The Antioxidant Capacity of Beverage Blends Made from Cocoa, Zobo and Ginger

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Abstract—The antioxidant capability of beverage blends made from cocoa, zobo and ginger with standard antioxidant assay procedures was investigated. The DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging capacity ranged from 21.2-25.8% in comparison with GSH of 37.1%. The ferric reducing ability was highest in the zobo drink and lowest in ginger. The superoxide scavenging capacity was also highest in the zobo drink followed by the drink with alkalized cocoa. The metal chelating power decreased as the level of zobo in the blends decreases. The chelating power of zobo and ginger were significantly lower than the natural and alkalized cocoa. The 100% zobo drink inhibited linoleic acid till the fifth day while natural and alkalized cocoa as well as the blend with 50% alkalized cocoa inhibited linoleic acid greatly till the sixth day. The finding describes the potential health benefit of the phytochemical antioxidants of cocoa:zobo:ginger beverage blends.

Keywords—antioxidant; cocoa; ginger; health benefit; zobo blend

I. INTRODUCTION

OXIDATION reactions are essential for life, but they generate reactive oxygen species that can cause significant damage to biological molecules, such as lipids, protein and DNA. Therefore, complex protection systems have evolved based on antioxidants that help to eliminate these dangerous molecules. Oxidative stress have been implicated in many human diseases, but dietary consumption of antioxidants have been strongly associated with reduced risk of chronic diseases such as cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts and age-related functional decline, in addition to other health benefits [1]-[2]. Plant foods especially fruits and vegetables are rich sources of antioxidant. The growing interest in the study of natural antioxidants has led to an increase in the market presence of what are now known as functional foods or nutraceuticals or health foods [3].

There is also an increase in the development and consumption of ready-to-drink beverages and it is imperative to ascertain the nutritional potential of these drinks.

Cocoa (*Theobroma cacao*) is one of the major cash crops in Nigeria. After harvest the cocoa seeds are fermented and dried and the embryonic tissue roasted and milled into chocolate liquor. When the cocoa butter from the chocolate liquor is pressed out, the left over is referred to as cocoa powder. Cocoa powder is laden with high antioxidant properties but rarely consumed due to its high astringency. Alkalization of the cocoa powder is usually carried out to mellow the flavor; however, the process has been shown to destroy the polyphenolic compounds in cocoa [4].

Roselle (*Hibiscus sabdariffa* Linn) is a herbal shrub plant grown for the calyces or petals of the flower which are used in the preparation of herbal drinks and beverages. In Nigeria, roselle is commonly referred to as 'Zobo' and the beverage produced from it is drunk not only in Nigeria but across Africa because of the antioxidant properties [5]. Aqueous extract of the petals have been reported to exhibit antihypertensive and cardioprotective effects in rats [6]. When the tea from dry roselle calyces was given to human, it was found to possess urocosuric effect [7].

Ginger (*Allium sativum*) has been used since ancient time. It contains unique organosulfur compounds [8], which provide its characteristic flavor and odor and most of its potent biological activity. It has been used in the treatment of wide variety of diseases, especially gastrointestinal disorders, such as constipation, diarrhea, anorexia, colic, dyspepsia, nausea, vomiting, and motion sickness [9]. The hypotensive, vasodilator, anti-inflammatory, antitumor, antilipedimic and antioxidant pharmacological effects of ginger have been reported [10]. In the present study, beverage blends generated from the mixture of cocoa:zobo:ginger were analyzed for their antioxidative activities and the ability to inhibit lipid peroxidation. Since there is no single antioxidant assay that can accurately reflect the antioxidant potency of any beverage we utilized multiple tests to measure their antioxidant potency.

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II. METHODOLOGY

A. Collection of samples

Zobo calyces and ginger rhizomes were purchased from Oba Market, Akure in Ondo State, Nigeria, while the Cocoa powder (alkalised and non alkalised) were obtained from Ile-oluji Cocoa Products Factory, Ile-Oluji, Ondo State Nigeria.

B. Chemicals and reagents

Linoleic acid, DPPH, BHT, GSH, and other antioxidant reagents were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA). Trichloroacetic acid (TCA) was obtained from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany), hydrogen peroxide, methanol and $FeCl_2$ were purchased from BDH Chemicals Ltd., (Poole, England).

C. Preparation of Cocoa-Zobo-Ginger Beverage

Zobo calyces were winnowed to remove unwholesome seeds, stones, sticks, pebbles and other unwanted plant materials. The sorted zobo calyces was dried in an air oven at 50^oC and milled. Ginger was peeled, dried using an air oven at 50^oC and milled using a Kenwood (USA) blender. Milled zobo calyces and ginger were added to the alkalized and non-alkalized cocoa powder at different ratios. Each mix was blended and sieved through 300 μm mesh sieve size. The powder was packaged in 'tea bags' and stored in a cool dry place for further analysis. The cocoa:zobo:ginger beverage mixing ratio used were 70:20:10, 60:30:10, 50:40:10 and 40:50:10.

D. DPPH Radical Scavenging Assay

The scavenging activity of the samples against DPPH was determined using previous method [11], with slight modification for 96-well clear flat bottom plate. The samples were dissolved (50 μg/mL) in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 μM. A blank control consisted of only DPPH and sodium phosphate buffer. Appropriate dilutions of the samples (100 μL) were mixed with 100 μL of DPPH solution and incubated at room temperature in the dark for 30 min. Thereafter, the absorbance of the sample (As) and control (Ac) was read at 517 nm. The scavenging activity of the fractions was compared with that of GSH at 0.05 mg/mL. The percent scavenging activity of GSH and the samples was calculated using the following equation:

DPPH Radical Scavenging Activity (%) = (Ac−As/Ac) x100.

E. Chelation of Metal Ions Assay

The metal chelating activity was measured using a slightly modified method [12]. A 1 mL aliquot of 25 μg/mL sample or GSH solution was combined with 0.05 ml of $FeCl_2$ (2 mM) and 1.85 mL double distilled water in a reaction tube. Thereafter, 0.1 mL of 5 mM Ferrozine (3-(2- pyridyl)-5,6-diphenyl-1,2,4 triazine-4′,4″-disulfonic acid sodium salt) solution was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min. An aliquot (200 μL) of the reaction mixture was removed and added to a clear bottom 96-well plate.

The control experiment contained all the reaction mixtures except the extracts, which was replaced with 1 mL of double distilled water. The absorbance of sample (As) and control (Ac) was measured using a spectrophotometer at 562 nm and the metal chelating activity of the sample was compared with that of GSH.

The percentage chelating effect (%) was calculated using the following equation: Metal chelating effect (%) = (Ac−As/Ac) x 100

F. Ferric Reducing Power Assay

Reducing power was measured according to an earlier reported method [13] with slight modifications. An aliquot (250 μL) of 1.0 mg/ml solution of the sample or GSH was prepared in 0.2 M sodium phosphate buffer (pH 6.6), mixed with 250 μL of buffer and 250 μL of 1% potassium ferricyanide solution dissolved in double distilled water. The control reaction contained buffer and ferricyanide only. Each resulting mixture was heated at 50°C and incubated for 20min. After incubation, 250 μL of 10% aqueous trichloroacetic acid was added. A 250 μL aliquot of the reaction mixture was then combined with 50 μL of 0.1% aqueous ferric chloride solution and 200 μL of double distilled water was also added. The mixture was allowed to stand at room temperature for 10 min and then centrifuged at 1000×g. A 200 μL aliquot of the supernatant was added to a clear bottom 96-well plate and the absorbance was measured at 700 nm.

G. Superoxide Scavenging Assay

The superoxide radical scavenging activity was determined according to an earlier method [14]. The sample (80 μL of 1 mg/mL solution) or GSH (1 mg/mL) were mixed with 80 μL of 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA in a clear bottom 96-well plate. Then, 40 μL of 1.5 mM pyrogallol dissolved in 10 mM HCl was added to each well. The reaction rate (ΔA/min)s was monitored as increase in absorbance at 420 nm for 4 min at room temperature. The control mixture, (ΔA/min)c contained the Tris-HCl buffer but no beverage fraction. The superoxide radical scavenging activities of the samples were calculated using the following equation:

Superoxide Radical Scavenging Activity (%) = (ΔA/min)c - (ΔA/min)s/ (ΔA/min)c x100

H. Inhibition of Linoleic Acid Oxidation

Linoleic acid oxidation was measured using the method described earlier [15]. Sample or standard compounds (GSH and BHT) at 50 μg/ml were dissolved in 1.5 mL of 0.1M sodium phosphate buffer (pH 7.0). The mixture was added to 1 mL of 50 mM linoleic acid dissolved in 99.5% ethanol. This was stored in a glass test tube and kept at 60 °C in the dark for 7 days. On a daily basis, an aliquot (100 μL) of the sample mixture was removed and mixed with 4.7 ml of 75% aqueous ethanol, 0.1 mL of ammonium thiocyanate (30%, w/v) and 0.1 mL of 0.02 M ferrous chloride dissolved in 1 M HCl. An aliquot (200 μL) of the resulting solution was added to a clear bottom 96-well plate and the degree of color development was measured using the spectrophotometer at 500 nm after 3 min incubation at room temperature.

I. Statistical Analysis

The results were analyzed using the statistical package for social sciences (SPSS) version 17.0 for Windows. All the data are expressed as means ± standard error (n = 3).

III. RESULTS AND DISCUSSION

A. DPPH Radical Scavenging Activities

DPPH is a stable free radical, and when it encounters proton-radical scavengers, the maximum absorbance at 517 nm fades rapidly. The antioxidant effect is proportional to the disappearance of DPPH in test samples by producing visually noticeable discoloration from purple to yellow. The DPPH scavenging activities were determined for all the blends of cocoa:zobo:ginger and reported as IC_{50} values (the antioxidant concentrations corresponding to 50% radical scavenging efficiencies). There was no significant difference in the DPPH radical scavenging values of natural and alkalized cocoa as well as zobo and ginger. The values ranged from 22.6% to 25.8%. All the various blends of cocoa:zobo:ginger also have values ranging from 20.9% to 25.4% (Fig. 1). The antioxidants in the beverage blends neutralized the free radical character of DPPH by transferring electron or hydrogen atoms to DPPH. [16]. The DPPH scavenging value of GSH, used as control, was however higher than all the various blends with a value of 37.4%. This notwithstanding, the values obtained for all the beverage blends showed that they have relatively high enough DPPH scavenging activities.
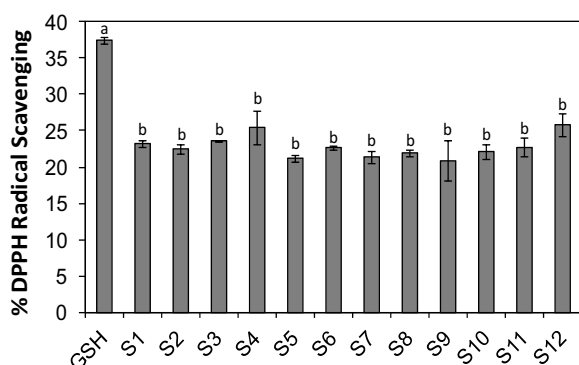

Fig. 1 The DPPH radical scavenging activities of cocoa:zobo:ginger beverage blends. Samples S1-S12 are the various mixing ratios where S1 (100N:0:0), S2 (40N:50:10), S3 (50N:40:10), S4 (60N:30:10), S5 (70N:20:10), S6 (100A:0:0), S7 (40A:50:10), S8 (50A:40:10), S9 (60A:30:10), S10 (70A:20:10), S11 (0:0:100), S12 (0:100:0), GSH; gluthatione, A; alkalized, N; natural. Different letters of the alphabet represent values which are statistically significant (P ≤ 0.05). Values are mean ± SE (n=3)

B. Superoxide Radical Scavenging Activities

Superoxides could be produced in large amounts by various biological processes. It is known to be very harmful to cellular components as a precursor of the most reactive oxygen species and it contributes to tissue damage and various diseases [17]-[18]. Apart from GSH that has a superoxide radical scavenging activity greater than 90%, zobo drink has the highest superoxide radical scavenging activity of more than 28% when compared to other beverage blends (Fig. 2). The alkalized cocoa (S6) and the blend with 50% alkalized cocoa (S8) both have high superoxide radical scavenging activity.

Although the natural cocoa has a very low superoxide radical scavenging activity, the blend containing 60% of the natural cocoa is rich in superoxide radical scavenging activity. The superoxide anion radical plays an important role in several pathophysiological conditions owing to its ability to transform into more reactive hydroxyl radicals. They are generated during a number of biological reactions. Superoxide anion radicals are generated in vivo when xanthine oxidase reduces molecular oxygen instead of nicotinamide adeninedinucleotide (NAD) under stress conditions. They have the ability to reduce transition metals and could promote oxidative reactions, lipid oxidation, cell membrane and DNA damages [19]-[20]. The scavenging potential would depend on the number and locations of the hydroxyl groups in the phenolic compounds present in the extracts. Superoxide dismutase catalyzes the neutralization of superoxide anion to hydrogen peroxide.
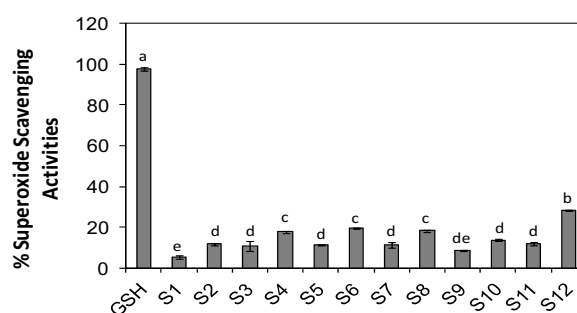

Fig. 2 Superoxide radical scavenging activities of cocoa:zobo:ginger beverage blends. Samples S1-S12 are the various mixing ratios where S1 (100N:0:0), S2 (40N:50:10), S3 (50N:40:10), S4 (60N:30:10), S5 (70N:20:10), S6 (100A:0:0), S7 (40A:50:10), S8 (50A:40:10), S9 (60A:30:10), S10 (70A:20:10), S11 (0:0:100), S12 (0:100:0), GSH; gluthatione, A; alkalized, N; natural. Different letters of the alphabet represent values which are statistically significant (P ≤ 0.05). Values are mean ± SE (n=3)

C. Ferric Reducing Antioxidant Power: FRAP

The reducing capacity of the beverage blends may serve as a significant indicator of the potential antioxidant activity [21]. The transformation of the Fe^{3+}/ferricyanide complex to the ferrous form at 700 nm was used to determine the ferric reducing power of the beverage blends with an increase in absorbance corresponding to high reducing power. Although the GSH used as control has the highest FRAP value of 2.4, the reducing power of 100% zobo was exceptionally higher than all the other beverage blends with a value of 1.4 (Fig 3).

The FRAP value of the natural cocoa (S1) was about 60% higher than the value obtained for the alkalized blend (S6). All the other blends of cocoa:zobo:ginger have values that were significantly lower than the 100% cocoa. Ginger (S11) also has an exceptionally low FRAP value of 0.07 as shown in Fig 3.
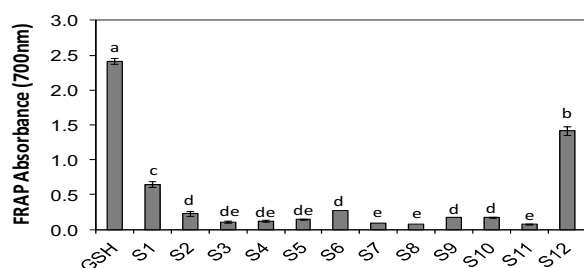
Fig. 3 Ferric reducing power of cocoa:zobo:ginger beverage blends. Samples S1-S12 are the various mixing ratios where S1 (100N:0:0), S2 (40N:50:10), S3 (50N:40:10), S4 (60N:30:10), S5 (70N:20:10), S6 (100A:0:0), S7 (40A:50:10), S8 (50A:40:10), S9 (60A:30:10), S10 (70A:20:10), S11 (0:0:100), S12 (0:100:0), GSH; gluthatione, A; alkalized, N; natural. Different letters of the alphabet represent values which are statistically significant (P ≤ 0.05). Values are mean ± SE (*n*=3)

D. Metal Chelating Power

Iron is an essential mineral required for normal physiology when available in the normal concentration, but an excess of it may result in cellular injury. The reactions leading to the generation of reactive oxygen species are catalyzed by transition metals such as Fe^{2+} and Cu^{2+}. Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [17], [22].The chelation of these metals could reduce the level of reactive oxygen species, retard lipid peroxidation and thus preserve the shelf life of food products. All the various blends of cocoa:zobo:ginger have very high metal chelating power. Although there were no significant difference between the blends with natural or alkalized cocoa, the chelating power of blends with 50A:40:10 (S9) and 40A:50:10 (S10) have lower values and were significantly different from others (Fig. 4). The chelating power of zobo and ginger were also significantly lower than the blends containing 100% natural or alkalized cocoa. There was however no significant difference in the chelating power of GSH and the 100% cocoa blends.
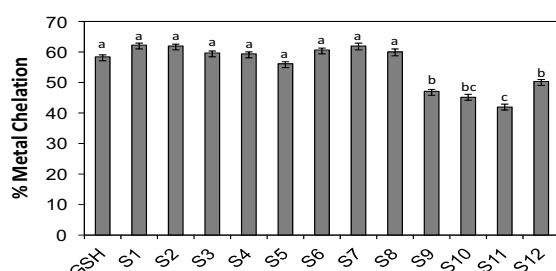

Fig. 4 Metal chelation effect of cocoa:zobo:ginger beverage blends. Samples S1-S12 are the various mixing ratios where S1 (100N:0:0), S2 (40N:50:10), S3 (50N:40:10), S4 (60N:30:10), S5 (70N:20:10), S6 (100A:0:0), S7 (40A:50:10), S8 (50A:40:10), S9 (60A:30:10), S10 (70A:20:10), S11 (0:0:100), S12 (0:100:0), GSH; gluthatione, A; alkalized, N; natural. Different letters of the alphabet represent values which are statistically significant (P ≤ 0.05). Values are mean ± SE (*n*=3)

E. Inhibition of Linoleic Acid Oxidation

Hydroxyl radicals can stimulate the free-radical chain reaction known as lipid peroxidation, which leads to the production of the toxic substances, lipid hydroperoxides and their aldehyde decomposing products, responsible for the rancidity of peroxidized food material [17]. Inhibition of lipid peroxidation can prolong the freshness of food products. The method used to determine lipid peroxidation involved the generation of a colored compound through the reaction of ammonium thiocyanate with ferric iron formed by the product of lipid oxidation of ferrous iron. The intensity of the generated colored compound is a measure of the extent of linoleic acid oxidation [23]. The entire beverage blends inhibited linoleic acid efficiently (Fig. 5A and 5B). The zobo peaked on day 5 before starting to decrease. Among the cocoa:zobo:ginger blends, sample S9 was able to inhibit linoleic acid till the 6th day (Fig 5B). Ginger started to decrease from day 4 while GSH inhibited linoleic acid till day 3. Both natural and alkalized cocoa effectively inhibited the oxidation of linoleic acid. The finding on GSH is similar to previously reported data, which showed that this compound gradually lost its ability to protect against linoleic acid oxidation after two days of incubation [23]. When GSH is oxidized into GSSG, the regeneration of GSH (antioxidant form) is not possible with increasing experimental time thus resulting in its decreased ability to inhibit lipid oxidation.
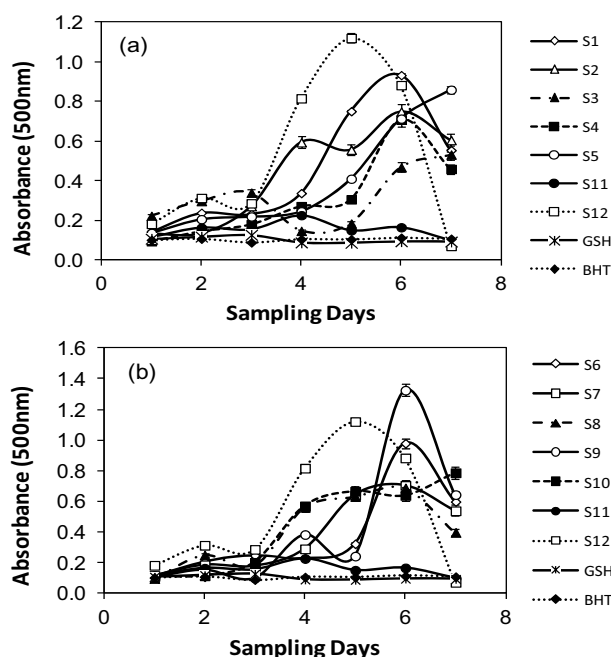

Fig. 5 Inhibition of Linoleic acid by the various blends of cocoa:zobo:ginger. (a) blends made with non-alkalized cocoa and (b) blends made with alkalized cocoa. Samples S1-S12 are the various mixing ratios where S1 (100N:0:0), S2 (40N:50:10), S3 (50N:40:10), S4 (60N:30:10), S5 (70N:20:10), S6 (100A:0:0), S7 (40A:50:10), S8 (50A:40:10), S9 (60A:30:10), S10 (70A:20:10), S11 (0:0:100), S12 (0:100:0), GSH; gluthatione, BHT; Butylated Hydroxyl Toluene; A; alkalized, N; natural. Values are mean ± SE (*n*=3)

IV. CONCLUSION

The present study showed that cocoa:zobo:ginger beverage blends have significant antioxidant activities and the various antioxidant mechanisms of these beverages may be attributed to a strong hydrogen-donating ability, a metal-chelating ability and their effectiveness as good scavengers of superoxide radicals.

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