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Antioxidant and biological activity of phenolic pigments from *Theobroma cacao* hulls extracted with supercritical CO_2

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Abstract

Theobroma cacao L. (Sterculiaceae) and cocoa-derived products are phenolics-rich food; these products are largely studied because of the antioxidant and antiradical in vitro properties of phenolic constituents. Cocoa hulls are the principal by-product of cocoa, separated from the cotyledons during the pre-roasting process or after the roasting process of *T. cacao* beans (de-hulling/de-husking step). This by-product is a matrix rich in fiber (namely insoluble, but also represented by pectins) and phenolics. Supercritical CO_2 is a powerful mild technology able to extract and fractionate from plant or animal foods without the use of organic solvent. This approach was used to extract some phenolics fractions from cocoa hulls. Only two recovered fractions, (150 bar, 50 °C, re-dissolved in acetone; 200 bar, 50 °C, re-dissolved in acetone), apparently free from (-)epicatechin, catechin and phenolic acids, showed protective action in an in vitro test (SH-SY5Y cells, differentiated to a neuronal phenotype using retinoic acid and then exposed to ischemic damage), similar to the action of cabergoline and vitamin E. We suggest the use of supercritical CO_2 for the isolation of bioactive fractions from cocoa hulls and an in vitro model as a useful model to study the antioxidant/antiradical properties of isolated phenolic pigments.

Keywords: *Theobroma cacao* L.; Antioxidant; Antiradical; Phenolics; Supercritical CO_2 extraction

1. Introduction

Cocoa-derived foods (cacao powders, chocolate, cocoa-related products) are phenolics-rich foods derived from the fermented, roasted and milled seeds of *Theobroma cacao* L. (Sterculiaceae). These products, consumed all over the world, are largely studied because of the antioxidant and antiradical in vitro properties of some phenolic constituents (phenolic acids, procyanidins, flavonoids) (Wollgast & Anklam, 2000a). Phenolics of cocoa (as well as those of other plant species) have been reported in many studies as bio-active compounds (antioxidant, antiradical, anticarcinogenic properties) (Ren, Qiao, Wang, Zhu, & Zhang, 2003; Sanbongi et al., 1998; Wollgast & Anklam, 2000b). Also the anti-microbial properties of cocoa phenolics against some food bacterial pathogens as well as against some cariogenic bacteria (Osawa et al., 1990) were previously shown. The anti-microbial activity is directly correlated to the property to penetrate the bacterial cell wall (Arlorio, Coïsson, Turri, & Martelli, 2000; Osawa et al., 1990). The in vivo bio-activity of cocoa phenolics (as well as phenolics from other foods like coffee and vegetables) was well studied. This activity is strictly

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correlated with their absorption and metabolism (Shahidi & Naczk, 2003). In 1994, the estimated per head consumption of cocoa/chocolate and chocolate confectionery in European Union ranged from 1.3 (Portugal) to 8.8 kg/year (Germany). Chocolate (particularly dark chocolate) can be seen as a relevant source for phenolic antioxidants. About bioavailability of polyphenols, above all flavonols, reliable data on the real content of polyphenols in food are still scarce. Recently, the flavonoids intake in Dutch diet (based on five flavonols and flavonons) was calculated as 23 mg/day. This estimation didn't include catechins and proanthocyanidins. Anyway, the bioavailability of flavonols (as well as all phenolics) is still largely discussed. For exemple, independently of the doses of chocolate and cacao ingested, only 0.5% of catechin was recovered in the free unbound form in the plasma and in the urine (Wollgast & Anklam, 2000b).

Phenolic compounds of cacao (*T. cacao* L.) belong to many classes of molecules: catechins, epicatechins, anthocyanins, pro-anthocyanidins, phenolic acids, condensed tannins, other flavonoids and some minor compounds (Sànchez-Rabaneda et al., 2003; Wollgast & Anklam, 2000a).

(-)Epicatechin is quantitatively the main compound of cocoa phenolics (approximately 35% of polyphenol content of unfermented Forastero cocoa beans). Total soluble phenolics of good fermented dried cocoa beans ranges from 2 to 6%, strictly depending on the variety as well as the geographic origin. Forastero typical content is about 6%; soluble phenolics content in Criollo cacao is about 2/3 of Forastero. Major content are often an index of bad fermentation. The fermentation step involves some changes in phenolic content of cocoa nibs: a strong decrease of total soluble phenolic content and the polymerisation of some constituent (above all (-)epicatechin with one other (-)epicatechin or with anthocyanidins, to form high molecular weight tannins) occurs (Shahidi & Naczk, 2003). Free low molecular weight polyphenols still present in chocolate are responsible of astringent and bitter taste. Some polyphenolic compounds are clearly involved in colour development of *T. cacao* beans, as well as other molecular classes (mainly proteins and reducing sugars involved in Maillard reactions) that act during the fermentation step and during the roasting process. "Cocoa red" is the common name of the typical pigment colour of "good fermented cocoa beans", obtained by mean of different kind of natural microbial fermentation. So, roasting process involves some changes in cacao nibs colour; also in this case some inter-variety difference exist (different phenolics and different natural colorant chemotypes).

The antioxidant properties of cocoa were largely studied during last years by mean of different approaches: chemical characterization of involved antioxidant species (HPLC, HPLC-MS) (Adamson et al., 1999), in vitro chemical studies (in order to study the ability to scavenging some stable radicals like $DPPH^{\cdot}$, $ABTS^{+\cdot}$) (Hatano et al., 2002), in vitro biological studies and nutrigenomic-based studies (bioavailability, in vivo interaction with cellular/molecular species) (Motohashi et al., 1999). Some bio-active properties of cocoa are strictly related to phenolic content as well as to some compounds from the Maillard reactions (non-enzymatic brown pigments).

Cocoa hulls are the principal by-product for cocoa industries, commonly used as secondary source of theobromine, caffeine and cocoa lipids (often not considered as "cocoa butter", because of their high acidity and high unsaponifiable content). Cocoa hulls are part of the cocoa bean, separated from the cotyledons together with the germ during pre-roasting process or after the roasting process of *T. cacao* (de-hulling/de-husking step).

Only few studies on *T. cacao* husks and hulls are developed (Martin-Cabrejas, Valiente, Esteban, Molla, & Waldron, 1994). Recently, because of their high content in pectin soluble fiber, a novel use of this by-product has been suggested (Arlorio, Coisson, Restani, & Martelli, 2001). Supercritical CO_2 is a powerful mild technology useful to extract and fractionate without the use of organic solvent. CO_2 in supercritical conditions was previously used to extract from cocoa nibs theobromine, lipids as well as some aromatic compounds. The studies about the extraction of phenolics from hulls by mean of supercritical CO_2 are still lacking.

Ischemia-induced neuronal degeneration is a good model to evaluate the in vitro activity of phenolic bio-active molecules. In fact, dopamine has a role in the ischemic damage; the dopamine released by damaged cells could be oxidized by non-enzymatic or enzymatic systems; both processes generate free radicals. Moreover, the dopamine released induces the glutamate release; also glutamate could be the reason of the secondary production of free radical species. A drug (cabergoline, an ergot-related compounds, a dopamine D2 receptor agonist) as well as an antioxidant bio-active molecule (vitamin E) both showed a protective antioxidant activity in an in vitro model, based on the use of human neuroblastoma cells (SH-SY5Y) (Miglio et al., 2004). This in vitro approach is a useful method to assess the bioactivity of antioxidant molecular species.

Aims of this work were (i) to apply the SFE (Supercritical Fluid Extraction) using CO_2 to extract some pigmented phenolic fractions from cocoa hulls, (ii) to characterize their composition by HPLC and (iii) to evaluate the anti-oxidative/protective effect using an in vitro model able to simulate the cellular ischemia.