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Food Additives In Commercial Cocoa Beverage Products and their Effects on Total Polyphenol Contents, Cellular Antioxidant and Anti-Inflammatory Activities

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Abstract

The study evaluated the uses of food additives in commercial cocoa beverages, and examined the effects of the food additives on their antioxidant and anti-inflammatory activities. The food additive lists of the cocoa beverage items on the shelves and chillers of ten randomly selected local supermarkets were recorded. The total flavonoid, polyphenol contents, and radical scavenging activity of the beverages were determined using the modified Dowd, Folin-Ciocalteu, and 1,1-diphenyl-2-picrylhydrazyl radical scavenging assays, respectively. Cellular experiments examined the inhibition of F_2-isoprostanes, lipid hydroperoxides, leukotriene B_4 productions, and myeloperoxidase activity by freshly isolated human neutrophils. The effects of food additives on the measured outcomes were evaluated. Food additives were added to 72% of the twenty five cocoa beverage products. Flavorings (60%), antioxidants (56%), pH regulators (40%), emulsifiers (36%), and colorings (4%) were added into these beverages. The cocoa beverages contained significant amounts of flavonoids, polyphenols, and radical-scavenging antioxidants. Their ethanolic extracts inhibited F_2-isoprostanes, lipid hydroperoxides, leukotriene B_4 productions, and myeloperoxidase activity from freshly isolated human neutrophils. After stratification by different food additive groups, the flavonoids, polyphenols contents, radical scavenging capacity, cellular inhibitions of F_2-isoprostanes, lipid hydroperoxides, leukotriene B_4, and myeloperoxidase activity were significantly increased by the beverages containing added antioxidants compared to those without. The other additive types did not influence the measured antioxidant and anti-inflammatory outcomes. Commercial cocoa beverages were shown to exert potential nutraceutical properties, such as antioxidant and anti-inflammatory activities. Selective food additives may exert profound effects on these properties by modulating the availability of flavonoids and polyphenols.

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Introduction

Consumers' affluence and increasing prevalence of lifestyle-related diseases, such as obesity and diabetes, encourage the consumers to selectively consume beverage products that support healthy lifestyles and potentially reduce the risk of chronic diseases. This group of modern consumers begins to pay attention to the nutraceutical properties of their beverages in addition to the nutritional ones. Chocolate, as a food or confectionery product, has been widely enjoyed by consumers, and therefore was extensively studied in the food science and nutrition domains. However, cocoa or chocolate-flavored beverages were comparatively less studied. Cocoa or chocolate-flavored beverage is a popular beverage preparation of the ground, roasted cocoa beans.[1] It is commonly enjoyed in different formats by consumers in various geographical regions and cultures. The results from several meta-analyses suggested health benefits from the consumption of cocoa.[2-4] However, little is known about the possible health benefits of cocoa beverage consumption. Numerous studies had associated antioxidant capacity of plant-based food, like fruits and vegetables to their flavonoid and polyphenol contents.[5-7] Similar to cocoaand other plant-based food products, the nutraceutical properties of cocoa beverages are likely contributed by their innate phytochemicals, such as flavonoids and polyphenols, via antioxidant and anti-inflammatory activities.[7,8] The flavonoid, polyphenol contents, antioxidant capacity, and anti-inflammatory activity of commercial cocoa beverage products are relatively unknown.

The food additives are a group of substances added to the food and beverage products to preserve flavor or enhance their tastes, appearances, stability, and other qualities, in order to meet the consumers' requirements. Recent studies had linked food additives, like preservatives and colorants, to detrimental physiological events.[9,10] Limited data are available on the uses of food additives in the manufacture of commercial cocoa beverage products. The effects of food additives on flavonoid, polyphenol contents, antioxidant capacity, and anti-inflammatory activity of commercial cocoa beverage products are also relatively unknown.

Cocoa beverage products are predominantly sold in the form of powder premix in Singapore. The study examined the presenceof food additives in these commercial cocoa beverage premix products. The same study also evaluated if the presence of food additives affects the total flavonoid, polyphenol contents, antioxidant capacity, cellular antioxidant and anti-inflammatory activity of these beverage products.

Materials and Methods

Chemicals and Materials

F_2-isoprostanes-d_4, F_2-isoprostanes, leukotriene B_4 (LTB_4), leukotriene B_4-d_4, and arachidonic acid (AA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Glucose, dextran 500, sodium carbonate, Folin–Ciocalteu's reagent, gallic acid, aluminium chloride,quercetin,1,1-diphenyl-2-picrylhydrazyl radical (DPPH), vitamin C, phorbol12-myristate 13-acetate (PMA), calcium ionophore, trypan blue, phosphate-buffered saline (PBS), pyridine, toluene, isooctane, hydrogens peroxide (50% by volume), guaiacol, xylenol orange, ammonium ferrous sulfate, 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,3,4,5,6-pentafluorophenylbromide, and bis (trimethylsilyl) trifluoroacetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethyl acetate, methanol, ethanol, and sulfuric acid were purchased from Tedia (Fairfield, OH, USA). Ficoll-paque was purchased from GE Healthcare (Uppsala, Sweden).

Food Additive Profiling

All the cocoa beverage powder premix products found on the shelves of ten randomly selected local supermarkets were included in this study. The supermarkets were selected through cluster sampling based on the geographical locations (two from The Central, North, South, East, and West of Singapore).The name of each beverage itemand the food additives used in its production declared on the product label were recorded by trained research personnel.

Total Polyphenol, Total Flavonoid, and Free Radical Scavenging Capacity Analysis

The nutraceutical contents of the commercial cocoa beverages were determined by measuring their total flavonoid, polyphenol contents and DPPH radical scavenging capacity. The cocoa beverage premix powder (10 g) was mixed with deionized water (10 mL) before extracting with 100% ethanol (2 x 5 mL).

The ethanolic extract was topped up to 10 mL to give a final concentration of 1 g/mL.

The total polyphenol content of the ethanolic extract was determined by a modified Folin-Ciocalteu assay.[11] The total polyphenol content was expressed as mg gallic acid equivalents (GAE)/ 100g beverage. The total flavonoid contents were measured by a modified Dowd colorimetric method.[12] The total flavonoid content was expressed as the quercetin-equivalence in 100 g beverage.

The radical scavenging capacity of the ethanolic extract was determined using DPPH radical scavenging assay.[13] The radical scavenging results were expressed in mg vitamin C/ 100 g.

Cellular Antioxidant and Anti-Inflammatory Studies

Human neutrophils were isolated from the neutrophil/ erythrocyte pellet of fresh human whole blood after Ficoll-Paque gradient centrifugation and dextran sedimentation of red blood cells.[14] The whole human blood was obtained in kind from the study researchers, as such human ethics approval is not required. The freshly isolated neutrophils were resuspended in phosphate-buffered saline at a concentration of $5x10^6$ cells mL^{-1}. Cell viability was assessed using trypan blue exclusion and was typically >98%.

The antioxidant and anti-inflammatory properties of the cocoa beverages were evaluated by measuring their abilities to inhibit the productions of F_2-isoprostanes (marker of oxidative stress), LPO (lipid hydroperoxides, marker of lipid oxidation), LTB_4, and MPO (myeloperoxidase) activity (markers of cellular inflammation) from freshly-isolated human neutrophils. Briefly, the freshly-isolated human neutrophils ($5x10^6$ cells/ mL in PBS, 1mL) were incubated with cocoa beverage ethanolic extract (10μL, final concentrations, 10 mg/mL) and AA (final concentration, 10 mmol/ L) at 37°C for 5 minutes before stimulation.The neutrophils were incubated with PMA (final concentration, 200 nmol/L) at 37°C for 15 minutes to stimulate the F_2-isoprostanes production. For LPO productions, the neutrophils were stimulated with AAPH (final concentration, 5 mmol/ L) at 37°C for 15 minutes. The neutrophils were incubated with calcium ionophore (final concentration, 200 nmol/L) at 37°C for 15 minutes to stimulate the cellular production of LTB4.[15] Positive control experiments were performed by incubating neutrophils with AA before activating with either PMA, AAPH, or calcium ionophore. Negative control experiments were carried out by incubating neutrophils with AA only. The supernatant from the cell suspension was collected and stored at -80°C before F_2-isoprostanes, LPO, and LTB4analysis. F_2-isoprostanes and LTB4 were quantified using stable isotope-labeled Gas Chromatography-Mass Spectrometry.[11, 15] The formation of LPO was quantified using the Ferrous Oxidation-Xylenol Orange assay.[16]

To examine the effects of the cocoa beverages on MPO activity, freshly isolated neutrophils ($1 \times 10_6$ cells/ mLin PBS) were incubated with their ethanolic extracts (final concentrations, 10 mg/mL) for 5 minutes at 37°C before the incubate was removed. The neutrophils were resuspended in fresh PBS and lyzed by sonication. Untreated neutrophils were used as positive controls. Functional MPO activity was determined by measuring its catalytic action on the oxidation of guaiacol in the presence of hydrogen peroxide.[17]

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (USA). Data were presented as mean ± standard deviation (SD). Differences between two groups were compared using two-sample independent t-tests. A significant difference was observed when $p<0.05$.

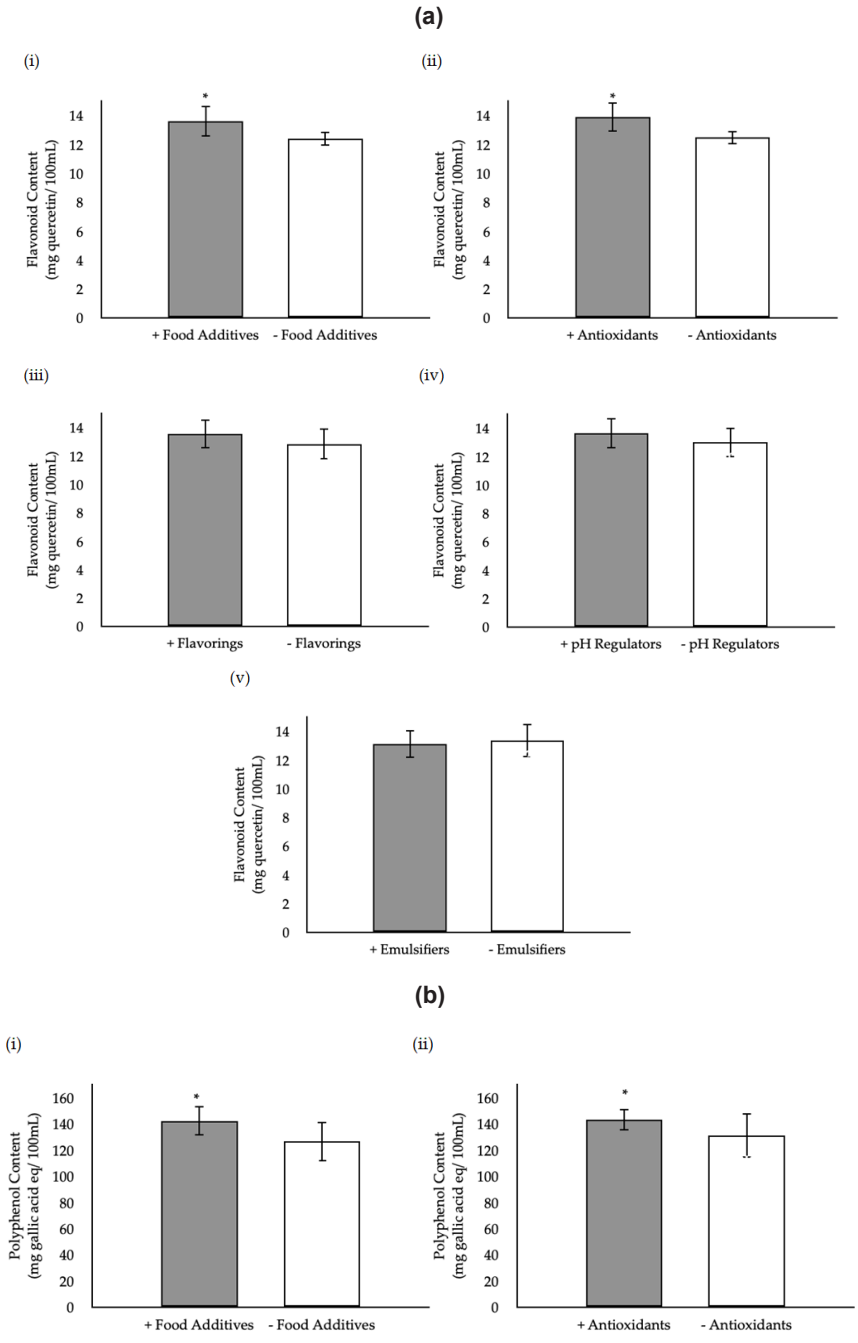
Results

Food Additive Profiling

Twenty five cocoa beverage powder premix products were included in this study. 28% of the studied cocoa beverages were free from food additives. Flavorings are the most prevalent additives present in 60% of these beverages. They were followed closely behind by antioxidants (56%), pH regulators (40%), and emulsifiers (36%). Colorings (4%) were less commonly added, and preservatives were totally absent.The specific antioxidants, colorings, emulsifiers, flavorings, and pH regulators were not stated on the product labels.

Total Polyphenol, Total Flavonoid, and Free Radical Scavenging Capacity Analysis

Significant concentrations of flavonoids (13.27±1.08 mg quercetin/ 100 mL) and polyphenols (137.8±14.0 mgGAE/ 100 mL) were present in the studied cocoa beverage products. The additive-free cocoa beverage products contained significantly lower concentrations of flavonoids and polyphenolsthan those with food additives (Figure 1a,b). After stratifying to different food additive groups, the presence of flavorings, pH regulators, and emulsifiers did not influence the concentrations of flavonoids and polyphenols in the cocoa beverages. Cocoa beverages with added antioxidants contained significantly higher concentrations of flavonoids and polyphenols than those without the designated additives (Figure 1a,b).



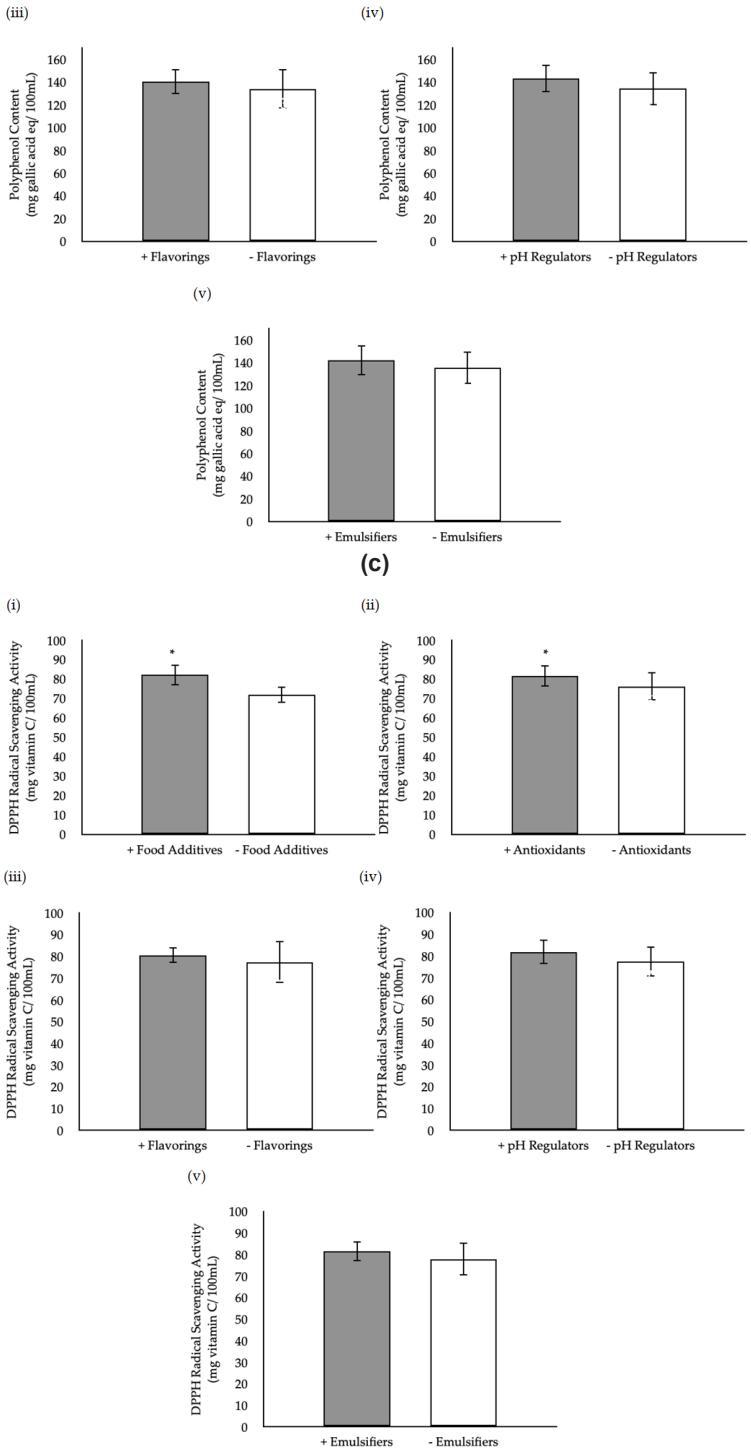
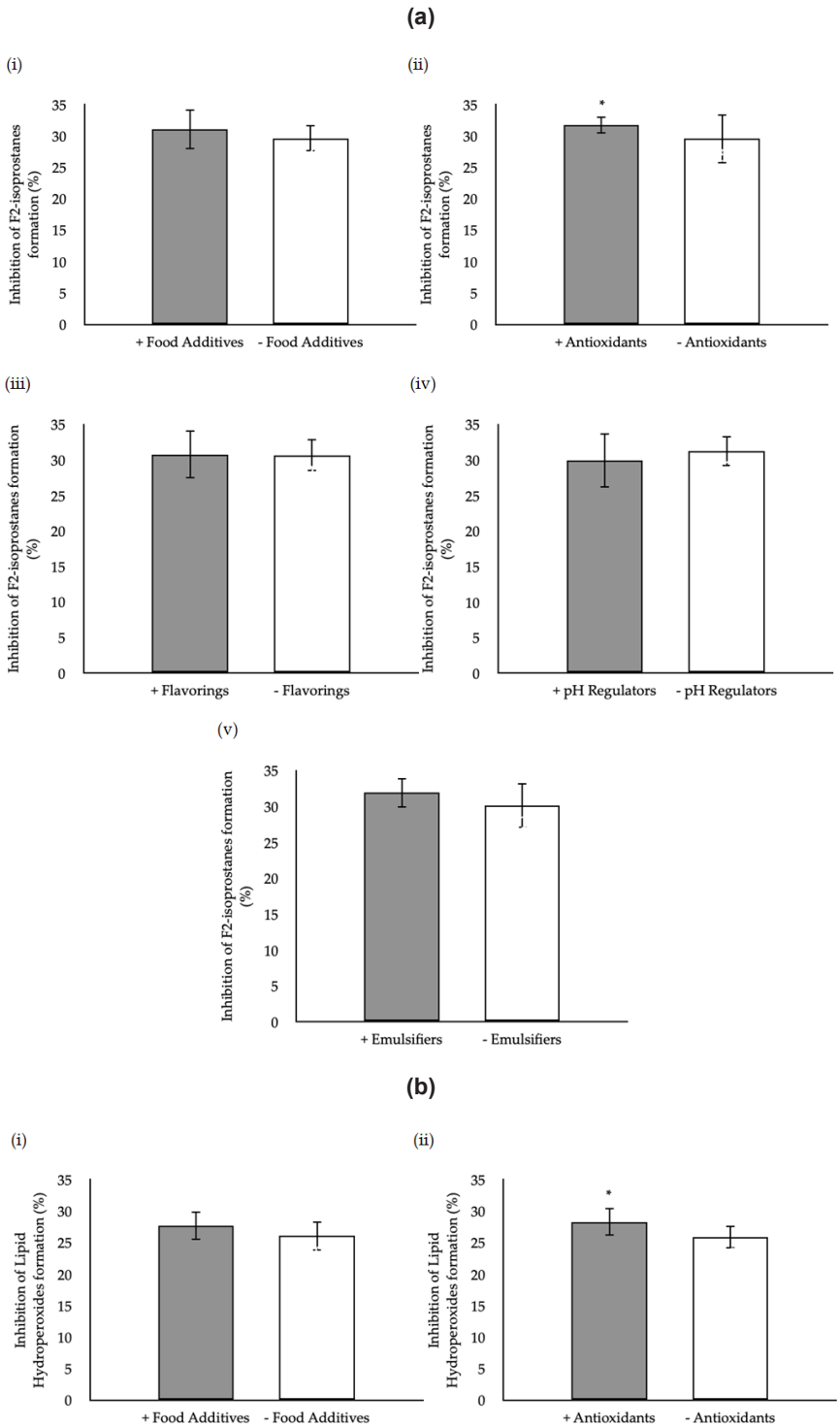
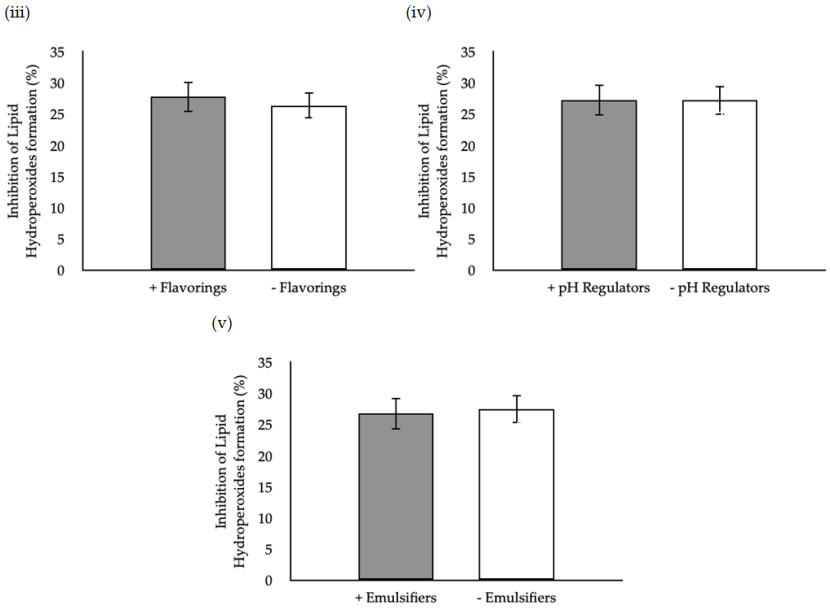
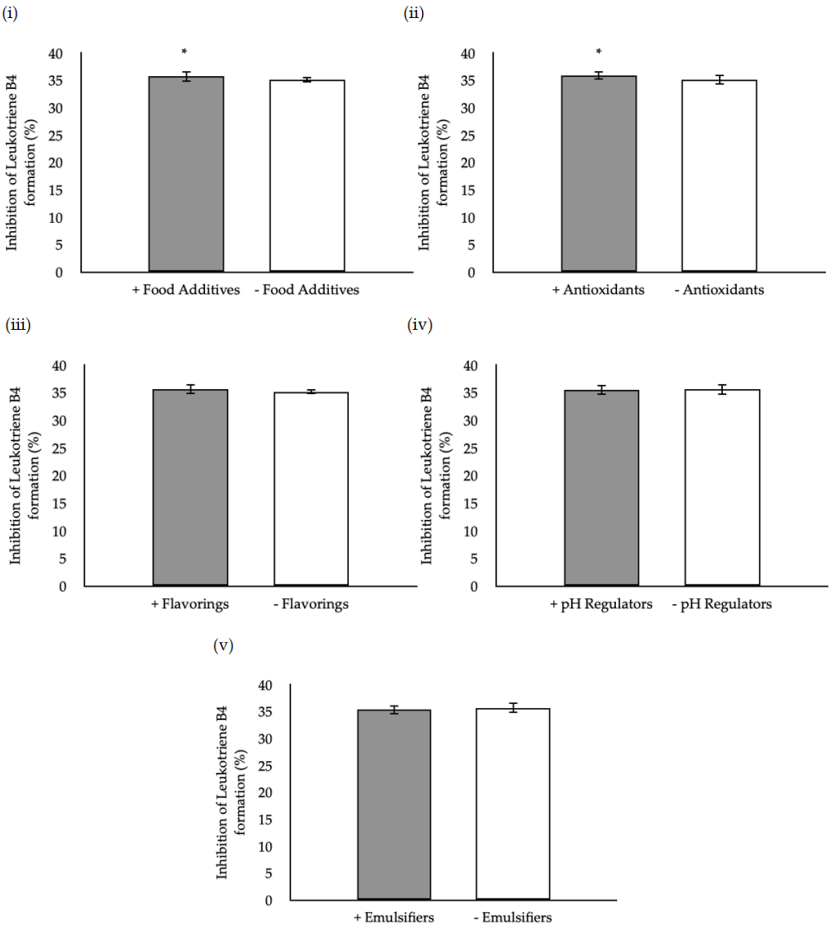


Fig. 1: Total (a) flavonoid (mg quercetin/ 100mL), (b) polyphenol (mg gallic acid equivalent/ 100mL) contents, and 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity (mean±SD) of cocoa beverage products (n=15) after stratification based on (i) food additives (with, +, n=18; without, -, n=7), (ii) antioxidants (with, +, n=14; without, -, n=11), (iii) flavorings (with, +, n=15; without, -, n=10), (iv) pH regulators (with, +, n=10; without, -, n=15), and (v) emulsifiers (with, +, n=9; without, -, n=16). * p<0.05 vs. – using two-sample independent t-test


(a)
(i)
Inhibition of F2-isoprostanes formation (%)
+ Food Additives
- Food Additives
(ii)
Inhibition of F2-isoprostanes formation (%)
+ Antioxidants
- Antioxidants
(iii)
Inhibition of F2-isoprostanes formation (%)
+ Flavorings
- Flavorings
(iv)
Inhibition of F2-isoprostanes formation (%)
+ pH Regulators
- pH Regulators
(v)
Inhibition of F2-isoprostanes formation (%)
+ Emulsifiers
- Emulsifiers
(b)
(i)
Inhibition of Lipid Hydroperoxides formation (%)
+ Food Additives
- Food Additives
(ii)
Inhibition of Lipid Hydroperoxides formation (%)
+ Antioxidants
- Antioxidants


(iii)
Inhibition of Lipid Hydroperoxides formation (%)
35
30
25
20
15
10
5
0
+ Flavorings
- Flavorings
(iv)
Inhibition of Lipid Hydroperoxides formation (%)
+ pH Regulators
- pH Regulators
(v)
Inhibition of Lipid Hydroperoxides formation (%)
+ Emulsifiers
- Emulsifiers

(c)


(i)
Inhibition of Leukotriene B4 formation (%)
40
35
30
25
20
15
10
5
0
*
+ Food Additives
- Food Additives
(ii)
Inhibition of Leukotriene B4 formation (%)
*
+ Antioxidants
- Antioxidants
(iii)
Inhibition of Leukotriene B4 formation (%)
+ Flavorings
- Flavorings
(iv)
Inhibition of Leukotriene B4 formation (%)
+ pH Regulators
- pH Regulators
(v)
Inhibition of Leukotriene B4 formation (%)
+ Emulsifiers
- Emulsifiers

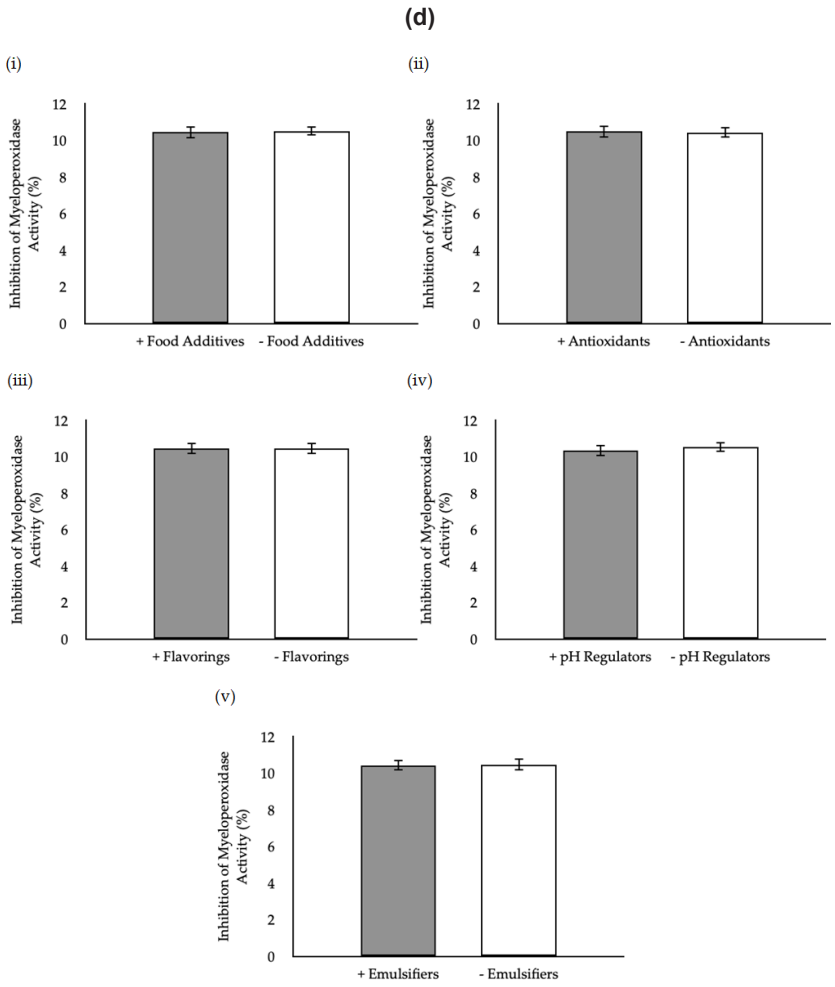


Fig. 2: Inhibition (%, mean±SD) of (a) F2-isoprostanes, (b) lipid hydroperoxides, (c) leukotriene B4 formations, and (d) myeloperoxidase activity in freshly isolated human neutrophils by the ethanolic extract of cocoa beverage products (n=15) after stratification based on (i) food additives (with, +, n=18; without, -, n=7), (ii) antioxidants (with, +, n=14; without, -, n=11), (iii) flavorings (with, +, n=15; without, -, n=10), (iv) pH regulators (with, +, n=10; without, -, n=15), and (v) emulsifiers (with, +, n=9; without, -, n=16). * p<0.05 vs. – using two-sample independent t-test

Correspondingly, the presence of food additives and antioxidants in cocoa beverages significantly increased DPPH radical scavenging capacity (Figure 1c). The other food additives did not influence DPPH radical scavenging activity.

Cellular Experiments

The F_2-isoprostanes, LPO, LTB4, and MPO activity inhibitory actions of cocoa beverage ethanolic extracts in human neutrophils are presented in Figure 2. The inhibitory activity was expressed as the percentage reduction in F_2-isoprostanes, LPO, LTB_4, and MPO activity compared to the untreated positive controls. None of the negative controls showed measurable F_2-isoprostanes, LPO, and LTB_4 productions.

The cocoa beverage ethanolic extracts decreased the cellular formations of F_2-isoprostanes, LPO, LTB_4, and MPO activity of the freshly isolated human neutrophils (Figure 2a-d). The presence of food additives in the cocoa beverages inhibited LTB_4 formation by activated human neutrophils to significantly greater extents when compared to the cocoa beverages without food additives (Figure 2c). The formation of F_2-isoprostanes, LPO, and MPO

activity were unaffected by the presence or absence of food additives. After stratifying to the different food additive types, the amounts of inhibition of F_2-isoprostanes, LPO, and LTB_4 formation in the freshly isolated human neutrophils were significantly increased by the presence of antioxidants (Figure 2a (ii), 2b(ii), and 2c(ii)) Added flavorings, pH regulators, and emulsifiers did not exhibited significant influence on the measured cellular activities.

Discussion

Nearly three-quarters of the commercial cocoa beverage powder premix products contained food additives. Consumers are avoiding products with food additives, especially food colorants and preservatives, as recent studies reported that these food additives may pose undesired health effects.[9,10] It becomes important for food manufacturers to justify, try to avoid or reduce the uses offood additives. Flavorings and antioxidants were the more prevalent food additives added into more than half of the cocoa beverages. Cocoa beverages offers unique flavor and aroma profiles that enable the easy identification. Flavorings are presumably used in cocoa beverage products to restore the flavors lost during the manufacturing processes and/or to enhance the original flavors.They may also be used to mask the undesirable roasted cocoa flavor. Antioxidants were likely added to prevent oxidation and photooxidation, to achieve shelf-life stability, and to maintain or enhance flavor, aroma, and color.[18] Stabilization of the beverage rheological property is an important process during the manufacture of the cocoa beverages, as evidenced by the presence of emulsifiersin the cocoa beverages. The pH regulators were likely added into the cocoa beverages to maintain a mildly acidic pH of 5-6 [19,20] to improve shelf-life stability and to reduce the alkalinity or bitter taste profiles. Color preservation was more likely achieved through the uses of antioxidants, in place of colorants. This was evidenced as less than 5% of the cocoa beverages had added colorings. The cocoa beverages have their unique natural colors, which have been universally accepted by their consumers. Adding food colorants into these beverages offers limited advantages to the sensory attributes of the final products. The absence or low prevalence of added colorings and preservatives are considered as welcome news to the consumers as recent studies had shown deleterious physiological effects of commonly used food colorants and preservatives.[9,10]

The ethanolic extracts of the cocoa beverages inhibited the formations of the markers of oxidative stress (F_2-isoprostanes and LPO) and inflammation (LTB_4 and MPO activity) by freshly isolated human neutrophils. The results of the DPPH radical scavenging activity, cellularinhibition of F_2-isoprostanes, and LPO productions affirmed that these commercial cocoa beverages are likelyto exert antioxidant property. Those from the MPO activity assay and LTB_4, cellular productions suggested that these beverages also exert anti-inflammatory activity. Cocoa possesses unique phytochemical compositions, especially the flavonoids and polyphenols,[21] which might contribute tothe demonstrated antioxidant and anti-inflammatory activities.[7] The study results showed that the commercial cocoa beverage premix products contained significant concentrations of flavonoids and polyphenols. The innate flavonoids and polyphenols in cocoa were previously shown to be retained in their beverages,[21] albeit of losses due to handling and processing. Therefore, the cocoa beverages may offer flavonoid- and polyphenol-associated nutraceutical benefits beyond the conventional macro- and micronutrients.[7] The study demonstrated that the food additives altered the antioxidant and anti-inflammatory properties of the cocoa beverages, dependent of the flavonoid and polyphenol contents. After stratification based on the types of food additives, similar significant difference in DDPH and cellular results were demonstrated only between cocoa beverages with and without added antioxidants.The added antioxidants may have prevented against the degradation of the innate cocoa flavonoids and polyphenols during the manufacturing processes[22] and thereby indirectly preserve the potential antioxidant and anti-inflammatory capacities.[22] Technically, the added antioxidants might directly contribute to the antioxidant and anti-inflammatory properties. The flavonoid and polyphenol molecules are required to be easilyoxidized to act as effective antioxidant[23] or possibly anti-inflammatory agents.[24] Unlike the antioxidants which are likely to stabilise the flavonoid and polyphenol molecules via oxidative-reductive mechanisms, emulsifiers and pH regulators may instead form relatively stable complexes with the flavonoid and polyphenol molecules,[25,26] and

make the latter less susceptible to oxidation and consequently decrease their antioxidant and anti-inflammatory efficacies.

The results and its accompanying discussion, although limited by the observational nature of the study, provided crucial information on the antioxidant and anti-inflammatory activities of real commercial cocoa beverage products, and the effects of food additives on these activities. More cellular, animal or human studies are required to ascertain the effects of food additives on the antioxidant and anti-inflammatory activities offered by these flavonoid- and polyphenol-rich beverages. Mechanistic studies are also required to elucidate the effects of specific food additives on their nutraceutical properties.

Conclusion

The commercial cocoa beverage powder premix products contained significant concentrations of flavonoids and polyphenols, and may offer antioxidant and anti-inflammatory benefits. The uses of food additives may exert profound and differential effects on the antioxidant and anti-inflammatory activities of cocoa beverages.

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Conflict of Interest

The authors declare no conflict of interest.

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