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Research Article

Comparison of Phytochemical Compositions of *Sorghum Bicolor* (L) Moench Red Flour and Pale Brown Leaves

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Abstract

There are increasing demands in plant derived natural food and their bioactive compounds with pharmaceutical, nutritional and health functional properties. No evidence exists regarding the bioactive and nutritive compounds of the red *S. bicolor* pale brown leaves which are consumed directly or indirectly as spice and herbs by humans and animals in some West Africa countries. We compared chlorophyll, carotenoids, phenolic acids, flavonoids, tannins, and fatty acid profile(s) of the flour and the pale brown leaves and their nutritional implication. The leaves were found to contain higher concentrations of total tannins (194.50-995.72mg/g), total phenolic (16.63-102.82mg/g), and flavonoids (0.20-0.36mg/g) and important essential fatty acids such as α-LA, EPA and DHA than the flour at P <0.05. These findings are the first of its kind in the leaves and suggest that red sorghum flour and its pale leaves may be a valuable health and nutrition promoting functional food that could fight against infectious and cardiovascular related illness in humans and animals.

Keywords: Bioactive; Health; Leaves; Nutrition; Phenolic; Sorghum flour

Introduction

Sorghum bicolor (L.) Moench is considered as the fifth most important cereal grain cultivated worldwide. The cereal is considered as an important staple food and medicinal crop consumed by over 750 million people in Sub-Saharan Africa, Latin America and India [1,2]. The grains have numerous benefits ranging from nutritional, chemical, pharmaceutical and food colorant [1,3-6].

In the USA, the cereal is used primarily for feeding animals and possibly ethanol production [7]. Although these uses of the grains are widely known, the leaf especially the pale brown type also have many potential uses which have not been exploited in the past partly because of lack of characterization of the leaf constituents and availability of ethnomedical information across countries. Furthermore, in Northern Ghana, the traditional folks use the leaves as herbal medicine for management of diseases such as anemia and iron deficiency [6].

Scientific information on phytochemical compounds composition of the Red *S. bicolor* pale brown leaves consumed in some African countries by humans and animals is scarce. Based on the numerous folk medicine benefits that the red *Sorghum bicolor* pale leaves provide to consumers in some West African countries (e.g., Ghana), a thorough characterization of the different stages of the color of the *S. bicolor* leaves used in different food preparations, herbs and feed formulations is warranted. In this present study, the levels of the phenolic acids, flavonoids, condensed tannins, chlorophylls, carotenoids, and fatty acid composition in the red *Sorghum bicolor* pale leaves were compared to the flour made from the sorghum grains that is used by people in Africa (especially in Ghana, Nigeria, Togo and Burkina Faso).

Materials and Methods

Samples collection and preparation of extracts

Sorghum bicolor (L) Moench pale brown dry leaves and sorghum gains were obtained from the upper east region (Bolgatanga) of Ghana. The pale leaves were harvested prior to complete maturations of the seeds, this part of the leave is different from the red leaves in that, the red leaves occur when the plant completely matures and ready for harvesting. Flour was made from the grain, and the pale leaves were ground using a coffee miller and sieved with a particle size less than 2mm for sub sampling for the various assays according to Abugri et al. [6]. All samples were stored in amber bottles prior to analysis.

All chemicals were of HPLC and ACS grade. Epigallocatechin, naringenin and apigenin were obtained from Santa Cruz biotechnology Inc. Santa Cruz, California, USA. Gallic acid, vallic acid, salicylic acids, Folin-Ciocalteu reagents were obtained from Sigma Aldrich USA.

A total of 30.0mL was used for extraction of compounds with a solvent mixture (Acetone: Hexane (HPLC grade)) in a ratio of 4:6. Methanol (LC-MS, HPLC, Spectrophotometry grade) and ethanol (99.9% AC reagent (2000 proof) all purchased from Fisher scientific, USA, were added to each test tube with 0.300g sample of flour or powdered leaves. The tubes were capped tightly with Teflon caps and incubated at 30°C for 20mins according to the procedure of Abugri et al., [6]. Samples were vortexed for 3mins and then centrifuged for 5mins using a clinical table top centrifuge (IEC Centra, CL2, International Equipment Company, Needham Heights,

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MA, USA) at 2000 rpm then filtered using Whatman No. 1 filter paper into new test tubes for various phenolics assays.

Total phenolic content determination

Total Phenolic Contents (TPC) were determined colorimetrically using Folin-Ciocalteu method as reported in Afify et al., [8] with modification. A quarter mL of sorghum flour/leaves was mixed with 0.250mL Folin-Ciocalteau reagent and 0.50mL of 2% sodium carbonate (Na_2CO_3) then the volume increased to 5mL with distilled water. After incubation in the dark for 30mins at room temperature the absorbance of the reaction mixture was measured at 725nm against a blank which was distilled water. Salicylic acid was used as a standard for calibration curve ($R^2 = 0.99$) based on previous literature about the presence of this phenolic acid in sorghum. Three independent samples were extracted and then analyzed in triplicates.

Total condensed tannins content determination

Total condensed tannins was determined according to the colorimetric procedures of Price et al., [9]. Briefly, a 1000µL of sorghum flour/leave was pipetted into a test tube followed by the addition of 5.0mL of vallin/HCl solution (v/v) reagent (0.5g vanillin in 4% methanol plus 1.5ml of 12M HCl solution (v/v)). After a thorough mixture the samples were kept in the dark for 20mins at room temperature. Absorbance was measured using Agilent /HP 8453 UV-Visible spectrophotometer at 500 nm. Ethanol, methanol and acetone-hexane were used as the blanks replacing the sample. Results are expressed as mg/g using Gallic Acid (GA) equivalents with $R^2 = 0.999$.

Total flavonoid content determination

The determination of total flavonoids was according to the procedure used by Abugri et al., [3] with modification. A 0.25mL of sample was mixed with 1.25mL of deionized water and 75µL of a 5% $NaNO_2$ solution. After 6min, 0.1mL of 10% $AlCl_3.6H_2O$ solution was added to the mixture and incubated for 5mins at room temperature. A 0.5mL of 1.0M NaOH was added to the sample followed by adding 2.5mL of deionized water. The mixture was then thoroughly mixed by vortexing and the absorbance of the light pink was measured at 510nm against a blank using Agilent /HP 8453 UV-Visible spectrophotometer. Results were expressed as mg/g of catechin hydroxide equivalent with $R^2 = 0.989$. Samples were analyzed in triplicates.

Total chlorophyll, carotenoids and chlorophyll content determination

The content of chlorophyll a and b, total carotenoids (xanthophyll and carotenes), **β-carotene** and lycopene were determined according to the method of Lichtenthaler and Buschmann [10]; Nagata and Yamashita [11]. The absorbance of the extracts was measured at 453, 505 and 663nm with Hewlett-Packard 8453 UV visible spectrophotometer. The results were expressed as mg of carotenoids/g of extract.

Fatty Acid Methyl Esters (FAMEs) preparation and Gas Liquid Chromatography (GLC) analysis

A procedure used by Abugri et al., [6] was used. Approximately 0.30g of flour/ leaves was weighed into a Pyrex culture test tube. A volume of 5.3mL of methanol plus 0.60mL of approximately 12M H_2SO_4 was added then incubated at 100 to 110°C for 30mins with an automated shaker at 100rpm. Sample test tubes were placed on ice for 5mins to cool then followed by addition of 4mL of hexane vortexed for 3mins using TXr vortex. Samples were centrifuged for 5mins using a clinical top table centrifuge (IEC Centra, CL2, International Equipment Company, Needham Heights, MA, USA) at 2000rpm. FAMEs were collected into GLC vials for analysis. The preparations were carried out in duplicate. Separations and quantification of individual fatty acids were carried out according to procedures presented by [6]. Individual fatty acids were identified by comparison of their retention times with the standard retention times using an external standard GLC 463. The results are reported as total fatty acid identified in percentages.

HPLC analysis of phenolic compounds

Quantitative analysis of the various phenolic compounds present in the samples were analyzed using Agilent 1100 series equipped with a Diode Array Detector (DAD) couple with UV-Visible Spectrophotometer. Samples were injected using an automated injector with an injection volume of 5.0µL with a flow rate of 0.5mL/min. The total run time was between 10-12mins per sample. The mobile phases were made up of 95 % methanol (B) and 5% HPLC water at pH = 3.01 (A), respectively. An Atlantis dC18 column (Waters Corporation, Ireland) with dimensions 4.6 x 250 mm and 5µm was used for identification and separation of phenolic acids and flavonoids using Agilent Chemstation software 3.1. Flavonoids and phenolic acids were identified and quantified by comparing their retention times with externals standards run at wavelengths of 210-270nm with a reference wavelength of 540nm. The amounts are reported as µg/g of sample used.

Statistical analysis

A completely randomized design with treatments arranged in a 2x2 factorial. The first factor was sample type (leaves or flour) and the second factor was extraction solvent (ethanol or methanol). The data were analyzed using SAS (SAS Inst. Inc., Cary, NC, USA). Where significant differences were observed, Tukey's multiple comparison procedure was used to separate the means. Level of significance was set at $p<0.05$.

Results and Discussion

Table 1, shows the individual fatty acids found in both leaves and flour. The fatty acids observed ranged from C6:0 to C24:0 which partially agreed with some previous studies in millet and sorghum varieties food, seed oils [12-14]. Among the individual fatty acids of the flour sample analyzed, linoleic acid (C18:2n6) alone constituted about 39.33% of the total fatty acids' and was observed to be the dominant fatty acid in the flour of the seed. This finding of predominance agreed with previous studies reported by [12] in sorghum seeds. Other fatty acids of interest both nutritionally and biochemically with appreciable amounts were C18:0 (2.63%), C18:1 (22.07%), and C16:0 (21:13%). These observations concurred with previous works in total lipid fractions using different solvents to characterized glycolipids, phospholipids and neutral lipids in two varieties of sorghum seeds (SSH3 and L187) [12]. In the case of the leaves, C16:0 (21.49%) was the chief saturated fatty acid with the highest percent among all the total identified fatty acids.

% FA	SBL	SBF
6:0	1.23 ± 0.78	1.24 ± 0.03
7:0	-	0.08 ± 0.01
8:0	0.32 ± 0.09	-
10:0	1.88 ± 0.30	-
11:0	0.33 ± 0.02	-
11:1	0.68 ± 0.29	-
12:0	1.13 ± 0.33	-
12:1	0.62 ± 0.15	0.35 ± 0.04
13:0	1.83 ± 0.34	-
14:0	0.46 ± 0.10	-
14:1	0.62 ± 0.10	-
15:0	0.38 ± 0.00	0.17 ± 0.04
15:1	1.74 ± 0.23	-
16:0	21.49 ± 3.51	21.13 ± 1.27
16:1	3.31 ± 0.52	0.16 ± 0.06
17:0	2.21 ± 0.43	0.19 ± 0.05
18:0	6.50 ± 0.95	2.63 ± 0.19
18:1	4.39 ± 0.55	22.07 ± 0.27
18:1n9 t	0.52 ± 0.19	1.38 ± 0.19
18:1n11	0.44 ± 0.09	0.95 ± 0.06
18:1n7	0.40 ± 0.11	1.85 ± 0.21
18:1n7t	0.76 ± 0.58	0.93 ± 0.02
18:2n6	5.14 ± 0.60	39.33 ± 0.99
18:3n6	0.46 ± 0.07	-
18:3n3	4.56 ± 0.62	1.34 ± 0.12
20:1n11	1.63 ± 0.09	-
20:0	-	1.08 ± 0.28
20:1n9	1.56 ± 0.34	-
20:1n15	1.83 ± 0.20	-
20:2n6	1.92 ± 0.14	-
20:3n6	0.77 ± 0.10	-
20:4n6	6.75 ± 1.21	-
20:3n3	0.69 ± 0.22	-
20:5n3	6.56 ± 1.95	-
C21:0	2.59 ± 0.90	-
22:2n6	1.39 ± 0.11	0.35 ± 0.12
22:5n3	-	1.37 ± 0.44
24:0	0.70 ± 0.03	2.73 ± 1.81
22:6n3	6.07 ± 0.28	0.55 ± 0.54

Table 1: Fatty acid(s) composition of *Sorghum bicolor* pale brown Leaves (SBL) and Red Sorghum Flour (SBF).

Means value plus standard deviation of two independent determinations reported in percent of fatty acid in *S.bicolor* red flour and pale brown leaves, - not detected or below detection limit.

Another, most interesting trend found was that the leaf contain higher ($p<0.05$) percent of omega 3s Polyunsaturated Fatty Acids (PUFAs) compared to omega 6 Polyunsaturated Fatty Acids (PUFAs). In the flour the trend was the reverse, which means that the leaves and the flour will be greater source of omega 3 and 6 fatty acids, respectively. A concise summary of the fatty acids biomarkers in both the pale leaves and the red flour is presented in figure 1. The omega 6 and omega 3 PUFAs content in the leaves will contribute greatly to alleviating cardiovascular related diseases based on its ratio of 2:1 which is the recommended ratio [15]. However, the flour had higher ratio of omega 6/omega 3 which was about 10:1, which is above the recommended ratio in diets but less than the typical western diets ratio of 20:1 or 30:1 [15]. The ratio in the flour could have been modified by postharvest issues, environmental factors, maturity stages, genetics, physiological and morphological differences [6,16]. Generally, there was significant differences between all lipid biomarkers measured (Figure 1). Higher amounts of Unsaturated Fatty Acids (UFAs) than Saturated Fatty Acid (SFAs) were found in all flour and leaves which indicates that both are good natural sources of unsaturated essential fatty acids for nutritional and medicinal application. We observed higher percent of C20:5n3 (EPA), C22:5n3 (DPA) and C22:6n3 (DHA) content in the leaves which were comparable to that of those found in *Catla catla* fish [17]. This trend was strange to found in a terrestrial plant like the leaves of *Sorghum bicolor*. We believe that it could have been attributed to environmental conditions and as well as extraction methodology drawbacks. However, we believed that the leaves will serve as important natural cheap source of omega 3 PUFAs for human nutrition.

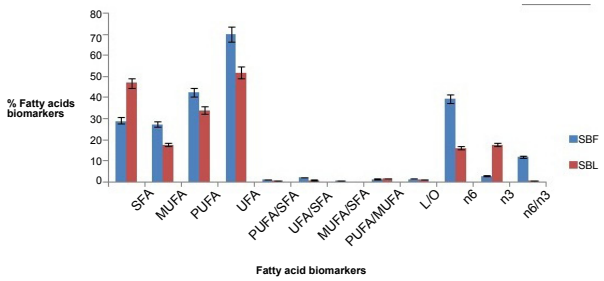

Figure 1: Total identified fatty acids biomarkers in *S. Bicolor* red Flour (SBF) and pale brown Leaves (SBL).

Table 2, shows total chlorophylls, carotenoids, ratio of chlorophyll a/b and total chlorophyll to carotenoids ratio obtained in both *S. bicolor* pale leaves and flour. Generally, the leaves were observed to contain chlorophyll a and b and carotenoids, the most abundant pigment detected in this current study in the pale leaf was carotenoids. These pigments could be used as food colorants and as drug coating agents.

Figures 2 and 3, depict total phenolic content, total condensed tannins content, and total flavonoid content using different solvents of extractions. It was observed that the pale leaves contain a higher ($p<0.05$) content of condensed tannins than the flour. In releasing the tannins the solvent of higher performance was methanol followed by ethanol. This observation has been reported previously by [18] in mushrooms that solvents have an impact on the amount of bioactive compounds released into solutions for spectroscopy and chromatographic analysis. A possible explanation for the low amounts of tannins found with the acetone-hexane mixture, is that the acetone-hexane mixture used in the extractions could have created significant isomerization and tatomerization of primary tannins (proanthocyanidins) resulting in significant structural changes and rendering them less reactive and might not provide high absorbance with the wavelength used in the colorimetric technique as compared to the methanol and ethanol extracts. This explanation is supported by [5] and [19] who reported that acetone forms pyrano-anthocyanins from anthocyanins via oxidative addition mediated reactions and

Sample type	Chlorophyll a	Chlorophyll b	Total Chlorophyll a	Total Carotenoids	Chl a/Chl b	T Chlorophyll/T Carotenoids
Pale brown leaves	2.43 ± 0.00	1.88 ± 0.01	4.31 ± 0.01	7.45 ± 0.04	1.29	0.58
Sorghum bicolor flour	2.81 ± 0.02	2.11 ± 0.18	4.92 ± 0.20	8.88 ± 0.05	1.33	0.55

Table 2: Chlorophylls and carotenoids, ratio content in *Sorghum bicolor* pale brown leaves and red Sorghum floor in mg/100g.

Mean values plus standard deviation of triplicate (3) sample determination, Chl a - Chlorophyll a; Chl b -Chlorophyll b; T Chlorophyll-Total Chlorophyll; T Carotenoids-Total Carotenoids

results in a shift of peaks absorbance wavelengths and makes it difficult to identify and quantify the total tannins amount in such solvents.

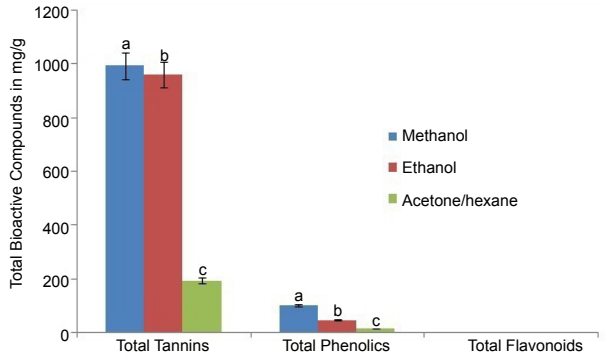

Figure 2: Effects of extraction solvent on total condensed tannins, phenolic and flavonoids content of *Sorghum bicolor* flour. Different letters represented in bar graph indicate means with significant difference at ($p<0.05$).

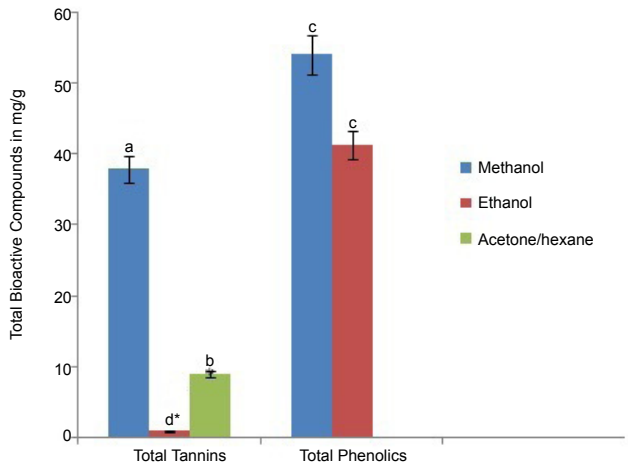

Figure 3: Effects of extraction solvent on total condensed tannins and total phenolics of *Sorghum bicolor* leaves.

(Note: values of flavonoids were in trace and were not reported). Bars with the same letter do not significantly differ ($p<0.05$).

It was interesting to observe that total phenolic content was best extracted using methanol then lastly ethanol. This trend could be attributed to the time of incubation used for the extraction [20], particle size and the strength of the different solvents used [6]. Total flavonoids were low in both flour and the leaves (Figures 2 and 3).

The high condensed total tannins content obtained indicates that both the leaves and seed flour contain greater amounts of anthocyanin, proanthrocyanidins and their derivatives which may have nutritional, pharmaceutical and medical implications. It is believed that most tannins are found in larger quantities in sources such as sorghum seed, wine, leaves and berries [3,5,20].

Table 3, summarizes the content of phenolic compounds present in the flour and the leaves respectively. It was observed that both samples contain hydrobenzoic acids, flavonols, hydroxycinnamic acids, and flavone. The most prominent ones were flavonoids (apigenin, naringenin and epigallocatechin) identified from two different extracts (methanol and ethanol). The flavonoids that were abundant in the pale red sorghum leaves were the apigenin, naringenin and epigallocatechin (Table 3). The absence of the common phenolic acids and flavonoids often reported in sorghum and its products of the solvents tested could be attributed to the complexity of their bounded sites and other biomolecules and the phenolic acids in a cell matrix [7]. This implies that the reaction conditions used might have not been appropriate to completely release these bound phenolics into the different solvents use for detection by HPLC. Another possible factor could be that the free forms of the phenolics were very low and hence below the detection limits of the instrument. It is also anticipated that some of the solvents used might have depleted or cause dimerization of the phenolic compounds and flavonoids resulting in different retention times elution outside that of the internal and external standard used.

The current results reported, especially that the major phenolic acid found in the sorghum leaves extract was gallic acid, which deviated from the red leaves reported in other studies [6]. The presence of flavonoids (narigenin, epigallocatechin gallate and apigenin) in the leaves will serve as good sources of antioxidants, anti-inflammatory, anti-allergic, anticancer and immune-modulator which can improve the immune system towards maintaining good health in consumers based on epidemiological evidence [7,22].

The findings obtained could be used as biochemical markers for the determination of best sorghum variety and quality of the cereal grains and leaves for human and animal consumption. This conjecture is supported by [23] who indicated that biochemical markers of grains are the key factors to consider for quality grains selection for food processing.

Conclusion

In summary the pale brown leaves and the flour of *Sorghum bicolor* have important biochemical, nutritional and health benefits when incorporated into humans and animals diets based on the unique bioactive compounds found in them. These findings can be used as a useful tool in selecting and screening sorghums varieties and sorghum plant parts with high phenolic compounds content and lipids for cultivation, and for nutraceuticals purposes. Lastly, further studies should be focus on high throughout put screening of both the leaves

Phenolic compound	Ethanol extract leaves	Methanol extracts leaves	Ethanol extracts flour	Methanol extracts flour
Hydroxybenzoic acids				
Gallic acid	221.43 ± 21.12[b]	1282.99 ± 21.12[a]	53.95 ± 21.12[b]	203.88 ± 21.12[b]
Flavones Apigenin	163.17 ± 6.69[a]	343.31 ± 6.69[a]	nd	nd
Flavanones Naringen	3830.50 ± 223.9[a]	nd	2650.93 ± 223.9[a]	nd
Flavonols (monomers/dimers) Epigallocatechin	886.47 ± 79.95[a]	1965.74 ± 79.95[b]	663.81 ± 79.95[a]	911.90 ± 79.95[a]

T**able 3:** Phenolic compounds in *S. bicolor* flour and pale brown leave in µg/g.

Mean values plus standard errors of duplicate (2) sample determination. Significant differences are denoted by difference in superscripts lower case letters with p<0.05; nd - not detected.

and the flour for comprehensive profile of all secondary metabolites present.

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