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Validation of coffee silverskin extract as a food ingredient by the analysis of cytotoxicity and genotoxicity

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ABSTRACT

The aim of the present study was to validate the food safety of CSE, by studying its effect on cytotoxicity (100–20000 μg/ml) and genotoxicity (10, 100 and 1000 μg/ml) and also to investigate its preventive potential (1, 10 and 100 μg/ml) against B(a)P induced DNA damage. Prior to analyses, the antioxidant capacity and the microbiological quality of CSE were tested. DNA damage (strand breaks and oxidized purines/pyrimidines) was evaluated by the alkaline single-cell gel electrophoresis or comet assay. HepG2 cells were pre-treated with CSE (1, 10 and 100 μg/ml) for 24 h followed by the addition of 100 μM B(a)P in presence of CSE for other 24 h. Detection of oxidized purines and pyrimidines was carried out using Formamidopyrimidine DNA glycosylase or Endonuclease III enzymes, respectively. Chlorogenic acid (CGA), the major antioxidant present in coffee, was used as a control. Treatment with 100 μM B(a)P significantly increased ($p < 0.05$) levels of DNA strand breaks and oxidized purine and pyrimidine bases. Treatment of HepG2 cells with CSE did not induce either cytotoxicity or genotoxicity. CSE significantly inhibited ($p < 0.05$) genotoxicity induced by B(a)P and the observed effect may be associated to its antioxidant capacity. CGA alone at the concentration present in CSE was effective against B(a)P. Thus, CGA seems to be a contributor to the preventive effect of CSE against B(a)P induced DNA damage in HepG2 cells. In conclusion, CSE presents potential as a natural sustainable chemoprotective agent against the chemical carcinogen B(a)P.

1. Introduction

The validation process for a novel food or ingredient established by the European legislation (EFSA Panel on Dietetic Products, N. and A., 2016;14(11):4594) comprises chemical characterization (toxic and health promoters), proposal for applications, *in vitro* assays, *in vivo* toxicity, *in vivo* bioactivity and human trials (Fig. 1). In 2016, EFSA published a guidance for preparing and presenting scientific data for novel food applications. This guidance indicates the requirements needed when submitting an application for the authorization of novel foods: physicochemical, biochemical properties and microbiological characterization as well as data on the compositional, nutritional, toxicological and allergenic properties of the novel food (EFSA Panel on Dietetic Products, N. and A., 2016). Information on genotoxicity is a key component in risk assessment of novel foods or ingredients. The purpose of genotoxicity testing for risk evaluation of substances in food is to identify compounds that could cause heritable damage in humans, to predict potential genotoxic carcinogens in cases where carcinogenicity data are not available, and to contribute to understand the mechanism of action of chemical carcinogens (Efsa Scientific Committee, 2011).

Coffee silverskin (CS) is a thin tegument of the outer layer of the two beans present in the coffee cherry. CS is the only by-product generated in the roasting process and it contains phytochemicals with antioxidant character such as chlorogenic acid (CGA). The extract (WO/2013/004873) prepared from Arabica coffee silverskin (CSE) is enriched in CGA and possesses high antioxidant power (del Castillo et al., 2016). The present study provides novel scientific information for supporting the usefulness of CSE as a sustainable natural source of bioactive compounds.

One of the mechanisms that leads to DNA lesions is oxidative stress associated with the activity of metabolic enzymes that can induce DNA strand breaks and oxidized bases (Xue & Warshawsky, 2005). Since CSE comes from a natural source and is known to possess high antioxidant

Abbreviations: CGA, chlorogenic acid; CSE, coffee silverskin extract; Endo III, Endonuclease III; Fpg, Formamidopyrimidine-DNA glycosylase

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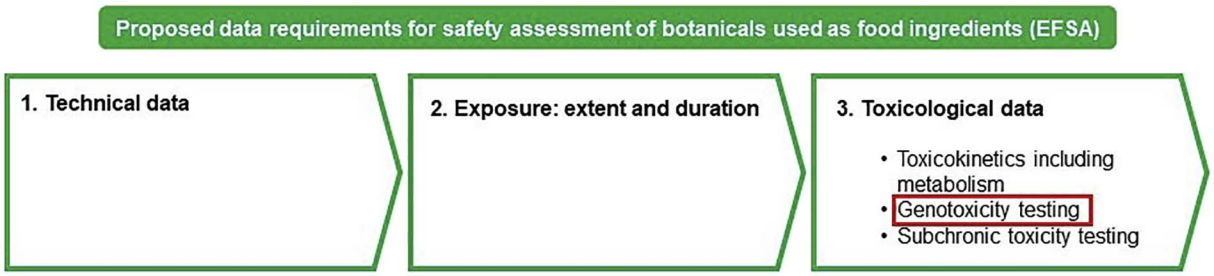

Fig. 1. Scheme of the proposed data requirements for safety assessment of botanicals used as food ingredients (EFSA).

power, this extract could protect cells from DNA damage when induced by an oxidative agent.

HepG2 cells, a human hepatoblastoma cell line, are widely used in the field of xenobiotic metabolism for the study of cytotoxic or genotoxic agents (Cao et al., 2007). Benzo(a)pyrene (B(a)P) is the most widely used model compound for studying the effects of carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Brinkmann et al., 2013). B(a)P is formed during incomplete combustion or pyrolysis of organic material and it is found in air, water, soils and in thermally processed foods and cigarette smoke (International Agency for Research on Cancer (IARC), 2012). B(a)P is a DNA-reactive chemical that interacts with DNA. Strand breaks or alkali labile sites, including abasic sites, may result from the action of reactive oxygen species (ROS) that arise during metabolism of food mutagens in cells (Haza & Morales, 2013).

The aim of the present study is to give new information related to CSE safety in order to use it as a food ingredient, as well as to provide scientific data to support a cause-effect relationship of the antioxidant power claim made on coffee and their derivatives. We also investigated the chemoprotective potential of CSE against B(a)P induced DNA damage in HepG2 cells and the contribution of CGA in CSE as a chemoprotective agent.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid (CGA), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), benzo(a)pyrene (B(a)P), dimethyl sulfoxide (DMSO) and low melting point agarose (LMP) were purchased from Sigma–Aldrich (St Louis, MO, USA). Formamidopyrimidine-DNA glycosylase (Fpg) and Endonuclease III (Endo III) were obtained from Trevigen Inc. (Gaithersburg, MD, USA). Culture medium and supplements required for the growth of HepG2 cells were purchased from Lonza (Lonza Group, Basel, Switzerland). MTT Proliferation Kit I was purchased from Roche (Indianapolis, IN, USA).

2.2. Preparation of soluble coffee silverskin extract

Coffee silverskin from Arabica variety was provided by Fortaleza S.A. (Spain). CSE was produced as described in the patent WO 2013/004873 (del Castillo et al., 2013). Briefly, 50 mg of coffee silverskin were added per H_2O milliliter. This mixture was stirred for 10 min at 100 °C, filtered and the filtrate was freeze-dried. Powdered CSE was prepared in aqueous solution, sterile filtered and added to medium to achieve final concentrations between 1 and 20,000 μg/ml. CSE chemical composition is described in Table S1 (Supplementary Material). CGA is the major antioxidant present in coffee; therefore, it was used as an antioxidant control.

2.3. Overall antioxidant capacity of coffee silverskin extract

The trapping capacity of cationic free radicals was evaluated using the method of radical $ABTS^{\bullet+}$ bleaching described by Re et al. (1999) and modified by (Oki, Nagai, Yoshinaga, Nishiba, & Suda, 2006) for its use in a microplate. Aqueous solutions of CGA (0.15–2.0 mmol/l) were used for calibration. Absorbance was measured in microplate using a UV–Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). All measurements were performed in triplicate and results were expressed as % CGA eq. (w/w).

2.4. Microbiological analyses

CSE was microbiologically analyzed to evaluate the safety of its use as a food ingredient. Count of (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores and (3) molds and yeasts were carried out. All assays were performed in sterile conditions and with previous solubilization of 10 g of CSE in BPW (90 mL) by using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30 °C 72 h; (2) pour plate, BHI agar medium, preincubation (80 °C, 10 min) and incubation at 37 °C 48 h; and (3) spread method, SDA with chloramphenicol and incubation at 25 °C 120 h. Results were expressed as colony forming units (CFU)/g.

2.5. Cell culture

Human hepatocellular carcinoma (HepG2) cells were purchased from the Biology Investigation Center Collection (CIB, Madrid, Spain). Only cells of passage 10–17 were used in the experiments. Cells were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v heat inactivated fetal calf serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin and 1% v/v L-glutamine. Cell cultures were incubated at 37 °C and 100% humidity in a 5% CO_2 atmosphere.

2.6. Cytotoxicity

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cell Proliferation Kit I, Roche, Indianapolis, IN, USA) to select non-toxic concentrations of CSE and CGA. First, HepG2 cells were cultured at a density of 1×10^5 cells per well of a 96-well plate for 24 h. Then, cells were treated with CSE (0–20,000 μg/mL) or CGA (0–10,000 μg/mL) for 24 h. Subsequently, cells were incubated in MTT Labeling Reagent for 4 h at 37 °C and then, 100 μl of solubilization solution were added. After 24 h, the optical density of each well was read at 620 nm (test wavelength) and 690 nm (reference wavelength) using a microplate reader. Experiments were carried out in triplicate ($n = 16$). Results were expressed as the percentage of viability (% SDH) with respect to the control (medium treated cells).

2.7. DNA damage

The comet assay was carried out according to the protocol of Olive et al. with minor modifications (Olive, Wlodek, Durand, & Banáth,