

Antihypertensive Effect of a Polyphenol-Rich Cocoa Powder Industrially Processed To Preserve the Original Flavonoids of the Cocoa Beans

ELENA CIENFUEGOS-JOVELLANOS,^{§,||} MARÍA DEL MAR QUIÑONES,^{#,||}
BEGOÑA MUGUERZA,[§] LEILA MOULAY,[§] MARTA MIGUEL,[#] AND AMAYA ALEIXANDRE*^{#,§}

[#]Departamento Farmacología, Facultad Medicina, Universidad Complutense, Avenida Complutense s/n, 28040 Madrid, Spain, and [§]Natraceutical Group, Autovía A-3, Salida 343, Camino de Torrent s/n, Quart de Poblet, 46930 Valencia, Spain. ^{||}E.C.-J. and M.Q. contributed equally to this study.

A natural flavonoid-enriched cocoa powder, commercially named CocioanOX and developed via a patented industrial process, was characterized and tested for a possible antihypertensive effect. The bioavailability of this polyphenol-rich cocoa powder developed at pilot scale was previously demonstrated in humans. The present results showed that this product was very rich in total procyanidins (128.9 mg/g), especially monomers, dimers, and trimers (54.1 mg/g), and mainly (–)-epicatechin (19.36 mg/g). The effect of a single oral administration of CocioanOX in spontaneously hypertensive rats (SHR) was evaluated at different doses (50, 100, 300, and 600 mg/kg). This product produced a clear antihypertensive effect in these animals, but these doses did not modify the arterial blood pressure in the normotensive Wistar–Kyoto rats. Paradoxically, the maximum effect in the systolic blood pressure (SBP) of SHR was caused by 300 mg/kg of CocioanOX. This dose brought about a decrease in this variable very similar to that caused by 50 mg/kg Captopril. It was also surprising that the maximum effect in the diastolic blood pressure (DBP) was caused by 100 mg/kg CocioanOX. The initial values of DBP and SBP were recovered in SHR, respectively, 24 and 48 h postadministration of the different doses of CocioanOX or Captopril. These results suggest that CocioanOX could be used as a functional ingredient with antihypertensive effect, although it would be also necessary to carry out bioavailability and clinical studies to demonstrate its long-term antihypertensive efficiency in humans.

KEYWORDS: CocioanOX; polyphenols; procyanidins; flavanols; epicatechin; hypertension; spontaneously hypertensive rats

INTRODUCTION

Hypertension is an important problem in our society given its high prevalence and its role as a critical cardiovascular risk factor. This pathology is a common and usually progressive disorder, which, if not effectively treated, has a high mortality rate. Epidemiological studies have shown an inverse association of flavonoid-rich diet consumption with the risk of hypertension and cardiovascular disease (1–5).

Cocoa and cocoa derivatives are known as significant sources of flavonoids, particularly of flavan-3-ols and procyanidins. Other examples of sources rich in this kind of flavanoids are wine and tea. However, cocoa has been shown to have the highest content of flavanols (6, 7). Several studies have published the quantitative determination of (+)-catechin, (–)-epicatechin, and procyanidins in cocoa products by normal- and reverse-phase liquid chromatography–mass spectrometry (8–11). Monomer units contribute most to the total procyanidin content in cocoa, (–)-epicatechin being the main component (12). Great interest is

focused on cocoa compounds for their possible beneficial health effects, and flavanols have made cocoa a candidate as a functional food. In fact, a recent epidemiological long-term study has reported a lowering effect of cocoa intake on cardiovascular mortality in elderly men (13). In addition, a prospective study in postmenopausal women demonstrated a borderline inverse correlation of chocolate intake and cardiovascular disease (14). Moreover, animal and human intervention studies have shown that cocoa polyphenols exhibit many health benefits (15, 16). In addition, the ingestion of cocoa beverage by Kuna Indians, a small population of Indians living in the San Blas Island, is related with a low prevalence of atherosclerotic diseases and no rise in blood pressure with age (17). These results are in agreement with data from human studies that showed the antihypertensive properties of cocoa polyphenols (18–23).

The botanical variety and manufacturing factors related to the processing of cocoa beans (such as postharvest handling, fermentation, drying, roasting, and alkalization treatment) affect, however, the flavonoid quantity and quality of a cocoa-based products. Kim and Keeney reported a 90% drop in the concentration of epicatechin after fermentation and drying (24).

*Author to whom correspondence should be addressed (telephone 34-91-3941475; fax 34-91-3941463; e-mail amaya@med.ucm.es).

Significant decreases in total flavonoid content after fermentation and roasting processes were observed in sun-dried cocoa beans (25). A decrease of 3–5-fold in procyanidin levels during fermentation was reported (26). In addition, it has been reported that high processing temperatures and longer roasting times reduce the content of polyphenols in cocoa. As a consequence of the alkalization process, losses of 36% for (+)-catechin, 67% for (–)-epicatechin, 69% for dimer B2, 67% for trimer C1, and 31% for tetramer D were registered (27). In addition, the procyanidin content reported by Gu et al. in Dutched and natural cocoa powder indicated degradation of procyanidins as a result of the alkali treatment (11). Therefore, there are many reported factors that can significantly reduce the polyphenol content in cocoa.

It is worth noting that the preservation of polyphenols during the cocoa manufacturing is important to exhibit the health effects associated with cocoa consumption. In a previous study a polyphenol-rich cocoa powder was demonstrated to have a high bioavailability in humans when it was administered in a milk drink (25). In addition, this cocoa powder was found to have values of the flavanols epicatechin and procyanidin B2, 8 times higher than the values of a conventional cocoa powder. Therefore, in the present study this polyphenol-rich cocoa powder, named *CocoanOX*, was produced by an innovative industrial patented process (28), and it was characterized for its procyanidin profile. In addition, the functionality of this cocoa powder was evaluated in a short-term study by using an experimental model of hypertensive rats.

MATERIALS AND METHODS

Procyanidin Profile of *CocoanOX*. Unfermented thermally treated with water and dried cocoa beans of CCN51 clone were prepared in Ecuador to produce different batches of *CocoanOX* (10–12% of fat) as previously described (28). The cocoa beans used were selected for its total polyphenols content prior to its processing to ensure a minimal content of 85 mg/g.

Total Polyphenol and Theobromine Determination. Total polyphenol content was determined in cocoa beans and in *CocoanOX* as previously described (25). Duplicate samples of 1 g were extracted with 100 mL of acetone/water (70:30; v/v) under reflux at 60 °C for 2 h, and the acetone was removed under vacuum at 45 °C. The total polyphenol content was determined according to the Folin–Ciocalteu spectrophotometric method (29), and it was expressed by using (+)-catechin as the standard. The results are calculated on a wet weight basis as mean values \pm standard deviation of the mean (SD).

Theobromine content in *CocoanOX* was also quantified in duplicate at 275 nm using the corresponding commercial standard by high-performance liquid chromatography with a diode array detector (HPLC-DAD) (30).

Total Procyanidin Extraction and Normal-Phase HPLC-MS Analyses. Twenty industrial batches of *CocoanOX* were characterized for their procyanidin profile (from monomers through polymers). A total procyanidin extract was obtained from *CocoanOX* according to a modified protocol of extraction (31). Samples (1 g) were extracted with 10 mL of extraction solvent (acetone/water/acetic acid, 70:29.5:0.5, v/v/v). The mix was vortexed for 30 s followed by sonication at 37 °C for 10 min. The extraction remained at room temperature for 50 min. The mix was vortexed for 30 s after 25 min. Fifty minutes later the tube was centrifuged at 1600g for 15 min. The solvents were evaporated to dryness under vacuum at 45 °C. The dried substance was dissolved in the solvent extraction, and the solution was filtered with a polypropylene filter unit (0.45 μ m) before it was injected into the column for normal-phase HPLC-MS analysis. Procyanidin monomers through polymers from *CocoanOX* extract samples have been identified and quantified by normal-phase HPLC as previously described (31). The results are expressed on a wet basis as mean values \pm SD.

Flavan-3-ol Extraction and Reversed-Phase HPLC-DAD Analyses. To determine the concentration of flavan-3-ol compounds present in *CocoanOX* [monomers of (+)-catechin and (–)-epicatechin and the dimers of procyanidins B2 (epicatechin-4 β -8-epicatechin) and

B1 (epicatechin-4 β -8-catechin)], a modified protocol of extraction (25) was performed. The cocoa powder sample was previously milled with a MS 50 laboratory mill. Approximately 0.5 g was dissolved in 5 mL of distilled water at 100 °C and agitated in a vortex for 1 min. Twenty milliliters of the extraction solution (methanol/HCl, 99.77:0.22, v/v) was added, and the extract was agitated in a vortex for 2 min. The homogenate was centrifuged at 1600g and 4 °C for 15 min in a Digicen20-R centrifuge (Oltoalresa, Spain). This procedure was repeated twice, and the supernatants were combined and concentrated under reduced pressure (35 °C) to remove the methanol. The remaining aqueous extract was made up to 50 mL with distilled water. One aliquot of 5 mL was diluted 4 times, and finally the sample extract was passed through a 0.45 μ m PTFE filter before 20 μ L was injected. Chromatographic separation was performed on an Agilent 1100 HPLC system equipped with a DAD, quaternary pump, column heater, and manual injector. Separation was carried out on a reverse-phase Zorbax Eclipse XDB-C₁₈ (150 \times 2.1 mm, 5 μ m) column at 35 °C. The mobile phase consisted of water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile (solvent B) applied at a flow rate of 0.6 mL/min. The gradient was as follows: 0–20 min, 6–10% B linear; 20–25 min, 10–13% B linear; 25–30 min, 13–15% B linear; 30–40 min, 15–10% B linear; 40–45 min, 10–6% B linear, followed by 10 min of re-equilibration of the column before a new injection. Flavan-3-ols, (+)-catechin, (–)-epicatechin, and procyanidins B2 and B1 were quantified at 280 nm using the corresponding commercial standards. The results are expressed on a wet basis as mean values \pm SD.

Reagents. Standards of (+)-catechin and (–)-epicatechin (Sigma, USA) and procyanidins B1 and B2 (Extrasynthese, France) were used for quantitative determinations. Organic solvents of HPLC grade were purchased from Scharlab (Spain) and Merck (Germany). Standard solutions were prepared daily and stored in the refrigerator at 4 °C and were previously filtered through 0.45 μ m PTFE filter (Teknokroma, Spain) before the injection in the column. Theobromine standard was purchased from Avocado-Panreac (Spain).

Experimental Procedure in Rats. General Protocol. In this study we have used 52 17–20-week-old male spontaneously hypertensive rats (SHR), weighing 314 \pm 3 g, and 10 17–20-week-old male normotensive Wistar–Kyoto (WKY) rats, weighing 337 \pm 6 g. All of these animals were obtained from Charles River Laboratories Spain. The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles and consumed tap water and a standard diet (A04 Panlab, Spain) ad libitum during the experiments. *CocoanOX* was dissolved in water and orally administered by gastric intubation, between 9 and 10 a.m. Distilled water was used as negative control, and Captopril (Sigma, USA) (50 mg/kg), a known antihypertensive drug, was given as positive control. Different doses of *CocoanOX* (50, 100, 300, and 600 mg/kg) were administered. The volume orally administered to the rats was always 1 mL/rat either of water or of the appropriate solution of *CocoanOX* or Captopril. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the rats by the tail cuff method before administration and 2, 4, 6, 8, 24, and 48 h postadministration. Before the measurement, the rats were kept at 38 °C for 10 min to detect the pulsations of the tail artery. The original method for measuring arterial blood pressure using the tail cuff provides only SBP values (32), but the equipment used in this study, LE 5001 (Leticia, Spain), has a high sensitivity pulse transducer coupled with an accurate micro-processor program and allows us to distinguish between SBP and DBP. To establish the values of SBP and DBP, five measurements were taken, and the average of all was obtained. To minimize stress-induced variations in blood pressure, all measurements were taken by the same person in the same peaceful environment. Moreover, to guarantee the reliability of the measurements we established a training period of 2 weeks before the actual trial time, and during this period the rats became accustomed to the procedure.

All of the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

Statistical Analysis. The results are expressed as mean values \pm standard error of the mean (SEM) for a minimum of eight rats and were analyzed by a two-way analysis of variance (ANOVA), using the GraphPad Prism software. In addition, to compare the different treatments and to assess the effect of time within each treatment, some data were also analyzed by a one-way ANOVA, and differences between the groups were assessed by the Bonferroni test. Differences between the means were considered to be significant when $P < 0.05$.

Table 1. Total Polyphenols of Cocoa Beans and Total Polyphenol and Procyanidin Contents of 20 Different Batches of CocoanOX

	total polyphenols ^a (mg/g)		procyanidins ^b (mg/g)											
	cocoa beans	cocoa powder	1-mers	2-mers	3-mers	4-mers	5-mers	6-mers	7-mers	8-mers	9-mers	10-mers	poly mers	total
mean	93.16	139.3	21.19	17.93	14.98	13.09	10.59	9.70	5.28	5.20	6.55	1.91	22.51	128.92
SD	4.15	15.4	2.58	2.01	1.61	2.05	1.49	1.72	1.30	1.54	2.60	1.60	40.29	19.99
% ^c			16	14	12	10	8	8	4	4	5	1	18	

^a Spectrophotometric method Folin–Ciocalteu. Results expressed as catechin equivalents. ^b Data obtained using normal phase HPLC. Data are average of duplicate tests. ^c Percentage of each procyanidin fraction (monomer through polymers) with respect to the total procyanidins.

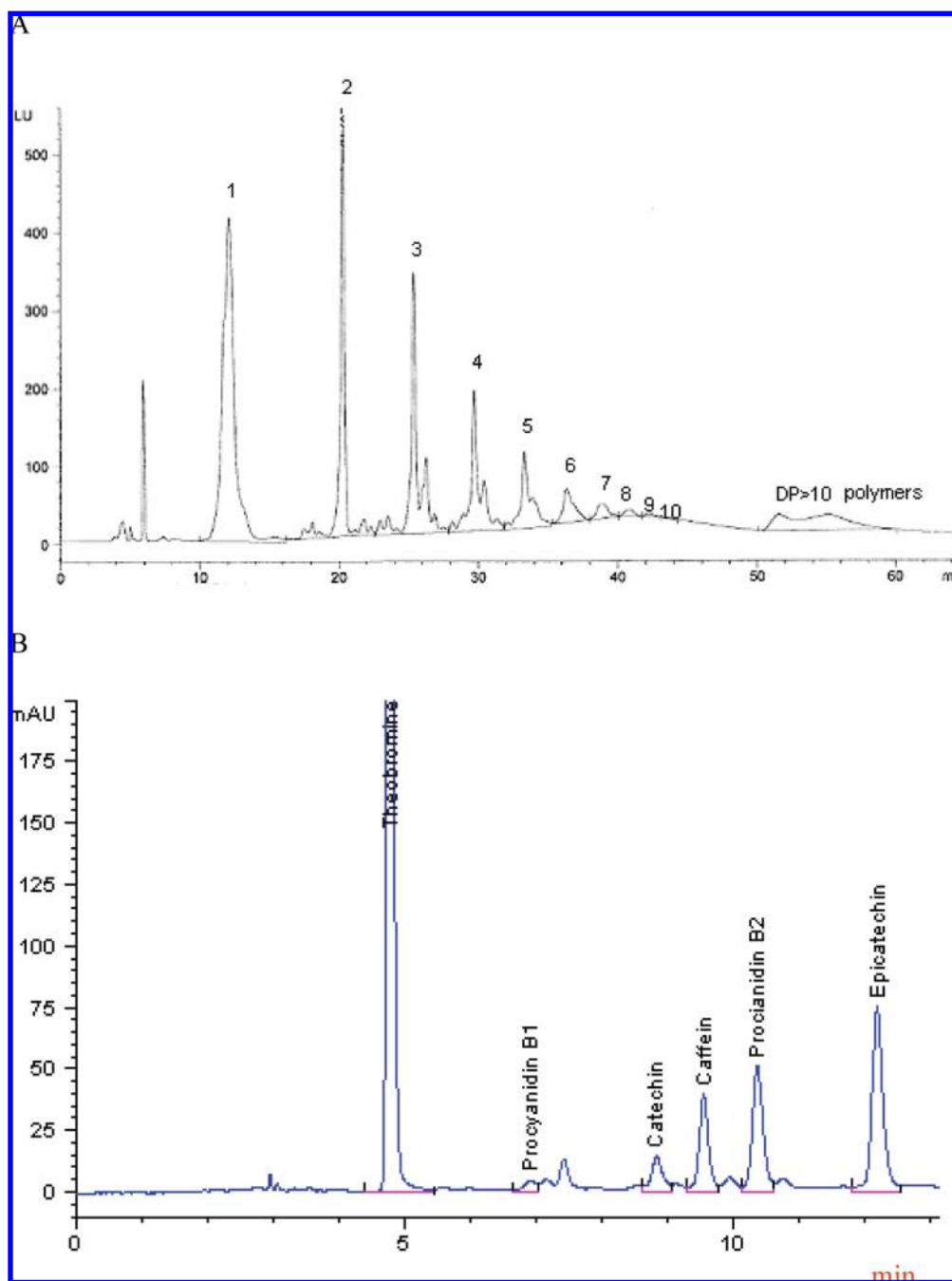


Figure 1. Normal-phase HPLC fluorescence chromatogram of procyanidins (A) and reverse-phase HPLC-DAD chromatogram of flavan-3-ols (B) of CocoanOX extracts. Numbers above peaks (A) denote degree of polymerization of procyanidins.

RESULTS

Total polyphenol contents of different batches of cocoa beans and CocoanOX and the corresponding procyanidin content of CocoanOX (monomers to >decamers) of this product are

presented in **Table 1**. The monomers through trimers contributed 42% of the total procyanidins in CocoanOX. **Figure 1A** shows the procyanidins (monomers through polymers) present in CocoanOX that were separated by using normal-phase HPLC-MS

fluorescence in accordance with the degree of polymerization. **Figure 1B** shows the flavan-3-ols that were separated by using reversed phase HPLC-DAD in this cocoa powder. Flavan-3-ol and theobromine contents were also characterized in CocoanOX, and the corresponding results are shown in **Table 2**.

CocoanOX produced an antihypertensive effect in SHR (**Figure 2**). The decrease in SBP was dose-dependent up to the dose of 300 mg/kg. The maximum decrease in the SBP was achieved 4 h postadministration of 300 mg/kg of CocoanOX.

Table 2. Concentrations of Theobromine and Flavan-3-ols (Milligrams per Gram) of the Industrial Batch of CocoanOX Used for the Rat Study^a

theobromine	15.95 ± 0.05
total flavan-3-ols ^a	42.64 ± 0.25
(+)-catechin	5.18 ± 0.09
(-)-epicatechin	19.36 ± 0.03
procyanidin B2	16.85 ± 0.06
procyanidin B1	1.25 ± 0.07

^a Results are expressed on a wet basis as mean ± SD (*n* = 2). ^b DAD-HPLC.

In fact, this dose of CocoanOX and 50 mg/kg of Captopril exhibited very similar effects in the SBP of the SHR group. The decrease in the SBP caused by 300 mg/kg of CocoanOX and the decrease in this variable caused by 50 mg/kg of Captopril were very similar. SBP decreased also in SHR after the administration of 50 mg/kg of CocoanOX or 100 mg/kg of CocoanOX, but the maximal effect in the SBP caused by these doses of CocoanOX was in both cases attained 8 h postadministration. Paradoxically, 600 mg/kg of CocoanOX had an effect on the SBP similar to that of 50 mg/kg dose. The maximum decrease in the DBP was achieved 4 h postadministration of 50 or 100 mg/kg of CocoanOX, and it was also surprising to observe that 50 and 100 mg/kg of CocoanOX caused a greater effect in the DBP of the SHR than 300 or 600 mg/kg of CocoanOX. The initial values of the DBP and SBP were not recovered until 24 and 48 h, respectively, postadministration of CocoanOX or Captopril. The administration of 300 mg/kg of CocoanOX did not modify SBP or DBP in normotensive WKY rats (**Figure 3**).

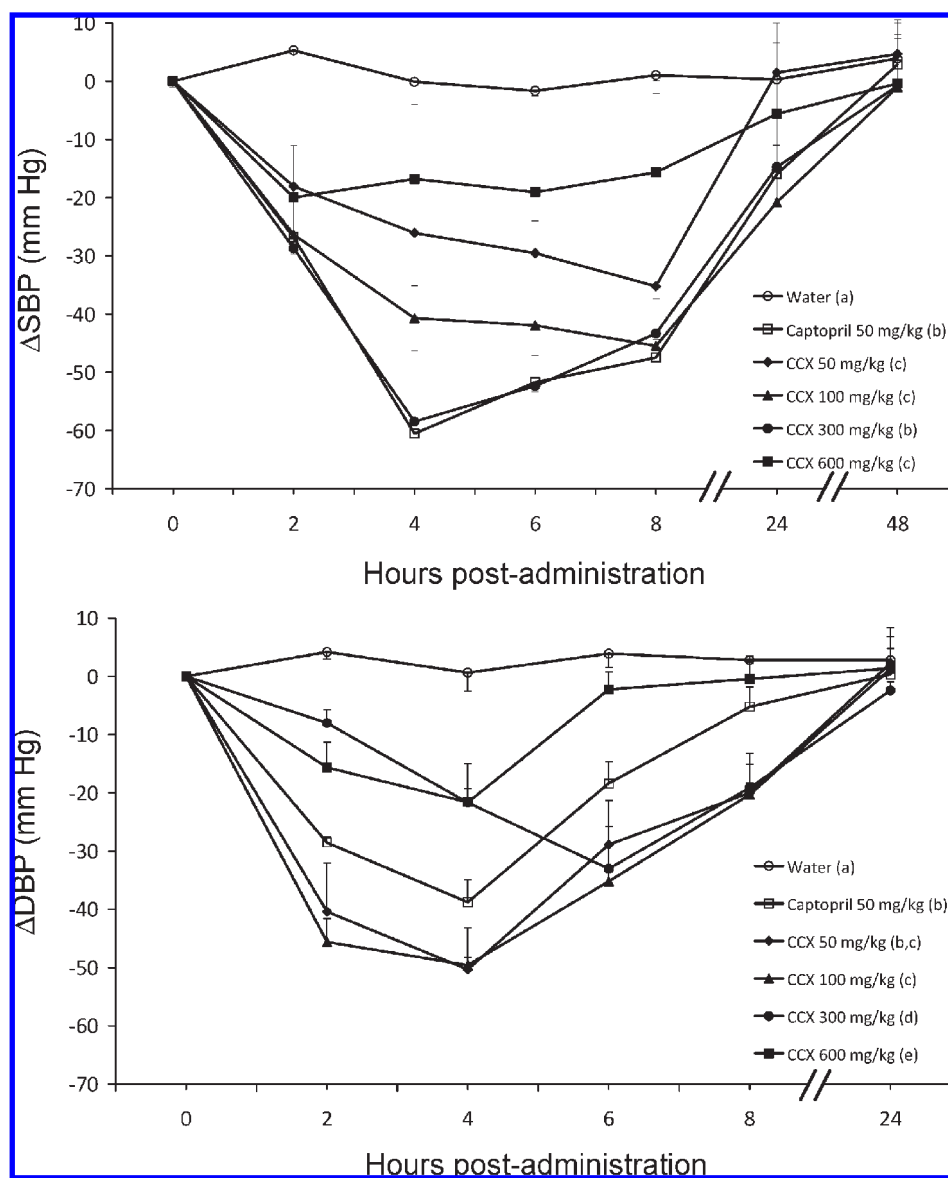


Figure 2. Decreases in systolic blood pressure (SBP) and diastolic blood pressure (DBP) caused in spontaneously hypertensive rats after administration of different products: water (○); Captopril (50 mg/kg) (□); or different doses of CocoanOX (CCX) of 50 mg/kg (◇), 100 mg/kg (▲), 300 mg/kg (●), and 600 mg/kg (■). Data are expressed as mean ± SEM. The experimental groups always have eight animals except in the case of water and Captopril, which had 10. Different letters represent statistical differences (*p* < 0.05). *P* was estimated by two-way ANOVA.

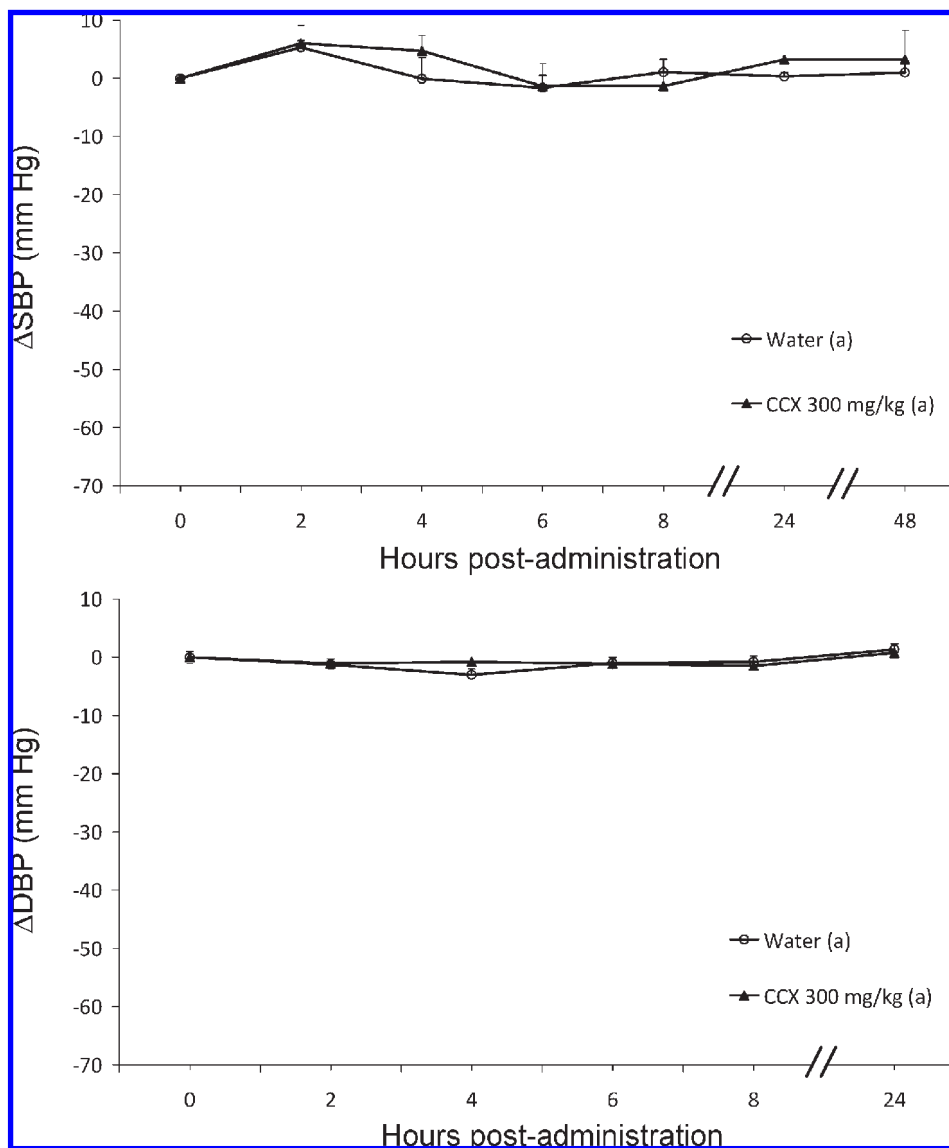


Figure 3. Decreases in systolic blood pressure (SBP) and diastolic blood pressure (DBP) caused in Wistar–Kyoto rats after administration of different products: water (○); 300 mg/kg CocoaOX (CCX) (▲). Data are expressed as mean \pm SEM. Both experimental groups have 10 animals. No statistical differences were observed by two-way ANOVA.

DISCUSSION

The results revealed a total polyphenol mean of 139.3 ± 15.4 mg/g CocoaOX in 20 batches of this cocoa powder. This value can be considered as very high in comparison to other cocoa derivatives such as chocolates (5–8.4 mg/g according to chocolate type) and standard cocoa powder (20 mg/g), although higher values of 70 mg/g have also been reported (33, 34). In addition, an important content of procyanidin oligomers was revealed also in CocoaOX. The content of procyanidin oligomers of our product is 3–6 times higher than values previously reported for natural cocoa powder (11, 35). However, it is also worth noting that this product contains 42% of monomers through trimers. Although polyphenol bioavailability is relatively poor (36), flavanols with low molecular weight are among the most bioavailable flavanoid compounds. In fact, cocoa flavonoids have been shown to be bioavailable in humans (37, 38). In addition to the monomeric flavanols, dimeric procyanidins have been identified in human plasma following consumption of a flavanoid-rich cocoa (39). In agreement with this, a previous study in humans demonstrated a high bioavailability of the compounds contained in a flavanoid-enriched cocoa

powder (25). On the other hand, it is reported that the healthy properties attributed to cocoa seem to be related to the high amount of monomeric and dimeric compounds (15).

In a study carried out by our group, the flavan-3-ol profile of a flavanoid-enriched cocoa powder obtained at laboratory scale revealed values of (–)-epicatechin and procyanidin B2 8 times higher than those of other conventional cocoa powders (25) and (–)-epicatechin values 11–300 times higher than those reported for different commercial dark chocolates (10). The industrial sample used to assess the antihypertensive effect in this study revealed values of flavan-3-ols, especially (–)-epicatechin, higher than those reported for other cocoa powder products (27). The high amount of this monomer is thought to be important because an increment in plasma (–)-epicatechin is accompanied by a dose-dependent increment in plasma antioxidant capacity (40, 41) and a dose-dependent decrease in plasma lipid oxidation (40). In fact, a recent study found that the effect of the flavanol-rich cocoa on vascular function in humans is mainly mediated by (–)-epicatechin (42). In addition, Flammer et al., in 2007, also observed that the immediate cardiovascular beneficial effects of dark chocolate were paralleled by a significant reduction of serum oxidative

stress and were positively correlated with changes in serum epicatechin concentrations (43). A functional effect of CoccoanOX was therefore expected.

In the present study, the antihypertensive effect of CoccoanOX was in fact assessed. A pronounced blood pressure lowering effect was observed when 300 mg/kg of body weight of CoccoanOX was administered to hypertensive rats. This dose of CoccoanOX had an effect similar to that of 50 mg/kg of Captopril in these animals, and this is important because this drug is known to be a very effective antihypertensive treatment in clinical practice and SHR represent nowadays the best experimental model for essential hypertension in humans (44). Both 300 mg/kg of body weight of CoccoanOX and 50 mg/kg of Captopril caused the maximal decrease of arterial blood pressure 4 h postadministration. Recent studies carried out in our laboratory revealed that (–)-epicatechin caused a decrease of arterial blood pressure in SHR that was also maximal 4 h postadministration (unpublished data). This flavanol may be therefore responsible for the antihypertensive effect of CoccoanOX. In fact, human studies have reported that epicatechin plasma concentrations can approach 1 μmol/L within 2 h after the consumption of flavonoid-rich chocolate (37, 40). In addition, the plasma half-life of (–)-epicatechin is relatively short (<24 h), and the plasma (–)-epicatechin concentration typically returns to baseline values within 6–8 h after the consumption of this cocoa flavonoid. Short- (45–51) and long-term (52–55) experimental studies associated the antihypertensive effect of other different polyphenols with nitric oxide mediated vasodilation (47, 48, 51, 55), angiotensin converting enzyme inhibition (46, 49), and the increase in the antioxidative status (45, 50, 52, 54).

Paradoxically, 600 mg/kg of CoccoanOX demonstrated a lower antihypertensive effect in SHR than lower doses of this cocoa powder. Nevertheless, different studies have demonstrated that a high quantity of polyphenols could exhibit pro-oxidant properties instead of antioxidant properties (56–58).

Although the antihypertensive effect of cocoa polyphenols seems clear, a possible blood pressure lowering effect of theobromine cannot be ruled out. In fact, this methylxanthine seems to be responsible for the decrease in blood pressure reported after the short administration of dark chocolate (59). It is well-known that the theobromine was commonly used to treat hypertension because of its ability to relax smooth muscle tissue and dilate blood vessels. Nevertheless, it is obvious that theobromine content in 600 mg/kg CoccoanOX is twice the content in 300 mg/kg CoccoanOX, and the group of SHR that received 300 mg/kg of CoccoanOX exhibited a higher decrease in blood pressure when compared to the 600 mg/kg CoccoanOX group. This paradox is not at all compatible with an effect mainly caused by theobromine because the blood pressure lowering effect of this methylxanthine is in principle dose dependent. Different data of this study support therefore that the blood pressure lowering effect exhibited by CoccoanOX would be mainly due to the presence of procyanidins as previously described by other researchers (20).

It is also important to point out that the administration of CoccoanOX to normotensive WKY rats did not change the arterial blood pressure of these animals. This indicates that the effect of CoccoanOX is specific to the hypertensive condition.

In conclusion, we have demonstrated the antihypertensive properties of the industrially processed natural flavonoid-enriched cocoa powder named CoccoanOX. The results obtained suggest that this product could be used as a functional food ingredient with potential therapeutic benefit in the prevention and treatment of hypertension. Our results also support that it can be also consumed without any risk in normotensive subjects. At the moment, we are conducting experiments aimed to establish their

long-term effect on the arterial blood pressure in rats and to better clarify the compounds and mechanisms that could be implicated in its antihypertensive activity. It is nevertheless clear that before routine clinical use of CoccoanOX, it would be also necessary to carry out clinical studies to demonstrate its long-term antihypertensive efficiency in humans.

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