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Evaluation of Antiradical Activity of Different Cocoa and Chocolate Products: Relation with Lipid and Protein Composition

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ABSTRACT Chocolate antioxidant properties are often claimed; however, they are frequently different from the parent natural sources due to the industry or artisan transformation. In particular, antioxidant property of chocolate and cocoa are not adequately taken into consideration by consumers who normally make use of this food just for its flavor and taste properties. In this study, we have investigated the antioxidant capacity and total phenolic content of cocoa nibs, cocoa masses, and corresponding chocolate bars with different percentages of cocoa from different origins. The antioxidant capacity of the different samples was measured by two different assays [1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) and ferric reducing antioxidant of potency (FRAP) tests]. The Folin–Ciocalteu reagent was used to assess the total phenolic content. The masses showed a higher antioxidant power than the nibs, and this has been attributed to the fact that in the nibs is still present the lipid part, which will form the cocoa butter. The influence of milk, whey, and soy proteins was also investigated. Our results showed that the extra dark cocoa bar, 100% cocoa chocolate, is the best in terms of total polyphenol content and in terms of antioxidant capacity according to the DPPH and FRAP tests. In addition, the bars of organic dark chocolate 80%, dark Tanzania 80%, and Trinidad 80% products are well performing in all respects. As highlighted by us, the antiradical properties of cocoa products are higher than many antioxidant supplements in tablets.

KEY WORDS: • ***DPPH and FRAP tests*** • ***Folin–Ciocalteu test*** • ***lipids*** • ***milk*** • ***phenolic content*** • ***proteins*** • ***soy*** • ***whey***

INTRODUCTION

POLYPHENOLS ARE IMPORTANT for human health for their biological properties and in the prevention of age-related diseases, including cardiovascular disease and cancer.[1] Dietary flavonoids derived from fruits, vegetables, red wine, and green tea decrease the risk of death from stroke.[2] Cocoa-derived products have been identified to be rich in flavonoids, particularly flavan-3-ol (-)-epicatechin (epicatechin) and its polymers[3,4] and they may act as potent antioxidants.[5,6] Recent research has indicated that the flavanols found in cocoa and chocolate products are associated with short- and long-term health benefits, including reduced oxidation of LDL cholesterol, reduced platelet aggregation, increased arterial blood flow, and decreased blood pressure.[7,8] The antioxidant activity of cocoa powder is well known, but it's physics and chemistry are complex, change occurs during the lifetime of the bean, and activity often depends on its processing.[9,10] Factors affecting the quality and quantity of cocoa and cocoa-based products, during production and manufacturing, are of great importance in delivering the best health effects; these factors could significantly reduce the polyphenol content and activity of the selected products. The content of polyphenols can vary greatly depending on the source of beans, primary and secondary processing conditions, and packaging and processing of chocolate making. Due to these factors, the ratio and types of polyphenols found in cocoa beans, as well as their activity, are unlikely to be the same as those found in the finished products.[11] In addition, alkalization (or dutching) of cocoa powder will reduce the overall polyphenol content and antioxidant activity.[3,12] Recipes also influence the properties of chocolate; the basic ingredients required for the manufacturing of chocolate are cocoa liquor, cocoa butter, sugar, other sweeteners, milk powder, and emulsifiers. Different percentages of nonfat cocoa solids and cocoa butter, sugar, and milk powder are used in making different types of chocolates, namely, dark chocolate, milk chocolate, and white chocolate; theoretically, the higher amount of nonfat cocoa solid indicates the higher phenolic content in the chocolates.[13,14] Several cocoa products (*i.e.*, dietetic bars) are enriched with proteins from different origin (*i.e.*, milk, soy, and whey) to obtain supplementation in sports and as a meal substitute. The aim of this study was to evaluate the polyphenol content and their antioxidant activity, in the presence of different proteins

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that may influence phenol availability, through the preparation of extracts from a selection of cocoa nibs, masses, and chocolate bars from different countries and with different percentages of cocoa content. To have common parameters, the chocolate bars were harvested from a single manufacturer who was also asked to provide the starting cocoa mass and cocoa nibs.

MATERIALS AND METHODS

General

Reactants, solvents, and standards samples were purchased from Sigma-Aldrich, Milan, Italy. Trolox [(S)-(2)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] was purchased from Sigma-Aldrich, Taufkirchen, Germany. Sample absorbance measurements (1,1-diphenyl-2-picryl-hydrazyl radical [DPPH], ferric reducing antioxidant of potency [FRAP], and Folin–Ciocalteu assays) were carried out on an ultraviolet-visible (UV-VIS) spectrophotometer (Shimadzu Italy, Milan) mod. UV-2600 provided with 60 mm integration sphere ISR-2600Plus.

Chocolates, cocoa masses, and cocoa nib samples

Chocolate bars and corresponding cocoa mass and cocoa nib samples kindly supplied by Chocolat Stella SA (Giubiasco, Switzerland) upon our randomized selection; the following extracts were obtained and analyzed for their antioxidant activity: cocoa nibs from Costa Rica, unspecified cocoa nibs, cocoa mass from Ecuador, cocoa mass from Peru, cocoa mass from Venezuela, milk chocolate, organic milk chocolate, milk chocolate 40% milk, organic dark chocolate 60% cocoa, organic dark chocolate 71% cocoa, dark chocolate 72% cocoa, organic dark chocolate 75% cocoa, organic dark chocolate 80% cocoa, extra dark chocolate 100% cocoa, Costa Rica dark chocolate 75% cocoa, Ecuador dark chocolate 65% cocoa, Madagascar dark chocolate 70% cocoa, Tanzania dark chocolate 80% cocoa, and Trinidad dark chocolate 80% cocoa. Organic chocolates are genetically modified organism (GMO) free and do not contain soy lecithin.

Extraction procedures

All the samples were grounded and then extracted at room temperature with methanol–water 50:50 v/v, by sonication for 30 min, and then centrifuged for 10 min at 5000 rpm (Thermo Electron Corporation, IEC CL31R Multispeed Centrifuge). The supernatant was removed and the solid residue was extracted again with acetone–water 70:30 v/v, for 30 min at room temperature. After centrifugation (10 min, 5000 rpm; Thermo Electron Corporation, IEC CL31R Multispeed Centrifuge), supernatants were collected and immediately analyzed to determine the antioxidant activity and the extractable polyphenol content determined by means of the Folin–Ciocalteu method.[15]

Folin–Ciocalteu assay

All the extracts were evaluated for polyphenol content by the Folin–Ciocalteu method adapted and optimized.[16] Gallic acid was used as the standard, and a calibration curve was obtained with solutions of 0, 50, 100, 150, 250, and 500 ppm. A 1.5-mL aliquot of water-diluted Folin–Ciocalteu reagent (1/15) was added to the extracts (20 μL). The mixture was incubated for 5 min at room temperature and 300 μL of sodium carbonate solution (200 g/L) was added. The mixture was then incubated for further 90 min at room temperature and the absorbance was measured at 765 nm by a UV-VIS spectrophotometer, against a blank similarly prepared, but containing distilled water instead of extract. In this way, the results were expressed as equivalent to milligrams of gallic acid equivalent (GAE) per gram of sample (mg of GAE/g of fiber).

Antioxidant activity assays

DPPH assay. DPPH assay was performed according to the method described by Wang *et al.*[17] To a DPPH methanolic solution (1.5 mL) was added 0.750 mL of extracts at different concentrations and the absorbance was measured by a spectrophotometer UV-VIS at 517 nm, inhibitory concentration 50% (IC_{50}) values expressed as μg/mL were determined by linear regression analysis of the results obtained at different concentrations of the sample.

FRAP assay. The ferric reducing ability of each standard solution was measured according to a modified protocol described by Guihua *et al.*[18] Samples were dissolved with the selected solvent (water and/or methanol). The reagent for analysis was freshly prepared by mixing the three following solutions in the reported ratio 10:1:1 (v:v:v): (1) 0.1 M acetate buffer (pH 3.6), (2) 2,4,6-tripyridyl-s-triazine (TPTZ) 10 mM in 40 mmol/hydrochloric acid (HCl), and (3) ferric chloride 20 mM. To a 1.9 mL of reagent was added 0.1 mL of sample extract or solvent when blank was performed. Readings at the absorption maximum (593 nm) were taken after 30 min using a UV-VIS spectrophotometer. The Trolox solution was used to perform the calibration curves. The FRAP values were expressed as μmol equivalents of Trolox for each gram of product.

Statistical evaluations

Relative standard deviations and statistical significance (Student's *t*-test; $P \leq .05$) were given, where appropriate, for all data collected. The one-way analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* Tukey's test were used for comparing the bioactive effects of different samples. All computations were made using the statistical software Statistica 6.0 (StatSoft Italia srl).

RESULTS

Cocoa nibs and cocoa masses

The total polyphenol content and antioxidant activities of the different types of cocoa nibs and masses studied are presented in Table 1. We can observe that the total polyphenol content of cocoa nibs from Costa Rica is higher than the content of the conventional cocoa nibs. In addition, the antioxidant power of cocoa nibs of Costa Rica is higher in both the DPPH and FRAP assays. The content of total polyphenols in the three different types of cocoa masses did

Table 1. Total Phenol Content and Antioxidant Assays of Cocoa Nibs and Cocoa Masses in Presence of Proteins of Different Origin

Samples	*Folin (mg gallic acid/g)*	*DPPH (IC50% mg/mL)*	*FRAP (μmol Trolox/g)*
Cocoa nibs from Costa Rica	30.9 ± 1.49	0.2 ± 0.01	175.87 ± 8.78
Cocoa nibs from Costa Rica + whey proteins	67.2 ± 3.21	0.28 ± 0.01	218.53 ± 10.51
Cocoa nibs from Costa Rica + soy proteins	42.6 ± 2.11	0.26 ± 0.01	232.19 ± 10.89
Cocoa nibs from Costa Rica + whole milk	90.2 ± 3.99	0.32 ± 0.02	218.34 ± 10.42
Conventional cocoa nibs	20 ± 0.98	0.47 ± 0.02	141.66 ± 6.98
Conventional cocoa nibs + whey proteins	51.8 ± 2.51	0.55 ± 0.02	147.09 ± 7.05
Conventional cocoa nibs + soy proteins	37.8 ± 1.76	0.56 ± 0.03	150.94 ± 7.35
Conventional cocoa nibs + whole milk	98.3 ± 4.82	0.67 ± 0.03	163.79 ± 7.96
Cocoa mass from Ecuador	36.7 ± 1.54	0.22 ± 0.01	245.9 ± 12.21
Cocoa mass from Ecuador + whey proteins	75.7 ± 3.65	0.24 ± 0.01	315.78 ± 15.65
Cocoa mass from Ecuador + soy proteins	61.8 ± 2.98	0.19 ± 0.01	307.2 ± 15.24
Cocoa mass from Ecuador + whole milk	77.9 ± 3.86	0.29 ± 0.01	265.48 ± 13.15
Cocoa mass from Peru	32.1 ± 1.32	0.14 ± 0.01	251.58 ± 12.46
Cocoa mass from Peru + whey proteins	64 ± 3.18	0.26 ± 0.01	281.11 ± 13.89
Cocoa mass from Peru + soy proteins	56 ± 2.64	0.2 ± 0.01	284.22 ± 14.05
Cocoa mass from Peru + whole milk	95 ± 4.57	0.27 ± 0.01	278.45 ± 12.54
Cocoa mass from Venezuela	35 ± 1.72	0.16 ± 0.01	223.25 ± 11.02
Cocoa mass from Venezuela + whey proteins	68.4 ± 3.21	0.24 ± 0.01	260.73 ± 12.78
Cocoa mass from Venezuela + soy proteins	49 ± 2.39	0.18 ± 0.01	276.7 ± 13.46
Cocoa mass from Venezuela + whole milk	90.2 ± 4.45	0.27 ± 0.01	263.31 ± 13.02
Whey proteins	25.2 ± 1.22	N.D.	0.78 ± 0.04
Soy proteins	9.9 ± 0.41	N.D.	4.64 ± 0.23
Whole milk	54.7 ± 2.68	N.D.	44.46 ± 22.12

Values are expressed as mean ± standard deviation ($n = 3$). IC50 value is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

DPPH, 1,1-diphenyl-2-picryl-hydrazyl radical; FRAP, ferric reducing antioxidant of potency; N.D., not determinable due to lack of activity.

not show significant differences and also the antioxidant power is comparable. With regard to the antioxidant activity, the masses have a higher power than the nibs, especially compared with the conventional ones, and this can be justified by the fact that in the nibs is still present the lipid part, which will form the cocoa butter, while the masses are nonfat part resulting from grinding the nibs and therefore most rich in polyphenolic content, as it is confirmed by the results of Folin–Ciocalteu (Table 1). Interestingly, the addition of proteins always increased FRAP, but in an

Table 2. Total Phenol Content and Antioxidant Assays of Chocolates

Samples	*Folin (mg gallic acid/g)*	*DPPH (IC50% mg/mL)*	*FRAP (μmol Trolox/g)*
Milk chocolate	15.13 ± 0.74	6.70 ± 0.32	82.24 ± 3.92
Organic milk chocolate	13.41 ± 0.65	7.72 ± 0.34	69.19 ± 3.41
Milk chocolate 40% milk solids	9.22 ± 0.39	3.82 ± 0.15	38 ± 1.52
Dark chocolate 72% cocoa with cocoa nibs	20 ± 0.12	0.55 ± 0.02	133.18 ± 6.43
Organic dark chocolate 60% cocoa	27.34 ± 1.34	0.43 ± 0.01	162.21 ± 7.92
Organic dark chocolate 71% cocoa	24.59 ± 1.19	0.36 ± 0.01	168.87 ± 7.95
Organic dark chocolate 75% cocoa	34 ± 1.54	0.39 ± 0.01	177.82 ± 8.11
Organic dark chocolate 80% cocoa	36.62 ± 1.62	0.36 ± 0.02	205.16 ± 10.19
Extra dark cocoa bar 100% cocoa	38.13 ± 1.48	0.28 ± 0.01	215.02 ± 10.25
Costa Rica dark chocolate 75% cocoa	30.45 ± 1.42	0.33 ± 0.01	190.45 ± 9.48
Ecuador dark chocolate 65% cocoa	26.18 ± 1.29	0.34 ± 0.01	162.77 ± 7.42
Madagascar dark chocolate 70% cocoa	29.17 ± 1.21	0.36 ± 0.02	181.83 ± 9.08
Tanzania dark chocolate 80% cocoa	28.72 ± 1.18	0.31 ± 0.01	219.04 ± 10.18
Trinidad dark chocolate 80% cocoa	25.83 ± 1.09	0.32 ± 0.01	213.04 ± 10.08
Organic baobab fruit 60% cocoa	23.4 ± 1.61	0.49 ± 0.06	109.77 ± 7.04
Duo dark chocolate with green tea	16.28 ± 1.49	0.81 ± 0.15	85.76 ± 7.34
Rice milk chocolate lactose free with cocoa nibs	18.76 ± 1.28	0.87 ± 0.27	74.29 ± 7.8
Traditionally made chocolate of Modica 75% cocoa	26.48 ± 1.65	0.32 ± 0.03	144.49 ± 16.31
Traditionally made chocolate of Modica 60% cocoa	21.69 ± 3.17	0.38 ± 0.01	140.71 ± 26.76

Values are expressed as mean ± standard deviation ($n = 3$).

unrelated manner with the increase in polyphenol content. This occurrence remains so far unexplained.

Chocolate bars

Table 2 presents the total polyphenol content and antioxidant activities for the different types of chocolate bars with different percentages of cocoa. A significant difference can be noticed between dark chocolate and milk chocolate for both the total phenol content and for the antioxidant activity. The milk chocolate is considerably lower than the dark one, as confirmed by the results of the three tests; these results can be justified by the lower amount of cocoa powder in milk chocolate. Regarding the dark chocolate samples, the worst both in terms of total phenol content and antioxidant activity was found to be the dark chocolate 72%, despite the higher percentage of cocoa contained compared with organic dark chocolate 60% and 71%, dark chocolate 65% from Ecuador, and dark chocolate 70% from Madagascar. This first remark reveals that it is not only the amount of cocoa used that is important, but also the quality and provenience affect the product properties. In general, for organic chocolates, by increasing the percentage of cocoa, the amount of total polyphenols enhances and the antioxidant capacity of the product increases in proportion. Regarding the chocolates produced from nibs from different sources, it can be observed that the different percentages of cocoa contained do not significantly influence the total content of polyphenols. In addition, the antioxidant capacity that emerged from DPPH does not show major differences. The FRAP test results, however, are mainly influenced by the percentage of cocoa present, whereas nonsignificant differences are noted on the basis of origin. Based on the results obtained from this study, we can affirm that the extra dark cocoa bar 100% cocoa chocolate is the best in terms of total polyphenol content and in terms of antioxidant capacity according to the DPPH and FRAP tests; even the bars of organic dark chocolate 80%, Tanzania dark chocolate 80%, and Trinidad dark chocolate 80% are good products in all respects.

CONCLUSION

In general, by decreasing the percentage of nonfat cocoa solids, a decrease in the antioxidant capacity associated with a lower content of total polyphenols can be observed. This fact is particularly evident analyzing the data of milk chocolate samples, which are the samples with a lower content of cocoa mass. Data related to the nibs and the masses, indicating that the masses of cocoa have a higher content in both antioxidants and total polyphenols, support that the major contribution to the antioxidant activity and the total polyphenol content is due to the nonfat cocoa solid. These results are explained by the fact that in the nibs is still present the lipid part, which will form the cocoa butter and this portion is of poor value for the antioxidant activity and therefore for healthy properties of the chocolate. The influence of different proteins (*i.e.*, whey, soy, and milk) was also investigated in view of their frequent association with cocoa and chocolate in low calorie dietetic/antihunger products. Interestingly, these proteins themselves were found endowed of interesting FRAP activity, not always related to the polyphenol content. As a final consideration emerging from this work, it should be noted that the overall antioxidative potency of cocoa and cocoa products is higher than many antioxidant vegetable extracts claimed for the increase of body oxidation defense.[19]

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AUTHOR DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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