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Hibiscus sabdariffa anthocyanins-rich extract: Chemical stability, *in vitro* antioxidant and antiproliferative activities

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ABSTRACT

Hibiscus sabdariffa calyx is a rich source of anthocyanins and other bioactive compounds but no study reported the effects of experimental conditions on the extraction of these chemical compounds. Therefore, the effects of time and extraction temperature on the bioactive compounds and antioxidant activity of *Hibiscus sabdariffa* calyx were evaluated. In addition, the effects of copigmentation and pH on the stability of anthocyanins were assessed and the cytotoxic effects (LC_{50}, IC_{50}, and GC_{50}) of the extracts were determined in relation to tumor cell lines - Caco-2, HepG-2, HCT8, and A549. The temperature significantly influenced the total anthocyanins and flavonoids contents. The interaction between time/temperature influenced the total phenolic content and ascorbic acid. The $t_{1/2}$ and the percentage of colour retention decreased markedly at temperatures above 80 °C. Variations in pH conserved the antioxidant activity of the anthocyanins, and the protonation-deprotonation process of the extract was reversible. The treatment of cells with purified anthocyanin extract or crude extracts at 5–800 μg mL^{-1} did not show significant cytotoxic effects on the cell lines, corroborating the chemical antioxidant effect of the extracts (DPPH assay). Cyanidin-3-glucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-sambubioside were identified in the extracts by LC-ESI-MS.

1. Introduction

Teas are used in different industrial sectors and of those that have been studied *Hibiscus sabdariffa* L. (Roselle) deserves special attention because of its chemical composition and functional properties (Higginbotham et al., 2014). There are more than 300 cultivated species of *Hibiscus* sp. and *Hibiscus sabdariffa* is widely consumed worldwide because of its considerable content of bioactive compounds, specifically flavonoids (Sindi et al., 2014).

Roselle calyxes have been used as a basis for infusions, jams, jellies, sauces and fermented products (Gradinaru et al., 2003). *In vitro* studies have already shown the ferric reducing antioxidant activity and free-radical scavenging activity in relation to 2,2-diphenyl-1-picrylhydrazyl, DPPH, and 2,2′-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals, and the oxygen radical absorbance capacity, ORAC, of the extracts from *H. sabdariffa* (Fernández-Arroyo et al., 2011; Mohd-Esa et al., 2010; Owoade et al., 2015). Furthermore, delfinidin-3-sambubioside has been shown to induce the apoptosis of HL-60 cells through a pathway of mitochondrial dysfunction that is mediated by reactive oxygen species. Beltran-Debon et al. (2010) observed that extract of *H. sabdariffa* protected mononuclear cells from H_2O_2-induced death. Studies have shown that extracts of *H. sabdariffa* have presented therapeutic effects regarding the prevention of atherosclerosis and oxidative stress, antibacterial, antioxidant, and hepato-protective effects, regulation of the lipid metabolism, as well as anti-diabetic and anti-hypertensive effects (Da-Costa-Rocha et al., 2014; Lin et al., 2015).

In terms of extraction, the yield of the target compound must be maximized, with the minimum of degradation. Anthocyanins are soluble in polar solvents and in the presence of HCl or organic acids (Naczk and Shahidi, 2004). The lower the particle size the greater the content of anthocyanins in the extract (Cissé et al., 2012). Factors such as temperature and time, as well as the type and volume of solvent, have an influence on the yield of anthocyanins. Mathematical models can accurately describe isolated effects as well as combinations of factors; they can also be used to maximize a functional extract (Zhang et al., 2006; Pedro et al., 2016). Heat treatment is a method that is used

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to preserve food; however, it is a factor that also affects the stability of anthocyanins, which are degraded when exposed to high temperatures. The flavylium cation is stable at acidic pH values but with increasing pH levels the anthocyanins become colourless, and at pH > 6 the rupture of the heterocyclic ring may occur (Patras et al., 2010). Thus, the study of the effect of temperature on extraction is relevant for technological purposes.

Intermolecular copigmentation can occur through the association of two or more anthocyanin molecules, and with phenolic compounds, proteins, sugars, nucleic acids, organic acids and minerals. The intramolecular association occurs when the pigment and the copigment are part of the same molecule. Anthocyanins derived from flowers present a higher degree of glycosylation and acylation, as well as greater stability, compared to those derived from fruits and vegetables (Castañeda-Ovando et al., 2009). These factors lead to effective protection against the nucleophilic attack of water molecules (Gradinaru et al., 2003; Grajeda-Iglesias et al., 2016).

Taking into consideration the fact that the food industry is constantly looking for natural colourings, with low toxicity and enhanced functional properties when regularly consumed as a part of a balanced and healthy diet, it is important to assess the effects of time and temperature on the extraction of the main compounds responsible for colour, i.e., anthocyanins. Consequently, in this work, the response surface methodology was used to evaluate the effects of time and extraction temperature on the bioactive compounds and antioxidant activity of *Hibiscus sabdariffa* calyx. In addition, the effects of copigmentation and pH on the stability of anthocyanins were assessed and the *in vitro* cytotoxicity properties of the extracts (Caco-2, HepG-2, HCT8, and A549 cells) were determined.

2. Materials and methods

2.1. Chemicals and cell lines

The solutions were prepared with ultra pure water (Millipore). The reagents were as follows: gallic acid, Folin-Ciocalteu's phenol reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), ethanol, chlorogenic acid, phytic acid, Amberlite®XAD7HP resin, DMEM/Ham-F12 glucose, and streptomycin penicillin/100 µg (Sigma-Aldrich, USA). Sodium carbonate, aluminium chloride, and HCl (Merck, Germany). Metaphosphoric, sulfuric, trifluroacetic and ascorbic acids, DTCS (2,4-dinitrophenyl hydrazine), thiourea, copper (II) sulfate, potassium hydroxide and sodium acetate (Vetec, Brazil). Formic acid and ethyl acetate (Synth, Brazil). Human hepatoma carcinoma cells (HepG-2), colorectal carcinoma epithelial cells (Caco-2), human colon carcinoma (HCT8), and lung adenocarcinoma epithelial cells (A549) were obtained from the Rio de Janeiro cell bank (BCRJ, Brazil).

2.2. Vegetal material and extraction procedure

The calyxes of *H. sabdariffa* L. were cultivated in São Paulo State (Brazil) in May 2015 and authenticated by Linea Verde Alimentos LTDA (Brazil). They were ground in an analytical mill (QUIMIS-Q298A21) and sieved to obtain 60 Tyler mesh particles. A 2^2 central, rotational, composite design was used to evaluate the effect of the combination of the factors of time and temperature. The temperatures were 40, 60 and 80 °C and the times were 10, 20 and 30 min; these values were established from the initial experiments (data not shown). The design was composed of 10 combinations (Table 1), with two central points to estimate the pure error. The experiments, including the extractions, were performed randomly and in true triplicates. The extraction procedure was conducted according to Lees and Francis (1972) and Pedro et al. (2016) using a thermostated cell, which was protected from light, under constant stirring. The extracts were analyzed up to 48 h after extraction.

2.3. Total anthocyanins, flavonoids, and total phenolics

The anthocyanins (λ = 535 nm) and flavonoids (λ = 374 nm) were quantified at pH = 1 according to Lees and Francis (1972) and the results were expressed as mg cyanidin-3-glucoside equivalents (CGE) 100 g^{-1} dry weight (mg CGE 100 g^{-1} DW) and mg quercetin equivalents (QE) 100 g^{-1} dry weight (mg QE 100 g^{-1} DW), respectively. Total phenolic content was determined in triplicate using the Folin–Ciocalteu method (Singleton et al., 1999). The absorbance was recorded at 761 nm and the content of phenolic compounds was determined from the standard curve of gallic acid (1.6–8.6 mg.L^{-1}, y = 10701x-74.769, R^2 = 0.9963). Data were expressed in mg of gallic acid equivalents per 100 g dry weight (mg GAE 100 g^{-1} DW).

2.4. Quantification of ascorbic acid

The ascorbic acid content was quantified, in accordance with Ramakrishnan and Suochana (2012), using the 2,4-dinitrophenylhydrazine-thiourea-Cu^{2+} assay. The absorbance was recorded at 520 nm, the ascorbic acid content was determined from the standard curve (0.01–0.12 mg.L^{-1}, y = 0.0107x + 0.0003, R^2 = 0.9959), and data were expressed as mg ascorbic acid per 100 g (mg AAE 100 g^{-1} DW).

2.5. Free-radical scavenging activity

The antioxidant activity was determined according to Brand-Williams et al. (1995). The DPPH solution (1.115 × 10^{-4} mol.L^{-1}) was prepared using ethyl alcohol and the reaction started when 150 µL of the extracts were added in a flask containing 3.85 mL of the DPPH. After 60 min at 25 °C, the antioxidant activity was quantified according to Equation (1):

$$Scavenging\ ability\ (\%) = \frac{A517\ nm\ blank - A517\ nm\ sample}{A517\ nm\ blank} \times 100 \quad (1)$$

2.6. Stability of anthocyanins

2.6.1. Effect of copigmentation in relation to temperature

The stability of the anthocyanins in relation to increasing temperature, both in the presence and absence of copigments, was evaluated at pH 3.0 according to the method of Reyes and Cisneros-Zevallos (2007). Phytic and chlorogenic acids, both at 4.0 × 10^{-4} mol.L^{-1} were also prepared. The solutions were suspended in three tubes, which were sealed, protected from light, and immersed in a thermostated bath (60, 70, 80, 90, and 100 °C); a glycerol bath was used for the test conducted at 100 °C. The absorbance was recorded, λ = 520 nm, at intervals of 30 min. Using the data, the percentage of colour retention (%R), rate constant (k), half life time ($t_{1/2}$), and activation energy (E_A) were calculated according to Pedro et al. (2016).

2.6.2. Effect of pH on stability and antioxidant activity

The effect of pH on the chemical stability of anthocyanins was conducted according to Pedro et al. (2016). The pH was monitored using a pH meter (MICRONAL B-474), and altered with 1.0 mol.L^{-1} NaOH. At each variation of 0.30 of pH (2.75–7.53) the absorbance was recorded, λ = 520 nm. A reverse spectrophotometric titration was subsequently performed with 1.0 mol.L^{-1} HCl and the absorbance was recorded at each variation of 1.0 pH. The effect of pH on the antioxidant activity relative to the DPPH radical was performed according to Brand-Williams et al. (1995). A solution of the *H. sabdariffa* extract was packed in the thermostated cell at 25 °C. The pH (2.5–7.5) was treated with 1.0 mol.L^{-1} NaOH. At each variation of 1.0 pH, the DPPH scavenging activity was assessed according to the method described in item 2.5, in which the extract (100 µL) was mixed with 4.90 mL of the DPPH solution (1.1158 × 10^{-4} mol.L^{-1}) and left to react for 60 min at