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ANTIOXIDANT CAPACITY OF CHOCOLATE MILK BEVERAGES

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Abstract

Health conditions such as cancer, atherosclerosis, and diabetes have been linked to oxidative damage caused by increased radical activity within the body. Antioxidants are stable, bioactive compounds that can neutralize the radicals they encounter, reducing the capacity of radicals to damage cellular structures. While existing evidence demonstrates the antiradical effects of dark chocolate, less is known about the antioxidant properties of chocolate milk beverages. This study aimed to examine the antioxidant activity of plant and animal-based chocolate milk, powder, and syrup samples compared to their non-chocolate milk counterparts. The 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical assay was used to compare the antioxidant capacity spectrophotometrically over time. The chocolate milk beverages all showed greater decreases in absorbance and increased antioxidant activity compared to their non-chocolate counterparts. This study demonstrates that the addition of chocolate to bovine and almond milk can boost antioxidant capacity, improving the disease-fighting potential of these chocolate-based beverages.

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Introduction

General aging and several diseases, including various cancers[1], atherosclerosis[2], diabetes[3], and Alzheimer's disease[4], have been linked to oxidative damage caused by increased free radical activity within the body. Metabolic processes such as the mitochondrial generation of energy through the electron transport chain and oxidative phosphorylation generate reactive oxygen species (ROS) as a natural byproduct[5]. These reactive species are often in the form of free radicals. Several ROS exist within the cell and extracellular space and have the potential to damage DNA, cellular membranes, and proteins[6]. Controlling ROS to manageable levels is imperative to reducing morbidity associated with oxidative stress.

Antioxidants are stable, bioactive compounds that can neutralize the radicals they come into contact with by donating or absorbing electrons, reducing the capacity of radicals to damage cellular structures[7]. It is well-documented that diets rich in antioxidants can help mitigate the effects of both internal and external causes of free radical induced oxidative damage[8–12]. Polyphenolic compounds are a large class of highly conjugated antioxidants, which may inhibit radicals directly, by neutralizing ROS, or indirectly, such as receptor-ligand interaction modulation and activation of antioxidant enzymes found within the body. Due to their antiradical properties, consumption of polyphenolic compounds, including those found in cereals, legumes, seeds, and cacao, has been associated with a decreased risk of cardiovascular disease and cancer[13].

Chocolate has been the subject of many studies on radical scavenging activity due to its high antioxidant content[14–16]. It is well established that chocolate samples with higher cocoa percentages have higher polyphenolic content and increased antioxidant activity than samples with lower cocoa content[17,18]. Since dark chocolate has a greater percentage of cocoa than milk chocolate, studies show that it consistently has greater antiradical potential than its milk chocolate counterpart[19,20]. Furthermore, clinical studies demonstrate that long-term and consistent intake of dark chocolate may reduce the risk of muscular injury[21], high blood pressure[22], colorectal cancer prevalence[18], inflammatory oxidative damage[23], and mononuclear blood cell DNA damage[24] in healthy individuals.

This pilot study aims to explore the antioxidant capacity of various plant and animal-based commercial chocolate milk, powder, and syrup beverages using the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical assay. The ABTS•+ radical assay was chosen because the initial electron transfer of the radical to the antioxidant occurs quickly due to low steric hindrance at the radical site; assays with greater steric hindrance may be difficult for polyphenolic compounds to access[25]. Additionally, an advantage of using ABTS•+ is that it is less prone to interference from colored samples, like chocolate milk, because it is measured at a high wavelength. It can also be used over a large pH range, which is particularly useful when examining food components[26]. Furthermore, ABTS•+ is useful for a wide variety of antioxidants because it can be neutralized by either the hydrogen atom transfer or single electron transfer mechanisms[27]. Based on previous studies involving chocolate[14–18], we hypothesized that the chocolate-based milk beverages would have greater antioxidant power than plain milk and that the dark chocolate beverages would have the highest antioxidant capacity.

Materials and Methods

Chemicals

The 10-mg ABTS tablets (Lot No. UA2660323) were purchased from Thermo Fisher Scientific®. Potassium persulfate (Lot No. A0465042) was acquired from Thermo Scientific®, and phosphate-buffered saline (PBS) tablets (Lot No. A0375116) were purchased from Acros Organics®. Concentrated radical solution was prepared ≥ 16 hours before testing by dissolving one 10-mg ABTS tablet with 1.215 mL Milli-Q® water, resulting in a 0.015 M ABTS solution. In a 10-mL volumetric flask, 0.0135g potassium persulfate was combined with Milli-Q® water to create a 0.005 M solution. Next, 1.215 mL of 0.015 M ABTS and 1.1215 mL of 0.005 M potassium persulfate were combined and vortexed for ≥ 60 seconds to create a 7.5 mM ABTS•+ radical solution. The 7.5

mM ABTS•+ was stored in the dark at 4ºC overnight. A 1X PBS solution was created with Milli-Q® water and was stored for 12 hours at 4ºC. On the day of testing, 250 mL of 0.075 mM ABTS•+ was prepared by diluting the radical solution with 1X PBS. This dilute ABTS•+ solution was stored on ice and covered to prevent light from degrading the radicals.

Instruments

Antioxidant capacity was determined spectrophotometrically using the BioMate™ 160 UV-Vis spectrophotometer (Thermo Fisher Scientific®, Waltham, Massachusetts, USA). The spectrophotometer was warmed for ≥ 30 minutes before measuring samples and was set to an absorbance of 734 nm. The chamber was zeroed with 1X PBS solution before samples were tested.

Food Samples and Sample Preparation

Milk samples, chocolate syrups, and chocolate powder were purchased from various grocery stores in central North Carolina, USA. Low fat cow milk and unsweetened almond milk were selected to compare the antioxidant effect of adding chocolate to animal-based milk versus plant-based milk. Name brands of all commercial food samples and nutrition information are listed in **Table 1**. Since macromolecules can interfere with spectrophotometric absorbance readings and antioxidant measurements[28,29], the low protein, carbohydrate, and fat content in almond milk make them ideal models for studying antioxidant activity. For this reason, almond milk was used as the plant-based milk for this pilot study.

Almond and cow milk samples were stored at 4ºC in a refrigerator until experimentation. The plain milk samples (cow and almond) were used as a control and as a base to prepare the chocolate milk beverages using the chocolate powder and different syrups. These samples were prepared using the instructions on the food label. For the powder, two tablespoons of powder were added to one cup (eight fluid ounces) of milk and mixed until homogenous. For the regular and dark chocolate syrups, one tablespoon of syrup was added to one cup (eight fluid ounces) of milk and mixed until homogenous. The pre-made commercial chocolate cow milk and chocolate almond milk samples did not require special preparation. Beakers containing all samples were kept refrigerated until ready for testing. The time lapse between sample preparation and antioxidant capacity measurement was not longer than two hours.

Table 1. Nutrition information obtained from each nutrition facts panel

Commercial Food Samples	Cal per Serving	Total Carbohydrate	Added Sugar	Total Fat	Protein
Milk Samples					
Food Lion® 1% Low-Fat Milk	110	13 g	0 g	2.5 g	8 g
Food Lion® Chocolate Low-Fat Milk	180	30 g	17 g	2.5 g	8 g
Blue Diamond® Almond Breeze Unsweetened Original Almond Milk	30	1 g	0 g	0 g	1 g
Blue Diamond® Almond Breeze Unsweetened Chocolate Almond Milk	40	2 g	0 g	3 g	1 g
Chocolate Powder & Syrups					
Nestle® Nesquik Chocolate Powder	50	12	10 g	0 g	0 g
Hershey's® Special Dark Chocolate Syrup	45	12 g	10 g	0 g	0 g
Hershey's® Genuine Chocolate Syrup	45	12 g	10 g	0 g	0 g

Antioxidant Capacity as Determined by Spectrophotometric Analysis

Radical scavenging activity was determined using the methods of Re et al. with minor adjustments[30]. In short, 2,000 µL of chilled 0.075 mM ABTS•+ was added to a 3-mL cuvette immediately before experimentation using a volumetric pipet. An amount of 5.0 µL of each milk sample was pipetted into the ABTS•+ solution and mixed by repetitive aspiration five times and with rapid stirring. The cuvette was immediately placed into the spectrophotometer and initial absorbance was measured followed by absorbance measurements every 30 seconds for five minutes. This process was repeated three times for each milk sample.

Data Analysis

The percent change in absorbance after five minutes for each of the three trials was calculated according to the following equation:

Percent change = [|final absorbance – initial absorbance| / initial absorbance] x 100

The mean percent change with standard deviation for each sample was calculated. The mean absorbance with respect to time was also plotted. Using absorbance data after five minutes ensured the stability and completion of the radical scavenging assay.

Results and Discussion

The assay used in this study measures the reduction and decolorization of the blue-green chromophore ABTS•+ to clear ABTS by antioxidants[31]. When ABTS•+ solution is combined with oxidizable substances, it is reduced to its colorless ABTS form[32]. The reduction of the radical solution is directly proportional to the decrease in absorbance. The higher the concentration of antioxidants, the faster and greater the reduction of blue-green ABTS•+ to clear ABTS solution. This inverse relationship between absorbance and antioxidant capacity was used to interpret the results of this study.

Percent decrease in absorbance and antioxidant capacity for all milk samples

For all samples tested, the antioxidant capacities of the chocolate-containing samples were higher compared to the plain milk controls for both the cow milk group and almond milk group. The antioxidant capacity was evaluated based on the percent change in absorbance. The mean percent change in absorbance over five minutes for each sample is shown in **Table 2**. Although the plain milk samples showed modest antioxidant capacity (15.3% for cow milk and 14.8% for almond milk), the cow milk chocolate bev-

Table 2. Percent change in absorbance over 5 min

Premade and Prepared Milk Samples	Percent Change
Cow Milk	
Plain	15.3 +/- 0.6
Premade Chocolate	27.4 +/- 1.0
Powdered Chocolate	25.8 +/- 1.5
Regular Chocolate Syrup	23.9 +/- 1.5
Dark Chocolate Syrup	24.3 +/- 1.9
Almond Milk	
Plain	14.8 +/- 2.0
Premade Chocolate	23.6 +/- 3.1
Powdered Chocolate	30.2 +/-1.3
Regular Chocolate Syrup	25.1 +/- 5.1
Dark Chocolate Syrup	27.3 +/- 4.1

*Percent change data is reported as mean with standard deviation based on three trials each.

erages ranged from 23.9% - 27.5% and almond milk chocolate beverages from 23.6% - 30.3%. These results support our first hypothesis that chocolate-based milk beverages would have greater antioxidant power than plain milk. For the cow milk group, the premade chocolate milk had the highest antioxidant capacity based on its greatest percent absorbance change (27.4% +/- 1.0%). In the almond milk group, the powdered chocolate mixture showed the greatest antioxidant capacity with a percent change in absorbance of 30.2% +/-1.3%. These results did not support our second hypothesis that the dark chocolate beverages would have the highest antioxidant capacity overall. For the almond milk group, this may be explained by the fact that the instructions for making chocolate milk with powder required two tablespoons of chocolate per one cup of milk, yet the dark chocolate syrup instructions suggest adding only one tablespoon of chocolate. Furthermore, it was easier to achieve quantitative transfer of the powder versus the syrup due to the viscous nature of the syrup. For the cow milk group, the commercial premade chocolate milk may contain more total chocolate or may contain a type of chocolate with a higher cacao content.

Antioxidant capacity of premade chocolate milk

The decrease in absorbance over five minutes was plotted for all samples to observe changes over time in more detail. When comparing the premade chocolate milk samples (**Figure 1**), both the chocolate cow milk and chocolate almond milk had a sharper decline in absorbance within the first 30 seconds compared to the plain control samples. The steeper the slope, the faster the free radicals are being neutralized by the antioxidants. The decrease in absorbance of the control plain milk samples demonstrates their natural antioxidant qualities. However, the premade chocolate milks resulted in a much sharper decline in absorbance, indicating a heightened rate of radical scavenging. These results are consistent with another study that found higher antioxidant levels in commercially available chocolate dairy milk compared to plain dairy milk[33]. To the best of our knowledge, there are no published studies that compare the antioxidant capacity of chocolate almond milk to non-chocolate almond milk. Based on our analysis, the results of the chocolate versus non-chocolate milk samples demonstrate that cacao polyphenolic compounds in chocolate likely enhance the antiradical properties of both animal and plant-based milk beverages.

Comparison of Powdered Chocolate Milk

The mean absorbance of the powdered chocolate milk samples versus the control plain milk samples can be visualized in **Figure 2**. Consistent with the commercial premade chocolate milk, there is a sharper decline in absorbance within the first 30 seconds for the beverages containing chocolate. In this group, the chocolate powder plus almond milk shows higher antioxidant capacity compared to the chocolate powder plus cow milk based on both the sharp initial decline in absorbance and the overall percent decrease over five minutes (30.2% for almond vs 25.8% for cow). Although both chocolate beverages showed higher antioxidant capacity compared to their respective controls (14.8% for plain almond and 15.3% for plain cow) it is interesting to note that the chocolate powder had a greater impact on improving antioxidant activity in almond milk compared to cow milk. It has been reported previously that proteins found in cow milk may interfere with polyphenolic compounds, reducing their antioxidant capacity[8]. This may account for the reduced impact we observed in the chocolate cow milk beverages prepared with powder and syrup compared to our low-protein almond milk beverages prepared with the same types of chocolate. Another possible explanation for the differences in the relative change in absorbance between chocolate and plain almond milk flavors compared to chocolate and plain dairy milk samples lies within the biochemical composition of each substance. Cow milk and dairy products are rich sources of various antioxidants, especially as a result of their high content of antiradical amino acids[34]. In particular, sulfur-containing amino acids, like cysteine and methionine, are the most readily available to scavenge radicals, followed by aromatic amino acids tyrosine and tryptophan, and the cyclic amino acid proline[35]. Additionally, cow milk contains fat-soluble and water-soluble vitamins in high concentrations, especially vitamins E, C, and β-carotene, which have intrinsic antioxidant properties[36]. While almond milk may contain some of these amino acids and vitamins, the concentrations of these antiradical substances may be lower in plain almond milk compared to plain cow milk. This may explain why adding chocolate to almond milk appears to have a greater impact on increasing antioxidant activity compared to adding chocolate to cow milk.

Comparison of milk prepared with regular and dark chocolate syrups

Another common way that chocolate milk can be prepared is by adding chocolate syrup to plain milk. Evidence shows that children who consume flavored beverages tend to drink more milk and lead healthier lifestyles[37]. In our study, the dark chocolate syrup showed a greater mean percent change in absorbance than the

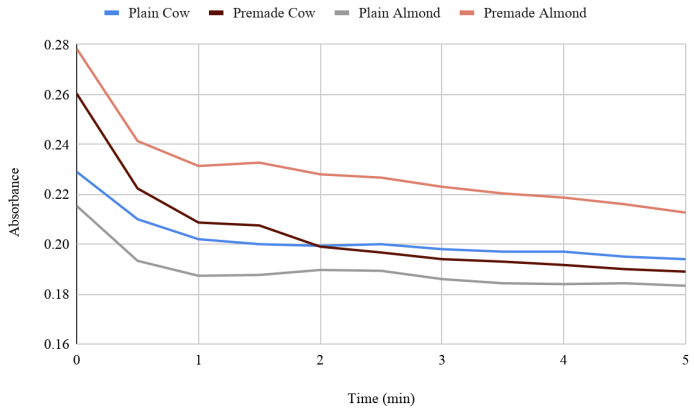

Figure 1. Relative antioxidant capacity of plain milk compared to commercial premade chocolate milk

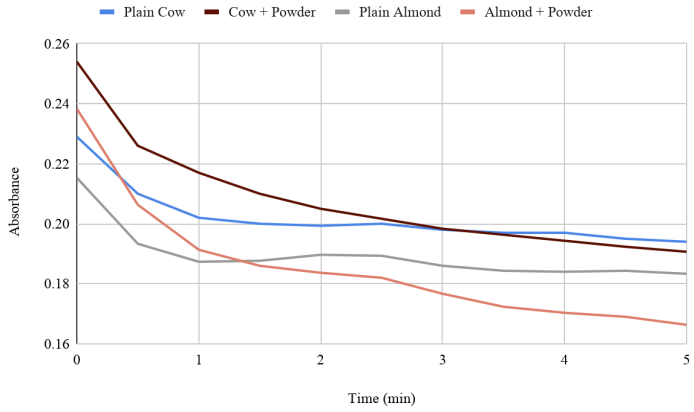

Figure 2. Relative antioxidant capacity of plain milk compared to chocolate milk made with powder

regular chocolate syrup (**Table 2**) for both the cow milk (24.3% for dark vs 23.9% for regular) and the almond milk (27.3% for dark vs 25.1% for regular). This is consistent with multiple studies that report higher antioxidant capacity for foods containing dark chocolate compared to other forms of chcolate[17,18,38]. For the dark chocolate syrup, the almond milk had a greater percent change in antioxidant capacity than the cow milk by a difference of 3.0%. Similarly, for the regular chocolate syrup, the added chocolate has a greater impact on the almond milk compared to cow milk by a difference of 1.2%. We cannot assume these results are statistically significant since we did not perform hypothesis testing. However, this pilot study shows a trend that adding chocolate to both plant and animal-based milk increases antioxidant capacity, and that adding chocolate to almond milk has a bigger impact on improving antioxidant capacity compared to adding chocolate to cow milk. This is consistent with our results for the powdered milk (**Table 2, Figure 2**). The change in absorbance over time was also plotted for milk samples containing syrup compared to controls (**Figure 3**). The sharper initial decline in absorbance can be observed when comparing the dark chocolate syrup samples to the regular chocolate syrup samples, although the difference is not as dramatic as we expected. The greater percent decrease in absorbance of the radical solution containing the dark chocolate syrup compared to the milk chocolate syrup respective to the control suggests that the increased cocoa content of the syrup contributes to the greater antioxidant activity of the milk. When comparing the antioxidant capacity of the powdered chocolate milk samples to the syrups, our results show that the powder is more effective compared to syrup in both animal and plant-based milk (**Table 2**). This is consistent with a previous study showing that commercially available chocolate syrups have lower levels of antioxidant activity compared to cocoa powder[39]. However, as mentioned earlier, the amount of chocolate powder added based on the food label instructions was higher compared to the syrups, which likely influenced the results in our investigation.

Conclusion

This pilot study demonstrates that the addition of chocolate to unsweetened almond milk and low-fat dairy milk may boost antioxidant capacity. Strengths of this study include the novelty of investigating antioxidant potential of both plant and animal-based milk combined with various forms of chocolate using realistic amounts of chocolate that may be consumed. Our study also used a simplified method to compare antioxidant capacity across common beverages. The study limitations include not using a standard such as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or vitamin C to obtain quantitative data to measure antioxidant capacity and not using samples from different batches or lot numbers to report inferential statistics for hypothesis testing. Future research should include an *in vivo* evaluation of antioxidant status with a wider variety of chocolate milk samples and other milk types, such as soy and oat milk. Furthermore, additional assays, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) or the oxygen radical absorbance capacity (ORAC) test can be used with the ABTS assay to validate the results. Based on our preliminary findings, we propose that adding chocolate to almond and cow milk may improve the disease-fighting potential of these beverages when consumed in moderation.

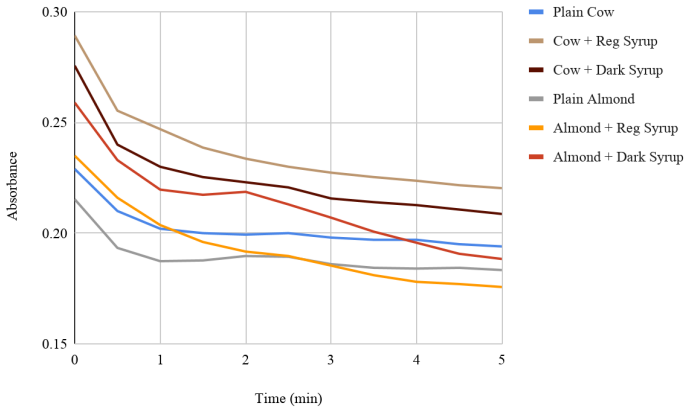

Figure 3. Relative antioxidant capacity of plain milk compared to chocolate milk made with dark chocolate syrup and regular chocolate syrup

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