Phytomedicine 17 (2010) 186–191

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

The aqueous extract of *Hibiscus sabdariffa* calices modulates the production of monocyte chemoattractant protein-1 in humans

R. Beltrán-Debón [a], C. Alonso-Villaverde [a,*], G. Aragonès [a], I. Rodríguez-Medina [b], A. Rull [a], V. Micol [c], A. Segura-Carretero [b], A. Fernández-Gutiérrez [b], J. Camps [a], J. Joven [a]

[a] *Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, IISPV, Universitat Rovira i Virgili, C/Sant Joan s/n, 43201 Reus, Spain*
[b] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Av/ Fuentenueva, 18071 Granada, Spain*
[c] *Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Avenida de la Universidad s/n, 03202 Elche, Spain*

ARTICLE INFO

Keywords:
Apoptosis
Chronic diseases
Hibiscus sabdariffa
Inflammation
Oxidation

ABSTRACT

Diet supplementation and/or modulation is an important strategy to significantly improve human health. The search of plants as additional sources of bioactive phenolic compounds is relevant in this context. The aqueous extract of *Hibiscus sabdariffa* is rich in anthocyanins and other phenolic compounds including hydroxycitric and chlorogenic acids. Using this extract we have shown an effective protection of cultured peripheral blood mononuclear cells from the cellular death induced by H_2O_2 and a significant role in the production of inflammatory cytokines. *In vitro*, the extract promotes the production of IL-6 and IL-8 and decreases the concentration of MCP-1 in supernatants in a dose-dependent manner. In humans, the ingestion of an acute dose of the extract (10 g) was well tolerated and decreased plasma MCP-1 concentrations significantly without further effects on other cytokines. This effect was not due to a concomitant increase in the antioxidant capacity of plasma. Instead, its mechanisms probably involve a direct inhibition of inflammatory and/or metabolic pathways responsible for MCP-1 production, and may be relevant in inflammatory and chronic conditions in which the role of MCP-1 is well established. If beneficial effects are confirmed in patients, *Hibiscus sabdariffa* could be considered a valuable traditional herbal medicine for the treatment of chronic inflammatory diseases with the advantage of being devoid of caloric value or potential alcohol toxicity.

Introduction

Anthocyanins are responsible for the attractive bright colors of some fruits and vegetables consumed in Western-type diets where they represent the most abundantly ingested flavonoids. Moreover, dietary supplementation with commercially available anthocyanin-rich extracts, especially from berries and grapes is currently advised by some investigators (Jing et al. 2008). However, the relationship between their chemical structures and corresponding biological activity and consequent putative health benefits is poorly understood (Jing et al. 2008). It is plausible that their effects are not uniquely elicited by their anthocyanin profiles, which may vary greatly, and that other accompanying phenolic compounds are involved. It is also possible that the combined actions of naturally occurring compounds may be more beneficial than the observed in isolated compounds. Therefore, research into plants that may be directly incorporated into normal diets may represent an alternative strategy.

The flowers of *Hibiscus sabdariffa* are rich in anthocyanins (Segura-Carretero et al. 2008) and are normally consumed throughout the world. Their extracts exert significant effects in animal and *in vitro* models (Chen et al. 2003; Haji Faraji et al. 1999; Hou et al. 2005; Lin et al. 2005; Mojiminiyi et al. 2007; Onyenekwe et al. 1999; Tseng et al. 1997; Tseng et al. 2000) but studies in humans are limited to their diuretic effect (Herrera-Arellano et al. 2004). We hypothesize that monocyte chemoattractant protein-1 (MCP-1), an emergent biomarker in the evaluation of inflammatory diseases, is involved in some of the potential benefits ascribed to this plant (Gonzalez-Quesada et al. 2009; Marsillach et al. 2005; Rollins 1996; Rull et al. 2007). In this study, we have extended our previous (Segura-Carretero et al. 2008) characterization of the phenolic compounds present in the *Hibiscus sabdariffa* extract (HSE) and, with the rationale that the

Abbreviations: DAD, diode array detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HSE, *Hibiscus sabdariffa* extract; IT, ion trap (analyzer); MCP-1, monocyte chemoattractant protein-1; MS, mass spectrometry; ORAC, oxygen radical absorbance capacity; PBMCs, peripheral blood mononuclear cells; TOF, time of flight (analyzer); UV, ultraviolet

* Corresponding author at: Servei de Medicina Interna, Hospital Universitari de Sant Joan, C/Sant Joan s/n, 43201 Reus, Spain. Tel.: +34 977 310 300; fax: +34 977 319 984.
E-mail address: cavillaverde@grupsagessa.com (C. Alonso-Villaverde).

0944-7113/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2009.08.006

HSE may reduce oxidative stress and inflammation (Kao et al. 2009; Marsillach et al. 2009), we have also explored the *in vivo* and *in vitro* effects in the production of selected cytokines.

Materials and methods

Characterization and identification of phenolic compounds

Sun-dried calices of *Hibiscus sabdariffa* were obtained from Guerle (Senegal). For analytical measurements, powdered plant material (650 g) was heated in 5 l of distilled water and the mixture was kept boiling for 5 min.

The infusion was filtered, cooled at room temperature, centrifuged and the supernatant was lyophilized and stored at 4 °C until use. The amount of lyophilized *Hibiscus sabdariffa* extract (HSE) obtained was 136 g (4.8:1).

For the HPLC analysis, 0.25 g of HSE were mixed with 5 ml of ultrapure water at room temperature obtaining a solution of 50 g/l freshly prepared for each analysis. The aqueous extract was stirred in a vortex for 10 min until diluted, filtered with single-use filter unit and directly injected into the HPLC system RRLC 1200 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a binary pump and a Zorbax Eclipse Plus C18 4.6 × 150 mm, 1.8 μm column. The compounds of the aqueous extract of *Hibiscus 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. The system was equipped with DAD and coupled to a TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an orthogonal electrospray interface (ESI G1607A; Agilent Technologies). UV–visible spectrophotometry delimited the class of phenolic compounds and the accurate mass measurements on the TOF spectrometer enabled the identification of the compounds in the extract. Finally, the fragmentation pattern obtained in MS/MS experiments performed with an Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) confirmed the proposed structures. Two optimized gradient programs were run in order to separate and identify the different families of compounds present in HSE. Polyphenols were successfully identified and analyzed with a gradient optimized for a negative ionization polarity mode while anthocyanins were identified with a gradient optimized for a positive ionization polarity mode.

In vitro studies

Freshly prepared for each experiment, 100 mg of extract were dissolved in 10 ml of RPMI medium, centrifuged at 1500*g* for 10 min and the supernatant filtered (22 μm). Heparinized whole blood was obtained from healthy volunteers and peripheral blood mononuclear cells (PBMCs) immediately separated using conventional Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Separated cells were washed twice with D-PBS (Gibco-Invitrogen, Carlsbad, CA, USA) followed by resuspension in 3 ml of pre-warmed (37 °C, 30 min) RPMI 1640, 1% penicillin/streptomycin and 10% fetal bovine serum (Sigma-Aldrich Inc, St. Louis, USA). Viable PBMCs (1×10^6) were treated with HSE according to the following protocols: (i) PBMCs were incubated simultaneously with HSE and 50 μM hydrogen peroxide (H_2O_2) at 37 °C in a humidified atmosphere of 5% CO_2 for 20 h; (ii) alternatively, after a pre-incubation of PBMCs with HSE for 24 h and subsequent washing with D-PBS, cells were incubated with 50 μM H_2O_2 alone under the same conditions for an additional 20 h. For each sample, the supernatants were kept at −80 °C until further assays. Apoptosis of cells was immediately determined by flow cytometry using the annexin V-FITC Apoptosis Detection Kit (MBL International, Woburn, MA, USA) following the manufacturer's instructions in an EPICS-MCL-XL flow cytometer (Beckman-Coulter, Fullerton, CA, USA).

Main characteristics of human volunteers

Ten ostensibly healthy adult volunteers (5 males and 5 females) between 23 and 50 years of age were recruited from the hospital staff for the study. Each participant gave written fully informed consent to participate in the study and the study procedures were approved and monitored by the Ethics Committee of the Hospital Universitari de Sant Joan de Reus. Participants were instructed to avoid the ingestion of polyphenol-rich foods or beverages (i.e. coffee, tea, juice, oil, chocolate, fruits and vegetables) for at least 2 days and to fast overnight before the experiments. Samples of freeze-dried HSE (10 g) dissolved in tap water were freshly prepared. The HSE was consumed by the participants, after which only water was allowed to be ingested for the following 3 h. Venous blood was obtained in EDTA tubes at 0, 1.5 and 3 h and plasma stored at −80 °C until further assays.

Other laboratory measurements and statistical analyses

Oxygen radical absorbance capacity (ORAC) measurements were performed as previously described by Ou et al. 2001 with minor modifications. Trolox (Sigma-Aldrich, Steinheim, Germany) was used as a standard and results were expressed as Trolox equivalents in micromoles per milliliter or gram. Assays were performed in a Synergy HT Multi-Mode microplate reader (BioTek instruments, Vermont, USA) in flat-bottom black-opaque microplates (Nunc, Langenselbold, Germany). The reaction was monitored for 3 h at intervals of 1 min. Interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured in supernatants and plasma aliquots using a multiplex cytometric bead-based assay (FlowCytomix Multiplex, BenderMedsystems, Austria).

Differences between two or more groups were compared using non-parametric tests. Differences were considered statistically significant when $p < 0.05$. All statistical analyses were carried out with the Statistical Package for Social Science version 17.0 (SPSS, Chicago, Illinois, USA).

Results

Hibiscus sabdariffa extract composition

The HSE antioxidant effect of all prepared extracts had similar values of ORAC of approximately 360 TE/g (μmol Trolox per 1 g of HSE). The identified and characterized compounds are shown in Table 1. Representative base peak chromatograms in both negative and positive polarity modes are also depicted in Fig. 1. The most representative anthocyanins present in the extract, delphinidin-3-sambubioside and cyanidin-3-sambubioside, are also accompanied by significant amounts of other phenolic compounds. Among them, the most abundant were hydroxycitric, hibiscus and chlorogenic acids, which may all have additional properties to be considered. Due to the unavailability of commercial standards for the identified compounds a first approach for quantification was measured and expressed in terms of equivalents of a compound with similar UV–vis absorption characteristics and chemical structure. Thus, the content of anthocyanidins, as delphinidin equivalents was 0.45% (w/w, peaks 15 and 16) and the flavonols, as quercetin equivalents