Journal of Agricultural Science; Vol. 8, No. 3; 2016
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

# Modulation of Bovine Wnt Signaling Pathway Genes by Cowpea Phenolic Extract

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Received: December 22, 2015 Accepted: January 29, 2016 Online Published: February 15, 2016

doi:10.5539/jas.v8n3p21 URL: http://dx.doi.org/10.5539/jas.v8n3p21

**Abstract**

The Wingless (Wnt) signaling pathway is a conserved pathway with essential roles in cellular and biological processes in mammals. Wnt signal transduction has been implicated in inflammation, innate immunity and homeostasis via Toll-like receptor and NF-κB pathways. Plant bioactive compounds are capable of modulating the Wnt signalling pathway, which can be either a canonical (β-Catenin dependent) or non-canonical (β-Catenin independent) mechanism. This study evaluated the effect of cowpea phenolic extract (CPE) on the expression and modulation of genes of the Wnt signaling pathway in cow blood. Whole blood collected from six Holstein-Friesian cows was treated with 10 µg/ml of the extract, and evaluated for packed cell volume (PCV), total count and viability of cells, and white blood cell differential count before and after treatment. Cowpea phenolic extract agonist activity in blood was measured using a Bovine toll-like receptor (TLR) 2, and TLR 4 ELISA kit. Total RNA was isolated from the blood cell pellet, reverse transcribed and used for real-time PCR to detect expression of 84 genes on the Cow Wnt signaling pathway array. The total cell-associated β-Catenin level was measured using a commercial ELISA kit. There was no treatment effect on PCV, total cell and viability ($P > 0.05$). The percentage of mononuclear cells were influenced by treatment, % monocytes ($P = 0.0136$) decreased and % lymphocytes ($P = 0.0114$) increased. Treatment with CPE activated cow blood cells, increased TLR2 release and total β-Catenin levels (6 ng/ml, $P < 0.05$), but TLR4 was not detected. Polyphenols from cowpea modulated the expression of Wnt signalling genes, especially canonical β-Catenin mediated pathway genes. Modulation of Wingless gene expression may be an important mechanism by which polyphenols in cowpea feed impact cellular immune response and homeostasis. Thus, further studies are needed to determine the association of CPE-mediated Wnt gene modulation on blood leucocytes subpopulations and animal health.

**Keywords:** bovine, blood, cowpea, leucocytes, Wnt signaling pathway, Wnt/β-catenin, phenolic compounds

## 1. Introduction

Cowpea (*Vigna unguiculata* L.Walp) is an important annual legume plant used for food. It is cultivated and consumed largely in Africa, Asia, and South America. Cowpea seed grains are rich in protein: 27% (Gupta et al., 2010). Cowpea with a comparable yield and digestibility to alfafa (Cook, 2005), has high nutritive value, a crude protein of 22%, and are fed to animals as forage, hay or silage (Etana et al., 2013). Using cowpea as protein supplement feed has been recommended for ruminants fed low-quality roughages for improved productivity (Etana et al., 2013; Gwanzura et al., 2012). Cowpea was used as one of the summer legumes for cow and calves on Bermudagrass creep grazing pastures (Pitman et al., 2015). Supplementing small ruminant feed with cowpea forage enhanced feed intake, increased average daily gain, nitrogen intake and digestibility and fecal nitrogen output (Baloyi et al., 2008; Koralagama et al., 2008). Use of cowpea as summer finishing forage in cattle resulted in greater marbling score and increased dressing percentage and consumer steak preference (Schmidt et al., 2013).

Cowpea contains phenolic compounds including phenol acids, flavonoids, and tannins (Cai et al., 2003). Due to their antioxidant, anti-inflammatory, and anticarcinogenic effects, these bioactive compounds pose an added health benefit to humans and animals (Lee & Yen, 2006; Balsano & Alisi, 2009; Folmer et al., 2014; Ojwang et al., 2015). In animal studies, flavonoids (Middleton et al., 2000) and condensed tannins (Min et al., 2004) have been reported to have benefits for ruminant health and productivity. For example, feeding tannin-rich forages to animals attenuates blotting, increases their resistance to gastrointestinal nematodes (Min & Hart, 2003; Min et al., 2004) and improves nutrition through increased availability of high-quality proteins to the small intestines (Barry et al., 2001). The health promoting benefits of cowpeas for human consumption have been studied using human cell lines and laboratory animal models against chronic diseases such as cancer (Gutiérrez-Uribe et al., 2011). The evaluation of cowpeas and their bioactive properties for use in livestock health has received less attention. Dietary bioactive substances influence animal health and well-being via activation of conserved gene pathways including the Wingless (Wnt) pathway (Logan & Nusse, 2004; Tarapore et al., 2012).

The Wnt gene family is comprised of numerous genes encoding highly conserved secreted glycoproteins related to the Wnt-1 proto-oncogene and to Drosophila wingless gene products (Wodarz & Nusse, 1998). Secreted Wnt proteins serve as ligands for Frizzled (Fzd) family membrane receptors together with co-receptors lipoprotein receptor–related protein (LRP) 5 and 6 to initiate signaling. Currently, 19 Wnt and 10 Fzd genes have been identified (Miller, 2002; He et al., 2004). The absence of Wnt protein secretion results in Axin/glycogen synthase kinase 3 β (GSK3β)/adenomatous polyposis coli (APC) complex degradation of phosphorylated cytoplasm β-Catenin (Liu et al., 2005). In vertebrates, the Wnt signaling pathway regulates various cellular processes such as cell fate, polarity, proliferation, movement, and stem cells hematopoiesis (Clevers & Nusse, 2012). Wnt proteins control cellular functions through two distinct pathways, canonical and non-canonical pathways; the former is β-Catenin dependent but the latter is not. The non-canonical pathway is subcategorized into two, planar cell polarity (PCP) and Wnt/$Ca^{2+}$ pathways (Logan & Nusse, 2004).

Wnt signaling lies at the heart of numerous biological processes including embryonic development, adipogenesis, myogenesis, disease progression as well as impacting meat quality (marbling) and production. Furthermore, Wnt signal transduction has been implicated in inflammation and innate immunity via Toll-like receptor and NF-κB pathways (Umar et al., 2009; Sun et al., 2005; Duan et al., 2007). Previous reports mostly but not limited to studies in cancerous cells have shown the effect of plant bioactive compounds in modulating the Wnt/B-catenin signaling pathway (Tarapore et al., 2012). The expression and regulation of Wnt signaling pathway genes in the peripheral blood cells of healthy cattle in response to phenolic extract fractions of cowpea have not been reported to the authors' knowledge. In this current study, the role of cowpea phenolic extracts in modulating the expression of 84 genes in the cow Wnt signaling pathway was investigated in bovine peripheral blood.

## 2. Materials and Methods

### *2.1 Animal Sampling and Blood Collection*

Six clinically healthy age-matched Holstein-Friesian cows (Body weight = 1662±79 kg) selected from the herd at the North Carolina Agricultural and Technical State University dairy farm were used. All protocol used were approved by the Institutional Animal Care and Use Committee. The animals used were under no medical and feed treatment during the study period. Twenty milliliters (20 mL) of blood were collected from the jugular vein of the cows in the morning prior to feeding and placed into vaccutainer tubes containing Acid Citrate Dextrose anticoagulant (BD Biosciences, San Jose, CA). Blood samples were kept on ice and processed with two hours.

### *2.2 Cowpea Phenolic Compounds Extraction*

The Mississippi silver variety of cowpea, a widely adapted variety with early and uniform maturity, resistant to *Fusarium* wilt, root knot nematodes, and other viruses was used for the study. Cowpea seeds were planted in pots (dimension are 6.3-inch depth × 6.5-inch diameter) in the greenhouse. Fresh leaves (100 g) were sampled from 30-day old plants and oven dried at 55 °C for 24 hours. The procedure followed for preparation of cowpea polyphenolic extracts was described by Adjei-Fremah, Jackai and Worku (2015). Total phenolic compound (TPC) was measured with the Folin-Ciocalteu method using gallic acid as standard (Singleton et al., 1999).

### *2.3 Evaluation of Endotoxin Levels*

All Solvents and diluents used in this study were tested for endotoxin with the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit following the manufacturer's protocol (GenScript, Piscataway, NJ). Ultrapure distilled water (Invitrogen, Camarillo, CA) had low endotoxin levels of 0.01 EU/ml and was used for the preparation of all solutions.

### 2.4 CPE Treatment of Bovine Peripheral Blood

Bovine whole blood ($10^7$ cells/mL, viable cells) was treated with 10 ug/mL CPE, and Untreated control samples were maintained in PBS. CPE-treated and control samples were incubated for 30 minutes at 37 $^oC$, 95% humidity and 5% $CO_2$. The treatment conditions followed was described by Worku and Morris, (2009). After incubation, the samples were centrifuged at 700 g for 5 mins at 4 $^oC$. The supernatant (plasma) was transferred into new tubes, and stored at -80 $^oC$ until further analysis. Plasma was also used to evaluate secretion of bovine Toll-like receptor 2 (TLR2) using a commercial ELISA kit. Tri-reagent (Sigma-Aldrich, St Louis, MO) was added to the cell pellet for total RNA isolation.

### 2.5 Blood Cell Analysis

Total cell count and viability in blood samples before and after CPE treatment was determined in the diluted blood (1:1000 PBS). Diluted samples were mixed with Trypan Blue and counts were performed using the TC20 automated cell counter (Bio-Rad, Hercules, CA). Blood packed cell volume (PCV) was determined using a microhematocrit centrifuge (Damon/IEC division). Thin blood smears were made on sterile glass slides prepared in triplicate for each animal. Wright staining was performed for histologic cell staining and white blood differentiation count. Cells were identified microscopically and enumerated. Counts of one hundred cells were evaluated for different white blood cell types.

### 2.6 Cell Lysate Preparation

To detect cellular secreted total β-Catenin levels, cell lysate was prepared after treatment of blood with CPE. The cell lysate preparation procedure followed was described by Adjei-Fremah et al. (2015). Blood cells were washed twice with PBS and then lysed using cell lysis buffer (Cell Signaling technology, Danvers, MA). The mixture was sonicated briefly on ice for 5 minutes. The samples were centrifuged at 700 g for 5 mins at 4 $^oC$ and supernatant (cell lysate) removed for detection and estimation of total β-Catenin levels.

### 2.7 Total β-Catenin Quantification

The β-Catenin (total) ELISA kit (Invitrogen, Camarillo, CA) was used to measure β-Catenin protein levels in the cell lysate for CPE-treated and control groups to determine canonical Wnt pathway activation, following manufacturer's protocol. The Enzyme Linked-Immuno-Sorbent Assay kit contains a monoclonal antibody specific for β-Catenin and detects and quantifies β-Catenin levels regardless of its phosphorylation state.

### 2.8 Detection and Quantification of TLR2 and TLR4 Secretion

A commercial bovine toll-like receptor 2 (TLR2) and TLR4 ELISA kit (CUSABIO) were used for detection and quantification of TLR2 and TLR4 concentration respectively in plasma of CPE-treated and control samples. Plasma sample (200 ul) was concentrated using the ProteoExtract$^{TM}$ protein precipitation kit (CALBIOCHEM). Concentrated samples were dissolved in 200 ul PBS for the detection of TLR2 and TLR4 as recommended by the manufacturer's (CUSABIO).

### 2.9 RNA Isolation and Quantitative RT-PCR

Total RNA was isolated using the Tri-reagent method (Sigma-Aldrich St. Louis, MO). The concentration and purity of RNA were measured with a Nanodrop spectrophotometer (Thermo-Scientific, Waltham, MA). The integrity of the isolated RNA was determined with Bioanalyzer (Agilent). Total RNA of the individual samples (0.5 µg each, RIN > 7) were pooled for cDNA synthesis using the $RT^2$ first strand kit (Qiagen). The cow Wnt signaling $RT^2$ Profiler™ PCR Array (Qiagen) with 84 test genes related to Wnt-mediated signals transduction was used. The genes profiled included genes associated with the canonical Wnt signaling, planar cell polarity, Wnt /$Ca^{2+}$ signaling and Wnt signaling negative regulation. Real-time PCR was performed using $RT^2$ SYBR Green Mastermix (Qiagen) on the CFX Connect real time system (Bio-rad). Real-time PCR workflow was per manufacturer's manual (Qiagen). All reactions were performed in triplicate. Real-time data analysis normalization was performed with Ct value of GAPDH and fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The student's t-test was used to determine the statistical significance of fold change in genes expressed.

### 2.10 Statistical Analysis

All data were analyzed using SAS 9.3 version (SAS Institute, Cary, NC). One way Analysis of variance (ANOVA) was performed on PCV, total cell and viability counts, white blood cell differential counts, total β-Catenin data and P-value < 0.05 was considered significant. All results are presented as mean ± SD.

## 3. Results

### *3.1 CPE Treatment of Bovine Cells*

Treatment of bovine blood *ex vivo* with CPE had no effect on total cell count and viability (P > 0.05). The average PCV observed was 32% and was not affected by treatment. However, the CPE treatment significantly changed the proportion of mononuclear cells in whole blood. Treatment significantly increased the percent lymphocytes (P = 0.0114) and decreased the % of monocytes (P = 0.0136) compared to the control group (Table 1). The percentage of neutrophils in blood was unaffected by treatment although animal variation in % neutrophils was observed after treatment with CPE (P = 0.0032). Secretion/release of TLR in plasma occurred following exposure to CPE. Treatment resulted in detection of TLR2 (51.56 ng/ml) in cow blood. Bovine TLR2 was not detected in the untreated control samples (Figure 1). Furthermore, bovine TLR4 was not detected in plasma of both CPE-treated and control groups.

Table 1. Analysis of bovine peripheral blood in Cowpea Phenolic Extract-treated and control

| Parameter | Control | CPE-treated | P-value |
|---|---|---|---|
| PCV (%) | 32±2.14 | 31±1.94 | NS |
| Total cell count (cells/ml) | $5.85×10^8±3.09×10^8$ | $5.13×10^8±2.05×10^8$ | NS |
| Total viable cells (cells/ml) | $4.87×10^7±2.06×10^7$ | $5.41×10^7±1.64×10^7$ | NS |
| White Blood cell differential Count (%) | | | |
| Lymphocytes | 79.33±6.32[b] | 83.056±3.05[a] | * |
| Neutrophils | 14.00±4.07 | 13.33±3.85 | NS |
| Monocytes | 5.67±3.92[a] | 3.11±1.90[b] | * |
| Eosinophils | 0.94±0.93 | 0.56 ±0.51 | NS |
| Basophils | 0.00±0.00 | 0.00±0.00 | NS |

*Note*. CPE = Cowpea Phenolic extract; PBS = Phosphate Buffer Saline; * for P < 0.05; NS: P > 0.05; a,b Values within a row with different superscripts differ significantly at P < 0.05.

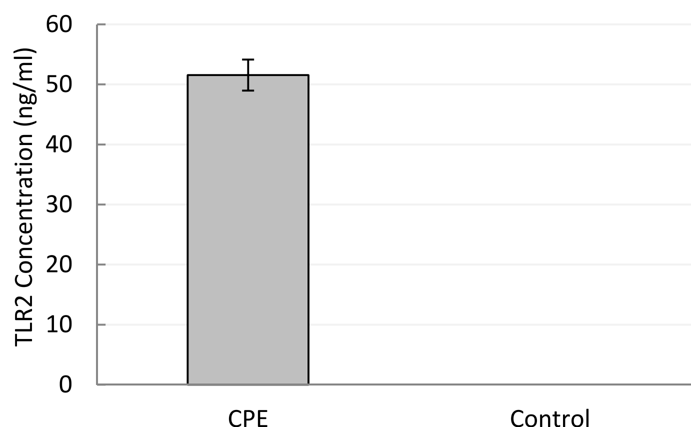


Figure 1. Bovine toll-like receptor 2 (TLR2) concentration measured in plasma samples following Cowpea Phenolic Extract treatment of bovine peripheral blood. TLR2 release was undetected in control group

### *3.2 Effect of CPE Exposure on Activation of the Wnt Signaling Pathway*

The Wnt signaling pathway activation was observed at the level of mRNA transcription in *ex vivo* CPE-treated bovine blood cells. Out of the 84 genes on the cow Wnt signaling pathway array, 60 genes were detected in untreated cow blood. Sixteen genes on the array were not detected; DKK3, DVL1, FGF4, FOSL1, FZD7, FZD8, SFRP1, WISP1, Wnt1, Wnt11, Wnt2b, Wnt5a, Wnt5b, Wnt7b, Dkk2 and KREMENS. Gene description, average Ct values, and fold changes for all 84 genes on the array are found on Appendix 1. Forty-two genes were expressed in both CPE and control group (Figure 2).

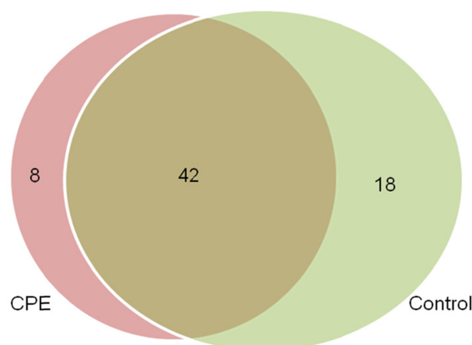


Figure 2. Venn diagram showing summary of the response of 84 genes on the cow Wnt siganling array in response to Cowpea Phenolic Extract (CPE). Forty-two genes were detected in both Cowpea Phenolic Extracts and control group, 18 genes were inhibited and 8 genes were newly expressed by Cowpea Phenolic Extracts

Treatment differentially impacted 28 genes, the level of expression/fold change of 19 genes was up-regulated, 9 genes were down-regulated and 14 genes were unchanged in expression. Treatment with CPE induced the expression of eight new genes in blood previously undetected in the control group. These genes were FOXN1, FZD9, PITX2, SFRP4, SOX17, Wnt3a, Wnt7a and Wnt8a. When compared to untreated samples, treatment with CPE inhibited the expression of eighteen genes in the Wnt pathway including APC, AXIN2, CCND1, CCND2, DAB2, DVL2, FZD1, MAPK8, MMP7, NKD1, PRICKLE1, RHOU, TCF7L1, Wnt10A, Wnt16, Wnt2, Wnt6, and MAP2K7 (Table 2). Among genes inhibited by CPE stimulation of Wnt pathway were planar cell polarity Wnt signaling related genes DVL2, MAP2K7, MAPK8, NKD1 and PRICKLE1; and Wnt/$Ca^{2+}$ pathway associated genes Wnt10a, Wnt16, Wnt2 and Wnt6 (Table 2).

The highest fold changes in expression were observed for genes associated with canonical Wnt signaling. The top five up-regulated genes were Dkk1 (17 fold), FZD4 (18 fold), FZD6 (12 fold), Wnt3 (12 fold) and VANGL2 (16 fold). Genes downregulated include MYC, NFATC1, PYGO1, RHOA, CBY1, and JUN. The Wingless protein with highest fold increase in expression was Wnt3. Genes involved in cell fate determination that were significantly up-regulated included DKK1 (30 fold), Wnt3 (17 fold), Wnt3a (expressed after CPE treatment). Cell growth and proliferation associated genes FOXN1, PIXT2 and Wnt3a were expressed after CPE treatment.

Table 2. Differentially expressed Wnt signalling pathway genes

| Wnt pathway category | Fold change |
|---|---|
| Canonical pathway genes | |
| Wnt3 | 12.00 |
| Wnt9a | 5.00* |
| Fzd3 | 4.00* |
| Fzd4 | 18.00* |
| Fzd6 | 12.00* |
| Lrp5 | 4.00* |
| Lrp6 | 5.00* |
| CTNNB1 | 1.00 |
| CTBP1 | 3.00* |
| AXIN | 6.00 |
| BCL9 | 3.00* |
| GSK3A | 1.00 |
| GSK3B | 1.00 |
| DKK1 | 17.00 |
| DKK4 | 6.00 |
| LEF1 | 1.00 |
| TCF7 | 1.00 |
| WIFI | 13.00* |
| Planar cell polarity genes | |
| DAAM1 | 0.23 |
| MAPK8 | Expressed in control only |
| PRICKLE1 | Expressed in control only |
| RHOA | 0.10 |
| RHOU | Expressed in control only |
| VANGL2 | 16.00* |
| NKD1 | Expressed in control only |
| Wnt/Ca2+ pathway genes | |
| NFATC1 | 0.33 |
| Wnt10A | Expressed in control only |
| Wnt16 | Expressed in control only |
| Wnt2 | Expressed in control only |

*Note*. * P-value < 0.05.

### *3.3 Total β-Catenin Levels*

Cellular associated β-Catenin protein was detected in bovine blood (Figure 3). However, increased β-Catenin levels (6 ng/ml, $P < 0.05$) were detected after CPE treatment relative to control group. There was variation in the β-Catenin levels among the different animals used ($P = 0.0084$). The assay used is unable to distinguish between active β-Catenin from phosphorylated B-catenin–It detects both in response to Wnt signaling. Thus, CPE increased the total cellular β-Catenin released in the bovine blood.

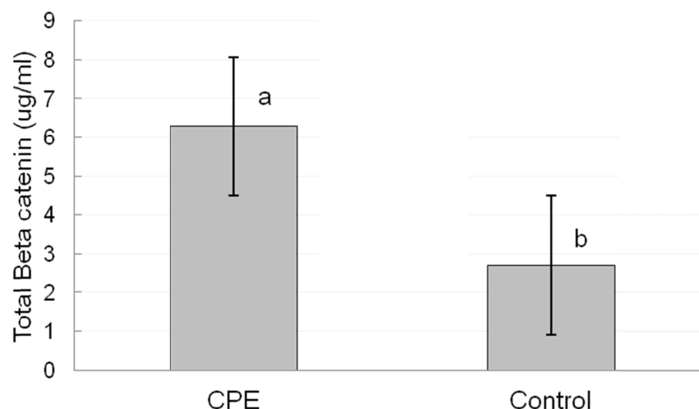


Figure 3. Total cell-associated β-Catenin measured in cell lysate of cowpea phenolic extract (CPE)-treated and control samples. Cowpea Phenolic Extracts activated the Canonical pathway, which is β-Catenin -dependent, in blood. β-Catenin proteins increased following Cowpea Phenolic Extracts treatment (n = 6, P < 0.05)

## 4. Discussion

The current study investigated the possible modulation of Wnt signaling genes in bovine blood in response to CPE. Plant metabolites such as phenolic compounds from feed sources are able to reach diverse tissues such as skeletal cells in muscle through blood. We demonstrated that *ex vivo* CPE treatment of bovine blood induced Wnt signaling through the modulation of β-Catenin expression, transcriptional activity and of the subsequent expression of Wnt target genes. In blood, CPE specifically targeted the canonical pathway and inhibited activation of the PCP and Wnt/$Ca^{2+}$ pathways. Wnt activation status was evident with fold increase in gene expression of Wnt3 ligand, Frizzled receptors Fzd3, Fzd 4 and Fzd 6, together with upregulation in expression of both co-receptors Lrp5/6.

Gene expression analysis showed CPE inhibition of APC which functions to remove phosphorylated β-Catenin (Xing et al., 2003). CPE activation of canonical pathway possibly stabilized β-Catenin and hence increased total β-Catenin protein levels, although no significant fold increase in nuclear β-Catenin-CTNNB1 gene expression was observed. Nevertheless, fold change in β-Catenin level, however low, are still capable of effecting transcriptional changes (Clevers & Nusse, 2012). β-Catenin influenced gene transcription of T cell factor (TCF) and lymphoid enhancing binding factor (LEF), although significant fold increase was not observed. CPE likely targets activation of the TCF/LEF pathway associated with T cell proliferation. The Wnt/β-Catenin signaling has been reported to regulate survival, proliferation and differentiation of hematopoietic cells (Logan & Nusse, 2004), and specifically relevant for lymphocytes differentiation (Reya et al., 2000; Stall & Clever, 2003). This provides a possible explanation for the observed *ex vivo* expansion of percentage lymphocytes numbers in blood following CPE treatment.

Within the immune system, Wnt signaling has been studied during lymphocytes development and in the context of hematopoietic stem cell biology (Staal et al., 2008; Reya et al., 2003). Here we demonstrated the Wnt/β-Catenin module may have an unexpected role as a regulator of lymphocytes in cow blood through a pathway that may involve TLR2. Previous work has shown that, TLR2 stimulation in macrophage resulted in actiavation of Wnt/β-Catenin (Bansal et al., 2011). Toll-like receptor 2 is an intergral membrane protein that recognizes both pathogen associate molecular patterns (PAMPs) and non-pathogenic microganisms/damage associated molecular patterns (Kawai & Akira, 2010). Engagement of TLRs triggers in inflammation response under pathogenic condition. In the present study, CPE treatment caused release of soluble TLR2 in cow blood under non-pathogenic condition. The antiinflammtory effect of cowpea phenolics extracts (Ojwang et al., 2015), may have severed as a negative regualtory mechanism in controllling TLR2 response to ensure system homeostatsis, with no effect on TLR4.

The effect of Wnt signaling activation on lymphocyte levels in cow blood by CPE was observed and this supports previous studies that have associated the canonical Wnt pathway with cell proliferation (Reya et al., 2003; Wang et al., 2006). In our work, Wnt3a which is associated with cell proliferation was induced after CPE treatment and this outcome may have similar implication previously reported by Willert et al. (2003). Treatment of hematopoietic stems cells with Wnt3a protein resulted in an increased self-renewal. In another study, Wnt signaling activation blocked effector T lymphocytes differentiation after treatment with either GSK3B inhibitor

TWS119 or Wnt3a. Their study demonstrated that for naïve-to-effector T cell differentiation in human T lymphocytes, the Wnt/β-Catenin system may act as a negative regulator (Muralidharan et al., 2011). Wnt signaling regulation of various aspects of hematopoiesis including stem cell establishment, regeneration, maintenance of homeostasis, and differentiation of cells in blood has been reported (Lento et al., 2013). Wnt signaling controlled early embryonic hematopoiesis in mice and dysregulated β-catenin has been implicated in leukemia (Kabiri et al., 2014). The hematopoietic effect of Wnt activation may be related to changes in leukocyte concentration and this was evident in our study with activation of cells and subsequent expansion of lymphocytes numbers, and fold increase in cell growth and proliferation genes expression after CPE treatment.

Treatment of bovine blood with CPE activated the cells, had no effect on PCV, total cell count and viability of cells. Our results indicated cell activation and increased mRNA transcriptional activity following CPE treatment. Plant-derived bioactive compounds such as tannins have been shown to activate blood cells such as gamma delta T cells, one of the primary lymphocytes that are key to innate immune response in bovine (Holderness et al., 2007). Our results provide insight into CPE activation of bovine blood cells and subsequently Wnt signaling. Further research is needed to elucidate the role CPE on activation of Wnt signaling in subpopulations of blood lymphocytes.

In cancerous cells, plant-derived phenolic compounds have been reported to inhibit Wnt signaling pathway (reviewed by Teiten et al., 2012). However, in this current study, we elucidated the potential role CPE on Wnt signaling in healthy bovine peripheral blood cells. Our results demonstrated that CPE induces Wnt signaling, increase β-Catenin expression levels, and functions to promote lymphocytes proliferation. Zhang et al. (2010), studied the effect of flavonoids from *Herba epimedii* on Wnt signaling using healthy human bone marrow-derived mesenchymal stem cells. Flavonoids from *Herba epimedii* were shown to induce Wnt signaling and an increase in β-Catenin expression was reported. Thus, CPE, activates and modulate Wnt signaling genes and may involve a crosstalk with the TLR pathway.

## 5. Conclusion

Cowpea serves as a source of nutrition for man and animals. The CPE extract evaluated in this study modulates a highly conserved signaling pathway essential to homeostasis and health. In the study we showed the *ex vivo* effect of cowpea-derived phenolic extract stimulates TLR2 release and modulate Wnt signaling, a key cellular pathway functioning in cell fate, specification, polarity, proliferation, movement and maintenance of homeostasis. Treatment of bovine peripheral blood cells with CPE specifically activated the canonical pathway which is β-Catenin dependent whiles inhibiting the expression of gene associated with the planar cell polarity and Wnt/$Ca^{2+}$ signaling pathways. The significant role of Wnt signaling in cellular and biological processes, elevates the potentials of cowpea as a feed supplement in the animal system for enhanced nutrition, development and health.

## Acknowledgements

The authors wish to acknowledge the support of Mr. Hamid Ismail, and Mr. Corey Burgess for his assistance during animal blood collection. This study was undertaken with the financial support from the United States Department of Agriculture project NC.X-271-5-13-120-1.

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## Appendix

Appendix 1. Cow WNT signalling array (Qiagen) contains 84 test genes. The gene names, decrription, average Ct values and fold change in gene expression are found below

| Position on array | Unigene | Refseq | Symbol | Description | CPE Average Ct | Control Average Ct | Fold change | p-value |
|---|---|---|---|---|---|---|---|---|
| A01 | Bt.9945 | NM_001128497 | AES | Amino-terminal enhancer of split | 34.00 | 31.32 | 1 | 0.2275 |
| A02 | Bt.11086 | NM_001075986 | APC | Adenomatous polyposis coli | 0.00 | 34.14 | | |
| A03 | Bt.21602 | NM_001191398 | AXIN1 | Axin 1 | 37.34 | 36.93 | 6 | 0.0812 |
| A04 | Bt.4412 | NM_001192299 | AXIN2 | Axin 2 | 0.00 | 37.30 | | |
| A05 | N/A | XM_002686086 | BCL9 | B-cell CLL/lymphoma 9 | 38.76 | 37.56 | 3 | 0.0438 |
| A06 | Bt.25271 | NM_001083475 | BTRC | Beta-transducin repeat containing | 36.02 | 34.46 | 3 | 0.0390 |
| A07 | Bt.88783 | NM_001046273 | CCND1 | Cyclin D1 | 0.00 | 38.00 | | |
| A08 | Bt.4895 | NM_001076372 | CCND2 | Cyclin D2 | 0.00 | 37.57 | | |
| A09 | Bt.65222 | NM_174711 | CSNK1A1 | Casein kinase 1, alpha 1 | 36.34 | 32.95 | 1 | 0.4114 |
| A10 | Bt.64603 | NM_174635 | CSNK2A1 | Casein kinase 2, alpha 1 polypeptide | 33.98 | 31.44 | 1 | 0.1908 |
| A11 | Bt.1780 | XM_002688450 | CTBP1 | C-terminal binding protein 1 | 34.41 | 32.79 | 3 | 0.0475 |
| A12 | Bt.33687 | NM_001076141 | CTNNB1 | Catenin (cadherin-associated protein), beta 1, 88kDa | 35.93 | 32.47 | 1 | 0.2150 |
| B01 | Bt.8208 | NM_001081588 | DAAM1 | Dishevelled associated activator of morphogenesis 1 | 37.24 | 32.78 | 0 | 0.0226 |
| B02 | Bt.15382 | NM_001193246 | DAB2 | Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) | 0.00 | 38.73 | | |
| B03 | N/A | XM_002693011 | DIXDC1 | DIX domain containing 1 | 36.19 | 32.90 | 1 | 0.3677 |
| B04 | Bt.13880 | NM_001205544 | DKK1 | Dickkopf homolog 1 (Xenopus laevis) | 38.19 | 39.33 | 17 | 0.0339 |
| B05 | Bt.18710 | NM_001100306 | DKK3 | Dickkopf homolog 3 (Xenopus laevis) | 0.00 | 0.00 | | |
| B06 | Bt.27892 | NM_001206601 | DVL1 | Dishevelled, dsh homolog 1 (Drosophila) | 0.00 | 0.00 | | |
| B07 | Bt.17972 | NM_001191382 | DVL2 | Dishevelled, dsh homolog 2 (Drosophila) | 0.00 | 40.64 | | |
| B08 | Bt.30467 | XM_002696250 | FBXW11 | F-box and WD repeat domain containing 11 | 37.73 | 34.78 | 1 | 0.3573 |

| Position on array | Unigene | Refseq | Symbol | Description | CPE Average Ct | Control Average Ct | Fold change | p-value |
|---|---|---|---|---|---|---|---|---|
| B09 | Bt.26327 | NM_001101985 | FBXW4 | F-box and WD repeat domain containing 4 | 36.75 | 33.45 | 1 | 0.4832 |
| B10 | Bt.62944 | NM_001040605 | FGF4 | Fibroblast growth factor 4 | 0.00 | 0.00 | | |
| B11 | Bt.17885 | NM_001205985 | FOSL1 | FOS-like antigen 1 | 0.00 | 0.00 | | |
| B12 | Bt.88317 | NM_001192452 | FOXN1 | Forkhead box N1 | 39.59 | 0.00 | | |
| C01 | N/A | XM_002698415 | FRAT1 | Frequently rearranged in advanced T-cell lymphomas | 37.32 | 32.60 | 0 | 0.0144 |
| C02 | Bt.121 | NM_174059 | FRZB | Frizzled-related protein | 39.74 | 40.07 | 10 | 0.0284 |
| C03 | Bt.26635 | NM_001101048 | FZD1 | Frizzled family receptor 1 | 0.00 | 39.50 | | |
| C04 | Bt.79602 | NM_001192964 | FZD3 | Frizzled family receptor 3 | 36.85 | 35.88 | 4 | 0.0308 |
| C05 | Bt.67879 | NM_001206269 | FZD4 | Frizzled family receptor 4 | 38.90 | 40.05 | 18 | 0.0456 |
| C06 | N/A | XM_005197510 | FZD5 | Frizzled family receptor 5 | 37.01 | 34.55 | 1 | 0.1992 |
| C07 | Bt.104004 | XM_863880 | FZD6 | Frizzled homolog 6 (Drosophila) | 39.09 | 39.71 | 12 | 0.0020 |
| C08 | Bt.105583 | NM_001144091 | FZD7 | Frizzled family receptor 7 | 0.00 | 0.00 | | |
| C09 | N/A | XM_005194311 | FZD8 | Frizzled family receptor 8 | 0.00 | 0.00 | | |
| C10 | N/A | XM_002698189 | FZD9 | Frizzled family receptor 9 | 40.94 | 0.00 | | |
| C11 | Bt.33944 | NM_001102192 | GSK3A | Glycogen synthase kinase 3 alpha | 40.71 | 38.00 | 1 | 0.2456 |
| C12 | Bt.48740 | NM_001101310 | GSK3B | Glycogen synthase kinase 3 beta | 34.42 | 31.70 | 1 | 0.2266 |
| D01 | Bt.11159 | NM_001077827 | JUN | Jun proto-oncogene | 38.38 | 30.00 | | |
| D02 | N/A | XM_002694622 | KREMEN1 | Kringle containing transmembrane protein 1 | 40.30 | 36.94 | 1 | 0.4120 |
| D03 | Bt.18467 | NM_001192856 | LEF1 | Lymphoid enhancer-binding factor 1 | 34.69 | 31.28 | 1 | 0.3099 |
| D04 | Bt.45360 | XM_002699405 | LRP5 | Low density lipoprotein receptor-related protein 5 | 37.68 | 36.70 | 4 | 0.0251 |
| D05 | Bt.60913 | XM_002687783 | LRP6 | Low density lipoprotein receptor-related protein 6 | 39.45 | 38.79 | 5 | 0.0539 |
| D06 | Bt.14050 | NM_001192974 | MAPK8 | Mitogen-activated protein kinase 8 | 0.00 | 34.57 | | |
| D07 | Bt.13092 | NM_001075130 | MMP7 | Matrix metallopeptidase 7 (matrilysin, uterine) | 0.00 | 40.81 | | |
| D08 | Bt.21164 | NM_001046074 | MYC | V-myc myelocytomatosis viral oncogene homolog (avian) | 32.95 | 28.20 | 0 | 0.0017 |
| D09 | Bt.45162 | NM_001166615 | NFATC1 | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 | 37.37 | 33.34 | 0 | 0.0105 |
| D10 | Bt.60075 | NM_001105453 | NKD1 | Naked cuticle homolog 1 (Drosophila) | 0.00 | 39.83 | | |
| D11 | Bt.43996 | NM_001193253 | NLK | Nemo-like kinase | 35.46 | 33.15 | 2 | 0.1781 |
| D12 | Bt.18496 | NM_001097991 | PITX2 | Paired-like homeodomain 2 | 40.12 | 0.00 | | |
| E01 | Bt.38004 | NM_001101208 | PORCN | Porcupine homolog (Drosophila) | 39.00 | 34.72 | 0 | 0.0643 |
| E02 | Bt.13264 | NM_001083636 | PPARD | Peroxisome proliferator-activated receptor delta | 37.90 | 36.65 | 3 | 0.0593 |

| Position on array | Unigene | Refseq | Symbol | Description | CPE Average Ct | Control Average Ct | Fold change | p-value |
|---|---|---|---|---|---|---|---|---|
| E03 | Bt.57449 | NM_001102534 | PRICKLE1 | Prickle homolog 1 (Drosophila) | 0.00 | 38.51 | | |
| E04 | N/A | XM_002690892 | PYGO1 | Pygopus homolog 1 (Drosophila) | 37.40 | 32.46 | 0 | 0.0005 |
| E05 | Bt.49678 | NM_176645 | RHOA | Ras homolog gene family, member A | 34.46 | 29.26 | 0 | 0.0002 |
| E06 | Bt.2846 | NM_001098147 | RHOU | Ras homolog gene family, member U | 0.00 | 36.86 | | |
| E07 | Bt.41723 | NM_001101076 | RUVBL1 | RuvB-like 1 (E. coli) | 31.69 | 30.64 | 4 | 0.0685 |
| E08 | Bt.5226 | NM_174460 | SFRP1 | Secreted frizzled-related protein 1 | 0.00 | 0.00 | | |
| E09 | Bt.3540 | NM_001075764 | SFRP4 | Secreted frizzled-related protein 4 | 37.12 | 0.00 | | |
| E10 | Bt.112292 | NM_001206251 | SOX17 | SRY (sex determining region Y)-box 17 | 39.08 | 0.00 | | |
| E11 | Bt.44634 | NM_001099186 | TCF7 | Transcription factor 7 (T-cell specific, HMG-box) | 36.02 | 32.27 | 1 | 0.0947 |
| E12 | N/A | XM_002691408 | TCF7L1 | Transcription factor 7-like 1 (T-cell specific, HMG-box) | 0.00 | 39.72 | | |
| F01 | Bt.3589 | NM_001098020 | TLE1 | Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) | 39.00 | 35.55 | 1 | 0.3267 |
| F02 | Bt.87234 | NM_001205875 | VANGL2 | Vang-like 2 (van gogh, Drosophila) | 38.43 | 39.42 | 16 | 0.1016 |
| F03 | Bt.63013 | NM_001075996 | WIF1 | WNT inhibitory factor 1 | 38.89 | 39.56 | 13 | 0.0115 |
| F04 | N/A | XM_002692603 | WISP1 | WNT1 inducible signaling pathway protein 1 | 0.00 | 0.00 | | |
| F05 | Bt.101628 | NM_001114191 | WNT1 | Wingless-type MMTV integration site family, member 1 | 0.00 | 0.00 | | |
| F06 | Bt.61102 | NM_001099078 | WNT10A | Wingless-type MMTV integration site family, member 10A | 0.00 | 38.79 | | |
| F07 | Bt.21876 | NM_001082456 | WNT11 | Wingless-type MMTV integration site family, member 11 | 0.00 | 0.00 | | |
| F08 | Bt.37171 | NM_001014949 | WNT16 | Wingless-type MMTV integration site family, member 16 | 0.00 | 37.64 | | |
| F09 | Bt.37360 | NM_001013001 | WNT2 | Wingless-type MMTV integration site family member 2 | 0.00 | 39.50 | | |
| F10 | Bt.27254 | NM_001099363 | WNT2B | Wingless-type MMTV integration site family, member 2B | 0.00 | 0.00 | | |
| F11 | Bt.112395 | NM_001206024 | WNT3 | Wingless-type MMTV integration site family, member 3 | 38.45 | 39.11 | 12 | 0.1081 |
| F12 | N/A | XM_002688509 | WNT3A | Wingless-type MMTV integration site family, member 3A | 39.48 | 0.00 | | |

| Position on array | Unigene | Refseq | Symbol | Description | CPE Average Ct | Control Average Ct | Fold change | p-value |
|---|---|---|---|---|---|---|---|---|
| G01 | Bt.88484 | NM_001205971 | WNT5A | Wingless-type MMTV integration site family, member 5A | 0.00 | 0.00 | | |
| G02 | Bt.6367 | NM_001205628 | WNT5B | Wingless-type MMTV integration site family, member 5B | 0.00 | 0.00 | | |
| G03 | Bt.27385 | NM_001205563 | WNT6 | Wingless-type MMTV integration site family, member 6 | 0.00 | 40.01 | | |
| G04 | Bt.69615 | NM_001192788 | WNT7A | Wingless-type MMTV integration site family, member 7A | 39.26 | 0.00 | | |
| G05 | N/A | XM_603482 | WNT7B | Wingless-type MMTV integration site family, member 7B | 0.00 | 0.00 | | |
| G06 | Bt.106446 | NM_001192370 | WNT8A | Wingless-type MMTV integration site family, member 8A | 40.29 | 0.00 | | |
| G07 | N/A | XM_002688510 | WNT9A | Wingless-type MMTV integration site family, member 9A | 39.33 | 38.73 | 5 | 0.0456 |
| G08 | Bt.17918 | NM_174739 | CBY1 | Chibby homolog 1 (Drosophila) | 37.75 | 33.58 | 0 | 0.0837 |
| G09 | Bt.24868 | NM_001082615 | DKK2 | Dickkopf homolog 2 (Xenopus laevis) | 0.00 | 0.00 | | |
| G10 | N/A | XM_002698709 | DKK4 | Dickkopf homolog 4 (Xenopus laevis) | 39.73 | 39.34 | 6 | 0.1599 |
| G11 | Bt.9084 | XM_002697867 | KREMEN2 | Kringle containing transmembrane protein 2 | 0.00 | 0.00 | | |
| G12 | N/A | XM_001254723 | MAP2K7 | Mitogen-activated protein kinase kinase 7 | 0.00 | 39.26 | | |
| H01 | Bt.14186 | NM_173979 | ACTB | Actin, beta | 30.42 | 26.73 | 1 | 0.0072 |
| H02 | Bt.87389 | NM_001034034 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | 31.89 | 28.91 | 1 | |
| H03 | Bt.49238 | NM_001034035 | HPRT1 | Hypoxanthine phosphoribosyltransferase 1 | 36.80 | 33.35 | 1 | 0.4253 |
| H04 | Bt.22662 | NM_001075742 | TBP | TATA box binding protein | 39.57 | 36.56 | 1 | 0.3511 |
| H05 | Bt.111451 | NM_174814 | YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | 34.35 | 30.26 | 0 | 0.0400 |
| H06 | N/A | SA_00137 | BGDC | Cow Genomic DNA Contamination | 0.00 | 0.00 | | |
| H07 | N/A | SA_00104 | RTC | Reverse Transcription Control | 24.88 | 25.51 | 12 | 0.0166 |
| H08 | N/A | SA_00104 | RTC | Reverse Transcription Control | 24.99 | 25.56 | 12 | 0.0137 |
| H09 | N/A | SA_00104 | RTC | Reverse Transcription Control | 25.29 | 25.46 | 9 | 0.0447 |
| H10 | N/A | SA_00103 | PPC | Positive PCR Control | 21.06 | 21.52 | 11 | 0.0207 |
| H11 | N/A | SA_00103 | PPC | Positive PCR Control | 20.69 | 21.35 | 13 | 0.0302 |

| Position on array | Unigene | Refseq | Symbol | Description | CPE Average Ct | Control Average Ct | Fold change | p-value |
|---|---|---|---|---|---|---|---|---|
| H12 | N/A | SA_00103 | PPC | Positive PCR Control | 21.08 | 21.49 | 10 | 0.0348 |

*Note*. 0.00 = means genes were undetected on the array (Fold change were not calculated for genes expressed singularly in either CPE or control group); CPE = Cowpea Phenolic Extracts (Fold change was calculated using the Livak's method).

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