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Original article

Interactions between bioactive components determine antioxidant, cytotoxic and nutrigenomic activity of cocoa powder extract

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ABSTRACT

Numerous studies have shown, rather disappointingly, that isolated bioactive phytochemicals are not as biologically effective as natural plant products. Such a discrepancy may be explained by the concept of food synergy, which was verified in this research for cocoa extract *versus* its major components with regard to cancer chemoprevention. The evaluation embraced the relationship between redox properties evaluated in cell-free systems with the aid of free radicals scavenging method and differential pulse voltammetry, and redox associated anticarcinogenic activities (cellular antioxidant activity, cytotoxicity, nutrigenomic activity) in human colon adenocarcinoma cell line exposed to either cocoa powder extract or artificial mixtures of cocoa bioactives at matching concentrations. In contrast to expectations, our results showed that the stepwise enrichment with antioxidants caused no gradual increase in the antioxidant activity of the model mixtures; also, these model mixtures did not reach the reducing potential of cocoa in the cell-free systems or cellular model employed. Further, the biological activities examined in colon adenocarcinoma cells did not alter in a stepwise manner that could reflect the gradual changes in composition of bioactive ingredients. In conclusion, the experiments presented here showed that the growing complexity of a mixture of phytochemicals seems to create a new redox bioactive substance rather than enrich the mixture with new activities, characteristic of the compound added. It follows that no simple, predictable relationship can be expected between the chemopreventive potential and the composition of real food items containing a complicated set of non-toxic redox active ingredients. Our observations suggest that the interactions between different bioactive compounds and food matrix components are cooperating factors determining the final bioactivity of foods.

1. Introduction

Cancer chemoprevention is considered a promising approach to cancer management, especially when it takes advantage of dietary sources of plant-based bioactive compounds to stop or at least to slow down carcinogenic processes [1–3]. Among phytochemicals, catechins have been particularly intensively studied over the past two decades in a variety of cancer models, and the results seem very encouraging [4–6]. The highest content of catechins among all foods on a dry weight basis is found in the cocoa powder contained in numerous frequently consumed products, *e.g.* chocolate. A combination of monomers and oligomers of catechins in conjunction with other bioactive compounds (anthocyanins, alkaloids, biogenic amines) makes cocoa a unique foodstuff appreciated by consumers of all generations for its attractive taste and aroma [7–9]. Recent MEAL (Mediterranean healthy Eating, Aging and Lifestyle) cohort study has pointed to chocolate as the main dietary source of catechins [10], which indicates that cocoa products may be regarded as an important contributor to the total dietary intake of these compounds. Although many mechanisms have been proposed to account for the beneficial effects of catechins, their antioxidant properties are typically cited as the most important factor [11–13]. These compounds have been demonstrated to effectively prevent the formation or to scavenge excessive amounts of reactive oxygen species (ROS), which might otherwise inflict severe cellular damage and promote cancerous growth [14]. Aside from neutralizing ROS, catechins can also affect the endogenous antioxidant defence systems of cells by modulating the activity of the secondary messengers and signal transduction pathways that control the expression of redox related genes

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Abbreviations

ALB	albumin
AOE	specific antioxidant energy
AOP	antioxidant power
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
C	(+)-catechin
CA	caffeine
CAA	cellular antioxidant activity
CCL5	chemokine (C–C motif) ligand 5
CE	cocoa powder extract
CYBB	cytochrome *b*-245
CYGB	cytoglobin
DHCR24	24-dehydrocholesterol reductase
DPPH test	test employing 1-diphenyl-2-picrylhydrazyl radicals
DPV	differential pulse voltammetry
E^0	standard reduction potential
EC	(−)-epicatechin
$E_{p,a}$	oxidation (anodic) peak potential *vs.* reference electrode
$E_{p,b}$	peak potential *vs.* standard hydrogen electrode
EPX	eosinophil peroxidase
EPXH2	epoxide hydrolase 2, cytoplasmic
GC	(+)-gallocatechin
GPX3	glutathione peroxidase 3
GPX7	glutathione peroxidase 7
GSR	glutathione reductase
GTF2I	general transcription factor IIi
HMOX1	heme oxygenase (decycling) 1
HSPA1A	heat shock 70 kDa protein 1A
M1-M8	model mixtures 1-8
MPO	myeloperoxidase
MT3	metallothionein 3
NUDT1	nudix type motif 1
PR	protocatechuic acid
PROB1	procyanidin B1
PTGS1	prostaglandin-endoperoxide synthase 1
q	charge density of the process
Q	quercetin
ROS	reactive oxygen species
RPLP0	ribosomal protein lateral stalk subunit P0
SEPP1	selenoprotein P, plasma, 1
SIRT2	sirtuin 2
STK25	serine/threonine kinase 25
TE	theobromine
TXNRD1	thioredoxin reductase 1
TXNRD2	thioredoxin reductase 2

[15]. Cancer cells constantly overproduce ROS, which are the key molecules for the activation of signalling pathways that stimulate proliferation. Thus, it can be expected that insufficient amount of ROS, resulting both from the antioxidant activity of catechins *per se* and from the reducing capacity of the endogenous antioxidant defence systems enhanced by these phytochemicals, may impair cancer cell growth.

The potential health benefits related to the redox properties of isolated catechins seem well documented; however, their contribution to the overall chemopreventive effects of real foods containing them has yet to be fully revealed. Our recent study suggests that other components of the food matrix strongly influence the overall bioactivity of polyphenols. The cellular response differs dramatically, even if cells are treated with the same concentration of a given phytochemical, isolated *vs.* found in real plant extract [16]. Also in the case of isolated bioactive components of cocoa, there is a substantial load of data (summarised in Ref. [15]) documenting that they are not as biologically effective as whole cocoa powder. Such discrepancies had already been noted, leading to the development of the concept of food synergy, which is defined as additive or more than additive influence of the combination of different food ingredients on human health [15].

Our research on the relationship between the antioxidant and chemopreventive activity of catechins [17] and the observed synergy of cocoa components [15] called for further investigation into the impact of individual cocoa bioactives on the modulation of the cellular response associated with redox status. Moreover, in our previous study [17], we successfully applied the electrochemical parameter, *i.e.* standard reduction potential (E^0), as the predictor of the bioactivity of isolated catechins in the cellular model. In the case of mixtures which mirror the complex food matrix ingested everyday by humans, it still remains unclear whether the redox properties described by chemical and electrochemical parameters may also be used to predict the impact of such mixtures of antioxidants on the redox biology of cells, as it has been demonstrated for pure compounds.

To resolve this question, the present study aimed to elucidate the relationship between the redox properties and the biological effects for model mixtures with growing complexity of bioactive compounds reflecting the composition of cocoa powder extract (CE). The additional objective was to find out to what extent the impact on cellular redox homeostasis observed for these model mixture(s) of pure compounds reflects the impact of cocoa extract, which represents a real food sample. The first step involved the quantitative and qualitative determination of phenolic composition in cocoa extract and was performed using HPLC-DAD-MS. The profiling of antioxidants in cocoa extract was performed by post-column derivatization with ABTS reagent. Based on these results, mixtures of the most abundant bioactive cocoa compounds were prepared to reveal potential interactions between individual components. The antioxidant properties of these mixtures and of the cocoa extract were initially determined by DPPH test, which in previous studies had exhibited the strongest correlation with electrochemical methods [17]. The electrochemical properties of the mixtures and the cocoa powder extract were then determined by differential pulse voltammetry (DPV). The biological experiments were carried out using the colon adenocarcinoma HT29 cell line recommended for nutritional studies by the National Centre for the Replacement, Refinement & Reduction of Animals in Research [18,19] as a model of the intestinal epithelium that may be exposed to high concentrations of antioxidants matching the ones in cocoa. The biological tests examined the impact of the studied samples on cell growth (MTT test), cellular antioxidant activity (CAA assay), and the expression of 84 redox-related genes (real-time PCR array-based technologies).

2. Materials and methods

2.1. Chemicals and reagents

The following standards were used for the study: (+)-catechin (C), (−)-epicatechin (EC), and (+)-gallocatechin (GC) from Extrasynthese (France) and proanthocyanidin B1 (PROB1), quercetin (Q), protocatechuic acid (PR), caffeine (CA), and theobromine (TE) from Sigma-Aldrich (USA). All standards used were characterized by the highest purity it was possible to obtain among the manufacturers. For the cocoa extract preparation and HPLC analysis, HPLC grade ethanol, formic acid, acetonitryle from Merck (Germany) were used. Water was purified with a QPLUS185 system from Millipore (USA). For spectrophotometric testing, 1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich (USA) and analytical grade methanol (POCH, Poland) were applied. In MTT tests, thiazolyl blue tetrazolium bromide (MTT) from Sigma-Aldrich (USA) was used. The OxiSelect™ Cellular Antioxidant Assay Kit was purchased from Cell Biolabs, Inc. (USA). QIAshredder, RNeasy Mini Kit, RNase-Free DNase set, RT^2 First Strand Kit, RT^2 SybrGreen qPCR

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Mastermix, and RT^2 Profiler PCR Arrays for Oxidative Stress (PAHS 0065) from Qiagen (Germany) were applied in the genomic studies. All reagents for the cell culture, such as PBS, McCoy's 5A medium, trypsin, L-glutamine, foetal bovine serum, and antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich (USA). For the electrochemical studies, sodium phosphate buffer (1 M) prepared by dissolving $Na_2HPO_4{\cdot}12H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ (Sigma Aldrich, USA) in deionized water was applied. To clean the working electrode and the electrochemical cell, a solution of 10 mM potassium permanganate (Sigma Aldrich, USA) in 95% H_2SO_4 (v/v) (POCH, Poland) was used. The reference electrode was stored in 3 M KCl (Sigma Aldrich, USA) dissolved in deionized water.

2.2. Preparation of cocoa powder extract and model mixtures

Cocoa powder was obtained from De Zaan company, the Netherlands (product code: N11 N). To obtain cocoa powder extract (CE), 100 mg or 200 mg (in the case of electrochemical determinations) of cocoa powder was suspended in 1 mL of 70% ethanol (v/v). The suspension was mixed vigorously for 1 min and centrifuged (13 000 rpm, 5 min, 25 °C). The collected supernatants were used in further tests.

The set of mixtures M1-M8, as presented in Table 1 (concentrations of compounds are given in Table 2), was prepared to evaluate the interactions between individual components, in search for any potential synergisms or antagonisms. Mixture M8 was assembled so as to reflect the composition of all main bioactive compounds detected in CE.

2.3. HPLC determination of bioactive compounds in cocoa powder extract

The quantitative and qualitative determination of phenolic compounds in cocoa powder extract was performed using the Agilent 1200 Series HPLC-DAD-MS (high-performance liquid chromatography with photodiode array and mass spectrometer detectors) system (Agilent Technologies, USA) as described earlier [20]. Chromatographic separation was carried out using a Kinetex PFP 100A column (4.6 × 150 mm, 5 μm). The separation of phytochemicals was performed at a flow rate of 0.8 mL/min using a mobile phase composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The injection volume of the extract was 10 μL. The linear gradient for solvent B was as follows: 0 min, 5%; 20 min, 50%; 25 min, 100%; followed by 5 min of column equilibration with 5% B. Absorbance spectra were recorded between 190 and 700 nm every 2 s with a bandwidth of 4 nm, while the chromatograms were monitored at 270 for catechins and methylxanthines, at 325 nm for phenolic acids, and at 360 nm for flavonol derivatives. The mass spectrometer (quadruple analyser) was equipped with an electrospray ionization interface (ESI, Agilent). MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 L/min; nebuliser pressure, 35 psig. The instrument was operated both in negative and positive mode, scanning from m/z 100–1000. Individual phenolic compounds were identified by comparing their retention times with those for standards, or on the basis of available literature data and mass spectra. The quantification of the analytes for which standards were available was performed with external calibration curves generated by integrating the area of absorption peaks, whereas for the analytes for which standards were lacking – by reporting the measured chromatographic area in the calibration equation of the reference standards: (+)-gallocatechin was quantified as (−)-epigallocatechin at 280 nm; quercetin derivatives were quantified as quercetin at 260 and 360 nm; procyanidin A, B, and C isomers were quantified as procyanidin A1, B2, and C1, respectively, at 280 nm.

2.4. Profiling of antioxidants by post-column derivatization

The profiling of antioxidants in cocoa powder extracts was performed by post-column derivatization with ABTS reagent as described earlier, with slight modifications [21,22]. The Pinnacle PCX derivatization instrument (Pickering Laboratories Inc., Mountain View, CA, USA) was used for the post-column addition of the ABTS reagent to HPLC eluate. The derivatization reagent was prepared by diluting ABTS stock solution (7 mM) with methanol in the ratio 3:7 (v/v). Derivatization was carried out at 130 °C with the flow rate of derivatization reagent set as 0.1 mL/min. The 0.5 mL (PTFE, 0.25 mm, 10 m) coil was used. The HPLC separation conditions were the same as described in section 2.3. The chromatograms obtained after derivatization were registered at 734 nm using a multiple-wavelength detector (Agilent 1200 series, MWD, USA).

2.5. Antioxidant activity by DPPH test

The determination of antioxidant activity of cocoa powder extract and mixtures M1-M8 was carried out by spectrophotometric assay employing DPPH radical as described previously [17,23]. Briefly, stock solution of DPPH radicals was diluted in methanol before measurements until absorbance amounted to 0.9 ± 0.05 at 515 nm. All reactions were carried out in 48-well plates at 37 °C. Mixtures M1-M8 and CE were diluted appropriately with 70% ethanol to achieve concentrations falling within the linear range of the assay. The DPPH solution (1 mL) was mixed with the dilutions of CE or the mixtures (30 μL) and the absorbance was measured at 515 nm after 10 min at 37 °C. All absorbance measurements were performed with the use of a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland). The antioxidant activity of samples was expressed as the stoichiometry values n_{10} calculated as described previously, with modifications [17]. The amount of radicals scavenged by the tested samples was calculated by using the Beer-Lambert law and the molar extinction coefficient of DPPH. The obtained data were used to generate linear dependence between the different volumes (μL) of tested samples (initial extract - 100%) present in the reaction mixtures and the number of scavenged μmoles of DPPH. The slopes of the straight lines obtained were then used for expressing the antioxidant activity and the mean of how many of μmoles of DPPH were scavenged by 1 mL of tested sample.

2.6. Antioxidant activity by differential pulse voltammetry

Differential pulse voltammetry (DPV) experiments were carried out with the Gamry Reference 600 potentiostat (Gamry Instruments, Inc., USA) using a three-electrode system. This consisted of a glassy carbon electrode (GC, 1.6 mm in diameter), platinum wire, and 3 M KCl Ag|AgCl electrode (type R-10/S, Hydromet S.C., Poland), which constituted the working, the auxilliary and the reference electrode, respectively. Before measurements, the surface of the working electrode was polished using an alumina suspension (MicroPolish Alumina, 0.05 μm particles, Buehler, USA) on microcloth pads (MF-1040, BASi, USA) and then rinsed with distilled water and methanol. The RE was stored in 3 M KCl and rinsed with water prior to use. The CE and mixtures M1-M8 were diluted using 1 M sodium phosphate buffer of

Table 1
The composition of mixtures (M1-M8) containing major bioactive compounds present in CE.

	M1	M2	M3	M4	M5	M6	M7	M8
(−)-epicatechin (EC)	x	x	x	x	x	x	x	x
(+)-catechin (C)	x	x	x	x	x	x	x	x
(+)-gallocatechin (GC)		x	x	x	x	x	x	x
procyanidin B1 (PROB1)			x	x	x	x	x	x
protocatechuic acid (PR)					x	x	x	x
quercetin (Q)				x		x	x	x
caffeine (CA)							x	x
theobromine (TE)								x

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Table 2
The amounts of main bioactive compounds identified in CE.

Peak no.	Compound	Content in CE [μg/mL]
1	unidentified	–
2	theobromine	1548 ± 23
3	protocatechuic acid	6.67 ± 0.29
4	procyanidin B1	19.20 ± 0.26
5	(+)-catechin	98.6 ± 6.8
6	caffeine	125.3 ± 4.2
7	(+)-gallocatechin	30.33 ± 0.57
8	(−)-epicatechin	268 ± 17
9	procyanidin C isomer	6.472 ± 0.011
10	clovamide	2.87 ± 0.24
11	procyanidin B isomer	5.4 ± 1.6
12	quercetin arabinoside	3.23 ± 0.11
13	quercetin	0.667 ± 0.080

pH = 7.4 as the supporting electrolyte. Prior to measurements, the diluted solutions were degassed (deoxidized) by a 3 min, high purity argon percolation. All DPV voltammograms were obtained at anodic polarization, in the range −0.15 to +0.8 V vs. the reference electorde. Scan rate of 100 mV/s, pulse height of 50 mV, pulse time of 0.1 s, and sample period of 0.5 s were set. All experiments were performed at 25 ± 0.01 °C. Temperature was controlled using an Ultra Thermostat (PolyScience, USA). Three independent replicates of DPV measurements were performed for each sample. Firstly, the charge density transferred in the oxidation process was calculated according to equation (1):

$$q = \frac{\int (I - I_{bckg})dt}{A_{WE}} [C \cdot cm^{-2}] \quad (1)$$

where: q is the value of the charge density transferred in the process, A_{WE} is the surface area of the working electrode (cm^2), and I is the value of current measured vs. the background current - I_{bckg} (A). The surface area of the working electrode had been determined in a separate experiment in which cyclic voltammetry of 1 mM $K_3[Fe(CN)_6]$ solution in 0.1 M KCl was performed.

The energetic significance of the transferred charge was assessed by determining *specific antioxidant energy* (AOE), expressed in units of specific energy (J/cm^2) and calculated according to equation (2):

$$AOE = E_{p,b} \cdot q \ [J \cdot cm^{-2}] \quad (2)$$

where $E_{p,b}$ is the absolute potential based on equation (3):

$$E_{p,b} = E_{p,a} + \varepsilon \quad (3)$$

where $E_{p,a}$ is the value of oxidation peak potential (V), ε is a correctional factor (accurate potential of the used reference electrode with diffusion potential) equal to 0.103 V, which was identified in separate measurements by titration of redox couples of known standard reduction potential: $FeCl_3$ (as an oxidant) and $Na_2S_2O_3$ (as a reducing agent) in aqueous solution. Such checks were performed periodically (every 3 months) during the whole project; the given value is valid for the period of the measurements discussed. Finally, the value of *specific antioxidant power* (AOP) was found from equation (4):

$$AOP = \frac{AOE}{t} [W \cdot cm^{-2}] \quad (4)$$

where t is the time span between the beginning of the oxidation peak and its end.

DPV voltammograms were analysed by SigmaPlot 13.0 software (Systat Software Inc., UK).

2.7. Cell culture

HT29 (human colon adenocarcinoma) cell line was purchased from ATCC collection. This cell line belongs to the ones recommended for nutritional studies by the National Centre for the Replacement, Refinement & Reduction of Animals in Research [18,19]. The cell culture was maintained in McCoy's medium supplemented with L-glutamine (2 mol/L), sodium pyruvate (200 g/L), foetal bovine serum (100 mL/L), and antibiotics (100 U/mL penicillin and 100 g/L streptomycin). The extract and mixtures of antioxidants were sterilized by being passed through Millex sterile R33 mm (0.22 μm) syringe-driven filters (Millipore). Cells were maintained at 37 °C under humidified atmosphere with 5% CO_2 in the Smart cell incubator (Heal Force). The cells used for experiments were derived from passages between 6 and 11. Cultured cells were regularly checked for mycoplasma contamination using Universal Mycoplasma Detection Kit from ATCC (USA).

2.8. Cytotoxicity assessment

To determine the growth inhibition of HT29 cells exposed to different mixtures of antioxidants or CE, MTT test was applied as described previously, with minor modifications [17]. Briefly, the exponentially growing cells were seeded in 96-well tissue culture plates (5×10^3 cells per well in 0.18 mL of medium) and were allowed to settle for 24 h at 37°C. Then, the cells were treated for 6, 24, or 72 h with 0.02 mL of different concentrations of the mixtures M1-M8 or CE; the latter was diluted to contain 30% ethanol. Using the quantitative data obtained by HPLC analysis, 30% ethanol extract was further diluted to obtain final concentrations of C and EC in cell culture in the range of 10 nM–50 μM. In turn, mixtures of bioactive cocoa components were initially prepared in 70% ethanol to reflect the concentrations of these compounds in CE according to the quantitative HPLC data (Table 2). Then, the mixtures were diluted so as to contain 10% ethanol. Final concentrations of C and EC in all mixtures applied to cell culture ranged from 10 nM to 100 μM. Control cells were treated with the corresponding solvent only.

After all exposure times (6, 24, 72 h), the medium was removed from the wells and replaced with 0.2 mL of fresh medium. After 72 h of total incubation time, 0.05 mL solution of MTT (4 g/L) was added per well and the cells were left for another 4 h at 37°C. Finally, the medium was aspirated from the wells and the formazan crystals, which had formed as a result of the action of mitochondrial dehydrogenases of living cells, were dissolved in 0.05 mL of DMSO. The absorption of the resultant solutions was determined at 540 nm with a TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland). Treatments were performed in four technical repetitions. Three independent replicates of each treatment were performed. Cytotoxicity was expressed as the growth inhibition of cells exposed to the tested antioxidants as compared to the control cells, treated with solvent, whose growth was regarded as 100%.

2.9. Cellular antioxidant activity

The cellular antioxidant activity of CE and mixtures was assessed in HT29 using a CAA assay (The OxiSelect™ Cellular Antioxidant Assay Kit, Cell Biolabs, Inc., USA) as described previously [17]. Briefly, cells were seeded in black 96-well plates designed for fluorescence measurements (3×10^4 cells per well in 0.2 mL of medium). The cells were allowed to settle for 24 h at 37°C and were then treated with 0.05 mL of 500 times diluted solution of 2′,7′-dichlorofluorescin diacetate provided with the kit, and with 0.05 mL of different concentrations of antioxidant samples for 1 h. The CE was diluted from 70% to 10% ethanol to contain 180 μM of C and EC. Final concentrations of C and EC in the cell culture treated with the diluted extract ranged from 0.1 μM to 90 μM. The mixtures of bioactive cocoa components were prepared in 70% ethanol and reflected the concentrations of these compounds in CE and were then diluted to contain 10% ethanol. Final concentrations of C and EC in the cell culture treated with the diluted mixtures ranged from 0.1 μM to 100 μM. Control cells were treated with the corresponding solvent only. Subsequent steps were carried out according to the

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manufacturer's procedure available from the website: https://www.cellbiolabs.com. Calculations were performed as described earlier [13].

2.10. Microarray analysis

HT29 cells were seeded in 24-well tissue culture plates (10^5 cells per well in 1.8 mL of McCoy's medium) and incubated for 24 h at 37 °C and 5% CO_2. After this time, the cells were treated for 24 h at 37 °C with 0.2 mL of different concentrations of CE or mixture M5 or M8. Both the CE and mixture M8 were prepared as for MTT test (described in section 2.8). Final concentrations of C and EC ranged from 1 μM to 10 μM. The cells used as negative controls were treated with the appropriate solvent only. Isolation of total RNA, reverse transcription, and real-time PCR analysis of the human oxidative stress PCR array – consisting of 84 genes involved in the oxidative stress response and antioxidant defence – as well as data analysis were all performed as described previously [17]. Three independent repetitions of each cell treatment were carried out.

2.11. Statistical analysis

All values are expressed as means ± SD of three independent experiments unless stated otherwise. An unpaired Student's t-test ($p \leq 0.05$) was used to evaluate the statistical significance of the determinations of antioxidant activity in the cell free system performed using DPV and DPPH assay, as well as those for the cellular model, performed using the CAA test. These statistical analyses were performed using the Prism 4.0 software package (GraphPad Software, Inc., USA). The statistical significance of the changes in gene expression between samples and controls was also evaluated by unpaired Student's t-test for each gene of interest; this was done using GenGlobe Data Analysis Center (Qiagen, USA). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Analysis of bioactive compounds in cocoa powder extract by HPLC

The health-promoting properties of cocoa have been attributed to the specific composition of phytochemicals, among which two main types of bioactive components can be distinguished. The first group embraces antioxidants, in particular flavan-3-ols in the form of monomers and oligomers of catechins. The most abundant monomers in cocoa powder are (+)-catechin and (−)-epicatechin, whose concentrations usually reach 0.2% and 0.7% (w/w), respectively [24]. Cocoa powder is also known to be a very rich source of various procyanidins. The second type of cocoa bioactives comprises the so-called psychoactive ingredients such as theobromine and caffeine, which do not exhibit redox properties. Caffeine content can amount to 0.28% (w/w) of cocoa powder, while theobromine even up to 2.21% (w/w) [24]. However, depending on the type of cocoa and the parameters of processing, the profile of the bioactive compounds in cocoa powder may vary considerably.

In the first step of the study, the profile of the bioactives present in the cocoa ethanolic extract (CE) was determined (Fig. 1). The amounts of the main compounds identified in CE are presented in Table 2. The psychoactive ingredients (caffeine and theobromine) quantitatively accounted for the majority of the detected bioactive compounds (Fig. 1 A, peaks no. 2 and 6, respectively). The other abundant bioactive components of CE were flavan-3-ols, specifically C, EC and GC (Fig. 1 A, peaks no. 5, 8 and 7, respectively). Among other flavan-3-ols, various oligomers of C and EC were detected (Fig. 1 A, peaks no. 4, 9 and 11, respectively). Other essential phytochemicals identified in CE, albeit occurring in much smaller concentrations, were protocatechuic acid, belonging to hydroxybenzoic acids, and quercetin, from the flavonols family (Fig. 1 A, peaks no. 3 and 13, respectively). The analysed sample also contained minor amounts of other compounds characteristic for cocoa, such as clovamide and quercetin arabinoside (Fig. 1 A, peaks no. 10 and 12, respectively). Unfortunately, peak no. 1 could not be identified due to the co-elution of several substances, whose UV–Vis and MS spectra were difficult to interpret. The complete separation of these

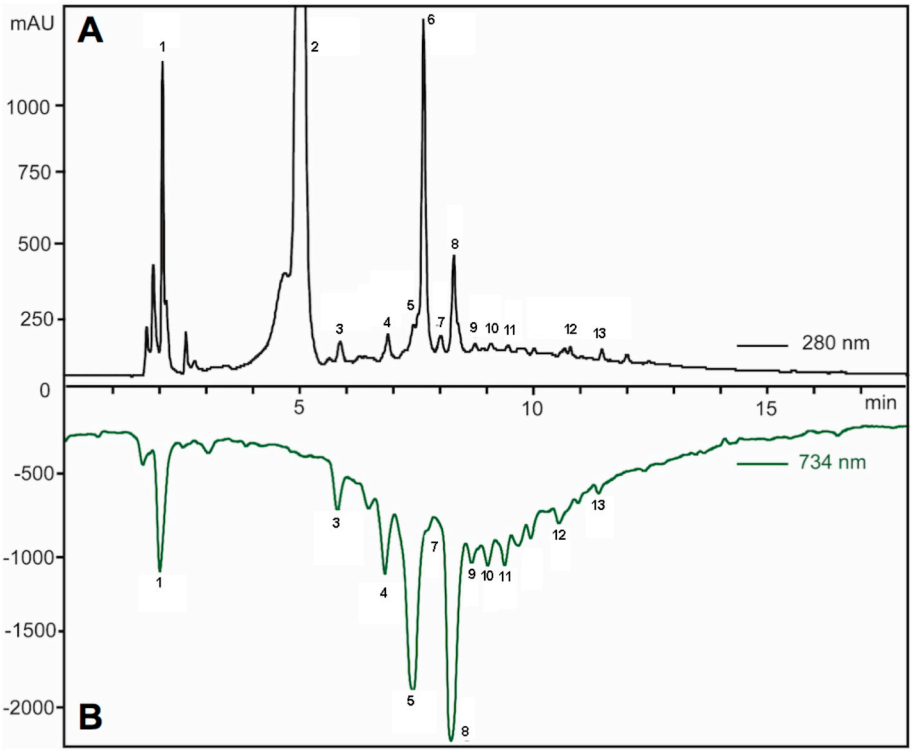

Fig. 1. Sample HPLC chromatograms of CE (chromatogram A at 280 nm) along with the profile of antioxidants detected online with ABTS reagent (chromatogram B at 734 nm). The major phytochemicals were identified based on MS and UV–Vis spectra and/or a comparison with appropriate standards. The peak numbers correspond to: **1**, unidentified compound (M − H) 191 m/z λmax = 280 nm; **2**, theobromine (M + H) 181 m/z, λmax = 280 nm; **3**, protocatechuic acid (M + H) 155 m/z, (M − H) 153 m/z, λmax = 295 nm; **4**, procyanidin B1 (M + H) 579 m/z, (M − H) 577 m/z, λmax = 280 nm; **5**, (+)-catechin (M + H) 291 m/z, (M − H) 289 m/z, λmax = 280 nm; **6**, caffeine (M + H) 195 m/z, λmax = 280 nm; **7**, (−)-epigallocatechin (M − H) 305 m/z, λmax = 280 nm; **8**, (−)-epicatechin (M + H) 291 m/z, (M − H) 289 m/z, λmax = 280 nm; **9**, procyanidin C isomer (M − H) 865 m/z, λmax = 280 nm; **10**, clovamide (M + H) 360 m/z, (M − H) 358 m/z, λmax = 320 nm; **11**, procyanidin B isomer (M + H) 579 m/z, (M − H) 577 m/z, λmax = 280 nm; **12**, quercetin arabinoside (M + H) 435 m/z, (M − H) 433 m/z, λmax = 360 nm; **13**, quercetin (M − H) 301 m/z, λmax = 360 nm.

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polar compounds eluting within 1–3 min was not possible under the chromatographic conditions used. Usually, in crude extracts of plant matrix, the polar fractions with a short retention time, often not separated under C-18 conditions, may contain a mixture of *e.g.* ascorbic acid and its derivatives, free amino acids, organic acids, and reducing sugars.

The chromatogram of the components of CE (Fig. 1 A) is accompanied by its "antioxidant fingerprint" (Fig. 1 B) obtained with the aid of HPLC coupled with post-column derivatization with ABTS radical, as described elsewhere [17]. The greatest contributions to the antioxidant properties of CE came from C, EC and GC (Fig. 1 B, peaks no. 5, 7 and 8). Among the identified oligomers of catechins, especially procyanidin B1, but also procyanidin B and C isomers showed substantial input into CE reducing capacity (Fig. 1 B, peaks no. 4, 9 and 11). On top of this, the analysed sample contained a large amount of other non-resolved substances, most probably oligomers of catechins, which, though occurring at very small concentrations, nevertheless increased markedly the total antioxidant activity of CE (Fig. 1 B). Their presence is manifested as a baseline drift along the chromatogram (Fig. 1 B, area between peaks 6 and 12 min). This type of redox-active plant matrix is commonly observed in analyses of natural samples [20,25]. The other major cocoa phytochemicals which influenced the total antioxidant capacity of CE were protocatechuic acid and quercetin (Fig. 1 B, peaks no. 3 and 13, respectively). Among other detected compounds, clovamide and quercetin arabinoside exhibited a slight reducing capacity (Fig. 1 B, peaks no. 10 and 12, respectively). In order to find out which of the major secondary metabolites have a particularly strong impact on the biological activity of the studied cocoa sample, a series of model mixtures from M1 to M8 was designed. The amounts of bioactive components identified in CE in 70% ethanol, which was used to prepare the mixtures, are presented in Table 3. Mixture M8 was assembled to reflect the concentrations of the eight most abundant bioactive components as identified in CE. The composition of mixture M8 was then gradually stripped of some of the bioactives (mixtures M7-M1). The compositions of mixtures M1-M8 are presented in Table 1 (Materials and Methods). What is more, it was not possible to obtain as high a concentration of theobromine in mixture M8 as it had been determined in CE (Table 2). The highest amount of this compound it was possible to dissolve was 1000 μM; that is almost 9 times lower than the concentration found in CE. Table 3 also presents the concentrations of these main bioactive compounds in diluted CE, which were used in biological studies (Table 3, (30% ethanol) column).

3.2. Antioxidant activity by DPPH test

The evaluation of the total antioxidant activity for the studied samples was performed with the aid of a batch DPPH test. This radical was selected because from among the various oxidants used in spectrophotometric assays ($ABTS^{+\bullet}$, Folin–Ciocalteu reagent, etc.) it showed the strongest correlation with the results of determinations of reduction potentials by cyclic voltammetry for a wide range of antioxidants [26], as well as with standard reduction potentials (E^0) of

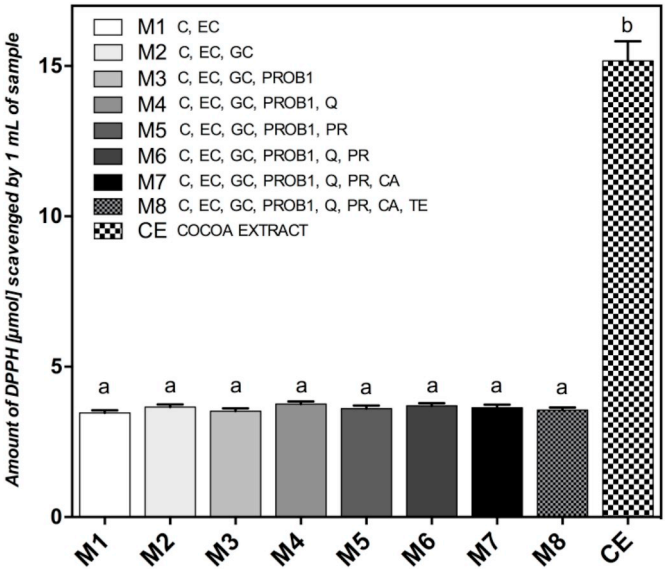

Fig. 2. The comparison of antioxidant activity expressed as *μmoles of DPPH scavenged by* 1 mL *of tested sample* calculated for all the samples studied based on the DPPH test. The results are means ± SD of three independent determinations. Different letters indicate significantly different values examined by unpaired Student's t-test ($p < 0.05$).

catechins measured by potentiometric titration [17]. In general, the antioxidant activity of a sample can be characterized by two factors. The first one is E^0, which expresses the ability of a compound to gain electrons. The second one is the rate of charge transfer during the oxidation process. In our experiments, the results of antioxidant activity determinations by DPPH test were expressed as a stoichiometry value (n_{10}), taking into account both the above-mentioned factors; it measures the number of oxidant molecules ($DPPH^{\bullet}$ radical) reduced by one molecule of antioxidant after 10 min of reaction. Thus, both the thermodynamic parameter (reduction potential) and the kinetic parameter (amount of product formed after 10 min of reaction) of the oxidation process are embraced. In the case of mixtures of compounds, we calculated how many *μmoles of DPPH were scavenged by* 1 mL *of tested sample* after 10 min of reaction. The values calculated for CE and the corresponding mixtures are presented in Fig. 2. Surprisingly, the increasing number of redox active compounds in the mixtures (M1-M8) did not affect their antioxidant activity. Contrary to expectations, the value of *μmoles of DPPH scavenged by* 1 mL *of tested sample* seems to summarize the antioxidant activity of analysed samples independently of the concentrations of redox active components. This may mean that the addition of small amounts of such antioxidants as GC, PROB1, Q, PC to the most redox active and about 10 times more abundant catechins had no impact on the total antioxidant activity of mixtures. The results indicated also that CE was the most efficient in scavenging the $DPPH^{\bullet}$ radical and stood out from any other mixture. This observation

Table 3
Recalculation of the concentrations of the main bioactive CE phytochemicals determined by HPLC as applied in the biological assays. The achievable concentration of theobromine in M8 is given in brackets. The concentrations of the two main antioxidants in CE are highlighted in boldface.

Compound	Content in CE (70% ethanol) [μM]	Content in CE (30% ethanol) [μM]	Content in CE containing 100 μM of C+EC(30% ethanol) [μM]
theobromine (TE)	8601 ± 132 (1000 μM in M8)	3687 ± 57 (430 μM in M8)	682 ± 11 (80 μM in M8)
Caffeine (CA)	646 ± 21	276.9 ± 9.2	51.2 ± 1.7
(−)-epicatechin (EC)	**924 ± 61**	**396 ± 26**	**73.3 ± 4.8**
(+)-catechin (C)	**340 ± 24**	**146 ± 10**	**26.9 ± 1.9**
(+)-gallocatechin (GC)	99.1 ± 1.9	42.48 ± 0.81	7.86 ± 0.15
Procyanidin B1 (B1)	33.28 ± 0.46	14.24 ± 0.20	2.633 ± 0.036
protocatechuic acid (PR)	43.3 ± 1.9	18.55 ± 0.80	3.43 ± 0.15
quercetin (Q)	2.21 ± 0.26	0.94 ± 0.11	0.175 ± 0.021

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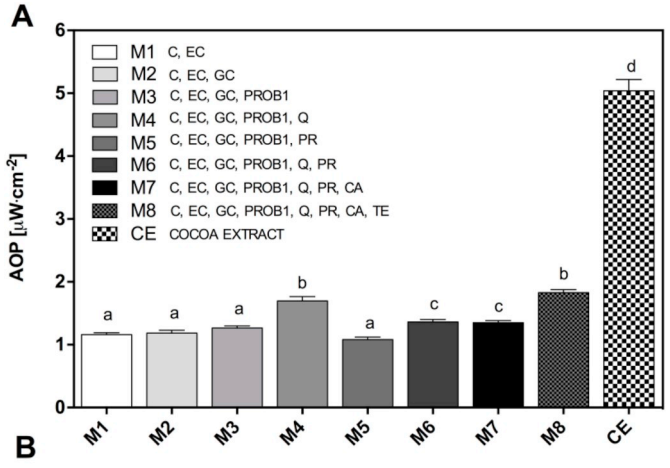

B

	M1	M2	M3	M4	M5	M6	M7	M8	CE
M1		0.7537	0.0585	**0.0026**	0.0899	**0.0126**	**0.0098**	**0.0005**	**0.0001**
M2	=		0.0695	**0.0005**	**0.0461**	**0.0073**	**0.0067**	**<0.0001**	**<0.0001**
M3	=	=		**0.0007**	**0.0037**	**0.0342**	**0.0335**	**<0.0001**	**<0.0001**
M4	>	>	>		**0.0002**	**0.0021**	**0.0016**	0.0546	**0.0001**
M5	=	<	<	<		**0.0001**	**0.0008**	**0.0005**	**<0.0001**
M6	>	>	>	<	>		0.7562	**0.0002**	**0.0001**
M7	>	>	>	<	>	=		**0.0001**	**0.0001**
M8	>	>	>	=	>	>	>		**0.0001**
CE	>	>	>	>	>	>	>	>	

Fig. 3. Values of antioxidant power (AOP) for mixtures M1 through M8 and CE, as determined by DPV, ($v = 0.1$ V s^{-1}). Different letters indicate a significant difference in the unpaired Student's t-test - **panel A**. The results are means ± SD of three independent measurements. **Panel B** - Probability values for pairs of mixtures compared by the unpaired Student's t-test. Values suggesting a statistically important difference ($p < 0.05$) between the two compared sets are given in boldface, while probability values $p \geq 0.05$ given in plain font signify the acceptance of the null hypothesis that the compared sets do not differ. The conclusions from the top right part of Fig. 3B and Table 3, reduced to relations >, <, =, are given in the lower left part of Fig. 3B. Explanations in the text.

contradicts the one made for mixtures, but confirms the strong impact postulated earlier of minor catechin oligomers suggested in the HPLC analysis on the cumulative antioxidant capacity of the CE sample [27].

3.3. Antioxidant power by DPV technique

Expressing the reducing activity of pure compounds through an electrochemical parameter, *i.e.*, the standard reduction potential, turned out to be a reasonable approach that made it possible to correlate biological observations with chemical properties [17]. The value of E^0 characterizes the ability of a compound to gain electrons (the higher the E^0, the weaker the antioxidant) and can be determined by, *e.g.,* potentiometric titration. However, in the case of mixtures of redox active components, it was not possible to evaluate the total antioxidant capacity using potentiometric titration. The most appropriate technique that could be applied for this purpose was voltammetry, where we decided to take three elements of total antioxidant capacity under consideration: a) the extent of the charge transfer (area under the curve, the larger the better), b) the rapidity of the charge transfer expressed by its time (the shorter the better), and c) the energy of the charge transfer reflected in the oxidation (anodic) peak potential ($E_{p,a}$, the larger the better). Such an approach enables the results of voltammetric determinations to be expressed in watts and to be related to the unit of the electrode surface area (see also Materials and Methods).

Among voltammetric techniques, differential pulse voltammetry (DPV) is the most appropriate for measuring total antioxidant activity of complex samples. In terms of the total antioxidant capacity evaluation, DPV prevails over other methods. Firstly, it allows to study the electrooxidation of pure compounds and their mixtures. Secondly, DPV is recommended for analysis of samples containing low concentrations of antioxidants as it is characterized by higher sensitivity than the classic cyclic voltammetry technique [28].

The antioxidant activity of M1-M8 mixtures measured by DPPH assay was virtually steady (Fig. 2). Therefore, in the next stage of our investigations, a parameter such as *antioxidant power* (AOP) of CE and mixtures M1-M8 was suggested (as discussed above) and measured. The results obtained by DPV technique for CE and the mixtures (M1-M8) are presented in Table 4. Considering that the higher scan rate implies a higher sensitivity of measurements, 100 mV/s was chosen as the highest scan rate, allowing us to obtain the appropriate shape of the oxidation peak [26].

The DPV examination revealed that the successive addition of components to mixtures affected their total AOP values, which had not been observed in DPPH test (Fig. 3A). The addition of GC to mixture M1 did not change the total AOP value of M2. In turn, presence of Q significantly increased the AOP of M4 as compared to M3. The addition of PR decreased the antioxidant activity of M5 and M6 as compared to M3 and M4. The presence of CA (M7) did not change the AOP, while supplementation with TE increased the reducing capacity of M8 in comparison to M7. Nonetheless, CE was characterized by the highest AOP value and the strongest antioxidant properties, which is in line with DPPH test.

3.4. Cytotoxicity by MTT test

The impact of CE and of the model mixtures M1-M8 on cell growth was studied by MTT test in HT29 cell line. The human colon adenocarcinoma HT29 cells were chosen as this cell line is recommended for nutritional studies by the National Centre for the Replacement, Refinement & Reduction of Animals in Research [18,19] and is a

Table 4
Values of oxidation peak potential vs. reference electrode ($E_{p,a}$), peak potential *vs.* standard hydrogen electrode ($E_{p,b}$), charge density of the process (q), specific antioxidant energy (AOE), peak time (t), and antioxidant power (AOP) for the model mixtures and CE, as determined by DPV ($v = 0.1$ V s^{-1}). The results are given as means ± SD of three independent determinations. If SDs are not given, it means three identical results were obtained.

Sample	$E_{p,a}$ [V]	$E_{p,b}$ [V]	q [μC·cm^{-2}]	AOE [μJ·cm^{-2}]	t [s]	AOP [μW·cm^{-2}]
M1	0.137 ± 0.006	0.240 ± 0.006	9.88 ± 0.44	2.304 ± 0.20	2.0 ± 0.1	1.161 ± 0.030
M2	0.130	0.233	11,99 ± 0.25	2.795 ± 0.059	2.4 ± 0.1	1.183 ± 0.046
M3	0.130	0.233	13.917 ± 0.062	3.243 ± 0.014	2.6 ± 0.1	1.265 ± 0.034
M4	0.130	0.233	20.44 ± 0.10	4.60 ± 0.20	2.7 ± 0.2	1.696 ± 0.071
M5	0.133 ± 0.006	0.236 ± 0.006	11.82 ± 0.17	2.812 ± 0.018	2.6 ± 0.1	1.084 ± 0.039
M6	0.130	0.233	14,97 ± 0.28	3.488 ± 0.066	2.6 ± 0.1	1.361 ± 0.040
M7	0.130	0.233	15.44 ± 0.15	3.598 ± 0.034	2.7 ± 0.1	1.351 ± 0.032
M8	0.130	0.233	20.136 ± 0.092	4.692 ± 0.021	2.6 ± 0.1	1.829 ± 0.048
CE	0.180 ± 0.010	0.280 ± 0.010	64.6 ± 1.5	18.30 ± 0.57	3.6 ± 0.1	5.04 ± 0.18

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representative model of alimentary tract epithelium, *i.e.* the tissue in direct contact with ingested food ingredients, such as the cocoa non-nutrient components. The cells were treated with the model mixtures and CE either containing CCE at physiological concentrations potentially occurring in blood (0.01–1 μM) or concentrations reachable in the alimentary tract (10–100 μM) after food ingestion. The dose response curves for 6, 24, and 72 h treatments are presented in Fig. 4.

The mixture of two main catechins identified in CE (C, EC) caused significant cell growth stimulation at low physiological concentrations (0.01–0.1 μM) for all exposure times, while higher concentrations did not impact cell proliferation. When mixture of C and EC was additionally enriched with (−)-gallocatechin (M2) and procyanidin B1 (M3), a similar biphasic tendency in dose response curves was observed. However, the stimulation of cell growth at physiological concentrations was hardly observed for mixtures M4-M8 or CE. The successive addition of compounds such as protocatechuic acid, quercetin, theobromine, or caffeine to mixture M3 suppressed the aforementioned effect on cell growth stimulation and the dose response curves held a constant course over the entire concentration range. A similar course of the dose-response curves was also seen for CE. In these cases, short exposure (6 h) to complex mixtures (M4, M5, M6, M7, M8) or CE did not inhibit the cell growth at any of the investigated concentrations, while prolonged exposures (24 and 72 h) inhibited cell growth, but by no more than 20%, regardless of concentration. Moreover, it was observed that disturbance in cell growth was more potent in the case of M4 containing catechins and quercetin, than with M5, supplemented with protocatechuic acid instead of quercetin.

3.5. Cellular antioxidant activity

To assess the impact of CE and model mixtures M1-M8 containing main cocoa bioactives on the efficiency of the endogenous antioxidant protection system of HT29 cells, the CAA assay was applied. In this method, the evaluation of cellular redox status relies on the ability of a sample containing antioxidants to inhibit the oxidation of the probe absorbed by cells to its fluorescent form. The decrease in probe oxidation, which is observed as the quenching of fluorescence, is a measure of the reducing capacity of an antioxidant in the cellular setting [29]. The results are expressed as so-called CAA values. A higher CAA value means better support of the endogenous antioxidant defence system of cells against oxidants.

As shown in Fig. 5, at the concentration of 0.1 μM CEC all samples, regardless of their composition, increased their cellular antioxidant capacity to the same extent. In the case of 1 μM, there was no clear-cut difference between model mixtures and CE in the protection of cells against oxidants. It seemed that exposure to physiologically relevant concentrations of both CE and mixtures M1-M8 did not have substantial impact on the cellular reducing status of cells. However, slight yet significant differences in CAA values were observed between the less and the more complex mixtures. At the concentration of 10 μM, exceeding the bioavailability of polyphenols, the enhancement of the cellular reducing status by CE began to significantly outpace the

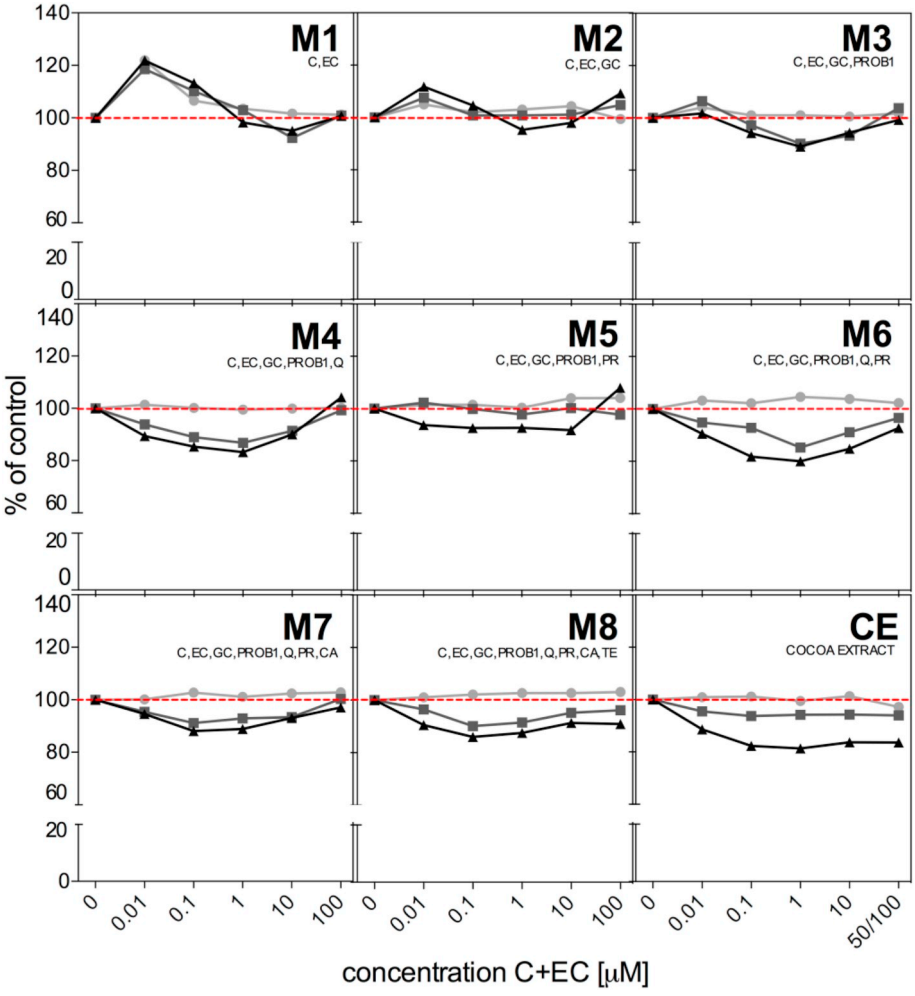

Fig. 4. Inhibition of growth of HT29 cells determined by MTT test after 6 (circles), 24 (squares) and 72 h (triangles) exposure to CE (0.01–50 μM CEC) or mixtures M1-M8 (0.01–100 μM CEC). Results represent means of three independent experiments carried out in triplicates (SD values were lower than 10%).

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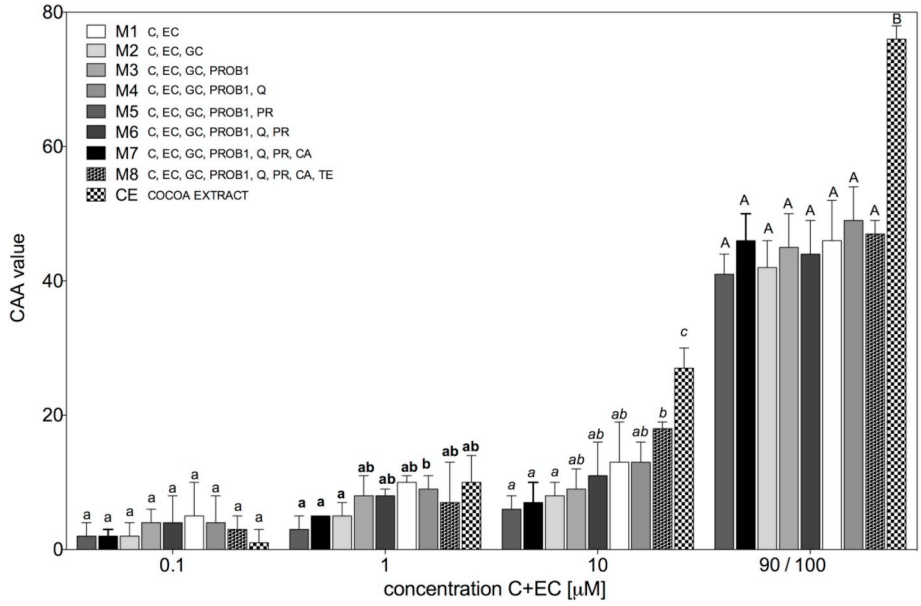

Fig. 5. Cellular antioxidant activity of CE (0.1–90 μM CEC) and mixtures M1-M8 (0.1–100 μM CEC). Results are means ± SD of three independent experiments carried out in triplicates. Different letters for the same concentration indicate a significant difference in unpaired Student's t-test ($p \leq 0.05$).

antioxidant impact of the tested mixtures. This effect was further escalated at 100 μM and CE became almost twice as efficient in cell protection against ROS as compared to artificial mixtures.

3.6. Microarray analysis

The present work also examined the influence of CE and two of the model mixtures, M5 and M8, on the expression of a wide range of redox related genes. This set of 84 genes (details in Table S1 in Supplementary Materials) comprises genes classified as those relevant for antioxidant activity, superoxide release and metabolism, activity of peroxidases and oxidoreductases, as well as being relevant for inflammation, apoptosis, regulation of cell cycle, and other processes associated with oxidative stress. Fig. 6 shows a heat map presenting the modulation of expression within the gene array investigated and the fold changes which reached statistical significance, determined for HT29 cells in response to 24 h incubation with CE and two model mixtures (M8, M5) at concentrations of CEC amounting to 1 or 10 μM. For comparison, the data for pure compounds (C, EC) at concentrations of 1 or 10 μM, borrowed from our previous work [17], are also included.

The obtained results showed that treatments with CE and with the richest model mixture, M8, had similar impact on the regulation of genes involved in redox control, while treatment with M5, containing only 5 components as well as pure compounds C or EC, modulated the expression of a different set of genes. Both CE and M8 at 1 and 10 μM CEC concentrations up-regulated the set of four genes: *EPHX2, HSPA1A*, *PTGS1* and *STK25*. Additionally, CE at 1 μM CEC also up-regulated gene *CYBB*, while at the higher concentration of CEC it enhanced also the expression of other genes important for cellular defence against ROS such as *GPX3* and *GTF2I*. In contrast to CE, mixture M8 up-regulated *GPX3* and *GTF2I* at the lower concentration of CEC. Furthermore, the set of genes regulated by the lower concentration of M8 was extended with *TXNRD1*, *TXNRD2*, *DHCR24*, *GSR*, *HMOX1*, *NUDT1* and *SIRT2*. The number of genes influenced by the M5 treatment was significantly lower. This mixture at 1 μM of CEC potentiated only the expression of CCL5. Among the genes up-regulated by M5 there was also *HSPA1A*, albeit the expression of this gene was enhanced only at 10 μM CEC. This concentration of M5 increased also the expression of *BNIP3*, *CYGB*, *GPX7*, *MPO* and *MT3*. The expression of only one gene in the panel, namely *EPX*, was down-regulated by M5 at both concentrations. In the case of isolated C, we found only three genes (*HSPA1A, ALB*, *CCL5*) out of 84 in the array to be significantly up-regulated at a concentration of 1 μM. In turn, EC increased the expression of *HSPA1A* at 10 μM, while at 1 μM it caused the up-regulation of *ALB*.

The elevated expression of *HSPA1A* observed for almost all the applied cell treatments with antioxidants (though not always reaching the level of statistical significance) may suggest infallible host protection against the polypeptide misfolding [30] which might result from the reductive stress leading to the breakage of disulfide bonds pivotal for the maintenance of the three dimensional structure of proteins. The *EPXH2* gene, whose expression was increased in the case of complex mixtures such as CE and M8, codes for a cytosolic epoxide hydrolase, *i.e.* an antioxidant enzyme involved in regulating cellular redox homeostasis [31]. An increase in expression caused by CE and M8 was seen for yet another member of the antioxidant enzyme gene family, namely *PTGS1*, which encodes prostaglandin synthase-2. The other gene up-regulated by CE and M8 was *STK25,* which codes for serine/threonine kinase 25, a protein activated by oxidative stress that induces apoptotic cell death [32]. The set of genes up-regulated by 1 μM CE was expanded to gene *CYBB*, which encodes NADPH oxidase (NOX2) subunit [33]. Additionally, at the higher concentration of CE, the expression of genes important for cellular defence against oxidative stress, such as *GPX3* and *GTF2I*, was also enhanced. The first one encodes glutathione peroxidase 3, which protects cells against oxidative damage by catalysing the reduction of peroxides by glutathione [34]. *GTF2I* acts as a general transcription factor and is involved in coordinating cell growth and division [35]. The increased expression of these two latter genes was also observed for M8 at 1 μM CEC. Within the group of genes up-regulated by M8 at 1 μM CEC there were also *TXNRD1*, *TXNRD2, DHCR24* and *GSR*, *HMOX1*, *NUDT1*, as well as *SIRT2*. *TXNRD1* and *TXNRD2* genes code for thioredoxin reductase 2, a mitochondrial protein that plays a key role in maintaining thioredoxin in a reduced state [36]. In turn, the enhanced expression of *DHCR24* may protect cells from oxidative stress by reducing caspase 3 activity during the apoptosis induced by elevated levels of oxidants [37]. Up-regulation of *GSR* is also critical for maintaining redox homeostasis in cells, because the encoded protein maintains high levels of reduced glutathione in the cytosol [38]. Another gene whose expression was increased by the 1 μM M8 mixture codes for heme oxygenase 1 (HMOX1), which cleaves the

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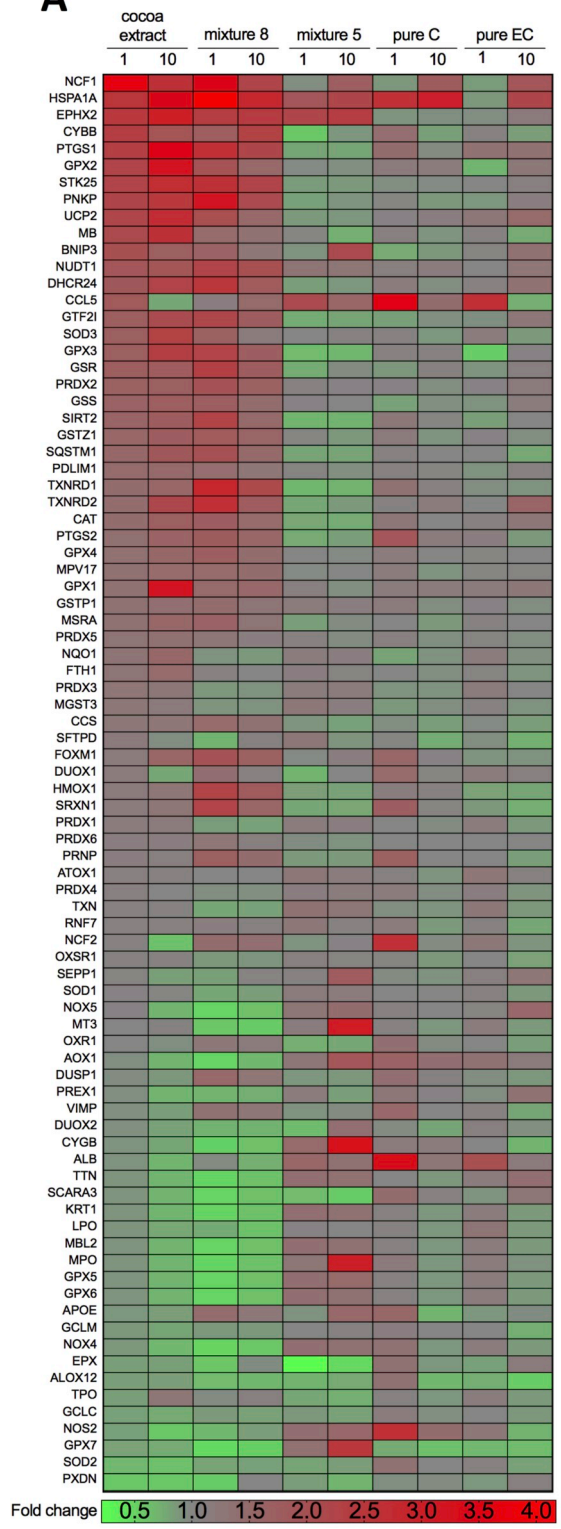
A



B

Gene	Fold change / *p* value									
	CE_1	CE_{10}	$M8_1$	$M8_{10}$	$M5_1$	$M5_{10}$	EC_1*	EC_{10}*	C_1*	C_{10}*
HSPA1A	2.58 0.030	2.99 0.003	4.17 0.002	2.80 0.042	n.s.	2.19 0.046	n.s.	2.19 0.019	2.63 0.040	n.s.
EPXH2	2.44 0.013	3.36 0.007	2.38 0.011	2.43 0.017	n.s.		n.s.		n.s.	
PTGS1	2.31 0.027	3.48 0.034	2.63 0.035	2.19 0.048	n.s.		n.s.		n.s.	
STK25	2.24 0.042	2.71 0.015	2.62 0.021	2.24 0.050	n.s.		n.s.		n.s.	
GPX3	n.s.	2.34 0.023	2.28 0.024	n.s.	n.s.		n.s.		n.s.	
CYBB	2.35 0.026	n.s.	n.s.	n.s.	n.s.		n.s.		n.s.	
GTF2I	n.s.	2.07 0.032	2.20 0.007	n.s.	n.s.		n.s.		n.s.	
TXNRD2	n.s.		2.89 0.007	n.s.	n.s.		n.s.		n.s.	
TXNRD1	n.s.		2.65 0.048	n.s.	n.s.		n.s.		n.s.	
DHCR24	n.s.		2.56 0.037	n.s.	n.s.		n.s.		n.s.	
GSR	n.s.		2.32 0.045	n.s.	n.s.		n.s.		n.s.	
HMOX1	n.s.		2.26 0.045	n.s.	n.s.		n.s.		n.s.	
NUDT1	n.s.		0.025 0.045	n.s.	n.s.		n.s.		n.s.	
SIRT2	n.s.		2.25 0.038	n.s.	n.s.		n.s.		n.s.	
CCL5	n.s.		n.s.		2.06 0.049	n.s.	n.s.		3.49 0.021	n.s.
ALB	n.s.		n.s.		n.s.		2.00 0.023	n.s.	3.30 0.002	n.s.
EPX	n.s.		n.s.		0.21 0.001	0.47 0.005	n.s.		n.s.	
BNIP3	n.s.		n.s.		n.s.	2.10 0.010	n.s.		n.s.	
CYGB	n.s.		n.s.		n.s.	3.25 0.001	n.s.		n.s.	
GPX7	n.s.		n.s.		n.s.	2.51 0.072	n.s.		n.s.	
MPO	n.s.		n.s.		n.s.	3.01 0.012	n.s.		n.s.	
MT3	n.s.		n.s.		n.s.	3.06 0.022	n.s.		n.s.	

*data taken from [12]
n.s. – not significant

Fig. 6. A – Heat map presenting the modulation of 84 oxidative stress response and antioxidant defence genes in HT29 cells after 24 h treatment with CE and two model mixtures M8 and M5 at concentrations of CEC amounting to 1 or 10 μM, as well as pure catechins EC and C at concentrations of 1 or 10 μM. B – statistically significant changes in the expression of genes as a result of indicated treatment. The results are calculated based on three independent experiments.

heme ring at the alpha methene bridge to form biliverdin. It exhibits cytoprotective effects because the excess of free heme sensitizes cells to undergo apoptosis [39]. *NUDT1* encodes the enzyme hydrolysing 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing the misincorporation of 8-oxo-dGTP induced by ROS during DNA replication or repair [40]. What may also have a positive effect on the redox status of cells is the up-regulation of *SIRT2*, encoding NAD-dependent protein deacetylase, which deacetylates internal lysines present in *e.g.* histones, alpha-tubulin, or some transcription factors; thus it participates in the modulation of crucial biological processes, such as cell cycle control,

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genomic integrity, cell differentiation, or metabolic networks, also at the level of the epigenome [41,42].

Further, M5 at 1 μM C + EC and C at 1 μM increased the expression of *CCL5*, which belongs to a group of inflammation-relevant genes. The protein encoded by it is classified as a chemotactic cytokine or chemokine, a secreted protein involved in immunoregulatory and inflammatory processes [43]. In turn, the higher dose of M5 enhanced the expression of *BNIP3*, *CYGB*, *GPX7*, *MPO*, and *MT3*. The first one codes for a protein that is a pro-apoptotic member of the Bcl-2 family. This gene is down-regulated in cancers, which correlates with resistance to chemotherapy and a worsened prognosis. The overexpression of this gene leads to the opening of the mitochondrial permeability transition pore, and cell death [44]. In turn, the cytoglobin gene (*CYGB*) functions as a tumour suppressor gene [45], while *GPX7* codes for glutathione peroxidase 7, which supports the antioxidant barrier of the cell by catalysing the reduction of peroxides by glutathione. *MPO* protein generates ROS endogenously by functioning as an antimicrobial enzyme, catalysing a reaction between H_2O_2 and chloride to generate hypochlorous acid, a potent oxidizing agent [46]. In contrast, *MT3* encodes a protein that plays an important role in zinc and copper homeostasis, as well as being a growth inhibitory factor [47]. The set of genes regulated by pure catechins, except *HSPA1A* and *CCL5*, contained also *ALB* coding for albumin being a part of the non-enzymatic antioxidant system, which, along with antioxidant enzymes, forms the line of defence against oxidation [45].

4. Discussion

This study examined the relationship between the redox properties of both natural cocoa powder extract (CE) and artificial mixtures of cocoa bioactive compounds and the redox associated biological effects in a model of the human alimentary tract – colon cancer HT29 cells – exposed to such cocktails of antioxidants. The objective of the study was to elucidate to what extent the impact on redox homeostasis exerted by the mixtures of purified antioxidants may reflect the activity of real food items containing a more complex composition of redox active ingredients on top of what is known as the food matrix.

At the initial stage of the study, the content of the main bioactive compounds in CE was determined using HPLC-DAD-MS analysis and on this basis the composition (in terms of qualitative and quantitative content) of eight model mixtures was designed (Table 1). The most complex one contained the eight most abundant cocoa bioactives (M8). The other seven had fewer and fewer of the less abundant components, while still always containing EC and C (M7-M1). This strategy aimed to spot the potential interactions between major bioactive cocoa phytochemicals. Caffeine and its catabolic product, theobromine, were the main electrochemically neutral bioactive compounds, which as food matrix components might nevertheless influence the overall biological effects of cocoa [48]. The most abundant phenolic antioxidants were EC and C, followed by GC, PROB1, PR, and Q. This is in accordance with observations reported in numerous studies that flavan-3-ols such as EC and C are the predominant compounds in cocoa powder and major modulators of the overall biological effects of cocoa [49–51]. Post-column derivatization with ABTS radical confirmed their prevalent individual (*i.e.* after separation) contributions to antioxidant activity of the CE sample (Fig. 1). However, when the mixtures M1-M8 and CE were investigated as wholes (without separation), a different picture emerged, especially in the case of redox properties, which suggests that growing complexity leads to new activity rather than just being the sum of the original activities of the separately existing compounds. This conclusion is argued for below on the basis of the study results.

The total redox properties of CE and M1-M8 were examined using a modified version of DPPH test that takes into account the kinetics of redox reaction, and whose results are expressed in the form of *μmoles of DPPH scavenged by* 1 mL *of tested sample*. In a previous study, we had found a high correlation between n_{10} and E^0 for catechins [17]. In the current experiment, DPPH test showed CE as having the highest antioxidant activity, but, surprisingly, did not reveal any differences between the artificial mixtures, despite their growing concentrations of antioxidants and qualitative complexity (Fig. 2). We therefore turned to a more sensitive electrochemical analysis using DPV, which is the most reliable technique for assessing the reducing capacity of complex mixtures [28]. To express the antioxidant activity of mixtures consisting of various bioactive compounds (such as M1-M8), an AOP parameter was calculated based on DPV measurements, making it possible to pinpoint the interactions between mixture components. Among the samples studied, CE exhibited the highest antioxidant activity also in this cell-free test (Fig. 3). However, DPV revealed variability in the impact of the individual components on the AOP parameter for the subsequent model mixtures. The addition of Q significantly increased the reducing power of the main catechins, despite the very small concentration of this compound. In turn, Q did not elevate the AOP of PR-containing mixtures, which suggests that these two antioxidants may have antagonistic effects. DPV also showed that the addition of redox neutral CA had no

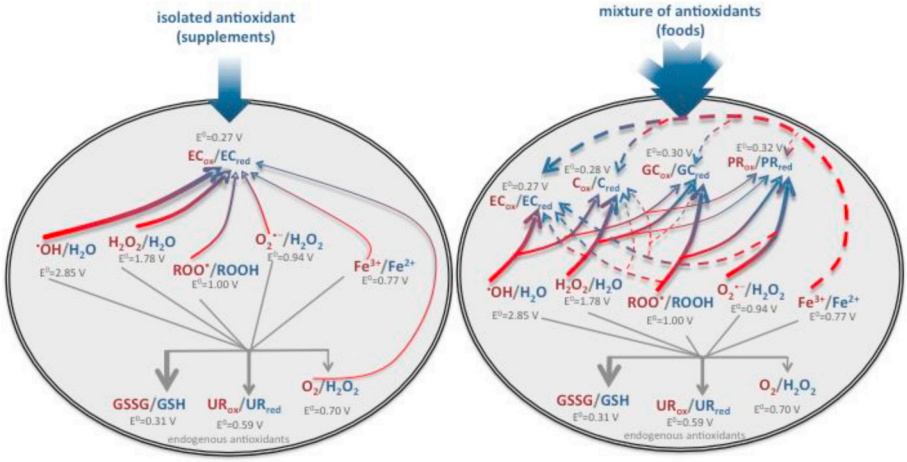

Fig. 7. A simplified scheme presenting the differential impact of single *vs.* mixed exogenous antioxidants on cellular redox status. In the case of exposure to individual purified antioxidants, this influence will be guided mainly by their standard reduction potentials (E^0) which are depicted for redox couples – oxidized and reduced forms (red/blue corresponding to ox/red). The strongest oxidant (highest E^0) will oxidize preferentially the strongest antioxidant (lowest E^0), *e.g.* treatment with an isolated epicatechin (EC) leads **in the first place** (thickest arrow) to the reaction of this antioxidant with the strongest oxidant ($^{\bullet}OH$). If the concentration of EC is high enough, after depletion of $^{\bullet}OH$, it will donate electrons to the oxidant characterized by the next highest E^0, *i.e.* $Cu^{3+} > H_2O_2 > ROO^{\bullet} > O_2^{\bullet-} > O_2$ (subsequent thinner arrows). As a result, the exogenous antioxidant supports the endogenous antioxidants (depicted at the bottom) of the cell. It follows that the exogenous antioxidants characterised by different E^0 make it possible to neutralize a wider variety of cellular oxidants **in parallel**, thus providing more efficient ROS depletion. In this situation, after depletion of a given ROS, the remaining antioxidants in the mixture will react with the strongest available oxidants (dashed lines). The acronyms EC, C, GC, PR refer to the polyphenols studied; GSSG to the oxidized form of GSH; UR to uric acid. The values of E^0 were taken form [17,54,55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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effect on the M7 antioxidant power, while the addition of TE had the effect that from all the model mixtures, the most complex one (M8) became the most potent in terms of antioxidant activity, which may suggest some interaction between antioxidants and the non-antioxidant theobromine. The markedly higher AOP of CE points to the importance of trace amounts of other redox active compounds, *e.g.* various non-resolved oligomers of catechins, quercetin isomers, caffeoyl aspartic acid, clovamide, or deoxyclovamid, which are known to contribute to the overall redox properties of cocoa [52].

The CAA assay, reflecting the impact of the studied samples on cellular antioxidant status, also showed that CE was the most efficient in cell protection against ROS (Fig. 5). However, it must be emphasized that under cellular conditions, as observed before [17], the concentrations of catechins (C + EC) in the studied solutions had to be above 10 μM to override cellular redox homeostasis and detectably increase the antioxidant capacity of the cells. Below this concentration, the antioxidant barrier of the treated cells was maintained at the control level. Therefore, it may be expected that in the situation of redox balance, the absorbed polyphenols from cocoa powder will only slightly, if at all, influence the redox status of different human tissues due to low bioavailability, which limits their internal exposure. However, intestinal epithelia, being in direct contact with the ingested food, may be exposed to much higher concentrations of catechins than those found in human plasma and thus be more effectively protected when redox homeostasis is disturbed [53]. Nonetheless, the question arises why CE offers so much more efficient antioxidant protection in comparison with the mixture containing its most abundant and strongest antioxidants at equivalent concentrations, *i.e.* M8. Fig. 7 suggests a possible explanation by pointing to the possibility of a parallel, thus quicker, neutralisation of a whole array of ROS by a complex mixture of antioxidants characterised by highly differentiated reduction potentials. Each antioxidant or its metabolite will react with a different cellular oxidant on the basis of their E^0; if two antioxidants have the same E^0, the kinetic factor will decide which antioxidant will preferentially react with a particular ROS. It is important to note that the reduced oxidants may include also *e.g.* oxidized forms of catechins, which in this way will be again available for the subsequent round of ROS neutralisation. It follows that the factors deciding about the final antioxidant potential of a mixture are not only the content or composition or bioavailability, but also, perhaps especially, the difficult-to-predict interactions between the reducing components, as seen for M1-M8. Therefore, mixtures of bioactive compounds, both natural and model, should be considered as individual redox active substances. Even minor alterations in the mixture composition may have significant consequences on the overall biological redox-associated effects. This may explain why observations made for pure phytochemicals isolated from their natural sources are not in line with the results obtained for mixtures, especially natural ones.

Another activity that is dependent on cellular redox homeostasis and that confounded expectations was cellular growth inhibition. The biphasic impact of mixtures M1-M3, containing only catechins, on HT29 cell growth did follow our earlier observations for pure catechins. The mixtures' physiologically achievable concentrations stimulated cellular growth. This phenomenon was then interpreted as the protective effect of catechins, which helps cancer cells to cope with the internal residual oxidative stress by restoring the optimal redox status [17]. At concentrations exceeding 1 μM, this stimulation was suppressed and the exposure to model mixtures gradually slowed down the growth of HT29 cells (Fig. 4). After the successive addition of further cocoa components to the model mixtures, the biphasic dose-dependence disappeared and the enhancement of growth inhibition was observed, though only in the case of prolonged exposures (24 and 72 h). Presumably, prolonged treatments deprived cells of ROS to such an extent that ROS-dependent signalling failed and proliferation became suppressed. However, the resulting cell growth inhibition was dose-dependent only for a narrow range of low concentrations; for higher ones, cell proliferation attained a constant level. Thus it may be concluded that complex mixtures of bioactive non-toxic compounds rather stabilize the level of cell growth than inhibit it in a dose dependent manner. It is known that cancer cells overproduce ROS molecules, which, on the one hand, are key for the activation of signalling pathways important for cell growth, but, on the other hand, may impair cell function due to oxidative damage. Redox balance is thus vital for cell survival. Neither oxidative stress, leading to oxidative damage, nor reductive stress, disabling cell growth, are favourable for cancer cells. Cocoa powder, with its unique combination of flavonoids and methylxanthines may prevent both these types of ROS dependent phenomena – proliferation and damage of the cellular structures, including DNA. The prevention of oxidative DNA damage by cocoa polyphenols was observed both *in vitro* and *in vivo* [56], while their significant antiproliferative effect was demonstrated in different human cancer cell lines [57,58]. An aqueous cocoa extract (100–150 μg/ml equivalent to 44–66 μM gallocatechin gallate) was reported to inhibit the proliferation of human prostate carcinoma cell lines [59]. The proantocyanidine fraction of cocoa extract caused a delay of the cell cycle in G2/M and consequently led to a 70% inhibition in the growth of human colon carcinoma cells [60]. *In vivo* cocoa catechins significantly reduced the incidence of lung cancer and thyroid gland adenoma induced in male rats [61]. All these observations are of interest from a pharmacological point of view as related to chemopreventive applications.

The pleiotropic biological properties of complex mixtures of antioxidants may also be explained based on their impact on the regulation of cellular redox homeostasis at the transcriptome level. The present study compared the influence of CE, M8, and M5, all containing a combination of bioactive components, but differing in their complexity and thus E^0 range, on the expression of a wide spectrum of genes associated with the antioxidant response system. In Fig. 6., these results are supplemented with the data from our earlier study [17] for pure cocoa catechins: catechin and epicatechin. The *HSPA1A* gene was the only one whose expression was significantly triggered by all samples investigated at least at one dose. Since all samples contained catechins, one explanation is that this particular group of polyphenols is responsible for *HSPA1A* gene up-regulation. However, an alternative interpretation seems more tempting. This gene codes for chaperon protein and such a result may suggest that in the situation of an altered cellular redox balance, the protection of three-dimensional native structures of proteins is a priority. In the case of exposure to antioxidants, this balance may be shifted towards a reduced state, leading to the more probable reduction of disulfide bridges to sulfhydryl groups, which may dramatically change the protein structure and thus function. HSPA1A chaperone is then needed to correct any occurring misfoldings.

As has already been mentioned, the up-regulation of *HSPA1A* gene expression was the only nutrigenomic effect shared by the all samples investigated. Otherwise, the vast differences in the sets of mostly up-regulated genes were clearly seen, as if samples used for these experiments were completely unrelated. The set of genes up-regulated by both CE and the most complex mixture M8 included the same additional five genes (*EPHX2*, *PTGS1, STK25, GPX3, GTF2I*) that may support redox balance maintenance (Fig. 6). However, the pool of modulated transcripts by M8 embraced also seven further proteins (DHCR24, GSR, HMOX1, NUDT1, SIRT2, TXNRD2, TXNRD3) that play key roles in building cellular defences against oxidants. It follows that CE probably contained some components that quenched the transcriptional activity of these genes.

In contrast, M5, composed of only catechins and protocatechuic acid, invoked yet another gene expression pattern and changed the expression of seven genes (*CCL5*, *EPX, BNIP3*, *CYGB*, *GPX7*, *MPO*, *MP3*), whose expression had not been significantly altered by either the more or the less complex samples. Compared to M8 or CE, mixture M5 is devoid of Q and alkaloids, which suggests that the presence of these substances induced the expression of genes observed only in the case of M8, and at the same time prevented the changes in gene expression

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observed in cells treated with M5. The least nutrigenomically active were pure catechins. It may be concluded that mixtures of antioxidants characterized by different E^0 may provide better cell antioxidant protection than isolated antioxidants, not only by virtue of more efficient chemical neutralisation of ROS, but also via activation of antioxidant genes. Strong antioxidants, individually or in mixtures such as CE, lead to direct chemical reduction of ROS, while weaker ones are able to induce endogenous antioxidant mechanisms. However, the expression of which genes becomes altered seems to depend on the structures of the antioxidants applied to cells and the interactions between them.

Our observations are thus in line with the food synergy concept, which is defined as additive or more than additive influence of different food ingredients on human health [15]. The synergistic effect of cocoa bioactives was reported in the case of anti-inflammatory effects. Cocoa extract decreased the secretion of monocyte chemoattractant protein 1 (MCP-1) and tumour necrosis factor alpha (TNFα) in activated NR8383 cells more effectively than it was observed for equivalent concentrations of (−)-epicatechin, which is the main antioxidant of cocoa [62]. Also the neuroprotective effects of cocoa appeared to be more pronounced in comparison to those of (−)-epicatechin when applied alone. These effects were seen as a decrease in ROS generation and modulation of c-Jun N-terminal kinase (pJNK) in a human neuroblastoma S-SY5Y cell line [63]. Similar observations were made with regard to oxidative stress biomarkers evaluated in a HepG2 cell line. Martin and co-workers (2008) reported that cocoa extract was more efficient in preventing lipid peroxidation than (−)-epicatechin [64,65].

In conclusion, the findings of our study support the idea of food synergy and demonstrate that the biological effects of samples with complex composition are not just a combination of the activities displayed by individual components. Contrary to intuition, the stepwise enrichment of the model mixtures with antioxidants at concentrations matching those in cocoa powder extract failed to even gradually increase the total antioxidant activity of the samples; also, these model mixtures did not reach the antioxidant potential of cocoa in the cell-free or cellular models. Neither other bioactivities tested in HT29 cells were altered in a stepwise manner that could reflect gradual changes in composition of bioactive ingredients. A similar discrepancy between the biological effects of extracts of berry fruits and of their major isolated component, anthocyanin cyanidin-3-*O*-glucoside, was observed in our other study [16]. In general, what we saw in both research projects, as predicted by the food synergy concept, is that no simple clear-cut relationship between the composition and content of the samples studied could be observed.

From the dietary chemoprevention standpoint, our results suggest that the current approach emphasizing the use of isolated bioactive food components is not likely to match the epidemiological observations made for the whole foods people ingest. This conclusion holds also for cocoa products for which human studies suggest health benefits. The results presented here provide a vital insight that had largely been missing so far: the understanding that to fully exploit the chemopreventive potential of cocoa, whether related to redox regulation of cellular functions or to other molecular mechanisms, the research should involve not individual ingredients, but complex mixtures matching natural composition.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.04.022.

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