# Effect of Ultrasonication on the Physicochemical Properties of Sorghum Kafirin and Evaluation of its Anti-inflammatory Properties *In Vitro*

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# DEDICATION

*To my family, thank you for your support.*

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# ABSTRACT

Sorghum is one of the hardiest, most drought resistent cereal grains known to exist, providing the bulk of nutritional content for many semi-arid regions and developing countries throughout Africa and southwestern Asia. It contains dense nutritional value, but due to inhibition factors within the grain, much of these nutrients are indigestible. Access to these nutrients, which have shown to contain high contents of bioactive molecules linked to the decrease of prevalence of chronic disease, must then be facilitated before consumption. In sorghum, these molecules could include complex carbohydrates, proteins and polyphenols. The overall objective of this study was to determine the effect of ultrasonication on kafirin's physicochemical properties and evaluate its effects on inflammatory response *in vitro.* Ultrasonication is a processing tool causing self-collapsing micro-cavitations disrupting structure and altering function. Kafirin was extracted and purified from sorghum flour using differential solubility extraction methods, concentrated and lyophilized. The effects of ultrasonication on the secondary structure of kafirin and its stability after ultrasonication against pepsin-pancreatin hydrolysis were measured. Ultrasonication altered the secondary structure of kafirin, increased its solubility and improved its antioxidant capacity. Kafirin was also evaluated for its ability to halt the production of pro-inflammatory cytokines through inhibition of reactive oxygen species production in THP-1 human macrophages primed with lipopolysaccharides (LPS). An association exists between overexpressed inflammatory response from aberrant activation of the inflammatory pathway and chronic diseases. LPS-treated THP-1 macrophages were treated with 50 and 100 μg

(microgram) kafirin/mL. The treatment of kafirin led to inhibition of the production of intracellular reactive oxygen species (ROS), disrupting the inflammatory signaling cascade and reducing the production of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α. These results showed that kafirin can inactivate the inflammatory response pathway by inhibiting intracellular ROS production. This study is the first to report on ultrasonication as a viable option for increasing the digestibility of kafirin as well as kafirin's potential as chemopreventive agent against chronic disease associated with aberrant inflammation.

# TABLE OF CONTENTS

**CHAPTER I: INTRODUCTION ..... 1**
HYPOTHESIS AND OBJECTIVE ..... 5
REFERENCES CITED ..... 7

**CHAPTER II: LITERATURE REVIEW ..... 10**
REFERENCES CITED ..... 21

**CHAPTER III: ULTRASONICATION AFFECTED THE PHYSICOCHEMICAL PROPERTIES OF SORGHUM KAFIRN AND ITS *IN* VITRO PEPSIN-PANCREATIN STABILITY ..... 29**
ABSTRACT ..... 30
INTRODUCTION ..... 31
MATERIALS AND METHODS ..... 33
RESULTS ..... 43
DISCUSSION ..... 48
REFERENCES CITED ..... 52
APPENDIX FIGURES ..... 56

**CHAPTER IV: SORGHUM KAFIRIN INHIBITED LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN THP-1 HUMAN MACROPHAGES ..... 71**
ABSTRACT ..... 72
INTRODUCTION ..... 72
MATERIALS AND METHODS ..... 76
RESULTS ..... 84
DISCUSSION ..... 87
REFERENCES CITED ..... 90
APPENDIX FIGURES ..... 93

**CHAPTER V: CONCLUSIONS ..... 105**

**VITA ..... 106**

# LIST OF FIGURES

## CHAPTER III

Figure 3.1. Effect of ultrasonication on kafirin ..... 55
Figure 3.2. Protein profile of ultrasonicated kafirin ..... 57
Figure 3.3. Secondary structure of ultrasonicated kafirin ..... 59
Figure 3.4. Anti-oxidant capacities of ultrasonicated kafirin..... 61
Figure 3.5. Protein profile of ultrasonicated PPH kafirin..... 63
Figure 3.6. SEC of ultrasonicated PPH kafirin..... 65
Figure 3.7. Degree of hydrolysis of ultrasonicated PPH kafirin ..... 67
Figure 3.8. Antioxidant capacities of ultrasonicated PPH kafirin ..... 69

## CHAPTER IV

Figure 4.1. Pro-inflammatory cytokines. ..... 92
Figure 4.2. Expression of p-65 ..... 94
Figure 4.3. Expression of c-JUN ..... 96
Figure 4.4. Expression of TLR-4 and p-ERK 1/2 ..... 98
Figure 4.5. Intracellular reactive oxygen species ..... 99
Figure 4.6. Proposed mechanism of action ..... 101

# CHAPTER I

# INTRODUCTION

Sorghum, a nutritionally dense cereal high in dietary fiber, proteins, and carbohydrates as well as vitamins and minerals, is the 5$^{th}$ most produced cereal crop in the world after rice, wheat, maize and barley (Taylor, Schober, & Bean, 2006). It plays a critical role as a major food source in semi-arid regions such as Africa and parts of Asia and remains a staple of the food industry (Rao, Birthal, Reddy, Rai, & Ramesh, 2006). Nutritional deficiencies in these regions, particularly caloric and protein deficiencies, are of major concern especially when considering the majority of those afflicted by malnourishment are children and pregnant or nursing women (Cahill-Ripley, 2012). In congruence, when facing the exponentially increasing global water shortage, sorghum proposes a plausible way to address this nutrition deficit due to its ability to withstand strenuous, minimal hydration growing conditions (Jan, 2014).

Notwithstanding, there is a limiting factor to sorghum's abundant nutritional value due in part to the nature of its physicochemical composition and structural morphology, both of which inhibit its digestibility. A multitude of factors such as origin, processing conditions, pH, ionic strength, and the presence of other ingredients all suppress sorghum's digestibility while exogenous factors such as polyphenols, lipids and cell wall components and endogenous factors such as other protein interferences are responsible for the creation of digestion resistant complexes (Awika Joseph, Piironen, & Bean, 2011). Included in this are anti-nutritional factors such as tannins which bind dietary proteins and digestive enzymes acting as catalysts of indigestibility (Staff, 2012).

In terms of consumption, the issue of digestibility is compounded during cooking by heat activation in which the insoluble, gluten-like based complexes form much more rapidly and robustly therefore making sorghum even less digestible (de Morais Cardoso, Pinheiro, Martino, Pinheiro, & Ana, 2017). This indigestible nature relates to its relatively low consumption rate in developed countries when compared to other cereal grains and, more so in places where it is consumed, it is not providing adequate nutritional balance (Taleon, Dykes, Rooney, & Rooney, 2012). Efforts are being made in terms of fortification for a more nutritionally rich and complex sorghum seed as well as the use of selective breeding practices and specialized cultivation techniques on sorghum with the goal of increased, post-processed digestibility (Dillon, Shapter, Henry, Cordeiro, Izquierdo, & Lee, 2007). This has led to recent emerging research on prolamin deposition and function. Several studies have shown that through combining deletion mutagenesis with current methods in genome and transcription profiling, cultivars of sorghum and subsequent variants are beginning to show a higher degree of digestibility and increased nutritional characteristics (Dillon, Shapter, Henry, Cordeiro, Izquierdo, & Lee, 2007; Holding, 2014). In another recent study, phenolic compounds and antioxidant activity was evaluated in sorghum bran, decorticated sorghum flour, and whole sorghum flour. The study disclosed total phenolic compounds, condensed tannins, total anthocyanins, and total flavonoids as well as highlighting specific flavonoids and antioxidant activity (Chung, Wong, Wei, Huang, & Lin, 1998; Moraes, Marineli, Lenquiste, Steel, Menezes, Queiroz, et al., 2015). A correlation of these results were drawn as a comparison to the glycemic index to allow the determination of which

form of the sorghum grain expresses the highest nutritional value (Moraes, et al., 2015). New research such as this is now catalyzing further directed studies specifically aimed towards increasing the digestible protein content through methods such as pre-hydrolysis (Adler Nissen, 1976; Zhao, Xiong, Selomulya, Chen, Zhong, Wang, et al., 2012). Hydrolyzed proteins are shown to have more abundant and improved functional properties than those of un-hydrolyzed proteins (Vioque, Sánchez-Vioque, Clemente, Pedroche, & Millán, 2000).

Further research has also shown that there is a potential for the use of these previously unstudied indigestible prolamin proteins as a regulator molecules useful in the inhibition and down regulation of expression of signal molecules generating inflammatory response (Holding, 2014). Inflammation is an innate immune response triggered by the presence of a foreign body or sterile stressor molecule such as pathogenic associated molecular patterns (PAMPs), which, through a cascade effect, lead to the transcription of inflammatory encoding genes (Aggarwal, 2014). These genes are actively transported out of the nucleus and finally the cell where they signal inflammatory proteins such as chemokines and cytokines (Chignard & Conference on Cytokines and Adhesion Molecules in Lung, 1996; Floege, Lüscher, & Müller-Newen, 2012; Guo, Callaway, & Ting, 2015). There is a complex extracellular regulatory mechanism that is in charge of mitigating the inflammasome-mediated responses as well as intracellular components which control the inflammasome assembly (Chanput, Mes, & Wichers, 2014; Eicke, Xiao, & Andrea, 2013; Guo, Callaway, & Ting, 2015; Veeranki, 2013). With chronic over-activation caused by irregular defensive cellular

response, unresolved inflammation and tissue damage can lead to a favorable environment for uncontrolled cellular growth (Staff, 2012). It is hypothesized that through the manipulation and regulation of this signaling platform it is possible to treat and prevent certain types of diseases associated with aberrant inflammation (Kazma, Mefford, Cheng, Plummer, Levin, Rybicki, et al., 2012). In sorghum, certain aspects of the prolamin storage protein kafirin has been linked to this type of controlled regulatory function and holds potential as an alternative form of chemoprevention against diseases caused by uncontrolled inflammation.

Aim 1 of this research was to determine the effect of ultrasonication on the physicochemical properties of sorghum kafirin. The isolation of kafirin was through a 60% solution of isopropanol leading to a purified kafirin protein. Samples were subjected to ultrasonication treatments of varying duration and amplitude, and analysis conducted on the secondary structure. To determine ultrasonication's effect on the digestibility of kafirin, the stability of kafirin against pepsin-pancreatin hydrolysis and its antioxidant capacity were analyzed.

Aim 2 of this study was to determine the effect of kafirin on lipopolysaccharide (LPS)-induced inflammation *in vitro* using THP-1 human macrophages. THP-1 cells were treated with varying concentrations of kafirin and LPS and the inhibition of pro-inflammatory cytokines was measured. To define the mechanism of action kafirin used to inhibit pro-inflammatory cytokine production, western blot techniques and the production of reactive oxygen species were employed.

# Hypothesis and Objectives

***Objective***

The overall objective is to determine the effect of ultrasonication on kafirin's physicochemical properties and evaluate kafirin's effects on inflammatory response *in vitro*.

***Central Hypothesis***

Ultrasonication can increase kafirin's digestibility and kafirin can reduce inflammatory response in THP-1 human macrophages.

***Aims***

***Aim 1. To determine the effect of ultrasonication on the physicochemical properties of sorghum kafirin.***

*Hypothesis: Ultrasonication will alter the physicochemical properties of sorghum kafirin which will positively impact its digestibility.*

1.1 To develop a method to isolate and purify the sorghum protein kafirin;

1.2 To evaluate the effect of ultrasonication on the secondary structure of kafirin;

1.3 To determine the effect of ultrasonication on the stability of sorghum kafirin against pepsin-pancreatin hydrolysis;

1.4 To measure the antioxidant activity of sorghum kafirin, ultrasonicated sorghum kafirin and their pepsin-pancreatin hydrolysis products.

***Aim 2. To determine the effects of kafirin on LPS-induced inflammatory response in THP-1 human macrophages.***

*Hypothesis: Kafirin will reduce inflammation in LPS-induced THP-1 human macrophages by reducing intracellular reactive oxygen species.*

2.1 To determine the effect of kafirin on the production of pro-inflammatory cytokines in THP-1 human macrophages treated with LPS;

2.2 To determine the mechanism of action by which kafirin inhibits LPS-induced inflammation in THP-1 human macrophages.

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# CHAPTER II

# LITERATURE REVIEW

## *Overview of Sorghum*

From a historical perspective, cereal grains have been a vital source of nutrition and calories dating back 10,000 years or more to parts of China and India. Sorghum itself was first domesticated sometime between 1,000 and 3,000 BCE. Today, cereal grains are still widely consumed throughout the world, but when considering developing countries specifically, a much higher rate of consumption is observed. It is estimated that roughly 60% of calories in developing countries come from cereal grains with values rising above 80% in the most underdeveloped regions (Ness, 2004). This stresses the need for abundant, high nutrition value cereal grains for regions such as Africa and Western Asia. In these regions, drought and minimal potable water availability are driving forces behind grain selection and production (Jan, 2014). For this reason, sorghum is seen as a plausible source of nutrition, granted a few caveats (Rao, Birthal, Reddy, Rai, & Ramesh, 2006).

Sorghum is a pseudo-cereal currently being investigated for a multitude of applications associated with its unique structural conformation, morphology, and physicochemical properties. The monocot seed structure found in sorghum is comprised of the bran (seed coating, various tissue layers), corneous (endosperm), and germ (embryo) with the monocot denoting its single embryonic cotyledon (Earp, McDonough, & Rooney, 2004). The nutritional composition is similar to most other cereal grains such as corn, wheat, and barley with around 360 calories per 100 grams (L. de Morais

Cardoso, S. S. Pinheiro, H. S. D. Martino, Sant Pinheiro, & H. M. Ana, 2017). Macronutrient composition is 72% carbohydrates, 4% fat, and 11% protein, and above average essential micronutrient mineral and vitamin content is present for niacin, thiamin, vitamin B6, iron (26% DV), and manganese (76% DV) (de MoraisCardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2015). Sorghum also contains rich phytochemical content which has been linked to significant impacts on human health. These phytochemicals, anthocyanins, tannins, phytosterols, policosanols, and phenolic compounds, give sorghum high antioxidant capacity and extensive research is being conducted on profiling and comparing these compounds for applications in current health concerns (Awika & Rooney, 2004; Awika Joseph, Piironen, & Bean, 2011; Frederico Barros, Awika, & Rooney, 2012; F. Barros, Dykes, Awika, & Rooney, 2013; Moraes, Queiroz, Shaffert, Costa, Nelson, Ribeiro, et al., 2012; Yang, Allred, Geera, Allred, & Awika, 2012). Sorghum tannins in *in vitro* studies have shown to inhibit enzymatic carbohydrate digestion in the stomach through sorghum sourced encapsulation methods to assist in insulin and blood glucose level regulation for type 2 diabetes (Links, Taylor, Kruger, Naidoo, & Taylor, 2016). Sorghum anthocyanins have shown to selectively inhibit the proliferation of human epidermal growth factor receptor 2 in patients with breast cancer (W. Liu, Xu, Wu, Liu, Yu, Chen, et al., 2013). Anthocyanins are among the few plant polyphenols which can be found in plasma sample and have shown promise in significantly decreasing the rate of cardiovascular disease (Wallace, 2012). Sorghum derived phenolic acid and fat-soluble compounds have shown to play a critical role in the promotion of beneficial gut microbiota and

parameters relating to obesity, oxidative stress, dyslipidemia, and hypertension (De Mesa-Stonestreet, Alavi, & Bean, 2010). The presence of these polyphenol groups along with sorghums bioactive compounds such as carotenoids and flavonoids are just now starting to be understood and utilized.

### *Sorghum Proteins and Kafirin*

Sorghum has a protein composition based on four main groups of proteins: albumin and globulin 10-30%, kafirin 50-70%, and glutelin 25-35%, with total crude protein contents ranging between 6-7% and total digestible protein content between 3.5-4% (Holding, 2014). Each of these groups vary in structure, composition, functional properties, and nature of solubility. Solubility is a key condition to note because this allows the proteins to be separated individually. Albumin has an amino acid content on the periphery body of the protein that has relatively high polarity, allowing it to easily dissolve in an aqueous solution (Landry, 1970). Globulins, more specifically “true” globulins referred to as euglobulins, are dissolved in the presence of low concentrations of salt ions (Landry, 1970). The salt ions break down the hydrophobic surface barrier surrounding the globulin which then allows binding. Kafirin, a water insoluble protein, is extracted by aqueous alcohols that cause denaturation and exposure of more hydrophilic interior amino acids (Landry, 1970). And finally glutelins, which are bound in carbohydrate matrixes formed in the presence of borate, become soluble after chelation of a various hydroxyl groups (Landry, 1970).

Kafirin, a prolamin, is the most abundant protein found in sorghum, comprising roughly 50-70% of the total protein content (Eicke, Xiao, & Andrea, 2013). Endoreduplicated cells create an excess of over-expressed duplicated genes in prolamin proteins which therefore become abundantly available in sources such as sorghum in the form of kafirin proteins (Belton, Delgadillo, Halford, & Shewry, 2006). The indigestible nature of kafirin, whose solubility is contingent on the presence of high levels alcohols such as ethanol, propanol, and in some cases methanol in order to cleave and inhibit cross-linking disulfide bonds and de-polymerize the protein, gives kafirin several distinct features. When kafirin is under reducing conditions, the kafirin polymers, oligomers, and dimers determined by the varying subclasses of the protein structure are reduced almost entirely into alpha, beta, and gamma monomers (Da Silva, Taylor, & Taylor, 2011). Because the majority of kafirin protein are in the cysteine rich $\gamma$-species, there is an overabundance of disulfide cross-linking as compared to typical $\alpha$-prolamins (Byaruhanga, Erasmus, Emmambux, & Taylor, 2007; Duodu, Nunes, Delgadillo, Parker, Mills, Belton, et al., 2002; Oom, Pettersson, Taylor, & Stading, 2008). Kafirin has been the focus of intense study recently on its application in biofilms and encapsulation based on key principles such as its hydrophobicity and indigestibility as well as being non-allergenic and minimally affected by mammalian proteases (J. Taylor, Anyango, Potgieter, Kallmeyer, Naidoo, Pepper, et al., 2015). One reason for this push is due to potential inherent risks through unintended chemical exposure from chemical coating and encapsulation methods that are not an issue with food sourced biomaterial. Kafirin has shown to be highly effective as both a drug delivery system and

oral nutraceutical (Lau, Johnson, Stanley, Mikkelsen, Fang, Halley, et al., 2015). *In vitro* analysis has already shown the potential of a sorghum derivative, condensed tannins, at inhibiting digestive amylases after being encapsulated in a sorghum kafirin micro-particle which allow survival at gastric pH and ensure tannin delivery to the small intestine (Janet Taylor, Bean, Ioerger, & Taylor, 2007). In terms of consumption as a food product, the issue of digestibility is compounded during cooking by heat activation in which the indigestible complexes form much more rapidly and robustly therefore making sorghum even less digestible (de MoraisCardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2015).Research on prolamin has recently shown that both the deposition and function can be altered through combining deletion mutagenesis along with current methods in genome and transcription profiling. This process alters its physicochemical state and has subsequently lead to variants with the capacities of increased digestibility and better expression of nutritional characteristics (Holding, 2014).

***Ultrasonication***

Ultrasonication refers to the process in which low-frequency sound waves are applied to a source with high power and at high intensity typically through an aqueous solution (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). Ultra-sonication is based on the principle of micro-cavitation in which microbubbles that form on the surface of a sonicated object generate vibration by immediately rupturing or imploding upon creation (Rozenberg, 1973). The utility of ultrasonication has grown in popularity within the food industry as a form of processing and acoustic energy is now being used to assist in

crystallization, freezing, bleaching, degassing, extraction, drying, filtration, emulsification, sterilization, cutting, etc. (Majid, Nayik, & Nanda, 2015). Ultrasonication, for the most part, is not considered a new form of processing to the food industry, but due to new technologies emerging it has seen a drastic and broadened increase in application. These new technologies have been shown to reduce processing time, facilitate faster energy and mass transfer, allow for lower processing temperatures, enhance specific constituent extraction, and increase production volume among others (Chemat, Zill-E-Huma, & Khan, 2011). It has the capability of inactivating enzymes and inhibiting or killing microorganisms while maintaining product integrity in most instances by affecting either the surface features including key signaling receptors and decreasing surface permeability (Chandrapala & Leong, 2015; Ghosh, Mukherjee, & Chandrasekaran, 2013). Ultrasonication has also exhibited the ability to induce conformational, morphological and structural changes in the form of $\alpha$-helix degradation and increased formation of $\beta$-sheets accelerating molecular motion and leading to protein aggregation (Jiang, Wang, Li, Wang, Liang, Wang, et al., 2014). It has shown to increase protein solubility while increasing free sulfhydryl content indicating a decrease in intermolecular interactions and partial unfolding (Hu, Wu, Li-Chan, Zhu, Zhang, Xu, et al., 2013).

***Inflammation***

Inflammation is the bodies response to a foreign substance or cellular stressor typically resulting from either an injury or infection (Aggarwal, 2014; Floege, Lüscher, &

Müller-Newen, 2012; Guo, Callaway, & Ting, 2015). Chronic inflammation leads to cellular malfunction and damage resulting in organ failure or unregulated cellular growth and proliferation (Aggarwal, 2014; Arthur, Perez-Chanona, Mühlbauer, Tomkovich, Uronis, Fan, et al., 2012; Breit, Wahl, & SpringerLink, 2001; Conway, Pikor, Kung, Hamilton, Lam, Lam, et al., 2016; Dannenberg, 2013; Leandro de Morais Cardoso, Soraia Silva Pinheiro, Hércia Stampini Duarte Martino, Sant Pinheiro, & Helena Maria Ana, 2017; Guo, Callaway, & Ting, 2015; Thornberry, 1994). Cytokines, or immunomodulating glycoproteins found in cells such as macrophages and lymphocytes, function as a signaling unit with highly specific binding properties which are released by a cell in the presence of an activating stimuli and attach to a correlating receptor of another cell thus completing the role of inflammatory mediator inside the body (Floege, Lüscher, & Müller-Newen, 2012). The signaling cascade caused by cytokine reception leads to specific cellular response such as proliferation or enzymatic release in order to promote cytokine function.

The role of cytokines in inflammation and inflammation's role in disease has been extensively researched. A host of gastrointestinal, respiratory, circulatory, reproductive, and skeletal/muscular diseases are linked to unbalanced cytokine expression and incorrect or overexpressed inflammatory response (Janet Taylor, Taylor, Belton, & Minnaar, 2009). One of the most pressing concerns in human health to date is the prevalence of metastatic forms of cancer (Debois, 2002). Typically, metastatic cancer is spread through the proliferation of principle cancer cells that become dislodged from the primary tumor source and then reattach to a new substrate in another location of the

body. The metastatic state of cancer is dependent upon this proliferation and is the focus of intense study. Research on multiple types of cancers has already been conducted. One study on cytokine Interleukin 6, a surrogate for chronic inflammation in prostate cancer, has been shown to promote proliferation and inhibit apoptosis through a host of pathways (Nguyen, Li, & Tewari, 2014). Another study has suggested that inflammatory tumor microenvironment is associated with facilitating the progression of tumor growth and metastasis through the formation of hypoxic environments, alteration of microRNA expression, and increasing stem cell phenotype in lung cancer (Dubinett & SpringerLink, 2015). It was shown that modifications to gut microbiota when interleukin-10-deficiency is present is directly related to inflammation in colorectal cancer suggesting that colitis and increased genotoxic capable microorganisms promote tumorigenesis (Arthur, et al., 2012), as well as a study supporting inflammation based adverse drug reactions to women treated with estrogen inhibitors for breast cancer was shown to decrease precursor IL-17RA and increase mature IL-17, IL-12, IL-12RB2, and IL-R2 expression along with NF-κB transcriptional activity indicating downstream cytokine expression alteration caused by specific T-Cell Leukemia 1A gene SNPs (M. Liu, Wang, Bongartz, Hawse, Markovic, Schaid, et al., 2011).

Most studies, like those listed above, utilize a surrogate to simulate cancer inflammation. In most instances, surrogate selection is based on gene expression profiling in which potential surrogates are pooled with relation to high correlation of cell line specific *in vitro* sensitivities. One of the most extensively studied and widely used surrogate cells is the human leukemia monocytic cell line THP-1 that functions as a

macrophage/monocyte model for the study of cellular functions, mechanisms, signaling pathways, and drug and nutrient delivery systems (Hjort, Brenyo, Finkelstein, Frampton, LoMonaco, Stewart, et al., 2003; Kramer & Wray, 2002; Sakamoto, Aikawa, Hill, Weiss, Taylor, Libby, et al., 2001; Ueki, Tabeta, Yoshie, & Yamazaki, 2002). In terms of inflammation, studies typically will use THP-1 cells as an *in vitro* model because they are readily activated by cell components such as lipopolysaccharide (LPS) stimulation resulting in inflammatory response (Chanput, Reitsma, Kleinjans, Mes, Savelkoul, & Wichers, 2012; Sharif, Bolshakov, Raines, Newham, & Perkins, 2007; Wichers Harry, Iacomini, Chanput, van Arkel, Ruthes Andrea, Smiderle Fhernanda, et al., 2011). This is based on the principle that inflammatory monocytes are not differentiated into macrophages until they reach the site of infection. THP-1 cells activated by LPS or inflammatory cytokines show a change in many inflammatory gene expressions. THP-1 macrophages exposed to LPS also result in the activation of the transcriptional factor NF-κB which is a regulatory molecule for the control of gene expression and triggers inflammation, cell proliferation, differentiation, and cellular migration that is mediated through chemokine and cytokine release (Chanput, Mes, & Wichers, 2014).

In the presence of LPS, a specific membrane bound toll-like receptor, TLR4, is activated. TLR4 acts as pathogen-pattern recognition receptor which recognizes pathogen associated molecular patterns (PAMPs). PAMPs such as gram negative bacterial lipopolysaccharide (LPS), a host biomolecule signaling infectious inflammatory response, or endogenous damage associated molecular patterns (DAMPs), such as necrosis tumor DNA. TLR4 reception and recognition only occurs with LPS priming and

transportation. A co-facilitator found on the surface of the cell membrane, CD14, is bound by a glycosylphosphatidylinositol anchor and acts as a holding unit of LPS. During activation, LPS binding proteins transfer the LPS to CD14 near the TLR4 receptor. In order for the TLR4 receptor to have access to LPS, the CD14 must first facilitate the transfer of LPS to a second co-facilitator, the myeloid differentiation protein 2 (MD2). MD2 portions of the LPS lipid A are utilized in order to achieve this as neither CD14 nor MD2 contain cytoplasmic tails capable of signal transduction. Congruently, a dual binding reaction occurs as negatively charged phosphate groups located along the diglucosamine backbone of Lipid A covalently bind with positively charged amino acid residues at the periphery face of MD2's binding pocket. At the same time, acyl chains incorporate with a separate hydrophobic pocket thus completing the LPS-MD2 intermediary. This intermediary is conjugated to the inside of the extracellular hook of TLR4 initiating dimerization with another TLR4. The TLR4 dimer, containing a 1:1:1 ratio of TLR4, LPS, and MD2, activates TLR4 signaling.

The following cascade event utilizes nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to transcriptionally generate inflammatory coded genes that signal and promote innate immune system inflammatory molecules such as chemokines and cytokines. First, upon stressor stimulation signaled by TLR4, reactive oxygen species (ROS) found in the cytosol of the cell initiate NF-κB phosphorylation. NF-κB is initially existing as a heterodimer bound by nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-$\alpha$), with 2 structural classes of protein involvement. Class I proteins contain the C-terminal portion encoding for trans-

repression and are comprised of complexes containing the protein p50, whereas Class II proteins' C-terminal portion encodes for the transactivation gene and is comprised of complexes containing p65. Both p50 and p65 contain N-terminal DNA binding domains (DBD) that allow IκB-α to induce binding. In order for nucleus permeation, NF-κB must be liberated from the IκB-α. Specific IκB-α kinases (IKK) catalyze the phosphorylation of IκB-α in the presence of ROS which then becomes unbound from NF-κB. Phosphorylated IκB-α is ubiquitinated during this process signaling proteasome activation and leading to its degradation. Active NF-κB is now capable of permeating the nucleus of the cell where it binds to DNA and transcribes inflammatory coding genes promoting the rise of pro-inflammatory regulators.

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# CHAPTER III

# ULTRASONICATION AFFECTED THE PHYSICOCHEMICAL PROPERTIES OF SORGHUM KAFIRIN AND ITS IN VITRO PEPSIN-PANCREATIN STABILITY

## Abstract

Sorghum is a nutritionally dense grain with a high content of biologically active components, however, most of these bioactives are unavailable to the body after consumption because they are trapped inside indigestible protein matrices. Ultrasonication is a processing tool used in the industry mainly as a supplementary tool for increasing extraction yields of various components from food, however, it has also been shown to increase the solubility of protein by breaking down bonds in the secondary and tertiary structures. The purpose of this study was to determine the effects of ultrasonication on the physicochemical properties of kafirin, the most abundant protein in sorghum, as well as its stability against pepsin-pancreatin hydrolysis (PPH). Sorghum gluten-like flour was sonicated at 20kHz ± 50Hz for 5 and 10 mins at 0, 20 and 40% amplitude and kafirin was extracted and purified using 60% isopropanol. In a separate experiment, sorghum gluten-like flour was ultrasonicated followed by PPH. Ultrasonication increased the soluble protein content of purified kafirin extract for 5 min from 6.6 μg/mL to 159.7 μg/mL for 20% amplitude and 128.8 μg/mL for 40% amplitude, respectively and for 10 min from 6.5 μg/mL to 87.8 μg/mL for 20% amplitude and 173.3 μg/mL for 40% amplitude, respectively. Ultrasonication altered the secondary structure of kafirin as evaluated by circular dichroism and Fourier-transform infrared red spectroscopy. In pepsin-pancreain hydrolysates, an increase of 14.7% was detected in size-exclusion chromatogram after pepsin-pancreatin hydrolysis for the 10 min treatment at 40% amplitude for molecules with molecular weight of between .075-.5 kDa. The degree of hydrolysis was increased after ultrasonication at 40% amplitude for

10 min by 17.7%, 127.6%, 346.6% as measured by o-phthaldialdehyde derivatization, trichloroacetic acid precipitation and hydrochloric acid hydrolysis, respectively. Antioxidant capacity was improved after ultrasonication at 40% for 10 min as measured by scavenging of 2,2-diphenyl-1-picrylhydrazyl and oxygen radical antioxidant capacity assays. Our results showed the potential of ultrasonication to improve digestibility and biological properties of sorghum flour.

## Introduction

Sorghum is a hardy cereal grain, most commonly found in semiarid regions such as northern Africa and western Asia (Rao, Birthal, Reddy, Rai, & Ramesh, 2006). In these areas it plays a critical role as a major economic resource and dietary staple (Rao, Birthal, Reddy, Rai, & Ramesh, 2006). However, sorghum is not just found in these locales, and is ranked as the 5$^{th}$ most produced cereal crop in the world after rice, wheat, maize, and barley. And although it is considered nutritionally dense, found to contain high concentrations of dietary fiber, proteins, and carbohydrates, its nutritional value is hindered by interfering molecules (Awika & Rooney, 2004; de Morais Cardoso, Pinheiro, Martino, Pinheiro, & Ana, 2017; Staff, 2012). Sorghum's own physicochemical composition and morphological structure promote the formation of indigestible complexes through a multitude of factors such as origin, processing conditions, pH, ionic strength, and the presence of other ingredients (Awika & Rooney, 2004). It has also been found that exogenous factors within sorghum such as polyphenols, lipids, and cell wall components as well as certain endogenous factors like ligand binding and

protein-protein bond formation can all lead to the formation of gastric tolerable, enzyme resistant complexes. All of these issues are compounded when discussing heat treatment, where upon cooking the sorghum grain accelerates complex formation further limiting its value as a nutritional source (Taleon, Dykes, Rooney, & Rooney, 2012). Thus, a need exists to increase the nutritional quality of sorghum.

Extensive research has already been done on germination practices for cultivating higher nutritional yield sorghum (Dillon, Shapter, Henry, Cordeiro, Izquierdo, & Lee, 2007; Duodu, Nunes, Delgadillo, Parker, Mills, Belton, et al., 2002; Gemenet, Leiser, Beggi, Herrmann, Vadez, Rattunde, et al., 2016). It is estimated that around 7,000 species of sorghum now exist. Despite this, refinement through selective breeding has yet to produce sorghum with significantly digestible portions of proteins, a key nutrient found missing in the diets of those who have high consumption of sorghum as a sole source of nutrition (Taleon, Dykes, Rooney, & Rooney, 2012). One proposed method to increase sorghum digestibility is through the process of ultrasonication wherein microbubble cavitation could lead to unraveling and denaturation of indigestible complexes. Sonication has been shown to lead to the deformation of $\alpha$-helix structures resulting in increased amounts of $\beta$-sheets in kafirin proteins. This suggests sonication can in fact lead to denaturation (Elbeshbishy, Hafez, & Nakhla, 2011; Hu, Wu, Li-Chan, Zhu, Zhang, Xu, et al., 2013; Jiang, Wang, Li, Wang, Liang, Wang, et al., 2014; Lin, Lee, Yoshimura, Yagi, & Goto, 2014; Pingret, Fabiano-Tixier, & Chemat, 2013). Kafirin is the most abundant protein in sorghum and it contains the periphery protein bodies heavy in cys-cys disulfide bonds responsible for its indigestibility, but, there are no

studies to date confirming the impact of sonication on kafirin digestibility. The purpose of this study was to determine the effects of ultrasonication on the physicochemical properties of sorghum kafirin and its stability against pepsin-pancreatin hydrolysis (PPH).

## Materials and Methods

### *Materials*

Whole grain, sweet, white sorghum flour was purchased from Amazon and was produced by Bob's Red Mill Natural Food, Inc. (Milwaukee, OR). All chemicals were purchased from ThermoFisher Scientific and VWR International unless otherwise specified.

### *Preparation of isolated and purified kafirin samples*

Kafirin was extracted from sorghum by first mixing 400 g of sorghum flour with 1.2 L of heptane for 2 h at room temperature to defat. The sample was centrifuged at 2,100 x g at 4 °C for 20 min after which the supernatant was discarded and the precipitate dried overnight. Albumin was extracted in a 10x vol of deionized (DI) water by stirring for 25 min at room temperature. The sample was centrifuged as above and the supernatant discarded. Globulin was extracted in a 10x vol of 0.5M NaCl solution by stirring for 1 h at room temperature. The sample was centrifuged as above, the supernatant discarded and the precipitate lyophilized. The lyophilized precipitate is called sorghum gluten-like flour. Ten gram of sorghum gluten-like flour were added to 10 mL of DI water. The

samples were separated based on treatment by ultrasonication at 20kHz ± 50Hz at 4°C at 0%, 20%, or 40% amplitude at either 5 min or 10 min. After ultrasonication treatment, samples were centrifuged as above and the supernatant discarded. Kafirin was then isolated from the precipitate in 10x vol of 60% isopropanol solution while being stirred for 4 h at room temperature. Samples were centrifuged as above and the precipitate discarded. The supernatant was distilled in a Buchi RotoVap R-200 (Switzerland) at 50°C and 25 mm Hg pressure. Samples were then frozen at -40°C and lyophilized. All samples were kept at -40 °C until analysis.

***Analysis of purified sorghum kafirin as affected by ultrasonication***

*Soluble protein concentration by Bicinchoninic Acid (BCA) Assay.* One mg of sample was added to either 1-mL of 60% isopropanol and vortexed at 3,000 rpm at room temperature until fully dissolved or 1 mL of Tris-buffered saline (TBS). The TBS samples were vortexed for 90 min and centrifuged at 20,000 x g at 4°C for 30 min. The supernatant was aliquoted and stored at -20 °C until analysis. In a 96 well plate, twenty five µL of TBS extract and varying concentrations of bovine serum albumin (BSA) standards from 0 to 250 µg/mL were plated and 225 µL of bicinchoninic acid reagent were added. After 30 min of incubation at 37°C the absorbance was read at 490 nm and the total soluble protein content was calculated using the generated BSA standard curve.

*Protein profile by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).* The TBS precipitate was re-suspended in 60% iso-2-propanol and vortexed at 3,000 rpm at room temperature until fully dissolved. Samples were stored at -20 °C until analysis. TBS supernatant and precipitate protein profiles were analyzed under reducing condition SDS-PAGE by loading 20 µg of protein to each well of a 4-20% Mini-Protean TGX gel (Bio-Rad Laboratories, Hercules, CA) and run at 100 volts for 100 min. Fifty mL Coomassie blue dye as stain was added to each gel and incubated overnight at room temperature with rotary shaking. Gels were destained in water until background became clear.

*Circular Dichroism (CD) Spectroscopy.* CD spectra were collected using a previously reported protocol (Xiao, Li, Li, Gonzalez, Xia, & Huang, 2015). Three hundred µL samples at a protein concentration of 0.5 mg/mL were filtered using 0.45 µm PVDF membrane (Millipore, Ireland) and transferred to 1 mm path length quartz cuvettes (model 110-QS, Hellma, USA) for analysis by an AVIV model 400 CD spectrometer. The baseline-subtracted representative spectra produced by each sample's 3 scans, measuring sample activity between the wavelengths of 190 and 260 nm at a band width of 5 nm, was plotted using the mean residual ellipticity (MRE) versus wavelength. Analysis was run through Dichroweb using the K2D program with the following parameters: input and output units were in millidegrees / theta (machine units), a wavelength step of .5 nm, a scaling factor of 1, and a residue reference of 109.1.

*Fourier Transform Infrared (FTIR) Spectroscopy.* Wavelength spectra were collected through FTIR and used to describe the secondary structure of kafirin. Samples were combined with a 1:1 ratio of potassium bromide and analyzed through OMNIC FTIR software. Programming was run with a Nexus model 670 FT-IR ESP spectrometer using a DTGS KBr detector and KBr beam splitter. Using KBr as the background, absorbance spectra were generated at a resolution of 4 with data spacing every 1.928 $cm^{-1}$ for 64 scans. Results were plotted as absorbance vs wavenumber $cm^{-1}$ after Fourier Transform Self Deconvolution with a bandwidth of 15.2 $cm^{-1}$ and enhancement set to 1.5.

*Antioxidant Capacity.*

*Oxygen radical antioxidant capacity (ORAC) assay.* In black 96 well plate, 20 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1 mg/mL and Trolox standards ranging from 0 to 240 µM were added to each well in triplicate. Fluorescein working solution was then added at 120 µL per well at a final concentration of 70 nM. The plate was covered and stored in the dark for 15 minutes at 37°C. After incubation a final concentration of 12 nM of 2,2'Azobis(2-amidonpropane) dihydrochloride was then added at 60 µL per well. Plates were read at 485 nm for excitation and 582 nm for emission with a sensitivity of 60 at 2 min intervals for 120 min using Synergy plate reader (BioTek, Winooski, VT). ORAC was calculated from the generated Trolox standard curve.

*Nitric Oxide Radical Scavenging Assay.* In a 96 well plate, 50 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1 mg/mL and 50 µL of water was added in 2 sets of triplicates. Sodium nitroprusside (100 mM, 25 µL) was added to first set and 25 µL of DI water was added to second set. The plates were incubated at room temperature for 2 h. All wells received 100 µL of Griess reagent (1:1 1% sulfanillic acid in 5% phosphoric acid, 0.1% N-(1-napthyl)-ethylenediamine dihydrochloride) and incubated at room temperature. After 15 min, absorbance was read at 550 nm. Results were presented as a percentage of nitric oxide production and calculated using the equation:

$$\%\ NO\ Production = \frac{(Sample\ Absorbance - Blank\ Absorbance)}{Control\ Solvent\ Average\ Absorbance}$$

*2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.* In a 96 well plate, 100 µL of DPPH solution (100 µM) and 100 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1mg/mL were added to each well in triplicate. Plates were incubated for 30 min in the dark and the absorbance was read at 517 nm. Results were presented as a percentage of DPPH production and calculated using the equation:

$$\%\ DPPH\ Production = \left(\frac{Sample\ Absorbance}{Control\ Solvent\ Average\ Absorbance}\right) * 100$$

**Effects of ultrasonication on the pepsin-pancreatin digestibility of sorghum kafirin**

*Pepsin-pancreatin in vitro* digestion was performed on whole sorghum flour by following previously reported protocol with some modifications (Cruz-Huerta, Fernndez-Tom, Arques, Amigo, Recio, Clemente, et al., 2015). Ten g of gluten-like sorghum flour were re-suspended in 200mL of DI water. Samples were ultra-sonicated at 4°C at 0%, 20%, or 40% amplitude for either 5 min or 10 min. The pH of the samples was adjusted to 2 and placed in a water bath shaking at 100 rpm until samples reached 37°C. Pepsin (75 mg) was added to each sample and they were placed back in the water bath. After 1 hour, the samples were removed and pH adjusted to 7.5. A 100 mL aliquot (pepsin samples) of each sample was then separated before adding 400 mg of bile and 50 mg of pancreatin to the remaining portion (pancreatin samples). Pancreatin samples were placed back in the water bath as above while pepsin samples were centrifuged at 23,000 x g at 4°C for 20 min. The pepsin precipitate was discarded and supernatant stored at 4°C. After 1 h, the pancreatin samples were transferred to a 75°C water bath for 10 min and centrifuged at 21,000 x g 4°C for 20 min. The pancreatin supernatant was collected and precipitate discarded. All samples were dialyzed in a membrane with a 3.5 kDa molecular weight cut-off (Spectrum Labs, Rancho Dominguez, CA), frozen, and lyophilized. All samples were stored at -40 °C until analysis.

***Analysis of sorghum flour pepsin-pancreatin hydrolysates as affected by ultrasonication***

*Soluble protein concentration by Bicinchoninic Acid (BCA) Assay.* One mg of sample was added to 1-mL of Tris-buffered saline (TBS) and vortexed at 3,000 rpm at room temperature until fully dissolved. The TBS samples were vortexed for 90 min and centrifuged at 20,000 x g at 4°C for 30 min. The supernatant was aliquoted and stored at -20 °C until analysis. In a 96 well plate, 25 µL of TBS extract and varying concentrations of bovine serum albumin (BSA) standards from 0 to 250 µg/mL were plated and 225µL of bicinchoninic acid reagent were added. After 30 min of incubation at 37°C the absorbance was read at 490 nm and the total soluble protein content was calculated using the generated BSA standard curve.

*Protein profile by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).* The TBS precipitate was resuspended in 60% iso-2-propanol and vortexed at 3,000 rpm at room temperature until fully dissolved. Samples were stored at -20 °C until analysis. TBS supernatant and precipitate protein profiles were analyzed under reducing condition SDS-PAGE by loading 20 µg of protein to each well of a 4-20% Mini-Protean TGX gel (Bio-Rad Laboratories, Hercules, CA) and run at 100 volts for 100 min. Fifty mL Coomassie blue dye as stain was added to each gel and incubated overnight at room temperature with rotary shaking. Gels were destained in water until background became clear.

*Size-Exclusion Chromatography.* Size exclusion chromatography was used to determine the samples' molecular weight profile. Samples were separated by a BioSep-

SEC-S2000 column (300 X 7.80mm, Phenomenex, Torrance, CA) using a model 1200 Agilent HPLC system (Agilent Technologies, Santa Clara, CA) equipped with autosampler (G1329A), quaternary pump (G1311A), vacuum degasser (G1322A), temperature controlled column (G1316A), and diode array detector (G1315D). 1 mg of sample was suspended in TBS and added to the mobile phase (45% acetonitrile in water with 0.1% trifluoroacetic acid) with a final protein concentration of 1 mg/mL. Prepared samples were filtered using 0.45 µm PVDF membrane and analyzed using the following parameters: an injection volume of 10 µL, a flow rate of 1.0 mL/min, running time of 20 min at room temperature and detection at 214 nm. Molecular weight for each peak was calculated from the generated standard curve using proteins and peptides with known molecular weight.

*Antioxidant Capacity.*

*Oxygen radical antioxidant capacity (ORAC) assay.* In black 96 well plate, 20 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1mg/mL and Trolox standards ranging from 0 to 240 µM were added to each well in triplicate. A final concentration of 70 nM of fluorescein working solution was then added at 120 µL per well. The plate was covered and stored in the dark for 15 minutes at 37°C. After incubation a final concentration of 12 nM of was then added at 60 µL per well. Plates were read at 485 nm for excitation and 582 nm for emission with a sensitivity of 60 at 2 min intervals for 120 min using Synergy plate reader (BioTek, Winooski, VT). ORAC was calculated from the generated Trolox standard curve.

*Nitric Oxide Radical Scavenging Assay.* In a 96 well plate, 50 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1mg/mL and 50 µL of water was added in 2 sets of triplicates. Sodium nitroprusside (100 mM, 25 µL) was added to first set and 25 µL of DI water was added to second set. The plates were incubated at room temperature for 2 h. All wells received 100 µL of Griess reagent (1:1 1% sulfanillic acid in 5% phosphoric acid, 0.1% N-(1-napthyl)-ethylenediamine dihydrochloride) and incubated at room temperature. After 15 min, absorbance was read at 550 nm. Results were presented as a percentage of nitric oxide production and calculated using the equation:

$$\%\ NO\ Production = \frac{(Sample\ Absorbance - Blank\ Absorbance)}{Control\ Solvent\ Average\ Absorbance}$$

*2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.* In a 96 well plate, 100 µL of DPPH solution (100 µM) and 100 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1mg/mL were added to each well in triplicate. Plate was incubated for 30 min in the dark and the absorbance was read at 517 nm. Results were presented as a percentage of DPPH production and calculated using the equation:

$$\%\ DPPH\ Production = \left(\frac{Sample\ Absorbance}{Control\ Solvent\ Average\ Absorbance}\right) * 100$$

*Degree of Hydrolysis.*

*Derivatization by orthophtaldehyde (OPA) assay:* In a 96 well plate, 10 µL of sample dissolved in 60% iso-2-propanol at a concentration of 1mg/mL and 200 µL of OPA reagent, consisting of 25 mL of sodium tetraborate (100 mM), 2.5 mL of 20% w/v sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL of methanol, 100 µL of 2-mercaptoethanol, and final volume adjustment to 50 mL total volume with distilled water, were added in triplicate. The plate was read immediately with detection at 340 nm.

*Soluble protein content in trichloroacetic acid (TCA):* In 1.5 mL Eppendorf tubes, 300 µL of sample and 300 µL of 20% w/v trichloroacetic acid were added in triplicate and allowed to incubate at room temperature for 30 min. After incubation, the samples were placed in a centrifuge cooled to 4°C and run for 10 min at 3,000 x g. The supernatant was collected and the soluble protein content was measured using the BCA protein assay method.

*Soluble protein content in hydrochloric acid (HCl):* In 1.5 mL Eppendorf tubes, 300 µL of sample and 300 µL of 2N hydrochloric acid were added in triplicate and allowed to incubate at 100°C for 4 h. The supernatant was collected and the soluble protein content was measured using the BCA protein assay method.

### *Statistical Analysis*

All experiments were conducted using independently created trials made in triplicate and reported using mean $\pm$ standard deviation. Analysis of the data was completed by the PROC GLM procedure of SAS Version 9.4 using the Tukey posthoc test with a confidence interval of 95% ($P < 0.05$) for means.

## Results

### *Effect of ultrasonication on extracted kafirin*

**Fig. 3.1** (All figures are listed at the end of this chapter as an appendix) Ultrasonication of sorghum gluten-like flour increased the amount of kafirin protein extracted and affected its morphological state as shown by direct imaging. Ultrasonicated samples were compared to a non-ultrasonicated control as a percent increase in total weight extracted **(Fig. 3.1a)**. The amount of kafirin extracted for 5 min treatments at 20% and 40% amplitude is 44.1 and 175.6% higher than control sample, respectively. At 10 min treatment, the amount of sorghum kafirin extracted is 422.9 and 274.2% higher than control for 20% amplitude and 40% amplitude, respectively. **Fig. 3.1b** shows representative images of purified kafirin from sorghum gluten-like flour with or without ultrasonication.

### *Effect of ultrasonication on protein solubility of purified kafirin*

The solubility of kafirin after ultrasonication and resuspension in TBS was measured by BCA and further confirmed with SDS-PAGE gel electrophoresis. Ultrasonication

increased the soluble protein content of treated samples **(Fig. 3.2a)**. Non-ultrasonicated sample has soluble protein concentration of 6.6 mg/g and ultrasonication at 20 and 40% amplitude for 10 min increased this value to 87.8 and 173.3 mg/g, respectively. **Fig. 3.2b** shows the electrophoretic profile of kafirin after 5 min and 10 min of ultrasonication, respectively, by its water soluble protein fractions dissolved in TBS, non-water solubilized protein fractions dissolved in 60% iso-2-propanol, and total protein fraction dissolved in 60% iso-2-propanol. No apparent difference on the SDS-PGAE profile was observed with respect to ultrasonication treatment.

***Effect of ultrasonication on the secondary structure changes of purified kafirin***

Ultrasonication increased the amount of unordered or random coils as well as decreased the $\alpha$-helix content for the 10 min treatment at 40% **(Fig. 3.3a)**. Ultrasonication also showed a shift in the peak height difference between the amide I and amide II bonds of kafirin with a 27.8% and 42.8% decrease in peak height for the 5 min treatment at 20% and 40% amplitude, respectively, and a 49.8% and 51.8% decrease in peak height for the 10 min. treatment at 20% and 40% amplitude, respectively **(Fig. 3.3b)**.

***Effect of ultrasonication on the antioxidant property of purified kafirin***

Antioxidants function as inhibitory molecules of potentially damaging oxidation caused by reactive oxygen species within the body. The antioxidant capacity of ultrasonicated sorghum kafirin was measured by its NO **(Fig. 3.4a)** and DPPH scavenging potential

**(Fig. 3.4b)** and its oxygen radical antioxidant capacity (ORAC) **(Fig. 3.4c)**. **Fig. 3.4a** shows that sonication for 5 min did not improve the capability of sorghum kafirin to scavenge NO free radicals. Sonication for 10 min at 40% amplitude significantly improved NO scavenging activity of sorghum kafirin. DPPH production was significantly reduced by purified kafirin ultrasonicated for 10 min at 20 and 40% amplitude by 36.7 and 54.3%, respectively (**Fig. 3.4b)**. **Fig. 3.4c** shows that sonication improved the ORAC value for kafirin for all treatments from 40.7 µM Trolox equivalent (TE)/mg (non-ultrasonicated) to 92.9, 94.0, 147.0 and 186.4 µM TE/mg for 5 min at 20% amplitude, 10 min at 20% amplitude, 5 min at 40% amplitude and for 10 min at 40% amplitude, respectively.

***Protein concentration and protein profile of pepsin-pancreatin hydrolyzed kafirin samples***

The proteolytic enzymes pepsin and pancreatin hydrolyze proteins and are useful for *in vitro* studies to simulate human digestion. The protein concentration of both pepsin and pepsin-pancreatin hydrolyzed sorghum kafirin products was determined by BCA as shown in **Fig. 3.5a** and **Fig. 3.5b**, respectively. **Fig. 3.5a** shows that pepsin hydrolysis significantly decreased the amount of soluble protein content after ultrasonication for both 5 min and 10 min. **Fig. 3.5b** shows that pepsin-pancreatin hydrolysis significantly decreased the amount of soluble protein content after ultrasonication for both 5 min treatments at 20% (18.6%) and 40% (8.3%), however, for the 10 min. treatments only the treatment at 20% had a significant decrease in the soluble protein content by 35.6%.

In **Fig. 3.5c**, SDS-PAGE gel profile confirmed the hydrolysis of the protein samples showing no protein bands above 10 kD for all samples.

***Molecular weight profile of pepsin-pancreatin hydrolyzed kafirin samples as determined by size exclusion chromatography***

Enzymatic protein hydrolysis leads to the formation of smaller molecular weight protein subunits. **Fig. 3.6a** shows the molecular weight profile as a chromatogram after pepsin-pancreatin hydrolysis for ultrasonicated kafirin at 5 min and 10 min treatments of 20% and 40% amplitude. Both of the chromatograms (5 min and 10 min) show a shift to the right for both treatment amplitudes (20% and 40%) as well as an increase in the total area below these lower molecular weight peaks indicating an increase in the amount of molecular content at these values. **Fig. 3.6b** shows the breakdown of molecular weight content for pepsin-pancreatin hydrolysis for all treatments. A trend appears for 20% amplitude and 40% amplitude individually, with 20% amplitude for both 5 and 10 min treatments having an increase of 27.9% and 29.7% increase in molecular weight content between 0.75 - 0.5 kDa, respectively. Similarly, 40% amplitude treatments for both 5 and 10 min also increased the content of 0.75 - 0.5 kDa content, however the increase was only 10.7% and 14.7%, respectively.

***Degree of hydrolysis of pepsin-pancreatin hydrolyzed kafirin samples***

Determination of the degree to which a protein can be hydrolyzed is measured by the available OPA content **(Fig. 3.7a)** as well as its soluble protein content after

precipitation with TCA **(Fig. 3.7b)** and hydrolysis with HCl **(Fig. 3.7c)**. **Fig. 3.7a** shows that OPA-derivatized content was significantly greater for all treatments with both 5 min treatments showing 11.2% and 17.3% increases for 20% and 40%, respectively. For 10 min treatments, a 17.2% and 17.7% increase was shown for 20% and 40%, respectively. The amount of soluble protein content after TCA precipitation also increased in ultrasonicated samples as shown in **Fig. 3.7b** with 5 min/20% amplitude at 24.8% and 5min/40% amplitude at 66.3%, and 10 min/20% amplitude at 26.2% and 10min/40% amplitude at 77.2%. **Fig. 3.7c** further confirms an increase in soluble protein content after HCl hydrolysis with increases of 40.3% and 150.3% for 5 min treatments at 20% and 40%, respectively, and 162.3% and 246.6% for 10 min treatments at 20% and 40%, respectively.

***Antioxidant capacity of pepsin-pancreatin hydrolyzed kafirin samples***

The antioxidant capacity of pepsin-pancreatin hydrolyzed sorghum kafirin was measured by its NO **(Fig. 3.8a)** and DPPH scavenging activity **(Fig. 3.8b)**, and its oxygen radical antioxidant capacity (ORAC) **(Fig. 3.8c)**. Ultrasonication did not improve the NO scavenging activity after hydrolysis **(Fig. 3.8a)**. **Fig. 3.8b** shows that ultrasonication improved the DPPH scavenging activity for all treatments with a decrease in DPPH production from 134.2% (non-ultrasonicated) to 57.1 and 67.2% for 5 min at 20% and 40% amplitudes, respectively. For 10 min, production of DPPH at 20% amplitude was decreased to 57.1% and for 40% was decreased to 64.2% of 109.0%. **Fig. 3.8c** shows that ultrasonication improved the ORAC value for kafirin for all

treatments from 198.2 µM TE/mg (non-ultrasonicated) to 246.9, 241.8, 246.0 and 241.9 µM TE/mg for 5 min at 20% amplitude, 10 min at 20% amplitude, 5 min at 40% amplitude and for 10 min at 40% amplitude, respectively.

## Discussion

Sorghum is one of the most drought resistant cereal grains known to exist and plays a critical role as a dietary staple in developing countries and major economic export of several developed countries including the United States (Ness, 2004; Rao, Birthal, Reddy, Rai, & Ramesh, 2006; Staff, 2012). Previous studies have shown the effects of ultrasonication as a processing tool for the elucidation of food components and improved digestibility of proteins, and a foundation of extensive research has already been conducted on the composition, structure, and bioactive characteristics of sorghum and sorghum kafirin (Bean, Ioerger, Smith, & Blackwell, 2011; Chandrapala, Oliver, Kentish, & Ashokkumar, 2012; Hu, et al., 2013; Jiang, et al., 2014; Lin, Lee, Yoshimura, Yagi, & Goto, 2014; Majid, Nayik, & Nanda, 2015). However, to our knowledge, no one has yet to study the effects of ultrasonication on sorghum kafirin to potentially increase its digestibility againsts simulated gastrointestinal PPH. Kafirin has been shown to have a dense concentration of bioactive components locked and inaccessible within insoluble protein matrices formed from a myriad of endogenous and exogenous factors (Awika & Rooney, 2004) such as disulfide crosslinking of periphery peptides that contain rich cysteine content (de MoraisCardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2015) and high contents of polyphenolic compounds such as tannins (Staff, 2012). Our study first

focused on the extraction and purification of sorghum kafirin proteins, in which a previously described method of kafirin precipitation was modified and improved upon (Landry, 1970). Kafirin was extracted from a gluten-like sorghum flour product with 60% iso-2-propanol after exposure to ultrasonication treatment at either 0%, 20%, or 40% amplitude at either 5 min or 10 min. Results showed that ultrasonication had a major impact on physicochemical and morphological characteristics of kafirin. There was a dramatic increase in yield for unhydrolyzed ultrasonicated kafirin, with improvement ranging from 50.3 g for the lowest ultrasonicated sample (5 min at 20% amplitude) to 182.5 g for the ultrasonicated sample (10 min at 20% amplitude) **(Fig. 3.1)**. Visually, easily definable differences exist between treatments levels as ultrasonication treatment time and intensity increased **(Fig. 3.1)**. Soluble protein content for the unhydrolyzed kafirin samples confirmed these results with ultrasonication proving to significantly increase the amount of soluble protein content for all treatments **(Fig. 3.2).** One potential explanation is the effect of ultrasonication on the secondary structure of kafirin. FTIR and CD were used to measure the changes on the secondary structure of kafirin and our results show an increase in unordered random coiling and β-sheet and a decrease in α-helix content **(Fig. 3.3)**. Previous studies have shown that ultrasonication can lead to reduction on the α-helix structures of proteins. For instance, low frequency sonication led to a marked decreased in α-helicity of chicken actomyosin (Saleem & Ahmad, 2016) and germin-like protein (Huang, Cheng, Hu, & Pan, 2015). Ultrasonication can also lead to increased intermolecular β-sheet as observed in silk-fibroin (Vu, Xue, Vuong, Erbe, Bennet, Palazzo, et al., 2016; Wang, Chen, & Zhang,

2015). These previous studies are in agreement to our observation on the effect of ultrasonication on the secondary structure of kafirin and indicate that ultrasonication is effective at disrupting secondary protein complexes of kafirin that partially contributes to its insoluble nature. Literature has shown that this data is congruent with denaturation and alteration of the secondary structure of proteins (Belton, Delgadillo, Halford, & Shewry, 2006; Xiao, Li, Li, Gonzalez, Xia, & Huang, 2015). In case of PPH samples, a significant decrease in soluble protein content for PPH samples after ultrasonication was observed **(Fig. 3.5)**. It is also known that lower molecular weight peptides as product of PPH are less available in a measurable form by BCA. We confirmed these results with an electrophoretic profile showing no available protein content greater than 10 kD **(Fig. 3.5)** as well as increased lower molecular weight content in ultrasonicated PPH samples **(Fig. 3.6)**. We then looked at the degree of hydrolysis for the ultrasonicated PPH kafirin samples. It was important to see how well the kafirin was able to be hydrolyzed after ultrasonication and PPH in order to determine the effect of ultrasonication on digestibility of sorghum gluten-like flour. Our data showed an increase in the soluble protein content for the ultrasonicated samples for both TCA and HCl hydrolysis as well as an increase in the OPA-derivatized products (**Fig. 3.7)**.The production of these low molecular weight peptides may be responsible for the increased antioxidant capacity for all ultrasonicated PPH samples as measured by DPPH scavenging and ORAC assays (**Fig. 3.8)**.

In summary, ultrasonication led to improvement in sorghum glutten-like flour digestibility by altering its secondary structure. In addition, ultrasonication also improved the

antioxidant property of purified kafirin and sorghum gluten-like flour after PPH. It is imperative that this form of processing be considered as not only a potential for increasing the nutritional value of sorghum, but also from a biomedical and neutracutical perspective for treatment and application possibilities.

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## Appendix Figures

**Fig. 3.1** a) Ultrasonication increased the yield of unhydrolyzed kafirin protein by weight as compared to the non-ultrasonicated sample. Of the sonicated samples, highest yield rate was 10 minutes, 20% amplitude at 182.5 grams and lowest was 5 minutes, 20% amplitude at 50.3 grams. b) Representative images of purified kafirin after ultrasonication. Values with different letter are significantly different from each other ($P < 0.05$).

**Fig. 3.1 continued**

**a)**

| Treatment | | Weight (grams) |
|---|---|---|
| Time, minutes | Amplitude, % | Average |
| 0 | 0 | 34.9±4.8e |
| 5 | 20 | 50.3±7.8d |
| 5 | 40 | 96.2±4.5c |
| 10 | 20 | 182.5±13a |
| 10 | 40 | 130.6±9.2b |

**b)**

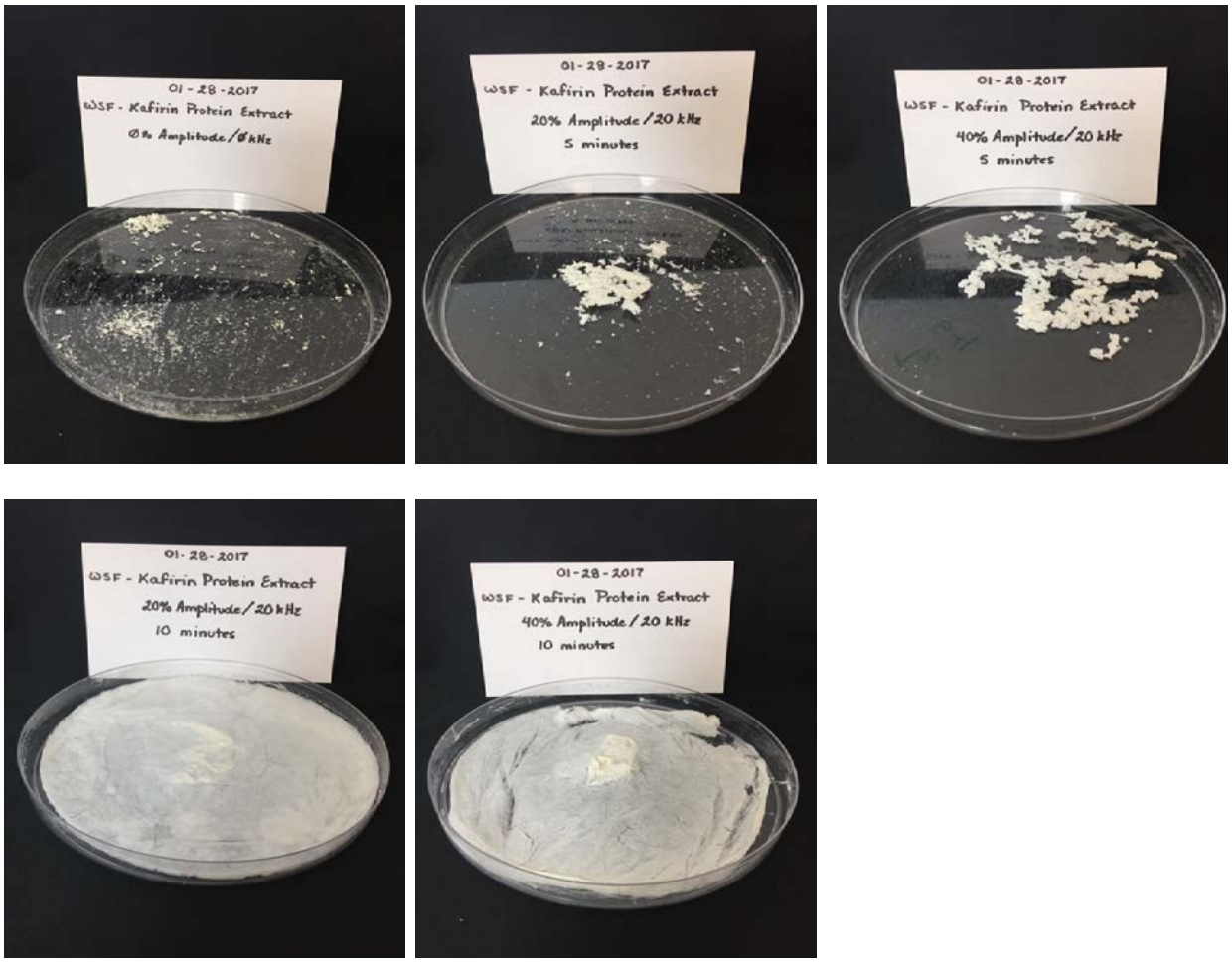
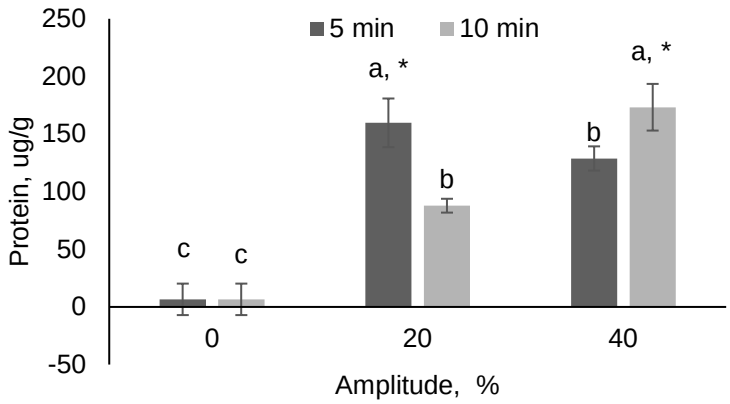


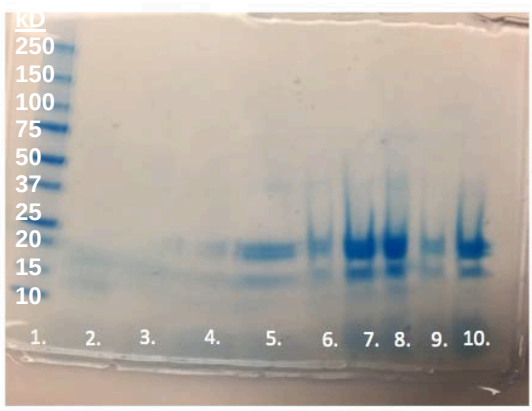
**Fig. 3.2** a) Sonication improved the solubility of extracted kafirin in TBS. Bars with different letters are significantly different from each other within the same ultrasonication time ($P < 0.0001$ for both 5 and 10 minute sonication times). Asterisk next to letter indicates significant difference between two sonication times at the same amplitude ($P < 0.0001$ at 5 minutes and $P = 0.0008$ at 10 minutes). b) Electrophoretic profile of sonicated kafirin extracted with TBS and 60% iso-2-propanol.

**Fig. 3.2 continued**

**a)**

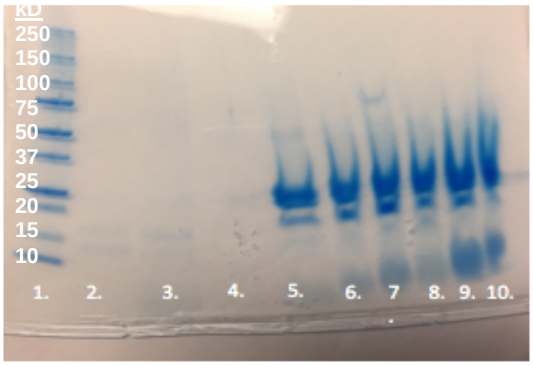



**b)**




5 min. ultrasonication

1. Molecular Weight
2. TBS Supernatant, 0%
3. TBS-Supernatant, 20%
4. TBS-Supernatant, 40%
5. TBS-Pellet in Propanol, 0%
6. TBS-Pellet in Propanol, 20%
7. TBS-Pellet in Propanol, 40%
8. Propanol, 0%
9. Propanol, 20%
10. Propanol, 40%




10 min. ultrasonication

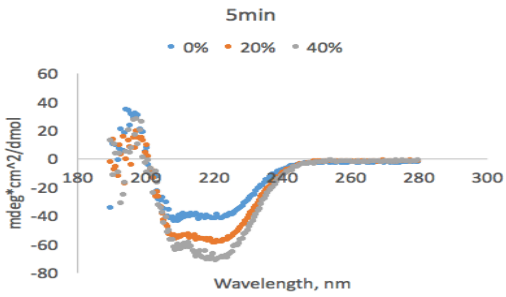
1. Molecular Weight
2. TBS Supernatant, 0%
3. TBS-Supernatant, 20%
4. TBS-Supernatant, 40%
5. TBS-Pellet in Propanol, 0%
6. TBS-Pellet in Propanol, 20%
7. TBS-Pellet in Propanol, 40%
8. Propanol, 0%
9. Propanol, 20%
10. Propanol, 40%

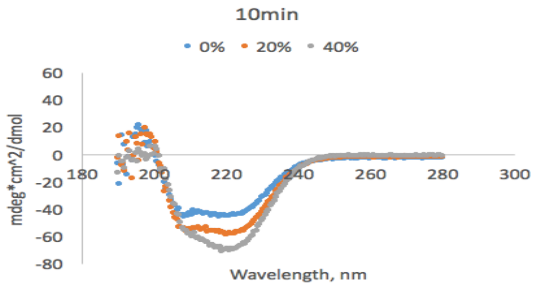
**Fig. 3.3** a) CD for all treatments showed a significant difference in $\alpha$-helix and unordered or random coil content after ultrasonication. Ultrasonication for the 10 min. treatment at 40% amplitude showed a 25% decrease for $\alpha$-helix content, a 23% increase in unordered or random coiling, and led to a deeper CD spectra of sorghum kafirin further confirming alterations to the secondary structure. Means within the same column with different letter(s) are significantly different from each other ($P < 0.05$). b) FTIR for all treatments showed a significant increase in peak height with the 10 min ultrasonication at 40% having a 51.8% increase. Means with different letter(s) are significantly different from each other ($P < 0.05$).

**Fig. 3.3 continued**

**a)**

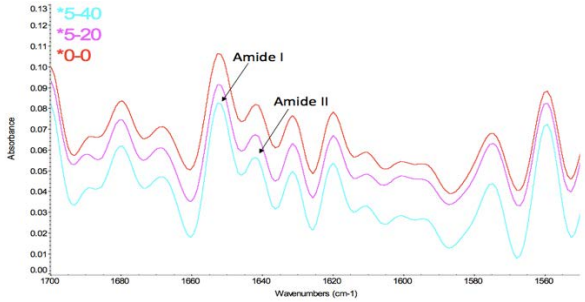
| Treatment | | | | |
|---|---|---|---|---|
| Time | Amplitude, % | α-helix | β-sheet | random |
| 0 min | 0 | 57±1.13a | 8±0.54c | 35±0.52c |
| 5 min | 20 | 41±1.53c | 20±0.39b | 39±1.73b |
| | 40 | 47±0.54b | 23±1.87a | 31±0.53d |
| 10 min | 20 | 43±2.02c | 20±1.65b | 37.±0.61b |
| | 40 | 32±1.11d | 10±1.62c | 58±0.98a |




**b)**

| Treatment | | |
|---|---|---|
| Time | Amplitude, % | Peak Height, % Difference |
| 0 | 0 | 100±7.8c |
| 5 | 20 | 127.8±2.1b |
| 5 | 40 | 142.8±2.6a |
| 10 | 20 | 149.8±6.4a |
| 10 | 40 | 151.8±3.8a |



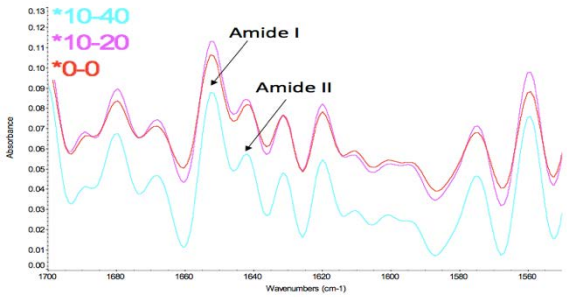
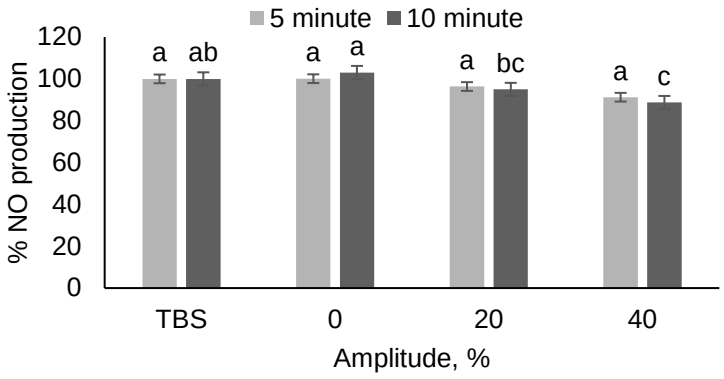



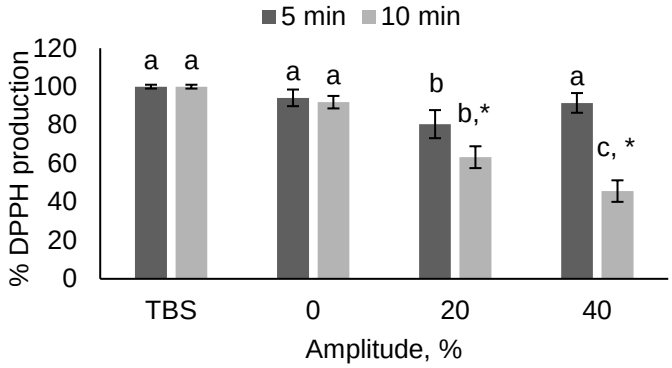
**Fig. 3.4** a) Sonication for 5 min did not improve the capability of sorghum kafirin to scavenge NO free radicals. Sonication for 10 min at 40% amplitude significantly improved NO scavenging activity of sorghum kafirin. Bars with different letter(s) are significantly different from each other within the same ultrasonication time ($P < 0.05$). b) Sonication improved the DPPH scavenging activity of sorghum kafirin. Bars with different letter are significantly different from each other within the same ultrasonication time ($P = 0.0003$ for 5 min and $P < 0.0001$ for 10 min). Asterisk next to letter indicates significant difference between two sonication times within the same amplitude. c) Sonication improved the ORAC value of sorghum kafirin. Bars with different letter are significantly different from each other ($P = 0.0012$ for 5 min and $P < 0.0001$ for 10 min). Asterisk next to letter indicates significant different between two sonication times at the same amplitude ($P = 0.0024$ at 5 min and $P = 0.0004$ at 10 min).

**Fig. 3.4 continued**

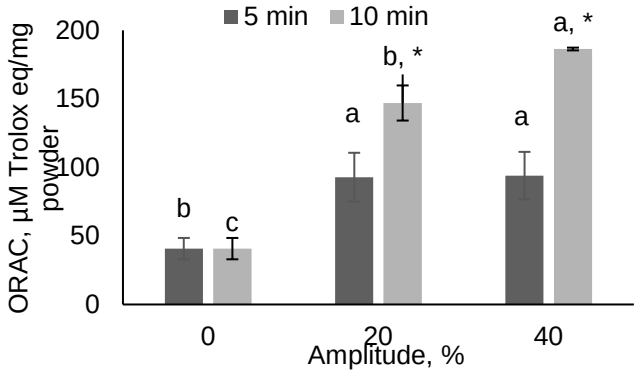
**a)**




**b)**

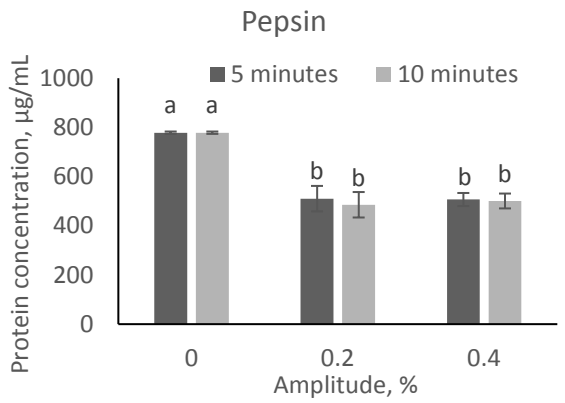



**c)**



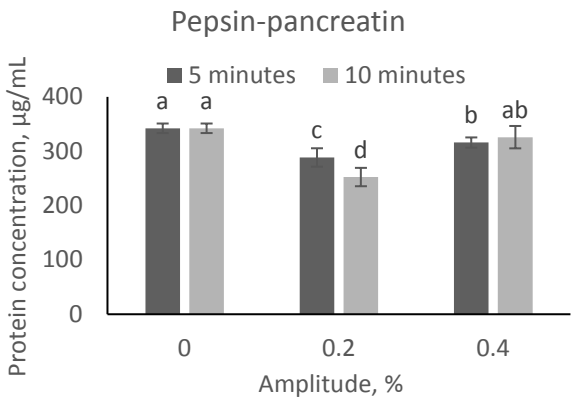
**a)**

Pepsin

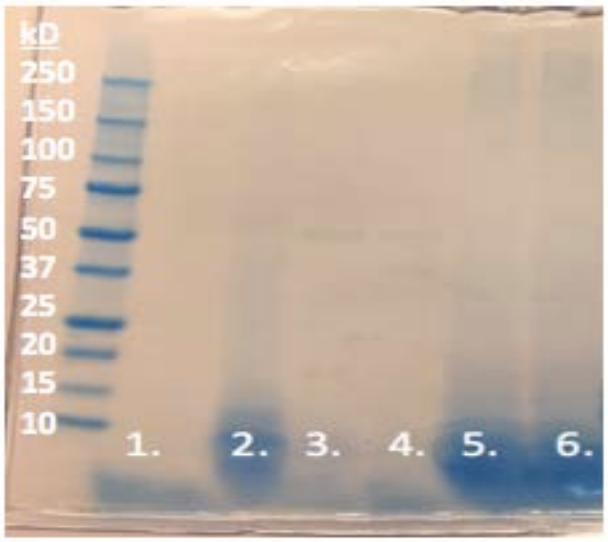



**b)**

Pancreatin




**c)**




1. Pancreatin Blank
2. 0 min, 0%
3. 5 min, 20 %
4. 5 min, 40 %
5. 10 min, 20 %
6. 10 min, 40%

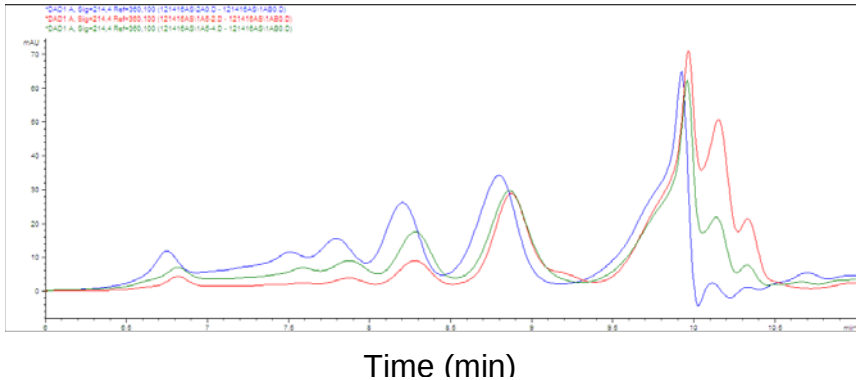
**Fig. 3.5** a) Ultrasonication reduced soluble protein content of pepsin and pepsin-pancreatin hydrolysates of sorghum gluten-like flour. Bars with different letter(s) are significantly different from each other ($P < 0.05$). b) Electrophoretic profile of pepsin-pancreatin hydrolyzed products confirming effects of hydrolysis with no significant content above 10 kDa.

**Fig. 3.6** a) Ultrasonication followed by pepsin-pancreatin hydrolysis increased the lower molecular weight content of the product as shown by a shift to the right for treatment peaks as well as an increase in total area below peaks on the far right side of the image. b) Molecular weight distribution graph quantifying shift in molecular weight profile of sorghum gluten-like pepsin-pancretain hydrolysates after ultrasonication.

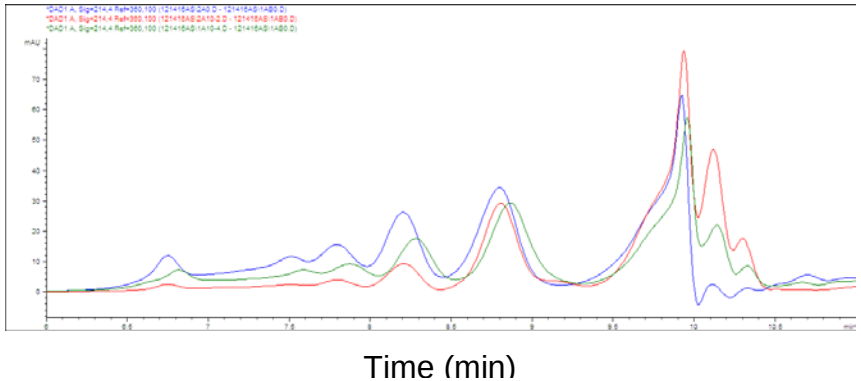
**Fig. 3.6 continued**

**a)**

5 minute




10 minute




**b)**

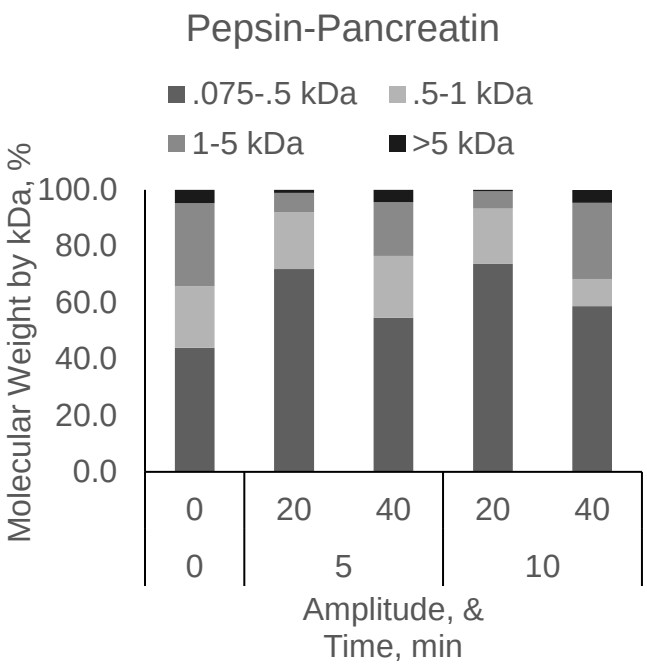
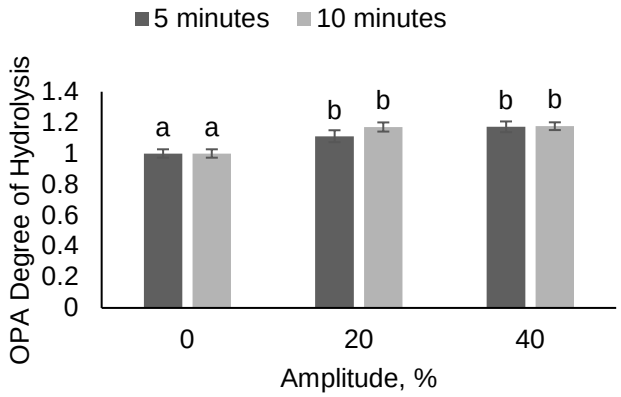


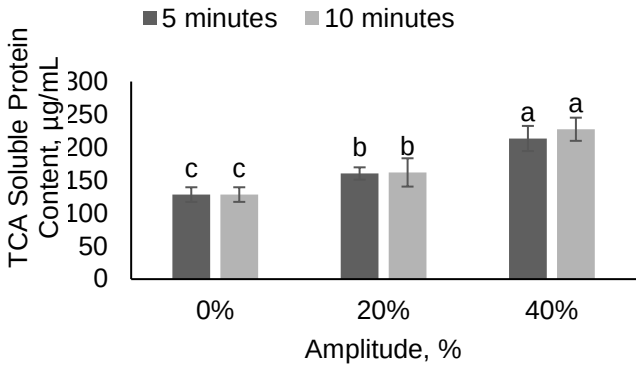
**Fig. 3.7** a) Ultrasonication prior to pepsin-pancreatin hydrolysis increased the degree of hydrolysis of sorghum gluten-like flour pepsin-pancreatin hydrolysates. Degree of hydrolysis was increased by 17.7% for the 10 min treatment at 40% amplitude (a) 77.3% (b) 246.6% (c) as measured by *o*-phthaldialdehyde derivatization, trichloroacetic acid precipitation and hydrochloric acid hydrolysis, respectively. Bars with different letter(s) are significantly different from each other within the same ultrasonication time ($P < 0.05$).

**Fig. 3.7 continued**

**a)**




**b)**




**c)**

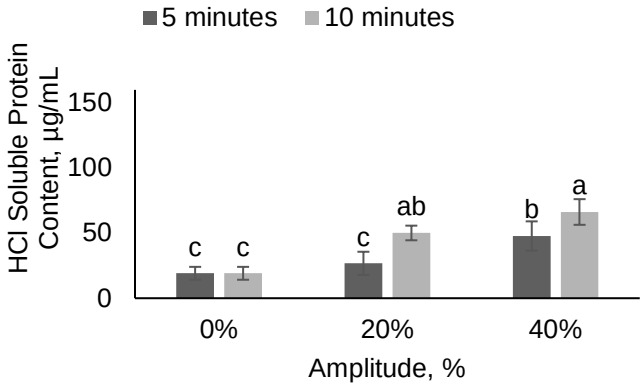
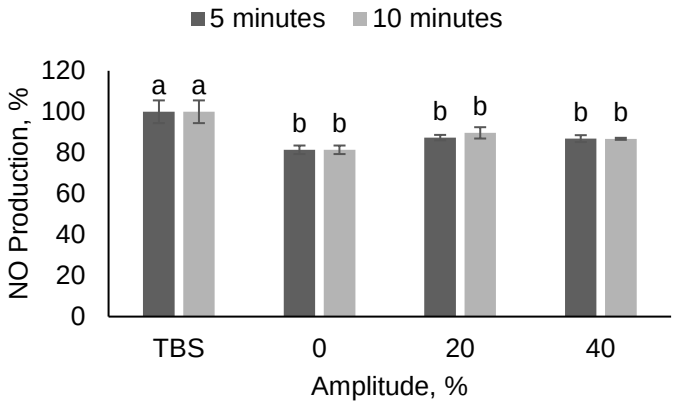


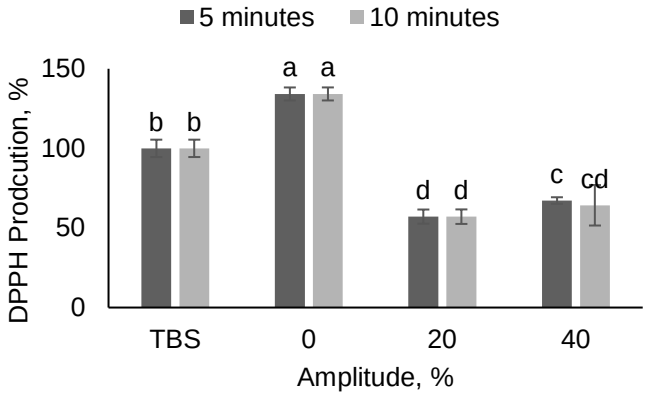
**Fig. 3.8** Antioxidant capacity of pepsin-pancreatin hydrolysates (PPH) of sorghum gluten-like four as affected by ultrasonication. Ultrasonication did not affect the NO scavenging activity of PPH (a). Ultrasonication increased DPPH scavenging activity of PPH (b). Ultrasonication increased the antioxidant capacity for oxygen radicals of PPH. Bars with different letter(s) are significantly different from each other within the same ultrasonication time ($P < 0.05$).

**Fig. 3.8 continued**

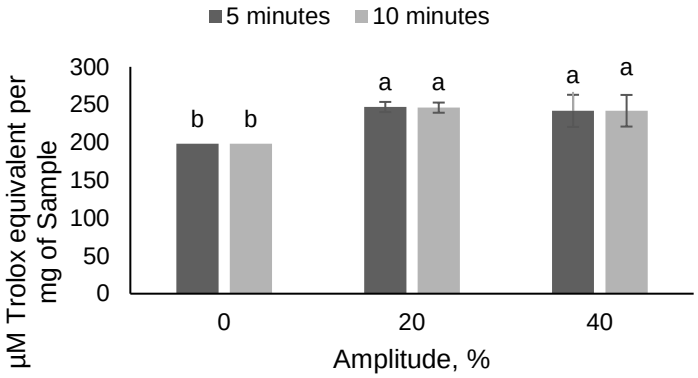
**a)**




**b)**




**c)**



# CHAPTER IV

# SORGHUM KAFIRIN INHIBITED LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN THP-1 HUMAN MACROPHAGES

## Abstract

Aberrant inflammation as a result of activation of the cell membrane periphery protein Toll-like receptor 4 and subsequent phosphorylation of signaling proteins facilitated by reactive oxygen species has been linked to a myriad of autoimmune and progressive diseases. Sorghum contains biologically active components with potential anti-inflammatory properties. The objective of this study was to determine the effects of kafirin on lipopolysaccharide (LPS)-induced inflammation in THP-1 human macrophages. THP-1 human monocytes were differentiated into macrophages by phorbol 12-myristate 13-acetate followed by treatment of LPS with or without 50 μg/mL or 100 μg/mL concentrations of kafirin. The presence of kafirin reduced the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by 28.3%, 74.0%, and 81.4%, respectively, at a concentration of 100 μg/mL. Kafirin reduced the production of intracellular reactive oxygen species leading to reduced phosphorylation of extracellular regulated kinase ½ and nuclear translocation of p65 and c-JUN transcription factors potentially explaining the observed reduction in the secretion of pro-inflammatory cytokines. Our results showed for the first time the anti-inflammatory property of kafirin purified from sorghum in LPS-induced THP-1 human macrophages.

## Introduction

Inflammation, an innate immune response by the body, is induced by stimulation from foreign substances or cellular sterile stressors typically rising from injury or infection. Inflammation is a necessary cellular response to the presence of typically damaging or

dangerous substances within the body. Correctly regulated inflammation is how the body fights disease and illness, heals and removes damaged or malfunctioning cells, and disposes of debris. However, unregulated inflammatory response can lead to a plethora of ailments such as autoimmune diseases, cancer, muscular and skeletal diseases, and cause damage to other body systems like the gastrointestinal tract, the respiratory and circular systems, and the function of reproductive organs (Arthur, Perez-Chanona, Mühlbauer, Tomkovich, Uronis, Fan, et al., 2012; Dubinett & SpringerLink, 2015; Liu, Wang, Bongartz, Hawse, Markovic, Schaid, et al., 2011; Nguyen, Li, & Tewari, 2014). Unfavorable cytokine expression is one of the most common methods in which many of these diseases grow, mature, and spread (Janet Taylor, Taylor, Belton, & Minnaar, 2009). In order to combat this growing health problem, research has begun to diverge from synthetic or manufactured drugs and therapies to a more holistic approach. The use of biomaterial as the basis for medicinal design, nutraceuticals, and supplements is a growing market. The use of bioactives, or food sourced compounds with added benefits beyond the scope of their nutritional properties, are at the heart of this trend.

Sorghum, hardy cereal grain most commonly found in semiarid regions such as northern Africa and western Asia, is considered nutritionally dense and found to contain high concentrations of dietary fiber, proteins, and carbohydrates (de Morais Cardoso, Pinheiro, Martino, Pinheiro, & Ana, 2017). However, its nutritional value is hindered by interfering molecules that form insoluble protein matrices (Awika & Rooney, 2004; Staff, 2012). One of the most effective bioactives found in sorghum's repertoire is the

prolamin protein, kafirin, which exists as the most abundant protein comprising roughly 50-70% of the total protein content (Eicke, Xiao, & Andrea, 2013). A process in which endoreduplication of certain cells exacerbates over-expressed duplicated genes in prolamin proteins. These genes, which typically have high binding affinity, become abundantly available as is the case for kafirin found in sorghum (Belton, Delgadillo, Halford, & Shewry, 2006). Kafirin's unique nature has led to a surge of research aimed at discovering and exploiting it for a variety of applications and aspects such as its use in the production of biofilms and its capacity for encapsulation. These studies are based on the hydrophobicity and indigestible nature of kafirin (Taylor, Anyango, Potgieter, Kallmeyer, Naidoo, Pepper, et al., 2015). However, the potential anti-inflammatory properties of sorghum kafirin, which exists in the form of bioactive, antioxidant rich polyphenolic compounds, is not completely understood.

Surrogate cell lines, such as the human leukemia monocyte THP-1, come in to play as useful tools in *in vitro* studies by simulating specific cellular signaling pathways. THP-1 is one of the most extensively utilized and studied cell lines for *in vitro* studies with prolific research supporting its efficacy as a surrogate (Hjort, Brenyo, Finkelstein, Frampton, LoMonaco, Stewart, et al., 2003; Kramer & Wray, 2002; Sakamoto, Aikawa, Hill, Weiss, Taylor, Libby, et al., 2001; Ueki, Tabeta, Yoshie, & Yamazaki, 2002). THP-1 cells have specific regulatory periphery proteins which respond to the presence of lipopolysaccharides (LPS) acting as molecular signaling units inciting the expression of pro-inflammatory cytokines both intra and extracellularly. For this to happen, LPS binds to the TLR4 receptor bound in the cellular membrane activating a signaling platform and

subsequent cascade effect. Reactive oxygen species (ROS) located inside the cytosolic fraction of the cell are stimulated to begin phosphorylation of highly specific inhibitory molecules, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which cleaves this molecule from NF-κB thus liberating it to transfer freely between the nucleus of the cell. NF-κB, which has now been translocated to the nucleus of the cell acts as a transcriptional factor for DNA and begins encoding for pro-inflammatory genes capable of increasing cytokine expression. These genes are then actively transported out of the cell where they trigger the inflammatory response. These pro-inflammatory cytokines act as constituents of a positive feedback loop in which LPS signaling is further enhanced. This cascade effect leads to the prolific quantities of inflammatory molecules associated with inflammatory based diseases. Due to the high specificity of this pathways activation, and several terminal or critical steps exist within it, it is plausible to regulate inflammatory expression through this pathways inhibition. One such way of doing this would be to inhibit production of intracellular ROS. In the absence of ROS, the inhibitory kinase molecules attached to NF-κB will not be cleaved and therefore it cannot enter the nucleus of the cell and induce transcription. The nature of ROS as an oxidizing agent dictates that antioxidants will be highly effective at inhibiting its activity potential through scavenging. Therefore, the purpose of this study was to determine the ability of kafirin to inhibit LPS-induced inflammation in THP-1 macrophages, and defining the mechanism by which kafirin inhibits inflammation.

## Materials and Methods

### *Materials*

THP-1 human leukemia monocyte cells were obtained from American Type Culture Collection (Manassas, VA), Roswell Park Memorial Institute-1640 (RPMI) growth media was obtained from Corning Inc. (Corning, NY), Fetal Bovine Serum (FBS) was obtained from Life Tech (Carlsbad, CA), Lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies (TLR4, ERK, phospho-ERK, beta-Actin, p65, c-JUN, Nucleolin) were obtained from Proteintech Group (ProteinTech Group, Chicago IL), and secondary antibodies (Goat anti-mouse 926-80010, goat anti-rabbit 926-80011) were obtained from LI-COR (Li-Cor Biosciences, Lincoln NE).

### *Preparation of isolated and purified kafirin samples*

Kafirin was extracted from sorghum by first mixing 400g of sorghum flour with 1.2L of heptane for 2 h at room temperature to defat. The sample was centrifuged at 2,100 x g at 4 °C for 20 min. after which the supernatant was discarded and the precipitate dried overnight. Albumin was extracted in a 10x vol of deionized (DI) water by stirring for 25 min at room temperature. The sample was centrifuged as above and the supernatant discarded. Globulin was extracted in a 10x vol of 0.5M NaCl solution by stirring for 1 h at room temperature. The sample was centrifuged as above, the supernatant discarded and the precipitate lyophilized. Ten gram of lyophilized sample were added to 10 mL of DI water. The samples were separated based on treatment by ultra-sonication at 4°C at

0%, 20%, or 40% amplitude at either 5 min or 10 min. After ultrasonication treatment, samples were centrifuged as above and the supernatant discarded. Kafirin was then isolated from the precipitate in 10x vol of 60% iso-2-propanol solution while being stirred for 4 h at room temperature. Samples were centrifuged as above and the precipitate discarded. The remaining supernatant was then distilled using a Buchi RotoVap at 50°C and 25 mm Hg pressure to remove any remaining isopropanol before being frozen to -40°C and lyophilized. All samples were kept at -40 °C until analysis.

***Cell culture and treatment***

THP-1 human leukemia monocyte cells were cultured using RPMI 1640 with a concentration of 10% FBS, 1% penicillin-streptomycin, 1% sodium pyruvate, 10mM HEPES, and 50 µM ß-mercaptoethanol. Proliferation was allowed to occur, followed by differentiation into macrophage-like cells as previously described (Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014) facilitated by a 162 nM solution of phorbol 12-myristate 13-acetate (PMA). A concentration of 500,000 cells/ml was added to the wells of either a 6 or 12 well plate and differentiation occurred over the following 24 hours at 37°C in a 5% $CO_2$/95% air incubator. After 24 h, differentiated cells were treated with kafirin at the concentrations of 50 µg/ml and 100 µg/ml, in the presence of 1 µg/mL LPS for 20 h with final concentration of propanol at 0.6%. For each trial, a negative control (0.6% propanol) containing only RPMI and a positive control (0.6% propanol) containing only RPMI and LPS were used. The concentration of kafirin did not

affect viability of THP-1 cells as determined by CellTiter 96® $AQ_{ueous}$ non-radioactive cell proliferation assay following manufacturer's protocol (Promega, Madison WI).

***Whole cell lysates preparation and cell culture supernatant collection***

Upon completion of treatment, the media supernatant was collected and the well plates were placed on ice. The cells were subsequently washed with 500 µL of ice cold PBS to remove all cellular debris. Immediately following washing, 100 µL of RIPA lysis buffer (protease inhibitor cocktail to RIPA 1:100) was added to each well and incubated for 5 minutes at 4°C. After incubation, the cells were dislodged using cell scrapers and the cell solution collected. Samples were then vortexed for 5 minutes at 4°C followed by centrifugation at 14,000 x g for 10 min at 4°C. The pellet was discarded and the cell supernatant was aliquoted into pre-chilled 1.5 mL Eppendorf tubes. Samples were analyzed using the Bradford method for protein concentration followed by denaturation with the addition of a 1:1 ratio of 5% ß-mercaptoethanol/Laemmli buffer solution and boiled for 5 min. Samples were stored at -20°C until analysis.

***Separation of cytosolic and nuclear fractions***

Upon completion of treatment, the media supernatant was collected and the well plates were placed on ice. The cells were subsequently washed with 500 µL of ice cold PBS to remove all cellular debris. Another 500 µL of ice cold PBS was added to each well and the cells were dislodged using a cell scraper. The cell suspension was then centrifuged for 5 min at 450 x g at 4°C and the supernatant discarded. For each sample, an aliquot

of 50 µL of lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, and 10mM KCl, pH 7.9 with 0.01 M DTT and 1% protease inhibitor cocktail) was added and the cell precipitate was gently re-suspended via pipette to avoid foam formation. The cell solutions were then incubated on ice for 15 min to allow the cells to swell. After incubation, a 10% solution of Triton-X100 was added to each sample and vigorously vortexed for 10 seconds. The samples were immediately centrifuged at 14,000 g for 30 sec. The supernatant containing the cytosolic fraction was discarded and the pellet placed on ice. For each sample, an aliquot of 30 µL of nuclear extraction buffer (20 mM HEPES, 1.5 mM MgCl2, 0.42 M NaCl, 0.2 mM EDTA, and 25% glycerol, pH 7.9 with 0.01 M DTT and 1% of protease inhibitor cocktail was added. The samples were then vortexed at 3,000 rpm and 4°C for 30 min. Samples were then centrifuged for 5 min at 21,000 x g and the supernatant (nuclear fraction) was transferred to pre-chilled Eppendorf tubes and the pellet discarded. Samples were analyzed using the Bradford method for protein concentration followed by denaturation with the addition of a 1:1 ratio of 5% ß-mercaptoethanol/Laemmli buffer solution and boiled for 5 min. Samples were stored at -20°C until analysis.

***Immunofluorescent staining of p-65 and c-JUN antibodies***

Upon completion of treatment, the media supernatant was discarded and the cells washed with 500 µL of PBS twice. To each well, 500 µL of 4% paraformaldehyde and allowed to incubate 15 min at room temperature. The wells were washed for 5 min 3 times each (all subsequent washes completed the same way) with 500 µL of PBS

before the addition of 500 μL of 0.01% Triton X100 and incubated for 10 min at room temperature. The wells were washed and 5% BSA solution was added to each well at a volume of 500 μL and incubated for 1 h at room temperature. The wells were washed and 200 μL of diluted primary antibody (p65: 1:200; c-JUN: 1:200) and incubated for 2 h at room temperature. The wells were washed and 200 μL of diluted secondary antibody (Alexa-Fluor 488-conjugated anti-rabbit: 1:500) and incubated for 1 h at room temperature in the dark. The wells were washed and 2 drops of 4',6-diamidino-2-phenylindole (DPPH) nuclear dye mounting medium was added to each well. The cells were cured overnight in the dark and cell imaging captured by fluorescence microscopy using an EVOS fluorescence microscope (Thermo Fisher Scientific, Waltham MA).

***Western blot***

SDS-PAGE was run under reducing conditions with a load volume of approximately 10 μg of protein in 4–20% Mini-Protean TGX gels (Bio-Rad Laboratories, Hercules, CA). Each gel was run at 200 V for 35 min. Once protein separation was complete, each gel was equilibrated using a blotting buffer containing a solution of 20% methanol in SDS-PAGE running buffer for 15 min. Transfer of the proteins onto Amersham™Hybond™ 0.45 μm PVDF membrane (GE Healthcare, Piscataway NJ) was done at 110 V for 60 min at 4 °C. Membranes were then blocked for non-specific binding with a solution of 5% non-fat dry milk in TBST for 60 min at room temperature. The membranes were then washed in 10 minute intervals 3 times each with TBST. Following this wash, membranes were incubated in primary antibody against TLR4, ERK, phospho-ERK,

beta-Actin, Nucleolin, p65, and c-JUN (Proteintech Group, Chicago IL) overnight at 4°C. After incubation, the membranes were once again washed in 10 minute intervals 3 times each in TBST. Secondary antibodies were then added at a 1:1000 dilution for 2 hours at 4°C (goat anti-rabbit and goat anti-mouse) (Li-Cor Biosciences, Lincoln NE). Chemiluminescense using a C-Digit blot scanner (Li-Cor Biosciences, Lincoln, NE) was accomplished after a final subsequent washing in 10 minute intervals 3 time with TBST using 1:1 mixture of WesternSure Premium stable peroxide and luminol enhancer (Li-Cor Biosciences, Lincoln, NE). All subsequent analysis done on these membranes was conducted after stripping for reprobing using a mild stripping buffer wash twice for 10 minutes. Mild stripping buffer was removed with the following PBS was (twice for 10 min) and TBST wash (twice for 5 min).

***Measurement of released interleukin-1ß, TNF-α and IL-6 and in the cell culture supernatant by ELISA***

*Interleukin-1ß.* Measurement of IL-1ß that was released into the supernatant was performed by an enzyme-linked immunosorbent Assay (ELISA), using a Human IL-1ß ELISA MAX™ Deluxe Set obtained from BioLegend (BioLegend, San Diego CA). Each well of an uncoated 96-well ELISA plate received 100 µL of detection antibody and allowed to incubate at 4°C for a minimum of 14 h. The wells were then washed with 300 µL of washing buffer (0.5% Tween 20 in PBS, PBST) four times before being blocked with 200 µL of assay diluent A to each well. The plates were placed on an orbital shaker at 50 rpm and allowed to incubate for 1 h at room temperature. Plates were once again

washed as previously described before receiving 50 µL of Assay Buffer D and 50 µL of diluted samples (1:50) with a standard in duplicate, placed on an orbital shaker at 50 rpm, and incubated at room temperature for 2 h. Plates were washed again before receiving 100 µL of diluted Detection Antibody to each well. Plates were placed on an orbital shaker at 50 rpm and incubated at room temperature for 1 h. Plates were washed again before receiving 100 µL of diluted avidin-horseradish peroxidase (HRP) solution to each well. Plates were placed on an orbital shaker and incubated at room temperature for 30 min. Plates were then washed 5 times allowing 30 seconds of soaking time in between each wash before receiving 100 µL of Substrate Solution F. Plates were incubated for 20 min in the dark and a final solution of 100 µL of 2 N $H_2SO_4$ was added to each well to stop the reaction. Plates were read at 450 nm immediately.

*TNF-α.* or *Interleukin-6*. Measurement of TNF-α or IL-6 that was released into the supernatant was performed by an enzyme-linked immunosorbent Assay (ELISA), using a Human TNF-α or IL-6 ELISA MAX™ Deluxe Set obtained from BioLegend (BioLegend, San Diego CA). Each well of an uncoated 96-well ELISA plate received 100 µL of detection antibody and allowed to incubate at 4°C for a minimum of 16 h. The wells were then washed with 300 µL of washing buffer (0.5% Tween 20 in PBS, PBST) four times before being blocked with 200 µL of assay diluent A to each well. The plates were placed on an orbital shaker at 50 rpm and allowed to incubate for 1 h at room temperature. Plates were once again washed as previously described before receiving 1000 µL of diluted samples (1:50 in assay diluent A) with a standard in duplicate, placed on an orbital shaker at 50 rpm, and incubated at room temperature for 2 h. Plates were

washed again before receiving 100 µL of diluted Detection Antibody to each well. Plates were placed on an orbital shaker at 50 rpm and incubated at room temperature for 1 h. Plates were washed again before receiving 100 µL of diluted avidin-horseradish peroxidase (HRP) solution to each well. Plates were placed on an orbital shaker and incubated at room temperature for 30 min. Plates were then washed 5 times allowing 30 seconds of soaking time in between each wash before receiving 100 µL of TMB Substrate Solution containing a 1:1 ratio of Substrate Solution A and B. Plates were incubated for 15 min in the dark and a final solution of 100 µL of 2 N $H_2SO_4$ was added to each well to stop the reaction. Plates were read at 570 and 450 nm immediately.

***Measurement of intracellular reactive oxygen species***

*Fluorescence microscopy.* THP-1 cells were treated as described previously. After the treatment, the plate was placed on ice and the supernatant was removed before the cells were washed with 500 µL PBS twice. After washing, 1 mL of 10 µM 2'7'-dichlorofluoroscein diacetate was added to each well and incubated at 37°C for 10 min in a 5% $CO_2$ incubator. After incubation, the dye was removed and the cells were washed with PBS twice before pictures were taken using a 20x magnification Green Fluorescent Protein (GFP) channel on an Evos microscope (Thermo Fisher Scientific, Waltham MA).

*Fluorescence spectrophotometry.* THP-1 cells were treated and harvested as described previously, and after the wash step, 200µl of trypsin-EDTA (ethylenediaminetetraacetic

acid) was added to each well and incubated for 5 minutes to detach cells from the plate. After incubation, 800µl of complete RPMI was added to inactivate trypsin, and the media containing the dislodged cells was collected and centrifuged at 500 x g for 5 min at 4 °C. The supernatant was then discarded and the pellet was re-suspended in 500 µL of PBS containing 10 µM 2'7'-dichlorofluoroscein diacetate and incubated for 30 minutes at 37°C in a 5% $CO_2$ incubator before being vortexed and 200 µL of the suspension was plated in duplicate on a 96-well plate and read with a fluorescence intensity at 485 nm/528 nm.

*Flow cytometry.* THP-1 cells are treated the same as described in fluorescence spectrophotometry, except the number of cells with green fluorescence and intensity are measured using an FL 1 channel (FITC) on a MACSQuant flow cytometer.

## Results

### Kafirin reduced the secretion of pro-inflammatory cytokines in cell culture supernatant

**Fig. 4.1** (all figures are listed in the appendix of this chapter) shows the effect of kafirin treatment on the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. THP-1 cells not treated with LPS and kafirin presented low secretion of these pro-inflammatory cytokines while treatment of LPS led to a significant increase in the amount of IL-1β, IL-6 and TNF-α secreted by THP-1 human macrophages. At 100 µg/mL kafirin treatment, IL-1β, IL-6 and TNF-α secretion was significantly reduced by 28.3% (**Fig. 4.1a)**, 74.0% (**Fig. 4.1b)** and 81.4% (**Fig. 4.1c)**, respectively.

**Kafirin inhibited translocation of p65 and c-JUN transcription factors**

THP-1 cells were analyzed by immunofluorescence microscopy to determine the effect of kafirin treatment on LPS-induced inflammation. As shown in **Fig. 4.2 and 4.3,** resting (untreated) THP-1 cells showed cytoplasmic expression of p-65 (**Fig. 4.2a)** and c-JUN (**Fig. 4.3a)**. Treatment of LPS led to increased nuclear staining of the cells for p65 and c-JUN. Treatment of LPS together with kafirin exhibited reduction in nuclear staining with more prominent effect at 100 µg/mL kafirin treatment. To validate this observation, THP-1 cells nuclei were harvested and analyzed for p65 and c-JUN expression using western blotting technique using nucleolin as internal control. As shown in **Fig. 4.2b,** low expression of p65 can be observed in the nucleus of resting THP-1 cells. Upon treatment of LPS, the expression of p65 was increased which was dose dependently reduced by kafirin treatment by 34.9% and 46.6% at 50 and 100 µg/mL kafirin treatment, respectively. The same validation was observed in c-JUN, reaching a statistically significant inhibition of 12.6% at 100 µg/mL kafirin treatment as compared to THP-1 macrophages treated with LPS alone.

**Kafirin reduced the phosphorylation of ERK ½ without affecting the expression of TLR-4**

LPS as a pro-inflammatory ligand binds to TLR-4 receptor leading to signaling cascades that ultimately lead to secretion of pro-inflammatory cytokines. As shown in **Fig. 4.4,** kafirin did not affect the expression of TLR-4 receptor in THP-1 human macrophages. To further elucidate the observed anti-inflammatory effect of kafirin, the expression of

phosphorylated ERK ½ (p-ERK) was measured. LPS treatment led to increased phosphorylation of ERK ½ and this was dose dependently decreased by kafirin treatment reaching 40.7% reduction 100 µg/mL treatment equivalent without significantly affecting the expression of total ERK and β-actin.

**Kafirin inhibited production of intracellular reactive oxygen species in LPS-induced THP-1 macrophages**

Intracellular reactive oxygen species are compounds that can lead to uncontrolled and aberrant inflammation. The amount of intracellular ROS was measured by fluorescence microscopy (**Fig. 4.5a),** fluorescence spectrophotometry (**Fig. 4.5b)** and flow cytometry (**Fig. 4.5c)** using 2'7'-dichlorofluoroscein diacetate dye that becomes brightly fluorescent green in the presence of ROS. Without LPS treatment, low green staining in THP-1 cells was observed by fluorescence microscopy using the GFP channel. This observation was validated by fluorescence spectroscopy and flow cytometry experiments. Upon LPS stimulation, green fluorescence in THP-1 human macrophages. Fluorescence spectrophotometry data indicate a 19.5 fold increase in the green fluorescence of LPS-treated cells as compared to untreated, resting THP-1 human macrophages (198.2 to 3874.2). LPS-treatment in the presence of 50 and 100 µg/mL kafirin reduced fluorescence intensity by 39.8 and 81.9%, respectively (**Fig. 4.5b)**. The same trend was observed in the measurement of intracellular ROS by flow cytometry using the FITC-channel of the flow cytometer (**Fig. 4.5c),** kafirin treatment at 100 µg/mL reduced intracellular ROS by 46.9%, 33.3% and 75.6% for percentage stained cells,

mean fluorescence intensity and median fluorescence intensity, respectively, as compared to LPS-treated THP-1 human macrophages.

## Discussion

Aberrant inflammation associated with unregulated cytokine expression has been linked to ailments and diseases ranging from autoimmune diseases and body system damage in the gastrointestinal tract and circulatory systems (Arthur, et al., 2012; Dubinett & SpringerLink, 2015; Liu, et al., 2011; Nguyen, Li, & Tewari, 2014). Inflammation has been directly related to cause of death for roughly 15% of cancer patients, and as life expectancy continues to rise and treatments not directed at inflammatory response become more available, a need exists to find a way to halt the activation of inflammatory encoding genes without inhibiting other cellular functions (Mantovani & Pierotti, 2008). Because the inflammatory response is the result of a cascade effect in which ROS facilitates phosphorylation of ERK ½ leading to translocation of pro-inflammatory transcribing NF-κB into the nucleus, blockage of this pathway is a potential strategy to prevent diseases associated with uncontrolled inflammation. Previous study have shown the bioactive properties of sorghum kafirin, supplementation of kafirin in the diet of rats led to improved lipid metabolosim and increased serum antioxidant potential (Cruz, Lopez, Aguilar, Garcia, Gorinstein, Romero, et al., 2015). To our knowledge, the anti-inflammatory property of sorghum kafirin is an unexplored area of study. Our study used a purified kafirin in LPS-induced inflammation in THP-1 human macrophages. LPS acts as the signaling molecule that binds to membrane bound TLR4 leading to the

activation of the inflammatory response cascade. During this activation process, production of intracellular ROS leads to the phosphorylation of inhibitory kinases attached to NF-κB as well as ERK. When NF-κB has been liberated from the inhibitory kinase and ERK has been phosphorylated to p-ERK, these signalings promote translocation of NF-κB into the nucleus. Once inside the nucleus, NF-κB will bind to DNA molecules and begin transcribing inflammatory encoding genes. These pro-inflammatory genes are then able to actively leave the cell where they facilitate the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Exposure of THP-1 cells to kafirin at concentrations of 50 μg/mL and 100 μg/mL resulted in a significant decrease in the secretion of these pro-inflammatory cytokines **(Fig. 4.1)**. Knowing that kafirin was, in fact, capable of suppressing the secretion fo these pro-inflammatory cytokines in THP-1 cells, we explore the potential mechanism of action involved through the observed antioxidant property of sorghum kafirin. Transcriptional factors and their preceding molecular constituents showed a decrease in expression for kafirin treated samples in the preence of LPS. p65, c-JUN, and p-ERK, which all exist as active molecules only after ROS generation, had a significant decrease in expression; for p65 47.6% **(Fig. 4.2c)**, for c-JUN 13.4% **(Fig. 4.3c)**, and for p-ERK 41.3% **(Fig. 4.4c),** all at concentrations of 100 μg kafirin/mL. In order to confirm the role of ROS in inhibiting the activation fo these protein signaling molecules, the effect of kafirin treatment on the production of intracellular ROS was evaluated. The data show that kafirn treatment led to reduction of intracellular ROS production promoted by LPS treatment in THP-1 human macrophages (**Fig. 4.5)**

In summary, our research clearly shows kafirin as an effective regulator of inflammatory expression in which its antioxidant property inhibit the production and activity of intracellular reactive oxygen species. The proposed mechanism of action (**Fig. 4.6)** is through inhibition of production of intracellular ROS. Reduction of intracellular ROS limited the phosphorylation of ERK ½ which led to reduced translocation of p65 NF-κB subunit. This reduced p65 nuclear translocation is responsible for the reduced secretion of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. To our knowledge, kafirin has never been explored in this capacity, and a mechanism of action has never been described for down regulating pro-inflammatory cytokine expression. Kafirin, acting as a naturally-occurring biomoleucle, should therefore be highly considered for future research focus as an anti-inflammatory nutraceutical.

***Acknowledgements***

This work is supported by the USDA National Institute of Food and Agriculture, HATCH 1010230 to VPD. Product names are necessary to report factually on available data; however, USDA neither guarantees nor warrants the standard of product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

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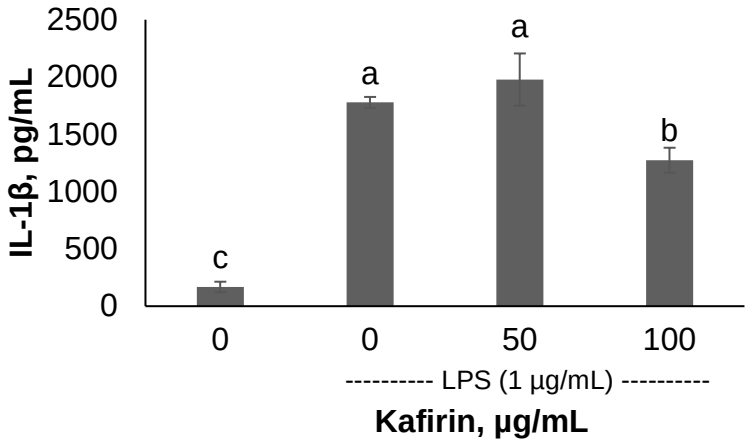
## Appendix Figures

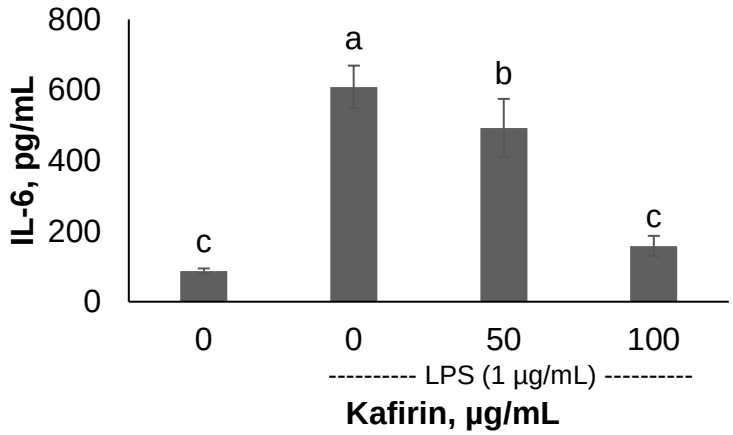
**Fig. 4.1.** Sorghum kafirin reduced the secretion of different pro-inflammatory cytokines in LPS-stimulated THP-1 human macrophages. Differentiated THP-1 cells were stimulated with 1 µg/mL LPS with or without sorghum kafirin for 24 h. At 100 µg kafirin/mL, IL-1β secretion was significantly reduced by 28.3% (a), IL-6 by 74.0% (b) and TNF-α by 81.4% (c). Bars with different letter are significantly different from each other ($P < 0.05$).

**Fig. 4.1 continued**

**a)**




**b)**




**c)**

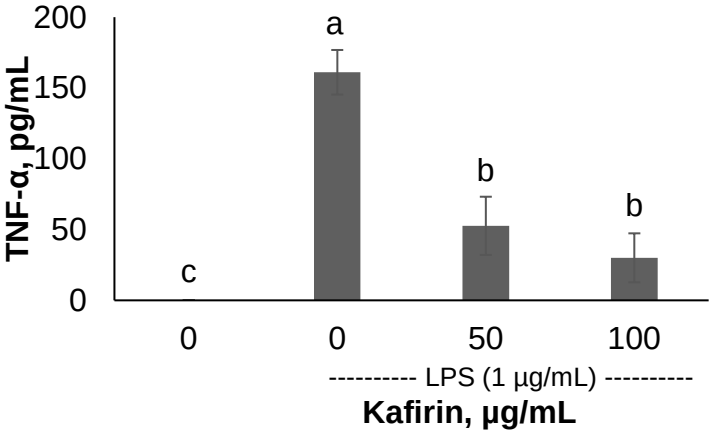
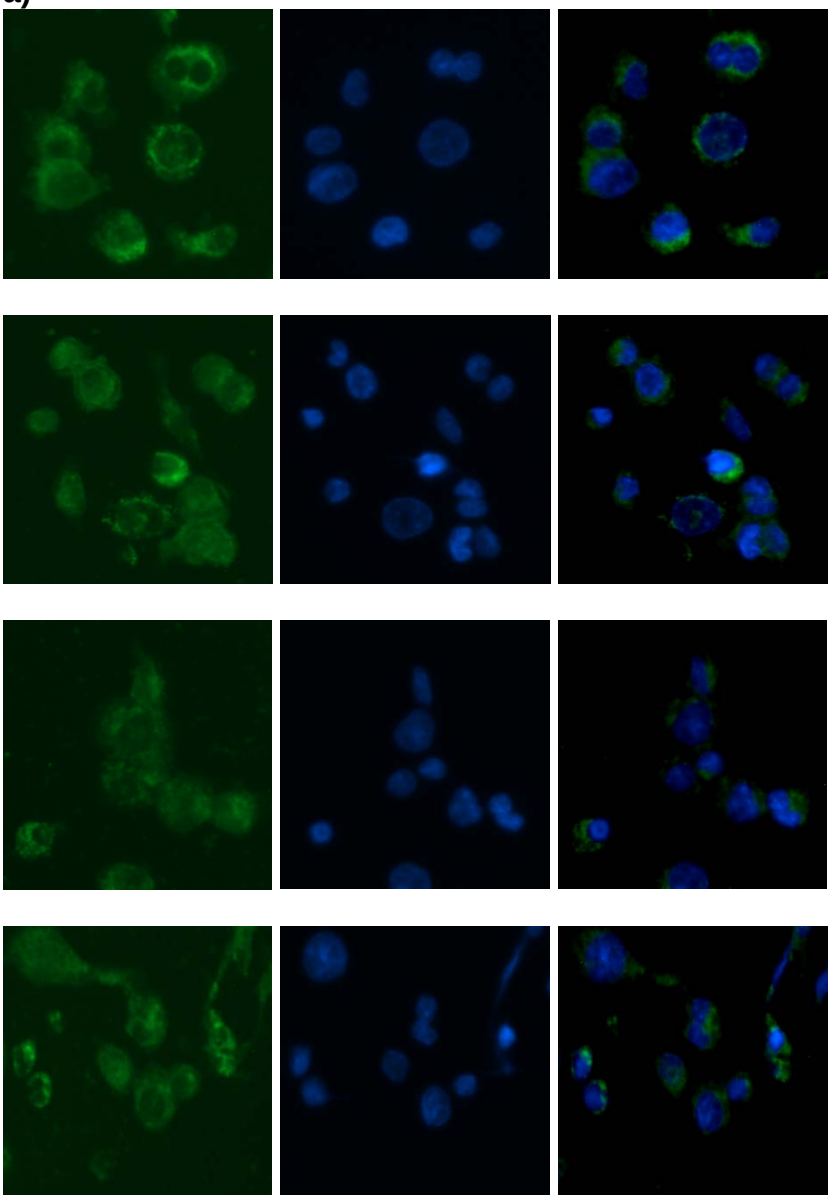


**Fig. 4.2.** Kafirin inhibited the expression of p65 in THP-1 cells as measured by immunofluorescence microscopy (a) and western blotting of nuclear lysates (b). Control panel shows that most THP-1 cells have low nuclear expression of p65 as shown by naked FITC-staining in the nucleus (a). Western blot profile confirming a decrease in presence of p65 after kafirin treatment (a). Bars with different letter are significantly different from each other ($P < 0.05$).

**Fig. 4.2 continued**

**a)**



Control

LPS-treated THP-1 cells

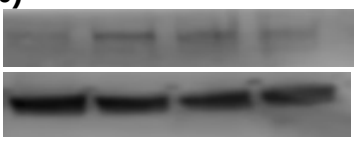
LPS + 50 µg/mL

LPS + 100 µg/mL kafirin

Alexa Flour-488 | DAPI | Merge

**Fig. 4.2 continued**

**b)**



**p65 (65 kDa)**

**nucleolin (110 kDa)**

0 0 50 100 µg/mL kafirin

---- LPS (1 µg/mL) ----

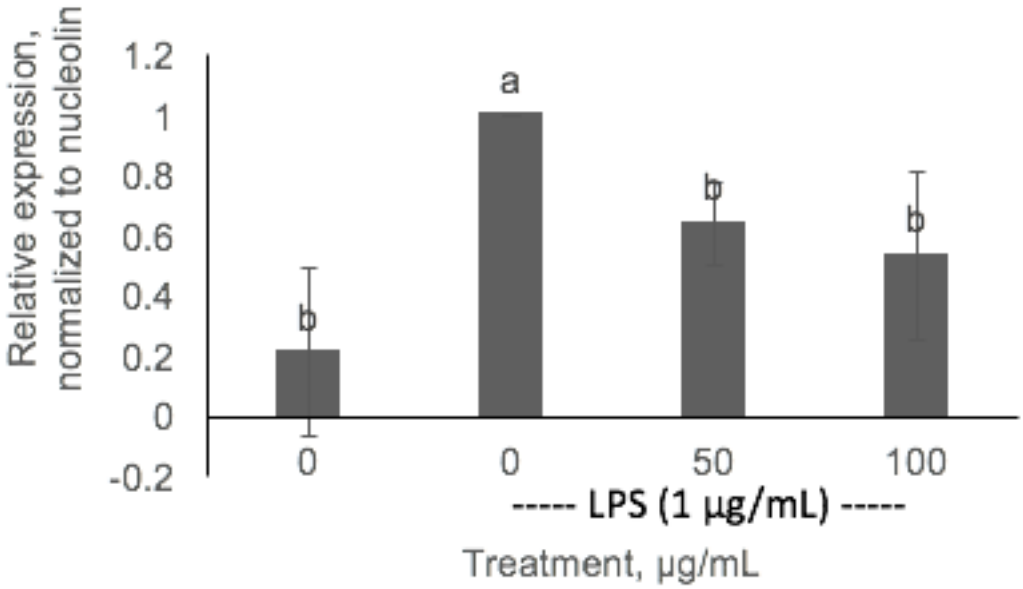
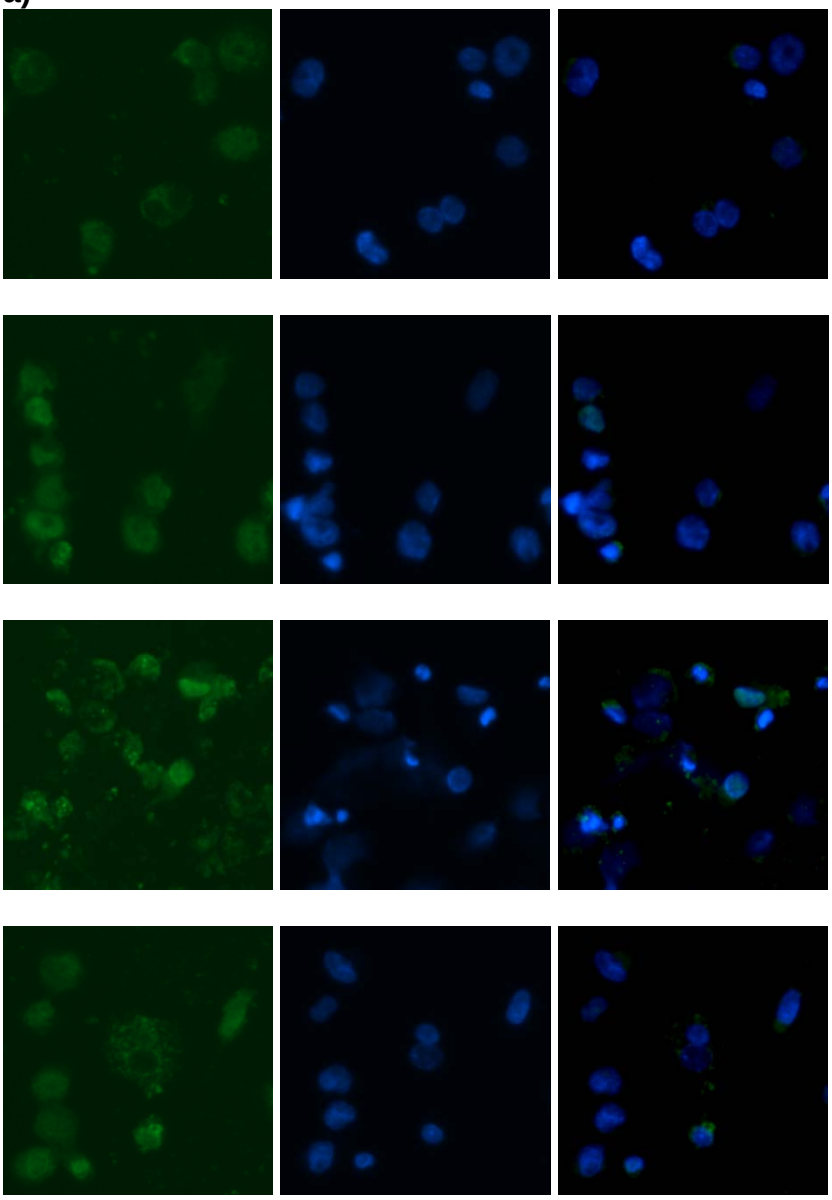


**Fig. 4.3.** Kafirin inhibited the expression of c-JUN in THP-1 cells as measured by immunofluorescence microscopy (a) and western blotting of nuclear lysates (b). Control panel shows that most THP-1 cells have low nuclear expression of c-JUN as shown by naked FITC-staining in the nucleus (a). Western blot profile confirming a decrease in presence of c-JUN after kafirin treatment (a). Bars with different letter are significantly different from each other ($P < 0.05$).

**Fig. 4.3 continued**

**a)**



Control

LPS-treated THP-1 cells

LPS + 50 µg/mL

LPS + 100 µg/mL kafirin

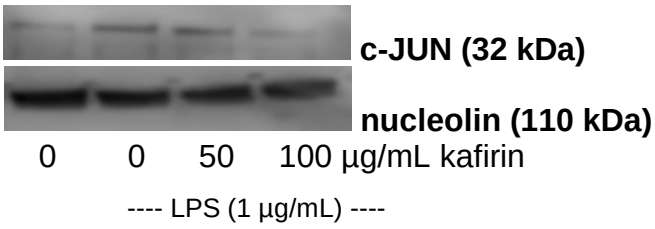
Alexa Flour-488

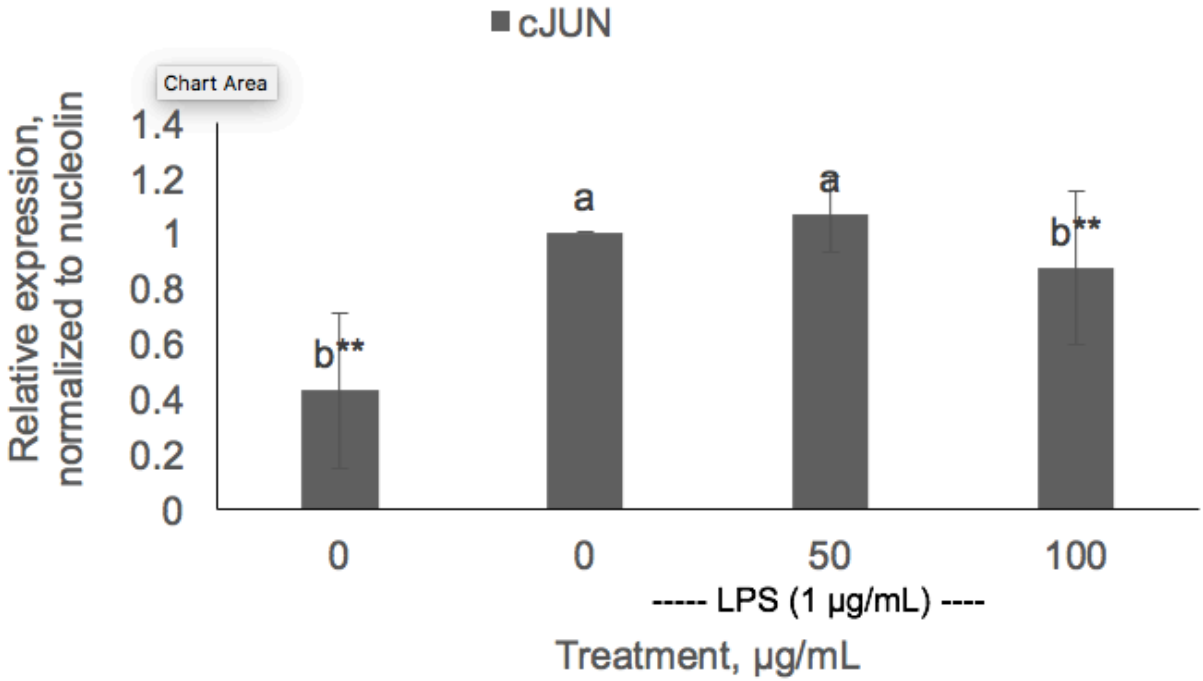
DAPI

Merge

**Fig. 4.3 continued**

**b)**



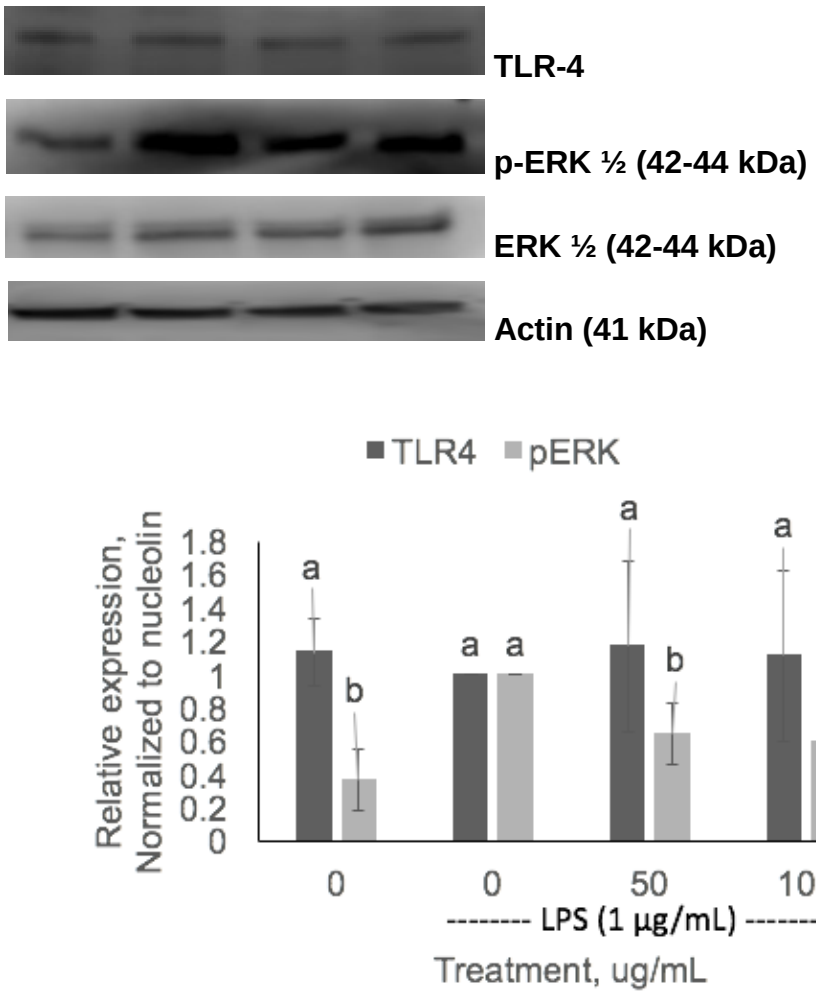
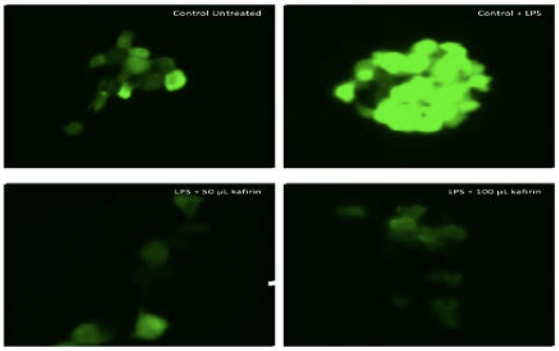



**Fig. 4.4** Kafirin did not reduce the expression of TLR-4 but inhibited the expression of p-ERK ½ in THP-1 human macrophages as measured by western blotting of whole cell lysates. Bars with different letter are significantly different from each other ($P < 0.05$).

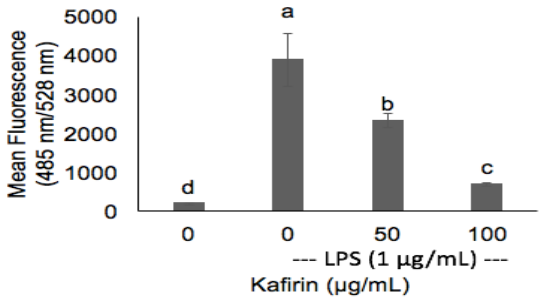
**Fig. 4.5.** Production of intracellular reactive oxygen species (ROS) was inhibited by kafirin. ROS was measured by fluorescence microscopy (a)**,** fluorescence spectrophotometry (b) and flow cytometry (c) Without LPS treatment, low green staining in THP-1 cells was observed by fluorescence microscopy using the GFP channel. Fluorescence spectrophotometry data indicate a 19.5 fold increase in the green fluorescence of LPS-treated cells when compared to resting non-LPS treated THP-1 human macrophages (198.2 to 3874.2). LPS-treatment in the presence of 50 and 100 µg/mL kafirin reduced fluorescence intensity by 39.8 and 81.9%, respectively (b). Kafirin treatment at 100 µg/mL reduced intracellular ROS by 46.9%, 33.3% and 75.6% for percentage stained cells, mean fluorescence intensity and median fluorescence intensity, respectively (c).

**Fig. 4.5 continued**

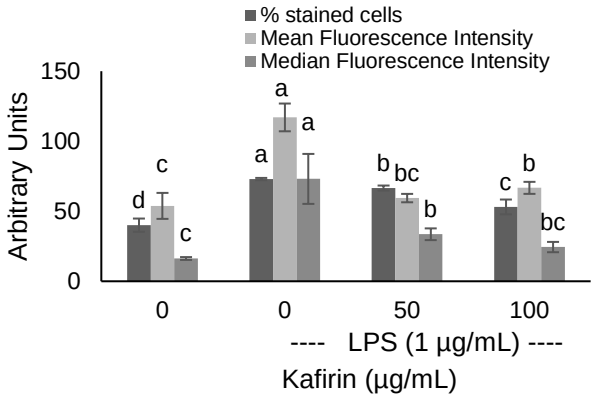
**a)**

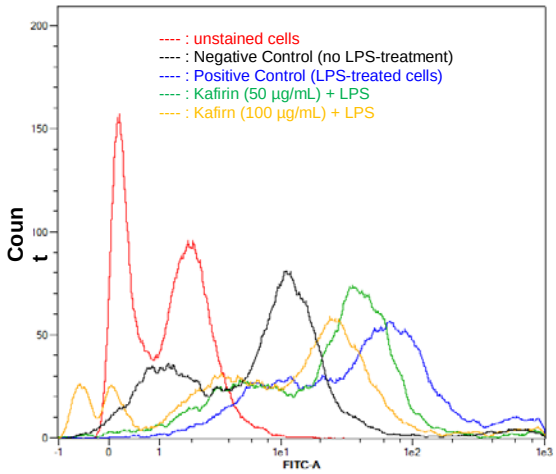



**b)**




**c)**



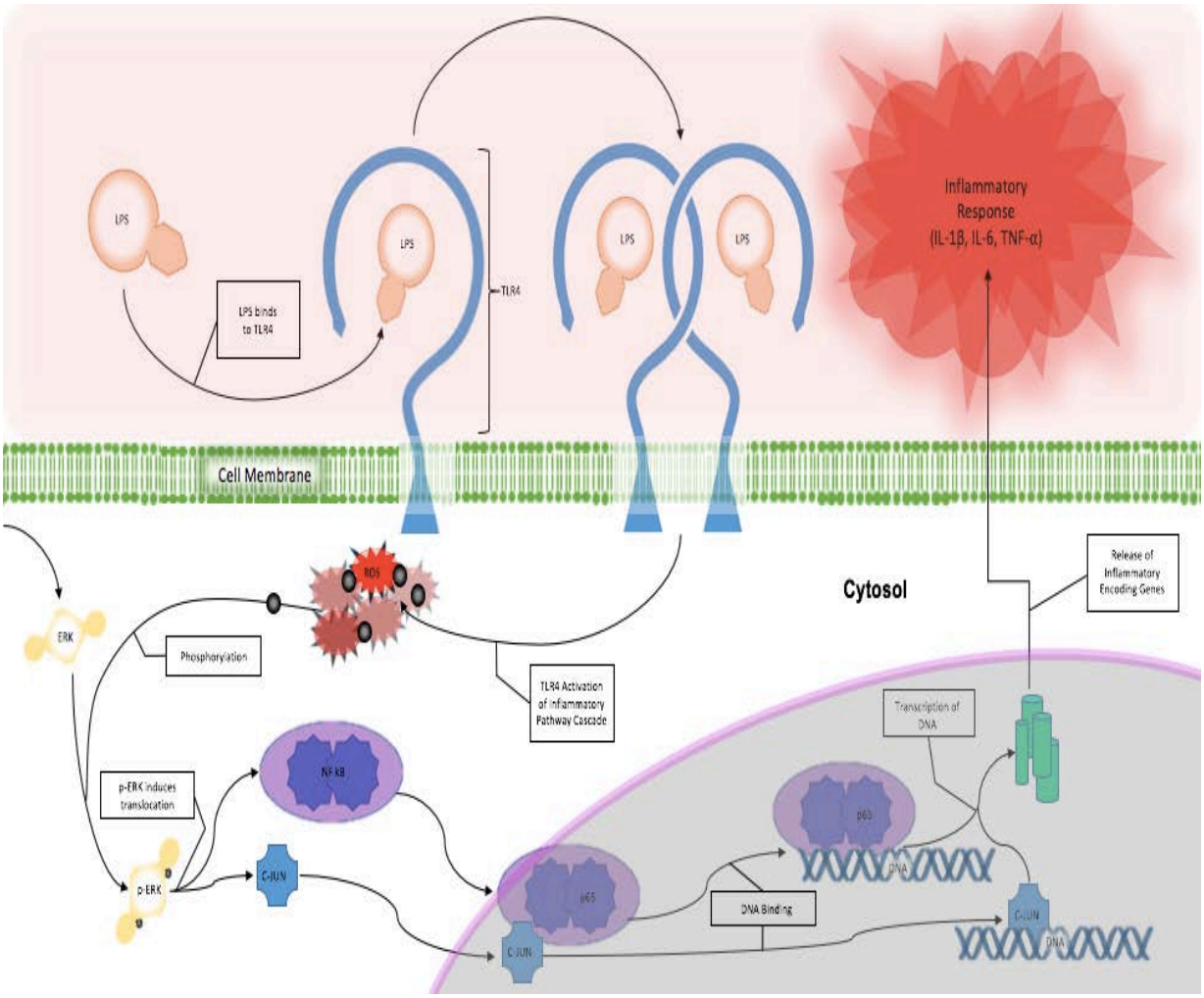



**Fig. 4.6** Mechanism of action for inhibition of production of pro-inflammatory cytokines.

# CHAPTER V

# CONCLUSIONS

Sorghum is the 5th most produced cereal in the world and US is the currently the number 1 producer. The most abundant protein in sorghum is a prolamin-type known as kafirin. Naturally, kafirn is a very hydrophobic protein and contains disulfide cross-linkings responsible for its reduced solubilty and digestibility. The objectives of this research were to determine the effect of ultrasonication on sorghum kafirin and evaluate the anti-inflammtory potential of kafirin using THP-1 human macrophages as an *in vitro* model. The results of this research, for the first time, showed that:

- Ultrasonication alters the secondary structure of purified sorghum kafirin;
- Ultrasonication prior to simulated gastrointestinal pepsin-pancreatin hydrolysis improves the digestibility of sorghum gluten-like flour;
- Sorghum kafirin inhibits inflammation in THP-1- human macrophages by reducing production of in  tracellular-reactive oxygen species.

In summary, the nutritional quality of sorghum can be improved to increase its acceptability as a food ingredient with health promoting property.

# VITA

Andrew Cullen Sullivan attended the University of Tennessee from Fall 2011 to Spring 2015 where he obtained a Bachelor of Science in Food Science. In the Fall of 2016, he began the pursuit of a Master of Science in Food Science from the University of Tennessee.