Correlation between Antimicrobial, Antioxidant Activity, and Polyphenols of Alkalized/ Nonalkalized Cocoa Powders

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Abstract: Many factors can influence antioxidative and antimicrobial characteristics of plant materials. The quality of cocoa as functional food ingredient is influenced through its processing. The main aim of this study was to test if there is difference in polyphenol content, antioxidant capacity, and antimicrobial activity between nonalkalized and alkalized cocoa powders. To estimate polyphenol and flavonoid content in cocoa samples the spectrophotometric microassays were used. Flavan-3ols were determined with reversed-phase high-performance liquid chromatography (RP-HPLC). Antimicrobial activity against 3 Gram positive bacteria, 4 Gram negative bacteria and 1 strain of yeast was determined using broth microdilution method. Total polyphenol content was 1.8 times lower in alkalized cocoa samples than in natural ones. Epicatechin/catechin ratio was changed due to the process of alkalization in favor of catechin (2.21 in natural and 1.45 in alkalized cocoa powders). Combined results of 3 antioxidative tests (DPPH, FRAP, ABTS) were used for calculation of RACI (Relative Antioxidant Capacity Index) and GAS (Global Antioxidant Score) values that were consistently higher in natural than in alkalized cocoa extracts. Obtained results have shown significant correlations between these values and phenolic content ($0.929 \leq r \leq 0.957$, $P < 0.01$). Antimicrobial activity varied from 5.0 to 25.0 mg/ml (MICs), while *Candida albicans* was the most sensitive tested microorganism. Cocoa powders subjected to alkalization had significantly reduced content of total and specific phenolic compounds and reduced antioxidant capacity ($P < 0.05$), but their antimicrobial activity was equal for *Gram-positive bacteria* or even significantly enhanced for *Gram-negative bacteria*.

Keywords: alkalization, antimicrobial activity, antioxidant activity, cocoa powder, polyphenol content

Practical Application: In this article, phenol content, antioxidant, and antimicrobial activity of alkalized and nonalkalized cocoa powders were described. Since the cocoa powder is very often the object of alkalization, the results here collected demonstrated epimerization flavan-3ols, reduction of phenol content, and antioxidant activity but equal or even enhanced antimicrobial activity of alkalized cocoa powders. Considering antioxidant and antimicrobial properties of analyzed cocoa powders, there are clear opportunities for the development of new cocoa-based functional foods.

Introduction

Theobroma cacao L. has become an important medicinal plant due to its unique chemical composition. Over the past decade the cocoa consumption has attracted considerable research attention because of its various health effects. Furthermore, antioxidant activity of cocoa (Jalil and Ismail 2008) was often reported, and there is some evidence on its antimicrobial activity (Summa and others 2008).

Cocoa powder is rich deposit of polyphenols (phenolic acids and flavonoids). Total polyphenol content of cocoa is significantly higher than in acai berries, blueberries, cranberries, and pomegranate, and consequently antioxidant activity is much higher (Crozier and others 2011). The cocoa powder total polyphenol content is reported to contain 30% of flavonoids (both flavanol monomers such as epicatechin and catechin, and procyanidin oligomers -dimmer to decamer). Given the significant presence of these bioactive compounds and their potential capacity to promote health benefits, cocoa powder is often considered as a functional food (Ackar and others 2013).

Beside antioxidative properties, antimicrobial activity of cocoa powder is also under consideration. Attempts to discover effective and nontoxic antimicrobial agents often focus on plant extracts. Extracts of rosemary, grape and papaya have been studied in terms of antimicrobial effect against various pathogenic bacteria (Moreno and others 2006; Sofi and others 2016). Cocoa bean is one of the possible sources of antibacterial substances. Actually, cocoa ethanolic extract showed antimicrobial activity by inhibiting growth of *E. coli* (Ariza and others 2014). Interestingly, spontaneous aerobic fermentation of cocoa pod husks is probably the reason for formation of different metabolites showing activity against *Gram negative bacteria*, *P. aeruginosa*, and *S. choleraesuis*, and against the basidiomycete fungus *M. perniciosa* and the yeast *S. cerevisiae* (Santos and others 2014). Cocoa antilisterial activity could be due to the positive additive and/or synergistic effects that exist between minor constituents and phenolic compounds present in cocoa extract (Bubonja-Sonje and others 2011).

Many factors can influence antioxidative and antimicrobial characteristics of plant materials. Cocoa powder is often further

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Table 1–Characteristics of the cocoa powders.

Cocoa powder	Manufacturer	Product code	Category	pH[a]	Color description
CP1	Gerkens-Holland	NA-54	Natural	5.73 ± 0.01	Light brown
CP2	De Zaan	11-N100	Natural	5.75 ± 0.02	Yellow-brownish
CP3	Nederland	PN11	Natural	5.72 ± 0.02	Light brown
CP4	Nederland	PNGH11	Natural	5.65 ± 0.06	Light brown
CP5	De Zaan	N11N	Natural	5.60 ± 0.04	Yellowish-brown
CP6	De Zaan	11-N-003	Natural	5.48 ± 0.03	Yellow-brownish
CP7	Nederland	PARR11	Alkalized	7.66 ± 0.01	Dark red
CP8	De Zaan	D-11-V	Alkalized	7.50 ± 0.02	Red
CP9	De Zaan	D-11-B	Alkalized	8.04 ± 0.05	Black
CP10	Nederland	PARN11	Alkalized	7.03 ± 0.01	Red/brown
CP11	De Zaan	D-11-MR	Alkalized	7.39 ± 0.04	Red

[a]Data are expressed as means± SD, $n = 3$.
CP, cocoa powder.

processed, such as being alkalized. Treatment with alkali, also known as Dutching, is very frequent modification of cocoa powder. This is a 180-y-old process that means washing with an alkaline solution which neutralizes cocoa's acidity to a pH of 7. The process of cocoa powder alkalization changes its characteristics by darkening its color, reducing the bitterness of its ingredients and increasing its solubility. Namely, Dutch processed cocoa has a more intense "chocolatey" flavor, while natural cocoa looks lighter in color and tastes slightly astringent. Alkalized cocoa powder is mainly used in making dessert sauces, stirred custards, ice cream, pudding, cocoa beverages, toppings, and the like. There are some reports on the influence of cocoa powder alkalization on polyphenol content and they indicate that polyphenol content is being significantly reduced (Miller and others 2008).

Because many factors during cocoa manufacturing process can affect its final properties and the functionality of the commercial products, the main aim of this paper was to test if there is difference in polyphenol content, antioxidant capacity, as well as antimicrobial activity between nonalkalized and alkalized cocoa powders. The second aim was to estimate the correlations between phenol content, antioxidant and antimicrobial activity of analyzed cocoa powders.

Materials and Methods

Samples

Eleven different commercial cocoa powders, 6 nonalkalized (natural) and 5 alkalized, were raw materials intended for confectionery industry and were obtained from leading Serbian chocolate manufactures. Each commercial cocoa powder was sampled in triplicate. Characteristics of the samples are presented in Table 1.

Chemicals

The various chemicals, standards and reagents used in this study were of analytical grade and were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Luis, Mo., U.S.A.) and Acros Organics (N.J., U.S.A.). Mueller-Hinton broth and Sabouraud dextrose broth were from Difco Laboratories (Md., U.S.A.). Antibiotic supplement was obtained from Oxoid Thermo Scientific (Hampshire, UK). The microbial strains were purchased from Microbiologics Inc. (St. Cloud, Minn., U.S.A.) as lyophilized microorganisms and were stored at 4 °C until the day of culturing them.

Sample preparation

Cocoa extracts were prepared according to the procedure described by Hammerstone and others (1999) with some modifications. Exactly 2.0 g of each cocoa product was extracted 3 times with 10 mL of *n*-hexane, in order to eliminate lipids from the samples. Defatted samples were air-dried for 24 h. Polyphenol-rich fraction was extracted from defatted cocoa products by acetone + distilled water + acetic acid (70 + 29.8 + 0.2 v/v/v). Extractions were carried out twice with 5 mL of solvent for 30 min in FALC ultrasonic bath (Treviglio, Italy). The mixtures were centrifuged (Janetzki T32 C, Wallhausen, Germany) for 10 min at 3000 rpm after each extraction and the supernatants were decanted. After filtration, the supernatants were combined. For antioxidant activity combined supernatants were filled up to obtain 10 mL in a flask. For antimicrobial activity solvent was evaporated under reduced pressure to dryness. Dry cocoa extracts residues were then weighted and dissolved in dimethylsulfoxid solution in concentration of 100 mg/mL.

pH determination

The pH of each dispersed cocoa powder suspension (1 part powder and 9 parts deionized water (Miller and others 2008)) was determined at room temperature (21.5 °C) using electrode SenTix 81 of a pH-Meter pH526 MultiCal® (WTW, Weilheim, Germany) placed directly into each suspension.

Determination of total polyphenol (TP) content

Total polyphenol (TP) content of cocoa extracts was determined using the rapid microtitre plate Follin-Ciocalteau method (Attard 2013). Briefly, 10 μL aliquot of sample (appropriately diluted) and gallic acid dilutions were pipetted in triplicate in wells of Micro Titre Plate (MTP). The repeated volumes of 1:10 diluted Folin–Ciocalteu's reagent (100 μL), and 1 M Na_2CO_3 (80 μL) were transferred to wells. The reaction mixtures were allowed 1h to incubate at room temperature in the dark and then were analyzed at 630 nm against a blank sample on MTP reader (BIOTEK, USA, ELx800 Absorbance Microplate Reader). Gallic acid was used as the standard and the results were expressed as mg Gallic Acid Equivalents (GAE) per gram of sample. Calibration curve ($y = 0.002x + 0.0003$) was prepared for the working solutions of Gallic Acid in the concentration range of 0 to 80 mg/mL and showed good linearity ($r = 0.9996$).

Determination of total flavonoid (TF) content

TF content of cocoa extracts was evaluated using a colorimetric assay with aluminum chloride (Zhishen and others 1999) and was

expressed as mg Catechin Equivalents (CE)/g. A 10 μL aliquot of sample (appropriately diluted) and catechin dilutions were added to 200 μL of distilled water and 30 μL of the 5% $NaNO_2$ solution in wells of MTP in triplicate. After 5 min at 37 °C, 30 μL of the 10% $AlCl_3$ solution was added and shaken up; then 6 min later, 20 μL of the 1M NaOH solution was added to the mixture. The reaction mixture was stirred and absorbance was measured on MTP reader at 490 nm as compared to the blank solution. A standard curve ($y = 0.0005x + 0.036$) was developed using a 1000 μM solution of catechin at intervals of 200 μM catechin concentration, and showed good linearity ($r = 0.9960$).

Quantitative determination of proanthocyanidins (PA)

The PA content of cocoa extracts was determined with butanol/HCl assay described by Porter and others (1986), with some modifications. In short, 0.2 mL of cocoa extract was mixed with 6 mL of n-BuOH-conc. HCl solution (70:30, v/v) and 0.3 mL of a 2% solution of $NH_4Fe(SO_4)_2$ x $12H_2O$ in 2 M HCl. Tube with carefully mixed solution were capped and heated for 45 min at 95 °C in Memmert water bath (Büchenbach, Germany). The sample was cooled to room temperature and the visible spectrum recorded at $\lambda = 550$ nm. The blank value of the BuOH-HCl-FeIII solvent was subtracted. The quantity of proanthocyanidins was determined from a standard curve of cyanidin chloride and expressed as mg Cyanidin Chloride Equivalents (CyE)/g of cocoa powder. The range 0.05 to 0.3 mg/mL of standard solutions was used for calibration curve with equation: $y = 3.7248x + 0.0129$ and it showed very good linearity ($r = 0.9987$).

HPLC determination of monomer flavan-3ols content

High pressure liquid chromatography (HPLC) analyses of monomer flavan-3ols was performed using Varian LC Star System with Star Solvent Delivery System 9010, Injector Rheodine 7125, Polychrom 9065 (UV-Diode Array Detector), $\lambda = 280$ nm (Palo Alto, Calif., U.S.A.). The extracts were filtered through 0.45 μm filter and separated on RP-LC column (Waters Spherisorb ODS. 250 mm × 4 mm. 5 μm I.D., Milford, Conn., U.S.A.) using the mobile phase: (A) water:acetic acid (97.5:2.5. v/v); (B) acetonitrile, in gradient elution: 0 min 97% A; 0 to 13 min 91% A; 13 to 18 min 89% A; 18 to 25 min 82% A; 25 to 45 min 70% A; and 45 to 50 min 97% A. The detection was performed with UV-PDA detector at 278 nm (Guyot and others 1998). Comparing UV spectra and retention times of the separated peaks with the retention times of the standards was used for the identification of the compounds. Flavan-3ols were quantified using the external standard method ((-)-epicatechine and (+)-catechine standards). Calibration curve for (-)-epicatechine was $y = 3E{+}07x - 129121$ ($r = 0.9999$). It was developed using a range 0.1 to 0.35 mg/mL of (-)-epicatechine. For (+)-catechine, calibration curve was $y = 2E{+}07x - 594309$ for range 0.1 to 0.35 mg/mL of (+)-catechine ($r = 0.9954$).

Antioxidant activity determinations

DPPH radical scavenging microassay. Antioxidant capacity of cocoa extracts was determined using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging microassay described by Melendez and others (2014) with some modifications. So, 7 μL of aliquoted samples (appropriately diluted) and Trolox dilutions were added to 193 μL of the DPPH radical solution in wells of MTP in triplicate and the reaction mixture was shaken vigorously. The free radical scavenging capacity was evaluated by measuring the absorbance at 490 nm after 1h of reaction at room temperature on MTP reader. Calibration curve ($y = 44.626x + 1.6538$), in the range 0.2 to 0.7 mmol Trolox mL^{-1} was used for the quantification of antioxidant activity showing good linearity ($r = 0.9922$). Results are expressed in μM Trolox Equivalents (TE)/g of cocoa powder.

FRAP microassay. The microplate ferric ion reducing antioxidant power (FRAP) assay was done according to Bolanos and others (2015) with some modifications. Firstly, the FRAP fresh working solution was prepared by mixing 10-volumes of 300 mM acetate buffer (pH 3.6) with 1-volume of 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl and 1-volume of 20 mM $FeCl_3x6H_2O$ solution and then warmed at 37 °C for 10 min prior to use. Aliquoted samples (20 μL) and Trolox dilutions were allowed to react with 280 μL of the FRAP working solution in 96-well microplate in triplicate. The mixtures were shaken and incubated at 37 °C for 30 min in dark conditions. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 630 nm using MTP reader. The antioxidant activity was calculated from calibration curve ($y = 1.0965x + 0.0486$) in the range 0.1 to 0.8 mmol Trolox mL^{-1} and with good linearity ($r = 0.9996$). Results are expressed in μM Trolox Equivalents (TE)/g of cocoa powder.

ABTS (TEAC) radical scavenging microassay. The Trolox equivalent antioxidant capacity (TEAC) of cocoa extracts was estimated by the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical action decolorization assay adapted to a microplate reader by Pastoriza and others (2011). Stock solutions of ABTS (7 mM) and potassium peroxodisulfate (2.45 mM) in phosphate buffer (pH 7.4) were prepared and mixed together in equal volumes. The mixture was left to react at room temperature, overnight (12 to 16 h) in the dark to allow free radical generation and was then diluted with phosphate buffer (1: 80, v/v). To determine the scavenging activity, 20 μL of aliquoted samples and Trolox dilutions and 280 μL of the ABTS radical solution were placed in 96-well microplate in triplicate and after exactly 6 min the absorbance readings were taken using MTP reader at 630 nm. Calibration curve ($y = 510.11x - 3.3025$) was developed using a range 0.2 to 1.5 mmol Trolox mL^{-1} and showed good linearity ($r = 0.9946$). The results were expressed as μM Equivalents of Trolox (TE) per gram of cocoa powder.

Relative antioxidant capacity index (RACI) and Global Antioxidant Score (GAS). Obtained antioxidant capacity results derived from different chemical methods (DPPH, FRAP, and ABTS) allowed calculating the Relative Antioxidant Capacity Index and Global Antioxidant Score. RACI value was created by averaging the standard scores transformed from the raw data. After data transformation, the standard scores (without any units) from various methods with different distributions will have similar normal distributions with a mean of 0 and variance of 1 (Sun and Tanumihardjo 2007). The standard score is calculated as follows:$(x - \mu)/\sigma$, where x is the raw data, μ is the mean, and σ is the standard deviation. Also for each cocoa powder, the average of 3 T-scores was taken for the value of GAS ranging from 0 to 3. *T-score* is calculated by the following equation: $T - score = (X - min)/(max - min)$, where *min* and *max*, respectively, represent the smallest and largest values of variable X among the cocoa powders of the same extract groups (Leeuw and others 2014).

Antimicrobial activity determinations

Microbial strains and preparation of the standardized microbial suspension. The antimicrobial activity of cocoa

Table 2–Total polyphenol, flavonoid, proanthocyanidin content and antioxidant capacity of cocoa powder extracts.

Cocoa powder	TP (mg GAE/g)	TF (mg CE/g)	PA (mg CyE/g)	DPPH (μM TE/g)	FRAP (μM TE/g)	ABTS (μM TE/g)
CP1	34.34 ± 1.99	28.27 ± 0.41	6.76 ± 0.78	253.3 ± 5.3	426.7 ± 2.9	260.0 ± 14.4
CP2	34.69 ± 1.46	24.64 ± 0.77	6.49 ± 0.89	322.6 ± 11.9	385.4 ± 3.2	253.8 ± 6.8
CP3	41.55 ± 1.71	27.64 ± 0.47	8.51 ± 0.95	352.8 ± 9.9	454.7 ± 2.1	262.3 ± 9.03
CP4	36.14 ± 2.79	28.40 ± 0.57	6.57 ± 0.52	386.6 ± 5.3	417.1 ± 1.6	250.7 ± 7.4
CP5	26.83 ± 3.25	22.90 ± 0.72	7.43 ± 087	328.5 ± 3.6	383.0 ± 3.3	251.5 ± 0.6
CP6	32.58 ± 2.38	21.45 ± 0.54	7.38 ± 0.66	306.0 ± 3.1	347.8 ± 1.6	227.4 ± 7.0
Mean ± SD	34.35 ± 4.79	25.55 ± 2.98	7.19 ± 0.77	324.5. ± 44.9	402.4 ± 37.9	250.9 ± 12.4
CP7	19.83 ± 1.61	16.18 ± 0.36	1.09 ± 0.05	198.8 ± 6.6	211.5 ± 2.1	149.8 ± 3.8
CP8	22.21 ± 1.43	16.13 ± 0.27	2.81 ± 0.12	267.2 ± 5.7	262.8 ± 2.3	169.1 ± 4.2
CP9	10.67 ± 2.36	8.01 ± 0.44	0.95 ± 0.08	128.1 ± 3.8	110.1 ± 1.1	112.6 ± 5.1
CP10	24.92 ± 1.47	18.40 ± 0.36	4.71 ± 0.51	271.2 ± 5.9	309.4 ± 2.1	227.7 ± 1.8
CP11	15.94 ± 0.99	13.12 ± 0.41	2.57 ± 0.32	180.4 ± 10.1	201.1 ± 1.6	149.3 ± 3.8
Mean ± SD	18.71 ± 5.58*	14.38 ± 4.02*	2.43 ± 1.53*	209.1 ± 60.7*	219.0 ± 74.7*	161.7 ± 42.2*

Data are expressed as the mean ± SD, $n = 3$.
*Statistically significant difference between natural and alkalized cocoa powders, $P < 0.05$.
SD, standard deviation; CP, cocoa powder; TP, total polyphenol; TF, total flavonoid; PA, proantocyanidins; GAE, gallic acid equivalents; CE, catechin equivalents; CyE, cyanidin chloride equivalents; DPPH-2, 2-diphenyl-1-picrylhydrazyl; FRAP, ferric ion reducing antioxidant power; ABTS (TEAC), trolox equivalent antioxidant capacity; TE, trolox equivalents.

polyphenol extracts was evaluated using 8 laboratory control strains of microorganisms. These included *Gram positive bacteria*: *Staphylococcus aureus* (ATCC (American Type Culture Collection) 25923), *Staphylococcus epidermidis* (ATCC 1228), *Bacillus subtilis* (ATCC 6633); *Gram negative bacteria*: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella abony* (NCTC [National Collection of Type Cultures] 6017); 1 strain of *yeast*: *Candida albicans* (ATCC 10231). The tested microbial strains were prepared by transferring a loopful of cells from the stock into tubes that contained 10 mL of Mueller-Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for yeast. After incubation for 24 h at 37 °C and 25 °C, a density of bacteria/yeast cultures was adjusted to 0.5 MacFarland turbidity standards and diluted with fresh Mueller-Hinton and Sabouraud dextrose broth in order to achieve optical densities corresponding to 2.5×10^6 colony forming units (CFU)/mL for bacteria and 1.2×10^7 CFU/mL for yeast and used as the inoculum.

Minimal inhibitory concentration (MIC). Minimal inhibitory concentrations of the cocoa extracts were determined using a standard microdilution technique in MHB according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2009). All microbial tests were performed in duplicate as well as 1 growth control and 1 sterility control for each test batch. The MIC of ampicillin and flavan-3ols (epicatechin and catechin) was individually determined in parallel experiments in order to control the sensitivity of the test organisms. MIC values were taken into consideration as the lowest concentration of extract (the highest dilution) that produced no visible microbial growth (at binocular microscope) in comparison with the control wells after incubation at 35 °C for 24 h (bacteria) or 48 to 72 h (yeast) (Savran and others 2016).

Statistical analyses

All determinations were performed in triplicate. Results are expressed as mean values with the corresponding standard deviations (SD). Homogeneity of variance was first assessed using Levene's test. Data were compared using Independent-samples *t*-test. Pearson's correlation coefficient was used to calculate the correlations between data. Statistical analyses were completed using the computer program SPSS (version 20, Chicago, Ill., U.S.A.) and a *P* value <0.05 was considered statistically significant.

Results and Discussion

Color and pH value of cocoa powders

Color and pH value of analyzed cocoa powders are shown in Table 1. When we examined natural cocoa powders, the average pH value was 5.61 without major differences between the groups, and the colors varied between different shades of light brown. As expected, all alkalized cocoa samples had pH values >7 with large variations, while 1 sample (CP9) had a high pH value of 8.04. Higher pH values of alkalized cocoa samples were accompanied by their intense colors that ranged from dark brown through red to black. These changes in pH values and colors observed in our samples correspond to changes reported by Miller and others (2008) who categorized process of alkalization as "light" (our sample CP10 correspond to this category), "medium" (samples CP8 and CP11), and "heavy" (samples CP7 and CP9). Several studies, including ours, suggest that a simple visual assessment of the cocoa powder color may provide information on the level of powder alkalization.

Polyphenol content of cocoa extracts

Table 2 presents the total polyhenol (TP), flavonoid (TF), and proanthocyanidin (PA) content determined in acetone extracts of tested 6 natural and 5 alkalized cocoa powders. Ultrasonic-assisted extraction was used because it gives significantly higher efficacy in comparison with the standard procedures (Carrillo-Hormaza and others 2016).

TP content ranged from 41.55 mg GAE/g in natural cocoa powder CP3 to 10.67 mg GAE/g in alkalized cocoa powder CP9, while TF content of the same extracts was 27.64 mg CE/g in CP3 and 8.01 mg CE/g in CP9. Since proanthocyanidins with flavan-3ols are the main flavonoids of cocoa products, it was of interest to analyze their content in different cocoa powders in this survey. The content of proanthocyanidins varied between 8.51 mg CyE/g in CP3 and 0.95 mg CyE/g in CP9 (Table 2).

Total polyphenol content was 1.8 times lower in alkalized cocoa samples than in natural cocoa samples. When we compared results for TF and PA content, a 0.6-fold lower values for average TF content, and 0.3-fold for average PA content in alkalized cocoa powders was noticed. The lowest values of TP, TF, and PA content were determined in alkalized cocoa powder CP9 that had the highest pH value and almost black color.

Table 3–Monomer flavan-3ol content in cocoa powder extracts.

Cocoa powder	Epicatechin (mg/g)	Catechin (mg/g)
CP1	1.36 ± 0.03	0.63 ± 0.01
CP2	1.37 ± 0.03	0.62 ± 0.007
CP3	1.81 ± 0.02	0.69 ± 0.01
CP4	1.33 ± 0.02	0.63 ± 0.01
CP5	1.46 ± 0.02	0.67 ± 0.03
CP6	1.29 ± 0.02	0.65 ± 0.03
Mean ± SD	1.44 ± 0.19	0.65 ± 0.03
CP7	0.55 ± 0.02	0.36 ± 0.009
CP8	0.65 ± 0.02	0.38 ± 0.01
CP9	0.30 ± 0.01	0.25 ± 0.02
CP10	0.75 ± 0.03	0.41 ± 0.02
CP11	0.56 ± 0.02	0.40 ± 0.02
Mean ± SD	0.54 ± 0.17*	0.37 ± 0.04*

Data are expressed as the mean ± SD, $n = 3$.
*Statistically significant difference between natural and alkalized cocoa powders, $P < 0.05$.
SD, standard deviation; CP, cocoa powder.

It is well known that polyphenols are unstable in alkaline conditions and oxidative degradation is likely to occur (Zhu and others 2003). Previous results concerning the change in total polyphenol content due to alkalization process support the conclusion that alkalization lowers the amount of polyphenols in cocoa, but the observed decrease varied significantly from 20.4% (Stanley and others 2015) to 60.5% (Miller and others 2008). Results obtained in this study showed on average 45.5% lower levels of total phenolics in alkalized compared with natural cocoa powders. Similar changes were registered for total flavonoid content in our study, but also in the study of Andres-Lacueva and others (2008). When it comes to proanthocyanidins, on average 67.1% lower levels in alkalized compared with natural cocoa powders were detected. These changes could result from 2 possible processes: their monomerization during the process of alkalization (Ortega and others 2008) or their transformation into quinones and further condensation and higher degree polymerization (Li and others 2014). Thus, the alkalizing process significantly influences the content of polyphenols, flavonoids and proanthocyanidins in final cocoa powders. It is interesting that regulations in the U.S. require labeling of cocoa powder that was subjected to alkalization as "cocoa treated with alkali" (FDA 2007). Obtained results suggest that mandatory labeling of cocoa powder type can give important information to the consumer about the quality and functionality of cocoa-based foods.

Concentrations of 2 major monomer flavan-3ols, epicatechin and catechin, are summarized in Table 3. A trend that has appeared for other polyphenol classes was also present in flavan-3ols, so alkalized cocoa powders contain less epicatechin and catechin. Interestingly, the reduction in the case of epicatechin was 62.5%, but in the case of catechin it was only 43.1%. Epicatechin/catechin ratio was changed in favor of catechin (2.21 in natural and 1.45 in alkalized cocoa powders). These patterns could be explained by epimerization, which occurs due to the effects of heat and alkali during the process of alkalization and that was previously reported for green tea catechins (Wang and Helliwell 2000). Second potential explanation is that under the conditions of alkalization process (high temperature, high pH) epicatechin as a cis-isomer is less stable than trans isomer catechin. Nakagawa (1967) has proved that tea catechin is more stable under the high temperature treatment, while Theppakorn (2016) has also proved tea catechin as more stable under the fermentation and drying conditions. Mazor Jolic and others (2011) have even reported increased catechin content because of an alkalization process as a result of epimerization. Epimerization process is disadvantageous, considering that there is data of a higher biological activity of epicatechin in final cocoa products (Donovan and others 2006).

Antioxidant activity of cocoa extracts

Clinical studies have shown that elevated levels of reactive oxygen species (ROS) or oxidative damage which they cause, can have direct connection with number of diseases (Halliwell and Gutteridge 2007). There is an inverse association between consumption of plant rich diet and the risk of these diseases and one of the reasons is high content of antioxidative compounds in plant material. In this respect, determining of the antioxidant capacity of different plant-derived products is the focus of research efforts. In the process of evaluating the antioxidant potential of certain foods and beverages, the best conclusion could be drawn with a combination of several tests that are based on different principles to demonstrate antioxidant potential of the analyzed material through different mechanisms of action (Mocan and others 2016; Llorent-Martınez and others 2016). DPPH, FRAP, and ABTS are the most common methods for determining in vitro antioxidant capacity. DPPH assay is based on the simplest mechanism of antioxidant protection (DPPH • Antioxidant + → DPPH-H +• Antioxidants). FRAP method is based on the ability of phenolic compounds, dissolved in water, to reduce Fe^{3+} to Fe^{2+}. In contrast to the inhibition of DPPH radical, which is suitable for antioxidants soluble in the organic solvents (ethanol, methanol), FRAP method determines the antioxidant properties of antioxidants soluble in water (Dai and others 2010). ABTS method is basically a measurement of the percentage of neutralization of ABTS radical in comparison with the Trolox standard. In this study, all 3 antioxidant assays were applied for the evaluation of antioxidant capacity of cocoa powders and the obtained results are shown in Table 2.

The results showed very strong correlations between all 3 assays as well as with TP, TF, PA, and flavan-3ols content ($0.856 \leq r \leq 0.980$, $P < 0.01$). They indicate that these 3 assays were appropriate for cocoa powder as a matrix. The cocoa polyphenol content affecting the antioxidant activity is dependent on the cocoa-processing techniques, such as fermentation, alkalization, roasting and even bean species used (Arteel and others 2000). By examining the antioxidant capacity in terms of alkalization process, we have demonstrated similar trend in different cocoa powders by applied assays. Importantly, antioxidant capacity was 1.6 (DPPH and ABTS) and 1.8 (FRAP) times lower in alkalized cocoa samples than in natural cocoa samples. Sample of alkalized cocoa powder CP9 that had the lowest polyphenol, flavonoid and proanthocyanidin content compared to other analyzed cocoa powders, consequently had the lowest antioxidant capacities measured with all 3 tests.

Combination of results obtained in 3 antioxidant assays enabled the calculation of Relative Antioxidant Capacity Index and Global Antioxidant Score. Since individual assays cover various segments of antioxidant action, their combination has certain advantages. We applied 2 statistical-mathematical models to provide a more complete profile of antioxidant capacity of natural and alkalized cocoa powders. As seen in Figure 1A, positive values of RACI have been ascribed to all natural while negative values of RACI have corresponded to alkalized cocoa powders. The same order was obtained with Global Antioxidant Score (GAS) (Figure 1B). GAS values were in the range between 2.14 and 2.87 in natural cocoa extracts and were consistently higher than in alkalized ones

(0 to 1.90). Significant correlations were found between these values and phenolic content ($0.929 \leq r \leq 0.957$, $P < 0.01$).

There are available data on RACI values of different vegetables (Sun and Tanumihardjo 2007) and cultivated plants from Serbia (Petrović and others 2016). In the study of Leeuw and others (2014), GAS values were used for comparison of antioxidant capacity of red wines. Therefore, analyzing the results of multiple antioxidant assays using several different statistical-mathematical models is a valuable novel method that opens new avenues in expression of antioxidant capacity of foods and beverages. Both of the calculated RACI and GAS values represent a ranking tool can be useful in the field of science and industry as well as for consumers.

Antimicrobial activity of cocoa extracts

The results for antimicrobial activity of natural and alkalized cocoa powder extracts are summarized in Table 4.

Analyzed extracts had antimicrobial activity against *Gram positive* and *Gram negative bacteria* as well as *Candida albicans,* determined using broth microdilution method, in the range of MICs from 5.0 to 25.0 mg/mL. Among tested microbial strains, yeast *Candida albicans* was the most sensitive microorganism with the lowest MIC value of 5.0 mg/mL. Obtained results for bacteria showed that analyzed cocoa extracts (predominantly of natural cocoa powders) had slightly higher potential against *Gram positive bacteria*. MIC values obtained for *Gram positive bacteria* have not shown correlations with different phenol (TP, TF, PA, flavan-3ols) content, while MIC values for *Gram negative bacteria* were in good correlations with mentioned phenol content ($0.634 \leq r \leq 0.777$, $P < 0.05$).

In order to evaluate the relative antibacterial efficacy of cocoa extracts the same test was performed for commercial antibiotic ampicillin, as well as for purified main polyphenol cocoa compounds, epicatechin and catechin. The results from Table 4 showed that efficacy of cocoa powder extracts was much lower than synthetic antibiotic (3.5×10^3 times) and purified flavan-3ols (70 times). On the other hand, if we compare obtained results with data for some herbal extracts from the literature, antimicrobial activity of cocoa powder extracts against *Staphylococcus aureus* and *Escherichia coli* is similar to the extracts of oregano, sage, clove, rosemary and celery, that are known as good antimicrobial agents in traditional medicine (Witkowska and others 2013).

When it comes to the process of alkalization, no significant differences between antimicrobial activity against *Gram positive bacteria* were observed. However, comparison of the MIC values obtained for *Gram negative bacteria* showed that alkalized cocoa powder extracts had enhanced antimicrobial activity in comparison with nonalkalized ones. This is the most important findings of this research. Our reported data suggest that distinct activities of cocoa powder are not consistently reduced by phenol losses as a result of alkalization process. A similar observation was reported by Ryan and others (2016), who demonstrated that the loss of native flavanols during cocoa roasting and fermentation is not necessarily connected with the reduction in the digestive

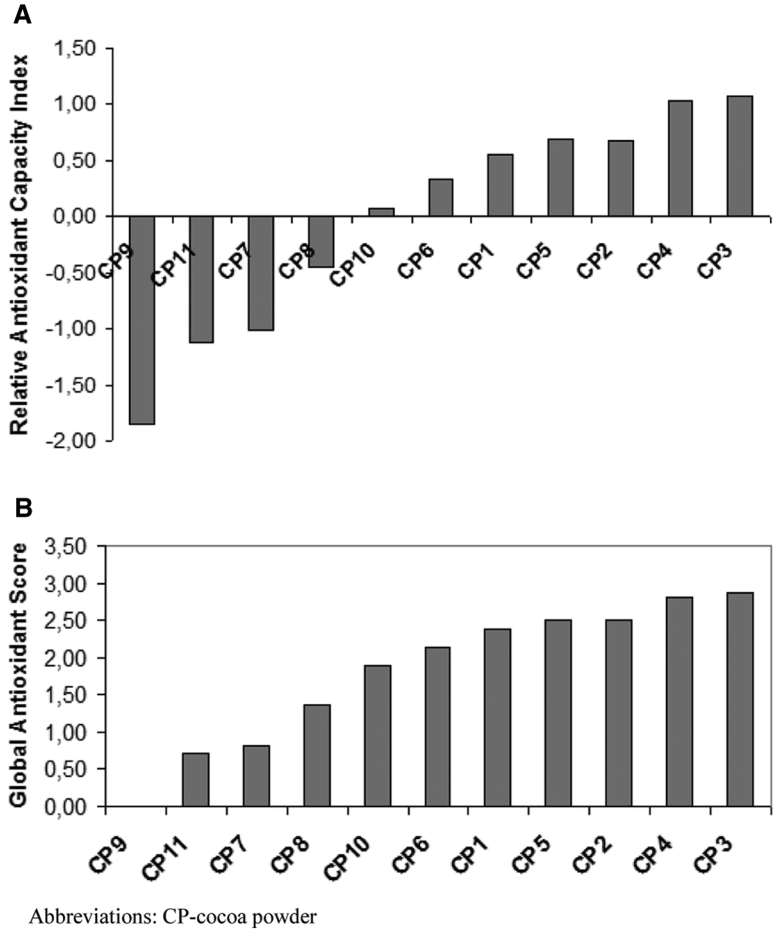

Figure 1–Relative Antioxidant Capacity Index (RACI) of cocoa powder extracts (A); Global Antioxidant Score (GAS) of cocoa powder extracts (B).

Table 4–Minimal inhibitory concentration – (MIC[a]) of cocoa powder extracts.

Cocoa powder	Staphylococcus aureus (ATCC 25923)	Staphylococcus epidermidis (ATCC 1228)	Bacillus subtilis (ATCC 6633)	Escherichia coli (ATCC 25922)	Klebsiella pneumoniae (ATCC 13883)	Pseudomonas aeruginosa (ATCC 27853)	Salmonella abony (NCTC 6017)	Candida albicans (ATCC 10231)
CP1	12.5	7.5	12.5	17.5	12.5	12.5	17.5	5.0
CP2	7.5	7.5	12.5	17.5	17.5	12.5	17.5	5.0
CP3	7.5	5.0	12.5	17.5	17.5	17.5	17.5	5.0
CP4	12.5	25.0	12.5	25.0	25.0	25.0	25.0	5.0
CP5	25.0	25.0	25.0	17.5	25.0	25.0	25.0	5.0
CP6	12.5	12.5	12.5	17.5	17.5	17.5	17.5	5.0
Mean ± SD	12.9 ± 6.4	13.7 ± 9.0	14.6 ± 5.1	18.7 ± 3.1	19.2 ± 4.9	18.3 ± 5.6	20.0 ± 3.9	5.0 ± 0.0
CP7	5.0	7.5	7.5	7.5	7.5	7.5	7.5	5.0
CP8	5.0	7.5	7.5	7.5	7.5	7.5	7.5	5.0
CP9	12.5	12.5	12.5	12.5	12.5	12.5	12.5	5.0
CP10	7.5	12.5	12.5	12.5	12.5	12.5	17.5	5.0
CP11	12.5	12.5	12.5	12.5	7.5	7.5	12.5	5.0
Mean ± SD	8.5 ± 3.8	10.5 ± 2.7	10.5 ± 2.7	10.5 ± 2.7*	9.5 ± 2.7*	9.5 ± 2.7*	11.5 ± 4.2*	5.0 ± 0.0
Ampicillin	0.5	1.5	1.8	2.0	4.9	7.4	n.t.	6.2^{Nys}
Epicatechin	500	250	250	500	250	500	500	125
Catechin	500	250	250	500	500	500	250	125

[a]Values given as mg/mL (for the cocoa extracts) and as μg/mL (for antibiotic and flavan-3ols).
*statistically significant difference between natural and alkalized cocoa powders, $P < 0.05$.
CP, cocoa powder; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; *Nys*, *Nystatin*; n.t., not tested.

enzymes inhibition. A potential explanation for these observations is the formation of new bioactive compounds during alkalization that have prominent antimicrobial but not antioxidant activity. Li and others (2012) found more than 80 volatile compounds in the cocoa mass after alkalization process. Some of them were studied for their antimicrobial characteristics. Namely, Lu and others (2016) have concluded that cyclic terpene limonene possess a higher antimicrobial potential against *Gram negative* compared with *Gram positive bacteria*. Further, aldehydes and ketones, flavor related components that increase after alkalization of cocoa powder, are known as electronegative compounds that may interfere in biological processes involving electron transfer and react with vital nitrogen components, for example proteins and nucleic acids and therefore inhibit the growth of the microorganisms (Dorman and Deans 2000). These data suggest a necessity for future detailed studies that would allow detection of new antimicrobial compounds potentially formed from cocoa powders during the alkalization process. Also, further studies are needed to elucidate the mechanisms of antibacterial capacity of cocoa extracts. Polyphenols were found to interact with bacterial cell membranes, resulting in disruption of the membrane functions or reducing membrane fluidity. It has been proved that flavan-3ols, epicatechin and catechin, are negatively charged and strongly bind to the positively charged lipid bilayer of *Gram positive bacteria* and their partitioning in the lipid bilayer membrane results in loss of cell structure and function leading to cell death (Koech and others 2013). Also, 1 possible mechanism of the bactericidal effects of polyphenols might involve the inhibition of enzymes or interference with the production of certain amino acids necessary for the bacterial growth.

Conclusion

From the results of this study it can be concluded that alkalized cocoa powders have lower polyphenol content and antioxidant activity in comparison to natural powders, while their antimicrobial potency is preserved or even enhanced by the alkalization process. All analyzed extracts are good antimicrobial agents for *Candida albicans* and this phenomenon requires further investigation on a larger set of different strains of this yeast. There are clear opportunities for the development of new cocoa-based functional foods with relevant antioxidant and antimicrobial properties, but the type of cocoa powder is of significant importance for the functionality of final products.

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Authors' Contributions

V. Todorovic was responsible for the elaboration of all the data and drafted the manuscript. M. Milenkovic and B. Vidovic contributed to the conception of the study and interpreted the results. Zoran Todorovic revised the manuscript. Sladjana Sobajic approved the final version of manuscript.

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