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A polyphenol-enriched cocoa extract reduces free radicals produced by mycotoxins

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ABSTRACT

Polyphenols are characterized by the presence of phenol units in the molecules. These compounds may show antioxidant ability by scavenging reactive oxygen species (ROS) of the free radical type. A polyphenol enriched cocoa extract (PECE) was obtained from cocoa seeds with 28% of procyanidins which were mainly epicatechin oligomers. PECE was very active as free radical scavenger against 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) radicals; and the tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) assay showed that the PECE might not be pro-oxidant. Thus it was considered a good candidate to be tested in *in vitro* models. It showed mild cytotoxic power on Hep G2 cells and induced ROS in a dose-dependent manner being weak oxidant only at high concentrations near the limit of solubility. The antioxidant properties were assayed in Hep G2 treated with the mycotoxins ochratoxin A (OTA) and/or aflatoxin B1 (AFB1). The PECE was not effective against AFB1 but it increased the cell viability and reduced significantly the amounts of ROS in cells treated with OTA or mixtures of AFB1 + OTA. These results are coherent with the role of oxidative pathways in the mechanism of OTA and indicate that polyphenols extracted from cocoa may be good candidates as antioxidant agents.

1. Introduction

Polyphenols are secondary metabolism products of plants and constitute one of the most numerous and widely distributed groups of natural antioxidants in the plant kingdom. They can be divided into at least 10 different classes depending on their basic structure, being the most important ones that of flavonoids (Wollgast and Anklam, 2000). Flavonoids act as antioxidants via several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes (Cos et al., 2004).

Red wine, cranberries, apples, tea and cocoa are among the richest food sources of flavonoids. Specifically, cocoa is rich in flavan-3-ol monomers, (−)-epicatechin (EC), (+)-catechin (C) and their related oligomers (procyanidins) (Osman et al., 2004). The mean estimated daily intake of polyphenols in the diet is 1 g/person/day (Scalbert et al., 2002). It has been estimated that about 90% of dietary polyphenols are bioaccesible in the small and in the large intestine (Saura-Calixto et al., 2007). Monomeric (epi)catechins are rapidly metabolized to glucuronide and methyl conjugates in the small intestine, absorbed into the bloodstream and then further conjugated (glucuronidated and sulfated) in the liver. Procyanidins that are not absorbed in the small intestine are partially depolymerized into monomeric units and the remaining polymers reach the colon where they are metabolized by colonic microbiota into smaller compounds such as phenolic acids, which pass into the bloodstream and are finally excreted in urine. An array of phenolic metabolites are absorbed in the intestine and may contribute significantly to antioxidant and other physiological effects in the gastrointestinal tract and other tissues (Spencer, 2000; Rios, 2003; Kwik-Uribe and Bektash, 2008; Urpí-Sardá et al., 2009; Touriño et al., 2011).

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are secondary products of several fungi metabolism mainly found in cereals, grapes, coffee, species, and cocoa and they belong to the most frequently occurring mycotoxins (Sedmíková et al., 2001). The IARC classified AFB1 and OTA as class 1 (human carcinogen) and class 2B (possible human carcinogens), respectively (IARC, 1987, 1993, 2002). AFB1 is

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bioactivated in the liver by P450 cytochrome and the resulting epoxide metabolite causes the DNA damage by adduct formation (McLean and Dutton, 1995). A secondary route for AFB1 metabolism involves ROS formation but the mechanisms are still unknown (Bedard and Massey, 2006). Due to the carcinogenic nature of AFB1, the authorities have not yet estimated a safe intake and advise the ALARA principle (as low as reasonable achievable). OTA is a potent nephrocarcinogenic compound in rodents but despite the controversy, it is considered an indirect genotoxic agent (Arbillaga et al., 2004, 2007; Mally et al., 2004, 2005; Mally and Dekant, 2005; Turesky, 2005). OTA mechanisms of action have not been clearly determined but apparently its ability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage (Ringot et al., 2006). Different official organizations have suggested safety PTDI between 5 and 17 ng of OTA per kg of bodyweight. With regard to AFB1 and OTA synergic behavior, it was found that OTA increased the mutagenicity of AFB1, and the mixture of both not only showed cytotoxic additive effects but also a slight increase in DNA fragmentation when compared to mycotoxins taken separately in Vero cells (El Golli-Bennour et al., 2010). In Hep G2 cells, the mixture induced more ROS than each mycotoxin alone but OTA reduced the AFB1 genotoxicity (Corcuera et al., 2011).

Recently, attention has been focused on inhibitory roles of natural constituents in suppressing or reducing the toxic action of mycotoxins, and results are not conclusive. In the case of AFB1, Muto et al. found that epicatechin (EC) partially inhibited the bioactivation of AFB1 in *Salmonella enterica serovar typhimurium* which expressed human CYPs (Muto et al., 2001). However, Lee et al. could not find any inhibition in the epoxide synthesis when AFB1 was mixed with mouse liver microsomal proteins and catechins (Lee et al., 2001). Yet a cocoa polyphenol extract was able to suppress acrylamide toxicity, which has the same bioactivation pathway as AFB1, by improving the redox status and by blocking the apoptotic pathway activated by acrylamide (Rodríguez-Ramiro et al., 2011). With regard to OTA, Costa et al. reported cytoprotective effects of catechins *in vitro* from OTA-induced cell damage and a good scavenging power according to inhibition of ROS production (Costa et al., 2007). Moreover, Baldi et al. (2004) found a small but significant protection (10%) of α-tocopherol towards OTA-induced cell death in bovine mammary epithelial cells (Baldi et al., 2004). However, Hundhausen et al. (2005) tested the effect of different polyphenols on OTA-induced cytotoxicity in Hep G2 liver cells, concluding that these compounds did not counteract OTA-induced cytotoxicity in working conditions (Hundhausen et al., 2005).

In this work, a polyphenol-enriched cocoa extract (PECE) has been prepared and evaluated as an antioxidant agent in a cell free system and in Hep G2 cells. Once the antioxidant activity was confirmed, its ability to reduce AFB1 and OTA cytotoxicity and ROS induction has been tested showing promising results.

2. Materials and methods

2.1. Chemicals

Cocoa beans were provided by a national cocoa import factory. Acetone, acid acetic and chloridric acid were purchased from Panreac (Barcelona, Spain). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%), 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) crystallized diammonium salt, (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin-3-*O*-gallate (ECG), OTA, AFB1 standards and cysteamine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4-β-(2-Aminoethylthio)catechin (Cya-Cat), 4-β-(2-aminoethylthio)epicatechin (Cya-EC), 4-β-(2-aminoethylthio)epicatechin-3-*O*-gallate (Cya-ECG) were synthesized and purified from grape extracts as previously described (Torres and Bobet, 2001). Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) and tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radicals were synthesized as described (Torres et al., 2003, 2007). For HPLC analysis, HPLC grade acetonitrile (Scharlab, Barcelona, Spain) and trifluoroacetic acid (TFA) (Fluorochem, Drebyshire, UK) were used. HPLC grade methanol was purchased from Merck (Darmstadt, Germany).

2.2. PECE preparation

OTA-free cocoa beans (20 g) were milled and lipidic compounds were removed with hexane. Next, theobromine and related compounds were discarded with ethanol:water:trifluoroacetic acid (15:84.9:0.1 v/v/v) (3 × 400 mL). Then, polyphenols were extracted with a mixture of acetone:water:acetic acid (70:29.98:0.02 v/v/v) (3 × 400 mL), the solid discarded by centrifugation and decantation and the acetone in the solution evaporated under vacuum. Finally, the polyphenolic extract was freeze-dried and kept in the dark in a N_2 atmosphere to avoid product oxidation.

2.3. Estimation of polyphenolic composition

The size and composition of the procyanidins were determined by HPLC analysis (Torres and Selga, 2003). Depolymerized sample was analyzed by HPLC-DAD using a Hitachi Lachrom Elite HPLC (San Jose, CA, USA) fitted to a Kromasil C18 column (25 × 0.4 cm, 5 μm) from Teknokroma (Barcelona, Spain). Components were eluted with a binary system 0.1% (v/v) TFA in HPLC grade water [A] and 0.1% TFA (v/v) in acetonitrile [B], gradient 9.6–20% [B] in 22 min, flow rate 1 mL/min. Selected wavelength for the polyphenol quantitative analysis was 214 nm.

Using this methodology, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of Cya (cysteamine) whereas the extension moieties were released as the Cya derivatives on the fourth position of the flavonoid system. Calibration curves of terminal units C and EC and extension units Cya-Cat and Cya-EC were plotted by analyzing 6 standards within the working range in triplicate. Then the terminal and extension units content in the cocoa extract were determined and the mean degree of polymerization (mDP) and the mean molecular weight of the polymer (mMW) were calculated as follows:

$$\mathrm{mDP} = \frac{\text{Total nmol}(\mathrm{Cat} + \mathrm{Ec} + \text{Cya-Cat} + \text{Cya-EC})}{\text{Total nmol ter min al units}(\mathrm{Cat} + \mathrm{Ec})}$$

$$\mathrm{mMW} = \frac{\text{Total ng}(\mathrm{Cat} + \mathrm{Ec} + \text{Cya-Cat} + \text{Cya-EC})}{\text{Total nmol ter min al units}(\mathrm{Cat} + \mathrm{Ec})}$$

2.4. Free radical scavenging activity

To study the free radical scavenging activity, the ABTS, DPPH, HNTTM, TNPTM methods described in Touriño et al. (2008) were applied (Touriño et al., 2008).

ABTS assay measures the total antioxidant activity, comparing antioxidant activity of the extract with that of Trolox. In this case, the concentrations of cocoa polyphenolic extract (50 μL) used to mix with the 1 mM ABTS solution (1950 μL) were 50, 62.5, 75, 100 and 125 μg/mL in methanol. The Trolox equivalent antioxidant activity (TEAC) of the fractions was expressed as μmol of Trolox equivalent to 1 mg of cocoa polyphenolic extract.

The free radical scavenging power was also evaluated against two other radicals: DPPH and HNTTM. These two radicals are used to calculate the stoichiometry of the redox reaction and to discriminate between hydrogen donation and electron-transfer mechanisms of radical scavenging. While the DPPH assay measures the combined hydrogen donation and electron-transfer capacity of the polyphenols, the HNTTM stable radical exerts its action exclusively by electron transfer. For both DPPH and HNTTM assays, the antiradical power (ARP) is expressed as the inverse of ED_{50} (μg of extract able to consume one-half the amount of free radical divided by μmol of initial DPPH or HNTTM). The concentrations of cocoa polyphenolic extract to be mixed with the radical solution were 20, 70, 100, 150, 200 and 300 μg/mL of methanol in the case of 60 μM DPPH and 0.5, 2.5, 5, 15, 35 and 50 μg/mL of chloroform:methanol 2:1 in the case of 120 μM HNTTM.

The specific reactivity of TNPTM radical with the most reducing positions (e.g. pyrogallol group) was compared with the hydrogen donation ability (DPPH) and global electron transfer capacity (HNTTM) in order to provide valuable information regarding the ability of some components to engage in putatively prooxidant/toxic effects involving electron transfer to oxygen. The concentrations of PECE to mix with the 80 μM TNPTM solution were 32.5, 65, 97.5 and 130 μg/mL of chloroform:methanol 2:1.

2.5. Cell cultures

Hep G2 (hepatocellular carcinoma epithelial cell line) (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It was kept in the laboratory satisfying the requirements for Good Laboratory Practice (GLP) conditions. A master bank was created on arrival and a working bank was created to perform the assays of this study, controlling the passing number, which never surpassed 20.

Cells were cultured in monolayer in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Prat de Llobregat, Barcelona, Spain) supplemented with 10% of fetal calf serum (FCS, Gibco) and 1% of antibiotics (10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Gibco). Cells were grown at 37 °C and 5% CO_2 in a humidified atmosphere.