EFFECT OF A COCOA POLYPHENOL EXTRACT IN SPONTANEOUSLY HYPERTENSIVE RATS

Quiñones M[1], Miguel M[2*], Muguerza B[3,4], Aleixandre A[1]

[1]Dpto. Farmacología, Fac. Medicina, U. Complutense, Avda. Complutense s/n, 28040 Madrid, Spain. [2]Instituto de Investigación en Ciencias de Alimentación (CIAL, CSIC-UAM) C/ Nicolás Cabrera, 9 28049, Madrid, Spain. [3]Natraceutical Group, Autovía A-3, Salida 343, Camino de Torrent s/n, Quart de Poblet, 46930 Valencia, Spain.

Present Address

[4]Dpto. Bioquímica y Biotecnología, U. Rovira i Virgili, C/ Marcel.li Domingo s/n 43007 Tarragona, Spain.

Running title: Cocoa extract in hypertensive rats

*Dra. Marta Miguel
Instituto de Investigación en Ciencias de Alimentación (CIAL, CSIC-UAM)
C/ Nicolás Cabrera, 9
28049, Madrid, Spain.
Telephone: 34-91-0017931
Fax: 34-91-0017905
E-mail: marta.miguel@csic.es

Abstract

In this study, we evaluated the short-term effect of a cocoa polyphenol extract (CPE), in spontaneously hypertensive rats (SHR). Male 17-22-week-old SHR were administered by intragastric gavage water, 50 mg/kg Captopril or CPE at different doses (13, 26, 80 and 160 mg/kg). The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded by the tail cuff method before the administration and also 2, 4, 6, 8, 24, 48 and 72 hours post-administration. Highly significant decreases in the SBP and in the DBP were observed when captopril or CPE was administered to SHR. The cocoa extract produced a dose dependent effect in the SBP of the SHR up to the dose of 80 mg/kg. Nevertheless this dose of CPE did not decrease the arterial blood pressure in the normotensive Wistar Kyoto rats. The decrease in the SBP caused by 80 mg/kg of CPE in the SHR (-39.1 ± 3.7 mm Hg) was maximum 6 hours post-administration, and the initial values of SBP were recovered 72 hours post-administration of this extract. Paradoxically, 160 mg/kg of the cocoa extract caused less antihypertensive effect than lower doses of CPE. In addition, the decrease in DBP was always more accentuated when the dose of CPE administered was lower. Our results suggest that CPE may be used as a functional food ingredient with beneficial effects for controlling arterial blood pressure.

Key word: Cocoa, Hypertension, Polyphenols, Spontaneously hypertensive rats.

Introduction

Cardiovascular disease is the most important cause of death in the industrial societies.[1-3] The intake of polyphenols has been inversely related with the reduced risk of this disease.[4-6] In fact, many epidemiological studies associate an increased consumption of foods and beverages rich in flavonoids, with a reduced risk of cardiovascular death.[7-9] Moreover, the use of products with a natural origin that may cause scarce side-effects, is an attractive possibility to be considered in treating several pathologies.[10,11]

Cocoa is one of the foods that possess major content in flavanols,[12,13] and the cardiovascular benefits of cocoa and cocoa derivatives has been extensively studied.[14-16] It was reported that cocoa consumption reduced cardiovascular mortality in the indigenous populations of Kuna islands,[17] and more recently cocoa consumption has also been associated with lower cardiovascular mortality.[18-20] Cocoa is rich in flavan-3-ols and procyanidins, being (-)-epicatechin the main component.[21] 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. [22,23]

The presence of these polyphenols is important for the healthy cocoa effects associated to its consumption. Moreover, it has recently been published that this kind of flavanoids are able of inducing a progressive, and sustained reduction in blood pressure when administered chronically to humans or to different rat models of hypertension, including spontaneously hypertensive rats (SHR) and L-NAME-treated rats.[24-26] Nevertheless, it should be have in mind that the cocoa flavanol ingestion will depend on the initial flavanol content of the cocoa beans and the manufacturing process.[27]

In previous works we have demonstrated the antihypertensive properties of a polyphenol rich cocoa powder, named CocoanOX™, after short[28] and long-term treatment,[29] and we have also studied the possible mechanisms implicated in their antihypertensive effect.[30] However, extracts are easier to handle in the food industry than other traditional functional ingredients, due to some of their physicochemical properties, such as their high solubility. Moreover, the organoleptic profile of the final application is usually not modified when extracts are used as food ingredients, and they could be added to many applications. Therefore, the aim of this study is to evaluate the possible short-term antihypertensive effect of a cocoa polyphenol extract (CPE) derived from a polyphenol rich cocoa powder, after single oral administration, in SHR.

Material and Methods

Cocoa polyphenol extract

The extract CPE used for this study was obtained from CocoanOX™ (Natraceutical SLU, Valencia, Spain), a polyphenol-rich cocoa powder produced from unfermented, blanch-treated, non-roasted cocoa beans which preserves polyphenol degradation.[31] The theobromine content of the extract, determined by High-Performance Liquid Chromatography (HPLC), was 90.6 mg/g. Total polyphenols, measured by Folin-Ciocalteu´s method and flavan-3-ols content (monomers and procyanidins B1 and B2), measured by HPLC-DAD according to Cienfuegos-Jovellanos et al.,[28] of CPE were 509.8 and 232.1 mg/g respectively. Table 1 shows these data and also the antioxidant capacity of this extract, expressed as µmol of Trolox equivalents (TE) per gram of CPE, that

was determined by the hydrophilic ORAC (H-ORAC) assay according to Ramos et al.[32] All the analysis has been performed in triplicate and the results are reported on wet basis.

Experimental Procedure in Rats.

In this study we have used twenty 17-20-week-old male SHR, weighing 314±3 g, and ten 17-20-week-old male normotensive Wistar-Kyoto (WKY) rats, weighing 337±6 g. All these animals were obtained from Charles River Laboratories Spain. The animals were maintained at a temperature of 23º C with 12 hour light/dark cycles, and consumed tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. CPE 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 CPE (13, 26, 80 and 160 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 CPE or Captopril. Systolic blood pressure (SBP) an diastolic blood pressure (DBP) were recorded in the rats by the tail cuff method,[33] before administration and 2, 4, 6, 8, 24, 48 and 72 hours post-administration. Before the measurement, the rats were kept at 38ºC for 10 minutes in order to detect the pulsations of the tail artery. To establish the value of SBP and DBP, five measurements were taken, and the average of all of them 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 two weeks before the actual trial time, and during this period the rats were accustomed to the procedure.

All 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 ± standard error of the mean (SEM) for a minimum of 8 rats, and were analyzed by a two-way analysis of variance (ANOVA), using the GraphPad Prism software. In addition, in order 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 was considered to be significant when $P < 0.05$.

Results

Figure 1 shows the changes in SBP and DBP obtained in SHR after the administration of the different assayed compounds. Before administration of the different products, the SHR showed SBP values of 216.9 ± 3.3 mm Hg (n=20) and DBP values of 158.8 ± 3.8 mm Hg (n=20). The values of SBP and DBP obtained after oral administration of bidistilled water were very similar to those obtained before its administration. Captopril caused a clear decrease in SBP and DBP in SHR. The maximum decrease in SBP and DBP caused by 50 mg/Kg of this drug were observed 4 hours post-administration. These variables returned to baseline 48 hours after the administration of Captopril. The oral

administration of CPE also result in a significant decrease of the SBP and the DBP in the SHR. The decrease in SBP caused by the extract was dose-dependent only up to the dose of 80 mg/kg. The change in the SBP caused by this dose of CPE was -39.1 ± 3.7 mm Hg. This decrease in SBP was the maximum decrease in this variable obtained with this extract, and it was observed 6 hours post-administration. Nevertheless, 72 hours post-administration of 80 mg/kg CPE, the values of SBP were very similar to those observed before the administration of this extract. The maximum decrease of SBP caused by 13 mg/kg CPE (-25.9 ±1.9 mm Hg) and the maximum decrease of SBP caused by 26 mg/kg of CPE (-28.6 ±4.5 mm Hg) were reached 4 hours post-administration. Paradoxically, the dose of 160 mg/kg of CPE had the lowest antihypertensive effect (-9.3 ± 2.7 mm Hg). In addition, the decrease in DBP was always more accentuated when the dose of CPE administered was lower. Therefore, 13 mg/kg CPE caused the maximum decrease in the DBP in the rats (-30.6 ±7.7 mm Hg), and this decrease in this variable was observed 4-6 hours post-administration of this dose of CPE.

The administration of CPE did not modify the arterial blood pressure in the normotensive WKY rats (Figure 2). This variable was similar in the WKY rats treated with this product and in the WKY rats administered water.

Discussion

The health benefits of cocoa reported in recent studies have increased the interest to obtain products with high cocoa polyphenol content.[34,35] A human study demonstrated that only 30 mg/day of flavan-3-ols (up to 5-mers) reduced blood pressure in humans.[24] According to previous results of our group an

important content of polyphenols was exhibit in CPE, in particular of low molecular weight procyanidins.[31] In fact, the concentration of total polyphenols and flavan-3-ols of CPE was very high when compared to other cocoa derivatives such as different cocoa powders or chocolates.[36-38] Moreover, the total flavan-3-ols content (monomers and procyanidins B1 and B2) and the (-)-epicatechin content of CPE were 2 and 7 times higher than the respective values previously reported for the original polyphenol rich cocoa powder.[28] The high content of flavan-3-ols, and in particular the high content in (-)-epicatechin, measured in CPE, point out that it should be possible to use a small amount of this extract to decrease arterial blood pressure. It should be noted that the doses of CPE selected for this study had the same (-)-epicatechin content as the doses of a polyphenol rich cocoa powder that had exhibited an antihypertensive effect.[28] Low molecular weight procyanidins are probably important component of CPE, because Cooper et al., in 2008, postulated that the healthy properties attributed to cocoa would be related to the high amount of monomeric and dimeric compounds.[39] In fact, the bioavailability of cocoa polyphenols is strongly related with their molecular size, and, in general, the smaller polyphenols (monomers and dimers) are found in higher concentration in the blood than other polyphenols. Polyphenol monomers and dimers have therefore more possibilities to reach their target organs in the body. A high amount of these compounds of low molecular weight in cocoa derivatives, specially for the monomer (-)-epicatechin, could also be accompanied by a dose-dependent increment in plasma antioxidant capacity[40,41] and by a dose-dependent decrease in plasma lipid oxidation.[40] This monomer is also considered responsible, at least in part, of many of the vascular beneficial

effects associated to cocoa consumption,[18, 20,29,42-44] and their antihypertensive effects after short-term treatment in SHR treated with this compound has been recently proved (unpublished dates).

MAR SEGÚN EL REFEREE HAY ALGUN TRABAJO QUE DESCRIBE EL EFECTO ANTIHIPERTENSIVO DE LA EPICATEQUINA ANTES QUE NOSOTROS ¿¿¿¿??? POR LO VISTO EN LA REF18 UN REVIEW DE CESAR FRAGA, MIRALO PLEASE, SI ES ASI PON ESA CITA AQUI, HABRÍA QUE CAMBIAR LA FRASE PERO ESO YA LO HAGO YO, ME MANDAS EL ABSTRACT PLEASE, Y DESPUÉS PONEMOS LA NUESTRA DE CÓMO QUE TAMBIEN LO HEMOS COMPROBADO CON UNPUBLHISED RESULTS.

Marta, es una revisión con algunos datos experimentales y lo publican en Enero del 2011[20]. Es **parecido al ensayo nuestro del L-NAME, pero utilizan ratas SD que tratan durante 6 semanas con L-NAME. A continuación hacen un tratamiento de 8 semanas con L-NAME y L-NAME+ EPI (0,4g /100g diet). Te mando el paper.**

It is well known that theobromine was commonly used to treat hypertension because of its ability to relax smooth muscle tissue and dilate blood vessels. In fact, this methylxanthine could be responsible for the decrease in blood pressure reported after the short-administration of dark chocolate.[45] In the present study, an antihypertensive effect of CPE was demonstrated, but theobromine is present in this extract only in a low concentration (90.6 mg/g) and could therefore hardly justify its antihypertensive properties. A recent study shows that a theobromine-enriched flavanol-rich cocoa with 979 mg of

theobromine could decrease central systolic blood pressure in healthy individuals. Nevertheless, the flavanol-rich cocoa with a natural dose of theobromine consisting in 106 mg of this methylxanthine did not significantly change it.[46] As in the study carried out with the original polyphenol rich cocoa powder,[28] in the present study, a dose dependent antihypertensive effect of CPE could not be demonstrated. On the contrary, we could observe that the highest dose of this polyphenolic compound (160 mg/kg) was not the most effective one to decrease arterial blood pressure. In this context, different studies have demonstrated that a high quantity of polyphenols could exhibit pro-oxidant properties instead of antioxidant properties.[47-49]

The lowering blood pressure effect exhibited by CPE would be mainly due to the presence of flavan-3-ols. As mentioned before, the doses of both products, the original polyphenol rich cocoa powder and the extract used in the present work, are equivalent in (-)-epicatechin content, but the potential contribution for the antihypertensive effect of other polyphenolic compounds, and the synergy between them, cannot be ruled out. It has been also published that polyphenols are able of inducing a progressive, and sustained reduction in blood pressure when administered chronically to humans or to different rat models of hypertension, including SHR and L-NAME-treated rats.[24-26] It is important to note that hypertension is a chronic pathology that requires chronic treatment, and the use of strategies with long lasting antihypertensive effects is always desirable. In this context, it is important to highlight that the decrease in arterial blood pressure caused by CPE lasted for a longer period of time than the antihypertensive effect previously reported for the original polyphenol rich cocoa powder. According with this idea, the antihypertensive properties of CPE

may be more favourable than the original cocoa powder for controlling high blood pressure levels.

The administration of 80 mg/kg of CPE to normotensive WKY rats did not change the arterial blood pressure of these animals. This indicates that the effect of CPE is specific for the hypertensive condition.

CPE presents an extraordinary antioxidant capacity (12134 μmol TE/g), which can be considered as a very high one when compared with the antioxidant capacity of other cocoa derivates such as milk chocolate, dark chocolate or unsweetened chocolate (74, 219 and 490 μmol TE/g respectively), or when compared with the antioxidant capacity of a natural cocoa powder (820 μmol TE/g), all of them analyzed by the H-ORAC method.[37] Moreover, the antioxidant capacity of CPE was much higher than those reported for other foods different from cocoa, such as cereals, legumes, vegetables, or fruits.[50] This suggests that an antioxidant mechanism could be implicated in the antihypertensive effect observed when CPE is administered. However, we cannot discard that other mechanisms of action are involved in this effect, and more studies are necessary in order to dilucidate the blood pressure lowering mechanisms of CPE.

In this paper, the antihypertensive effect of CPE in SHR has been demonstrated. Our results suggest that this extract could be used as a functional food ingredient with potential therapeutic benefit in the prevention and treatment of hypertension. In particular, CPE could be useful for prehypertensive patients who do not need the prescription of blood pressure-lowering medications so far, and we want also to point out that our results clearly support that it can be consumed by normotensive subjects without

promoting any change in arterial blood pressure. Undoubtedly clear advantages are applicable to CPE, and this extract could be used preferentially to the original polyphenol rich cocoa powder as a food antihypertensive ingredient. However, further studies after long-term treatment are necessary in animals and humans before to use CPE as antihypertensive functional ingredient. In addition, studies to elucidate the mechanisms implicated in the antihypertensive effect of CPE should also be carried out. In fact, we are currently conducting some studies using young SHR, in order to investigate if CPE is useful to prevent the development of hypertension and to clarify the mechanisms implicated in their antihypertensive effect.

288 Acknowledgements

289

290 This study was supported by Natraceutical Group (36/2007 U.C.M. Project). We
291 also thank Manuel Bas Caro, Technician in Pharmacology, for his excellent care
292 of the rats, and Yolanda Castilla for her helpful technical assistance. In addition,
293 Miguel M. holds a Ramon and Cajal work contract.

294 References

295

296 1 American Heart Association: Heart Disease and Stroke Statistics: 2004
297 Update. Dallas, TX, American Heart Assosiation., 2003.
298 2 J. Müller-Nordhorn, S. Binting, S. Roll and S.N. Willich, An update on regional
299 variation in cardiovascular mortality within Europe, *Eur Heart J.,* 2008, **29**,
300 1316-26.
301 3 K. L. Thomas, A. F. Hernandez, D. Dai, P. Heidenreich, G.C. Fonarow, E. D.
302 Peterson and C. W. Yancy, Association of race/ethnicity with clinical risk
303 factors, quality of care, and acute outcomes in patients hospitalized with
304 heart failure, *Am Heart J.*, 2011, **161**, 746-54.
305 4 L. Yochum, L. H. Kushi, K. Meyer and A. R. Folsom, Dietary flavonoid intake
306 and risk of cardiovascular disease in postmenopausal women, *Am J*
307 *Epidemiol.*, 1999, **149**, 943-49.
308 5 M. Galleano, O. Pechanova and C.G. Fraga, Hypertension, nitric oxide,
309 oxidants, and dietary plant polyphenols, *Curr Pharm Biotechnol.*, 2010, **11**,
310 837-48.
311 6 F. Perez-Vizcaino and J. Duarte, Flavonols and cardiovascular disease, *Mol*
312 *Aspects Med.,* 2010, **3,** 478-94.
313 7 Z. Liu, L. P. Ma, B. Zhou, L. Yang and Z. L. Liu, Antioxidative effects of green
314 tea polyphenols on free radical initiated and photosensitized peroxidation of
315 human low density lipoprotein, *Chem Phys Lipids.,* 2000, **106**, 53-63.
316 8 K. J. Joshipura, F. B. Hu, J. E. Manson, M. J. Stampfer, E. B. Rimm, F. E.
317 Speizer, G. Colditz, A. Ascherio, B. Rosner, D. Spiegelman, and W. C.

318 Willett, The effect of fruit and vegetable intake on risk for coronary heart
319 disease, *Ann Intern Med.*, 2001, **134**, 1106-14.
320 9 P. M. Kris-Etherton and C. L. Keen, Evidence that the antioxidant flavonoids
321 in tea and cocoa are beneficial for cardiovascular health, *Curr Opin Lipidol.,*
322 2002,**13,** 41-9.
323 10 M. Miguel, B. Muguerza, E. Sánchez, M. A. Delgado, I. Recio, M. Ramos
324 and M. A. Aleixandre, Changes in arterial blood pressure in hypertensive
325 rats caused by long-term intake of milk fermented by Enterococcus faecalis
326 CECT 5728, *Br J Nutr*., 2005, **94**, 36-43.
327 11 Z. Y. Chen, C. Peng, R. Jiao, Y. M. Wong, N. Yang and Y. Huang, Anti-
328 hypertensive nutraceuticals and functional foods, *J Agric Food Chem.,* 2009,
329 **57**, 4485-99.
330 12 K. W. Lee, Y. J. Kim, H. J. Lee and C. Y. Lee, Cocoa has more phenolic
331 phytochemicals and higher antioxidant capacity than teas and red wines, *J.*
332 *Agric. Food Chem*., 2003, **51**, 7292-95.
333 13 C. Manach, A. Scalbert, C. Morand, C. Rémésy and L. Jiménez,
334 Polyphenols: food sources and bioavailability, *Am J Clin Nutr.*, 2004, **79**,
335 727-47.
336 14 E. L. Ding, S. M. Hutfless, X. Ding and S. Girotra, Chocolate and prevention
337 of cardiovascular disease: a systematic review, *Nutr Metab (Lond).,* 2006, **3**,
338 2.
339 15 K. A. Cooper, J. L. Donovan, A. L. Waterhouse and G. Williamson, Cocoa
340 and health: a decade of research, *Br J Nutr.*, 2008, **99**, 1-11.
341 16 M. Galleano, P. I. Oteiza and C. G. Fraga, Cocoa, chocolate, and
342 cardiovascular disease, *J Cardiovasc Pharmacol.,* 2009, **54**, 483-90.

17 N. K. Hollenberg, G. Martinez, M. McCullough, T. Meinking, D. Passan, M. Preston, A. Rivera, D. Taplin and M. Vicaria-Clement, Aging, acculturation, salt intake, and hypertension in the Kuna of Panama, *Hypertension.*, 1997, **29,** 171-76.

18 B. Buijsse, E. J. Feskens, F. J. Kok and D. Kromhout, Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study, *Arch Intern Med.*, 2006, **166**, 411-17.

19 P.J. Mink, C. G. Scrafford, L. M. Barraj, L. Harnack, C. P. Hong, J. A. Nettleton and D. R. Jacobs, Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women, *Am J Clin Nutr*., 2007, **85**, 895-909.

20 C. G. Fraga, M. C. Litterio, P. D. Prince, V. Calabró, B. Piotrkowski, M. Galleano, Cocoa flavanols: effects on vascular nitric oxide and blood pressure, *J Clin Biochem Nutr.*, 2011, **48**, 63-7.

21 J. Wollgast and E. Anklam, Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification, *Food Res Int.,* 2000, **33**, 423-47.

22 I. C. Arts, P. C. Hollman and D. Kromhout, Chocolate as a source of tea flavonoids, *Lancet.*, 1999, **354**, 488.

23 K. W. Lee, Y. J. Kim, H. J. Lee and C. Y. Lee, Cocoa has more phenolic phytochemicals and higher antioxidant capacity than teas and red wines, *J Agric Food Chem.,* 2003, **51**, 7292-95.

24 D. Taubert, R. Roesen, C. Lehmann, N. Jung and E. Schömig, Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide, *JAMA.,* 2007, **298**,49-60.

25 S. Desch, D. Kobler, J. Schmidt, M. Sonnabend, V. Adams, M. Sareban, I. Eitel, M. Blüher, G. Schuler and H. Thiele, Low vs. higher-dose dark chocolate and blood pressure in cardiovascular high-risk patients, *Am J Hypertens*., 2010, **23**, 694-700.

26 M. O. Kane, N. Etienne-Selloum, S. V. Madeira, M. Sarr, A. Walter, S. Dal-Ros, C. Schott, T. Chataigneau and V. B. Schini-Kerth, Endothelium-derived contracting factors mediate the Ang II-induced endothelial dysfunction in the rat aorta: preventive effect of red wine polyphenols, *Pflugers Arch.*, 2010, **459**, 671-79.

27 J. Wollgast and E. Anklam. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification, *Food Res Int.,* 2000, **33,** 423-47.

28 E. Cienfuegos-Jovellanos, M. Quiñones, B. Muguerza, L. Moulay, M. Miguel and A. Aleixandre, Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans, *J Agric Food Chem.*, 2009, **57**, 6156-62.

29 M. Quiñones, D. Sánchez, B. Muguerza, L. Moulay, S. Laghi, M. Miguel and A. Aleixandre, Long-term intake of a soluble cocoa fiber product attenuates the development of hypertension in spontaneously hypertensive rats, *Food Chem.*, 2010, **122**, 1013-19.

30 M. Quiñones, D. Sánchez, B. Muguerza, M. Miguel and A. Aleixandre, Mechanisms for antihypertensive effect of CocoanOX, a polyphenol-rich cocoa powder, in spontaneously hypertensive rats, *Food Res Int.,* 2011, **44**, 1203-08.

31 I. Andujar, M. C. Recio, R. M. Giner, E. Cienfuegos-Jovellanos, S. Laghi, B. Muguerza and J. L. Ríos, Inhibition of Ulcerative Colitis in Mice after Oral Administration of a Polyphenol-Enriched Cocoa Extract Is Mediated by the Inhibition of STAT1 and STAT3 Phosphorylation in Colon Cells, *J Agric Food Chem.,* 2011, **59**, 6474-83.

32 S. Ramos, L. Moulay, A. B. Granado-Serrano, O. Vilanova, B. Muguerza, L. Goya and L. Bravo, Hypolipidemic effect in cholesterol-fed rats of a soluble fiber-rich product obtained from cocoa husks, *J Agric Food Chem.*, 2008, **56**, 6985-93.

33 R. D. Buñag, Validation in awake rats of a tail-cuff method for measuring systolic pressure, *J Appl Physiol.,* 1973, **34**, 279-82.

34 F. A. Tomás-Barberán, E. Cienfuegos-Jovellanos, A. Marin, B. Muguerza , A. Gil-Izquierdo, B. Cerda, P. Zafrilla, J. Morillas, J. Mulero, A. Ibarra, M. A. Pasamar, D. Ramón and J. C. Espín, A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans, *J Agric Food Chem.*, 2007, **55**, 3926-35.

35 G. Schinella, S. Mosca, E. Cienfuegos-Jovellanos, P. A. Pasamar, B. Muguerza, D. Ramón and J. L. Ríos, Antioxidant properties of polyphenol-rich cocoa products industrially processed, *Food Res Int.*, 2010, **43**, 1614-23.

414 36 J. A. Vinson, J. Proch and L. Zubik , Phenol antioxidant quantity and quality
415 in foods: cocoa, dark chocolate, and milk chocolate, *J Agric Food Chem.,*
416 1999, **47**,4821-24.
417 37 L. Gu, S. E. House, X. Wu, B. Ou and R. L. Prior, Procyanidin and Catechin
418 Contents and Antioxidant Capacity of Cocoa and Chocolate Products, *J*
419 *Agric Food Chem.,* 2006, **54**, 4057-61.
420 38 K. B. Miller, D. A. Stuart, N. L. Smith, C. Y. Lee , N. L. McHale, J. A.
421 Flanagan, B. Ou and W. J. Hurst, Antioxidant activity and polyphenol and
422 procyanidin contents of selected commercially available cocoa-containing
423 and chocolate products in the United States, *J Agric Food Chem.*, 2006, **54**,
424 4062-68.
425 39 K. A. Cooper, E. Campos-Giménez, D. Jiménez-Alvarez, A. Rytz, K. Nagy
426 and G. Williamson, Predictive relationship between polyphenol and nonfat
427 cocoa solids content of chocolate, *J Agric Food Chem.,* 2008, **56**, 260-65.
428 40 D. Rein, S. Lotito, R. R. Holt, C. L. Keen, H. H. Schmitz and C. G. Fraga.
429 Epicatechin in human plasma: in vivo determination and effect of chocolate
430 consumption on plasma oxidation status, *J Nutr.,* 2000, **130**, 2109S-14S.
431 41 M. Serafini, R. Bugianesi, G. Maiani, S. Valtuena, S. De Santis and A.
432 Crozier , Plasma antioxidants from chocolate, *Nature.*, 2003, **424**,1013.
433 42 H. Schroeter , C. Heiss, J. Balzer, P. Kleinbongard, C. L. Keen, N. K.
434 Hollenberg, H. Sies, C. Kwik-Uribe, H. H. Schmitz and Kelm M, (-)-
435 Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular
436 function in humans, *Proc Natl Acad Sci U S A.,* 2006, **103**,1024-29.
437 43 M. Quiñones, B. Muguerza, M. Miguel and A. Aleixandre, Evidence that nitric
438 oxide mediates the blood pressure lowering effect of a polyphenol-rich

cocoa powder in spontaneously hypertensive rats, *Pharmacol Res.,* 2011, **64**, 478-81.

44 I. Ramirez-Sanchez, L. Maya, G. Ceballos and F. Villarreal, (-)-epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways, *Hypertension.,* 2010, **55**,1398-1405.

45 C. J. Kelly, Effects of theobromine should be considered in future studies, *Am J Clin Nutr.*, 2005, **82**, 486-87.

46 B. van den Bogaard, R. Draijer, B. E. Westerhof, A.H. van den Meiracker, G.A. van Montfrans, and B-J. H. van den Bor, Effects on peripheral and central blood pressure of cocoa with natural or high-dose theobromine, *Hypertension.,* 2010, **56**, 839-846.

47 N. Cotelle, Role of flavonoids in oxidative stress, *Curr Top Med Chem.,* 2001, **1**, 569-90.

48 S. Azam, N. Hadi, N. U. Khan and S. M. Hadi, Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties, *Toxicol In Vitro.,* 2004, **18**, 555-61.

49 M. Lahouel, S. Amedah, A. Zellagui, A. Touil, S. Rhouati, F. Benyache, E. Leghouchi and H. Bousseboua, The interaction of new plant flavonoids with rat liver mitochondria: relation between the anti- and pro-oxydant effect and flavonoids concentration, *Therapie*., 2006, **61**, 347-55.

50 G. E. Adamson, S. A. Lazarus, A. E. Mitchell, R. L. Prior, G. Cao, P. H. Jacobs, B. G. Kremers, J. F. Hammerstone, R. B. Rucker , K. A. Ritter and H. H. Schmitz, HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity, *J Agric Food Chem.,* 1999, **47**, 4184-88.

464 **Figure legends**

465

466 **Figure 1**. Decrease in systolic blood pressure (SBP) (A) and diastolic blood
467 pressure (DBP) (B) caused in spontaneously hypertensive rats after the
468 administration of different products. Water (○), Captopril (50 mg/kg) (□) or
469 different doses of CPE: 13 mg/kg (♦), 26 mg/kg (▲), 80 mg/kg (●) and 160
470 mg/kg (■). Data are expressed as mean ± SEM. The experimental groups
471 always have a minimum of 8 animals. Same letters indicate no statistical
472 differences ($p>0.05$). P estimated by two-way ANOVA.

473

474 **Figure 2.** Decrease in systolic blood pressure (SBP) (A) and diastolic blood
475 pressure (DBP) (B) caused in Wistar-Kyoto rats after the administration of water
476 (○), or 80 mg/kg CPE (●). Data are expressed as mean ± SEM. The
477 experimental groups always have a minimum of 8 animals. No statistical
478 differences were observed.

479

480 **Table 1.** Theobromine, total polyphenol content, Flavan-3-ols and antioxidant
481 capacity of the cocoa polyphenol extract studied.

Compounds	mg/g wet matter*
Theobromine	90.6 ± 0.2
Total polyphenol content [1]	509.8 ± 4.0
Epicatechin	133.5± 1.1
Catechin	10.8 ± 1.0
Procyanidin B1	9.1 ± 0.2
Procyanidin B2	78.7 ± 1.2
Antioxidant capacity [2]	12134 ± 379

482

483 * The values are expressed as the mean ± SD (n=3)

484 [1] Measured by Folin-Ciocalteu´s method

485 [2] Hydrophilic ORAC (H-ORAC) assay, expressed as µmol of Trolox equivalents
486 (TE) per gram of CPE

487

488

Figure 1

A)

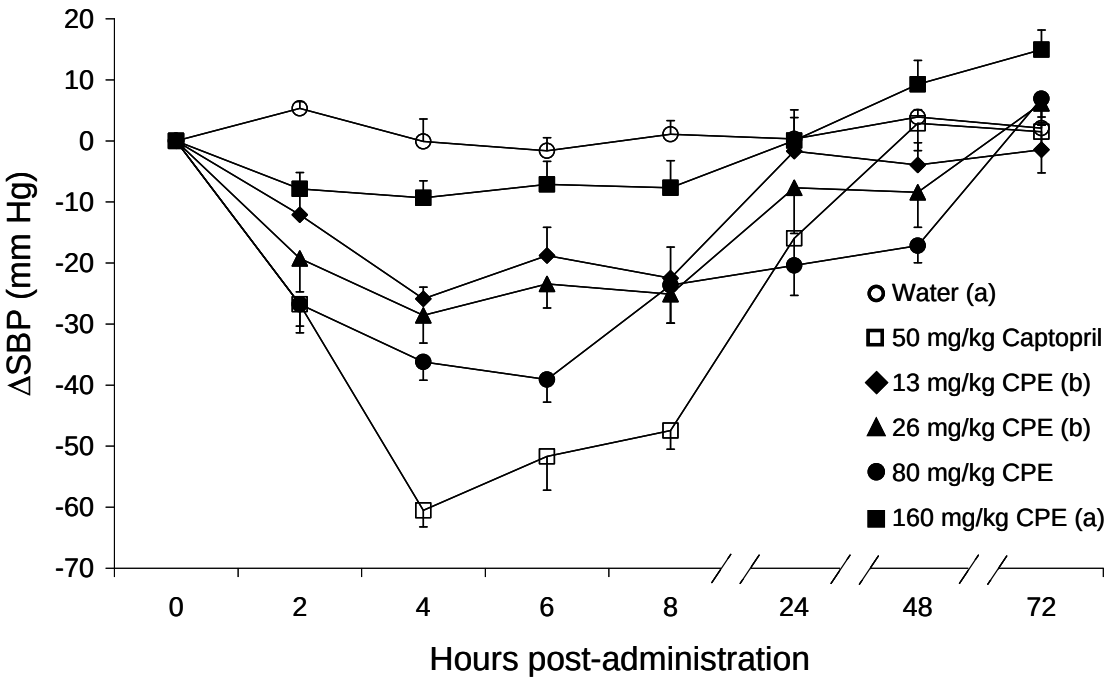

20
10
0
-10
-20
-30
-40
-50
-60
-70
ΔSBP (mm Hg)
0
2
4
6
8
24
48
72
Hours post-administration
Water (a)
50 mg/kg Captopril
13 mg/kg CPE (b)
26 mg/kg CPE (b)
80 mg/kg CPE
160 mg/kg CPE (a)

Figure 1

B)

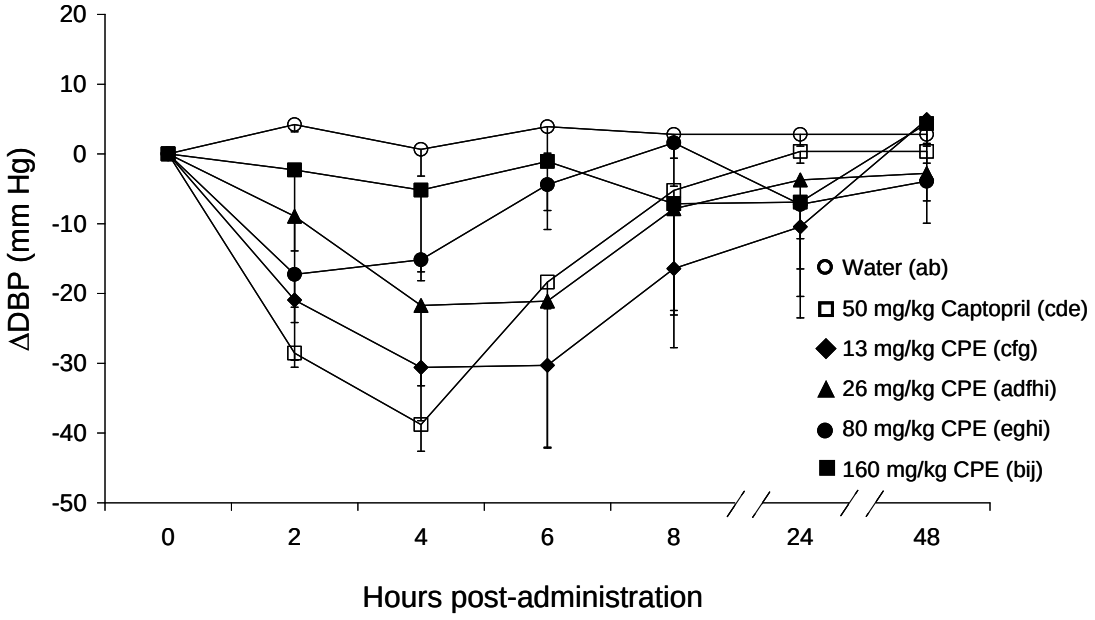
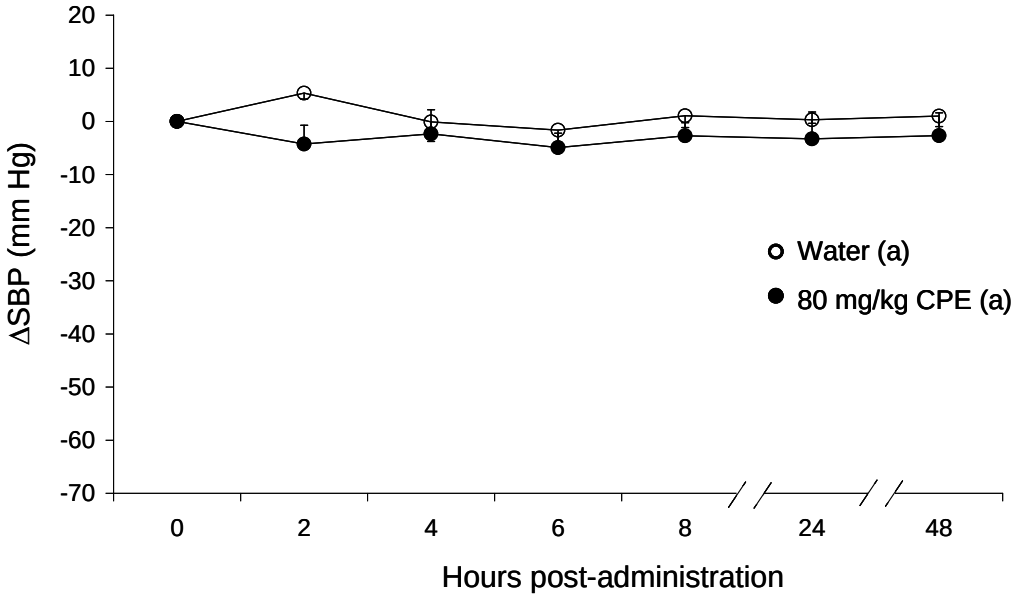

20
10
0
-10
-20
-30
-40
-50
ΔDBP (mm Hg)
0
2
4
6
8
24
48
Hours post-administration
Water (ab)
50 mg/kg Captopril (cde)
13 mg/kg CPE (cfg)
26 mg/kg CPE (adfhi)
80 mg/kg CPE (eghi)
160 mg/kg CPE (bij)

Figure 2

A)


20
10
0
-10
-20
-30
-40
-50
-60
-70
ΔSBP (mm Hg)
0
2
4
6
8
24
48
Hours post-administration
Water (a)
80 mg/kg CPE (a)

564 **Figure 2**

565 **B)**

566

567

568