Potential for preventive effects of cocoa and cocoa polyphenols in cancer

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[1] **Abbreviations:** AhR, aryl hydrocarbon receptor**;** AKT/PKB, protein kinase B; AOM, azoxymethane; AP, Acticoa power; AP-1, activator protein-1; ARE, antioxidant response element; CDK, cyclins-dpendent kinase; CLPr, cacao liquor proanthocyanidins; COX-2, cyclooxygenase-2; CYP, cytochrome P450; DEN, diethylnitrosamine; EC, (-)-epicatechin; DMBDD, 2,2'-dihydroxy-di-n-propylnitrosamine; DOC, deoxycholic; ERK, extracellular regulated kinase; DMBA, dimethylbenz[a]anthracene; FAK, focal adhesion kinase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-tranferases; IκB, inhibitor of κB; HUVEC, human endothelial cell; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associating protein-1; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinase; MEK, mitogen activated protein kinase kinase; MNU, N-methylnitrosourea; NF-κB, nuclear factor kappa B; Nrf2, nuclear-factor-E2-related factor 2; 8-oxodG, 8-oxo-7,8-dihydro-2´-deoxyguanosine; PhIP, 1-methyl-6-phenylimidazo [4,5-b]-pyridine; PI3K, PI-3-kinase, phosphatidylinositol-3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PB2, procyanidin B2; ROS, reactive oxygen species; SOD, superoxide dismutase; *t*-BOOH, *tert*-butylhydroperoxide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TNFα, tumour necrosis factor α; Topo , topoisomerase; VEGF, vascular endothelial growth factor.

Abstract

Prevention of cancer through the diet is receiving increasing interest, and cocoa because of its polyphenolic compounds has become an important potential chemopreventive and therapeutic natural agent. Cocoa and its main polyphenols have been reported to interfere at the initiation, promotion and progression of cancer. Cocoa flavonoids have been demonstrated to influence several important biological functions *in vitro* and *in vivo* by their free radical scavenging ability or through the regulation of signal transduction pathways to stimulate apoptosis and to inhibit inflammation, cellular proliferation, apoptosis, angiogenesis and metastasis. Nevertheless, these molecular mechanisms of action are not completely characterized and many features remain to be elucidated. The aim of this review is to provide insights into the molecular basis of the potential chemopreventive activity of cocoa and its polyphenolic components by summarizing cell culture and animal models studies, as well as interventional and epidemiological studies on humans.

Keywords: Cocoa, cancer, inflammation, antioxidant defenses, cell death, survival/proliferation pathways.

Highlights

- Cocoa promotes changes in redox-sensitive signaling pathways involved in the expression of many genes and cell functions.

- Cocoa may prevent chronic inflammatory response and oxidative damage and thus affect carcinogenesis.

- Cocoa has been demonstrated to increase serum antioxidant status and apoptosis of cancer cells, theoretically reducing cancer risk.

- Research to date suggests that moderate cocoa or dark chocolate consumption may reduce cancer risks.

1.- Introduction

Carcinogenesis is generally a slow process and often takes decades from tumor initiation to diagnosis, offering a considerable time frame for chemopreventive approaches. Chemoprevention is defined as the use of specific natural (dietary) or synthetic agents to prevent, delay, or slow the carcinogenic process (Kelloff et al., 2006). Accumulating epidemiological and experimental studies suggest that a high consumption of fruits and vegetables and the intake of certain non-nutrients that are present in foods reduce the risk of different cancers (Ramos, 2008, Surh, 2003). Therefore, the identification of dietary components as potential cancer chemopreventive agents in the form of functional foods or nutraceuticals has become an essential subject for study in current research. This is the case for polyphenols, natural dietary compounds present in fruits and vegetables, which have attracted a great deal of interest because of their potential ability to act as highly effective chemopreventive agents (Ramos, 2008). In addition, the low toxicity and the very few adverse side effects linked to polyphenols consumption give them potential advantages.

Cocoa, the dried and fermented seeds derived from *Theobroma cacao*, has been consumed since 1100 B.C. by ancient civilizations such as the Mayans and Aztecs (Hurst et al., 2002). In the 16th century cocoa was introduced into Europe by Hernan Cortes and, three centuries later, Conrad van Houten developed cocoa powder as we know it today (Dillinger et al., 2000, Rössner, 1997). Cocoa powder is a rich source of fiber (26–40 %), proteins (15–20 %), carbohydrates (about 15 %) and lipids (10–24%) and it contains minerals and vitamins (Ramiro-Puig and Castell, 2009). Out of more than 200 compounds found in the cocoa bean that are thought to be beneficial for the human body, research is mainly focused on polyphenols, particularly the flavanols, that are so abundant in this ancient plant (Visioli et al., 2009). Cocoa has the highest flavanol content of all foods on a weight basis and is a significant contributor to the total dietary intake of flavonoids (Lee et al., 2003, Rusconi and Conti, 2010,

Vinson et al., 1999). Principally, cocoa contains high amounts of flavonoids (-)-epicatechin (EC), (+)-catechin and their dimers procyanidins B2 (PB2) and B1, although other polyphenols such as quercetin, isoquercitrin (quercetin 3-O-glucoside), quercetin 3-O-arabinose, hyperoside (quercetin 3-O-galactoside), naringenin, luteolin and apigenin have also been found in minor quantities (Sánchez-Rabaneda et al., 2003).

Cocoa is a rich source of antioxidants; in a study that measured the total concentration of redox compounds in 1,113 different foods, of the 50 foods with the highest antioxidant capacity, 5 were cocoa based (Halvorsen et al., 2006). Besides, cocoa and derivatives are widely consumed worldwide due to the highly attractive organoleptic characteristics. Indeed, cocoa products constitute a larger proportion of the diet of many individuals than green tea, wine, or soy beans (Arts et al., 2001, Tabernero et al., 2006). The mean intake of catechins and procyanidins estimated for USA is higher than the estimated intake of other flavonoids (Gu et al., 2004). Chocolate consumption contributed 2 to 5 mg of daily catechin intake out of an estimated total of 50 mg per day in a report from the Netherlands (Arts et al., 2001). For the Spanish diet, it was estimated that cocoa products account for 10% of the total antioxidant capacity of dietary intake (Tabernero et al., 2006).

Health effects derived from cocoa flavonoids depend on their bioavailability (absorption, distribution, metabolism, and elimination), a factor which is also influenced by their chemical structure (Manach et al., 2005). In this regard, different studies have shown the absorption of catechin, EC and dimeric procyanidins after the intake of different cocoa by-products by animals and humans (Lamuela-Raventós et al., 2005, Urpi-Sarda et al., 2009). In particular, monomeric flavonoids are absorbed in the small intestine and they and their metabolites (eg, methylated, sulfated, and glucuronidated compounds), which could also be bioactive, are rapidly detected in plasma at concentrations in the range of nM to μM (Baba et al., 2000, Holt et al., 2002, Roura et al., 2005, Uhlenhut and Högger, 2012) and urine (Tsang et al., 2005).

Accordingly, absorbed flavonoids are widely distributed and can be detected in lymphoid organs, including the thymus, spleen and mesenteric lymphoid nodes, as well as in the liver and testes at different concentrations (Urpi-Sarda et al., 2010). In contrast, procyanidins (dimers and trimers) and large proanthocyanidins appear to be 10- to 100-fold less absorbed (Manach et al., 2005, Serra et al., 2010); therefore, their beneficial effects could be restricted to the gastrointestinal tract where they may have an important local function (Ramiro-Puig and Castell, 2009). In addition, oligomers and polymers of flavanols that are not absorbed through the gut barrier could be metabolized by the intestinal microbiota into various phenolic acids of low molecular weight, which are more bioavailable, and might be well absorbed through the colon (Urpi-Sarda et al., 2009, Urpi-Sarda et al., 2010). Interestingly, recent findings have demonstrated that some of these microbial metabolites derived from cocoa consumption also possess biological properties (Monagas et al., 2010).

The present review will focus on the molecular basis of the potential chemopreventive activity of cocoa and their polyphenolic components. Firstly, this paper summarizes recent *in vitro* studies which have evaluated the potential anti-carcinogenic properties of cocoa and their components and the molecular mechanism involved. Although *in vitro* studies will provide a hint to potential cancer preventive effects *in vivo,* chemopreventive efficacy of natural products can only be demonstrated in animal models or human intervention studies. Therefore, in the second part, investigations on the effect of cocoa in various animal models of carcinogenesis are also presented. Finally, this review briefly describes the current evidence on the link between cocoa and cancer occurrence, based on interventional and epidemiological studies on humans.

2.- Studies in cell culture

Cell culture studies constitute a useful tool to elucidate the molecular mechanisms of action of cocoa extracts and its polyphenolic compounds in different cancer cell lines. In this regard, it should be mentioned that pure compounds are metabolized and circulate in the plasma, although the free aglycone is accumulated in tissues as a consequence of a conjugation-deconjugation cycle; thus, pure phenolic compounds seem not to be bioactive unless deconjugation occur in the cell, being the free aglycone the final effector, as already reported for quercetin (Perez-Vizcaino et al., 2012). Moreover, intracellular and bound phenolic metabolite concentrations can be higher than plasma levels and be bioactive even when plasma concentrations are in the nM range (Uhlenhut and Högger, 2012).

2.1- Antioxidant and detoxifying effects

Cells are continuously threatened by the damage caused by free radicals and reactive oxygen species (ROS), which are produced during normal oxygen metabolism or induced by exogenous damage (Finkel and Holbrook, 2000). In physiological conditions, there is a balance between ROS generation and neutralization, and to this equilibrium contribute the antioxidant-defense mechanisms of the body, which include enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) and non-enzymatic defenses (glutathione, acid ascorbic, etc.) (Finkel and Holbrook, 2000). In pathological situations, this balance can be altered and an accumulation of free radicals at the early stages and in the progression of cancer might lead to an oxidative cellular damage or to an alteration in a signaling pathways since ROS may act as signaling molecules (Finkel and Holbrook, 2000). Thus, foods containing antioxidants such as vegetables, fruits, soy products, cocoa and tea that counteract ROS are expected to be protective in cancer causation and development.

Flavonoids can prevent the DNA-damage caused by free radicals or carcinogenic agents acting through different ways: (i) direct radical scavenging, (ii) chelating divalent cations involved in

the Fenton reaction (Nakagawa et al., 2004), (iii) modulation of enzymes related to oxidative stress (GPx, SOD, nitric-oxide synthase, lipooxygenase, xanthine oxidase, etc.) and (iv) alteration of procarcinogenic metabolism by inhibiting phase-I drug-metabolizing enzymes (cytochrome P450, CYP) or activating phase II conjugating-enzymes (glucuronidation, sulfation, acetylation, methylation and conjugation).

2.1.1.- Protective effects against oxidative stress

Cocoa has a potent antioxidant capacity as compared with other products, and this property has been related to its flavonoid content (Lee et al., 2003). Thus, a cocoa extract and its main flavonoid, EC, exerted a similar neuroprotective effect by diminishing intracellular ROS production in H_2O_2/Fe^{2+}-stimulated SH-SY5Y cells (Ramiro-Puig et al., 2009) (Table 1). In this line, a cocoa phenolic extract and PB2 counteracted acrylamide-induced cytotoxicity by inhibiting glutathione (GSH) consumption and ROS generation, and increasing the levels of gamma-glutamyl cysteine synthase and glutathione-S-transferase (GST) in Caco-2 cells (Rodriguez-Ramiro et al., 2011b). Similarly, a cocoa phenolic extract and EC induced beneficial changes in the antioxidant defense system in a human hepatic cell line (HepG2) (Granado-Serrano et al., 2007, Martín et al., 2008, Martín et al., 2010b); both substances prevented or delayed conditions which favored cellular oxidative stress. In particular, the cocoa polyphenolic extract increased the activity of GPx and glutathione reductase (GR) via extracellular regulated kinase (ERK) activation (Martín et al., 2010a), and the pretreatment of cells with the cocoa polyphenolic extract completely prevented cell damage and enhanced activity of the antioxidant enzymes GPx and GR induced by the pro-oxidant *tert*-butylhydroperoxide (*t*-BOOH). Moreover, depletion of GSH and ROS over-production caused by *t*-BOOH in HepG2 cells were prevented by the pretreatment with the cocoa polyphenolic extract (Martín et al., 2008). Accordingly, EC is also a powerful radical scavenger. EC reduced the cytotoxicity induced by *t*-BOOH (Granado-Serrano et al., 2009, Martín et al., 2010b) and

conferred a protection against oxidative stress by preventing *t*-BOOH-induced increase of ROS, malondialdehyde, GPx and GR activities as well as the decrease of GSH in HepG2 cells (Martín et al., 2010b, Murakami et al., 2002). EC also protected cells from oxidative insults by preventing hydroxyl radical formation in the presence of copper (Azam et al., 2004), and played a role in modulating oxidative stress in lead-exposed cells through the downregulation of ROS generation, decrease of intracellular calcium and prevented the alteration of the mitochondrial membrane potential (Chen et al., 2002, Chen et al., 2003). Moreover, EC and one its major metabolites *in vivo*, 3´-*O*-methyl epicatechin, protected human fibroblasts against oxidative stress induced by H_2O_2 (Spencer et al., 2001b) and UVA light (Basu-Modak et al., 2003).

Cocoa and its main flavanols prevent the oxidative damage to DNA. In calf thymus DNA, procyanidins isolated from cocoa, EC and (+)-catechin inhibited 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodG) production in a concentration- and time-dependent manner. EC was more efficient than catechin with respect to inhibiting 8-oxodG formation, although monomer, tetramer, and hexamer fractions were equally effective in inhibiting 8-oxodG formation when assayed at 10 µM monomer equivalent concentration, suggesting that both epimerism and degree of oligomerization are important determinants for the antioxidant activity of flavan-3-ols and procyanidins (Ottaviani et al., 2002). EC and (+) catechin also protected HepG2 cells against oxidative DNA damage induced by N-nitrosamines and heterocyclic amines as reduced DNA strand breaks, the formation of endonuclease III and oxidized purines and pyrimidines (Delgado et al., 2009, Haza and Morales, 2011).

2.1.2.- Molecular mechanisms related to the cellular protective effect

One possible mechanism by which cocoa and its phenolic compounds might exert their protective effect towards oxidative stress-induced damage may be through the modulation of phase I and II enzyme activities. A polyphenolic cocoa extract increased CYP1A1 mRNA and protein levels and enzymatic activity in MCF-7 and SKBR3 breast cancer cells (Oleaga et al.,

2012). In particular, the CYP1A1 transcriptional activation by the polyphenolic cocoa extract was mediated through aryl hydrocarbon receptor (AhR) binding to xenobiotic response elements within the CYP1A1 promoter in MCF-7 cells. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells, which suggest that the interaction between estrogen receptor α and AhR upon incubation with the polyphenolic cocoa extract lead to CYP1A1 induction in breast cancer cells (Oleaga et al., 2012). In this regard, (+)-catechin moderately inhibited CYP1A1 activity induced by heterocyclic amines, whereas EC showed a weaker effect on the inhibition of the activity of CYP1A1 in HepG2 cells (Haza and Morales, 2011). However, (+)-catechin induced the greatest increase in the activity of the phase II metabolizing enzyme UDP-glucuronyltransferase (Haza and Morales, 2011). Similarly, EC increased NADPH cytochrome *c* reductase activity in MCF-7 breast cancer cells (Rodgers and Grant, 1998).

PB2 dimer and to a lesser extent EC and related metabolites [3′-O-methyl epicatechin, 4′-O-methyl epicatechin, and (−)-epicatechin glucuronide] inhibited the activity of NADPH-oxidase in cultures of human umbilical vein endothelial cells (Steffen et al., 2008, Steffen et al., 2007). Interestingly, flavanols and procyanidins of cocoa were able to alter the cellular oxidant production as a result of inhibiting the binding of a ligand to its receptor. This is the case for tumor necrosis factor (TNF)-α, whose binding to TNFα receptor 1 leads to the activation of NADPH-oxidase and to the subsequent superoxide anion production (Yang and Rizzo, 2007). In intestinal cells, EC, (+)-catechin, B2 dimers, and hexameric procyanidins decreased the transient increase in oxidants associated with TNFα-triggered signaling (Erlejman et al., 2008).

Upregulation of cytoprotective enzymes by therapeutic agents to prevent damage by ROS and xenobiotic electrophiles is a strategy for cancer chemoprevention. The Kelch-like ECH-associated protein 1 (Keap1) and its binding partner, transcription factor NF-E2-related factor-2 (Nrf2), are chemopreventive targets because of their role in regulating the antioxidant response

element (ARE), which is present in the promoter region of many antioxidant genes, in response to oxidative stress and exposure to electrophiles. Modification of the sensor protein Keap1 by electrophiles such as the cocoa and its phenolic compounds can direct Nrf2 accumulation in the nucleus and subsequent ARE activation. In this regard, it has been described that EC induces Nrf2 translocation and phosphorylation via ROS generation, through the modulation of proliferation/survival pathways in human HepG2 cells (Granado-Serrano et al., 2010). EC also increased GSH content and stimulated Nrf2 via AKT (protein kinase B) in astrocytes (Bahia et al., 2008). Accordingly, catechin decreased lipid peroxidation and ROS, and increased the activity of GPx, GR total sulfhydryl groups and the expression of Nrf2 and heme oxygenase-1 in intestinal Int-407 cells (Cheng et al., 2012). PB2 also evoked a dose-dependent increase in GPx, GR and GST, which could be related to an improved cell response to an oxidative challenge (Rodriguez-Ramiro et al., 2011a). Hence, Caco2 cells treated with PB2, and then submitted to an oxidative stress induced by *t*-BOOH showed a reduced ROS production, restricted activation of caspase 3 and higher viability than cells only submitted to the stressor (Rodriguez-Ramiro et al., 2011a). PB2 also showed a protective effect against the oxidative injury induced by *t*-BOOH in colonic cells through the upregulation of the expression of GSTP1 via a mechanism that involved ERK and p38 mitogen-activated protein kinase (MAPK) activation and Nrf2 translocation (Rodrıguez-Ramiro et al., 2011).

Procyanidin dimers and trimers isolated from cocoa can provide protection against the attack of oxidants and other molecules that challenge the integrity of the bilayer by interacting with membrane phospholipids, presumably with their polar headgroup (Verstraeten et al., 2005). Similarly, a hexameric procyanidin fraction isolated from cocoa interacted with Caco-2 cell membranes preferentially at the water–lipid interface without affecting their integrity, and it inhibited the deoxycholic (DOC)-induced cytotoxicity, oxidant generation, NADPH oxidase

activation, loss of monolayer integrity and DOC-triggered increase in cellular calcium (Da Silva et al., 2012, Erlejman et al., 2006).

2.1.3.- Pro-oxidative effect

Despite these well-defined antioxidant characteristics, flavonoids can become pro-oxidants under certain conditions, such as high flavonoid concentrations and the presence of redox-active metals (Sakano et al., 2005). PB2 inhibited the formation of 8-oxodG in the human leukemia cell line HL-60 treated with an H_2O_2-generating system. In contrast, a high concentration of PB2 increased the formation of 8-oxodG in HL-60 cells (Sakano et al., 2005). Experiments with calf thymus DNA also revealed that PB2 decreased 8-oxodG formation by Fe(II)/H_2O_2, whereas PB2 induced DNA damage in the presence of Cu(II), and H_2O_2 extensively enhanced it. This suggests that PB2 exerts both antioxidant and pro-oxidant properties by interacting with H_2O_2 and metal ions (Sakano et al., 2005).

2.2- Anti-inflammatory effects

The association between inflammation and cancer involves key inflammatory mediators such as nuclear factor kappa B (NF-κB), TNFα, cyclooxygenase-2 (COX-2), etc. These proteins are also related to cell proliferation, anti-apoptotic activity, angiogenesis and metastasis (Table 2) (Ramos, 2008).

Cocoa phenolic extract inhibited the inflammatory mediator prostaglandin E2 in human intestinal Caco-2 cells (Romier-Crouzet et al., 2009). Similarly, a cocoa polyphenolic extract dose-dependently diminished the secretion of monocyte chemoattractant protein 1 and TNFα, this effect being greater than that produced by equivalent concentrations of EC (Ramiro et al., 2005). Interestingly, cocoa extract added prior to monocyte activation with lipopolysaccharide (LPS) resulted in a significantly greater inhibition of TNFα secretion. Both cocoa extract and EC decreased TNFα, interleukin (IL) 1α and IL-6 mRNA expression, and cocoa phenolic

extract also diminished NO secretion in a dose-dependent manner and with a greater effect than that produced by EC (Ramiro et al., 2005). Accordingly, two cocoa extracts inhibited phorbol 12-myristate 13-acetate (PMA)- induced superoxide production, IL-1 and IL-6 release in human monocytes (Zeng et al., 2011), and cocoa polyphenols also dose-dependently inhibited xanthine oxidase activity and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide-anion generation in cultured human promyeolcytic leukemia HL-60 cells (Lee et al., 2006). In mouse epidermal cells, cocoa polyphenols inhibited TNFα-induced phosphorylation of AKT and ERK, as well as the activation of downstream kinases (p70 kDa ribosomal protein S6 kinase and p90 kDa ribosomal protein S6 kinase) and suppressed the TNFα-induced MEK1 (mitogen activated protein kinase kinase-1) activity and the phosphatidylinositol-3-kinase (PI3K) activity via binding PI3K directly, suggesting a chemopreventive potential against pro-inflammatory cytokine-mediated skin cancer and inflammation (Kim et al., 2010). Correspondingly, (+)-catechin and EC inhibited nitrite and TNFα production in LPS-stimulated macrophages and murine aorta endothelial cells, respectively (Guruvayoorappan and Kuttan, 2008, Schroeder et al., 2001). More recently, a cocoa phenolic extract significantly reduced the increase in inflammatory markers such as IL-8 secretion, COX-2 and inducible nitric oxide synthase (iNOS) expression induced by the pro-inflammatory agent TNFα in Caco-2 cells (Rodrıiguez-Ramiro et al., 2012). In this study, cocoa phenolic extract selectively decreased both the phosphorylation of c-Jun N-terminal kinase (JNK) and the nuclear translocation of NK-κB induced by TNFα, indicating that this pathway could be an important mechanism contributing to the reduction of intestinal inflammation.

EC and procyanidins can inhibit NF-κB at different levels in the activation pathway. A decrease in cell oxidants that are involved in NF-κB activation is a potential mechanism of modulation by these compounds. Thus, hexameric procyanidins inhibited TNFα-induced NF-κB activation [inhibitor of κB (IκB) phosphorylation and degradation, p50 and RelA nuclear translocation,

and NF-κB–DNA binding], iNOS, and cell oxidant increase in Caco-2 cells (Erlejman et al., 2008). These effects occurred because hexameric procyanidins can inhibit NF-κB activation by interacting with the plasma membrane of intestinal cells, and through these interactions preferentially inhibit the binding of TNFα to its receptor and the subsequent NF-κB activation (Erlejman et al., 2008). PB2 also inhibited the binding of NF-κB proteins, i.e., RelA and p50, to its κB DNA consensus sequence in whole cells, nuclear fractions, and purified chemical systems (Mackenzie et al., 2008, Mackenzie et al., 2004, Mackenzie et al., 2009). Thus, procyanidins B1 and B2 inhibited TNFα and PMA-induced transactivation of NF-κB and NF-κB–DNA nuclear binding in Jurkat T cells, whereas none of these effects were observed with A1 and A2 procyanidins, suggesting that B-type dimeric procyanidins can provide anti-inflammatory benefits due to their ability to reduce NF-κB binding to the DNA (Mackenzie et al., 2009). To a lesser degree, EC can also interact with NF-κB and inhibit TNFα-stimulated and constitutive NF-κB activation in T lymphocytes (Jurkat) and Hodgkin lymphoma cells, respectively (Mackenzie et al., 2004, Mackenzie and Oteiza, 2006). However, in HepG2 cells, EC transiently stimulated the NF-κB pathway: increased NF-κB(p65) levels and IκB kinase, induced NF-κB nuclear translocation and NF-κB-binding activity, as well as IκB phosphorylation and degradation (Granado-Serrano et al., 2010). The induction of the redox-sensitive transcription factor NF-κB was connected to ERK, which is involved in the control of hepatic cell survival and proliferation, pointing out to the role of EC in the promotion of cell protection and survival pathways (Granado-Serrano et al., 2010).

2.3.- Effects on apoptosis and proliferation

In chemoprevention, suppression of cell proliferation and induction of differentiation and apoptosis are important strategies, with the induction of programmed cell death currently considered as one relevant target in a preventive approach.

2.3.1.- Cell cycle

Deregulated cell cycle and resistance to apoptosis are hallmarks of cancer (Ramos, 2008). Cell cycle control is a highly regulated process that involves the modulation of cell cycle regulatory proteins, including cyclins (cyclin A, B, D, or E), cyclin-dependent kinases (CDKs) (CDK 1, 2, 4, or 6), and CDK inhibitors, such as p21, p27, p53, and phosphorylated retinoblastoma (pRb) (Ramos, 2008). Any alteration of cell cycle-specific proteins can affect and/or block the continuous proliferation of cancer cells. In addition, cell cycle checkpoints, such as G1/S and G2/M, are also important targets for cocoa and its polyphenols (Table 3).

Type II topoisomerases (topo II) are enzymes that transiently modify the DNA topology to allow DNA replication, transcription and chromosomal recombination and segregation. Thus, inhibition of topoisomerases leads to cellular accumulation of DNA strand breaks and can result in cell death, which is the desired effect of anti-cancer drugs (Lanoue et al., 2009). Limited topo II inhibition and low cellular toxicity were observed for a procyanidin-rich cocoa extract in leukemia cells (Lanoue et al., 2009). The dimers (B2, B5 and a mix of both) were the most active inhibitors of topo II and proliferation in HL-60 and Raji cells lines (Lanoue et al., 2009).

Cocoa-derived pentameric procyanidin (pentamer) caused G0/G1 cell cycle arrest in human breast cancer MDA MB-231, MDA MB-436, MDA MB-468, SKBR-3, and MCF-7 cells and in benzo(a)pyrene-immortalized 184A1N4 and 184B5 cells, whereas normal human mammary epithelial cells in primary culture and spontaneously immortalized MCF-10A cells were resistant (Ramljak et al., 2005). Pentamer caused depolarization of mitochondrial membrane in MDA MB231 cells but not in MCF-10A cells. In addition, in MDA MB-231 cells, pentamer induced a specific dephosphorylation, without changes in protein expression, of key G1-modulatory proteins, such as Cdc2, forkhead transcription factor and p53, although expression and phosphorylation of retinoblastoma protein decreased after pentamer treatment (Ramljak et al., 2005). These results showed that breast cancer cells are selectively susceptible to the

cytotoxic effects of pentameric procyanidin, and suggested the inhibition of cellular proliferation. Accordingly, a natural and synthetic pentameric EC-derived procyanidin inhibited the growth of breast cancer MDA MB 231 cells through the induction of cell cycle arrest in G0/G1 phase, the subsequent cell death being more likely necrotic rather than apoptotic (Kozikowski et al., 2003). Similarly, procyanidin-enriched extracts from cocoa caused growth inhibition with a blockade of the cell cycle at G2/M phase in human colonic Caco-2 cells (Carnésecchi et al., 2002), and EC induced S phase arrest in the cell cycle progression in LoVo colon cancer cells (Tan et al., 2000). Flavanol dimers B1-B4 decreased growth of the androgen-sensitive (LnCaP) and androgen-resistant (DU145) human prostate cancer cell lines, and PB2, oleylated or not, displaced testosterone from membrane androgen receptors and induced DU145 tumor xenograft regression (Kampa et al., 2011). In addition, procyanidin B3 inhibited acetylation-dependent prostate cell proliferation and expression of cell-cycle control genes (cyclin E and D1), subsequently increasing cell death in LNCaP cell line (Choi et al., 2011).

EC seemed to be inactive to induce growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization in the metastatic human prostate cancer DU145 cells (Chung et al., 2001, Ravindranath, M.H. et al., 2006), but it reduced proliferation of the poorly differentiated HH639 epithelial ovarian cancer cells (Ravindranath et al., 2006).

2.3.2.- Apoptosis

Apoptosis induction may be considered one of the important targets in a preventive approach against cancer. This programmed-cell death is a complex process that involves the active participation of affected cells in a self-destruction cascade and is defined by a set of characteristic morphological features such as membrane blebbing, shrinkage of the cell and nuclear volume, chromatin condensation and nuclear DNA fragmentation due to endonuclease activation (Ramos, 2007, Ramos, 2008). At the molecular level, apoptosis shows characteristic biochemical hallmarks, and two effector mechanisms: (a) extrinsic, mediated by death receptors

CD95/Fas/ Apo1, TNF receptor 1, TNF receptor 2, and death receptors 3 to 6, and (b) intrinsic (mitochondria-mediated) (Ramos, 2007). In the mitochondria, propagation of the apoptotic signal is regulated by proteins such as Bcl-2 family members (Bcl-2, Bcl-x_L, and Bcl-w), which exert anti-apoptotic effects, and Bid, Bad, Bak, Bax, and Bim, which exert pro-apoptotic effects (Ramos, 2007). Both cascades converge in a common executor mechanism involving DNA endonucleases, which cleave regulatory and structural molecules and activated proteases (caspases) and lead to the cellular death (Table 4) (Ramos, 2007, Ramos, 2008).

Procyanidin induced apoptosis in prostate cancer PC-3 cells by altering the mitochondrial membrane potential, but when the incubation time was extended the necrotic rate was higher than that of the apoptosis (Shang et al., 2009). Accordingly, procyanidin B2 decreased expression of the NF-κB-regulated anti-apoptotic proteins such as Bcl-x_L, Bcl-2, X-linked inhibitor of apoptosis protein and cellular FLICE-inhibitory protein (cFLIP), leading to a decreased cell viability, although it did not significantly affect the progression of the cell cycle, or parameters of apoptosis in Hodgkin´s lymphoma cells (KM-H2, L-428, L-540 and L-1236 cells) (Mackenzie et al., 2008). Importantly, PB2 did not affect cell viability of Daudi B lymphoblast cells and Jurkat T leukemia cells, both lacking constitutive NF-κB activation. This suggests the involvement of B2-mediated NF-κB inhibition in Hodgkin´s lymphoma cell survival and the low toxicity of this procyanidin to other cells (Mackenzie et al., 2008). In neurons and fibroblasts treated with H_2O_2, EC glucuronide did not affect the peroxide-increased caspase-3 activity, whilst EC and 3´-*O*-methyl EC reduced the peroxide-induced caspase-3 activation (Spencer et al., 2001a, Spencer et al., 2001b). In this line, the protection elicited by 3´-*O*-methyl EC was not different from that of EC, suggesting that the hydrogen-donating antioxidant activity is not the primary mechanism of protection (Spencer et al., 2001b). Similarly, phenolic-rich extracts of both unroasted and roasted cocoa prevented the programmed cell death induced by celecoxib in MLP29 liver cells, i.e.: diminished the number

of cells in the sub-G1 peak and the activation of Bax, partially restored the levels of Bcl-2, and decreased the released cytochrome *c* in the cytosol and the activation of caspase 3 (Arlorio et al., 2009). Moreover, the protective effect of cocoa against liver cytotoxicity provoked by celecoxib was probably attributable to induction of the autophagic process, as shown by enhanced Beclin 1 expression and accumulation of monodansylcadaverine in autolysosomes, suggesting that apoptosis was prevented by stimulating autophagy (Arlorio et al., 2009). Hexameric procyanidins also delayed the DOC-induced Caco-2 cell apoptosis (Da Silva et al., 2012).

Interestingly, a synergistic effect has been reported to induce apoptosis by combination of drugs and/or natural compounds and cocoa phenols (Ramos, 2007). In this line, EC showed a major synergistic effect on the induction of apoptosis in gastric cancer MKN-45 cells treated with epigallocatechin-3-gallate (Horie et al., 2005) and in human lung cancer PC-9 cells (Suganuma et al., 1999). Similarly, the combination of curcumin with EC increased the inhibition of cell growth as compared to curcumin or EC alone, as well as the apoptosis rate and the expression of related genes to the programmed cell death such as GADD153 and GADD45 in PC-9 cells (Saha et al., 2010).

2.3.3.- Proliferation/survival

The most important signaling pathways regulating cell proliferation and survival involve PI3K/AKT, growth factor receptors/Ras/MAPKs, and NF-κB, which also importantly contributes to the inflammatory process, as mentioned above (Table 5) (Ramos, 2008).

Cocoa phenolic compounds can interact with signaling proteins and modulate their activity. A cocoa extract and EC exerted a neuroprotective effect by down-regulating proteins involved in apoptosis such as p38 and phosphorylated JNK (Ramiro-Puig et al., 2009). Moreover, a cocoa procyanidin fraction and PB2 inhibited TPA-induced neoplastic transformation of JB6 P+ mouse epidermal cells, COX-2 expression, phosphorylation of MEK and p90 ribosomal s6

kinase and attenuated activator protein-1 (AP1) and NF-κB stimulations (Kang et al., 2008). In this study, it was demonstrated that the cocoa procyanidin fraction and PB2 inhibited the kinase activity of MEK1 and directly bound with MEK1. PB2 and a cocoa polyphenolic extract also protected colonic Caco-2 cells against acrylamide-induced cytotoxicity by blocking the activation of the apoptotic pathway as prevented the increase in the levels of p-JNK induced by the toxicant, whereas ERK seemed to play an indirect protective role through the promotion of cell proliferation and survival signaling (Rodriguez-Ramiro et al., 2011b). Similarly, hexameric procyanidins prevented oncogenic events initiated by DOC through the interaction with Caco-2 cell membranes, and inhibited the DOC-promoted activation of AKT, ERK and p38, as well as the downstream transcription factor AP-1 (Da Silva et al., 2012). In monocytes, PB2 dimer decreased the endotoxin-induced expression of COX-2, through the inhibition of MAPKs (p38, JNK, and ERK) and NF-κB (Zhang, et al., 2006). However, PB2 and EC did not have an obvious effect on Caco-2 and SW480 colon carcinoma cells, whereas PB2 promoted cell growth in SW480 cells by increasing p-AKT and p-ERK levels (Ramos et al., 2011). This different response depends on the distinct chemical structure of the compound and the different degree of cell differentiation: it ranges from no effect of any of the flavanols on Caco-2 cells to a survival/proliferative effect of PB2 on SW480 cell line. This highlights the importance of an holistic approach to the study of the biological effects of phytochemicals and calls for caution on the conclusions derived from their studies (Ramos et al., 2011). In addition, it has been suggested that the capacity of procyanidins to interact with the cell membrane and to prevent those cell membrane-associated events can in part explain their effects (Da Silva et al., 2012).

EC had no prominent effects on the HepG2 cell rate (Ramos et al., 2005), it did not induce the caspase cascade for apoptosis or affect the expression of pro-apoptotic Bcl-2 proteins, such as Bax or Bcl-x, but induced a sustained activation of the major survival signals (Granado-Serrano et al., 2007). In this regard, EC induced a time-dependent regulation of survival/proliferation

pathways in HepG2 cells (Granado-Serrano et al., 2009). EC-induced cell survival was a rapid event that was accompanied by early and sustained activation of AKT and ERK, as well as protein kinase C (PKC)-α, in concert with unaltered JNK levels and early inactivation of key death-related signals like PKC-δ. All these suggest that EC induces cellular survival through a tight regulation of survival/proliferation pathways that required the integration of different signals and persists over time (Granado-Serrano et al., 2009).

2.4.- Effects on angiogenesis and metastasis

Angiogenesis, the formation and growth of new blood vessels from preexisting microvasculature (Ramos, 2008), is a key stage in tumor growth, invasion, and metastasis (Table 6).

Cocoa polyphenols inhibited TNFα-induced up-regulation of vascular endothelial growth factor (VEGF) by reducing TNFα-induced activation of the nuclear transcription factors AP-1 and NF-κB, which are key regulators of VEGF expression (Kim et al., 2010). Similarly, (+)-catechin inhibited tumour-specific angiogenesis by regulating the production of pro- and anti-angiogenic factors such as pro-inflammatory cytokines, nitric oxide, VEGF, IL-2 and tissue inhibitor of metalloproteinase-1 (Guruvayoorappan and Kuttan, 2008). In addition, in (+)-catechin-treated animals the TNFα production, the proliferation, migration and tube formation of endothelial cells as well as the microvessel outgrowth in the rat aortic ring assay were inhibited (Guruvayoorappan and Kuttan, 2008). Accordingly, it has been reported that the chemical modification of EC by its acylation improved the anti-cancer and anti-angiogenic activities of this flavanol (Matsubara et al., 2007). EC conjugated with fatty acid (acyl-catechin) strongly inhibited DNA polymerase activity, HL-60 cell growth and angiogenesis. EC conjugated with palmitic acid ((2R,3R)-3′,4′,5,7-tetrahydroxyflavan-3-yl hexadecanoate, epicatechin-C16) was the strongest inhibitor in DNA polymerase α, β, λ and angiogenesis

assays. Epicatechin-C16 also suppressed human endothelial cell (HUVEC) tube formation on reconstituted basement membrane, suggesting that it affected not only DNA polymerase activity but also the signal transduction pathways needed for the tube formation in HUVECs (Matsubara et al., 2007).

Metastasis involves the interplay of extracellular matrix degradation, proteolysis, cell adhesion, cell migration, angiogenesis, and invasion. Dietary polyphenols have also been reported to interfere with cancer cell adhesion and movement processes through multiple mechanisms (Ramos, 2008). Inhibition of gap-junction intercellular communication is strongly related to tumorigenesis (Ramos, 2008). Cocoa polyphenol extracts dose-dependently attenuated H_2O_2-induced inhibition of gap-junction intercellular communication in rat liver epithelial cells (Lee et al., 2010). Cocoa polyphenol extracts also inhibited the H_2O_2-induced phosphorylation and internalization of connexin 43, the accumulation of ROS and activation of ERK through the direct binding to MEK1, to inhibit its activity (Lee et al., 2010). Importantly, EC arrested growth of murine mammary 4T1 cancer cells, decreased ROS generation, downregulated metalloprotease-9 and inhibited invasion of tumor cells into embryonic stem cell-derived and into vascularized tissues (Günther et al., 2007).

3.- Studies in animal models

Animal studies offer a unique opportunity to assess the contribution of the physiological effects of consumption of cocoa and cocoa components in different models of carcinogenesis.

Before considering application of a compound or product for prevention of cancer, toxicological and safety issues also have to be considered. Hence, no evidence of carcinogenicity was found in a chronic study of dietary doses as high as 5% of cocoa powder in rats (Tarka et al., 1991). On the contrary, extracts of cocoa have been shown to decrease the mutagenicity of carcinogens assayed by different tests. Cocoa liquor polyphenols showed an

inhibition of the mutagenic effect of the heterocyclic amines in the Ames test (Yamagishi et al., 2000, Yamagishi et al., 2002). Likewise, in the micronucleus test in mice, cocoa liquor polyphenols administered orally reduced the occurrence of micronucleated cells after the injection of the potent DNA crosslinker mitomycin C (Yamagishi et al., 2001).

Although current data are limited, cancer prevention by cocoa and its major components has been studied in different animal models for genetic and chemically-induced cancer, including mammary, pancreatic, lung, thyroid, prostate, leukemia, hepatic and colorectal cancers. Most of these studies have been conducted with cocoa polyphenol extracts or with pure compounds, whereas two of them have been conducted with a cocoa enriched diet (Table 7). The mechanisms involved in this protective effect of cocoa and its flavonoids appear at different stages of the carcinogenesis process, both in phases of tumor initiation and promotion and in tumor progress and metastasis. The following is a review of studies on the individual cancer types (Table 7).

3.1.- Mammary and pancreatic cancers

Cacao liquor is a major ingredient of chocolate and cocoa that is abundant in polyphenols, including catechins and their oligomers B-type proanthocyanidins (Natsume et al., 2000). Cacao liquor proanthocyanidins (CLPr) exerted inhibitory effects on the mutagenicity induced by diverse heterocyclic amines such as 1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) (Yamagishi et al., 2000, Yamagishi et al., 2002). Yamagishi et al. (2002) investigated the potential protective effect of CLPr on PhIP-induced mammary and pancreas carcinogenesis in female Sprague–Dawley rats. CLPr were incorporated into the basal diet at doses of 0.025% or 0.25% during the initiation or the post-initiation period of PhIP-induced carcinogenesis. They found that the incidences of pre-neoplastic lesions in the exocrine pancreas were dose-dependently decreased by CLPr given during the initiation period, although the multiplicities in

each group were not significantly different. On the other hand, the incidences, multiplicities, and volumes of fibroadenomas or adenocarcinomas in mammary tumors showed a tendency to decrease at the dose of 0.25% CLPr (post-initiation) although without statistical significance. This different organ effect might be due to dissimilar concentrations of CLPr in the target tissues. Altogether, CLPr diet administered during the initiation period of PhIP-induced carcinogenesis was able to inhibit the initiation stage of pancreatic but not mammary carcinogenesis. Irrespective of the mechanisms involved, oxidative stress is implicated in the initiation phases of carcinogenesis (Reuter et al., 2010); therefore, due to the amount of antioxidant flavonoids in CLPr (Natsume et al., 2000), their administration during the initiation period seems to provoke an inhibitory effect against oxidative stress associated with the initiation stage of carcinogenesis.

3.2.- Lung and thyroid cancers

CLPr administered in the post-initiation period was also effective in suppressing lung and thyroid carcinogenesis (Yamagishi et al., 2003). In this study, the inhibitory effect of CLPr against tumorigenesis was examined in F344 male rats using a multi-organ initiation model. The multi-organ carcinogenesis model using diethylnitrosamine (DEN), N-methylnitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine, dimethylhydrazine and 2,2'-dihydroxy-di-n-propylnitrosamine as initiators (DMBDD treatment) is a useful tool for screening chemopreventive as well as tumorigenic activities of chemicals (Ito et al., 1996). CLPr was incorporated into the basal diet at a dose of 0.025% or 0.25%, starting 1 week after the final carcinogen treatment. At the end of the experimental period (36 weeks), significant reduction of adenocarcinoma development in the lungs and a tendency for a decrease in thyroid lesions were found, without any adverse effects in any major organ. Furthermore, the fact that CLPr decreased the development of carcinomas in the lung but not that of hyperplasias and adenomas

suggested some influence on progression from adenoma to carcinoma. Interestingly, the DMBDD group treated with the highest dose of CLPr further exhibited a higher survival rate than the DMBDD alone group. This effect seems to be the consequence of a delay in tumor development in the CLPr group, as evidenced by a significantly lower incidence of lung tumors, and albeit non-significant, lower incidences of pituitary, Zymbal's gland and urinary bladder tumors, kidney nephroblastomas and angiosarcomas.

3.3.- Prostate cancer

The effects of cocoa polyphenols have also been studied against experimental prostate carcinogenesis in male Wistar–Unilever rats (Bisson et al., 2008). In this study, a polyphenolic extract obtained from a polyphenolic-enriched cocoa powder (Acticoa powder –AP-) was used. AP is a cocoa that contains a high percentage of flavanols preserved thanks to a patented process developed by Barry Callebaut (Louviers, France). The polyphenolic-enriched cocoa extract was given orally at doses of 24 and 48 mg/kg body weight starting two weeks before induction of prostate carcinogenesis and throughout the experiment (36 weeks). The induction of prostate tumors was performed using the direct acting chemical carcinogen MNU followed by chronic androgen stimulation with testosterone (Boileau et al., 2003). The administration of 24 mg/kg of AP extract before the initiation and the promotion phases of cancer induction significantly reduced the incidence of prostate lesions in rats. However, the number of preneoplastic and neoplastic lesions observed in rats given 48 mg/kg of AP extract was higher than the observed in the group treated with 24 mg/kg. The reasons for this result could be that a higher dose of cocoa rich-polyphenolic extract may involve a mechanism of regulation such as a negative feedback that might enhance the development of prostate tumors rather than inhibiting them. Finally, similar to what has been found with cocoa liquor procyanidins

(Yamagishi et al., 2003), the preventive treatment of rats with AP extract at the low dose of 24 mg/kg was associated with greater longevity.

3.4.- Leukemia

One of the major cocoa constituent, EC, has also showed to possess anti-carcinogenic activity *in vivo*. Papiez et al. (2011) have recently demonstrated that EC exert genotoxic and necrotic effects in Brown Norway rat myeloid leukemia cells. These properties may have utility in anticancer therapy against acute myeloid leukemia. The Brown Norway rat myeloid leukemia model used in this work was developed after intravenous inoculation with 10^6 splenic-derived leukemia cells in Brown Norway rats. EC was given by gavage at a dose of 40 mg/kg body weight for 22 consecutive days from the second day after inoculation. The results indicated that EC was able to induce selective necrotic cell death in leukemia bone marrow. Likewise, there was a tendency for an increase of early apoptotic cells. On the contrary, EC induced neither necrosis nor apoptosis of non-leukemia cells. Thus, it could be suggested that EC will be helpful by inducing death in cancerous cells which are resistant to apoptosis, while inducing no toxicity in healthy cells.

3.5.- Hepatic cancer

The first animal study on the chemopreventive effects of a cocoa diet was performed by Granado-Serrano et al. (2009). Male Wistar rats were treated with DEN, a potent hepatotoxic agent, to induce liver hepatocarcinogenesis (Köhle et al., 2008, Sivaramakrishnan et al., 2008, Sundaresan and Subramanian, 2008). The basal diet was supplemented with 16% powdered Natural Forastero cocoa. To test both the preventive and the potential therapeutic activity of the cocoa-rich diet, the dietary intervention started either before or after the induction of hepatotoxicity. At the end of the intervention period (6 weeks), it was observed that the cocoa

diet protected the liver against the oxidative damage induced by DEN through the modulation of the activities of several Phase I (GR, GPx and catalase) and Phase II (GST) enzymes. More interestingly, the protective effect of cocoa also involved the regulation of key proteins of cell signaling cascades, which play a main role in the promotion of carcinogenesis. In particular, cocoa exerted an anti-apoptotic effect in the liver of DEN-treated animals that was associated with the prevention of both JNK and caspase-3 activations. The cocoa-diet also prevented the DEN-induced increase of the survival/proliferation signal PI3K/AKT, which has been considered a critical factor in the aggressiveness of hepatocellular cancer and has been associated with poor prognosis in liver cancer (Parekh and Rao, 2007, Schimtz et al., 2007). In detail, cocoa diet completely counteracted the hepatic damage induced by DEN in both pre-and post-treated animals, as phase I and II enzymes showed comparable values to those of animals only fed with cocoa or to control rats, and JNK levels were similar to untreated animals (Granado-Serrano et al., 2009). In addition, caspase-3 activity was not modified in animals fed with cocoa before DEN injection when compared to control rats, whereas animals receiving cocoa after DEN injection showed values of caspase-3 activity in between the levels of untreated animals and rats only injected with DEN. Altogether, these effects indicated a potential attenuation of the post-necrotic proliferation induced by DEN and a reduction of the number of initiated cells.

3.6.- Colon cancer

Cocoa has a high concentration of polyphenols such as procyanidins that are poorly absorbed in the intestine where they may have an important antioxidant and anti-inflammatory local function; therefore, they could have a major role in the prevention of colorectal cancer onset and development. Accordingly, the potentially important role of cocoa and their phenolic compounds for colon cancer prevention was first demonstrated by Weyant et al. (2001). In this

study, a genetic model of multiple intestinal neoplasia, the C57BL/6J Min/+ (Min/+) mice, was used. Min/+ mice have one mutated allele of the adenomatous polyposis coli (*Apc*) gene and spontaneously develop multiple intestinal tumors (Su et al., 1992); therefore, it is considered a reasonable murine model of inherited (familial adenomatous polyposis) and sporadic colorectal cancer in humans. Using this model, the authors demonstrated that the cocoa flavonoid catechin added to a diet in concentrations of 0.1 and 1%, was able to diminish the formation of intestinal tumors by 75 and 71%, respectively. Further mechanistic studies linked this effect to (+)-catechin-induced changes in integrin-mediated intestinal cell-survival signaling, including structural alteration of the actin cytoskeleton and decreased focal adhesion kinase (FAK) tyrosine phosphorylation. FAK has been implicated in the regulation of cell migration, one of the earliest changes in adenoma development (Mahmoud et al., 1999), suggesting that (+)-catechin could prevent the progression of initiated enterocytes to the adenoma stage.

More recently, the chemopreventive ability of a cocoa rich-diet on colon carcinogenesis has been studied in detail in male Wistar rats using the well-defined azoxymethane (AOM)-induced colon cancer model (Rodrıiguez-Ramiro et al., 2011). Administration of the colon-specific carcinogen AOM to rodents provokes the development of aberrant crypt foci, pre-neoplastic lesions in the colon that may progress into cancer later on (Pritchard and Grady, 2011). Animals were fed with a cocoa-enriched diet (12%) starting two weeks before the carcinogenic induction and throughout the experimental period (8 weeks). This study demonstrated that a cocoa-rich diet could prevent the early stage of chemically induced colorectal cancer in rats. Cocoa feeding significantly reduced AOM-induced colonic aberrant crypt foci formation and crypt multiplicity. At the molecular level, the chemopreventive effects elicited by cocoa were due at least in part to its anti-oxidative and anti-inflammatory properties. Indeed, cocoa feeding was able to prevent oxidative stress by reverting to control values the diminished levels of GSH and the activities of GPx, GR and GST provoked by the toxicant. The cocoa-rich diet also prevented

the subsequent increase in AKT and ERK phosphorylation induced by AOM and the decrease in the levels of the proliferative marker cyclin D1 as well as cell proliferation. In addition, cocoa polyphenols suppressed intestinal inflammation induced by AOM through the inhibition of NF-κB signaling and the downregulation of the pro-inflammatory enzyme expressions of COX-2 and iNOS (Rodrıiguez-Ramiro et al., 2012). Finally, cocoa was also able to induce apoptosis as another complementary mechanism of chemoprevention during the progression of carcinogenesis through the upregulation of Bax levels and the downregulation of Bcl-x_L (Rodrıiguez-Ramiro et al., 2012).

4.- Studies in humans

Although animal studies have indicated cancer preventive activity of cocoa, extrapolation of the results to the human situation is difficult. Exposure of humans to low doses of endogenous or exogenous carcinogens and tumor promoters may occur constantly and life-long. Additionally, the response of humans to carcinogens and chemoprotective agents may be influenced by genetic polymorphisms, changes in DNA methylation and epigenomic events (Milner, 2006). Indeed, epidemiologic studies of cocoa intake and cancer risk are few, and those assessing mortality by cancer provide only weak support for a benefit of cocoa. Consequently, confirmation of cancer preventive efficacy in humans requires large and long-lasting controlled clinical trials.

4.1.- Epidemiologic studies

Substantial laboratory data suggest that flavonoid-rich food could help to prevent cardiovascular disease and cancer (Andújar et al., 2012). Since cocoa is one of the richest sources of flavonoids, populations with a diet rich in cocoa products should show a significant protection against these two pathologies.

Perhaps the most prominent case in support of the cancer preventive effect of cocoa in humans is the Kuna tribe in Panama. The Kuna living in the San Blas district of Panama drink a flavanol-rich cocoa as their main beverage, contributing more than 900 mg/day and thus probably have the most flavonoid-rich diet of any population (Bayard et al., 2007). Examination of death certificates to compare cause-specific death rates from year 2000 to 2004 in mainland Panama and the San Blas islands where Kuna live, indicated that the rate of cardiovascular disease, diabetes mellitus and cancer among island-dwelling Kuna was much lower than in mainland Panama. This comparatively lower risk among Kuna from two of the most common causes of morbidity and mortality possibly reflects a very high flavanol intake. However, there are many risk factors and an observational study cannot provide definitive evidence (Bayard et al., 2007).

Also in support of the anticancer effect of cocoa is a case-control study carried out in Spain (Garcia-Closas et al., 1999). The authors reported an inverse relation between the incidence of gastric cancer and flavonoid consumption. Additionally, data from the Iowa Women's Study, established an inverse epidemiological relation between catechin consumption and the incidence of rectal cancer in post-menopausal women (Arts et al., 2002).

Nevertheless, some human studies have failed to show a beneficial impact of cocoa intake in the rate of cancer appearance. A French study showed no significant association between a high chocolate dietary pattern and any stage of colorectal disease ranging from polyps, to adenomas, and colorectal cancer (Rouillier et al., 2005). An adenoma study in North Carolina also failed to observe a significantly lower prevalence of adenomatous polyps and colorectal cancer with chocolate consumption (McKelvey et al., 2000). Panelli et al. (1989) examined changes in mortality from urinary bladder cancer in Italy during the years 1950-81 in relation to changes in smoking habits and in coffee, cocoa and tea consumption. The authors found an increase of mortality throughout this period that was related to occupational exposures and to high-tar-

content tobacco smoking, but consumption of coffee, cocoa and tea did not seem to be related to the increase in bladder cancer risk in Italy.

In the Zutphen study, a prospective cohort study of 806 men aged 65 to 84 years at baseline, catechin intake was not associated with epithelial cancer or lung cancer when the models were adjusted for confounders (Arts et al., 2001). In a clinical trial carried out in Greece with cases (820) and controls (1,548) no significant association was found between flavanol consumption and the incidence of breast cancer (Peterson et al., 2003). In addition, in a recent update including data from 1965 to 2011, the authors showed a lack of solid evidence supporting a prevention of oral cancer by flavonoid intake (Varoni et al., 2012).

Finally, there are a number of human studies that have shown a negative effect of cocoa intake on cancer incidence. In a case-control study in Burgundy (France), chocolate was identified as a risk factor for colorectal cancer (Boutron-Ruault et al., 1999). A correlation study on the association of disease rates with the dietary practices in various geographical areas has suggested that exposure to cocoa during prenatal life or childhood may be associated to the risk of both hypospadias and testicular cancer in the offspring (Giannandrea, 2009). However, the association between male reproductive diseases and consumption of cocoa has been linked to reproductive toxicity of cocoa theobromine (EFSA, 2008, Wang et al., 1992). In any case, the overall association at least may serve to stimulate further epidemiological and experimental studies.

4.2.- Intervention studies

Research on the incidence of flavanol intake and cancer risk in human subjects has been mostly focused on green and black tea consumption (Yang et al., 2009). Cocoa flavanols, monomeric catechins and polymeric procyanidins, are similar to those in tea and they also decrease the adverse effects of ROS. However, they have different chemical structures and different

metabolic functions, therefore, antimutagenic and anticancer properties reported for tea phenolics could not be attributed to cocoa flavanols. There are properly no human intervention studies attempting to show a correlation between cocoa intake and cancer prevention, but a few human intervention trials indicate that cocoa favorably affects intermediary factors in cancer progression (Maskarinec, 2009).

Chronic inflammation and oxidative stress are significant contributing factors to carcinogenesis. ROS can damage DNA and interfere with DNA repair, leading to mutations that favor uncontrolled cell growth and replication. Thus, the antioxidant activity of cocoa flavanols is of particular interest for its potential influence on the initiation stage of carcinogenesis. In this line, Spadafranca et al. (2010) have demonstrated that dark chocolate consumption significantly improved DNA resistance to oxidative stress. In this study healthy subjects were assigned to a daily intake of 45 g of dark chocolate or white chocolate for 14 days and oxidative damage to mononuclear blood cells DNA was reduced in the dark chocolate group 2 h after consumption; 22 h later the effect disappeared.

In vitro and *ex vivo* studies support an anti-inflammatory effect of cocoa flavanols, but these effects have not always been replicated *in vivo* (Selmi et al., 2008). Thus, cocoa consumption reduced NF-κB activation in peripheral blood mononuclear cells in healthy voluntaries (Vázquez-Agell et al., 2011), but biomarkers of inflammation, including IL-6, were unaffected in patients at high risk of cardiovascular disease consuming cocoa powder (Monagas et al., 2009). Overall, an increasing number of human trials have assessed changes in markers of oxidative stress and inflammation with cocoa products (reviewed by Maskarinec, 2009). However, whether modification of any of these parameters will correlate with a lower incidence of appearance or development of specific cancers is highly debatable.

5.- Conclusion

This review addresses the potential anti-carcinogenic mechanisms of action that have been so far identified for cocoa and its main phenolic compounds, as well as the feasibility that they could occur *in vivo*. In general terms, those cellular mechanisms include the modulation of the redox status and multiple key elements in signal transduction pathways related to cell proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis. Studies performed in animals have demonstrated that cocoa and its main phenolic components can prevent and/or slow down the initiation-progression of different types of cancers such as cancer of prostate, liver, colon, leukemia, etc. In addition, several human intervention studies have reported some favorable changes in biomarkers assessing antioxidant status. However, caution is needed when attempting to extrapolate the *in vitro* observations to *in vivo* animal tumor models and, most importantly, to humans. Cocoa products deserve further investigations since the molecular mechanisms of action are not completely characterized and many features remain to be elucidated. Additionally, more extensive, well-controlled clinical trials are needed to fully evaluate the potential of cocoa in terms of optimal dose, route of administration and cancer targets. Overall, these studies suggest that the daily consumption of small amounts of flavanols and procyanidins from cocoa or chocolate, in conjunction with usual dietary intake of flavonoids from mixed food sources, could offer a natural therapeutic approach to improve individual health status including potential efficient cancer prevention with minimal toxicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1.- Cocoa and cocoa polyphenols modulation of cellular antioxidant status and phase I and II enzymes[a].

Polyphenol	Concentration	System studied	Effects	Reference
Cocoa	5-30 µg/mL	SH-SY5Y (neuroblastoma)	↓ H_2O_2/Fe^{2+} induced ROS production	Ramiro-Puig, et al., 2009
	10 µg/mL	Caco-2 (colon)	↓ acrylamide-induced GSH depletion, ↓ ROS generation, ↑ γ-GCS, ↑ GST	Rodriguez-Ramiro, et al., 2011
	0.05-50 µg/mL	HepG2 (hepatoma)	↓ *t*-BOOH-induced death, ↓ GSH depletion, ↓ MDA, ↓ ROS generation, ↓ GPx and GR activation	Martin, et al., 2008
	0.05-50 µg/mL	HepG2 (hepatoma)	↑ GPx and GR	Martin, et al., 2010a
	0.25 µg/ mL	MCF-7, SKBR3 (breast)	↑ CYP1A1 mRNA, protein and activity, ↑ binding to XRE	Oleaga, C. et al, 2012
Polymers procyanidins[b]	0.1-100 µM (0.03-29 µg/mL)	Calf thymus DNA	↓ UVC-induced 8-oxodG	Ottaviani, et al., 2002
	2.5-60 µM (0.73-17.4 µg/mL)	Caco-2 (colon)	(hexamer) ↓ TNF-induced oxidation	Erlejman, et al., 2008
	2.5-20 µM (0.73-5.81 µg/mL)	Caco-2 (colon)	(hexamer) ↓ DOC-induced cytotoxicity, ↓ oxidant generation, ↓ NADPH oxidase, ↓ Ca^{2+}	Da Silva, et al., 2012; Erlejman al., 2006
Procyanidin B2	10 µM (5.79 µg/mL)	Caco-2 (colon)	↓ acrylamide-induced GSH depletion, ↓ ROS generation, ↑ γ-GCS, ↑ GST	Rodriguez-Ramiro, et al., 201
	0.1-100 µM (0.06-57.9 µg/mL)	HUVEC (umbilical vein endothelial)	↓ NADPH oxidase	Steffen, et al., 2008
	60 µM (34.71 µg/mL)	Caco-2 (colon)	↓ TNF-induced oxidant formation	Erlejman, et al., 2008
	10-30 µM (5.79-17.37 µg/mL)	Hl-60 (leukemia)	↑↓ H_2O_2-induced 8-oxodG formation, ↓ H_2O_2/Fe^{2+}-induced DNA damage, ↑ H_2O_2/Cu^{2+} induced DNA damage	Sakano, et al., 2005
	1-10 µM (0.6-5.79 µg/mL)	Caco-2 (colon)	↑ GPx, GST and GR, ↓ *t*-BOOH-induced ROS production and ↓ caspase activation	Rodriguez-Ramiro, et al., 201
	1-20 µM (0.6-11.57 µg/mL)	Caco-2 (colon)	↑ GST, ↑ Nrf2 translocation	Rodriguez-Ramiro, et al., 201

Epicatechin	12.4-100 µM (3.6-29 µg/mL)	SH-SY5Y (neuroblastoma)	↓ H_2O_2/Fe^{2+}-induced ROS production	Ramiro-Puig, et al., 2009
	50-100 µM (14.5-29 µg/mL)	HepG2 (hepatoma)	↓ *t*-BOOH-induced death, ↓ GSH depletion, ↓ ROS generation, ↑ GPx and GR	Martin, et al., 2010b
	2.5-25 µM (0.73-7.26 µg/mL)	HepG2 (hepatoma)	↓ *t*-BOOH-induced lipid peroxidation, ↓ GSH and tocopherol depletion, ↑ GPx	Murakami, et al., 2002
	2.5-300 µM (0.73-87 µg/mL)	Calf thymus DNA	↓ Cu^{2+} induced radical formation	Azam, et al., 2004
	5-100 µM (1.45-29 µg/mL)	HepG2 (hepatoma), PC12 (pheochromocytoma)	↓ Pb^{2+} induced cell death, ↓ ROS formation, ↓ mitochondrial dysfunction, ↑ Ca^{2+}dysregulation, ↓ lipid oxidation, ↓ membrane fluidity	Chen, et al., 2002 and 2003
	0.1-100 µM (0.029-29 µg/mL)	Calf thymus DNA	↓ UVC-induced 8-oxodG	Ottaviani, et al., 2002
	10-50 µM (2.9-14.5 µg/mL)	HepG2 (hepatoma)	↓ N-nitrosamine-induced DNA damage	Delgado, et al., 2009
	10-50 µM (2.9-14.5 µg/mL)	HepG2 (hepatoma)	↓ heterocycle amine-induced DNA damage, ↓ CYP1A1 activity	Haza . and Morales, 2011
	25-100 µM (7.26-29 µg/mL)	MCF-7 (breast)	↑ NADPH cytochrome c reductase activity	Rodgers, and Grant, 1998
	0.1-100 µM (0.029-29 µg/mL)	HUVEC (umbilical vein endothelial)	↓ NADPH-oxidase ↓ TNF-induced oxidant formation	Steffen, Y., 2007 and 2008 Erlejman, et al., 2008
	120 µM (34.8 µg/mL)	Caco-2 (colon)	↑ Nrf2 translocation	Granado-Serrano, et al, 2010 Bahia, et al., 2008
	10 µM (2.9 µg/mL)	HepG2 (hepatoma)	↑ Nrf2, ↑ GSK	
	0.1-0.3 µM (0.029-0.087 µg/mL)	Astrocytes and neurons (primary culture)		
3´O-methyl epicatechin	1-30 µM (0.3-9.15 µg/mL)	Cortical neurons (primary culture), dermal fibroblasts	↓ H_2O_2-induced cell death and caspase-3 activity	Spencer, et al., 2001b

	1-30 µM (0.3-9.15 µg/mL)	FEK4 (skin fibroblasts)	↓ UVA-induced oxidative damage	Basu-Modak, et al., 2003
	0.1-100 µM (0.03-30.5 µg/mL)	HUVEC (umbilical vein endothelial)	↓ NADPH-oxidase (= 4´-O-methyl EC and EC-glucuronide)	Steffen, et al., 2008
Catechin	0.1-100 µM (0.029-29 µg/mL)	Calf thymus DNA	↓ UVC-induced 8-oxodG	Ottaviani, et al., 2002
	10-50 µM (2.9-14.5 µg/mL)	HepG2 (hepatoma)	↓ N-nitrosamine-induced DNA damage	Delgado, et al., 2009
	10-50 µM (2.9-14.5 µg/mL)	HepG2 (hepatoma)	↓ heterocycle amine-induced DNA damage, ↓ heterocycle amine-induced CYP1A1 activity, ↑ UGT activity	Haza, and Morales, 2011
	120 µM (34.8 µg/mL)	Caco-2 (colon)	↓ TNF-induced oxidant formation	Erlejman, et al., 2008
	100 µM (29 µg/mL)	Int-407 (intestine)	↓ lipid peroxidation, ↓ ROS formation, ↑ GPx, ↑ GR, ↑ Nrf2, ↑ HO-1	Cheng, et al., 2012

[a] The arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters. In certain cases, opposing results have been obtained since the studies were carried out in different cell types (non-tumorigenic, different cancer cell lines, etc.) and/or the final effects may depend on the dose and time of treatment with the phenolic compound.

[b] Concentration in µg/mL was calculated as epicatechin equivalents.

Table 2.- Cocoa and cocoa polyphenols modulation of proteins involved in the inflammatory process[a].

Polyphenol	Concentration	System studied	Effects	Reference
Cocoa	50 µM GAE (14.5 µg/mL)	Caco-2 (colon)	↓ PGE2	Romier-Crouzet, et al., 2009
	5-80 µg/mL	ELY.BU.OU6 (thymoma)	↓ MCP1, ↓ TNF-α, ↓ IL1α, ↓ IL6, ↓ LPS-induced TNF-α secretion, ↓ NO	Ramiro, et al., 2005
	0.1-10 µg/mL			
	0.5-20 mg/L	Monocytes	↓ PMA-induced superoxide production, ↓ IL1α, ↓ IL6	Zeng, et al., 2011
	10 µg/mL	HL-60 (leukemia)	↓ XO- and TPA-induced superoxide production	Lee, et al., 2006
		Caco-2 (colon)	↓ TNF-induced IL-8, COX-2, iNOS and NFκB activation	Rodriguez-Ramiro et al., 2012
Polymers procyanidins	2.5-60 µM (0.73-17.4 µg/mL)	Caco-2 (colon)	(hexamer) ↓ TNF-induced NFκB activation and iNOS	Erlejman, et al., 2008
Procyanidin B2	1.7-50 µM (1-29 µg/mL)	Jurkat T, H-RS, Daudi (Hodgkin lymphoma)	↓ NFκB binding	Mackenzie, et al., 2004, 2008 a 2009
	50 µM (29 µg/mL)	Jurkat T (lymphocyte)	↓ TNF- and PMA-induced NFκB activation (=PB1)	Mackenzie, et al., 2009
Epicatechin	200-400 µM (58-116 µg/mL)	ELY.BU.OU6 (thymoma)	↓ MCP1, ↓ TNF-α, ↓ IL1α, ↓ IL6, ↓ NO	Ramiro, et al., 2005
	1.7-8.6 µM (5-25 µg/mL)	Macrophages (primary culture)	↓ LPS-induced nitrite and TNFα production	Guruvayoorappan, and Kuttan, Schroeder, et al., 2001
	1-10000 µM (0.29-2900 µg/mL)	Aortic endothelial	↓ LPS-induced nitrite and TNFα production	Mackenzie, et al., 2004 and 20
	1.7-17.2 µM (4.93-49 µg/mL)	Jurkat T (lymphocyte)	↓ TNF-stimulated NFκB	Granado-Serrano, et al, 2010
	10 µM (2.9 µg/mL)	HepG2 (hepatoma)	↑ NFκB	
Catechin	1.7-8.6 µM (5-25 µg/mL)	Macrophages (primary culture)	↓ LPS-induced nitrite and TNFα production	Guruvayoorappan, and Kuttan, Schroeder, et al., 2001
	1-10000 µM (0.29-2900 µg/mL)	Aortic endothelial	↓ LPS-induced nitrite and TNFα production	

[a] The arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters.

[b] Concentration (μM) was in gallic acid equivalents (GAE).

Table 3.- Cocoa and cocoa polyphenols modulation on cell cycle[a].

Polyphenol	Concentration	System studied	Effects	Reference
Polymers procyanidins	0.25-5 µM (0.073-1.45 µg/mL)[b]	Raji (lymphoma), HL-60 (leukemia)	↓ Topo II (= B2, B5 and B2+B5)	Lanoue, et al., 2009
	100 µg/mL	MDA-MB231, MB231, MDA-MB468 (breast)	(pentamer) G0/G1 arrest, ↓ p-Cdc2, ↓ p-FOXO, ↓ p-p53, = pRb	Ramljak, et al., 2005
	25-100 µg/mL	MBA-MB 231 (breast)	(synthethic pentamer) G0/G1 arrest	Kozikowski, et al., 2003
	5-100 µg/mL	Caco-2 (colon)	G2/M arrest	Carnésecchi, et al., 2002
	0.1-100 nM (0.06-57.9 ng/mL)	LNCaP, DU145 (prostate)	(dimers B1 and B4) ↓ cell growth	Kampa, et al., 2001
	25-100 µM (14.5-57.9 µg/mL)	LNCaP, PC-3 (prostate)	(Procyanidin B3) ↓ cell growth, ↓ cyclin E, ↓ cyclin D1	Choi, et al., 2011
Procyanidin B2	0.1-100 nM (0.06-57.9 ng/mL)	LNCaP, DU145 (prostate)	↓ cell growth	Kampa, et al., 2001
Epicatechin	5-1000 µM (1.45-290 µg/mL)	LoVo (colon)	S arrest	Tan, et al., 2000
	50-100 µM (14.5-29 µg/mL)	DU145 (prostate), HH639 (ovary)	= ↓ growth, apoptosis, mitochondrial depolarization	Chung, et al, 2001, Ravindrana al., 2006

[a] The arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters. In certain cases, opposing results have been obtained since the studies were carried out in different cell types and/or the final effects may depend on the dose and time of treatment with the phenolic compound.

[b] Concentration in µg/mL was calculated as epicatechin equivalents.

Table 4.- Cocoa and cocoa polyphenols modulation of the apoptotic process[a].

Polyphenol	Concentration	System studied	Effects	Reference
Cocoa	0.5 mg/mL	MLP29, AML12 (liver)	↓ celecoxib-induced sub-G1 peak, ↓ Bax, ↑ Bcl-2, ↓ cytochrome c release, ↓ caspase-3 activity, ↑ Beclin-1	Arlorio, et al., 2009
Polymers procyanidins	300 µg/mL 2.5-20 µM[b] (0.73-5.8 µg/mL)	PC-3 (prostate) Caco-2 (colon)	↓ Ψm (hexamer) ↓ DOC-induced apoptosis	Shang, et al., 2009 Da Silva, et al., 2012
Procyanidin B2	2.5-50 µM (1.6-28.92 µg/mL)	Daudi, H-RS (lymphoma Hodgkin)	↓ $Bclx_L$, ↓ Bcl-2, ↓ XIAP, ↓ cFLIP	Mackenzie, et al., 2008
Epicatechin	30 µM (8.7 µg/mL)	FEK4 (skin fibroblasts)	↓ H_2O_2 induced caspase-3	Spencer, et al., 2001b
	50-300 µM (14.5-87 µg/mL) (+ 100 µM, 45.84 µg/mL)	KATO-III, MKN-45 (gastric)	(+ EGCG) ↑ apoptosis induction	Horie, et al., 2005
	100-200 µM (29-58 µg/mL) (+ 10-20 µM, 3.7-7.4 µg/mL)	A549, PC-9 (lung)	(+ curcumin) ↓ cell growth, ↑ apoptosis rate, ↑ GADD153, ↑ GADD45	Saha, et al., 2010
3´O-methyl epicatechin	10-30 µM (3-9.15 µg/mL)	Cortical neurons (primary culture), dermal fibroblasts FEK4 (skin fibroblasts)	↓ H_2O_2-induced caspase-3 activity	Spencer, et al., 2001a and 2001
EC-glucuronide	10-30 µM (2.9-8.7 µg/mL)	Cortical neurons (primary culture)	↓ H_2O_2-induced caspase-3 activity	Spencer, et al., 2001a

[a] The arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters.

[b] Concentration in μg/mL was calculated as epicatechin equivalents.

Table 5.- Cocoa and cocoa polyphenols modulation on cell survival/proliferation[a].

Polyphenol	Concentration	System studied	Effects	Reference
Cocoa	5-30 µg/mL	SH-SY5Y (neuroblastoma)	↓ p38, ↓ JNK	Ramiro-Puig, et al., 2009
	0.05-50 µg/mL	HepG2 (hepatoma)	↑ ERK	Martin, et al., 2010a
	10 µg/mL	Caco-2 (colon)	↓ acrylamide-induced p-JNK	Rodriguez-Ramiro, t al., 2011b
Polymers procyanidins[b]	17-138 µM (5-40 µg/mL)	JB6P+ (epidermal)	↓ TPA-induced neoplastic transformation, ↓ COX-2, ↓ p-MEK, ↓ p90s6 kinase, ↓ AP-1, ↓ NFκB	Kang, et al., 2008
	2.5-20 µM (0.73-5.81 µg/mL)	Caco-2 (colon)	(hexamer) ↓ DOC-induced AKT, ERK, p38 and AP-1	Da Silva, et al., 2012
Procyanidin B2	8.6-69 µM (5-40 µg/mL)	JB6P+ (epidermal)	↓ TPA-induced neoplastic transformation, ↓ COX-2, ↓ p-MEK, ↓ p90s6 kinase, ↓ AP-1, ↓ NFκB	Kang, et al., 2008
	10 µM (5.79 µg/mL)	Caco-2 (colon)	↓ acrylamide-induced p-JNK	Rodriguez-Ramiro, et al., 2011
	10 µM (5.79 µg/mL)	Caco-2 (colon)	↑ ERK, ↑ p38	Rodriguez-Ramiro, et al., 2011
	10-100 µM (5.79-57.9 µg/mL)	THP-1 (monocytes)	↓ endotoxin-induced COX-2, ↓ p38, ↓ JNK, ↓ ERK, ↓ NFκB	Zhang, et al., 2006
	10-50 µM (5.79-28.93 µg/mL)	SW480 (colon)	= ↑ proliferation, = ↑ p-AKT, = ↑ p-ERK	Ramos, et al., 2011
Epicatechin	12-100 µM (3.6-29 µg/mL)	SH-SY5Y (neuroblastoma)	↓ p38, ↓ JNK	Ramiro-Puig, et al., 2009
	10-50 µM (2.9-14.5 µg/mL)	Caco-2, SW480 (colon)	= proliferation, = p-AKT, = p-ERK	Ramos, et al., 2011
	1-1000 µM (0.29-290.5 µg/mL)	HepG2 (hepatoma)	= Bax, = Bclx, ↑ p-AKT, ↑ p-ERK, ↑ PKCα, = JNK, ↓ PKCδ	Ramos, et al., 2005, Granado-Serrano et al., 2007 and 2010

[a] The arrows indicate an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters. In certain cases, opposing results have been obtained since the studies were carried out in different cell types (non-tumorigenic, different cancer cell lines, etc.) and/or the final effects may depend on the dose and time of treatment with the phenolic compound.

[b] Concentration in μg/mL was calculated as epicatechin equivalents.

Table 6.- Cocoa and cocoa polyphenols modulation on angiogenesis and metastasis[a].

Polyphenol	Concentration	System studied	Effects	Reference
Cocoa	5-40 µg/mL 10-100 µg/mL	JB6P+ (epidermal) RLE (liver)	↓ TNF-induced VEGF, ↓ AP-1, ↓ NFκB ↓ H_2O_2-induced inhibition of gap-junction phosphorylation and internalization of connexion 43, ↓ ROS generation, ↓ ERK, ↓ MEK1, = p38	Kim, et al., 2010 Lee, et al., 2010
Epicatechin	1-100 µM (0.29-29 µg/mL) 10 µM (2.9 µg/mL)	HL-60 (leukemia) CGR8 (embryonic stem cells)	(EC-acylated and EC-C16) ↓ DNA polymerase and angiogenesis ↓ growth, ↓ ROS generation, ↓ MMP-9, ↓ angiogenesis	Matsubara, et al., 2007 Günther, et al., 2007
Catechin	17-86 µM (5-25 µg/mL)	B16F.10 (melanoma)	↓ NO, ↓ VEGF, ↓ IL2, ↓ TIMP-1, ↓ TNF production, ↓ pro-inflammatory cytokines	Guruvayoorappan, and Kuttan, 2008

[a] The arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different analysed parameters.

Table 7.- Summary of cancer preventive effects of cocoa and cocoa flavonoids in animal models

Cancer type	Model (carcinogen)	Intervention dose	Duration[a]	Main outcomes[b]	Reference
Mammary Pancreatic	Female Sprague-Dawley rats (PhIP)	Procianidims of cocoa liquor (0.025% or 0.25%)	48w (d) or (p)	↓ Incidence and multiplicity of pancreatic lesions	Yamagishi, M. et al., 2002
Lung Thyroid	Male F344 (DMBDD)	Procianidims of cocoa liquor (0.025% or 0.25%)	36w (p)	↓ Incidence and multiplicity of carcinomas in lung and thyroid	Yamagishi, M. et al., 2003
Prostate	Male Wistar-Unilever rats (MNU+ TP)	Cocoa rich-polyphenolic extract (24 or 48 mg/kg body weight)	36w (b-d-p)	↓ Incidence of prostate tumours at 24 mg/kg body weight.	Bisson, J.F. et al., 2008
Leukaemia	Brown Norway rat (leukaemia cells)	(−)-Epicatechin (40 mg/kg body weight)	3w (p)	↑ Necrosis of leukaemia bone marrow cells	Papiez, M. et al., 2011
Hepatic	Male Wistar rats (DEN)	Cocoa rich-diet (16%)	6w (b-d-p) or (p)	↓ Post-necrotic proliferation and reduced initiated cells.	Granado-Serrano, A.B. et al., 2009
Colon	Female C57BL/6J-Min/1 mouse	(+)-Catechin (0,1% or 1%)	10w	↓ Intestinal tumour number	Weyant, M.J. et al., 2001
Colon	Male Wistar (AOM).	Cocoa-rich diet (12%)	8 w (b-d-p)	↓ pre-neoplastic lesions (aberrant crypt foci)	Rodriguez-Ramiro, I. et al., 2012

[a]**w**, weeks; dietary intervention before (**b**), during (**d**), post (**p**) carcinogen treatment.

[b] The arrow indicate an increase (↑) or decrease (↓).