

## Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine

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Black tea, green tea, red wine, and cocoa are high in phenolic phytochemicals, among which theaflavin, epigallocatechin gallate, resveratrol, and procyanidin, respectively, have been extensively investigated due to their possible role as chemopreventive agents based on their antioxidant capacities. The present study compared the phenolic and flavonoid contents and total antioxidant capacities of cocoa, black tea, green tea, and red wine. Cocoa contained much higher levels of total phenolics (611 mg of gallic acid equivalents, GAE) and flavonoids (564 mg of epicatechin equivalents, ECE) per serving than black tea (124 mg of GAE and 34 mg of ECE, respectively), green tea (165 mg of GAE and 47 mg of ECE), and red wine (340 mg of GAE and 163 mg of ECE). Total antioxidant activities were measured using the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays and are expressed as vitamin C equivalent antioxidant capacities (VCEACs). Cocoa exhibited the highest antioxidant activity among the samples in ABTS and DPPH assays, with VCEACs of 1128 and 836 mg/serving, respectively. The relative total antioxidant capacities of the samples in both assays were as follows in decreasing order: cocoa > red wine > green tea > black tea. The total antioxidant capacities from ABTS and DPPH assays were highly correlated with phenolic content ( $r^2 = 0.981$  and  $0.967$ , respectively) and flavonoid content ( $r^2 = 0.949$  and  $0.915$ ). These results suggest that cocoa is more beneficial to health than teas and red wine in terms of its higher antioxidant capacity.

**KEYWORDS:** Cocoa; black tea; green tea; red wine; phenolic phytochemicals; ABTS radicals; DPPH radicals; vitamin C equivalent antioxidant capacity (VCEAC).

### INTRODUCTION

Free radicals cause degenerative human diseases such as cancer, heart disease, and cerebrovascular disease through multiple mechanisms. Recently, natural foods and food-derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage. Vitamin C is one of the most popular and least toxic antioxidant components in food and has been most popularly used as a dietary supplement to prevent oxidative stress-mediated diseases. However, the contribution of vitamin C to the total antioxidant activity of fruits is generally <15% (1). Therefore, many efforts have been invested to elucidate the potential health benefits of dietary phenolic phytochemicals that have stronger antioxidant activities than vitamin C.

Black and green teas, red wine, and cocoa are consumed widely and are known to be rich in phenolic phytochemicals.

In particular, theaflavin (TF), epigallocatechin gallate (EGCG), resveratrol, and procyanidin in black tea, green tea, red wine, and cocoa, respectively, have been considered as major chemopreventive agents mainly due to their strong antioxidative activities. A recent report suggested that drinking black tea has benefits equal to those of having green tea in terms of their antioxidant capacity, because the TF found in black tea possesses as much antioxidant potency as catechins present in green tea (2). However, whereas catechins are major antioxidants in green tea, TF represents a smaller proportion of black tea. Similarly, resveratrol, a phytoalexin found in red wine, has been considered as a major component responsible for anticarcinogenic activity (3–5) but is present at minimal levels in red wine (only 1.5 mg/L), whereas flavonoids such as catechin and epicatechin are present at levels of 191 and 82 mg/L, respectively (6). Therefore, most of the beneficial effects of red wine are attributable to phenolic phytochemicals other than resveratrol. Chocolate has also been reported to be a good source of dietary catechins, second only to black tea in a study on a Dutch population (7). The same report showed that the monomeric catechins were considered as antioxidants in only black tea and chocolate. Because catechins are major antioxidants in green tea, but not

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in chocolate and black tea, the results may not reflect substantial total phenolic phytochemical contents and antioxidant capacities of black tea and chocolate. Thus, investigations into the antioxidant capacity of food should consider the overall concentrations and compositions of diverse antioxidants, because the total antioxidant capacity of food is due to the combined activity of diverse antioxidants including phenolics, rather than being attributable to any particular phenolics.

We suggested recently that a 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay is better for evaluating the antioxidant activity of phenolic phytochemicals than the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, because it can be used in both organic and aqueous solvent systems, employs a specific absorbance at a wavelength remote from the visible region, and requires a short reaction time. We also recommended that antioxidant activity of phenolic phytochemicals be expressed on the basis of the weight of vitamin C equivalents (VCEAC) using the ABTS radical scavenging assay (8). To further validate the method, the total antioxidant capacity of foods including mixtures of antioxidants needs to be investigated. Therefore, the present study investigated the phenolic and flavonoid contents and total antioxidant capacities of cocoa, black tea, green tea, and red wine.

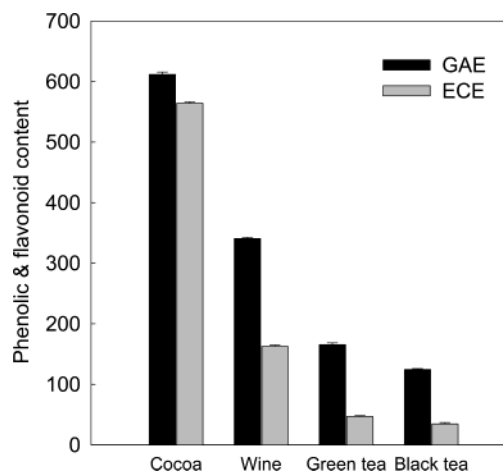
## MATERIALS AND METHODS

**Chemicals.** Gallic acid, epicatechin, vitamin C, ABTS as diammonium salt, DPPH, and Folin-Ciocalteu phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). All other chemicals used were of analytical grade (Fisher, Springfield, NJ).

**Sample Preparation.** The serving size of each beverage was defined as follows: commercial cocoa powder manufactured using a nonalkalized process from Ghanaian cacao beans (7.3 g, 2 tablespoons in accordance with the manufacturer's instructions) was dissolved in 200 mL of distilled water (ddH<sub>2</sub>O) at 100 °C; commercial black tea (2 g bag) and green tea (2 g bag) were each extracted with 200 mL of ddH<sub>2</sub>O at 100 °C for 2 min (according to the manufacturer's instructions); and 140 mL of red wine (Merlot, California) as one serving size (14). The samples then were centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont, Biomedical Products Department, Wilmington, DE) at 12000g using a GSA rotor for 5 min, and the resulting supernatants were used as the final samples.

**Total Phenolic Content.** The total phenolic phytochemical concentration was measured using the Folin-Ciocalteu method. Briefly, 1 mL of appropriately diluted samples and a standard solution of gallic acid were added to a 25 mL volumetric flask containing 9 mL of ddH<sub>2</sub>O. A reagent blank using ddH<sub>2</sub>O was prepared. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of a 7% Na<sub>2</sub>CO<sub>3</sub> solution was added with mixing. The solution was then immediately diluted to a volume of 25 mL with ddH<sub>2</sub>O and mixed thoroughly. After incubation for 90 min at 23 °C, the absorbance relative to that of a prepared blank at 750 nm was measured using a spectrophotometer (Hitachi). The total phenolic contents of the samples are expressed in milligrams per serving of gallic acid equivalents (GAE). All samples were prepared in five replications.

**Total Flavonoid Content.** The total flavonoid concentration was measured using a colorimetric assay developed by Zhishen et al. (8, 9). Briefly, 1 mL of appropriately diluted sample was added to a 10 mL volumetric flask containing 4 mL of ddH<sub>2</sub>O. At time zero, 0.3 mL of 5% NaNO<sub>2</sub> was added to each volumetric flask; at 5 min, 0.3 mL of 10% AlCl<sub>3</sub> was added; at 6 min, 2 mL of 1 M NaOH was added. Each reaction flask was then immediately diluted with 2.4 mL of ddH<sub>2</sub>O and mixed. Absorbances of the mixtures upon the development of pink color were determined at 510 nm relative to a prepared blank. The total flavonoid contents of the samples are expressed in milligrams



**Figure 1.** Total phenolic and flavonoid contents of cocoa, black tea, green tea, and red wine, expressed as milligrams of GAE and ECE per serving, respectively.

per serving of epicatechin equivalents (ECE). All samples were prepared in five replications.

**ABTS Radical Scavenging Activity.** A method developed by van den Berg et al. was used with slight modification in this experiment (8, 10): 1.0 mM AAPH was mixed with 2.5 mM ABTS as diammonium salt in phosphate-buffered saline solution (100 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl). The mixture was heated in a 68 °C water bath for 13 min. The concentration of the resulting blue-green ABTS radical solution was adjusted to an absorbance of  $0.650 \pm 0.020$  [mean  $\pm$  standard deviation (SD)] at 734 nm. Various concentrations of the sample solution of 20  $\mu$ L were added to 980  $\mu$ L of the resulting blue-green ABTS radical solution. The mixture was incubated in darkness in a 37 °C water bath for 10 min, and the decrease of absorbance at 734 nm was measured. A control solution consisted of 20  $\mu$ L of 50% methanol and 980  $\mu$ L of ABTS radical solution. Stable ABTS radical scavenging activities of the samples are expressed in milligrams per serving of VCEAC. The radical stock solution was freshly prepared each day.

**DPPH Radical Scavenging Activity.** The method of Brand-Williams et al. was used with slight modifications in this experiment (8, 11). DPPH radical was dissolved in 80% aqueous methanol. Various concentrations of 0.1 mL of sample solution were added to 2.9 mL of the DPPH radical solution. The mixture was then shaken vigorously and allowed to stand at 23 °C in the dark for 30 min, at which time the decrease in absorbance at 517 nm was measured using a spectrophotometer (Hitachi). A control solution consisted of 0.1 mL of 50% aqueous methanol and 2.9 mL of DPPH radical solution. The radical stock solution was freshly prepared each day.

**Antioxidant Capacity.** The antioxidant capacity of the samples was measured and calculated as VCEAC according to the method described earlier (8). Briefly, vitamin C standard curves that correlate the concentration of vitamin C and the amount of absorbance reduction caused by vitamin C were obtained using the ABTS scavenging assay. Absorbance reductions of the samples at 734 nm were also measured at various concentrations by the ABTS assay. Determination of VCEACs of the samples at various concentrations was made using vitamin C standard curves. The median effective dose (EC<sub>50</sub>) of the samples was calculated on the basis of the dose-response curve. The absorbance reduction of the samples was correlated to that of vitamin C standards with the results calculated as milligrams of vitamin C equivalents per serving. All data are presented as mean  $\pm$  SD for at least five replications for each prepared sample.

## RESULTS AND DISCUSSION

**Figure 1** shows that cocoa contained much higher levels of total phenolics (611 mg of GAE) and flavonoids (564 mg of ECE) per serving than black tea (124 mg of GAE and 34 mg of ECE, respectively), green tea (165 mg of GAE and 47 mg

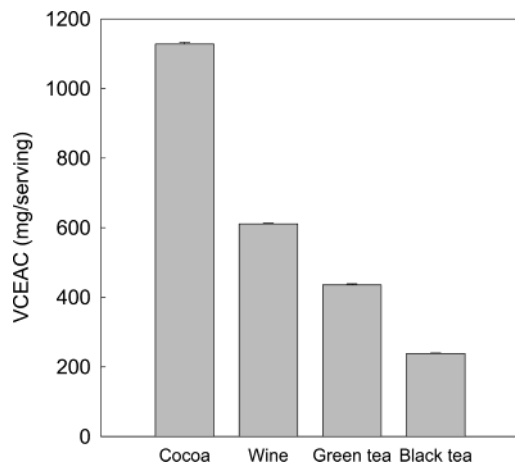


Figure 2. VCEACs of cocoa, black tea, green tea, and red wine per serving evaluated by ABTS radical scavenging assay. Error bars = SD,  $n \geq 5$ .

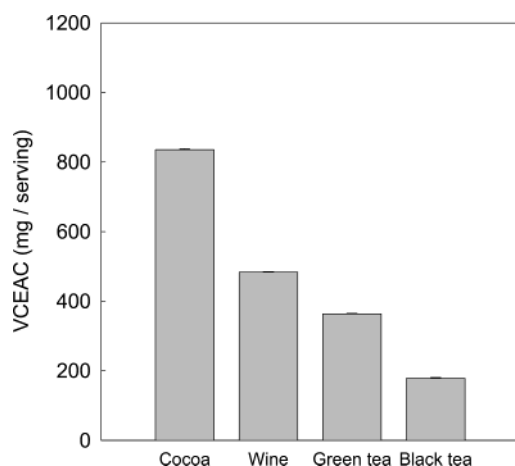


Figure 3. VCEACs of cocoa, black tea, green tea, and red wine per serving evaluated by DPPH radical scavenging assay. Error bars = SD,  $n \geq 5$ .

of ECE), and red wine (340 mg of GAE and 163 mg of ECE). Most of the phenolics in cocoa are flavonoids, whereas phenolics other than flavonoids predominate in green and black teas. Consistent with our previous results for pure phenolic phytochemicals (8), the antioxidant activities of all the tested samples were dose-dependent in both assays (data not shown). Cocoa exerted the highest antioxidant activity: VCEACs of 1128 and 836 mg per serving in ABTS and DPPH radical scavenging assays, respectively (Figures 2 and 3). The relative total antioxidant capacities of the samples per serving in both ABTS and DPPH assays were as follows in decreasing order: cocoa > red wine > green tea > black tea. The total antioxidant capacities from ABTS and DPPH assays were highly correlated with phenolic content ( $r^2 = 0.981$  and  $0.967$ , respectively) (Figure 4) and flavonoid content ( $r^2 = 0.949$  and  $0.915$ ) (Figure 5). The different VCEAC values indicated that the DPPH assay underestimates by ~30% the antioxidant capacity as compared to the ABTS assay. A similar methodological difference has been reported previously (8, 12), and these differences may be due to absorbance interruption at 517 nm by other compounds in the DPPH assay. Furthermore, the ABTS assay is sensitive, requires a short reaction time, and can be used in both organic and aqueous solvent systems. Therefore, the ABTS assay may be preferable over the DPPH assay for evaluating the total antioxidant capacity of antioxidants and food.

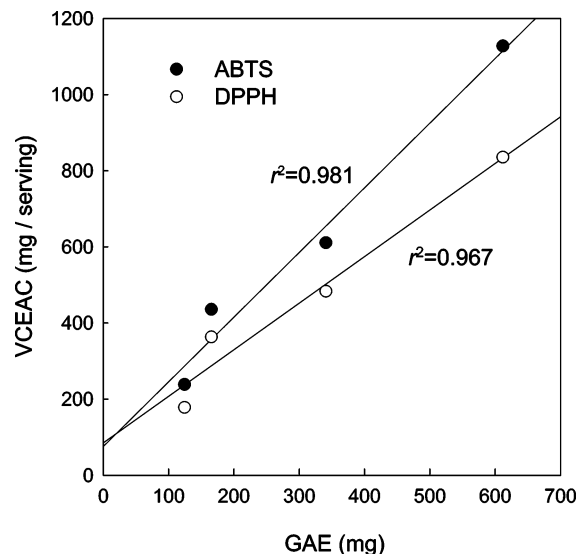


Figure 4. Relationship between total phenolic contents and VCEACs of cocoa, black tea, green tea, and red wine per serving evaluated by ABTS and DPPH radicals, respectively. Error bars = SD,  $n \geq 5$ .

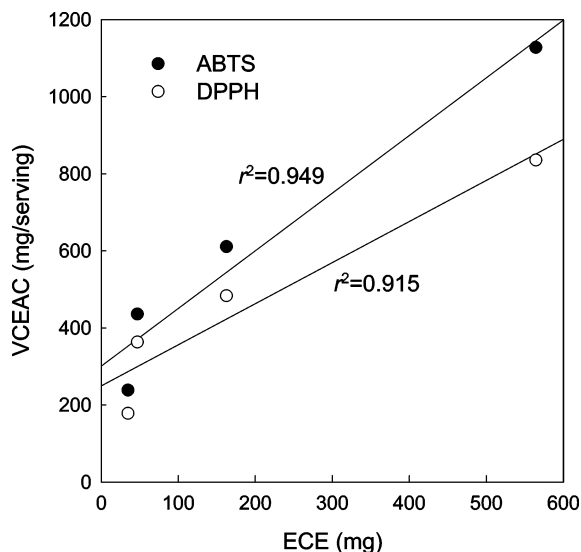


Figure 5. Relationship between total flavonoid contents and VCEACs of cocoa, black tea, green tea, and red wine per serving evaluated by ABTS and DPPH radicals, respectively. Error bars = SD,  $n \geq 5$ .

Many studies have considered fruits, vegetables, and teas as the major sources of dietary antioxidative phenolics, but our results also demonstrate the importance of cocoa. Arts et al. (7) reported that chocolate and black tea may contribute significantly to the total dietary catechin intake of the Dutch population (20 and 55%, respectively). They suggested that black tea is a better source of catechins such as (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin gallate, and EGCG. Because the major phenolics in black tea and chocolate are not catechins but thearubigins and procyanidins, respectively (13), it is important to determine the total contents of antioxidants in tea and chocolate.

The extraction method of antioxidants also affects the total phenol and flavonoid contents and antioxidant capacities. Waterhouse et al. (14) reported that cocoa powder had 20 mg of GAE per gram when extracted with 95% aqueous methanol and that a cup of hot cocoa had 146 mg of GAE. Consistent with this, we found that cocoa powder contained 24 mg of GAE per gram when extracted with 80% aqueous methanol (unpub-

lished data). Because cocoa extracted with aqueous methanol is not the type normally consumed, the total phenolic and flavonoid contents of hot cocoa extracted with 95% aqueous methanol may not represent the actual amounts consumed (14). Therefore, various factors such as experimental conditions, sample preparation methods, and physiological relevance of the assays should be considered in the evaluation of antioxidant activity (15).

Gallic acid and EGCG, the major antioxidants in tea, have shown strong antioxidant activity but also could act as pro-oxidants (16, 17). This pro-oxidant activity is thought to be directly proportional to the total number of hydroxyl groups, and gallic acid and EGCG including multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in a Fenton system [see review (18)]. Some reports have suggested that a high dose of gallic acid and EGCG induces cellular DNA damage (16, 17, 19, 20). Long et al. (21) also showed that the addition of gallic acid and EGCG to commonly used cell culture media led to the generation of substantial amounts of H<sub>2</sub>O<sub>2</sub>, which may cause cellular DNA damage. However, the amount of H<sub>2</sub>O<sub>2</sub> generated by cocoa rich in procyanidins was much lower than that generated by black and green teas (22). Furthermore, procyanidins are known to have protective effects against inflammation (23) and the inhibition of gap junction intercellular communication (GJIC) (24), which may be linked to carcinogenesis and, particularly, tumor promotion. However, high doses of EGCG did not show protective effects against inhibition of GJIC by H<sub>2</sub>O<sub>2</sub> (25). We also found that gallic acid did not affect GJIC (data not shown). These results thus indicate that cocoa procyanidins may possess more beneficial effects than green tea phenolics.

Even though a bar of chocolate exhibits strong antioxidant activity, the health benefits are still controversial because relatively large amounts of saturated fats are present. However, a cup of hot cocoa has a much lower level of saturated fats (0.3 g per serving) than a bar of chocolate (8 g per 40 g bar). Thus, our results demonstrate that cocoa has a high flavonoid content and substantial antioxidant capacity: on a per-serving basis 4–5 times stronger than that of black tea, 2–3 times stronger than green tea, and almost 2 times stronger than red wine.

#### ABBREVIATIONS USED

AAPH, 2,2'-azobis(2-amidinopropane)dihydrochloride; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC<sub>50</sub>, concentration of samples required to quench free radicals by 50%; SD, standard deviation; VCEAC, vitamin C equivalent antioxidant capacity.

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