

# Regulation of Cellular Redox Homeostasis by (–)-Epicatechin and Cocoa Extracts—A Pilot Study <sup>†</sup>

Vanja Todorovic <sup>1,\*</sup>, Monika Baranowska <sup>2</sup>, Sladjana Sobajic <sup>1</sup> and Agnieszka Bartoszek <sup>2</sup>

<sup>1</sup> Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia; sobajic04@yahoo.com

<sup>2</sup> Faculty of Chemistry, University of Gdansk, Narutowicza 11/12, 80-233 Gdansk, Poland; bbmmonik@gmail.com (M.B.); agnieszka.bartoszek@pg.edu.pl (A.B.)

\* Correspondence: vanja.todorovic@hotmail.com; Tel.: +381-638-455-591

<sup>†</sup> Presented at Natural Products and the Hallmarks of Chronic Diseases—COST Action 16112, Luxembourg, 25–27 March 2019.

Published: 16 April 2019

**Abstract:** Cocoa polyphenols play an important role in protection against diseases in which oxidative stress is implicated as a causal or contributing factor. The main aim of this study was to elucidate the influence of different cocoa extracts and main cocoa bioactive compound (–)-epicatechin on cell growth and support of antioxidant cellular barrier in colon adenocarcinoma cell line model (HT29). Results show that the tested cocoa bioactivity results from concerted interactions between epicatechin and other components. Hence, cocoa is a very good example which supports the food synergy concept that is attracting growing interest.

**Keywords:** cellular antioxidant activity; cocoa extract; (–)-epicatechin; food synergy

## 1. Introduction

Living in an oxygenated environment has forced the evolution of effective cellular strategies to take advantage of endogenous sources of reactive oxygen species (ROS), which are generated as a consequence of metabolic activities. ROS regulate several signalling pathways affecting a variety of cellular processes. However, many environmental stimuli generate high levels of ROS that can perturb the normal redox balance and increase the risk of chronic diseases. Therefore, the enhancement of an endogenous antioxidant defence system through dietary supplementation with antioxidants seems to present a reasonable approach to reduce undesirable oxidative stress. However, epidemiological studies suggest that regular consumption of products rich in antioxidants is more effective in the decreasing risk of chronic diseases than isolated antioxidants [1]. The reasonable explanation to these observations may be the food synergy concept, which is defined as additive influence of different food ingredients on modulation of biological processes being involved in redox homeostasis. To fully understand this concept, it is necessary to investigate the influence of both isolated antioxidants and whole food products on the redox biology of cells.

A product characterized by very high content of antioxidants is cocoa powder, thus it seems to be an important contributor to the total dietary intake of antioxidants. Cocoa has been shown to protect against diseases in which oxidative stress is implicated as a contributing factor [2]. Over 10 % of the weight of cocoa powder consists flavan-3-ols, that makes it one of the most concentrated source of (–)-epicatechin, (+)-catechin, and procyanidins. Among all bioactive compounds of cocoa, (–)-epicatechin has been pointed out as the most active ingredient [3]. It has been revealed, however, that cocoa extract exhibits stronger redox-related activities in cell culture than the equivalent concentration of the main cocoa antioxidant, (–)-epicatechin. Cocoa extract appears to be more

efficient in modulating redox dependent processes, such as anti-inflammatory and neuroprotective processes, in comparison to equivalent concentrations of (-)-epicatechin [4].

Once the synergy of cocoa bioactives was revealed, the need of further investigation of its impact on regulation of cellular redox status arose. The objective of this study was to elucidate the impact of main cocoa antioxidant, namely (-)-epicatechin, and cocoa extract on cell growth and support of cellular antioxidant barrier in colon adenocarcinoma cell line (HT29) as a cellular model of alimentary tract.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

In MTT test, thiazolyl blue tetrazolium bromide (MTT) from Sigma-Aldrich (St. Louis, MO, USA) was applied. The OxiSelect™ Cellular Antioxidant Assay Kit was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). All reagents for cell culture were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified with a QPLUS185 system from Millipore (Billerica, MA, USA).

### 2.2. Preparation of Cocoa Extract

This investigation was conducted on two natural cocoa powders (CE1—product code PNGH11; CE2—PARN12) obtained from leading Serbian chocolate manufactures and one commercial standardized cocoa extract purchased from Oryza oil & fat chemical co., ltd, Japan.

To obtain cocoa extract, 100 mg of cocoa powder was suspended in 1 mL of 70% ethanol (*v/v*). The suspension was vortexed for 1 min and centrifuged (13,000 rpm, 5 min, 25 °C). The supernatants were used in MTT and CAA tests.

### 2.3. Cell Culture

HT29 cell line was purchased from the ATCC culture collection (Manassas, VA, USA). The cell culture was maintained in McCoy's medium supplemented with L-glutamine (2 mol/L), sodium pyruvate (200 g/L), fetal bovine serum (100 mL/L) and antibiotics (100 U/mL penicillin, 100 g/L streptomycin). Cells were maintained at 37 °C under humidified atmosphere with 5% CO<sub>2</sub> in the Smart cell incubator (Heal Force, Shanghai, China).

### 2.4. MTT Test

To determine the impact on growth of HT29 cells MTT test was used as described previously [5]. Briefly, exponentially growing cells were seeded in 96-well plates (5 \* 10<sup>3</sup> cells/0.18 mL of medium) and allowed to settle for 24 h at 37 °C. Then the cells were treated for 24 h with 0.02 mL of different concentrations of cocoa extracts. 70% ethanol extract was diluted to 30% ethanol extract. Final concentrations of (+)-catechin and (-)-epicatechin in cell culture ranged from 10 nM to 50 μM. Control cells were treated with the corresponding solvent only.

Following incubation, the medium was removed from the wells and replaced with 0.2 mL of fresh medium. The cells were incubated at 37 °C until 24 h. After this time, 0.05 mL solution of MTT (4 g/L) was added to well and. The plate was left for another 4 h at 37 °C. Then, medium was removed from wells and 0.05 mL of DMSO per well was added. The absorption of solutions was determined at 540 nm with TECAN Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). Treatments were performed in four technical replications. Three independent experiments for each treatment were performed. The impact on cell growth was expressed as growth inhibition of cells exposed on tested antioxidants compared to control cells treated with solvent only whose growth was regarded as 100%.

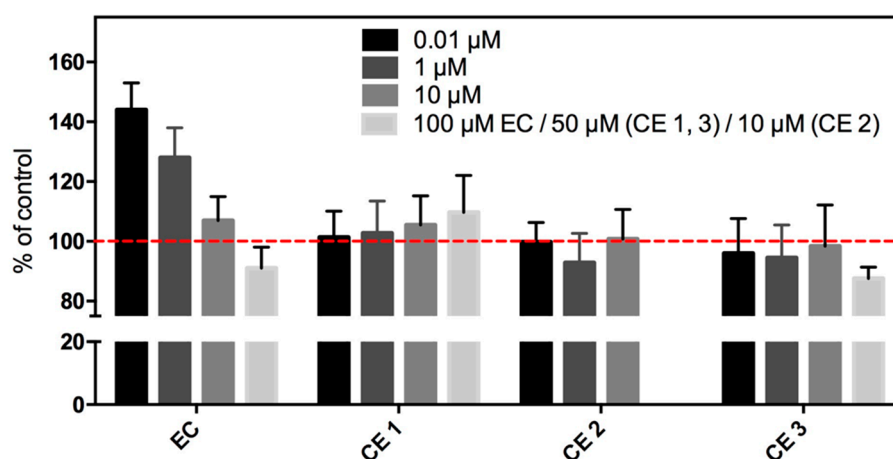
### 2.5. CAA Test

The cellular antioxidant activity of EC and extracts in HT29 cells was studied using a CAA assay. The cells were seeded in black 96-well tissue culture plates with a transparent base (3 × 10<sup>4</sup>

cells/0.2 mL of medium). The cells were allowed to settle for 24 h at 37 °C, then were treated with 0.05 mL of different concentrations of samples for 1 h. Final concentrations of investigated samples ranged from 1  $\mu$ M to 90  $\mu$ M of (+)-catechin and (–)-epicatechin. Subsequent steps were performed strictly according to the manufacturer's procedure available from the website: <https://www.cellbiolabs.com>. Treatments were performed in four technical replications. Emission of fluorescence at 538 nm in cell cultures was measured every 5 min for 1 h after excitation at 485 nm using TECAN Infinite M200 plate reader (Männedorf, Switzerland). Three independent experiments for each treatment were performed. The calculation of cellular antioxidant activity expressed as CAA value has been described previously [5]

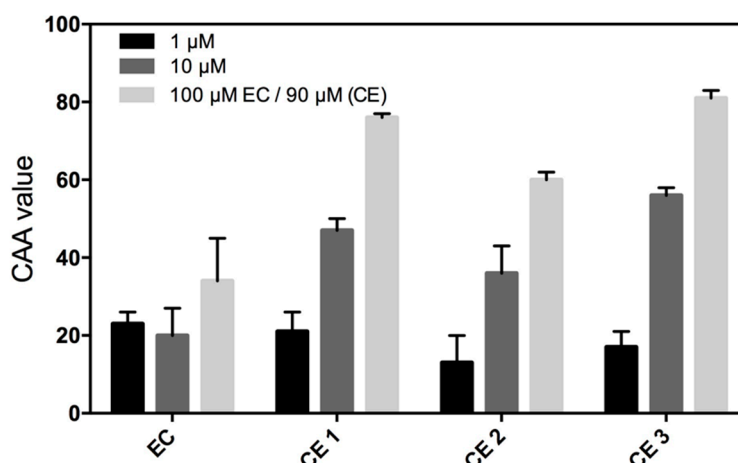
### 3. Results

This study examined the impact of cocoa extracts (CE 1, CE 2 and CE 3) and main cocoa bioactive compound, that is (–)-epicatechin (EC), on regulation of redox status of cell. In the initial stage, the impact on HT29 cell growth was assessed (Figure 1). Our earlier study showed that physiological concentrations of (–)-epicatechin (0.01–1  $\mu$ M) caused significant growth stimulation of HT29 cells, while concentration of 10  $\mu$ M maintained cell growth at the control level. The higher doses of main antioxidant of cocoa inhibited cell growth [5]. In the case of cocoa extracts, equivalents of physiological EC concentrations (0.01–1  $\mu$ M) did not cause cell growth stimulation. It turned out, that cocoa extracts maintained cell growth at control level at the broad range of investigated concentrations, both physiological and those relevant only for intestinal epithelium (Figure 1).



**Figure 1.** Biological activity of cocoa extracts and equivalent concentrations of (–)-epicatechin in terms of cytotoxicity evaluated in HT29.

In cellular antioxidant activity (CAA) test, cocoa extracts exhibited similar as EC antioxidant protection at low physiological concentrations up to 10  $\mu$ M. Interestingly, cocoa extracts were the most efficient in cell protection against ROS, in particular at the highest concentration (100  $\mu$ M) (Figure 2).



**Figure 2.** Biological activity of cocoa extracts and equivalent concentrations of (–)-epicatechin in terms of cellular antioxidant activity evaluated in HT29.

#### 4. Discussion

The objective of this study was to elucidate whether the impact on redox homeostasis observed for pure (–)-epicatechin, the main antioxidant and attributed active component of cocoa, will be comparable to the biological activity of cocoa extracts containing equivalent concentration of this compound. It appeared that the natural mixture of bioactive compounds, even if containing equivalent concentration of EC, exhibited different impact on cancer cell growth, than pure compound. In comparison to isolated EC, cocoa extracts did not stimulate cell growth at physiological concentrations. Because experiments were performed in colon adenocarcinoma cell line, this observation can have a crucial meaning for anticarcinogenic efficacy of catechin based prevention. Interestingly, at physiological concentrations, the support of cellular antioxidant barrier is similar for both the pure compound and cellular extracts. Higher doses of natural mixtures of bioactive compounds offered much better protection against oxidants than the pure compound. It seems that fraction of oligomeric catechins may play an important role in cellular antioxidant activity at concentrations relevant for intestine epithelia.

From the results of this study, it can be concluded that cocoa bioactivity in term of chemoprevention is a result of interactions between various components. Therefore, impact of (–)-epicatechin on cellular antioxidant activity is enhanced by other cocoa compounds and cocoa as a full matrix represent a good candidate, in aiding the understanding of the food synergy concept.

**Author Contributions:** Conceptualization, A.B. and S.S.; Methodology, M.B.; Investigation, M.B. and V.T.; Writing—Original draft preparation, M.B. and V.T.; Writing—Review and editing, A.B. and S.S.

**Funding:** This work is based upon work from COST Action NutRedOx-CA16112 supported by COST (European Cooperation in Science and Technology) and National Science Centre (Poland) in a programme MAESTRO 6 (application number 2014/14/A/ST4/00640).

**Conflicts of Interest:** The authors declare no conflict of interest.

#### References

1. Liu, H.R. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *J. Nutr.* **2004**, *134*, 3479S–3485S, doi:10.1093/jn/134.12.3479S.
2. Katz, D.L.; Doughty, K.; Ali, A. Cocoa and chocolate in human health and disease. *Antioxid. Redox Signal* **2011**, *15*, 2799–2811, doi:10.1089/ars.2010.3697.
3. Panneerselvam, M.; Tsutsumi, Y.M.; Bonds, J.A.; Horikawa, Y.T.; Saldana, M.; Dalton, N.D.; Head, B.P.; Patel, P.M.; Roth, D.M.; Patel, H.H. Dark chocolate receptors: Epicatechin-induced cardiac protection is dependent on delta-opioid receptor stimulation. *Am. J. Physiol. Heart Circ. Physiol.* **2010**, *299*, H1604–H1609, doi:10.1152/ajpheart.00073.2010.

4. Koss-Mikołajczyk, I.; Baranowska, M.; Todorovic, V.; Albin, A.; Sansone, C.; Andreatti, P.; Cherkaoui-Malki, M.; Lizard, G.; Sobajic, S.; Bartoszek, A. Prophylaxis of non-communicable diseases: Why fruits and vegetables may be better chemopreventive agents than dietary supplements based on isolated phytochemicals? *Curr. Pharm. Des.* just accepted.
5. Baranowska, M.; Suliborska, K.; Chrzanowski, W.; Kusznierevicz, B.; Namieśnik, J.; Bartoszek, A. The relationship between standard reduction potentials of catechins and biological activities involved in redox control. *Redox Biol.* **2018**, *17*, 355–366, doi:10.1016/j.redox.2018.05.005.



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).