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Analysis of Phenolic Content and Antioxidant Properties of Selected Cowpea Varieties Tested in Bovine Peripheral Blood

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Abstract: Cowpea is an important grain legume with a dual purpose function as a food and feed resource. Cowpea contains phenolic compounds that are beneficial to human and animal health. We evaluated the phenolic content, condensed tannin content and antioxidant capacities of methanol extracts of seed and leaf of seven varieties commonly used in food and animal feed. The total phenolic content and condensed tannins were quantified using the Folin-Ciocalteu and Vanillin-HCL method respectively. The effects of cowpea phenolic extract on total antioxidant capacity, glutathione peroxidase and superoxide dismutase activity was evaluated *in vitro* in bovine blood. Overall, the methanol extracts of the leaves contained high concentrations ($p < 0.0001$) of total phenolic content (290.51 ± 38.02 mg GAE/g) compared to seed extracts (118.10 ± 71.96 mg GAE/g), although high antioxidant capacity was observed in both extracts. In addition, a positive correlation was found between total phenol and tannins content and antioxidant capacity of the extracts. Treatment with cowpea phenolic extract increased ($p < 0.0001$) the total antioxidant capacity in cow blood (5.33 ± 0.27 mM UAE) relative to controls (1.62 ± 0.10 mM UAE). The enzymatic activities of GSH-Px and SOD were also increased. Our findings, suggest the potential of cowpea polyphenols to reduce oxidative stress in livestock production. Results of the present study showed that leaf and seeds of cowpea possess rich amounts of natural antioxidants and can be further explored for their possible use as a natural additive in food or use in pharmaceutical industries and in animal feed.

Keywords: Cowpea, Polyphenols, Antioxidants, Cow Blood, SOD, GSH-Px

Introduction

Cowpea is an important grain legume native to sub Saharan Africa, are typically grown in semi-arid regions in Africa, Asia, Europe, South America and some parts of the United States. The cowpea plant serves as food source to millions of people globally and as animal feed (Singh *et al.*, 2003). The seeds are nutritious, contains 25% protein and 64% carbohydrate, vitamins and minerals (Chinma *et al.*, 2008; Phillips and McWatters, 1991). Cowpea is fed to animals as forage, hay or silage. Cowpea forage has high nutritive qualities with crude protein of about

22% and therefore is highly recommended as a supplementary protein feed for animals on low quality diets (Gwanzura *et al.*, 2012; Paduano *et al.*, 1995). Studies reported that cowpea haulms could be used to sustain animal growth and milk production in lactating dairy cattle during the dry season without health challenges (Anele *et al.*, 2010; 2011). Some cowpea cultivars have dual purpose production for dry seed grains and use as forage. Several crop improvement programs over the past two decades have produced dual purpose cowpea varieties with wider adaptability, drought resistance, insect pest resistance and increase grain and forage yields (Hall, 2004).

Cowpea contains significant amounts of phenolic compounds including phenol acids, flavonoids and tannins (Cai *et al.*, 2003; Rochfort and Panozzo, 2007; Ojwang *et al.*, 2012). The predominant phenolic acid in cowpea is protocatechuic acid (Cai *et al.*, 2003). Ojwang *et al.* (2013), characterized the proanthocyanidin profile in cowpea, catechin and (epi) afzelechin were identified as the major flavan-3-ol unit. The phenolic compositions in both raw and cooked forms of cowpea seed have been identified and their inhibitory potentials against oxidative DNA damage have been tested (Nderitu *et al.*, 2013). Cowpea seed oils have been analyzed for their phenolic content and free radical scavenging properties (Nadeem Asghar *et al.*, 2013). The total phenolic content in cowpea varieties is dependent on the seed coat phenotypes (Nzaramba *et al.*, 2005).

Cowpea phenolic compounds have health benefits for humans and animals due to their antioxidant, anti-inflammatory (Ojwang *et al.*, 2015), anticancer (Gutiérrez-Urbe *et al.*, 2011) and anti-atherogenic (Cui *et al.*, 2012) properties. The antioxidant capacity of phenolic compounds in different cowpea varieties has been reported. Phenolic constituents and their antioxidant activity function to protect cells from oxidative stress which has been implicated in the etiology of various degenerative disorders in humans (Mathers 2002; Siddhuraju and Becker, 2007; Ojwang *et al.*, 2015) and inflammation in cows (Gyenai *et al.*, 2012). Endogenous antioxidant enzymes such as glutathione peroxidase (GSH-Px) and Superoxide Dismutase (SOD) and catalase function to combat free radicals such as superoxide ions (O_2^-) and hydroxyl radicals (OH), as well as non free-radical species such as hydrogen peroxide (H_2O_2) (Celi, 2011). Thus, due to its use in human nutrition and interest in evaluation of health benefits the phenolic compounds in cowpea seeds have been the subject of many studies.

Cowpea leaves and husk have high nutritive values, in addition the cowpea leaves contain polyphenols (Gwanzura *et al.*, 2012; Chikwendu *et al.*, 2014). Variation in polyphenols may be important in selective preferences for feed by animals. Furthermore, polyphenols in leaves may have beneficial effects for animal health. Researchers in Florida (Foster *et al.*, 2009) have tested cowpea forage as a supplement for sheep fed bahiagrass hay compared to other legume hays or soybean meal. Further development to improve forage cowpea for specific systems can improve their utilization as a supplemental feeds for livestock, thus it is important to evaluate varieties in the US and their food polyphenols for health as well as animal feed. To define the usefulness of cowpea derived forages for animal nutrition and health, the composition and activity of cowpea derived forages needs to be

evaluated. The activity and benefits of phenolic in cowpea leaves used as forage in livestock health and production have received little attention. Hence, the main objectives of this study were to (a) individually evaluate the Total Phenolic Content (TPC), Condensed Tannin content (CT) and antioxidant capacity of the leaves and seed methanol extracts of selected cowpea varieties commonly used in the Unites States and (b) to determine the effect of extracts of Mississippi Silver (MS), a variety commonly grown in the southern U.S. on antioxidant status in cow blood.

Materials and Methods

Cowpea Varieties and Seed Flour Preparation

Seven cultivars of cowpea (Table 1); Texas cream, Mississippi silver, Early scarlet, CT pinkeye, CB#46, Red Bisbee and Rough et Noir, commonly grown in the U.S, were evaluated in this study. The seeds were purchased from Southern exposure seed exchange (Mineral, VA). Seeds from the cowpea varieties were ground in a coffee blender and sieved twice through a 60 mm mesh. The cowpea seed flour was stored at 4°C until used for analysis.

Greenhouse Planting and Leaf Flour Preparation

Cowpea seeds were planted in the greenhouse at North Carolina Agricultural and Technical State University. Leaves were sampled from 30-day-old plants for phenolic content and condensed tannin content analysis. The leaves were oven-dried at 55°C for 24 h, ground and sieved using 60 mm mesh. The cowpea leaf powder was stored 4°C until used for analysis. Cowpea fresh leaf powder was prepared by grinding 0.5 g of harvested leaves in liquid nitrogen. The fresh leaf powder was stored at -80°C until used.

Extraction and Quantification of Total Phenolic Content and Condensed Tannins

Cowpea seed, fresh and dry leaf flour (0.5 g) was weighed into 15 mL polypropylene conical bottom centrifuge tubes and 10 mL of 80% methanol (w/w) was added. The mixture was shaken continuously for 2 h at 37°C in an incubator shaker. The tubes were centrifuged at 4500 rpm for 15 min. The supernatant was sieved through Whatman paper no. 4.

The total phenolic content in seed and leaf extracts of the seven varieties were measured using the Folin-Ciocalteu method (Singleton *et al.*, 1999). Aliquot (0.5 mL) of each extract was mixed with 10% (v/v) Folin reagent (2.5 mL) and 7.5% (w/v) sodium carbonate (2.0 mL). The mixture was incubated in a water bath at 40°C for 30 min. Absorbance was measured at 765 nm using an Evolution 60S UV-VIS spectrophotometer (Thermo-

Scientific, Waltham, MA). Gallic acid was used as standard. Total phenolic compound was expressed as mg per Gallic Acid Equivalent (GAE) per gram.

The condensed tannins content in seeds and leaves extracts were measured using the vanillin-HCL method (Price *et al.*, 1978), catechin was used as standard. The cowpea condensed tannins content were expressed as mg of Catechin Equivalent (CE) per gram.

Antioxidant Properties Evaluation Using DPPH

Antioxidant capacity of the extracts was measured in all seven varieties using 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical (Brand-Williams *et al.*, 1995). Briefly, the extracts were mixed with DPPH solution, incubated in the dark at room temperature for 30 min and absorbance read at 517 nm on an Evolution 60S UV-visible spectrophotometer (Thermo-Scientific, Waltham, MA). A decrease in absorbance of DPPH solution connotes an increase in radical scavenging activity of DPPH. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as a standard. A standard curve was generated from known concentrations and absorbance reading of Trolox. The total antioxidant property was expressed as micromol Trolox Equivalent (TE)/g.

Animal Sampling and Blood Collection

Six female Holstein Friesian cows (at same stage of lactation) at the North Carolina Agricultural and Technical State University dairy farm were used for the experiment. The experimental protocol used was approved by the university's animal care and use committee. Ten milliliters (10 mL) of blood was collected aseptically from the jugular vein of the cows and placed into vacutainer tubes containing Acid Citrate Dextrose anticoagulant (BD Biosciences, San Jose, CA). Blood samples were maintained on ice until used.

Preparation of Cowpea Phenolic Extracts (CPE)

The Mississippi Silver (MS) cowpea variety commonly grown in Southern U.S, widely adapted with medium maturity (70-79 days), high yielding genotype, resistant to *Fusarium* wilt, root knot nematodes (*Melodogyne spp*), cowpea Mosaic virus and other viruses was selected for the *in vitro* animal study (Dadson *et al.*, 2005). Seeds of MS were planted in the greenhouse, leaves were collected from 30-day-old plants and oven dried at 55°C for 24 h. Methanolic extract was prepared from MS dry leaves powder (0.5 g). The phenolic extract of MS were concentrated and dried using a vacufuge (Eppendorf) to evaporate the methanol and the actual dry weight of the crude extract was determined (6.7 mg). The crude phenolic extracts were resuspended in 1ml of Phosphate Buffered Saline (PBS).

Treatment of Bovine Blood Cells with Cowpea Extract

The concentration of blood cells was adjusted to ensure only viable cells was used. Whole blood (10^7 cells/mL of viable cells) was treated with 10 $\mu\text{g mL}^{-1}$ of cowpea phenolic extract. Untreated samples were maintained in Phosphate Buffered Saline (PBS) as control. The samples were incubated for 30 min at 37°C, 95% humidity and 5% CO₂. At the end of incubation, the samples were spun down at 4500 rpm for 5 min at 4°C using a centrifuge (5810R, Eppendorf). The supernatant (plasma) was transferred into new tubes and stored at -80°C until further analysis. The total protein concentration in plasma was determined using the Pierce Bicinchoninic assay kit (Thermo-Scientific, Waltham, MA). Total antioxidant level and SOD in plasma were determined using commercial kits as described below.

Cell Lysate Preparation

To determine the cell associated antioxidant capacity, cell lysate was prepared after treatment of blood with CPE. Briefly, the peripheral blood cells were washed twice with PBS (pH = 7.4) and then lysed with a cell lysis buffer (Cell Signaling technology, Danvers, MA). The mixture was sonicated briefly on ice for 5 min. The samples were centrifuged at 700 g for 5 min at 4°C and supernatant (cell lysate) removed for determination of total antioxidant capacity and cellular activity of GSH-Px and SOD using commercial kits as described below.

Total Antioxidant Capacity Assay

The endogenous antioxidant activation by CPE in blood was determined in cell lysate and plasma using the OxiSelect™ TAC assay kit following the manufacturer's protocol (Cell Biolabs Inc., San Diego, CA). The Cell Biolabs' OxiSelect™ Total Antioxidant Capacity (TAC) assay measures the total antioxidant capacity of biomolecules from samples through a single electron transfer mechanism. The TAC assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid. Cell lysate and plasma were analyzed separately with the assay buffer containing copper ion reagent and incubated for 5 min. The absorbance was read at 490 nm on a microplate reader (Epoch, BioTek). A standard curve ($r^2 = 0.99$) using known concentrations of uric acid was used determine the blood total antioxidant capacity in cell lysate and plasma.

Gluthathione Peroxidase Detection

The effect of CPE on antioxidant enzymatic activity of glutathione peroxidase (GSH-Px) in blood

was determined with the glutathione peroxidase (GSH-Px) cellular activity assay kit following the manufacturer's protocol (Sigma-Aldrich, St Louis, MO). This assay is an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GSH-Px.

Superoxide Dismutase Assay

The effect of cowpea phenolic extract on SOD antioxidant enzymatic activity in blood was evaluated in both cell lysate and plasma using the Calbiochem® Superoxide Dismutase Assay Kit II per manufacturer's manual (EMD Millipore).

Statistical Analysis

All data were analyzed using SAS 9.3 version (SAS Institute, Cary, NC). One way Analysis of Variance (ANOVA) was performed on TPC, CT, DPPH free radical scavenging activity, plasma protein concentration, total antioxidant capacity, SOD, GSH-Px activity and p-value <0.05 was considered significant. To evaluate relationship if any between seed TPC and leaf TPC, the Pearson's correlation analysis was performed. Results are presented as mean ± SD.

Results

Phenolic Compound in Seed and Leafs

Table 1 shows seed phenotypic characteristics for the cowpea varieties used for the study. As expected there was variation (p<0.0001) among seed types of the cowpea varieties for total phenolic content, condensed tannins content and antioxidant capacity of the extracts (Table 2). The seed total phenolic content among the varieties ranged from 46.48 mg GAE/100g (Texas cream) to 269.39 mg GAE/100g (Mississippi silver). Condensed tannins amounts were high in Mississippi silver seeds (0.22 mg/CE/100g) while low content was observed in Early scarlet variety (0.22 mg/CE/100g). Low TPC levels were measured in white seed coat colored varieties-Texas cream,

early scarlet, CT Pinkeye and CB#46. Condensed tannins were undetected in Texas cream and CB #46 and much lower CT levels were recorded in Early scarlet. Seed extracts of all the cowpea varieties showed high antioxidant capacity although variations were observed. The free radical scavenging activity of the phenolic compounds among the cowpea varieties ranged from 53.20 to 344.58 µm Trolox Equivalent (TE)/100g. Methanolic extract of Mississippi silver seeds had higher antioxidant capacity compared to the other varieties.

Similarly, there was variation among leaf samples from the different cowpea varieties for total phenolic content, condensed tannins and their free radical scavenging activity (p<0.0001). The phenolic content, condensed tannins and their free scavenging activity in leaf were higher in dry samples relative to fresh samples. The total phenolic compounds in fresh leaves ranged from 92.84 mg GAE/100g (Texas cream) to 126.61 mg GAE/100g (Early Scarlet). Generally, low levels of CT were observed in all fresh leaves samples ranging from 0.13-0.22 mg CE/100g compared to high CT content in dry leaves samples (0.30-0.52 mg CE/100g). Early scarlet variety leaves either as fresh or dry form recorded the highest TPC and antioxidant capacity (Table 3 and Table 4). Dry leaves of MS and CB#46 varieties had the highest CT content (0.5 and 0.52 mg CE/100g respectively). Six of the tested varieties showed correlation between seed and leaf TPC. A positive correlation (r = 0.83) between seed TPC and leaf TPC was observed for CT Pinkeye variety. A negative correlation (r = 0.75) was observed between seed TPC and leaf TPC for the Red Bisbee variety. All other varieties had moderate correlation. No correlation was observed between seed TPC and leaf TPC of Rough *et* Noir variety (Table 6).

In summary, phenolic composition and condensed tannins content in cowpea leaves was significantly higher (p<0.0001) compared to seeds, however, the free scavenging activity of DPPH was similar among extracts- seed and leaf (Table 5).

Table 1. Description of phenotypic characteristic of seeds of cowpea varieties commonly grown in the Southern U.S. and their breeding sources

Cowpea variety	Seed characteristics	Breeder
Texas cream 40	Cream, Wrinkled, Yelloweye	Texas A and M College, Department of Hort., College Station
Mississippi silver	Brown, Wrinkled, Whiteeye	Mississippi State University, State College
Early Scarlet	Cream, Wrinkled, Pinkeye	University of Arkansas, Fayetteville, AR
CT Pinkeye	Cream, Wrinkled, Pinkeye	C.T. Smith Company, Pleasanton, Texas
Red Bisbee	Red, Smooth, Whiteeye	Originated in Bisbee, Arizona
Rough et Noir	Black, Smooth, Whiteeye	-
CB# 46	Cream, Smooth, Blackeye	University of California, Davis and the California Agriculture Experimental Station

Source: Richard Fery (ed.) USDA/ARS vegetable laboratory, Charleston, SC. Vegetable cultivar descriptions for North America, southern pea (cowpea) <http://cuke.hort.ncsu.edu/cucurbit/wehner/vwegcult/southernpea.html>

Table 2. Total phenolic compound, condensed tannins and antioxidant capacity of seed extracts of selected cowpea varieties

Cowpea variety	Total Phenolic compound (mg GAE/100 g)	Condensed tannins (mg CE/100g)	Antioxidant capacity (μ m TE/100g)
Texas cream40	46.48 \pm 0.78 ^f	ND	53.20 \pm 1.26 ^g
Mississippi silver	269.39 \pm 1.35 ^a	0.22 \pm 0.01 ^a	344.58 \pm 1.77 ^a
Early scarlet	78.34 \pm 2.22 ^e	0.03 \pm 0.01 ^c	89.16 \pm 0.61 ^e
CT pinkeye	53.69 \pm 0.18 ^g	0.16 \pm 0.003 ^b	72.90 \pm 0.40 ^f
Red bisbee	113.84 \pm 2.26 ^d	0.13 \pm 0.002 ^b	129.40 \pm 0.74 ^d
Rough et noir	145.34 \pm 2.93 ^b	0.16 \pm 0.005 ^b	174.25 \pm 0.71 ^b
CB# 46	119.61 \pm 2.48 ^c	ND	136.41 \pm 2.11 ^c

ND = Not Detected, Means with the same letter are not significantly different at $p < 0.05$

Table 3. Total phenolic content, condensed tannins and antioxidant capacity of fresh leaf extracts of selected cowpea varieties

Cowpea variety	Total Phenolic content (mg GAE/100 g)	Condensed tannins (mg CE/100g)	Antioxidant capacity (μ m TE/100g)
Texas cream40	92.84 \pm 1.73 ^d	0.19 \pm 0.017 ^c	88.16 \pm 0.08 ^c
Mississippi silver	94.49 \pm 1.69 ^d	0.13 \pm 0.015 ^d	78.62 \pm 2.14 ^d
Early Scarlet	126.61 \pm 4.41 ^a	0.13 \pm 0.006 ^d	163.79 \pm 0.46 ^a
CT Pinkeye	118.53 \pm 0.79 ^b	0.14 \pm 0.01 ^d	135.95 \pm 8.62 ^b
Red Bisbee	89.80 \pm 2.39 ^e	0.22 \pm 0.02 ^b	70.54 \pm 1.01 ^e
Rough et Noir	102.72 \pm 1.95 ^c	0.17 \pm 0.005 ^c	82.95 \pm 3.08 ^d
CB# 46	93.41 \pm 1.14 ^d	0.27 \pm 0.03 ^a	81.25 \pm 0.17 ^d

Means with the same letter are not significantly different at $p < 0.05$

Table 4. Total phenolic content, condensed tannins and antioxidant capacity of dry leaf extracts of selected cowpea varieties

Cowpea variety	Total Phenolic content (mg GAE/100g)	Condensed tannins (mg CE/100g)	Antioxidant capacity (μ m TE/100g)
Texas cream40	304.99 \pm 6.70 ^b	0.42 \pm 0.02 ^c	145.33 \pm 0.001 ^c
Mississippi silver	297.67 \pm 4.70 ^c	0.50 \pm 0.023 ^{ab}	178.83 \pm 1.18 ^b
Early scarlet	324.07 \pm 2.63 ^a	0.38 \pm 0.006 ^d	208.45 \pm 2.01 ^a
CT pinkeye	207.78 \pm 3.03 ^e	0.30 \pm 0.012 ^e	137.01 \pm 1.65 ^d
Red bisbee	327.43 \pm 4.03 ^a	0.47 \pm 0.014 ^b	126.05 \pm 1.34 ^e
Rough et noir	292.84 \pm 2.01 ^c	0.42 \pm 0.025 ^c	146.14 \pm 1.69 ^c
CB# 46	278.76 \pm 3.13 ^d	0.52 \pm 0.022 ^a	119.91 \pm 0.74 ^f

Means with the same letter are not significantly different at $p < 0.05$

Table 5. Summary of total phenolic content, condensed tannins and their antioxidant capacity analyzed in cowpea seed and leaf extracts

Cowpea part	Total Phenolic content (mg GAE/100g)	Condensed tannins (mg CE/100g)	Antioxidant capacity (μ m TE/100g)
Leaf	290.51 \pm 38.02 ^a	0.43 \pm 0.072 ^a	151.68 \pm 93.04 ^a
Seed	118.10 \pm 71.96 ^b	0.11 \pm 0.079 ^b	142.85 \pm 29.80 ^a

Means with the same letter are not significantly different at $p < 0.05$

Table 6. Correlation analysis between total phenolic content in seed and leaf extracts of selected cowpea varieties

Cowpea variety	Seed TPC Vs leaf TPC ^a
Texas cream40	-0.420
Mississippi silver	-0.470
Early scarlet	-0.330
CT pinkeye	0.830
Red bisbee	-0.750
Rough et noir	0.006
CB# 46	0.220

^aTPC = Total Phenolic Content

Effect of CPE on Total Protein and Antioxidant Activity in Blood

Total protein concentration in plasma significantly increased ($p < 0.0001$) in CPE treated blood relative to control group (Fig. 1). To evaluate if CPE antioxidant capacity enhances bovine cellular antioxidant activity, a TAC kit was used. The TAC assay measures the cellular reductive activity that converts copper (I) to copper (II) through a single electron transfer mechanism. The endogenous antioxidant level was significantly high ($p < 0.0001$) in cell lysate (5.33 ± 0.27 mM UAE) compared to plasma (1.15 ± 0.10 mM UAE) following CPE treatment (Fig. 2). The plasma TAC measured for

the control group was negligible (0.013 ± 0.010 mM UAE). There was variation in TAC in blood from the different cows used ($n = 6$; $p < 0.0001$).

The cellular antioxidant enzymatic activity of glutathione peroxidase (GSH-Px) significantly increased ($p < 0.05$) in CPE treated samples (9.1 ± 0.89 units mL^{-1}) compared to control (0.14 ± 0.03 units mL^{-1}) (Fig. 3). The GSH-Px cellular activity in blood varied among the different cows used ($p = 0.0341$).

The cellular activity of SOD was detected in blood after CPE treatment (0.10 ± 0.004 $\mu\text{g mL}^{-1}$) and SOD activity was not observed in the control group. The activity of SOD was not detected in plasma samples.

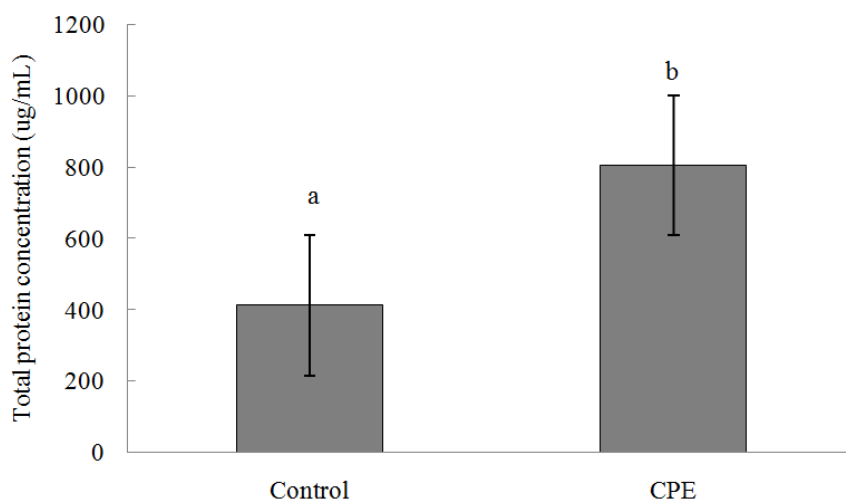


Fig. 1. Total protein concentration measured in plasma after CPE treatment of bovine peripheral blood. The CPE treatment increased plasma protein concentration. Bars with different superscripts differ ($p < 0.05$)

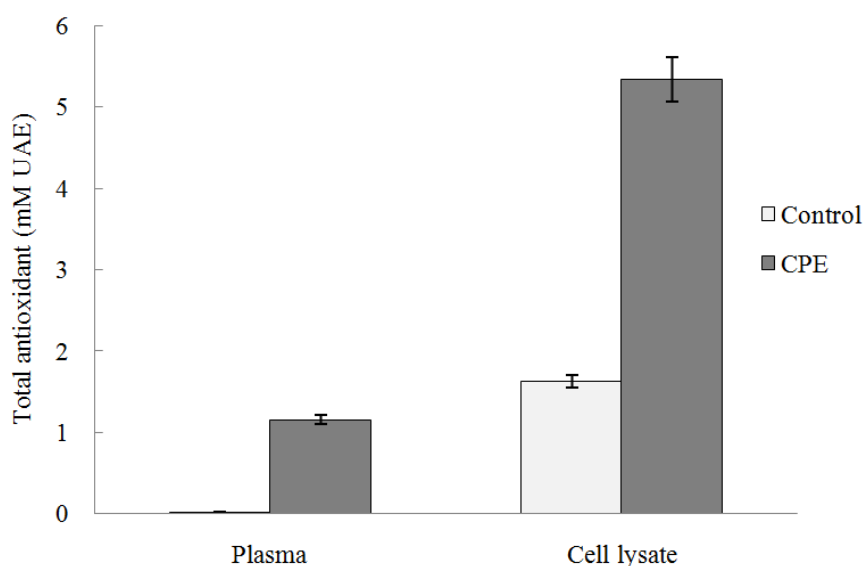


Fig. 2. Total Antioxidant Capacity (TAC) in cow blood cell lysate and plasma for Cowpea Phenolic Extracts (CPE) treated and control samples. UAE is uric acid equivalent

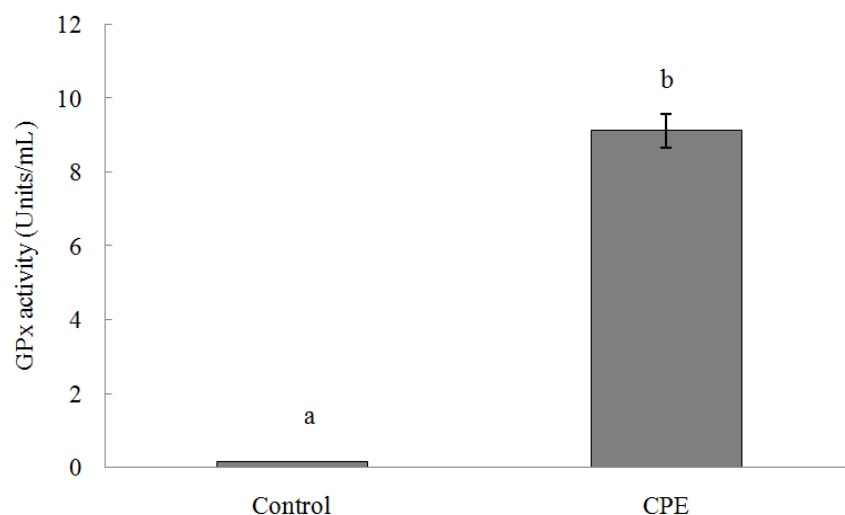


Fig. 3. Glutathione Peroxidase (GSH-Px) cellular activity in cow blood for CPE treated and control groups. Treatment with CPE increase cell associated GSH-Px enzymatic activity. Bars with different superscripts differ ($p < 0.05$)

Discussion

Dual purpose cowpea varieties supply protein in the human diet and in fodder for livestock, as well as bringing nitrogen into the farming system through biological fixation. The current study analyzed the phenolic composition in cowpea seeds and leaves from seven commonly used commercial varieties in the U.S. Cowpea varietal differences for TPC, CT and DPPH free radical scavenging activity were observed. The seed coat color variation among the varieties used was associated with variation in TPC and CT similar to previous reports (Nzaramba *et al.*, 2005; Ojwang *et al.*, 2012). Varieties with high TPC and CT also had corresponding antioxidant capacity. Previous studies have also reported that the antioxidant activity of different extracts was dependent on the level of phenolic content (Jayaprakasha *et al.*, 2003).

The levels of TPC and CT in seeds were not directly related to their content in leaves in our results presented. Phenolic composition in plants have been reported to vary both quantitatively and qualitatively with differences in plant organ, plant age, season and growing condition (Levin, 1971). In the present study leaves from 30-day-old plants were sampled for TPC and CT determination and therefore the observed difference may have been influenced by maturity stage of the plants. A similar phasic differential total phenolic content in cowpea was observed by Chon (2013). Dry seed extracts showed high TPC compared to 7-day-old seedlings (Chon, 2013). Our study findings showed that cowpea leaves contain greater amounts of TPC and CT in dried compared to fresh leaves, this may be an important consideration in use of cowpea for animal feed either as hay or silage.

Polyphenols are known antioxidants with impact in blood (Tedesco *et al.*, 2000). There is lack of information regarding the effect of cowpea polyphenols on oxidative stress in cow blood. In the present study, *in vitro* CPE treatment led to increased blood total antioxidant capacity. Plant derived polyphenols modulate the antioxidants and antioxidant enzymatic activities in blood and tissues (Halliwell and Chirico, 1993; Duthie *et al.*, 2000; Dai and Mumper, 2010). In rats, treatment with catechins significantly increased plasma and urine TAC (Simos *et al.*, 2012). The enzymatic activities of SOD and GSH-Px increased in blood after treatment with combine extracts of beet or broccoli (Sarhan *et al.*, 2014) and extracts from *Silybummarianum* and *Prunella vulgaris* (Škottová *et al.*, 2004). In cow neutrophils, Gyenai *et al.* (2012) showed that tomato polyphenols may play a role in animal immunity by modulating the expression of proinflammatory genes. Phenolic compounds in cowpea may modulate antioxidant enzymes activity and the overall antioxidant capacity in cow blood. In the present study CPE treatment activated the cellular SOD and increased GSH-Px activity in cow blood. The role of SOD and GSH-Px is intertwined to ensure intracellular antioxidant defense against oxidative stress (Halliwell and Chirico, 1993). The enzyme SOD is the first line of antioxidant defense against Reductive Oxygen Species (ROS) and SOD catalyzes the dismutation reaction of superoxide radical anion (O_2^-) to hydrogen peroxide (Zelko *et al.*, 2002). The enzyme GSH-Px functions to directly remove the hydrogen peroxide generated by SOD (Dröge, 2002). Cowpea extract showed antioxidant properties in cow blood, thus this suggest a possible role of CPE in regulating

oxidative stress in cow blood. Furthermore, use of cowpea as feed in cows in addition to the nutrition benefits may help improve their health status.

Oxidative stress plays a key role in several pathological conditions connected with animal production, reproduction and welfare (Lykkesfeldt and Svendsen, 2007). Oxidative stress affects the health status of animals as well as product quality such as milk and meat (Castillo *et al.*, 2005; 2006). Lowered antioxidant status is predominant in ruminants during mastitis, metritis, retained placenta, acidosis, ketosis and milk fever conditions (Lykkesfeldt and Svendsen, 2007; Celi, 2011). Plant-derived phenolic substances have antioxidant capacity that contributes to reducing oxidative stress. The use of antioxidant-rich plant parts such as Yerba Mate (*Ilex paraguariensis*) and moringa leaves has been presented as a strategic goal in animal nutrition (Celi, 2013; Makkar *et al.*, 2007; Rochfort *et al.*, 2008). A study conducted by Santos *et al.* (2014) showed that addition of citrus pulp rich in flavonoids, increased the total polyphenols and antioxidant amount in milk.

Other studies have suggested the possibility of dietary antioxidant transfer to animal products. Feeding phenolic compounds from propolis extract to dairy cows significantly increased the antioxidant capacity and improved milk quality (Aguiar *et al.*, 2014). A similar study where citrus pulp was used as feed for dairy cow, resulted in increased total polyphenols, flavonoid concentration and ferric reduction antioxidants power in milk (Santos *et al.*, 2014). Exploration of plant natural products such cowpea polyphenols as feed additives in animal nutrition presents a unique opportunity to improve feed efficiency, promotes growth in livestock and has less public health and safety concerns compared to use of antibiotics (Wallace, 2004). Our study likewise may suggest the possibility of cowpea antioxidant transfer to cow blood. These results will contribute to use of cowpea as supplement feed to impact milk and meat antioxidant capacity and as well as the overall quality of products. Therefore, feed sources with good supply of natural antioxidant such as cowpea can potentially reduce oxidative stress burden in animals during lactation.

Conclusion

In summary, this study showed that cowpea varieties have variation in total phenol content, condensed tannin content and antioxidant capacity. The parts of the cowpea plant (seed or leaf) also showed variation for TPC and CT. Processing of the cowpea leaves either as fresh or dry impacted the TPC, CT and antioxidant capacity. In preparing cowpea ingredients for food/feed these factors may be

considered; variety, seed, leaf- fresh or dry. Phenolic extracts of Mississippi silver variety had antioxidant effect in bovine blood cell lysate and plasma. We conclude that MS variety may have the potential to modulate the physiology, health and production of dairy cow if used as a feed source or supplement. As livestock systems change, a continued and perhaps, increasing role of cowpea can be envisioned. Characterization of cowpea varieties and their use for animal feed may maximize the use of the plant as feed and food for sustainable livelihood.

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Author's Contributions

Sarah Adjei-Fremah: Performed the experiment, statistically analyzed the data and prepared the manuscript.

Mulumbet Worku: Designed and supervised the study and assisted in preparation of the manuscript

Louis EN Jackai: Assisted with the study plan, provided the cowpea materials and partial student funding for the research.

Conflict of Interest

The authors declare no conflict of interest.

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Abbreviation and Units

CE-Catechin equivalent
CPE-Cowpea phenolic extract
GAE-Gallic acid equivalent
GSH-Px-Gluthathione peroxidase
MS-Mississippi silver
SOD-Superoxide dismutase
TAC-Total antioxidant capacity
TE-Trolox equivalent
UAE-Uric acid equivalent
mM-millimole
 μ M-micromole
 μ g-microgram