

Full title

EFFECT OF ACIDIC CONDITION ON PHENOLIC COMPOSITION AND ANTIOXIDANT POTENTIAL OF AQUEOUS EXTRACTS FROM SORGHUM (*SORGHUM BICOLOR*) BRAN

Short title

ANTIOXIDANT POTENTIAL OF AQUEOUS EXTRACTS FROM SORGHUM BRAN

Names and affiliations of authors

JEREMIAH S. SHELEMBE^{1,2}, DUNCAN CROMARTY³, MEGAN BESTER⁴, AMANDA MINNAAR¹, KWAKU G. DUODU^{1,5}

¹Department of Food Science, ³Department of Pharmacology, ⁴Department of Anatomy, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa;

²Department of Consumer Science, Faculty of Agriculture, University of Swaziland, Luyengo, Swaziland

⁵Corresponding author: Kwaku Gyebi Duodu. Address: Department of Food Science, University of Pretoria, Private bag X20, Hatfield 0028, South Africa; Tel.: +27 (012) 420 4299; Fax +27 (012) 420 2839; E-mail address:

gyebi.duodu@up.ac.za

ABSTRACT

The effect of extraction under acidic condition (pH2) on phenolic compounds and antioxidant potential of aqueous extracts from condensed tannin sorghum bran was investigated. The protective effect of extracts prepared with water (WE) and acidified water (pH 2) (AWE) against free radical-induced red blood cell hemolysis and low density lipoprotein (LDL) oxidation was

determined. The % yield (grams of freeze dried aqueous extract/100 g of dry sample weight), total phenolic, flavonoid content and DPPH radical scavenging activity were significantly higher in AWE than WE. AWE had 19% higher overall content of identified phenolic acids and flavonoids than WE. AWE showed a significantly higher degree of protection against erythrocyte membrane oxidation than WE. Both extracts showed protective effect against LDL oxidation. However, there were no significant differences in tannin content and oxygen radical absorbance capacity (ORAC) values between the extracts. Extracting sorghum bran under acidic condition may release bound phenolic compounds thereby improving the health-promoting benefits of extracts from sorghum bran.

Keywords: Sorghum, Bran, Antioxidant activity, Phenolics, LDL oxidation, Erythrocyte hemolysis

PRACTICAL APPLICATIONS

The study shows that aqueous extracts with higher phenolic content, antioxidant activity and potential health benefits can be prepared from condensed tannin bran under acidic condition. Using water as a solvent may be preferred over organic solvents because it is cheaper, environmentally friendly and does not require special handling. The aqueous extracts from sorghum bran may be used exogenously as antioxidant functional food ingredients in the formulation of functional foods with potential health-promoting properties. This could improve the economic value of sorghum grain.

Abbreviations: WE, water extract; AWE, acidified water extract

INTRODUCTION

Sorghum (*Sorghum bicolor*) is an important cereal crop in semi-arid and arid regions of the world, especially in parts of Africa where it is a staple food (Svensson et al., 2010). More than 42% of global production of sorghum is utilized for food (Léder, 2004). Sorghum bran, a byproduct of sorghum milling is an underutilized source of phytochemicals yet it contains high levels of diverse phenolic compounds (Awika et al., 2003). Bran from condensed tannin sorghum varieties has higher antioxidant activity compared to fruits such as blueberries and strawberries, and may be regarded as an excellent source of natural antioxidants with potential health benefits (Awika and Rooney, 2004). The bran can be incorporated into other types of cereal-based foods such as cookies and breads to increase their antioxidant activity and fiber levels (Awika et al., 2005) thereby improving their health benefits. The economic value of sorghum as a crop can be improved through extraction of bioactive compounds which may be used in the formulation of foods with improved functional or health promoting properties.

Some phenolic compounds such as ferulic, caffeic acid and coumaric acids in plant materials are bound to cell wall polysaccharides (Smith and Hartley, 1983; Madhujith and Shahidi, 2009) therefore unavailable for extraction and absorption in the gastrointestinal tract. Alkaline, acidic or enzymatic hydrolysis methods have been reported to release phenolic compounds from bound forms (Madhujith and Shahidi, 2009). Phenolic compounds are usually extracted with organic solvents such as methanol, ethanol, acetone or their aqueous mixtures (Krygier et al., 1982). However organic solvents may be toxic to human health (Tsuda et al., 1995) therefore, aqueous extraction may be the preferred method for preparation of extracts for use as antioxidant functional food ingredients. In this study, the effect of extraction of condensed tannin sorghum bran under aqueous acidic condition was investigated. The aim was to determine if the acidic

condition would increase phenolic compound content and antioxidant activity of extracts for use as functional antioxidant food ingredient. Acidification to pH 2 using HCl was considered to be a safer and more physiologically relevant method that may be used to increase the phenolic content of aqueous extracts as these conditions do exist in the upper digestive tract of man. HCl is also relatively inexpensive and food grade forms exist and this provides a more practical approach for preparing extracts with improved phenolic content for potential exogenous use as antioxidant food ingredients. The protective effect of the extracts against biomembrane oxidative damage was evaluated using 2,2'-azobis [2-methylpropionamidine] dihydrochloride (AAPH)-induced human red blood cell (RBC) hemolysis as a biological model system and against copper-catalyzed low density lipoprotein (LDL) oxidative damage in relation to their phenolic composition and antioxidant activity was determined.

MATERIALS AND METHODS

Materials

Sodium hydroxide pellets, 32% hydrochloric acid, sodium chloride, Folin Ciocalteu's phenol reagent, sodium carbonate, sodium nitrite, aluminium chloride, vanillin, methanol, ethyl acetate, glacial acetic acid, acetonitrile, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium chloride, thiobarbituric acid, copper sulphate, ethylenediaminetetraacetate (EDTA), trichloroacetic acid were purchased from Merck (Johannesburg, South Africa). (+)-Catechin, LDL (human low density lipoprotein), fluorescein sodium salt, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azobis [2-methylpropionamidine] dihydrochloride (AAPH), (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Trolox), protocatechuic acid, *p*-hydroxybenzoic acid,

caffeic acid, *p*-coumaric acid, ferulic acid, taxifolin and naringenin were purchased from Sigma Aldrich (Johannesburg, South Africa).

Sorghum bran preparation

Condensed tannin sorghum grain (PAN 3860) was hand cleaned and sorted to remove diseased, broken grains and foreign materials. The grain was decorticated using a sorghum dehuller (Rural Industries Innovation Center, Kanye, Botswana) for 5 min to obtain a bran yield of approximately 7%. Bran was separated from de-hulled grain by sieving through a 1000 μm aperture test sieve. The bran was ground into powder using a Waring blender to pass through a 500 μm aperture test sieve, packed under vacuum and stored at 4°C until extraction.

Preparation of aqueous extracts from sorghum bran

Aqueous extracts from sorghum bran were prepared with water adjusted to pH 2 (acidified water extract, AWE) or water only (WE) according to the method described by Liyana-Pathirana and Shahidi (2005) with some modifications. The AWE was prepared by mixing 10 g sample with 150 mL distilled water and stirring for 30 min using a magnetic stirrer. The solution was adjusted to pH 2 using 1 M HCl and incubated with constant shaking in a water bath at 37°C for 30 min. The mixture was adjusted to pH 6 using 1 M NaOH and incubation continued in a shaking water bath at 37°C for 30 min. WE was prepared by mixing 10 g sample with 150 mL distilled water containing 0.3 g NaCl (equivalent to the amount produced by the reaction between 1 M HCl and 1 M NaOH added in AWE) and stirring for 30 min using a magnetic stirrer. Extraction was further carried out in a shaking water bath for 60 min. Both water and acidified water extracts were centrifuged at 7500 $\times g$, 25°C for 15 min. The supernatant was decanted and freeze dried at -40°C and -80 KPa for 5 days using freeze drier model Instruvac

13KL (Air and Vacuum Technologies, Johannesburg, South Africa). Extracts were weighed and stored at -20°C in sealed zip-lock bags until analyzed. Yield was reported as grams of freeze dried crude extract per 100 g of dry sample weight.

Total phenolic content

Freeze dried aqueous extracts were dissolved in distilled water (25 mg/mL) and total phenolic content was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Absorbance was read at 760 nm using a T80+ UV-VIS spectrophotometer (PG Instruments, Leicestershire, UK). Total phenolic content was expressed as mg catechin equivalent per gram of sample (mg CE/g) on dry weight basis, using a catechin standard calibration curve.

Total flavonoid content

Total flavonoid content was determined using a spectrophotometric method as described by Ali-Farsi and Lee (2008) with some modification. Freeze dried extracts were dissolved in water to obtain a 5 mg/ml concentration solution. Thirty microlitres of extract solution or catechin standard solution was placed in a 96 well plate. Twenty microlitres of 36 mM sodium nitrite solution, 20 µL of 94 mM aluminium chloride solution and 100 µL of 0.5 M sodium hydroxide solution were added. Absorbance was read at 450 nm on a 96 plate reader model EL x800, (Bio-tek Instruments Inc, Winooski, USA). Catechin standard was used to make a calibration curve. Results were expressed as mg catechin equivalent per gram of sample (mg CE/g) on dry weight basis.

Condensed tannin content

The condensed tannin content was determined using the vanillin-HCl assay (Price et al., 1978). The freeze dried aqueous extracts were dissolved in methanol at a concentration of 0.35 mg/mL

and filtered through Whatman no. 4 filter paper. Absorbance was measured at 500 nm using a T80+ UV-VIS spectrophotometer (PG Instruments, Leicestershire, UK). Sample blank readings were subtracted from sample absorbance results. Results were reported as mg catechin equivalent/g (mg CE/g) of dry sample weight.

Determination of phenolic composition by HPLC/MS

Freeze dried extracts were hydrolyzed according to the method as described by Svensson et al. (2010) with some modification. Freeze dried extract (20 mg) was dissolved in 5 mL of 2 M HCl, sealed and heated in a shaking water bath at 98°C for 1 h. The reaction mixture was allowed to cool and then extracted twice with 5 mL ethyl acetate using a separation funnel. Fractions were pooled and then evaporated to dryness in a rotary vacuum evaporator model Rotavapor RE 120 (Büchi Labortechnik AG, Flawil, Switzerland) at 30°C. The residue was re-dissolved in 1 mL methanol, filtered through a 0.2 µm PTFE filter and stored at 4°C until analysis.

HPLC condition: The HPLC system (Waters Associates, Milford, USA) consisted of a model 1525 binary pump, model 2487 dual wavelength absorbance detector and a YMC-Pack ODS AM-303 (250 x 4.6 mm i.d., 5 µm particle size) reverse phase column (Waters Associates, Milford, USA). The HPLC conditions were according to Kim et al. (2007). Mobile phase A was 0.1% acetic acid in HPLC water and mobile phase B was 0.1% acetic acid in acetonitrile. Solvents were delivered in a linear gradient as follows: 8-10% B (2 min), 10-30% B (25 min), 30-90% B (23 min), 90-100% B (2 min), 100-8% B (2 min) and 8% B (7 min). Total run time was 61 min. Injection volume was 20 µL and flow rate was 0.8 mL/min and phenolic compounds were detected at 280 nm and data acquired by Breeze system software (Waters Associates, Milford, USA).

HPLC-DAD/ESI-MS conditions: The system consisted of an Agilent 1100 series HPLC system with degasser, auto sampler, binary pump, column heater and photo diode array detector (Agilent Technologies, Santa Clara, CA, USA). A YMC-Pack ODS AM-303 (250 x 4.6 mm i.d., 5 μ m particle size) reverse phase column (Waters Associates, Milford, USA) was used. The HPLC system was coupled to a triple quadrupole / linear ion trap mass spectrometer, model 4000 Q TRAP LC/MS/MS system (AB SCIEX, Ontario, Canada). Injection volume was 20 μ L and solvent system running conditions were the same as the HPLC conditions except that column temperature was set at 40°C and detector wavelength at 190 - 600 nm. The operating conditions for the mass spectrometer were set as follows: The source temperature was maintained at 400°C, ion spray voltage at -4000 V (negative mode). Nitrogen at a pressure of 35 psi was used as a nebulizer gas (GS1) and at 40 psi as a heating gas (GS2) and at 23 psi as a curtain gas (CUR). Declustering (DP) and entrance potential (EP) were set at -60 and -10 V, respectively. The mass of compounds was scanned from m/z 70 Da to m/z 1200 Da in 0.75 s and the data acquired and monitored by Analyst software, version 1.5 (Applied Biosystems / MDS Analytical Technologies Instruments, Ontario, Canada).

DPPH antioxidant capacity

The extracts were analyzed for their DPPH free radical scavenging capacity according to Brand-Williams et al. (1995). A 0.57 mM DPPH working solution in methanol was adjusted to an absorbance value of 1.1 at 515 nm with 3.4 mM DPPH stock solution or methanol. Sample extract in methanol (0.5 mg/mL) or trolox standard solution (150 μ l) were placed into sealable tubes, 2850 μ L of DPPH working solution was added, tubes sealed and covered with foil. Tubes were shaken for 60 min and absorbance was measured at 515 nm using a T80+ UV-VIS

spectrophotometer (PG Instruments, Leicestershire, UK). Results were expressed as μmol trolox equivalent per gram ($\mu\text{mol TE/g}$) on a dry weight basis.

Oxygen radical absorbance capacity assay

The method of Ou et al. (2001) was used to measure the oxygen radical absorbance capacity of the extracts with some modifications. Freeze dried extracts were dissolved in distilled water to a concentration of 0.15 mg/mL. Fluorescein stock solution (0.2 mM) was prepared with 0.2 M phosphate buffer saline (PBS) pH 7.4 containing 150 mM NaCl. Fluorescein working solution was prepared by mixing 120 μL of 0.2 mM fluorescein stock solution, 5 mL PBS pH 7.4 and 45 mL distilled water. Sample extract solution or trolox standard solution (10 μL) was added into a 96 well plate. A fluorescein-AAPH mixture was prepared by mixing 4 mL of 74 mM AAPH solution with 16 mL fluorescein working solution, and 200 μL of this mixture was immediately added into each well containing extract or trolox standard. Negative control was prepared by adding 200 μL fluorescein working solution and positive control by adding 200 μL fluorescein-AAPH mixture into well containing 10 μL distilled water. The plate was placed in a FLUOstar OPTIMA multifunctional plate reader (BMG LABTECH, GmbH, Germany) set at 37°C. Fluorescence at 485 nm excitation and 520 nm emission (Macdonald et al., 2006) was recorded at every 5 min interval for 245 min. Area under fluorescence decay curve (AUC) was calculated using Microcal Origin software, version 6 (Microcal Software, Inc, MA USA) and results reported as μmol trolox equivalent per gram ($\mu\text{mol TE/g}$) on dry weight basis.

Effect of extracts on AAPH-mediated erythrocyte hemolysis

Human red blood cells were obtained from the Department of Pharmacology of the University of Pretoria. Assay was according to Tang and Liu (2008) with some modifications. Red blood

cells were washed 3 times by mixing with phosphate-buffered saline (PBS) (pH 7.4 containing 8.1 mM Na₂HPO₄, 1.9 mM NaHPO₄, 3 mM KCl and 137 mM NaCl) solution in a ratio of 1:1 and centrifuged at 1184 x g for 3 min and supernatant discarded. Freeze dried extracts were dissolved in distilled water and diluted into three concentrations 100, 200, and 400 µg/mL. For treatments 100 µL PBS solution, 10 µL red blood cells, 40 µL extract solution and 40 µL of 110 mM AAPH solution prepared in PBS were added into 1.5 ml eppendorf tubes. For sample controls 140 µL PBS, 10 µL of red blood cells and 40 µL extract solution were added into each tube. Positive controls were prepared by adding 140 µl PBS, 10 µl red blood cells and 40 µl of 110 mM AAPH solution into tubes. Negative controls were prepared by adding 180 µl PBS and 10 µl red blood cells. Tubes were sealed, mixed and incubated at 37°C for 16 h. Thereafter tubes were mixed and centrifuged at 1184 x g for 3 min. Supernatant (50 µL) was transferred into a 96 well plate and absorbance read at 405 nm on a 96 well plate reader Bio Tek ELx 800 (Biotek Instruments Inc, Winooski, VT, USA).

Effect of extracts on copper-catalyzed LDL oxidation

The effect of extracts against copper-catalyzed human low density lipoprotein (LDL) oxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) (Rüfer and Kulling, 2006; Xu et al., 2007). Freeze dried extracts were dissolved in distilled water and diluted into three different concentrations 100, 250 and 500 µg/mL. For treatments, 2 µL of LDL solution (5.5 mg/mL), 168 µL 0.02 M phosphate buffer saline (PBS) solution and 10 µL sample extract or trolox solution were added into eppendorf safety lock tubes and oxidation was initiated by adding 20 µl of 100 mM copper sulphate solution. Positive controls were prepared by adding 2 µL LDL solution, 178 µl PBS and 20 µL copper sulphate solution. Negative controls were prepared by adding 2 µL LDL solution, 198 µL PBS solution. Tubes were sealed

and incubated in a water bath at 37°C for 3 h. After incubation, 200 µL of 10 mM EDTA, 200 µL of 20% (w/v) trichloroacetic acid solution and 200 µL of 0.67% (w/v) thiobarbituric acid solution in 0.2 M NaOH were added and sealed. Tubes were then heated at 80°C for 30 min in a water bath and after cooling they were centrifuged at 1500 x *g* for 15 min and supernatant transferred into 1 mL cuvette. Absorbance was measured at 532 nm using a T80+ UV-VIS spectrophotometer (PG Instruments, Leicestershire, UK).

Statistical analysis

Results from two independent experiments performed in duplicate were expressed as means ± standard deviations. Data were analyzed using t-test to compare two means and one-way analysis of variance (ANOVA) and post hoc testing using Fishers LSD test for multiple comparisons of means to determine significant differences between means. Statistica 8.0 program (StatSoft, Inc, Tulsa, OK) was used for statistical data analysis and significance was accepted at $p \leq 0.05$.

RESULTS AND DISCUSSION

Yield and phenolic content

The effect of extraction under acidic condition on yield and phenolic content is shown in Table 1. Acidified water extracts (AWE) had 15 and 9 % higher total phenolic and total flavonoid content, respectively, compared to water extracts (WE). Liyana-Pathirana and Shahidi (2005) reported a two fold increase in yield and 2 – 5 fold increase in total phenolic content of extracts from soft and hard wheat, as a result of extraction under simulated gastric pH conditions. According to these authors the increase was attributed to the release of phenolic compounds

esterified to non-starch polysaccharides such as arabinose and xylose under simulated gastric pH conditions. Although condensed tannin content of the AWE was higher than that of WE, the contents were not significantly different statistically. The total phenolic content (5.8-6.7 mg CE/g) of WE and AWE from sorghum bran was higher than that reported for extracts from wheat bran (181 -243 μg ferulic acid equivalent/g deffated flour or 0.181 – 0.234 mg/g), although standards used were different. The sorghum used in this work was a tannin-containing type and the extracts from the sorghum bran contained condensed tannins as shown in Table 1. Condensed tannins have more phenolic hydroxyl groups compared to simple phenolics therefore samples containing condensed tannins tend to have higher total phenolic content (Hagerman et al., 1998; Chiremba et al., 2009) compared to the wheat bran used by Liyana-Pathirana and Shahidi (2005) which does not contain condensed tannins. The lower increase (15%) in total phenolic content of AWE from sorghum bran as a result of extraction under acidic condition compared to the 2-5 fold (100-400%) increase reported for extracts from wheat bran in the study by Liyana-Pathirana and Shahidi (2005) could be due to differences in chemical composition and properties (Chen and Blumberg, 2008) of the bran from the two cereals. Recovery of phenolics in both extracts was high due to the presence of condensed tannins thereby minimizing the differences in total phenolic content between the two treatments which would explain the lower increase in total phenolic content. Nevertheless the slight increase in phenolic content does suggest enhanced extraction of free, esterified phenolic compounds and possibly release of bound phenolic compounds under acidic condition. The levels of condensed tannins in the WE and AWE were 17 and 12 fold lower respectively than levels reported in 70% aqueous methanol extracts from Sumac (brown sorghum) bran which averaged 175 mg CE/g of dry sample weight (Awika et al., 2005). The lower levels compared to the reported organic solvent extract could be due to the

relatively lower solubility of tannins in aqueous medium. Chen and Blumberg (2008) reported larger extraction efficiencies (18-fold) of phenolic compounds in acidified methanolic extracts compared to aqueous gastrointestinal simulated extracts indicating that organic solvents are more efficient in extracting phenolic compounds than aqueous medium. The higher extraction efficiency could be due to the use of acidified methanol which enhanced extraction of free, esterified phenolic compounds and possibly released bound phenolic compounds as well.

Phenolic compound profile

Eleven phenolic compounds in the extracts were identified by HPLC/MS (Table 2). Compounds 1, 3, 5, 8, 9 and 11 were positively identified by comparing their retention times and mass spectra to those of authentic phenolic standards and they were quantified using calibration curves prepared from the standards. Compounds 2, 4, 6, 7 and 10 were identified by comparing their *m/z* values to literature values and their concentrations were estimated using calibration curves of chemically related standards. Five of the compounds were phenolic acids namely; protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid and gentisic acid. The first four phenolic acids have been previously identified in aqueous methanolic extracts from sorghum grain (Hahn et al., 1983; Svensson et al., 2010). Gentisic acid has been reported in acidified acetonitrile extracts from Korean sorghum varieties (Chung et al., 2011) and aqueous methanol extracts from Sudanese sorghum cultivars (Awadelkareem et al., 2009). Three flavonoids (taxifolin, eriodictyol and naringenin) were also identified in the aqueous extracts, and these have been previously identified in organic extracts from sorghum grain (Gujer et al., 1986; Svensson et al., 2010). Caffeoylglycerol and the phenolic aldehydes, protocatechuic aldehyde and *p*-hydroxybenzaldehyde were also identified. Protocatechuic aldehyde and caffeoylglycerol have been reported in aqueous methanolic extracts from sorghum grain

(Svensson et al., 2010) and the *p*-hydroxybenzaldehyde was only identified by the most abundant molecular ion at *m/z* 121.

Acid treatment resulted in a significant increase in eight of the phenolic compounds and the other three namely; protocatechuic acid, *p*-hydroxybenzoic acid and gentisic acid did not show a significant increase. The increase in phenolic acids ranged from 13% for caffeic acid to 36% for ferulic acid. Flavonoid content increased by 34, 41 and 48 % for naringenin, eriodictyol and taxifolin, respectively. The sum of all phenolic compounds of the AWE was 19% higher than that of WE. The increase could be a result of release of bound phenolic compounds under the acidic condition (Liyana-Pathirana and Shahidi, 2005) because a significant proportion of phenolic compounds are present in bound form in cereals (Madhujith and Shahidi, 2009). The phenolic acids; ferulic acid, caffeic acid, *p*-hydroxybenzoic acid and protocatechuic acid were found to exist mainly in bound forms in most sorghum varieties (Hahn et al., 1983). However in some sorghum varieties protocatechuic acid was also shown to be equally distributed between free and bound forms according to the authors. The increase may also have been due to enhanced extractability of free and esterified phenolic compounds as a result of acidification (Liyana-Pathirana and Shahidi, 2005) of the sorghum bran samples. Acidification may help disintegrate cell walls thus facilitating solubilization and diffusion of phenolic compounds from plant material (Campos et al., 2008).

Antioxidant activity

The DPPH radical scavenging activity of the AWE was significantly higher than that of WE (Table 3). There was no significant difference in the ORAC values between treatments. However the mean for the AWE was slightly higher than that of the WE. The DPPH free radical scavenging activities of AWE and WE were 42 and 51 fold, respectively and lower than that of

Sumac (brown sorghum) bran extracts which was 716 $\mu\text{mol TE/g}$ on dry weight basis (Awika et al., 2005). Furthermore the ORAC values for AWE and WE were 23 and 28 fold, respectively and lower than those reported for 70% methanol extracts from Sumac bran which was 3124 $\mu\text{mol TE/g}$ on dry weight basis (Awika et al., 2005). This could be a result of poor solubility of phenolic compounds in water compared to organic solvents such as acetone and methanol (Ali-Farsi and Lee, 2008).

Effect of aqueous extracts on AAPH-induced in vitro human red blood cell hemolysis.

The protective effect of the extracts against free radical-induced red blood cell hemolysis was investigated using AAPH, a thermolabile radical starter that generates peroxy radicals ($\text{ROO}\cdot$) at a constant rate (Abuja et al., 1998). This radical induces oxidative stress on the red blood cell membrane resulting in hemolysis. The results showed that both extracts protected red blood cells against the peroxy radical in a dose dependent manner (Fig. 1). Protection was more than 40% even with the WE (WE+RBC+AAPH) at low concentration (100 $\mu\text{g/ml}$) which had the least phenolic content and antioxidant activity compared to the positive control (RBC+AAPH). AWE showed better protection compared to WE and the differences were significant ($p < 0.05$) at all three treatment concentrations. However the dose response effect was not linear. The higher protective effect of AWE could be a result of the significantly higher levels of phenolic compounds compared to the WE. Phenolic compounds may scavenge the chain propagating peroxy radical thus acting as chain-breaking antioxidants. Condensed tannins in the extracts may also provide protection by accumulating at the membrane surface through hydrogen bonding to polar groups of membrane phospholipids thus preventing access of the deleterious peroxy radicals to the hydrophobic region of the bilayer (Verstraeten et al., 2003). The results

suggest that phenolic compounds in the extracts have a potential to reduce oxidative stress on cell biomembrane.

Effect of aqueous extracts on copper-catalyzed in vitro human LDL oxidation

The results in Fig. 2 showed that both aqueous extracts at 500 µg/mL concentration inhibited TBARS formation in copper catalyzed human LDL oxidation and there were no significant differences between the AWE and WE treated samples and the negative control at this concentration. At the two lower concentrations (100 and 250 µg/mL) for both AWE and WE extract-treated samples, protection was low ranging from 12 to 20% compared to the positive control and there was also no significant difference between treatments. Liyana-Pathirana and Shahidi (2005) reported a significant protective effect of extracts prepared under simulated gastric pH conditions compared to water extracts from soft and hard wheat against LDL oxidation. This was probably because of the large increase (2-5 fold) in phenolic content that they observed as a result of simulated gastric pH treatment. However, in our experiment the increase in phenolic content was only 15% and there was no significant increase in condensed tannin content and ORAC values as a result of acid treatment. Antioxidants may delay or inhibit LDL oxidation by scavenging lipid peroxy radicals, chelating copper ions and stabilizing LDL structure through interaction with apolipoprotein B and preventing binding of copper ions to the particles (Rüfer and Kulling, 2006). LDL oxidation is implicated in the pathogenesis of coronary heart disease (Regnström et al, 1993), therefore inhibition of LDL oxidation may reduce the risks associated with this disease.

The antioxidant activities of AWE were significantly higher than that of WE as measured with the DPPH radical scavenging and the AAPH-induced erythrocyte hemolysis assay. However ORAC and TBARS assay did not show any significant differences between the treatments. The

former assays could have been influenced more by the total phenolic content which showed significant differences and the latter assays by the condensed tannin contents which were not significantly different between treatments. Xu and Chang (2008) reported that DPPH correlated strongly with total phenolic content than ORAC values, demonstrating that different antioxidant activity assays may respond differently to the same extract. The differences were said to be due to differences in reaction mechanisms, where DPPH uses the electron transfer mechanism whereas ORAC uses the hydrogen transfer mechanism.

CONCLUSION

The results suggest that the phenolic compound content of aqueous extracts from condensed tannin sorghum bran can be improved by extracting under acidic condition. Extraction under acidic condition results in an extract with higher DPPH free radical scavenging activity and protective effect against free radical induced erythrocyte hemolysis. However it does not result in a significant increase in condensed tannin content, ORAC value and protective effect of the extract against copper catalyzed LDL oxidation. Although the phenolic content and antioxidant activity of the aqueous extracts were lower than reported for organic extracts, they still show significant protection of biological molecules against free radical and copper-induced oxidative damage. This suggests that aqueous extraction which is more compatible with food systems may be a preferred method compared to organic extraction. The freeze dried aqueous extracts could be used as an antioxidant functional food ingredient in the formulation of functional food products with health-promoting properties. The low levels of condensed tannins in the aqueous extracts may be an advantage because high levels may have adverse effects on the sensory

characteristic of foods such as astringency and bitterness (Dlamini et al., 2007) and they may also reduce protein digestibility by binding to proteins (Butler et al., 1984).

REFERENCES

- ABUJA, P. M., MURKOVIC, M. and PFANNHAUSER, W. 1998. Antioxidant and prooxidant activities of elderberry (*Sambucus nigra*) extract in low-density lipoprotein oxidation. *J. Agric. Food Chem.* 46, 4091-4096.
- ALI-FARSI, M. A. and LEE, C. Y. 2008. Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chem.* 108, 977-985.
- AWADELKAREEM, A. M., MURALIKRISHNA, G., EL TINAY, A. H. and MUSTAFA, A. I. 2009. Characterization of tannin and study of in vitro protein digestibility and mineral profile of Sudanese and Indian sorghum cultivars. *Pak. J. Nutr.* 8, 469-476.
- AWIKA, J. M. and ROONEY, L. W. 2004. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* 65, 1199-1221.
- AWIKA, J. M., MCDONOUGH, C. M. and ROONEY, L. W. 2005. Decorticating sorghum to concentrate healthy phytochemicals. *J. Agric. Food Chem.* 53, 6230-6234.
- AWIKA, J. M., ROONEY, L. W., WU, X., PRIOR, R. L. and CISNEROS-ZEVALLOS, L. 2003. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *J. Agric. Food Chem.* 51, 6657-6662.

- BRAND-WILLIAMS, W., CUVELIER, M. E., and BERSET, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* 28, 25-30.
- BUTLER, L. G., RIEDL, D. J., LEBRYK, D. G. and BLYTT, H. J. 1984. Interaction of proteins with sorghum tannins: mechanism, specificity and significance. *J. Am. Oil Chem. Soc.* 61, 916-920.
- CAMPOS, D., CHIRINOS, R. AND PEDRESCHI, R. 2008. Extraction, recovery and purification of natural antioxidants from Andean crops. *Elec. J. Env. Agricult. Food Chem.* 7, 3221-3225.
- CHEN, C. Y. and BLUMBERG, J. B. 2008. In vitro activity of almond skin polyphenols for scavenging free radical and inducing quinone reductase. *J. Agric. Food Chem.* 56, 4427-4434.
- CHIREMBA, C., TAYLOR, J. R. N. and DUODU, K. G. 2009. Phenolic content, antioxidant activity, and consumer acceptability of sorghum cookies. *Cereal Chem.* 86, 590-594.
- CHUNG, I., KIM, E., YEO, M., KIM, S., SEO, M. and MOON, H. 2011. Antidiabetic effects of three Korean sorghum phenolic extracts in normal and streptozotocin-induced diabetic rats. *Food Res. Int.* 44, 127-132.
- DLAMINI, N. R., TAYLOR, J. R. N. and ROONEY, L. W. 2007. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. *Food Chem.* 105, 1412-1419.
- GUJER, R., MAGNOLATO, D. and SELF, R. 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* 25, 1431-1436.

HAGERMAN, A. E., RIEDL, K. M., JONES, G. A., SOVIK, K. N., RITCHARD, N. T., HARTZFELD, P. W. and RIECHEL, T. L. 1998. High molecular weight plant phenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* 46, 1887-1892.

HAHN, D. H., FAUBION, J. M. and ROONEY, L. W. 1983. Sorghum phenolic acids, their high performance liquid chromatographic separation and their relation to fungal resistance. *Cereal Chem.* 60, 255-259.

KIM, M., HYUN, J., KIM, J., PARK, J., KIM, M., KIM, J., LEE, S., CHUN, S. and CHUNG, I. 2007. Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. *J. Agric. Food Chem.* 55, 4802-4809.

KRYGIER, K., SOSULSKI, F. and HOGGE, L. 1982. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem.* 30, 330-334.

LÉDER, I. 2004. Sorghum and millets. In *Cultivated plants, primarily as food sources*, (G. Füleky, ed), in *Encyclopedia of Life Support Systems (EOLSS)*, pp. 66-84, Eoloss Publishers, Oxford.

LIYANA-PATHIRANA, C. M. and SHAHIDI, F. 2005. Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. *J. Agric. Food Chem.* 53, 2433-2440.

MADHUJITH, T. and SHAHIDI, F. 2009. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chem.* 117, 615-620.

OU, B., HAMPSCH-WOODILL, M. and PRIOR, R. L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Agric. Food Chem. 49, 4619-4626.

PRICE, M. L., VAN SCOYOC, S. and BUTTLER, L. G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. 26, 1214-1218.

REGNSTRÖM, J., STRÖM, K., MOLDEUS, P. and NILSSON, J. (1993). Analysis of lipoprotein diene formation in human serum exposed to copper. Free Radical Res Com, 19, 267-278.

RÜFER, C. E. and KULLING, S. E. 2006. Antioxidant activity of isoflavones and their major metabolites using different in vitro assays. J. Agric. Food Chem. 54, 2926-2931.

SINGLETON, U. L. and ROSSI, J. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. Am. J. Enol. Viticult. 16, 144-158.

SMITH, M. M. and HARTLEY, R. D. 1983. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. 118, 65-80.

SVENSSON, L., SEKWATI-MANANG, B., LUTZ, D. L., SCHIEBER, A. and GANZLE, M. G. 2010. Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) Moench). J. Agric. Food Chem. 58, 9214-9220.

TANG, Y. and LIU, Z. 2008. Chemical kinetic behavior of chlorogenic acid in protecting erythrocyte and DNA against radical induced oxidation. J. Agric. Food Chem. 56, 11025-11029.

TSUDA, T., MIZUNO, K., KAWAKISHI, S. and OSAWA, T. 1995. Supercritical carbon dioxide extraction of antioxidative components from tamarind (*Tamarindus indica* L.) seed coat. *J. Agric. Food Chem.* 43, 2803-2806.

VERSTRAETEN, S. V., KEEN, C. L., SCHMITZ, H. H., FRAGA, C. G. and OTEIZA, P. I. 2003. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer. *Free Radical Biol. Med.* 34, 84-92.

XU, B. and CHANG, S. K. C. 2008. Characterization of phenolic substances and antioxidant properties of food soybeans grown in the North Dakota-Minnesota region. *J. Agric. Food Chem.* 56, 9102-9113.

XU, B. J., YUAN, S. H. and CHANG, S. K. C. 2007. Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. *J. Food Sci.* 72, S167-S177.

FIGURE CAPTIONS**FIG. 1.**

EFFECT OF WATER (WE) AND ACIDIFIED WATER (AWE) EXTRACTS FROM CONDENSED TANNIN SORGHUM BRAN ON AAPH-INDUCED *IN VITRO* HUMAN RED BLOOD CELL (RBC) HEMOLYSIS. BARS ARE MEANS OF FOUR DETERMINATIONS FROM TWO INDEPENDENT EXPERIMENTS. ERROR BARS REPRESENT STANDARD DEVIATION FROM THE MEAN. BARS WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($P < 0.05$).

FIG. 2.

EFFECT OF WATER (WE) AND ACIDIFIED WATER (AWE) EXTRACTS FROM SORGHUM BRAN ON COPPER-CATALYZED *IN VITRO* HUMAN LOW DENSITY LIPOPROTEIN (LDL) OXIDATION AS MEASURED BY THE TBA ASSAY. BARS ARE MEANS OF FOUR DETERMINATIONS FROM TWO INDEPENDENT EXPERIMENTS. ERROR BARS REPRESENT STANDARD DEVIATION FROM THE MEAN. BARS WITH THE SAME LETTERS ARE NOT ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$).

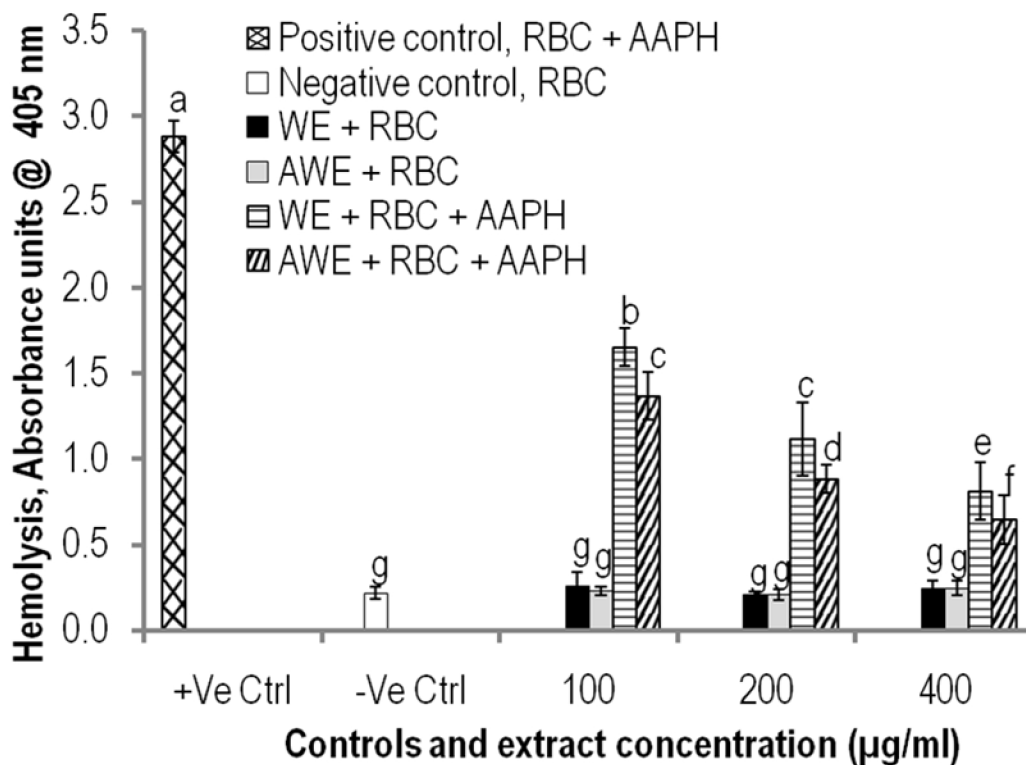


FIG. 1.

EFFECT OF WATER (WE) AND ACIDIFIED WATER (AWE) EXTRACTS FROM CONDENSED TANNIN SORGHUM BRAN ON AAPH-INDUCED *IN VITRO* HUMAN RED BLOOD CELL (RBC) HEMOLYSIS. BARS ARE MEANS OF FOUR DETERMINATIONS FROM TWO INDEPENDENT EXPERIMENTS. ERROR BARS REPRESENT STANDARD DEVIATION FROM THE MEAN. BARS WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($P < 0.05$).

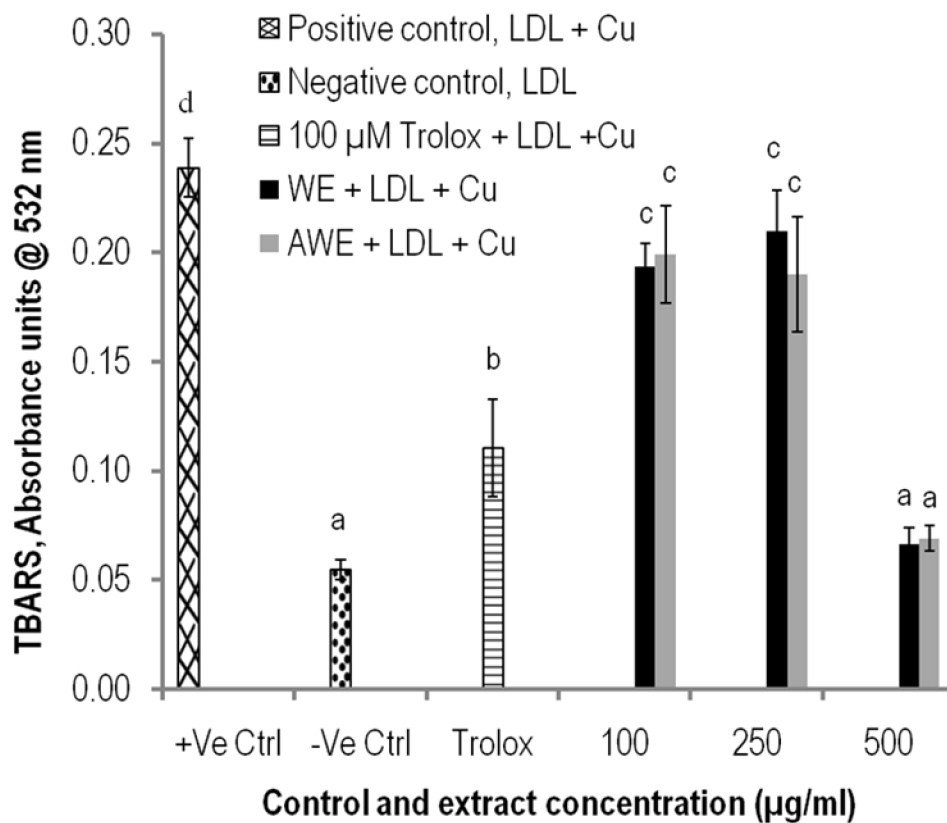


FIG. 2.

EFFECT OF WATER (WE) AND ACIDIFIED WATER (AWE) EXTRACTS FROM SORGHUM BRAN ON COPPER-CATALYZED *IN VITRO* HUMAN LOW DENSITY LIPOPROTEIN (LDL) OXIDATION AS MEASURED BY THE TBA ASSAY. BARS ARE MEANS OF FOUR DETERMINATIONS FROM TWO INDEPENDENT EXPERIMENTS. ERROR BARS REPRESENT STANDARD DEVIATION FROM THE MEAN. BARS WITH THE SAME LETTERS ARE NOT SIGNIFICANTLY DIFFERENT ($P < 0.05$).

TABLE 1. EFFECT OF ACIDIC EXTRACTION CONDITION (pH 2) ON YIELD, PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACTS FROM CONDENSED TANNIN SORGHUM BRAN

Extract	% Yield	Total phenolic (mg CE/g)	Total flavonoid (mg CE/g)	Tannins (mg CE/g)
WE	9.6a* ± 0.0	5.8a ± 0.1	4.3a ± 0.2	10.5a ± 2.0
AWE	10.4b ± 0.1	6.7b ± 0.1	4.7b ± 0.2	13.9a ± 2.0

Results are means ± standard deviations of four determinations from two independent experiments on dry sample weight basis.

*Means within the same column followed by different letters are significantly different ($p < 0.05$) using t-test.

CE, Catechin equivalent; WE, water extract; AWE, acidified water extract.

TABLE 2. EFFECT OF ACIDIC EXTRACTION CONDITION (pH 2) ON PHENOLIC COMPOUND PROFILE OF AQUEOUS EXTRACTS (WE, WATER EXTRACT; AWE, ACIDIFIED WATER EXTRACT) FROM CONDENSED TANNIN SORGHUM BRAN

Peak No.	t_R (min)	Compound Identity	Mw	m/z [M-H] ⁻ (% intensity)	Fragment ion m/z (%intensity)	Compound concentration ^a	
						WE (µg/g)	AWE (µg/g)
1	10.7	Protocatechuic acid	154.10	153 (100)	109 (31)	282 a ^b ± 13	280 a ± 30
2	13.8	Protocatechuic aldehyde ^c	138.12	137 (100)	-	179 a ± 32	240 b ± 30
3	14.9	<i>p</i> -Hydroxybenzoic acid	138.12	137 (100)	93 (61)	369 a ± 41	384 a ± 51
4	16.2	Caffeoylglycerol ^d	254.08	253 (100)	-	78 a ± 4	113 b ± 15
5	16.5	Caffeic acid	180.20	179 (100)	135 (60), 121 (15)	148 a ± 17	172 b ± 6
6	18.7	<i>p</i> -Hydroxybenzaldehyde ^e	122.12	121 (100)	-	49 a ± 8	95 b ± 7
7	21.4	Gentisic acid ^d	154.14	153 (100)	-	6 a ± 2	6 a ± 1
8	23.0	Ferulic acid	194.19	193. (100)	-	11 a ± 2	17. b ± 2
9	24.0	Taxifolin	304.25	303.8 (100)	285 (15)	39 a ± 2	69 b ± 3
10	32.6	Eriodictyol ^f	288.25	287.5 (100)	151 (14), 135 (7)	78 a ± 10	134 b ± 12
11	35.4	Naringenin	272.26	271.2 (100)	151(11)	100 a ± 9	153 b ± 15
		Total				1339 a ± 98	1663 b ± 97

^aResults are means ± standard deviation of four determinations from two independent experiments on dry weight basis

^bMeans with different letters in the same row are significantly different at $p \leq 0.05$. ^cValues expressed as protocatechuic acid equivalent. ^dValues expressed as caffeic acid equivalent. ^eValues expressed as *p*-hydroxybenzoic acid equivalent

^fValues expressed as taxifolin equivalent

TABLE 3. EFFECT OF ACIDIC EXTRACTION CONDITION (pH 2) ON ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACTS FROM CONDENSED TANNIN SORGHUM BRAN

Extract	DPPH ($\mu\text{mol TE/g}$)	ORAC ($\mu\text{mol TE/g}$)
WE	14.0a \pm 0.8	111.1a \pm 20.1
AWE	16.9b \pm 1.1	134.9a \pm 13.5

Results are means \pm standard deviations of four determinations from two independent experiments on dry sample weight basis.

*Means within the same column followed by different letters are significantly different ($p < 0.05$) using t-test.

$\mu\text{mol TE}$, micromolar trolox equivalent; WE, water extract; AWE, acidified water extract.