Carbohydrates (g/100 g dw)	87 ± 1	Lauric acid (C12:0)	0.47 ± 0.03
Energy (kcal/100 g dw)	373 ± 2	Myristic acid (C14:0)	1.24 ± 0.01
Sugars Content (g/100 g dw)		Pentadecanoic acid (C15:0)	0.84 ± 0.01
Fructose	4.6 ± 0.3	Palmitic acid (C16:0)	27.73 ± 0.02
Glucose	6.5 ± 0.6	Palmitoleic acid (C16:1)	1.32 ± 0.04
Sum	11.1 ± 0.9	Heptadecanoic acid (C17:0)	1.28 ± 0.06
Organic acids Content (g/100 g dw)		Stearic acid (C18:0)	4.46 ± 0.01
Oxalic acid	1.81 ± 0.05	Oleic acid (C18:1n9)	9.1 ± 0.1
Malic acid	9.10 ± 0.09	Linoleic acid (C18:2n6)	32.65 ± 0.07
Shikinic acid	0.356 ± 0.002	α-Linolenic acid (C18:3n3)	15.76 ± 0.04
Fumaric acid	0.043 ± 0.002	Arachidic acid (C20:0)	1.02 ± 0.05
Sum	11.31 ± 0.04	Eicosenoic acid (C21:0)	0.21 ± 0.02
Tocopherols (mg/100 g dw)		Behenic acid (C22:0)	1.40 ± 0.02
α-Tocopherol	39.19 ± 0.06	Tricosanoic acid (C23:0)	0.67 ± 0.01
β-Tocopherol	0.76 ± 0.06	Lignoceric acid (C24:0)	1.08 ± 0.02
Sum	39.95 ± 0.01	SFA	41.2 ± 0.1
		MUFA	10.4 ± 0.2
		PUFA	48.4 ± 0.1

SFA- Saturated fatty aids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids.

major fatty acid (32.65%) in the sample and, consequently, contributes to the higher levels of PUFA. This molecule displays health-promoting benefits in cancer prevention, reduction in body fat causing decrease in obesity, anti-inflammatory properties and resolving the severity of atherosclerosis and diabetes (Yang et al., 2015). Other PUFAs have been reported to display multiple physiological functions such as promoting normal human metabolism, immunomodulation and prevention of cardiovascular diseases and cancer (Pelliccia et al., 2013).

In sum, this study will provide a complete chemical characterization, due to the fact that to the best of our knowledge, neither sugars, nor tocopherols or fatty acids have been previously described in *H. sabdariffa*.

3.2. Phenolic characterization

Peak characteristics, tentative identities and phenolic compounds quantification are presented in Table 2. A chromatographic profile of the anthocyanin profile of *H. sabdariffa* hydroethanol extract is presented in Fig. 1.

Compounds 4 (5-*O*-caffeoylquinic acid), 7 (quercetin-3-*O*-rutinoside), 8 (quercetin-3-*O*-glucoside) and 9 (kaempferol-3-*O*-rutinoside) were positively identified by comparison with authentic standards, as also by their MS fragmentation pattern, retention time and UV-VIS characteristics. Compound 4 was previously identified by Peng et al. (2011) in the methanol extract of *H. sabdariffa* dried calyx. Beltran-Debon et al. (2010), reported the presence of compounds 7 and 8 in infusions prepared from the calyces of *H. sabdariffa*, while Herranz-López et al. (2012) described the presence of compound 9 in aqueous and ethanol extracts prepared from dried calyces of *H. sabdariffa*.

Compound 1 ([M-H]$^-$ at m/z 353) was identified as 3-*O*-caffeoylquinic acid based on its fragmentation pattern, yielding a base peak at m/z 191 (deprotonated quinic acid) and an ion at m/z 179 [caffeic acid-H]$^-$ with an intensity of 47% of the base peak, as reported by Clifford, Johnston, Knight, and Kuhnert (2003); Clifford, Johnston, Knight, and Kuhnert (2005). Similarly, compound 3 ([M-H]$^-$ at m/z 353) was tentatively identified according to its MS^2 fragmentation as 4-*O*-caffeoylquinic acid (Clifford et al., 2003; Clifford et al., 2005). Meanwhile, compound 2 ([M-H]$^-$ at m/z 127) consisted of a 5-(hydroxymethyl) furfural, taking into account the findings described by Zhen et al. (2016), who studied the phenolic compounds profile of *H. sabdariffa* leaves. These same authors also reported the presence of the different

chlorogenic acids described above (neochlorogenic, chlorogenic and cryptochlorogenic acids).

Compounds 5 ([M-H]$^-$ at m/z 611) and 6 ([M-H]$^-$ at m/z 595) presented UV spectra (λ$_{max}$ 350–354 nm) and the production of a unique fragment ion at m/z 317 ([myricetin-H]$^-$) and 301 ([quercetin-H]$^-$), releasing − 294 μ, that could correspond to a pentosyl (− 132 μ) and hexosyl (− 162 μ) moiety. Therefore, these compounds were tentatively identified as myricetin-*O*-pentosylhexoside and quercetin-*O*-pentosylhexoside, respectively.

The anthocyanin compounds (peaks 10–13) were identified taking into account the identifications performed by Abdel-Moemin (2016) and Sinela et al. (2017), being identified as delphinidin-3-*O*-sambubioside (peak 10), delphinidin-3-*O*-glucoside (peak 11) and cyanidin-3-*O*-sambubioside (peak 13). The most abundant compounds present in both extracts were 5-(hydroxymethyl) furfural and delphinidin-3-*O*-sambubioside, as also described by Abdel-Moemin (2016) and Sinela et al. (2017). According to several studies, these two compounds have been previously reported to have bioactive potential. 5-(Hydroxymethyl) furfural has shown pharmacological effects, such as antioxidant, antiischemic, and antityrosine enzyme effects, improving blood rheology, and affecting the role of glycyrrhizin metabolism (Zhao et al., 2013). Also, delphinidin has demonstrated several biological activities, such as antioxidant, antimutagenesis, anti-inflammatory and anti-angiogenic properties. The mechanism of action of delphinidin acts through the vascular endothelial growth factor receptor-2 phosphorylation inhibition, platelet-derived growth factor ligand/receptor signaling, cancer cell proliferation and modulation of Met receptor phosphorylation (Patel, Jain, & Patel, 2013). Nonetheless, to the best of our knowledge, myricetin glycoside derivatives and 5-(hydroxymethyl) furfural (the main compound) were not previously described in *H. sabdariffa* dried calyx.

3.3. Bioactive properties

The antioxidant activity of *H. sabdariffa* hydroethanol and infusion extracts was evaluated using four different in vitro assays. The results are shown in Table 3. Both extracts revealed antioxidant potential, thus presenting statistically significant differences (*p-value* < 0.05) between both extracts in all the assays. The highest antioxidant activity (lowest EC$_{50}$ values) for DPPH and reducing power was obtained for infusions (1.26 and 0.54 mg/mL, respectively). Otherwise, the

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)	λ_{max} (nm)	[M-H]⁻ (m/z)	MS² (m/z)	Tentative identification	Hydroethanolic (mg/g extract)	Infusion (mg/g extract)	t-Students test p-value
Non-anthocyanins								
1	4.89	328	353	191(100),179(80),173(5),161(16),135(20)	3-O-Caffeoylquinic acid[1]	2.6 ± 0.1	2.88 ± 0.03	0.005
2	5.23	285,330sh	127	109(100)	5-(hydroxymethyl)furfural[2]	5.75 ± 0.08	4.8 ± 0.2	< 0.001
3	6.9	328	353	191(78),179(34),173(100),161(5),135(10)	4-O-Caffeoylquinic acid[1]	1.44 ± 0.08	1.00 ± 0.02	< 0.001
4	7.34	328	353	191(100),179(87),173(3),161(5),135(12)	5-O-Caffeoylquinic acid[1]	1.53 ± 0.06	1.14 ± 0.01	< 0.001
5	13.2	350	611	317(100)	Myricetin-pentosylhexoside[3]	0.961 ± 0.001	0.951 ± 0.003	0.001
6	16.14	354	595	301(100)	Quercetin-pentosylhexoside[4]	1.031 ± 0.002	0.987 ± 0.001	< 0.001
7	17.95	356	609	301(100)	Quercetin-3-O-rutinoside[4]	1.07 ± 0.01	1.00 ± 0.01	< 0.001
8	19.15	350	463	301(100)	Quercetin-3-O-glucoside[5]	tr	tr	–
9	21.27	347	593	285(100)	Kaempferol-3-O-rutinoside[6]	tr	tr	–
					Total phenolic acids	5.602 ± 0.004	5.01 ± 0.02	< 0.001
					Total flavonoids non-anthocyanins	4.007 ± 0.003	2.94 ± 0.01	< 0.001
					Other compounds	5.75 ± 0.08	4.8 ± 0.2	< 0.001
Peak	Rt (min)	λ_{max} (nm)	[M + H]⁺ (m/z)	MS² (m/z)	Tentative identification	Hydroethanol extract (mg/g extract)	Infusion (mg/g extract)	t-Students test p-value
Anthocyanins								
10	30.9	525	597	303(100)	Delphinidin-3-O-sambubioside[7]	7.03 ± 0.04	7.0 ± 0.2	0.535
11	33.4	527	465	303(100)	Delphinidin-3-O-glucoside[7]	1.54 ± 0.01	1.296 ± 0.001	< 0.001
12	37.2	518	581	287(100)	Cyanidin-3-O-sambubioside[7]	4.40 ± 0.02	4.08 ± 0.07	< 0.001
					Total anthocyanins	12.96 ± 0.06	12.3 ± 0.3	0.007

tr-traces; nd-not detected. Calibration curves used: 1-chlorogenic acid (y = 168823x − 161,172; R^2 = 0.999); 2-p-hydroxybenzoic acid (y = 208,604x + 173,056; R^2 = 0.999); 3-myricetin-3-O-glucoside (y = 23,287x − 581,708; R^2 = 0.999); 4-quercetin-3-O-rutinoside (y = 13,343x + 76,751; R^2 = 0.999); 5-quercetin-3-O-glucoside (y = 34,843x − 160,173; R^2 = 0.999); 6-kaempferol-3-O-rutinoside (y = 11,117x + 30,861; R^2 = 0.999);7-cyaniding-3-O-glucoside (y = 243,287x − 1E + 06; R^2 = 0.995).

hydroethanol extract revealed the highest antioxidant activity (1.95 and 0.26 mg/mL, respectively), for the β-carotene bleaching and TBARS formation inhibition assays (both related with lipid peroxidation processes).

In order to obtain this beneficial effect with the consumption of this plant as an infusion, and considering the highest mentioned EC_{50} values (results obtained from the inhibition of β-carotene bleaching inhibition) and extraction yields, a portion of 6.2 mg of *H. sabdariffa* calyces/mL would be necessary. Therefore, consumers when preparing a tea cup of *H. sabdariffa* calyces in the normal recommended doses (1 g in 100 mL, as suggested in tea packaging labels), will be incorporating 1.6 times the EC_{50} value obtained for antioxidant activity, which could provide this bioactivity. Regarding the extract, to obtain the beneficial effects, its consumption could be obtained by its incorporation in a food product, in which the highest concentration obtained in the antioxidant activity, could be used to estimate the amount to be incorporated in the product.

Different authors have previously described in vitro and in vivo antioxidant properties of *H. sabdariffa* extracts using different extraction solvents. Tsai, McIntosh, Pearce, Camden, and Jordan (2002) tested this action in water and methanol extracts, while Borrás-Linares et al. (2015) used a maceration with acidified ethanol. Mohd-Esa, Hern, Ismail, and Yee (2010) evaluated the aqueous extract and 80% (v/v) methanol extract, by studying the β-carotene bleaching inhibition and the DPPH radical-scavenging activity, while Sindi, Marshall, and Morgan (2014) used water, methanol, ethyl acetate or hexane extract and described a strong correlation between the antioxidant activity and the phenolic content.

To the best of our knowledge, none of the previous mentioned studies used an ethanol/water extraction mixture, which is considered a green solvent. It should be also be highlighted that, in the present study, no toxicity (tested in porcine liver cells) was observed for *H. sabdariffa* hydroethanol and infusion extracts, up to the maximal tested concentration of 400 μg/mL (Table 3). These assays were performed according with the National Cancer Institute guidelines.

The results on antibacterial and antifungal activities of *H. sabdariffa* hydroethanol and infusion extracts are presented in Table 4. The samples were tested against a panel of eight bacteria and fungi strains,
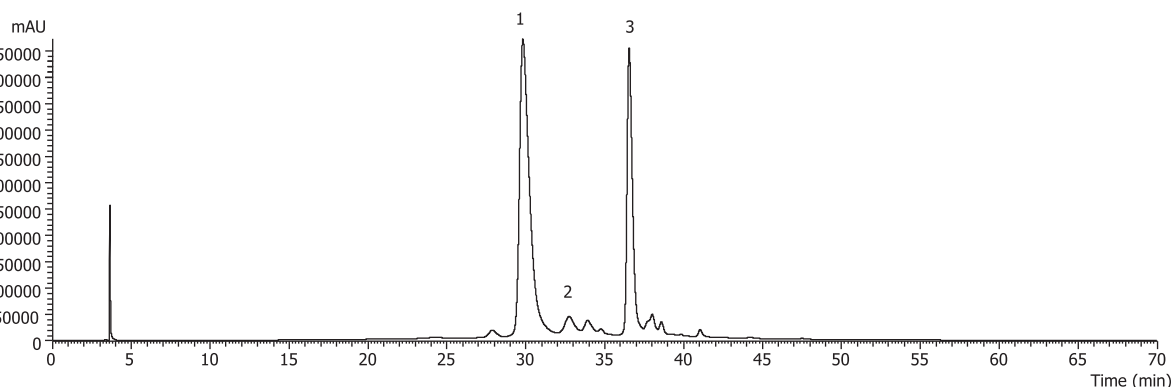

Fig. 1. Anthocyanins profile of *Hibiscus sabdariffa* L. hydroethanolic extract, recorded at 520 nm.

Table 3
Antioxidant activity of *Hibiscus sabdariffa* L. lyophilized hydroethanolic and infusion extracts (mean ± SD).

	Hydroethanolic	Infusion	t-Students test p-value
Antioxidant activity (EC$_{50}$ values, mg/mL)			
DPPH scavenging activity (RSA)	1.37 ± 0.04	1.26 ± 0.05	< 0.001
Reducing power (RP)	0.603 ± 0.002	0.54 ± 0.01	< 0.001
β-Carotene bleaching inhibition (CBI)	1.95 ± 0.05	4.2 ± 0.2	< 0.001
TBARS formation inhibition (LPI)	0.26 ± 0.01	0.27 ± 0.01	0.015
Hepatotoxicity (GI$_{50}$ values, mg/mL)			
PLP2	> 0.4	> 0.4	–

EC$_{50}$ values: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox (positive control) EC$_{50}$ values: 41 μg/mL (reducing power), 42 μg/mL (DPPH scavenging activity), 18 μg/mL (β-carotene bleaching inhibition) and 23 μg/mL (TBARS inhibition). GI$_{50}$ values: Extract concentration that inhibited 50% of the net cell growth in PLP2. Ellipticine (positive control) GI$_{50}$ values: 2.29 μg/mL.

Table 4
Antibacterial (MIC and MBC mg/mL) and antifungal (MIC and MFC mg/mL) activities of *Hibiscus sabdariffa* L. lyophilized hydroethanolic and infusion extracts.

Antibacterial activity

		B.c.	M.f.	S.a.	L.m.	E.c.	En.cl.	P.a.	S.t.
Hydroethanolic	MIC	0.15	0.20	0.15	0.15	0.20	0.15	0.15	0.15
	MBC	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Infusion	MIC	–	–	0.20	0.20	–	0.30	0.20	0.20
	MBC	–	–	0.30	0.30	–	–	0.30	0.30
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	0.30	0.10	0.15	0.30	0.075	0.20	0.15	0.20
	MFC	0.45	0.15	0.30	0.45	0.15	0.45	0.30	0.45
Infusion	MIC	0.3	0.3	–	0.3	0.3	–	0.3	0.3
	MFC	–	–	–	–	–	–	–	
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 concentration.

particularly selected on the basis of their relevance to public health.

The hydroethanol extract revealed the same bactericidal effect for all the tested bacteria, but the lowest MIC (minimum growth inhibitory concentrations) values were observed against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Salmonela typhimurium*. No growth inhibitory activity was observed for the infusion extract against *Bacillus cereus*, *Micrococcus flavus* and *Escherichia coli*, and no bactericidal effect was provided on *Enterobacter cloacae*, despite the capacity to inhibit its growth.

The hydroethanol extract showed antifungal activity against all the tested strains with fungicide effects in all cases. *Trichoderma viride* was the most susceptible strain to this extract (MIC = 0.075 mg/mL and MFC = 0.15 mg/mL). The same fungicidal effect was obtained for

Aspergillus versicolor (0.15 mg/mL). Otherwise, no fungicidal activity was achieved by the *H. sabdariffa* infusion for any of the studied strain, despite its capacity to inhibit the growth of *Aspergillus fumigatus*, *A. versicolor*, *A. niger*, *Trichoderma viride*, *Penicillium ochrochloron* and *P. verrucosum* strains (MICs = 0.3 mg/mL).

The differences observed for the antimicrobial activity using the hydroethanolic extract and infusion could be attributed to the different concentrations of the identified phenolic compounds present in both extracts, which can also be ascribed to the different solvents' polarity used to obtain the extract. Moreover, in general both antibacterial and antifungal properties revealed a similar concentration range of inhibition.

A portion of 0.67 mg of *H. sabdariffa* dry calyces/mL would be necessary to obtain the beneficial effects of the infusion, considering the highest mentioned MFC values and extraction yield, and taking into account the previously mentioned considerations. Therefore, consumers when preparing a tea-cup of infusions will be incorporating 15 times the necessary concentration to provide this bioactivity. As mentioned above to obtain this effect, for example in a food product, the extract, would have to be incorporated taking into account the highest obtained concentration (e.g. MFC), obtained in the antimicrobial activity in order to estimate the amount to be incorporated in the product.

There are previous reports on the antimicrobial activity of *H. sabdariffa* calyce extracts obtained with several kinds of extracts. Higginbotham, Burris, Zivanovic, Davidson, and Stewart (2014) evaluated the antimicrobial potential of aqueous extracts, while Borrás-Linares et al. (2015) used an acidified ethanol extract, and Abdallah (2016) tested this action in methanolic extracts. Moreover, Wong, Lim, and Chan (2010) also described its antimicrobial activity against Gram-positive and Gram-negative bacteria, using methanol extracts of leaves and calyces of different Hibiscus species. Elmanama, Alyazji, and Abu-Gheneima (2011) studied the methanol and water extracts of *H. sabdariffa*, obtained by a Soxhlet extraction, against a panel of fungi and bacteria, revealing a higher antifungal effect.

In sum, the antimicrobial properties of *H. sabdariffa* calyces could be attributed to the richness in phytochemical metabolites, such as phenolic acids (protocatechuic acid) and anthocyanins (delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside), as also described by other authors (Gutiérrez-Alcántara et al., 2016; Liu, Tsao, & Yin, 2005).

4. Conclusions

Overall, medicinal plants intervene in different systems of the organism providing several preventive and therapeutic actions, due to the presence of a diversity of nutritional and bioactive compounds. *H. sabdariffa* revealed the presence of several interesting compounds, such as tocopherols, phenolic acids and flavonoids, including three different anthocyanins. Furthermore, lyophilized hydroethanolic and infusion extracts prepared from this plant exhibited antioxidant, antibacterial and antifungal properties. The obtained results highlight the potential of this species to be used as a source of bioactive and natural colouring ingredients for exploitation in food and pharmaceutical industries, among others, besides its value as functional herbal beverage.

Acknowledgements

Conflict of interest

The authors declare they have no conflict of interest.

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