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Quantification of the polyphenolic fraction and *in vitro* antioxidant and *in vivo* anti-hyperlipemic activities of *Hibiscus sabdariffa* aqueous extract

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ABSTRACT

In the present study the quantification of the polyphenolic fraction, anthocyanins and other polar compounds, the antioxidant capacity and the anti-hyperlipemic action of the aqueous extract of *Hibiscus sabdariffa* has been achieved. Seventeen compounds were successfully quantified either by HPLC-DAD or HPLC-ESI-TOF-MS. Six of them were directly quantified by their corresponding standards, whereas the rest were indirectly quantified as equivalents using standards of similar compounds. The antioxidant capacity have also been estimated by comparing different assays, i,e, Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and measurement of thiobarbituric acid reacting substances (TBARS). *H. sabdariffa* showed high reducing capacity in FRAP assay and significant capability to scavenge peroxyl radicals in the ORAC assay. Nevertheless, the extract exhibited poor efficacy to inhibit peroxyl radicals in lipid systems. The plant extract also exhibited the capacity to decrease serum triglyceride concentration on hyperlipemic mouse model.

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1. Introduction

The *Hibiscus sabdariffa* L. (family: *Malvaceae*), usually named bissap, karkade or roselle is a tropical plant commonly used as local soft drink. It is highly appreciated all over the world for the particular sensation of freshness conveyed. Traditionally, it has been used effectively against hypertension, inflammation, and liver disorders (Wang et al., 2000). Previous studies showed that *H. sabdariffa* possesses anti-tumoral, anti-oxidant and anti-hyperlipemic activities (Chen et al., 2003; Hou, Tong, Terahara, Luo, & Fujii, 2005; Kao et al., 2009; Lin, Huang et al., 2007; Lin, Lin et al., 2007; Tseng et al., 1997, 2000). Recently, it was reported that the extract of *H. sabdariffa* inhibited the LDL oxidation and lowered serum triglycerides, cholesterol and LDL-cholesterol in animal models (Chen et al., 2003; Lin, Huang et al., 2007; Lin, Lin et al., 2007). Histological examination revealed that it could reduce foam cell formation and inhibit VSMC proliferation and migration, suggesting the anti-atherosclerotic effect of *H. sabdariffa*. In addition, studies on humans show the anti-hypertensive and anti-inflammatory effects of *H. sabdariffa* consumption (Beltrán-Debón et al., 2010; Herrera-Arellano, Flores-Romero, Chavez-Soto, & Tortoriello, 2004). The brilliant red color and unique flavor make it a valuable food product. The anthocyanin pigments that create the color (Tsai & Ou, 1996) are responsible for the wide range of coloring in many foods. The *H. sabdariffa* petals are potentially a good source of antioxidant agents as anthocyanins (Segura-Carretero et al., 2008). Overall, there is now increasing evidence that antioxidants in the human diet are of major benefit for health and well-being. The antioxidant properties of *H. sabdariffa* and other hibiscus species have been widely studied (Büyükbalci & El, 2008; Oboh & Rocha, 2008; Vankar & Srivastava, 2008).

In this work we focused on quantifying the phenolic fraction, anthocyanins and other polar compounds in the aqueous extract of *H. sabdariffa*. This quantification was achieved using two detection systems, i.e. RP-HPLC coupled to DAD or RP-HPLC coupled to ESI-TOF-MS. Recently, the qualitative characterization of the compounds present in the aqueous extract of *H. sabdariffa* was carried out successfully (Rodríguez-Medina et al., 2009). These methods were also suitable for the quantification of these substances. To evaluate the antioxidant capacities of foods, numerous *in vitro* methods have been developed and reviewed. However, there has not been a consensus for the preferred method. ORAC (Oxygen Radical Absorbance Capacity),

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TBARS (Thiobarbituric Acid Reactive Substances), TEAC (Trolox Equivalent Antioxidant Capacity) and FRAP (Ferric Reducing Antioxidant Power) assays are among the more popular methods that have been used (Wua et al., 2004). Advantages and disadvantages of these methods have been fully discussed in several reviews (Frankel & Meyer, 2000; Prior, Wu, & Schaich, 2005; Sánchez-Moreno, 2002; Strube, Haenen, Van Den Berg, & Bast, 1997). In this work we have studied the antioxidant capacity of *H. Sabdariffa* aqueous extract using four different methods TEAC and FRAP (based on electron transference) vs. TBARS and ORAC (based on hydrogen atom transference) (Huang, Ou, & Prior, 2005). To further assess *H. sabdariffa* aqueous extract bioactivity, it was administered as sole drinking fluid to mice fed with a high fat-high cholesterol diet in order to assay its anti-hyperlipemic effects.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Solvents were previously filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The standards, for the calibration curves, chlorogenic acid, quercetin 3-rutinoside, quercetin 3-glucoside, kaempferol 3-O-rutinoside and kaempferol 3-(*p*-coumarylglucoside), quercetin, 4-hydroxycoumarin and delphinidin-3-sambubioside were purchased either from Fluka, Extrasynthese (Genay Cedex, France) or Polyphenols (Polyphenols Laboratories AS, Hanaveien Sandnes, Norway). The stock solutions containing these analytes were prepared in methanol (Lab-Scan). The reagents to measure the antioxidant capacity, EYPC (egg yolk phosphatidylcholine), AAPH (2,2′-Azobis (2-methyl-propionamine) dihydrochloride), BHT (butylhydroxytoluene), TEP (1,1′,3,3′-tetraethoxypropane), SDS (Sodium Dodecyl Sulfate), TBA (thiobarbituric acid), TPTZ (Tripyridyltriazine), ABTS (2,2‵-azinobis (3-ethylbenzothiazoline-6-sulphonate), fluorescein, Trolox and ferric sulfate were purchased from Sigma-Aldrich. Sodium acetate, ferric chloride, sodium chloride, hydrochloric acid, sulphuric acid, acetic acid, chloroform, ethanol, TRIS (Tris(hydroxymethyl)aminomethane) were purchased from Panreac (Barcelona, Spain).

2.2. Sample preparation

The *H. sabdariffa* plant was originally from the village of Guerle in Senegal. It was kindly provided by Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan (Reus, Tarragona, Spain). The dry calices from the plant were manually mill grounded and mixed with ultrapure water, up to a concentration of 25 g/l, stirred it in vortex until dissolved, filtered with units of single use Filters Millex (Millipore, Bedford, MA, USA) and directly injected into the HPLC system for the direct and indirect quantifications. On the other hand, the aqueous extract of *H. sabdariffa* for the different antioxidant capacity determinations was conveniently diluted in order to comply with the working range of each spectrophotometric method.

2.3. Instrumentation

HPLC analyses were performed with a RRLC 1200 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump with Zorbax Eclipse Plus C_{18} 4.6 × 150 mm, 1.8 μm column. Prefilters were used as precolumn, RRLC in-line filters, 4.6 mm, 0.2 μm supplied by Agilent Technologies. The mobile phase flow rate was 0.5 ml min^{-1}. HPLC was equipped with DAD and coupled to a TOF mass spectrometer equipped with an orthogonal electrospray interface ESI (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative mode and positive mode of ionization. (MS-Instrument: microTOF, ESI-TOF mass spectrometer) (Bruker Daltonik GmbH, Bremen, Germany). Fluorescence (ORAC and TBARS) and absorbance (FRAP and TEAC) measures were carried out on a spectrofluorimeter Polarstar Omega (BMG Labtechnologies, GMBH; Offenburg, Germany) (Thermostated at 37 °C for the ORAC assay).

2.4. Chromatografic, UV and spectrophotometric conditions

The compounds of the aqueous extract of *H. sabdariffa* were separated by the C18 column at room temperature at a flow rate of 0.5 ml/min and the injection volume was 10 μl for both gradient elution programs. The use of the prefilters as guard column also provided some protection against decomposition and blocking of the working column. The linear gradient used for the analysis, separation and identification of the polyphenols, hydroxycitric acid and its lactone (gradient program 1) was as follow: Mobile phases: A: water/ACN (acetonitrile) 90:10 (1% HCOOH) and B: ACN. The linear gradient elution program was run as stated: 0 min, 5% (B); 20 min 20% (B); 25 min 40% (B); 30 min 5% (B); 35 min, isocratic of B 5%. Anthocyanins, due to their acid–base equilibrium, needed lower pH to be resolved, and a different chromatographic method (gradient program 2) was employed. Solvents that constituted the mobile phases were: A, water (10% HCOOH) and B, ACN. The applied elution conditions were: 0 min, 0% (B); 13 min 20% (B); 20 min 30% (B); 25 min 80% (B); 30 min 0% (B); 35 min, isocratic of B 0%. The DAD coupled to the HPLC system was set in a spectrum range starting at 190 nm and ending at 950 nm. The excitation and emission wavelengths were 485 and 520 nm respectively for the ORAC assay, whereas these sets were 500 and 530 nm for the TBARS determination. The absorbance wavelength for FRAP and TEAC assays were 593 and 734 nm respectively.

2.5. ESI-TOF-MS conditions

TOF-MS transfer parameters were optimized by direct infusion experiments with Tunning mix (Agilent Technologies). The trigger time was set to 53 s (50 s for setting transfer time and 3 s for pre-pulse storage time), corresponding to a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters were capillary, 4500 V gas heater temperature, 200 °C; drying gas flow, 7 l/min; nebulizing gas pressure, 1.5 bar and the spectra rate was 1 Hz. At this stage the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the ESI-micro-TOF detector had to be 0.25 ml/min in order to obtain reproducible results. The TOF mass spectrometer was equipped with an ESI interface operating in both, negative and positive, polarity modes. To tune the detector to optimal conditions calibration was performed with sodium formate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% (v/v) of formic and acetic acids) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior the polyphenol identification. The accurate mass data for the molecular ions were processed using the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formula by using the GenerateMolecularFormula™ editor. The GenerateFormula™editor uses the sigmaFit™algorithm, CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue™) for increased confidence in the suggested molecular form (Bruker Daltonik, Technical Note 008; Rodríguez-Medina et al., 2009). The use of isotopic abundance patterns as a single further constraint removes >95% of false candidates. This orthogonal filter can condense

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