Total Phenolic Contents and Antioxidant Potential of Soya Bean and Maize and their Beverages *In vitro*

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Abstract

The present investigations sought to correlate the total phenolic contents (TPC) with antioxidant potential, using *in vitro* antioxidant evaluation models, of unprocessed soya bean (SB) and its industrial processed beverages (SBB1 and SBB2) as well as unprocessed maize (SM) and its industrial processed beverages (MBB1 and MBB2). The TPC and antioxidant potential of the samples were measured using standard spectrophotometric methods. The radical scavenging capacity index (SCI50) defined the concentration, in µg/mL, of the sample required to scavenge 50% of the investigated radicals. Likewise, AP50 defined the concentration, in µg/mL, of the sample required to reduce 50% of ferric ion. The TPC of SB, SBB1 and SBB2 was within the range of 0.97 ± 0.02-2.86 ± 0.02 mg gallic acid equivalent per gram dry sample, and TPC of SM, MBB1 and MBB2 were in the increasing order: SM>MBB1>MBB2. The TPC of SB, SBB1 and SBB2 and their corresponding SCI50 against NO–, H_2O_2 and •−OH gave correlation coefficients between the range: -0.77227-0.338172 units, whereas their corresponding AP50 gave a strong positive correlation. The TPC of SM, MBB1 and MBB2 and their corresponding SCI50 against NO–, H_2O_2 and •−OH gave correlation coefficients between the range: 0.040672-0.51799 units, whereas their corresponding AP50 showed a strong negative correlation. The study revealed that antioxidant potential was intertwined with the combinatorial antioxidant peculiarities of the various samples.

Keywords: Antioxidants; Beverages; Maize; Soya bean; Total phenolic contents

Introduction

Phenolic compounds are of considerable importance in human diets because of their capacities to scavenge/neutralize cytotoxic reactive oxygen and nitrogen species (RONS) by donating electrons or hydrogen atoms to radicals [1-4]. In addition, phenolic compounds chelate transition metals such as Cu^{2+}, Co^{2+} and importantly, Fe^{2+}, which have been implicated in the generation of highly reactive hydroxyl radicals via the Fenton reaction [2,5]. Fruits, vegetables and beverages are the major sources of phenolic compounds and contribute to the dietary intake of natural antioxidants [6,7]. Phenolic compounds are generally synthesized via the pentose phosphate, phenylpropanoid and shikimate pathways [2,8,9]. These compounds possess aromatic rings bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer [4,9,10]. For instance, the flavonoids (including anthocyanins) and isoflavonoids bear the carbon skeleton C_6-C_3-C_6 structure and account for more than half of over 8,000 different phenolic compounds [1,3,6,9]. In addition, tannins or tannic acids are water-soluble polyphenols that are present in many plant foods [11,12] and may exhibit antioxidant activity [13]. Bioactivity investigations have shown that the molecular structures, in particular, number of hydroxyl groups and nature of substituent groups in the aromatic rings of phenolic compounds are the major determinant of their antioxidant properties [1,14], which is often referred to as the structure-activity relationships of phenolic compounds. The chemical diversity, classification and medicinal potential of phenolic compounds as well as their inherent toxicity concerns have exhaustively been reported [2-4,6,7,15-18].

The soya bean (Glycine max) in Europe, also called soybean in North America, is a leguminous vegetable of the pea family that is grown in the tropical, subtropical and temperate climates. High quality protein and lipids are abundant in soya bean, which qualifies it as a very nutritive plant. Accordingly, the Food and Agricultural Organization (FAO) classified the plant as an oil seed rather than a pulse [19]. The carbohydrates and energy values of soya bean have been reported [20]. Reports also showed that daily intake of processed soya bean appeared to be one of several beneficial factors responsible for the health and longevity of Japanese people [21]. Global soya bean demand is increasing, not only for use as an oil crop and feeds for livestock and aquaculture, but as a nutritious beverage for human consumption as well as feedstock for industrial materials and biofuel [22]. Soya beans are usually processed into soya-based infant formula, dairy product substitutes, meat alternatives, low-cost substitutes for meat and poultry products [23-26] as well as production of vodka. However, soya bean meals often contain anti-nutritional factors like trypsin inhibitors, saponins, phytoestrogens, glucinins, goitrogens, lectins and urease [27,28]. Based on recent production estimates, Brazil (90 million metric tons), United States of America (89.5 million metric tons), Argentina (52.6 million metric tons) and China (15 million metric tons) accounted for worldwide major producers of soya bean in 2014 [29].

Maize (Zea mays) or corn is a cereal crop that is grown widely throughout the world in a range of agro-ecological environment. There are about 500 species of maize exist in different colours, textures and grain shapes and sizes. Although maize is a grain crop, it is usually consumed as vegetable. The grain is rich in vitamins A, C and E, carbohydrates, essential minerals, dietary fiber and proteins [30]. On a large scale, maize could be processed into biofuel [30] and as a source of starch for the production alcoholic and non-alcoholic beverages as

well as feeds for livestock [26,30]. According to 2007 reports, FAO estimated that 158 million hectares of maize were harvested worldwide. Africa harvested 29 million hectares, of which Nigeria was the largest producer in sub-Sahara Africa [31]. The United States of America is the world largest producer of maize, followed by China, Brazil, Mexico and Argentina [30,32,33]. Maize and cornmeal are important staple foods for more than 1.2 billion people in sub-Sahara Africa. Maize can be eaten roasted, boiled or fried. Popcorn consists of kernels of certain maize varieties that explodes when heated forming fluffy pieces that are eaten as snacks.

Previous survey showed that the food, wine and agricultural products processing industries cause the generation of substantial quantities of phenolic-rich products and by-products, which could be valuable natural sources of antioxidants [1,34]. Phenolic compounds mostly present in human diets include phenolic acids, flavonoids and tannins [3,35]. Some of these products have been the subject of investigations and have proven to be sources of dietary antioxidants [1,36]. In view of the increasing consumption of industrial processed plant foods, either due to dietary preference or for convenience, the present investigations sought to correlate the total phenolic contents (TPC) with antioxidant potential, using *in vitro* antioxidant evaluation models, of unprocessed soya bean and maize with their corresponding industrial processed beverages commonly sold in Nigerian markets.

Materials and Methods

Collection of samples

The commercially available soya bean-based beverages (SBB1 and SBB2) and maize-based beverages (MBB1 and MBB2) were purchased at the Relief Market, Owerri, Imo State, Nigeria. The manufacturer's label showed that the beverages were within 30 days shelf-life from the date of production. Soya bean (SB) and the sweet corn variety (SM) (*Z. mays* var. *saccharata*) were harvested during the wet season, on the 16th of August, 2015, from Ofkaja Farm at Uruagu-Nnewi, Anambra State (Latitude 6°20′N; Longitude 7°00′E), Nigeria, which lies on the rainforest belt. The samples were transported to the laboratory within 24 h, identified and authenticated by Dr. Mbagwu at the Herbarium of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. The samples have voucher numbers IMSUH 223 and IMSUH 198 for the SB and SM, respectively.

Compositions of beverages

The major compositions of SBB1, SBB2, MBB1 and MBB2 according to the manufacturers' labels are presented in Table 1.

Samples	Compositions/ingredients per 100 g
SBB1	Carbohydrates (65.5 g), protein (13.0 g), fat (6.6 g), dietary fiber (7.2 g), sodium (580 mg), iron (9.0 mg), calcium (455 mg), vitamin A (1166 IU).
SBB2	Carbohydrates (64.2 g), protein (15.0 g), fat (9.0 g), dietary fiber (7.0 g), sodium (210 mg), potassium (570 mg), calcium (400 mg), phosphorus (260 mg), linoleic acid (3.4 g), vitamin A (1500 IU), ash (2.3 g)
MBB1	Carbohydrates (80.0 g), protein (7.5 g), fat (0.8 g), dietary fiber (3.0 g), sodium (0.7 g), iron (14.0 mg), vitamin A (450 µg), vitamin B1 (1.2 mg), vitamin B2 (1.3 mg), vitamin B6 (1.5 mg), vitamin B12 (0.85 µg), folic acid (190 µg).
MBB2	Carbohydrates (85.0 g), protein (6.0 g), fat (1.1 g), sodium (270 mg), iron (16 mg), phosphorous (140 mg), zinc (7.0 mg), vitamin C (50.0 mg), vitamin D (8.5 µg), niacin (7.0 mg), vitamin E (5.0 mg), pantothenic acid (3.1 mg), thiamin (0.50 mg), vitamin A (608 µg), folic acid (80 µg).
SBB1, SBB2: Soya Bean-Based Beverages; MBB1, MBB2: Maize-Based Beverages	

Table 1: Major compositions of soya bean- and maize-based beverages.

Preparation of samples

The grains of SB and SM were removed manually from the cobs and pods respectively. Next, samples of SB and SM as well as that of their beverages were dried separately in an oven (Gallenkamp Oven 300 plus series, England) at 60°C until a constant weight was achieved. The separate samples were ground into powder using the Thomas-Willey milling machine (ASTM D-3182; India). A 2.0 g of each ground sample was transferred into corresponding conical flasks containing 10 mL of 99.0% CH_3OH (BDH, U.K). The suspensions were shaken thoroughly and allowed to stand for 30 min, after which the mixtures were filtered using Whatman № 1 filter paper. The extracts were concentrated and recovered in a rotary evaporator (Büch Rotavapor R-200) for 12 h at 50°C, for each procedure, under reduced pressure [37]. The yields were calculated to be as follows: SB=12.7% (w/w), SM=13.1% (w/w), SBB1=11.6% (w/w), SBB2=11.5% (w/w), MBB1=11.8% (w/w) and MBB2=11.1% (w/w). The separate extracts were reconstituted in corresponding 10 mL phosphate buffered saline (PBS) solution, osmotically equivalent to 100 g/L PBS; pH=7.4 (90.0 g NaCl, 17.0 $Na_2HPO_4.2H_2O$ and 2.43 g $NaH_2PO_4.2H_2O$). Portions of the individual extracts were measured for TPC. Serial dilutions of the extracts in the order of 20, 40, 60 and 80 mg/mL were prepared and used for measurement of their antioxidant activities *in vitro*.

Total phenolic content

TPC of the extracts were determined using the Folin-Ciocalteu method as previously described [38]. A 0.1 mL of 20-80 mg/mL (w/v) of the extracts were added to corresponding 1.0 mL of 7% Na_2CO_3 solutions and mixed thoroughly. Next, 0.1 mL of Folin-Ciocalteu reagent was introduced into the mixtures. The final mixture volume was made up to 2.5 mL using distilled water and was allowed to stand for 90 min during intermittent shaking. The absorbance of the mixture was measured at λmax=750 nm using a spectrophotometer (Digital Blood Analyzer; SPECTRONIC 20; Labtech, LabX, Bay Street, Midland, ON, Canada). The TPC of the samples were obtained by comparing the absorbance with that of standard gallic acid calibration curve, and expressed as milligram of gallic acid equivalent per gram (mg GAE/g) of dry weight of the extracts.

Antioxidant potential *in vitro*

The antioxidant potential of the extracts, which defined the capacity of the extracts to scavenge nitric oxide radical, hydrogen peroxide, hydroxyl radical and ferric reducing antioxidant power were measured as previously described [39].

Nitric oxide radical

The procedure was according to previous methods [40] but with minor modifications. Reaction mixtures containing of 2.0 mL of 10 mM NaN_3 in phosphate buffered saline (pH=7.4) and 1.0 mL of various concentrations (20-80 µg/mL) of the extracts were incubated at 25°C for 150 min. Next, 1.0 mL of 0.33% sulfanilic acid in 20% glacial CH_3COOH was added to 0.3 mL of the incubated solution and was allowed to stand for 5 min. A 0.5 mL of 0.1% (w/v) napthylethylenediamine dihydrochloride was added to the mixture and incubated at 25°C for 30 min. The absorbance was measured at λ_{max}=540 nm using quercetin as blank [37]. The nitric oxide scavenging capacity index (NOSCI) of the extracts was calculated thus:

$$NOSCI\% = \left(1 - \frac{Absorbance_{Test}}{Absorbance_{Blank}}\right) \times 100 \text{ Equation 1}$$

The NOSCI% was expressed as SCI50, which is defined as the concentration (µg/mL) of the extract required to scavenge 50% of NO−.

Hydrogen peroxide: Measurement of hydrogen peroxide scavenging potential was according to the methods of Banerjee et al. [41] but with minor modifications. Separate volumes of 50 µL of 1.0 mM H_2O_2 and 100 µL of various concentrations (20-80 µg/mL) of the extracts were incubated at 25°C for 30 min. A 0.85 mL FOX Reagent (100 µM xylenol orange, 250 µM ammonium ferrous sulphate and 25 mM H_2SO_4) was added to the reaction mixtures and allowed to stand at 25°C for 30 min. The absorbance of ferric-xylenol orange complex of the mixtures were measured against a blank at λ_{max}=560 nm.

The hydrogen peroxide scavenging capacity index (HPSCI) of the extracts was calculated thus:

$$HPSCI\% = \left(1 - \frac{Absorbance_{Test}}{Absorbance_{Blank}}\right) \times 100 \text{ Equation 2}$$

The HPSCI% was expressed as SCI50, which is defined as the concentration (µg/mL) of the extract required to scavenge 50% of H_2O_2.

Hydroxyl radical

The procedure was carried out as previously described [42] but with minor modification. Briefly, the reaction mixture containing 100 µL of 28 mM 2-deoxyribose, 500 µL of various concentrations (20-80 µg/mL) of the extracts in phosphate buffer (pH=7.4), 200 µL of 200 µM $FeCl_3$ in 1.04 mM aqueous EDTA (1:1, v/v), 100 µL of 1.0 mM H_2O_2, and 100 µL of 1.0 mM ascorbic acid was incubated at 37°C for 1 h. The reaction was terminated by the addition of 1.0 mL of 28% trichloroacetic acid (TCA). A 1.0 mL of 10% thiobarbituric acid (TBA) was added and the mixture was again incubated on a water bath at 80°C for 20 min. After cooling to 25°C, the absorbance the mixtures were measured at λ_{max}=532 nm against a blank.

The hydroxyl radical scavenging capacity index (HRSCI) of the extracts was calculated thus:

$$HRSCI\% = \left(1 - \frac{Absorbance_{Test}}{Absorbance_{Blank}}\right) \times 100 \text{ Equation 3}$$

The HRSCI% was expressed as SCI50, which is defined as the concentration (µg/mL) of the extract required to scavenge 50% of • −OH.

Ferric reducing antioxidant power

The ferric (Fe^{3+}) reducing antioxidant power was measured as previously reported [43] but with minor modifications. Equal volumes (0.5 mL) of various concentrations (20-80 µg/mL) of the extracts and 1.0% $K_3Fe(CN)_6$ with 0.2 M phosphate buffer (pH=6.6) were mixed and incubated at 50°C in a water bath for 20 min. TCA (0.5 mL) was added to the mixture and centrifuged at 3000 rpm for 10 min. Finally, 0.5 mL of the supernatant was mixed with equal volume of distilled water and 0.1 mL of 0.1% $FeCl_3$ solution. The reaction mixture was left to stand at 25°C for 10 min and the absorbance measured at λ_{max}=700 nm against a blank.

The ferric reducing antioxidant power (FRAP) of the extracts was calculated thus:

$$FRAP\% = \left(1 - \frac{Absorbance_{Test}}{Absorbance_{Blank}}\right) \times 100 \text{ Equation 4}$$

The FRAP% was expressed as AP50, which is defined as the concentration (µg/mL) of the extract required to reduce 50% of $FeCl_3$.

Statistical analysis

The data collected were analyzed by the analysis of variance (ANOVA) procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 (2006 version). Correlation coefficients were determined using Excel Software (Microsoft, 2010 version).

TPC (mg GAE/g dry sample)					
SB	SBB1	SBB2	SM	MBB1	MBB2
2.86 ± 0.02	0.97 ± 0.02	1.71 ± 0.05	1.35 ± 0.01	0.33 ± 0.01	0.17 ± 0.01

TPC: Total Phenolic Content; SB: Soya Bean; SBB1, SBB2: Soya Bean-Based Beverages; MBB1, MBB2: Maize-Based Beverages. The values are mean (X) ± S.D of three (n=3) determinations. All values of the means are significantly different at p<0.05 according to LSD.

Table 2: Total phenolic contents of soya bean and maize and their beverages.

Results

Table 2 showed that the TPC of SB, SBB1 and SBB2 was within the range of 0.97 ± 0.02-2.86 ± 0.02 mg GAE/g dry sample, in which the TPC of the samples were in the increasing order: SB>SBB2>SBB1.

Furthermore, the TPC of SM, MBB1 and MBB2 showed significant difference ($p<0.05$) and were in the increasing order: SM>MBB1>MBB2. Overall, the TPC of the various samples showed significant difference ($p<0.05$).
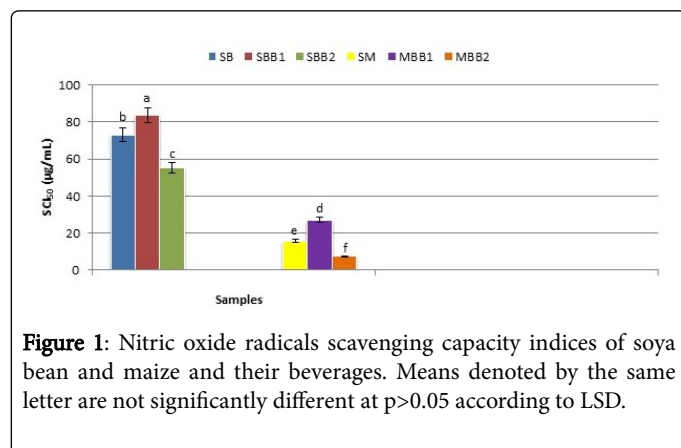

Figure 1: Nitric oxide radicals scavenging capacity indices of soya bean and maize and their beverages. Means denoted by the same letter are not significantly different at $p>0.05$ according to LSD.

Figure 1 showed that SCI50 of SB against NO– was significantly ($p<0.05$) lower than that of SBB1. Additionally, SCI50 of SBB2 against NO– was significantly lower ($p<0.05$) than that of SB. In the same order described above, the SCI50 of SM against NO– was significantly lower ($p<0.05$) than that of MBB1 but significantly higher ($p<0.05$) than that of MBB2. Generally, Figure 1 showed that SM and its industrial processed products (i.e. MBB1 and MBB2) gave significantly lower ($p<0.05$) SCI50 against NO– than those of SB and its industrial processed products (i.e. SBB1 and SBB2).
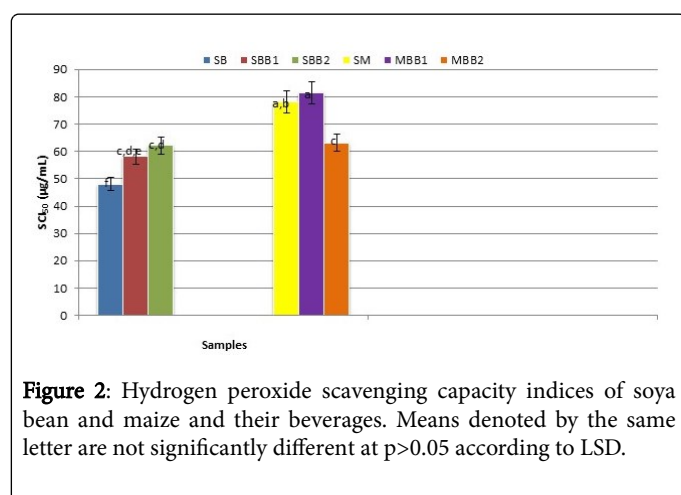

Figure 2: Hydrogen peroxide scavenging capacity indices of soya bean and maize and their beverages. Means denoted by the same letter are not significantly different at $p>0.05$ according to LSD.

The SCI50 of SB against H_2O_2 was significantly lower ($p<0.05$) than those of SBB1 and SBB2 (Figure 2). Furthermore, the SCI50 of SBB1

and SBB2 against H_2O_2 showed no significant difference ($p>0.05$). Conversely, SCI50 of MBB2 against H_2O_2 was significantly lower ($p<0.05$) than those of SM and MBB1, in which the SCI50 of SM and MBB1 against H_2O_2 showed no significant difference ($p>0.05$).

Overall, SCI50 of SB, SBB1 and SBB2 against H_2O_2 were significantly lower ($p<0.05$) than those of SM and MBB1. However, SCI50 of MBB2 against H_2O_2 was not significantly different ($p>0.05$) from those of SBB1 and SBB2.
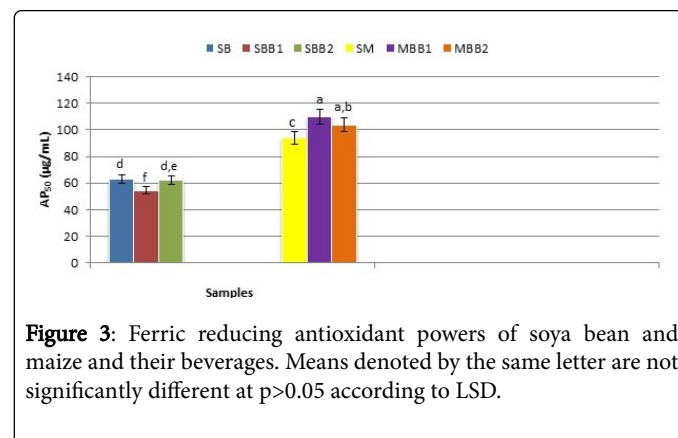

Figure 3: Ferric reducing antioxidant powers of soya bean and maize and their beverages. Means denoted by the same letter are not significantly different at $p>0.05$ according to LSD.

Figure 3 showed that AP50 of SB was not significantly different ($p>0.05$) from that of SBB2; whereas that of SBB1 was significantly lower ($p<0.05$) than those of SB and SBB2. Likewise, AP50 of MBB1 and MBB2 showed no significant difference ($p>0.05$) whereas that of SM was significantly lower ($p<0.05$) than those of MBB1 and MBB2.

Generally, AP50 of SB, SBB1 and SBB2 were significantly lower ($p<0.05$) than those of SM, MBB1 and MBB2.
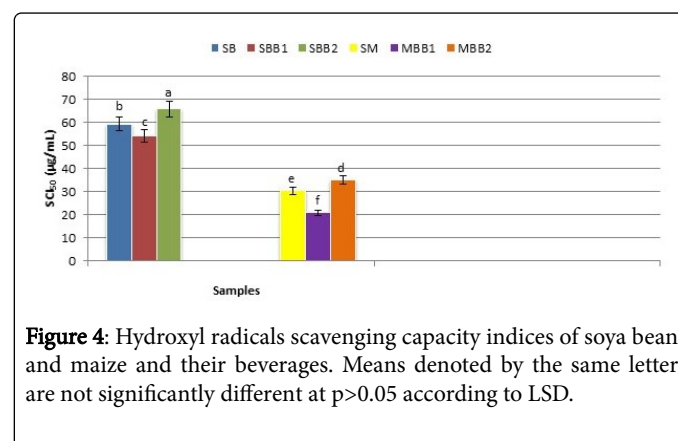

Figure 4: Hydroxyl radicals scavenging capacity indices of soya bean and maize and their beverages. Means denoted by the same letter are not significantly different at $p>0.05$ according to LSD.

Figure 4 showed that SCI50 of SB, SBB1 and SBB2 against •−OH were significantly different ($p<0.05$) and was in the increasing order: SBB2>SB>SBB1. Similarly, SCI50 of SM, MBB1 and MBB2 against •−OH was in the increasing order: MBB2>SM>MBB1; $p<0.05$. Overall, SCI50 of SM, MBB1 and MBB2 against •−OH were significantly lower ($p<0.05$) than those of SB, SBB1 and SBB2.

Correlation coefficient (r)		
Parameters	Soya bean and its beverages	Maize and its beverages
Nitric oxide radicals	-0.24949	0.040672
Hydrogen peroxide	-0.77227	0.517909
Ferric reducing antioxidant power	0.863087	-0.87017
Hydroxyl radicals	0.338172	0.064258
Soya bean and its beverages: SB, SBB1 and SBB2; Maize and its beverages: SM, MBB1 and MBB2.		

Table 3: Correlation between total phenolic contents and antioxidant capacity indices of soya bean and maize and their beverages.

The TPC of SB, SBB1 and SBB2 and their corresponding SCI50 against NO–, H_2O_2 and •−OH gave correlation coefficients between the range: -0.77227 to -0.338172 units (Table 3). Additionally, the TPC of SB, SBB1 and SBB2 and their corresponding AP50 gave a strong positive correlation.

The TPC of SM, MBB1 and MBB2 and their corresponding SCI50 against NO–, H_2O_2 and •−OH gave correlation coefficients between the range: 0.040672-0.51799 units, whereas TPC of SM, MBB1 and MBB2 and their corresponding AP50 showed a strong negative correlation.

Discussion

The present study showed that SB and SM with their corresponding industrial processed beverages exhibited variations in their TPC, in which TPC of the unprocessed samples were relatively higher than those of their corresponding processed samples (Table 2). Previous studies had reported that the impact of processing and storage of plant foods may alter their comparative TPC, as observed in berry processing where myricetin and kaempferol contents were noted to be more prone to losses than quercetin [1,44]. Likewise, processing raw apples of different cultivars into juices and sauces at different temperatures (20-50°C) caused between 19% and 53% losses in TPC in the finished products [45]. The losses or reductions in TPC in the beverages were attributed to commercial processing procedures as previously reported [3,46,47]. Aside the impacts of processing and storage on TPC, intrinsic factors like genus, and species and cultivars differences in conjunction with extrinsic factors like agronomic practices, environmental conditions also dictate the TPC of plant foods [1,3,47,48].

Phenolics are powerful antioxidants [2,5] and have been proven to be more potent antioxidants than ascorbic acid, α-tocopherol and the carotenoids [3,49]. Earlier reports had shown that the levels of phenolic compounds in plant foods could be a major determinant of their antioxidant potential [1,50]. Intuitively, unprocessed samples that contain relatively high TPC ought to exhibit proportionately greater antioxidant activities than their corresponding processed samples, exemplified by the greater capacity of SB to scavenge H_2O_2 than SBB1 and SBB2 (Figure 2), which contained comparatively lower TPC (Table 2). Additionally, extracts with relatively high TPC ought to exhibit greater FRAP than those that contained lower TPC (Figure 3). However, the paradox whereby the industrial processed samples, that contained relatively lower TPC, exhibited greater radical scavenging capacities compared with the unprocessed samples was as a result of fortification of the products with antioxidant vitamins A, C and E as

indicated in Table 1, which conformed with previous reported [51,52]. The relatively wide differentials in correlation coefficients between TPC and antioxidant activities of the various samples, which corroborated previous results [53-55], were obvious indications that non-phenolic antioxidant components acted in synergy with phenolic compounds of the samples at varying capacities in neutralizing the radicals *in vitro*. According to previous reports, the total antioxidant potential of foodstuffs did not depend on their absolute antioxidants contents but, to a large extent, on the outcomes of synergic and redox interactions among the various radical neutralizing molecules in the products [53,56,57]. Additionally, intrinsic factors such as the molecular configuration of phenolics [4,58] and type of cultivar from which the phenolic compounds are sourced [59-61] as well as the presence of interfering elements and antioxidant antagonist, for the most part, define the antioxidant capacities of phenolic compounds [54,55].

For instance, the capacities of SB, SBB1 and SBB2 to scavenge H_2O_2 as well as FRAPs of SM, MBB1 and MBB2 were largely dependent on their TPC as typified by their corresponding strong negative correlations. Conversely, FRAPs of SB, SBB1 and SBB2 were not dictated, to large extent, by their TPC as indicated by the strong positive correlation between their TPC and AP50 (Table 2). Previous reports had noted that in the presence of non-phenolic antioxidants, the antioxidant activities of phenolic compounds were not mutually exclusive but acted in synergy with their co-antioxidants, and phenolic compounds were also involved in the regeneration of essential antioxidant vitamins in biologic systems [2,62,63].

Conclusion

From general principles and definition, the experimentally derived SCI50 or AP50 are inversely proportional to antioxidant activity of the sample. The *in vitro* antioxidant systems used in the present study revealed that SB and SM with their beverages exhibited differential FRAP indices as well as antioxidant activities against NO–, H_2O_2 and •−OH and, for the most part, their antioxidant potential was intertwined with the combinatorial antioxidant peculiarities of the various samples. On a point of caution, measurement of antioxidant activities of plant foods applying *in vitro* models, in many instances, do not always present reproducible outcomes using *in vivo* evaluation methods.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgement

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