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Critical Review

Cocoa and cocoa bean shells role in human health: An updated review

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ABSTRACT

Cocoa is derived from the seeds of *Theobroma cacao* L., an evergreen tree typical of tropical regions. It contains numerous phytochemicals, with polyphenols representing the largest groups of compounds inside the seed, and has been implicated in numerous biological properties, such as antioxidant, antiproliferative, antiapoptotic, anti-inflammatory, anti-cancer. Moreover, cocoa has been investigated in different health conditions, including heart diseases, dyspepsia, nervous system diseases, circulation problems, and many others. Given its high consumption in many countries all over the world, it is important to know and understand its effects on human health. In addition, the cocoa bean shell, a by-product of the process of cocoa preparation, has been gaining remarkable interest due to its high content of phytochemicals. This review summarizes the available literature and works on the health benefits of cocoa and cocoa bean shells. Moreover, the current review focuses on studies investigating their possible therapeutic roles in cancer and the underlining potential mechanisms of action.

1. Introduction

Cocoa (*Theobroma cacao* L.) is mostly produced in West African countries, mainly the Ivory Coast and Ghana (60 % out of the world's total cocoa), but is usually processed in the European Union (40 % in 2014), Indonesia, United States, and Brazil (ICCO, 2020b). It is an important commodity in the world and the main ingredient in chocolate manufacture. However, the whole cocoa fruit including husk, shell, and pod can be utilized to produce a wide product range, such as animal feed, cocoa butter, and powder, soft drinks, alcohol, jam, confectionary, and cosmetics (ICCO, 2020a). Each variety provides beans with specific sensorial characteristics related to its origin, environmental conditions, and fermentation (Chetschik et al., 2018), and in this respect, hundreds of volatile compounds (alcohols, carboxylic acids, aldehydes, ketones, esters, pyrazines) have been identified as odor-active components in cocoa (Aprotosoaie et al., 2016). After harvesting, cocoa beans undergo fermentation and drying, after which shelling provides nibs and shells, the latter being the first subproduct (Tan and Kerr, 2018). Nibs can be roasted and milled to obtain cocoa liquor, an excellent source of bioactive compounds (Oliviero et al., 2009; Talbot et al., 2018), such as polyphenols, mainly flavonoids (flavanols, procyanidins, and anthocyanins), and methylxanthines (caffeine and theobromine), which gives

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two products after pressing, *i.e.* cocoa butter and cocoa cake, prior to obtaining cocoa powder from cocoa cake milling. Notably, cocoa butter composition is affected by the genotype, the cultivation conditions and post-harvest techniques and its percentage have a significant impact on chocolate manufacturing (Aprotosoaie et al., 2016). In cocoa butter, the fatty acids are triacylglycerols, most of which are palmitic and stearic acids (Segall et al., 2005); in this respect, the fat component in chocolate affects many parameters during processing, storage and consumption, and in addition to two aforementioned saturated fatty acids includes the two major unsaturated acids oleic and linoleic acid (Melo et al., 2020). Dark chocolate is also an excellent source of magnesium, iron, zinc, and selenium (Cinquanta et al., 2016).

Taking into account that the population has been aging and the cancer incidence also increases with age (Crocetti et al., 2012), within the novel therapeutics and targeted therapies for cancer, chocolate has an important impact on human health and plays a role as a potential functional food (Rusconi and Conti, 2010). Indeed, in a recent review, chocolate consumption was found to be associated with reduced risk of cardiovascular disease death, acute myocardial infarction, stroke, and diabetes, as well as flow-mediated vessel dilatation and markers of insulin resistance (Veronese et al., 2019). The latter effects may be elicited by flavonoids protecting against cardiovascular disease, acting as antioxidant, antiplatelet, and anti-inflammatory agents (Corti et al., 2009), and against adiposity, acting as a fat-reduction agent (Smith et al., 2020).

Based on the goals of the present review to discuss the beneficial properties of cocoa and cocoa chocolate on human health, the following sections focus on cocoa composition, bioactive compounds and the related functions, bioavailability, consumption, and diseases, antiproliferative, antiapoptotic and chemopreventive effects. Finally, the main remarks, as well as future perspectives and challenges, have been expressed.

2. Cocoa and cocoa bean shell

Cocoa is defined as the food of the Gods, indeed the scientific term "theobroma cacao" (literally "food of the gods") was indicated by Carlo Linnaeus in the 18th century, due to its numerous properties attributed to cocoa by the peoples of Central America (Montagna et al., 2019). Now it is known that cocoa is a complex food that contains more than 350 different components and possesses unquestionable health effects (Wickramasuriya and Dunwell, 2018). This section is mainly focused on cocoa and cocoa bean shell composition, phenolic content, bioavailability, and their health-promoting effects through the following subsections.

2.1. Cocoa and cocoa bean shell composition

Cocoa is a dried and thoroughly fermented product of cocoa beans obtained from *Theobroma cacao* (McShea et al., 2009), which are composed of lipids, fibers, minerals carbohydrates, proteins, and many bioactive compounds including polyphenols and methylxanthines (Table 1) (Katz et al., 2011).

The International Cocoa Organization (ICCO) reported that more than 4000 tons of cocoa beans are expended/roasted each year around the World (ICCO, 2020a). Therefore, an enormous amount of cocoa bean shells (CBS) is produced as a residue by the cocoa industry. However, several studies have investigated the nutritional value of CBS, which were found to be similar to that of cocoa butter. CBS were further examined to extract their phenolic compounds (mainly catechins, epicatechins, and procyanidins), dietary fibers, methylxanthines such as theobromine (3,7-dimethylxanthine), and lipids (Table 2) (Arlorio et al., 2005; El-Saied et al., 1981; Lecumberri et al., 2007; Richards and Wailes, 2012). In the study of Rojo-Poveda et al. (2019), 20.9 g protein, 2.3 g fat, 7.85 g carbohydrates, 55.1 g dietary fiber were quantified per 100 g of dried CBS (Rojo-Poveda et al., 2019). Additionally, in the study

Table 1
General compositions of cocoa bean powder.

Macronutrients	Fiber Content	Phytochemicals
(Caprioli et al., 2016)	(Lecumberri et al., 2007)	(Aranaz et al., 2019; Ávila-Gálvez et al., 2019; Caprioli et al., 2016)
24.4 %–43.4 % Carbohydrates	≈60 % of the dry Cocoa sample	Flavanols (−)-Epicatechin ≈16,868 mg/kg extract Epicatechin glucoside ≈951 mg/kg extract Catechin glucoside ≈1623 mg/kg extract (+)-Catechin ≈5782 mg/kg extract Procyanidins; Dimer fraction of procyanidin B2 ≈10,116 ± 1191 mg/kg extract
11.7 %–13.4 % Proteins		Flavonols Quercetin ≈139 mg/ kg extract Quercetin-3-arabinoside ≈826 mg/ kg extract Quercetin 3-*O*-glucuronide ≈801 mg/ kg extract
24.6 %–43.1 % fats; Stearic acid (C18:0) Oleic acid (C18:1, n-9) Palmitic acid (C16:0)		Methylxanthines Caffeic acid ≈2.1 mg/g dried extract Theobromine ≈4.7–11.6 mg/g dried extract Theophylline

Table 2
General compositions of cocoa bean shells per 100 g.

Macronutrients	Fiber content	Minerals	Phytochemicals
(Rojo-Poveda et al., 2019)	(Rojo-Poveda et al., 2019)	(Bonvehí and Jordà, 1998)	(Hernández-Hernández et al., 2019)
7.85 g Carbohydrates	55.1 g Dietary fiber	Potassium (0.21−1.82 g)	Theobromine
2.3 g Fat		Magnesium (0.20−1.29 g)	Catechin
20.9 g Protein		Calcium (0.09−0.51 g)	Epicatechin
		Phosphorus (0.15−1.00 g)	
		Copper (12.1−66.2 g)	
		Zinc (17.3−75.3 g)	

of Okiyama et al. (2018), CBS were found as a substantial source of flavanols and alkaloids when extracted by pressurized liquid extraction technique using ethanol (Okiyama et al., 2018). Moreover, CBS were also identified as a good source of minerals including potassium, magnesium, calcium, phosphorus, copper, and zinc (Bonvehí and Jordà, 1998).

In the case of phytochemicals, while theobromine was identified as major methylxantine component, catechin and epicatechin were indicated as the dominant polyphenols in CBS from different cocoa genotypes (Hernández-Hernández et al., 2019). According to the analysis of HPLC-UV, the majority of alkaloids found in cocoa and cocoa byproducts consisted of theobromine and caffeine (Bartella et al., 2019).

In the study of Caprioli et al. (2016), the nutritional composition analysis of cocoa seeds from five different regions in Cameroon was reported as follows: fats (24.6–43.1 %), carbohydrates (24.4 %–43.4 %), and proteins (11.7 %–13.4 %). According to high-performance thin-layer chromatography analysis, the polyphenol content of the cocoa beans among the different regions in Cameroon was detected in the following concentration ranges: 1.1–2.1 mg/g dried extract for caffeic acid, 4.7–11.6 mg/g dried extract for theobromine and 1.1–142.9 mg/g dried extract for (-)-epicatechin. Moreover, carboxylic acids such as acetic acid were reported with the highest concentration among the

other volatile components in all cocoa samples by the technique of headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry. The fatty acid profile of those cocoa samples showed that stearic acid (C18:0), oleic acid (C18:1, n-9), and palmitic acid (C16:0) were found in cocoa at abundant amounts in the range of 37.6–39.5 %, 30.4–33.2 %, 24.8–28.2 %, respectively (Caprioli et al., 2016). Moreover, Lecumberri et al. (2007) showed that nearly 60 % of the dry cocoa sample was composed of total dietary fiber including 17 % soluble dietary fiber and mostly insoluble dietary fiber (Lecumberri et al., 2007).

In the study of Dang and Nguyen (2019), maturity stages and fermentation showed a significant effect on bioactive compounds of the cocoa bean, an increase in bioactive compound content *via* maturation, and the reduced amounts of caffeine and theobromine due to the increase of fermentation duration. Additionally, this study demonstrated that the number of proanthocyanidins, the amount of caffeine and theobromine, as well as antioxidant capacity, of cocoa beans increased when cocoa beans were harvested at an advanced maturity stage; however, the concentrations of the latter alkaloid compounds were significantly decreased by fermentation with or without pectinase for 3–7 days. Overall these results indicated that fermentation duration and maturity interacted with the production of bioactive compounds in cocoa beans (Dang and Nguyen, 2019). Polyphenols are the major biologically active phytochemicals found in cocoa beans and will be considered specifically in section 2.2.

It is reported that at least 3 different varieties of cocoa are available: Criollo, Forastero, and Trinitario. Even though Criollo and Forastero are considered the main varieties (genotypes), based on bean morphology and geographical origin (Criollo from Central America and Forastero from Amazon basin), Trinitario was more recently originated in Trinidad as a natural hybridization between Criollo and Forastero (Kongor et al., 2016; Motamayor et al., 2003). From an economical point of view, the 3 varieties are considered in worldwide trade, but only Forastero variety is commonly used in commercial production.

Different analyses reported that the geographical origin of cocoa beans and chocolate were recognized (Afoakwa et al., 2008; Cambrai et al., 2010). This reflects the classical flavor of cocoa which is obtained especially from post-harvest treatments and not only by peculiar cocoa biochemical composition. Numerous factors can affect cocoa phytochemicals, *i.e.* cocoa variety, tree cultivation conditions, climatic conditions, bean maturity, harvest time, and storage after harvest, among the most important (Dang and Nguyen, 2019; Oracz et al., 2015). Of note that technical processing and especially high temperatures can negatively alter cocoa polyphenol composition, reducing quality and sensory properties in addition to a decrease in health benefits of cocoa products (Urbańska et al., 2019). Thus an approach that lessens any operation in cocoa production is highly desirable and urgently needed. Nonetheless, producers, manufactures, researchers still focus prevalently on cocoa varieties and environmental aspects. However, the final flavor quality is based on the "perfect synergy" between technological processes and agricultural methods and their interaction/combination is a work in progress. Consequently which factors can affect cocoa and chocolate taste are not completely understood. For example, recent work showed that after consuming dark chocolates (thus analyzing the released aroma in the nose of subjects during consumption) the botanical origin of cocoa beans was better reflected in the final chocolates than their geographical origin (Acierno et al., 2019). Also, inter-individual differences in volunteers were more significant than cocoa bean differences. The study investigated 10 identical chocolates manufactured with beans from different botanical and geographical origins, using the nose-space profiles analyzed by quadrupole interface time-of-Flight (Qi-ToF)-mass spectrometry. The authors reported that future direction in chocolate consumption will have to investigate how human sensory perception is influenced by cocoa beans botany rather than their geographical origin.

In addition, an International Board has been created to standardize cocoa production and manufacturing (ISCQF, 2021). This fact underlines that even today it is difficult to assess the quality and flavor of cocoa and probably an international consensus is effectively needed to have a certified, high-quality, safe, healthy, and sustainable product.

2.2. Cocoa polyphenols and health-promoting effects

Polyphenols are molecules that are mainly provided by plants, which show various beneficial health effects like defensive properties against pathogens and microorganisms, the ability to scavenge free radicals, and preventive effects against various degenerative diseases such as cancers and cardiovascular diseases (Scalbert et al., 2002). According to literature, among the variety of polyphenols detected in cocoa, epicatechin, catechin, procyanidin B_2, quercetin, and protocatechuic acid were major polyphenols found in this plant species that have been assessed for their beneficial and protective effects on human health and diseases (Fig. 1) (Table 3).

2.2.1. Antioxidant and anti-inflammatory activity of cocoa polyphenols

Lee et al. (2003) investigated the total phenolic and flavonoid concentration of cocoa, black tea, green tea, and red wine. The phenolic and flavonoid content of the cocoa per serving was 611 mg of gallic acid equivalents (GAE) and 564 mg of epicatechin equivalents (ECE), respectively. As a result, the highest amount of phenolic and flavonoid content was detected in cocoa as compared to black tea (124 mg of GAE and 34 mg of ECE), green tea (165 mg of GAE and 47 mg of ECE), and red wine (340 mg of GAE and 163 mg of ECE). Moreover, ABTS and DPPH radical scavenging activity assays indicated the highest antioxidant activity in cocoa (Lee et al., 2003). An *in vitro* study by Martins et al. (2020) investigated the possible protective effects of cocoa flavanols against oxidative stress on EA.hy926 human endothelial cell line. In the co-treatment assay, cells were treated with 100 μM tertbutylhydroperoxide (t-BOOH) to achieve a culture model of oxidative stress with cocoa phenolic extract (2.5, 5, 10, and 20 μg/mL) or epicatechin (2.5, 5, 10, and 20 μM) simultaneously for 18 h. In the pre-treatment assay, cells were primarily treated with tested doses of cocoa polyphenolic extract and epicatechin for 18 h and after 200 μM t-BOOH for 4 h. The results of the study demonstrated that cocoa flavanols and mainly epicatechin have a reductive effect on oxidative stress by limiting ROS generation and strengthening the antioxidant defense response (Martins et al., 2020). In the *ex vivo* and *in vivo* study by Vinson et al. (2006), the phenolic antioxidant content determined using the Folin-Ciocalteau assay of chocolate products from different brands revealed that dark chocolate contained a significantly higher amount of phenolics than other chocolate products such as milk chocolate.

In a double-blind, randomized, placebo-controlled, and crossover clinical trial conducted by Barrera-Reyes et al. (2019), the antioxidant effect of high-polyphenols cocoa consumption was demonstrated on the plasma of healthy humans and gene expression profile of peripheral mononuclear cells (PBMCs). The trial was conducted on twenty healthy, young participants that warned about avoiding polyphenol-rich products at least 24 h before taking the cocoa supplement. The supplementation was given as a single dose (2 h) in the form of high-polyphenol cocoa powder or maltodextrins (hydrolyzed polysaccharide) as a placebo. Blood samples were collected under fasting conditions and two h after treatment intake, and then plasma and PBMCs were isolated. The transcriptomic profile of PBMCs was analyzed using microarray techniques, qPCR, and Ingenuity Pathway Analysis. As a result of cocoa consumption, various genes related to inflammatory cytokines and redox balance showed a moderate differential expression in PBMCs. The gene expression change after cocoa consumption was converged with the reduced production of reactive oxygen species, modulated inflammatory response by reducing leukocyte activation as well as calcium mobilization according to the gene and network analysis. Moreover, the (-)-epicatechin derived metabolites in plasma were quantified by ultra-high performance liquid chromatography-tandem mass

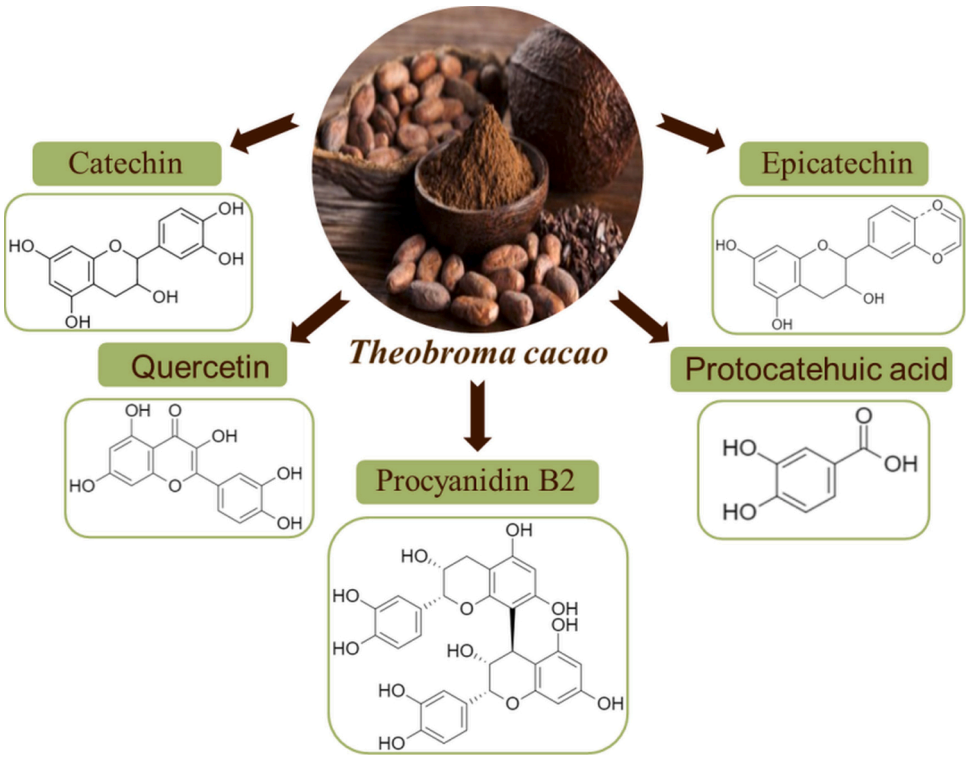

Fig. 1. Major polyphenols found in cocoa.

spectrometry (UHPLC–MS/MS) after two h of cocoa consumption. The concentration of (-)-epicatechin-derived metabolites reportedly increased after cocoa supplementation, though the antioxidant activity did not change in plasma. Therefore, the latter study suggested that there was no relation between the (-)-epicatechin metabolite concentration and the antioxidant activity of cocoa powder (Barrera-Reyes et al., 2019).

2.2.2. *Preventive and curative effects of cocoa polyphenols on insulin resistance and obesity*

The *in vivo* study of Aranaz et al. (2019) investigated the beneficial effects of cocoa intake on metabolic syndrome, obesity, liver steatosis, and lipid metabolism. The main phenolic compounds were procyanidins which consist of oligomers and polymers of (-)-epicatechin (16, 868 ± 2047 mg/kg extract) and (+)-catechin (5782 ± 455 mg/kg extract), their glucosides (951 ± 43.3, 1623 ± 160 mg/kg extract, respectively), and the dimer procyanidin B_2 (10,116 ± 1191 mg/kg extract). Quercetin arabinoside, quercetin glucoside, and protocatechuic acid were also detected in a sufficient amount in the extract. Male Wistar rats were separated into four groups; one group was fed with a control diet while the other three groups were fed with a high fat/high sucrose diet for 70 days. Two high fat/high sucrose diet groups were supplemented with 14 or 140 mg/kg of cocoa extract each day. As a result of this study, it has been reported that safe doses (14 and 140 mg/kg) of cocoa extract decreased body-weight gain, visceral adiposity, and adipocyte size by down-regulating adipogenesis-key proteins. Furthermore, beneficial effects on insulin resistance, glucose intolerance, and liver steatosis were indicated since the triglyceride content and the insulin levels were reduced significantly in cocoa extract supplemented groups as compared to non-supplemented animals (Aranaz et al., 2019).

2.2.3. *The role of cocoa polyphenols in cardiovascular diseases*

Male, weanling, Syrian Golden hamsters were fed with a high saturated fat and cholesterol diet for the animal atherosclerosis study. Rats were supplemented with a mixture that included 1 g (low dose, 0.1 %) or 10 g (high dose, 1.0 %) cocoa powder. Accordingly, in the rats fed with cocoa powder, no significant weight gain was reported. It was revealed that cholesterol levels were decreased by 36 % upon cocoa supplementation at both doses. Both cocoa doses increase HDL (12 % and 23 %, respectively) and decreased LDL levels (by 62 % and 66 %, respectively) in a dose-dependent manner, and reduced atherosclerosis significantly by 40 % and 36 %, respectively. Results of the animal atherosclerosis study proved the beneficial effects of chocolate consumption to prevent atherosclerosis by decelerating the process (Vinson et al., 2006). Moreover, the low dose of cocoa decreased the plasma triglyceride level by 34 %. A double-blind, randomized, placebo-controlled trial was conducted on twenty subjects with congestive heart failure (Flammer et al., 2012). Patients consumed 40 g of flavanol-rich chocolate or 28.4 g of cocoa-free control chocolate. Endothelial, platelet, and baroreceptor functions were measured after 2 h (after intake of 1 bar), 2 weeks, and 4 weeks (after intake of 2 bars, daily) for the assessment of both short-term and long-term effects. Analysis indicated that the flavanol-rich chocolate improves vascular function in congestive heart failure patients by preventing platelet function.

2.2.4. *Antimicrobial and antiviral effects of cocoa and CBS polyphenols*

The antimicrobial effects of cocoa and CBS can be deduced from that of its polyphenols (Daglia, 2012; Pinto et al., 2021). Cocoa and CBS contain about 1–8 % of polyphenols as dry weight, (Nsor-Atindana et al., 2012; Zumbé, 1998) and, as above mentioned, these molecules possess numerous health benefits, including antioxidant, anticancer, and anti-inflammatory effects among the most common. Different literature works analyzed the antimicrobial effects of polyphenols individually rather than polyphenols extracted from cocoa. Consequently, the reader is referred to other reviews for a more comprehensive framework on the antimicrobial effects of polyphenols (Álvarez-Martínez et al., 2020; Bubonja-Šonje et al., 2020; Daglia, 2012; Olszewska et al., 2020). Nonetheless, some works explored the antimicrobial potential of both cocoa and CBS.

The antibacterial activity of cocoa has been studied in different microorganisms. The bacteria *Cronobacter sakazakii* was challenged with cocoa powder 5% (w/v) in milk formulas (Pina-Pérez et al., 2011). The

Table 3
Cocoa polyphenols and health-promoting effects.

Reference	Type of study	Product/ Compound(s), Treatment Dose and Duration	Result(s)
Oxidative stress and inflammation			
Lee et al., 2003	*In vitro* ABTS, DPPH radical scavenging activity assays	Cocoa, black tea, green tea, and red wine	Highest antioxidant activity determined in cocoa.
Martins et al., 2020	Cell culture study with EA.hy926 cell line (oxidative stress model)	t-BOOH (100 or 200 μM, 4 or 18 h), cocoa phenolic extract (2.5, 5, 10, and 20 μg/mL, 18 h) or epicatechin (2.5, 5, 10, and 20 μM, 18 h)	Cocoa flavanols and epicatechin showed reducing effect on ROS generation.
Barrera-Reyes et al., 2019	Clinical trial on healthy, young participants (n = 20)	A single dose of high-polyphenol cocoa powder or maltodextrins (2 h)	Reduced ROS production, leukocyte activation and calcium mobilization
Insulin resistance and obesity			
Aranaz et al., 2019	*In vivo* study with male Wistar Rats (4 groups)	(High fat/high sucrose diet for 70 days) cocoa extract (14 or 140 mg/kg, each day for 70 days)	Reduced triglyceride content and insulin levels
Cardiovascular diseases			
Vinson et al., 2006	*In vivo* study with male, weanling, Syrian Golden hamsters (atherosclerosis model)	(High saturated fat and cholesterol diet) cocoa powder (1 g (0.1 %) or 10 g (1%))	Increased HDL (12 %, 23 %) and decreased LDL (62 %, 66 %) levels; reduced atherosclerosis (40 % and 36 %)
Flammer et al., 2012	Clinical trial on CHF patients (n = 20)	Flavanol-rich chocolate (40 g) or cocoa-free control chocolate (28.4 g)	Improved vascular function by preventing platelet formation (2 h or 2 weeks or 4 weeks)
Antimicrobial and antiviral activities			
Unten et al., 1991	*In vitro*	Ethanol/acid CBS extract (31.2–250 μg/mL)	Antiviral against HIV and influenza virus
Ooshima et al., 2000	*In vitro* and *in vivo*	CBS extract (2, 1, 0.5 and 0.1 mg/mL), (73 days)	Anticariogenic
Kim et al., 2004	*In vitro*	CBS extract in 50 % (v/v) aqueous/ acetone	Anticariogenic
Matsumoto et al., 2004	*In vitro* and *in vivo* (human) (n = 28)	CBS extract (1 mg/mL), (4 days)	Antiplaque
Summa et al., 2008	*In vitro*	raw, pre-roasted, and roasted cocoa (>100 μg/mL)	Bactericidal in all considered strains (*Bifidobacterium lactis* Bb-12, *B. bifidum* B 7.1, *Enterobacter cloacae, E. coli* O157:HH7)
Sakagami et al., 2008	*In vitro*	Lignin fractions of CBS, (1 mg/mL)	No antibacterial activity
Pina-Pérez et al., 2011	*In vitro*	Cocoa powder in milk (1%, 2.5 % and 5% (w/v))	Bacteriostatic (*Cronobacter sakazakii*)
Nsor-Atindana et al., 2012	*In vitro*	CBS extract	Bacteriostatic
Prayoga et al., 2013	*In vitro*	fermented and unfermented dry cocoa beans	Bacteriostatic (*S. aureus* and *S. typhimurium*)
Ariza et al., 2014	*In vitro*	Cocoa ethanolic extract	Bacteriostatic (*E. coli*)
Yumas, 2017	*In vitro*	CBS extract (0.25 %, 0.5 %, 1%, 1.5 %, 2%)	Bacteriostatic (*Streptococcus mutans*)
Diniardi et al., 2020	*In vitro*	CBS extract (5 mg/mL)	Antibacterial

authors reported that cocoa had a bacteriostatic effect rather than an inhibitory effect and could be potentially useful for increasing the shelf life of pasteurized milk supplemented with cocoa. Furthermore partially fermented dry cocoa beans and unfermented dry cocoa beans were used in the treatment of *S. aureus* and *Salmonella typhimurium*. The authors reported that MIC was 25 and 100 mg/mL respectively, with a bacteriostatic effect rather than a bactericidal effect (Prayoga et al., 2013). *E. coli* was challenged with cocoa ethanolic extract and a MIC of 835 mg/mL was determined (Ariza et al., 2014). However, this bacterial growth suppression was counterbalanced by fragmentation of the *E. coli* cells observed using scanning electron microscopy. Another work investigated different strains, *i.e. Bifidobacterium lactis* Bb-12, *B. bifidum* B 7.1, *Enterobacter cloacae*, *E. coli* ATCC 8439, *E. coli* O157:HH7 ATCC 43888, treated by raw, pre-roasted, and roasted cocoa (Summa et al., 2008). These different extracts however did not guarantee a different result, as only a concentration of 100 μg/mL (independent of the fraction and the roasting stage) showed inhibitory effects. In addition, the growth-reducing effect was observed for the non-pathogenic *B. bifidum*, thus it seems that cocoa (raw, pre-roasted, and roasted) can be poorly useful. The same work also studied the antimutagenic effect of these cocoa preparations by the *Salmonella* microsome assay, using strains TA98, TA100, and TA102. Neither a mutagenic effect nor an effective reduction in revertants in the cocoa fractions was observed, as previously demonstrated for cocoa powder (Brusick et al., 1986), but not for cocoa liquor in which derived polyphenols showed antimutagenic effects (Yamagishi et al., 2000). In this last case, extracted and concentrated cocoa polyphenols could aid in reducing mutagenic action induced by heterocyclic amines in the *Salmonella* microsome assay. Therefore, in summary, cocoa's antimicrobial effects are more bacteriostatic than bactericidal, but indubitably more research is necessary to confirm these data.

CBS was investigated for antimicrobial activity on *Streptococcus mutans* MT8148R and *Streptococcus sobrinus* 6715 and in rats infected by these mutants (Ooshima et al., 2000). CBS was able to inhibit glucosyltransferase (GTF) that plays a key role in dental caries and reduces dental plaque and caries development in animals, suggesting an anti-cariogenic potential. *Streptococcus mutans* was also evaluated and an MIC of 2.5 mg/mL determined (Yumas, 2017). Moreover, CBS was effective against GTF for the prevention of tooth decay (anticaries activity) at a high level (Kim et al., 2004). Similarly, CBS blocked *Streptococcus mutans* MT8148 in saliva-coated hydroxyapatite and on an orthodontic wire, suggesting an antiplaque activity also in humans (Matsumoto et al., 2004). Negative results, *i.e.* no antibacterial activity, were obtained for different bacteria, including *S. mutans*, *Actinomyces viscosus*, *Fusobacterium nucleatum,* and *Porphyromonas gingivalis* treated by the lignin fractions from CBS (1 mg/mL) (Sakagami et al., 2008). Differently, in *E. coli*, *S. aureus*, *Salmonella* spp., and *B. cereus*, CBS extracted by different methods (acetone, ethanol, methanol, water) showed weak antibacterial power, with acetone extract having the highest antimicrobial activity (MIC 0.937 mg/mL for *E. coli*) (Nsor-Atindana et al., 2012). More recently MIC was evaluated in *E. coli* with a

result of 5 mg/mL for CBS extracted by microwave-assisted extraction method (with ethanol 1:4 w/v), in contrast with previous work (Diniardi et al., 2020). The conflicting results on MIC can be imputable to different extraction methods (*i.e.* polyphenols concentration), to the strain of *E. coli,* and original material. Furthermore, CBS has been studied against the fungus *Fusarium oxysporum* (pathogen on tomatoes) (Rachmawaty et al., 2018). The authors showed a minimum growth inhibitory concentration of 0.68 cm in acetone/water extract of CBS at 2% by the agar diffusion method.

The effects of CBS have been also tested as a potential antiviral substance. In 1991 CBS was tested for the inhibition of HIV absorption in human T-cell leukemia virus I MT-2 and MT-4 (highly sensible to HIV-1) (Unten et al., 1991). Cells were treated with 31.2–250 μg CBS/mL. In addition, syncytium formation between uninfected and HIV-infected human T-lymphoblastoid cells (MOLT-4 cells) was blocked by CBS. Also, the lignin fraction of CBS has been evaluated: in the MTT assay for MT-4 cells, CBS showed a high selectivity index against HIV (SI = 30–10000, where SI = 50 % cytotoxic concentration, CC_{50}/50 % effective concentration, EC_{50}) (Sakagami et al., 2008). Moreover, the same work found that CBS and its lignin fraction exerted anti-influenza virus activity in infected MDCK cells (Madin-Darby Canine Kidney cells). A complex picture emerges from antimicrobial data on cocoa and CBS, but a fixed point is the polyphenol activity in such products. In general, when they are more abundant, they can more effectively modulate microorganism growth. Certainly, more efforts are required to better characterize the potential and useful products of cocoa.

2.3. Bioavailability of cocoa

Various studies have demonstrated the potential beneficial and protective effects of cocoa on human health and against numerous chronic diseases. Besides that, understanding the bioavailability and metabolism of cocoa/bioactive compounds including phenolic compounds and methylxanthines is an important point to elucidate their specific functions and additive or synergistic interactions which may help to develop more effective cocoa products (Oracz et al., 2020). (-) Epicatechin, which is one of the mainly found phenolics in cocoa, is absorbed in small intestines and its metabolism is reported to be affected by various factors including nutrient-nutrient interactions, food processing, and also by biological factors such as metabolic enzyme polymorphisms (Cifuentes-Gomez et al., 2015). A table summarizes the updated literature on cocoa bioavailability in human clinical trials (Table 4).

A randomized, cross-over study was conducted by Vitaglione et al. (2013) on twelve healthy volunteers to investigate the human bioavailability of cocoa flavanols and phenolic acids in different formulations of cocoa-nut cream (CC). Two different CCs were prepared as control cream containing 20 % (w/w) and ten prototypes of polyphenol-enriched CC containing 0.5−2.5% (w/w) polyphenols range. The polyphenol-enriched CC was processed with free cocoa polyphenol extract (FPC) or encapsulated with gastric-resistant high-amylose maize starch (EPC). Volunteers followed a polyphenol-free diet 3 days before and over the experiment day. After 12 h of fasting, participants had consumed 99 g of one out of the three CCs in each test day, randomly. The trial was repeated for all three types of CCs with a 1 week washing period between them. According to analysis within six h after CCs consumption, the mean epicatechin serum concentration in blood samples taken from subjects who had consumed FPC was significantly higher (by 13.9-fold) than samples collected from subjects who had ingested EPC, which indicated that the gastric absorption and rapid plasma clearance of epicatechin from cocoa decreased by encapsulation by a gastric absorption resistant biomaterial. Moreover, it was observed that the phenolic acid concentration in urine from FPC-consuming subjects was significantly higher compared to that of the CC and EPC consuming subjects within six h after consumption. Overall, this study indicated that cocoa polyphenol supplementation raised the phenolic acid concentration in the circulatory system (Vitaglione et al., 2013).

A randomized, controlled, crossover and single-blind study was conducted by Martínez-López et al. (2014) to investigate the bioavailability of methylxanthines present in soluble cocoa products. An insoluble cocoa powder that includes naturally occurring methylxanthines (CC) and a cocoa product enriched in methylxanthines (CC-MX) were prepared and supplied to 13 healthy individuals who were not on any medication or supplementation for ten days. The individuals consumed 15 g of CC and 25 g of CC-MX in milk which delivered 94.35 and 256 mg total methylxanthines content respectively. Blood samples were collected 0.5, 1, 2, 3, 4, 6, 8 h after consuming the cocoa drinks, while urine samples were collected at different time intervals such as 2–0, 0–4, 4–8, 8–12, and 12–24 h. Plasma concentrations of methylxanthines were higher in subjects who had consumed the CC-MX compared to the CC, and a dose-response effect was observed. Similarly, the concentration of urinary metabolites of methylxanthines after supplementation with CC-MX was higher compared to the consumption of CC with a dose-response effect. Collectively, this study suggested that the strong dose-response effect of CC-MX methylxanthines in cocoa demonstrated high bioavailability and rapid urinary excretion in humans over 24 h (Martínez-López et al., 2014).

A clinical study by Rodriguez et al. (2015) was conducted on 80 healthy children to determine theobromine levels in urine after consumption of cocoa products. Children were separated into four groups according to their consumption of cocoa containing products and their food intake was recorded during the day. These groups consumed only cocoa containing products, only cocoa powder, chocolate including cocoa powder, or no cocoa/chocolate as control. Theobromine, magnesium, and oxalate levels in diluted urine samples were quantified for all of the four groups. The urinary theobromine levels were directly related to the amount of cocoa consumption, though there was no significant difference in oxalate and magnesium levels (Rodriguez et al., 2015).

An *in vivo* study was conducted by Borges et al. (2016) on Sprague Dawley rats to investigate the bioavailability of [2-^{14}C] (–)-epicatechin.

Table 4
Bioavailability of cocoa in humans.

Reference	Subject	Supplementation type, dose	Collected sample(s) and collection time (after supplementation)	Outcome(s) compared to control group(s)
Vitaglione et al. (2013)	12 healthy adults	polyphenol-enriched cocoa-nut creams (free or encapsulated) containing 0.5−2.5% (w/w) polyphenols or control cream, 99g	Blood (1, 2, 4, and 6 h); urine (–2, 2–4, 4–6, 6–8, 8–10, 10–24 h)	higher epicatechin (13.9 fold) in blood samples with free polyphenol enriched supplementation
Martínez-López et al. (2014)	13 healthy adults	15 g of CC (94.35 mg methylxanthines) or 25 g of CC-MX (256 mg methylxanthines) in milk	Blood (0.5, 1, 2, 3, 4, 6, 8 h); urine (2–0, 0–4, 4–8, 8–12, and 12–24 h)	higher methylxanthine in both blood and urine samples of CC-MX group
Rodriguez et al. (2015)	80 healthy children	only cocoa containing products or only cocoa powder or chocolate including cocoa powder or no cocoa/chocolate (C)	Urine (12 h-day and 12 h-night samples)	Increased theobromine conc. due to cocoa consumption
Gómez-Juaristi et al., 2019	13 healthy adults	conventional soluble cocoa (15 g) or flavanol-rich soluble cocoa (25 g) in semi-skimmed milk	Blood (0.5, 1, 2, 3, 4, 6, 8 h); urine (-2–0, 0–4, 4–8, 8–12, and 12–24 h)	Flavanol metabolites in plasma (10) and urine (30)

Rats were fed with 500 μL of a solution of [2-^{14}C] (–)-epicatechin to achieve an intake of 1.3 μmol. Urine, feces, and blood samples were collected during 72 h post-supply. After the feeding period, 3 rats were killed *via* CO_2 narcosis and the gastrointestinal tract was removed. Feces and tissues were extracted for HPLC-RC-MS analysis and ^{14}C-metabolites were identified *via* HPLC-MS^2 with a radioactivity monitor. Recovery of radioactivity in urine and feces was quantified as 78 % and 19 %, respectively. According to the radioactivity recovery quantification, it was concluded that orally supplemented (–)-epicatechin was efficiently absorbed and was bioavailable in rats (Borges et al., 2016).

A clinical study carried out by Sansone et al. (2017) consisted of three randomized, double-masked crossover and one with 4 parallel crossover studies conducted on 47 healthy volunteers. A randomized, double-masked, 2-arm crossover design was conducted on 5 healthy volunteers for study 3 to assess the modulatory roles of methylxanthines on plasma concentration and urinary excretion of the structurally related (2)-epicatechin metabolites (SREMs). Drinks containing 820 mg/75 kg BW of cocoa flavanols and 125 mg/75 kg BW were consumed by subjects on 2 separate days. After supplementation, blood and 0 to 24-h urine samples were collected from the subjects. Furthermore, a single-center, randomized, double-masked crossover study was conducted on 6 healthy volunteers (study 4) to investigate the effects of pure theobromine and caffeine on plasma concentrations and urinary excretion when they were consumed together with (2)-epicatechin. Drinks including 75 mg (2)-epicatechin were consumed with or without 400 mg theobromine and 26 mg caffeine by volunteers on 2 separate days. Zero to 24-h urine samples and before (0 h) and after (1, 2, 4 h) blood samples were collected from subjects. (2)-Epicatechin metabolites in urine and plasma were quantified *via* HPLC. A significant increase in SREMs was observed after the consumption of cocoa flavanols and methylxanthines together, compared to the cocoa flavanols consumption alone in study 3. Likewise, in study 4, a significant increase was observed after both consumptions; (2)-epicatechin only and together with theobromine and caffeine. It was concluded that the interaction between cocoa methylxanthines and flavanols affected the absorption of flavanols (Sansone et al., 2017).

A randomized, single-blind comparative study was conducted on 13 healthy participants to investigate the bioavailability of flavanols. Two soluble cocoa products were used: conventional soluble cocoa (CC) and flavanol-rich soluble cocoa (CC-PP). Participants were warned not to consume cocoa products and polyphenol-rich products three days before the experiment which was carried out in two days, separated by two weeks. Cocoa products (15 g of CC or 25 g CC-PP) in 200 mL of semi-skimmed milk were consumed by volunteers after an overnight fast. Blood samples (0.5, 1, 2, 3, 4, 6, and 8 h) and urine samples (-2–0, 0–4, 4–8, 8–12, and 12–24 h) were collected and analyzed *via* high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (HPLC-ESI-QToF-MS) to identify the metabolites after cocoa consumption. Ten metabolites were identified in plasma and 30 in urine. The urinary excretion of flavanol metabolites was quantified as 35.3 % and 34.6 % after CC and CC-PP consumption, respectively. Therefore, the bioavailability of flavanols was demonstrated as moderate. Also, it was indicated that the flavanols from the soluble cocoa products were absorbed to a certain extent and metabolized comprehensively in humans (Gómez-Juaristi et al., 2019).

Another randomized clinical trial conducted by Avila-Galvez et al. (2019) investigated the metabolic profiling of polyphenols and methylxanthines received from dietary plant extracts including cocoa on breast cancer patients that were diagnosed with biopsy-confirmed breast cancer. The mixture of dietary plant extracts including 37 diff ;erent phenolics (473.7 mg), theobromine, and caffeine (19.7 mg) was supplied to 17 patients for 2 ± 6 days until surgery in capsule form three times a day. Urine and blood samples were collected from the patients just before the surgery. During the resection surgery, samples were removed from malignant and normal breast tissue in each patient. According to UPLC-ESI-QTOF-MS analysis, a total of 101 metabolites including 90 phenolics and 11 methylxanthines was detected in urine samples and 69 of them were found in plasma of patients who consumed the capsules. However, only a total of 39 and 33 metabolites were detected in normal and malignant breast tissues, respectively. Glucoronidated and sulfated phenolic metabolites were detected in both normal and malignant tissues, however, there were no conjugated methylxanthine metabolites in either tissue. Furthermore, concentrations of the polyphenolic metabolites especially resveratrol 3-O-sulfate and 5-(3, 4 -Dihydroxyphenyl)-γ -valerolactone 3 -sulfate (DHPV-3 –sulfate) were significantly higher by 1.5-fold and 2.5-fold in malignant tissue compared to the normal tissue, respectively. For methylxanthine metabolites, the concentrations of 7-methylxanthine, 3-methylxanthine, theobromine, caffeine, and theophylline were higher by 5, 2, 2, 1.8, and 1.5 fold, respectively, in malignant tissue compared to the normal tissue. Collectively, these results indicated that the conjugation profiling of dietary polyphenols and methylxanthines in malignant tissue was not significantly different from normal tissue. However, there is a need to elucidate this topic with a more detailed and developed trial design (Ávila-Gálvez et al., 2019).

3. Cocoa consumption and human diseases

According to the statistical report of the ICCO, the worldwide cocoa bean production was recorded as 3993 thousand tonnes for 2015–2016 and 4733 thousand tonnes for 2016–2017, indicating an 18.5 % increase (Organization, 2017). The major reason for the remarkable cocoa consumption is that the cocoa plant, whose origin can be traced back tens of thousands of years ago, is now widely used in food industries related to the production of chocolates, sweets, pastries, beverages, confectionery, and dairy products. Besides the popular food products of cocoa, it can be presented in diverse non-food products including pharmaceuticals or cosmetics (Hii and Borém, 2020). Recently, several clinical trials have highlighted the potential beneficial effects of cocoa and cocoa polyphenols/methylxanthines for human health including the improvement of the endothelial function, reversing age-dependent cardiovascular risk factors, and increasing vascular function (Heiss et al., 2015; Sansone et al., 2015; Schroeter et al., 2006). Besides, recent studies also focused on the chemotherapeutic effects of cocoa and its bioactive compounds against the adverse symptoms of various chronic diseases. For instance, the cognitive functions and blood oxygen level-dependent response were improved in patients with Type 1 diabetes after the consumption of cocoa flavonols (Decroix et al., 2019). Camps-Bossacoma et al. (2018), investigated the immunoregulatory role of cocoa on Lewis rats fed a 10 % cocoa–enriched diet, 0.25 % theobromine diet (one of the major methylxanthines in cocoa), or a standard diet as the control group. Accordingly, serum IgG, IgM, and IgA, and intestinal sIgA concentrations of rats fed with cocoa diet were detected lower than the control group. Moreover, spleen and thymus weights of rats fed with cocoa or theobromine were lower than rats fed with a standard diet, and also the lymphocytes phenotype of these organs were modified after cocoa and theobromine diet. Furthermore, it was suggested that the decrease in lymphoid tissue relative weights after one week of cocoa or theobromine diet could be caused by the inhibition of lymphocyte proliferation (Camps-Bossacoma et al., 2018). Its neuroprotective effect was also demonstrated by *in vitro* and *in vivo* studies. Treatment of rat pheochromocytoma PC12 cells with epicatechin and catechin found in cocoa protected cells against amyloid beta-induced neurotoxicity (Heo and Lee, 2005). Supplementation of *Theobroma cacao* stem bark aqueous extract reversed the DOX-induced oxidative stress in brain tissues of rats (Kosoko et al., 2017). It was suggested that cocoa bean extract and especially cocoa procyanidin, procyanidin B_2, have an antiapoptotic effect in neuronal cells and, therefore, cocoa may be proposed as a potential nutritional supplement for neuroprotection in neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Chidambaram et al., 2020; Cho et al., 2009). Moreover, high-dose

artemether/lumefantrine-induced hepatotoxicity was reduced after cocoa powder administration in Non-Malarious Guinea Pigs (Asiedu-Gyekye et al., 2016). Celecoxib-induced apoptosis in MLP29 liver cells was inhibited by pre-treatment of *Theobroma cacao* L. phenolic extract indicating the hepatoprotective effect of cocoa (Arlorio et al., 2009). Furthermore, cocoa powder supplementation promoted intestinal health by enhancing glucose homeostasis, intestinal integrity, the abundance of *Lactobacillus* and *Bifidobacterium* species, and regulating the pro-inflammatory cytokine production, while improving immune response against influenza virus infection (Álvarez-Cilleros et al., 2020; Jang et al., 2015; Kamei et al., 2016). Besides all these health benefits of cocoa consumption, cocoa has been extensively studied for their preventive and ameliorative effects on cancer development and progression. In addition to earlier studies proving the potential anti-cancer effects of cocoa consumption due to its components (Martín et al., 2016; Pandurangan et al., 2015a), new research studies mainly focused on preventive/therapeutic effects of cocoa consumption on cancer development and progression (Martín et al., 2016; Saadatdoust et al., 2015; Zamanian-Azodi and Rezaei-Tavirani, 2019). Due to the recent interest in this field; the following sections give attention to these beneficial effects.

3.1. *Antiproliferative and antiapoptotic effects of cocoa and cocoa phenolics: in vitro and in vivo studies*

Oxidative stress contributes to both the development and progression of carcinogenesis by affecting signal transduction pathways that regulate cell survival and proliferation, so antioxidant bioactive compounds are currently being extensively studied to elucidate their promising anticancer effects (Martín et al., 2016). Although cocoa products have been largely consumed and incorporated into dietary supplements, clinical trials evaluating the anticancer efficacy in terms of chemopreventive and therapeutic perspectives are limited (Maskarinec, 2009). *In vitro* and *in vivo* studies mentioned below showed that cocoa diet or cocoa polyphenols, in particular procyanidins, showed antiproliferative, apoptotic, and chemopreventive effects with its highly potent antioxidant and anti-inflammatory effects (Fig. 2).

The flavanol content of cocoa is essentially composed of (-)-epicatechin and (+)-catechin and their related procyanidin oligomers. In the study of Carnesecchi et al. (2002), the antiproliferative effects of cocoa powder, flavanols, and procyanidins obtained from the crude extract were assessed primarily *in vitro* on Caco-2 human colonic cancer cells. The highest growth inhibition effect (75 %) was obtained in Caco-2 cells treated with procyanidin-enriched (PE) extracts at the concentration of 50 μg/mL consisting of 941 mg/g of flavanols and procyanidins during 10 days. However, while procyanidin (CE) extract including 501 mg/g of flavanol and procyanidin led to only 25 % cell growth suppression, there were no growth inhibitory effects in cells treated with cocoa powder (CC) samples including 141 mg/g of flavanol and procyanidin. These results indicated that procyanidin may play an important role in the antiproliferative effect of cocoa as a bioactive compound. On the other hand, Annexin V-FITC and propidium iodide (PI) analysis with PE-treated cells showed that procyanidin caused non-apoptotic cell death and DNA fragmentation and significantly increased the number of lysed cells by four-fold after six h according to the lactate dehydrogenase (LDH) assay. Furthermore, the activity of rate-limiting enzymes (ODC and AdoMetDC) in polyamine synthesis was significantly and time-dependently decreased after one and three days of the treatment of PE extract by 25 % and 45 % for ODC activity and 15 % and 47 % for AdoMetDC activity, respectively. Accordingly, the results relevant to the reduced spermidine content and high level of N^1–acetyl spermidine accumulation supported that the antiproliferative effect of procyanidins or cocoa flavanols may be mediated through the polyamine synthesis pathway in colon cancer cells (Carnésecchi et al., 2002).

In the study of Jourdain et al. (2006), cocoa polyphenols extract was found to have a selective antiproliferative effect on prostate cancer cells *in vitro*. Non-metastatic 22Rv1 and metastatic DU145 prostate cancer cell lines were treated with cocoa, β-sitosterol, which is the most common phytosterol, and also with the combination of the latter two at the concentration range of 0.2–1.562 × 10^{-3}% in culture media. Cocoa and the combined treatment groups showed remarkable apoptotic morphological changes with an increased number of rounded cells and loss of adherent cells in the cultures of nonmetastatic cells after 48 h of incubation. However, the apoptotic morphological change was less apparent in the metastatic cell line, while RWEP-1 normal prostate cells were not affected. Moreover, nonmetastatic prostate cancer cells were more sensitive to cocoa treatment than other cell lines, in a dose- and time-dependent manner (Jourdain et al., 2006).

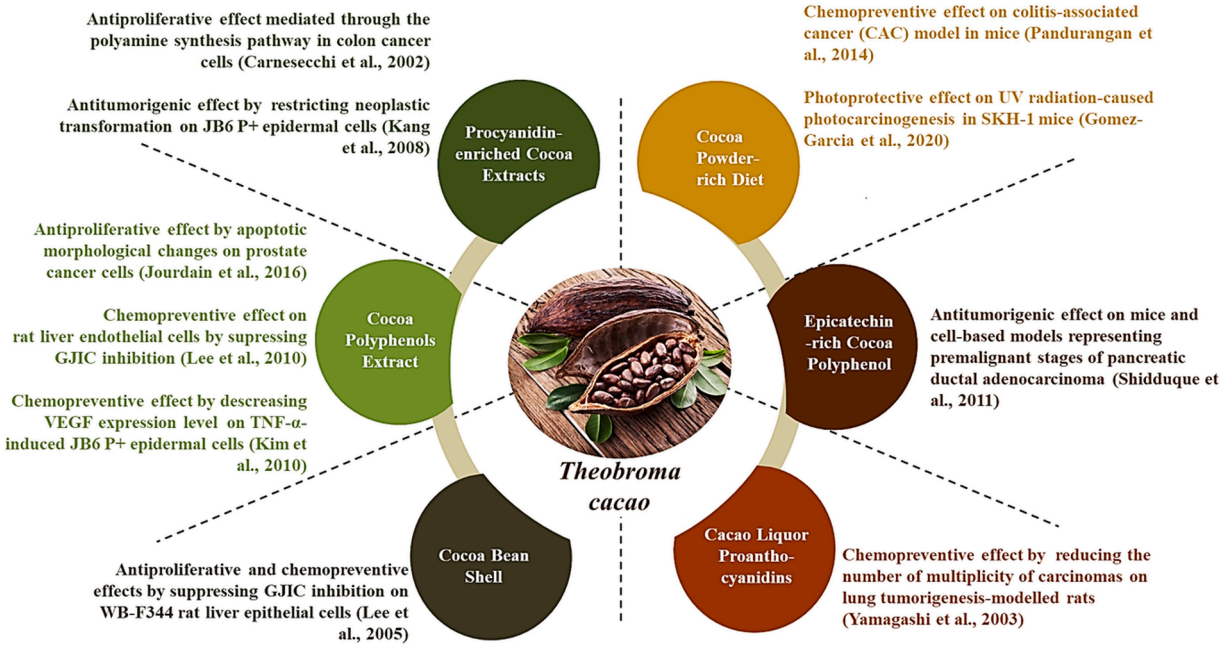

Fig. 2. Summary of the antiproliferative, apoptotic, and chemopreventive effects of cocoa on different *in vitro* and *in vivo* cancer models.

Ramiro et al. (2011) exhibited the first evidence about the anti-proliferative and apoptotic effects of cocoa on early events of colon carcinogenesis *in vivo*. The experimental design was conducted on four groups of male Winstar Han rats that are composed of two control and two cocoa-rich diet groups. The treatment period was eight weeks and at the second and third weeks rats were also exposed to azoxymethane (AOM) to create colonic preneoplastic lesions or saline. Interestingly, the body weights of rats fed with cocoa-rich diet were significantly decreased (around 10 %) compared to the control group fed with a standard diet. The number of crypt multiplicity of AOM-induced colonic aberrant crypt foci (ACF) significantly decreased especially for the four crypts numbers or more in the colon samples of rats fed with the cocoa-rich diet compared to the control group. Moreover, cocoa suppressed the oxidative stress at the levels of MDA and lipid peroxidation end product, and also reversed the antioxidant defense enzyme levels, *i.e.* glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST), in colon tissues of rats fed with cocoa. Proliferating cell nuclear antigen (PCNA) immunohistochemistry and TUNEL technique demonstrated that cocoa decreased levels of PCNA-positive cells and increased the apoptotic index. In addition, cocoa supplementation led to alleviated protein expression levels of p-ERKs, p-AKT, cyclin D1 and Bcl-xL (members of the Bcl-2 protein family), while increasing the protein expression level of Bax and caspase-3 activity in colon tissues of AOM-induced rats compared to the control (Rodríguez-Ramiro et al., 2011).

3.2. Chemopreventive effects of cocoa and cocoa phenolics: in vitro and in vivo studies

As a by-product of the chocolate industry, CBS was evaluated for its cancer chemoprevention role *in vitro* by Lee et al. (2005). CBS and cocoa bean (CB) were extracted with 50 % acetone, ethanol, or methanol and these extracts were fractioned with different percentages of ethanol. The ratio of phenolic phytochemicals to the total weight was quantified in a higher range of 2.3–14.0 % in 60 % ethanol fraction of CBS compared to the CB by the range of 1.1–6.3 %. Additionally, 60 % ethanol fraction of CBS which was extracted with 50 % acetone using an adsorption column packed with styrene-based porous resin showed the maximal yield by 43 % (w/w) (Lee et al., 2005). Suppression of gap-junction intercellular communication (GJIC) is one of the key biochemical indexes examined in cancer progression (Trosko and Chang, 2000). In the scrape-loading/dye transfer assay, 1 μg/mL of the aforementioned CBS fraction effectively suppressed the H_2O_2-induced GJIC inhibition by 66 % in WB-F344 rat liver epithelial cells, which was reported as more effective as compared to 10 μg/mL of vitamin C, taking into account that the positive control resulted in just 35 % inhibition. However, it was revealed that the protective effect of CBS was not due to its radical (H_2O_2) scavenging activity, but mainly their mechanisms of action. Moreover, the antiproliferative effect of CBS was evaluated on DNA synthesis of HepG2 liver cancer cells, SNU1 stomach cancer cells, and SNUC2A colon cancer cells by using ^{3}H-thymidine uptake assay. As a result, dose-dependent inhibition of proliferation was detected in three cell lines treated with both 50 % ethanol and acetone extract of CBS in the concentrations of 400 and 800 μg/mL. The results of these anticarcinogenic assays suggested that polyphenol extracts or fractions of CBS can be developed as a potential chemopreventive product, though there is a need to elucidate the mechanisms of action of the polyphenols from CBS on cancer cell proliferation pathways (Lee et al., 2005).

Another *in vitro* study demonstrated the molecular mechanism behind the suppressive effect of cocoa polyphenols on H_2O_2-induced GJIC inhibition *in vitro* by Lee et al. (2010). Cocoa polyphenol extract (CPE) was obtained from 50 g of commercial cocoa powder which was refluxed with 500 mL of 50 % (v/v) aqueous ethanol for 6 h. Rat liver endothelial (RLE) cells were exposed to 100 μM of H_2O_2 after being treated with CPE at 10, 50, and 100 μg/mL doses. According to the number of communicating cells, H_2O_2-induced GJIC inhibition was dose-dependently suppressed by CPE while relative p-ERK protein level and internalization of a regulating protein of GJIC, connexin 43, were effectively restrained with pre-treatment of CPE at 50 μg/mL in H_2O_2-exposed rat liver endothelial (RLE) cells. Furthermore, *ex vivo* kinase and pull-down assays demonstrated that CPE inhibited MEK1 activity (mitogen-activated protein kinase kinase-1), plays an important role in cellular transformation and tumor development (Cowley et al., 1994) *via* direct attachment on cells pretreated with CPE for 30 min and then exposed to H_2O_2 for 30 min. Collectively, this study suggested that CPE may be a chemopreventive agent as a potent direct MEK1 inhibitor (Lee et al., 2010).

In the study of Kang et al. (2008), the molecular mechanism of the chemopreventive potential of procyanidin fraction extracted from the cocoa powder was reported, as well as the particular active phytochemicals of cocoa, procyanidin B_2, and theobromine. JB6 P tumor-promotion-sensitive mouse epidermal (JB6 P+) cells were exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA) to stimulate the tumorigenic neoplastic transformation. JB6 P + cells were co-treated with TPA and cocoa procyanidin fraction (CPF) at the concentrations of 5, 10, 20, and 40 μg/mL, procyanidin B_2 or theobromine at the concentrations of 20, 40, 80, and 160 μg/mL in soft agar for 14 days. According to the number of colonies obtained by anchorage-independent transformation assay, except theobromine, CPF showed a dose-dependent inhibition of neoplastic transformation, while 40 μg/mL of procyanidin B_2 inhibited TPA-induced neoplastic transformation by 93 % in JB6 P + cells. TPA-induced up-regulation of COX-2 was significantly suppressed at protein expression level by the treatment of CPF and procyanidin B_2 in JB6 P + cells. The measurement of luciferase activity from the cells transfected with COX-2, AP-1, or NF-KB luciferase reporter plasmid revealed that CPF and procyanidin B2 inhibited TPA-induced COX-2 promoter activity *via* suppressing the activation of AP-1 and/or NF-KB in a dose-dependent manner. Furthermore, this study suggested that CPF and procyanidin B_2 exerted an antitumorigenic effect on JB6 P + cells by restricting the neoplastic transformation through blocking of MEK/ERK/p90RSK signaling pathway (Kang et al., 2008).

Because of the detrimental contribution of TNF-α to tumor development, Kim et al. (2010) investigated the chemopreventive potential of CPE on TNF-α-induced JB6 P + cells. CPE was obtained from 50 g of commercial cocoa powder and then eluted with 60 % (v/v) aqueous ethanol by using a styrene-based adsorption resin column. JB6 P + cells were treated with 4 ng/mL of TNF-α for 18 h after pre-treatment with CPE at the concentrations of 5, 10, and 20 μg/mL. CPE dose-dependently decreased the level of TNF-α- induced vascular endothelial growth factor (VEGF) expression which was associated with the pathology of tumorigenesis as a key regulator of angiogenesis. Luciferase activities of transcription factors NF-kB and AP-1 regulating VEGF expression, significantly increased 2.5 and 2 fold, respectively, by the exposure of 4 ng/mL of TNF-α for four h. However, 10 and 20 μg/mL of CPE significantly attenuated their relative transactivation levels as compared to TNF-α treated cells. Furthermore, this study revealed that the suppressive effect of CPE on TNF-α-induced VEGF expression was accompanied by the inhibitory activities of PI3K (phosphoinositide 3-kinase) and MEK1 which finally led to inhibition of downstream phosphorylation of extracellular signal-regulated kinase (ERK) and Akt in JB6 P + cells (Kim et al., 2010).

In the *in vivo* study of Yamagashi et al. (2003), a multi-organ carcinogenesis model was developed in rats to investigate the chemopreventive effect of cocoa liquor proanthocyanidins (CLPr) on lung tumorigenesis. Lung carcinogenesis was triggered in rats by a combined treatment which was abbreviated as DMBDD for four weeks. One week afterwards, rats received 0.025 % or 0.25 % doses of CLPr diet until the 36th week. The relative kidney weights of DMBDD-treated rats were significantly increased by 0.88 ± 0.30 %, while their total body weights were decreased by 317.8 ± 13.5 g compared to that of the control group (0.58 ± 0.04 % and 396.8 ± 8.5 g, respectively). Additionally, the

number of dead rats at the end of the experiment due to exposure to DMBDD was reduced with the treatment of CLPr. Furthermore, the percentage and multiplicity of carcinomas in DMBDD exposed rats were reduced with CLPr treatment in a dose-dependent manner (Yamagishi et al., 2003).

In the study of Shidduque et al. (2011), cell-based models representing premalignant stages of pancreatic ductal adenocarcinoma (PDAC) were developed by constitutively activating the Kras gene to investigate the chemopreventive effect of epicatechin-rich cocoa polyphenols *in vitro* and *in vivo*. Liquid chromatography (LC)–(MS)/MS quantified 625 nmol/g of epicatechin, 376 nmol/g of catechin, and 292 nmol/g of epicatechingallate from 0.5 mg/mL of cocoa-polyphenols (CP) solution. Tumorigenic and non-tumorigenic pancreatic ductal epithelial (PDE) cells were treated with 0.001–0.1 % of CP which were prepared in water and epicatechin only for 48 h. After that, the IC_{50} values of CP for nontumorigenic and tumorigenic cells survival were reported as 0.042 % and 0.028 %, while the IC_{50} values of epicatechin were predicted as 0.075 and 0.05 %, respectively. Moreover, proliferation, guanosine triphosphate (GTP)-bound Ras protein, as well as the level of p-Akt and NF-KB transcriptional activity were reduced by CP and epicatechin treatments in premalignant and malignant Kras-activated PDE cells, while normal PDE cells were not affected in this way. Furthermore, 25 mg/kg of CP were supplied orally to athymic nude mice which were inoculated with Kras-PDE cells to generate tumors for 3-days per week. Accordingly, the average tumor volume in mice that were treated with CP (377 mm^3) was remarkably lower than the control-treated with water (1000 mm^3) after 6 weeks of inoculation, and also the tumor growth volumes were significantly different at the end of 10 weeks (Siddique et al., 2012).

Chronic colonic inflammation has been implicated as a critical risk factor in cancer worldwide (Eaden et al., 2001; Jemal et al., 2011). An *in vivo* study by Pandurangan et al. (2015a) investigated the protective effect of cocoa on mice that were treated with azoxymethane (AOM)/-dextran sulfate sodium (DSS) to induce colitis-associated cancer (CAC) model. After AOM/DSS stimulation, mice were treated with a 5% or 10 % cocoa diet for 62 days that led to a significant reduction in disease activity index by 2–3 folds compared with only AOM/DSS-induced mice. Additionally, the cell proliferation index of each group was quantified by immunohistochemical expression of Ki-67 protein as a marker. Accordingly, colon tissues from mice treated with a 5% and 10 % cocoa diet after AOM/DSS induction, showed a remarkable decrease of Ki-67 positive cells. Besides, the antioxidant effect of cocoa was exhibited by the improved activity of colonic enzymic and non-enzymic antioxidant levels. Cocoa administration also significantly suppressed the AOM/DSS induced-gene expression of two major inflammatory mediators, iNOS and COX-2 in mouse colon tissues. In addition to these protective effects of cocoa against CAC, the Nrf2/Keap1 pathway was also activated by a 10 % cocoa diet in the CAC mouse model *via* promoting cytosolic Nrf-2 protein level and gene expression of its downstream targets including NQO1 and UDP-GT (Pandurangan et al., 2015a).

Recently, Gomez-Garcia et al. (2020) suggested that oral administration of cocoa extract may protect against ultraviolet radiation-caused photocarcinogenesis in SKH-1 mice. Animals were pre-treated with 228 mg of cocoa extract/kg body weight daily for two weeks before starting the carcinogenic UV radiation exposure mainly consisting of UVA radiation. The dietary supplementation was continued for 29 weeks, while UV irritation lasted for 80 sessions. At the end of the experiment, microscopic analysis showed that cocoa extract supply remarkably suppressed the development of carcinoma. Furthermore, the level of mutated p53 expression was significantly reduced, while the intensity values of two important tissue remodeling matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 were improved in the cocoa extract-treated group compared to the control group, as detected by semi-quantitative immunohistochemical analysis (Gómez-García et al., 2020).

4. Future perspectives and conclusions

Cocoa, chocolate, and cocoa bean shells obtained from *Theobroma cacao* are useful for preventing and treating several human diseases, thanks to the significant content in polyphenols such as catechin, epicatechin, procyanidin B_2, quercetin, and protocatechuic acid. Cocoa and cocoa derivatives are rich in alkaloids, *i.e.* theobromine and caffeine, and minerals including potassium, magnesium, calcium, phosphorus, copper, and zinc. Indeed, cocoa has been shown to possess higher antioxidant activity compared to black tea, green tea, and red wine, by limiting ROS generation *in vitro* experiments (Martins et al., 2020). In addition, cocoa supply can impact human health: its use resulted in cholesterol level decrease, thus reducing atherosclerosis, inhibition of lymphocyte proliferation and lymphoid tissue growth, alleviation of glucose intolerance and liver steatosis, by reduction of the triglyceride content and the insulin levels (Camps-Bossacoma et al., 2018). The cocoa antioxidant activity also proved to affect the gene expression profile related to both peripheral mononuclear cells and inflammatory cytokines and redox balance (Barrera-Reyes et al., 2019). Cocoa polyphenols/methylxanthines showed beneficial effects on endothelial function, cardiovascular risk factors, Parkinson's and Alzheimer's neurodegenerative diseases, intestinal diseases, colon and prostate cancer, and ultraviolet radiation-caused photocarcinogenesis. Moreover, cocoa could help in fighting pathogenetic microorganisms that affect human health and nutrition and this road is wide open for investigation especially in food preparation.

The dark side of cocoa chocolate is sugar, *i.e.* sucrose that is added in amounts typically at 15–35 % range. This can rapidly increase the glycemic index post-prandially, with a high impact on energy intake and health consequences (obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease, *etc.*). Accordingly, as sugar consumption has become a worldwide concern, different approaches tried to diminish the amount of sucrose in the chocolate, without modifying cocoa flavor (Bandy et al., 2021; Tolve et al., 2021). In particular, the replacement of sucrose with maltitol, sucralose, and stevia seems the most frequently used, strategies without an effect on energy intake (de Medeiros et al., 2019; Saraiva et al., 2020). However, the great challenge of producers and traders is to preserve the classic aroma of chocolate without the amount of sucrose, especially for those people used to consuming sweet chocolate.

It is noteworthy that together with the interesting properties of cocoa, CBS also showed stimulating properties. Based on the reported works above, the valorization of CBS is a promising goal due to its significant content of bioactive compounds and the relevant beneficial activity on human health. Trying to better analyze and explore CBS activities both in preclinical and clinical studies will guarantee a successful way to create new potential curative treatments and diet products for the market.

Author statement

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The authors declare no conflict of interest.

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