Optimal extraction method and immunological effects of sorghum polyphenols

by

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B.S., Kansas State University, 2015
B.S., Kansas State University, 2019

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Food, Nutrition, Dietetics and Health
College of Health and Human Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2022

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Abstract

Sorghum is a cereal grain with immense nutraceutical polyphenolic compounds that have demonstrated multiple beneficial qualities such as anti-proliferative, anti-cancer, anti-diabetic, anti-obesity, anti-inflammatory, and apoptotic effects. While there is a large amount of diversity within sorghum varieties in both quantity and composition of polyphenols, it is often found to possess high levels of tannins that may interfere with polyphenol extraction and detection. Here, the extraction process was evaluated, and multiple steps varied to determine the optimal procedure and solvent to be used. A combination of 70% ethanol and 5% citric acid solvent was found to be optimal, as it extracted some of the highest levels of polyphenols while minimizing effects on the bioactivity of the polyphenols on Human colorectal adenocarcinoma HCT15 and human hepatocellular carcinoma HEP2 cells. The extracts were then used to treat RAW 264.7 cells to evaluate their immunological effects. Both the HP (High Polyphenol; accession number, PI570481) and SC84 (high polyphenol brown sorghum) extracts demonstrated significant changes in cytokine production and DNA expression. The HP extract was also found to increase autophagy.

In chapter 1, the nutritional and nutraceutical properties of sorghum polyphenols are explored. The different varieties and their basic structures are discussed, and the potential health qualities such as anti-cancer and anti-inflammation effects are evaluated. In chapter 2, the process of extracting total polyphenols from sorghum bran is evaluated and optimized. The effects of sorghum polyphenols on inflammation and the immune system are evaluated in Chapter 3 using LPS and IFN Y activated RAW 264.7 cells. RAW 264.7 cells showed significant increase in interleukin 6 (IL-6) and interleukin 10 (IL-10) when exposed to

polyphenolic extracts from both sorghum lines. The RAW 264.7 cells also demonstrated structural changes by developing multiple large vacuoles over time. Finally, both sorghum varieties increased LC3 II production in the RAW 264.7 cells, indicating an increase in autophagy. These results indicate that sorghum polyphenols modulate the immune system and inflammatory response. They also provide support that sorghum polyphenols have anti-proliferative effects, and the optimization of food grade total polyphenolic extraction assay will be beneficial for quantification of total polyphenolic content in future research as well as dietary supplement development.

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Acknowledgements

I would like to thank the members of my committee, Dr. Weiqun Wang, Dr. Stephanie Shames and Dr. Dmitriy Smolensky for their guidance and support. I would like to give particular thanks to Dr. Dmitriy Smolensky for his work in overseeing my research and his guidance in helping me through this project. His assistance was integral throughout this process. Finally, I would like to thank all the professors who have imparted their knowledge on me.

Chapter 1 - Understanding the nutritional and nutraceutical properties of sorghum

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This literature review has been accepted to be published as a book chapter.

http://dx.doi.org/10.19103/AS.2021.0087.21

Contribution of Sarah Cox: Writing the entire manuscript, assisting in reviewing and editing the final version

Abstract: Sorghum is a globally grown cereal. Many sorghum varieties contain high levels of polyphenolic compounds with potential health benefits. With a growing interest in using diet as a preventative measure against chronic diseases, the benefits of sorghum need to be examined. This chapter discusses current research on sorghum and its bioactive compounds, particularly the diversity of polyphenolic compounds present in sorghum. The effects of the phenolic compounds against cancer, their anti-inflammatory properties, anti-obesity effects and effects on gut

microbiome are discussed. The chapter also discusses anti-nutritional effects of sorghum polyphenols as well as the effects of processing on bioactive compounds and bioavailability.

Keywords: Sorghum, polyphenols, bioactivity, anti-proliferative, inflammation, obesity

Contents:

1 Introduction

Sorghum bicolor is a drought resistant crop grown as a dietary staple around the world, particularly in Africa and Asia (Rooney, 2018a, 2018b; Taylor & Duodu, 2018). However, it remains relatively little used as a food source in many developed countries. While sorghum is used primarily for cattle feed and biofuel production in the United States, increased interest is now being shown in its use as a food due to its potential health benefits. Sorghum has a wide variety of bioactive compounds. These include polyphenols, proteins, lipids and fiber, all of which have been demonstrated to show potential health benefits such as anti-cancer and anti-inflammatory effects (Girard & Awika, 2018). Sorghum has also shown the potential to affect the microflora of the gastrointestinal tract by selectively increasing or decreasing different types of bacteria (de Morais Cardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2017).

Numerous studies have shown the potential anticancer effects of sorghum, particularly in preventing colorectal cancer(Awika, 2017; de Morais Cardoso et al., 2017; Vanamala, Massey, Pinnamaneni, Reddivari, & Reardon, 2018). Research has also explored the anti-inflammatory effects of sorghum polyphenols. Sorghum polyphenols have also shown benefits in treating obesity and diabetes(Xiong, Zhang, Warner, & Fang, 2019). While there are some anti-nutritional effects associated with sorghum polyphenols, research is being undertaken to minimize those impacts. This chapter discusses these topics in more detail.

2 Variation in the nutritional value of sorghum grain

Sorghum has a wide range of macronutrients, micronutrients and bioactive compounds that show promising health benefits. Sorghum has, for example, high levels of tannins and other polyphenols that have strong antioxidant properties (Civáň, 2019). These bioactive compounds and health benefits are listed in table 2. The composition of these compounds can vary by the variety of sorghum and by the environment in which the sorghum is grown. In one study ten wild genotypes of sorghum were gathered to analyze their micronutrient composition in comparison to domestic varieties. Several genotypes were found to have high protein and iron content and one wild genotype was found to be particularly high in zinc (Abdelhalim, Kamal, & Hassan, 2019). The total phenolic content, protein digestibility, and mineral content of 16 varieties of raw sweet sorghum flour from Sudan have been compared. Almost no significant differences were observed between the samples regarding protein digestibility. Total phenolic content varied among the samples with a maximum of 12.98 mg/g and a minimum of 0.89mg/g of total phenolic acids. Tannins were between 12.97 to 1.58 mg/g among the raw sorghum flour samples (ELnasikh et al., 2020). 35 Ethiopian sorghum varieties, including white, brown and red pigmented grain, were also examined for mineral and chemical content, nutritional and anti-

nutritional effects. Significant variation was found among the samples in minerals including iron, which ranged from 2.262 to 14.08mg/100g, Phosphorous ranged from 367.965 ppm to 112.554mg/100g, and magnesium ranged from 207.53 to 62.06mg/100g. Tannin levels ranged from 1.36 to 3337.2 mg/100g within the samples (Tasie & Gebreyes, 2020).

A wide diversity of nutrients was found in 390 accessions of sorghum with a range of 1.0 to 4.3% fat, 8.1 to 18.8% protein and 61.7 to 71.1% starch. Durra and bi-color durra sorghum from India and Ethiopia had the greatest protein and fat content while kafir-type sorghum from the USA, India, and South Africa had the highest starch but the lowest protein content (Rhodes et al., 2017). In a survey of sorghum varieties from around the world, 381 accessions were evaluated for their polyphenolic content. 55% of evaluated samples contained proanthocyanidins and 13% contained 3-deoxyanthocyanins, with 6% of samples containing both (Rhodes et al., 2014). When 50 different sorghum varieties from Burkina Faso were evaluated for their phenolic content, most varieties had less than 0.25% w/w content of tannins, with red varieties having the highest total phenolic content (Mamoudou H. Dicko et al., 2002).

3 Polyphenols in sorghum

As the previous section suggests, sorghum grains have a wide range of polyphenols, which can differ by both composition and quantity in each sorghum variety. Polyphenolic compounds have strong anti-proliferative and apoptotic effects on cancer cells and reduce pro-inflammatory cytokines. Sorghum polyphenols also reduce fat storage, increase glucose tolerance, and modify levels of bacteria in the gut. Sorghum polyphenols can be used to reduce inflammation and to help treat HIV positive individuals.

Polyphenols present in sorghum include the following groups:

- phenolic compounds

- tannins
- flavonoids

Sorghum possesses both bound and free forms of phenolic compounds. Bound phenols are esterified to cell walls and represent a significant portion of the phenols in sorghum (Luthria & Liu, 2013). Ferulic acid is the most common of the bound phenolic acids present in sorghum and is a derivative of cinnamic acid, whilst other commonly bound polyphenols in sorghum are caffeic, sinapic and p-coumaric acids (Awika & Rooney, 2004).

Tannins are a second type of polyphenolic compounds in sorghum. Tannins fall into two distinct categories: hydrolysable or condensed tannins. In sorghum the majority of tannins present are condensed tannins, which can inhibit nutrient absorption (Butler, Riedl, Lebryk, & Blytt, 1984). Also known as proanthocyanidins, tannins are associated with some anti-nutritional effects such as inhibition of protein digestibility (Elmaki, Babiker, & El Tinay, 1999). A third type of polyphenolic compound in sorghum are flavonoids. Subgroups of flavonoids include:

- anthocyanidins
- flavones
- flavanones
- flavanonols

3-deoxyanthocyanidins are the most common type of anthocyanins in sorghum (Awika, Rooney, & Waniska, 2004). 3-deoxyanthocyanins are associated with darker pigmented sorghum grains, with black sorghum showing the highest levels (Dykes, Seitz, Rooney, & Rooney, 2009). 3-deoxyanthocyanin levels are also significantly higher in sorghum grown under direct sunlight. Luteolin and apigenin are flavones which are commonly found in sorghum (Bradwell, Hurd,

Pangloli, McClure, & Dia, 2018). A common flavanone present in sorghum is naringenin (Dykes et al., 2009). Taxifolin is also a common flavanonol found in sorghum (Dykes & Rooney, 2006).

Black and brown sorghum varieties were found to have the highest total phenolic and proanthocyanidin content. Sorghum varieties with high antioxidant activity are characterized by a wide variety of polyphenols rather than a specific phenol (Rao et al., 2018). Red sorghum was found to have a significantly higher content of polyphenols than white sorghum, though a wide range of polyphenol content was reported for both types (Aruna et al., 2020). White sorghum was found to have three times higher levels of ferulic acid than red sorghum, with p-coumaric and protocatechuic acids two times higher; however, red sorghum had twice as much caffeic and gallic acids present. Flavonoid content was higher in white sorghum, but red sorghum had higher levels of naringenin, vitexin and apigenin (Przybylska-Balcerek, Frankowski, & Stuper-Szablewska, 2019).

Comparing different sorghum grains for their polyphenol and antioxidant content, a strong correlation was found between antioxidant activity and total polyphenol content. White sorghum has the lowest levels of polyphenols while red and brown brewing sorghum grains have the highest levels. Most free phenols are protocatechuic acid and taxifolin, with ferulic acid the most common bound phenol (S. Shen et al., 2018). Raw pigmented sorghum flour is higher in kafirin and tannin than white sorghum and cooked pigmented sorghum has greater resistant starch than white sorghum (Rocchetti et al., 2020). In a sample set of 50 varieties of sorghum grain, most had less than 0.25% tannins with red sorghum having the highest phenolic content of the varieties studied (Mamoudou H. Dicko et al., 2002). When assessing the utilization of white sorghum in the making of tortilla chips by mixing corn flour with increasing amounts of sorghum flour, total phenolic content increases with the increased addition of sorghum flour.

However antioxidant levels had no significant change between the mixtures, potentially due to production conditions (Gutiérrez-Salomón, Aguilar-Raymundo, & Barajas-Ramírez, 2020). The impact of germination on the total polyphenolic content of 50 sorghum varieties was assessed and showed that un-germinated sorghum varieties had the highest phenolic content while levels of proanthocyanidins, 3-deoxyanthocyanidins and flavan-4-ols decreased by germination. (Mamoudou H Dicko, Gruppen, Traoré, van Berkel, & Voragen, 2005).

While sorghum is a particularly rich source of polyphenols, other grains have a variety of polyphenols as well. A summary of sources can be found in table 1. When comparing old and modern Tunisian durum wheat varieties, modern wheat was found to have higher total phenolic content, with a range of 177.48 to 272.62 mg gallic acid/ 100g dry weight. Ferulic acid was the most common phenolic acid found in the wheat samples (Boukid et al., 2019). Ferulic acid is higher in wheat seed coats than the endosperm, with greater quantities being found in wheat bran than flour (Klepacka & Fornal, 2006). 20 varieties of colored barley grown in southern Italy were evaluated for their phenolic content. Total phenolic content of the samples ranged from 1929 $\mu g\ g^{-1}$ to 2917 $\mu g\ g^{-1}$ with insoluble bound phenolic acids making up 88.3% of total phenolic content. There were significant differences in antioxidant activity, among the samples, with a maximum of 13.4 $\mu mol\ g^{-1}$ (Suriano et al., 2018). Evaluation of the polyphenolic content in six varieties of Thai rice, including white, red and purple rice, found that a red rice variety had the highest total phenol content and a purple rice variety had the highest anthocyanin content. Anthocyanins were too low to be measured in white rice, which also had the lowest total phenol content (Ratseewo, Warren, & Siriamornpun, 2019). Commercial oats were found to have a total phenol content up to 25.05 mg per 40g dry weight. The most common polyphenol was ferulic acid (Soycan et al., 2019).

The polyphenols of grains other than sorghum have also been found to have health benefits. When extracted and enriched, avenanthramides, a unique polyphenol found in oats prevented the release of pro-inflammatory cytokine interleukin-8 (IL-8) which affects inflammatory response by reducing recruitment of inflammatory cells (Sur, Nigam, Grote, Liebel, & Southall, 2008). Phenolic extracts from eight varieties of Australian pigmented rice were measured for their total phenol content and antioxidant ability prior to *in vitro* treatment of murine C3H10T1/2 multipotent cells to evaluate their effects in modulating adipogenicity (a factor in obesity). Peroxisome proliferator-activated receptor gamma (PPARγ), which regulates adipocyte differentiation in fat deposition, was also reduced by red, purple and brown rice (Callcott, Santhakumar, Strappe, Luo, & Blanchard, 2018). Mice fed a high dose black barley diet had reduced total cholesterol and low-density lipoprotein cholesterol as well as increased high-density lipoprotein cholesterol in comparison to high fat diet mice. Mice that were fed the black barley extract also had increased antioxidant gene expression(Y. Shen et al., 2016).

4 Nutraceutical properties of sorghum: anti-cancer and anti-inflammatory effects

There has been a considerable amount of research into the effects of sorghum polyphenolic compounds on cancer. Black and brown sorghum as well as pigmented rice were found to have significant anti-proliferative effects on SW480 colon cancer cells; black sorghum increased p53 activity as well as increasing effector caspases 3 and 7, indicating apoptotic effects (Rao, Chinkwo, Santhakumar, Johnson, & Blanchard, 2019). In an *in vitro* study (using tissue culture) conducted on two cancer cell lines, HepG2 and CaCo2, polyphenols were extracted from sorghum from a panel of 15 genotypes. Extracts with a high polyphenol content induced

apoptosis and cell cycle arrest, thus demonstrating anti-proliferative effects on cancer cells (Smolensky et al., 2018).

When breast cancer cell line MCF-7 was treated with anthocyanins extracted from red sorghum bran using a methanol-based solvent, the anthocyanin rich extract induced morphological changes to the MCF-7 cells which inhibited cancer cell viability (Devi, Kumar, & Das, 2011). Hwanggeumchal sorghum has been found to slow tumor growth caused by breast cancer MCF-7 and MDA-MB 231 cells in mice. Additionally, cells treated in vitro with the hwanggeumchal sorghum extract experienced a down regulation of STAT5b and STAT3 signaling pathways, and induced cell cycle arrest. Compared to the results of similar treatments using wheat, millet and panicum, sorghum was shown to be the most effective in suppressing oncogenic proteins (Park et al., 2012). The anti-cancer effects of a sorghum ethyl-acetate extract were evaluated using PC3M prostate cancer cells, showing the extract as having an antiproliferative effect dependent on time and dose of extract, as well as apoptotic effects. After seeding prostate cancer cells into mouse prostates and treating them for 5 weeks with the extract, tumors were significantly smaller in a dose dependent manner. Metastases were also significantly reduced depending on dose (Ryu et al., 2017).

Multiple variations of food-grade extraction solvents and temperatures have been evaluated to improve phenolic extraction from sorghum bran using a novel high polyphenol strain as well as other commercially available varieties. A 70% ethanol and 5% citric acid solvent was shown to be the most effective extraction solvent and had the highest anti-proliferative effect on colon cancer cells. Higher temperatures during the extraction process decreased the anti-proliferative properties of the extracts. When compared to green tea extract, the high polyphenol variety of sorghum was more effective at preventing cancer cell

proliferation, despite lower total polyphenols (Cox et al., 2019). While extruded red sorghum was found to have antiproliferative effects on colon cancer, white sorghum had hyper-proliferative effects on colonic epithelial cells. Both red and white extruded sorghum also reduced body weight in the rats through increased satiety (Llopart et al., 2017).

Sumac sorghum has high levels of proanthocyanins and moderate levels of flavonoids, while black sorghum lacks significant proanthocyanins but possesses high levels of 3-deoxyanthocyanins (Hargrove, Greenspan, Hartle, & Dowd, 2011). The proanthocyanins in sumac sorghum inhibit both amylase and aromatase in non-malignant mouse colonocytes. Amylase breaks down starch and its inhibition has the potential to lower blood sugar, while aromatase is an important enzyme in breast cancer because it converts androgens to estrogens, affecting the growth of cancerous cells. The same study found that black and white sorghums had higher quantities of flavonoids compared to red sorghum (Yang, Allred, Geera, Allred, & Awika, 2012). When the effect of sorghum polyphenols was evaluated against cells expressing alpha and beta estrogen receptors, red sorghum had no significant anti-cancer properties, while white and black sorghums were able to significantly reduce cancer cell count. Strong synergistic effects were found among the flavonoids apigenin and naringenin in activating estrogen receptors (Yang, Allred, Dykes, Allred, & Awika, 2015).

There have been many studies to optimize the process by which sorghum polyphenols are extracted. The use of ultrasound-assisted extraction was (UAE) was found to be more efficient than conventional solvent extraction in extracting polyphenols. Extracts that were obtained using UAE had higher levels of antiproliferative activity against HEPG2 cells compared to those extracted using conventional solvent extraction (Luo et al., 2018).

The impact of phenolic compounds on inflammation is another area of interest. In an *in vitro* study using derived human macrophages, co-treated with LPS, Kafirin protein extract from sorghum was shown to reduce multiple proinflammatory cytokines and reactive oxygen species (Sullivan, Pangloli, & Dia, 2018b). In a seven week-long randomized simple blind clinical study, patients with chronic kidney disease undergoing regular dialysis treatment were fed either a probiotic milk and extruded sorghum mixture, or a pasteurized milk and extruded corn mixture once every day. Patients who consumed the probiotic milk with extruded sorghum mixture showed a reduction in inflammation and oxidative stress as well as lower levels of malondialdehyde (a biomarker of oxidative stress) and increased levels of superoxide dismutase (an important antioxidant) potentially due to the high levels of zinc present in the sorghum as well as the phenolic compounds present (Lopes et al., 2018). In a clinical study, pasta made with red sorghum whole grain flour, control non-sorghum pasta and white whole grain flour sorghum pasta were fed to human participants. Participants who ate the pasta containing red sorghum flour had higher antioxidant activity two hours after consumption than white sorghum and non-sorghum groups (Khan, Yousif, Johnson, & Gamlath, 2015).

Both black and sumac sorghum bran extracts were found to reduce inflammation in mice (Burdette et al., 2010). When oxidative stress was induced in human epithelial cells via hydrogen peroxide treatment, a 2-hour exposure to higher concentrations of black sorghum extract exerted antioxidant and anti-inflammatory effects in epithelial cells (Francis, Rao, Blanchard, & Santhakumar, 2019). To evaluate the impact of the addition of sorghum flour in high fat diets, rats were fed three different types of sorghum flour as part as part of a high fat diet for 35 days. The BRS 310sorghum flour sample, a red tannin-free sorghum, caused a reduction in TNF-a

expression in adipose tissue as well as in thiobarbituric acid reactive substances (TBARS) (Moraes et al., 2012).

Another potential use for sorghum is utilizing the leaves and stalk of the plant, instead of using only the grain for health benefits. This would not only reduce environmental waste but also increase income for producers. Jobelyn™ is a brand of supplement made from sorghum leaf powder. This supplement has been evaluated for potential anti-inflammatory and immune system effects in human polymorphonuclear cells and mononuclear cells. The supplement was found to decrease the formation of reactive oxygen species and decrease cell migration due to inflammatory chemo-attractants as well as increasing natural killer cell activation in vitro in CD3-CD56+ NK cells (Benson et al., 2013).

The anti-inflammatory effects of Jobelyn have also been evaluated *in vivo*. Inflammation was induced in rats via intraplantar injection of carrageenan, a known inflammation activator, which causes swelling. When carrageenan treated rats were fed 50-200mg/kg Jobelyn, there was a reduction of leukocytes and malondialdehyde at the site of inflammation, demonstrating a distinct anti-inflammatory effect (Umukoro, Oluwole Oluwafemi, Eduviere Anthony, Adrian Omogbiya, & Ajayi Abayomi, 2015). A clinical human study conducted with Jobelyn, which evaluated its effects on CD4+ cells counts in HIV positive patients, demonstrated that, when combined with the use of antiretrovirals, Jobelyn could significantly and more rapidly improve CD4 cell counts and increase hemoglobin when compared to treatment with antiretrovirals alone (Ayuba, Jensen, Benson, Okubena, & Okubena, 2013). In separate studies, polyphenols extracted from red sorghum leaves were also found to inhibit the parasite *Toxoplasma gondii* (Abugri, Witola, Jaynes, & Toufic, 2016). Studies of extraction methods showed that acetone, ethanol

and methanol were found to extract similar amounts of polyphenols and flavonoids, while water extracted significantly less polyphenols than the 70% aqueous solvents (Tugli et al., 2019).

5 Nutraceutical properties of sorghum: controlling obesity, antimicrobial effects and impact on gastrointestinal function

Another interesting health benefit which is currently being explored is the effects of sorghum in controlling and reducing obesity. When extruded sorghum was added to a high fat diet and fed to male Wistar rats, markers of fatty liver disease and lipogenesis in the liver were decreased. Quantification of liver gene expression via RT-QCR showed that mRNA expression of PPAR-α, a protein which regulates lipid metabolism via fatty acid oxidation, increased in the liver of the sorghum fed rats. Another mechanism of decreased fatty liver in the rats was reduced expression of sterol regulatory element binding protein 1c, which regulates lipid synthesis and development of fatty tissue (de Sousa et al., 2018). When evaluating raw or extruded sorghum bran added to a high fat diet in male Wistar rats, both raw and extruded sorghum bran addition to the diet reduced fat storage, insulin levels and interleukins 1B, 4 and 6 when compared to a high fat diet without sorghum bran (Salazar-López et al., 2020). Obese rats fed fractions of brown sorghum whole grain flour, bran or decorticated flour, in addition to a high fat diet for 12 weeks, had an increase in glucose tolerance and insulin resistance, as well as a decrease in liver fat (Moraes et al., 2018). The sorghum fed rats had increased glucose tolerance and decreased adipose tissue and adiposity, accompanied by reduced expression of fatty acid synthase (a lipogenic enzyme) and increased expression of lipoprotein lipase (a triglyceride breaking enzyme) in adipose tissues. Feeding sorghum in high fat diets also resulted in a decrease of pro-inflammatory TNF-α and increase of anti-inflammatory interleukin-10 (Arbex et al., 2018).

There have also been a number of human clinical studies on the impact of sorghum phenolic compounds on obesity. In a single blind, randomized crossover trial, overweight men consumed either extruded sorghum or extruded wheat for two eight-week periods. Subjects who consumed sorghum had a greater reduction in waist circumference, BMI and bodyweight when compared to subjects who consumed wheat. It is important to note that the sorghum diet contained higher levels of dietary fiber due to sorghum having higher fiber levels than wheat. (Anunciação et al., 2019).

Antimicrobial effects are also associated with the grain portion of sorghum. When evaluating the anti-microbial properties of polyphenolic extracts of Algerian sorghum samples, total polyphenols ranged from 301.89 to 3214.46 mg GAE/ 100g of sorghum flour. They were found to be moderately to highly effective at inhibiting microbial growth, particularly in gram positive bacteria (Farida, Messaoud, Bernard, & Salah, 2020). The increase in pro-inflammatory cytokines seems to have inhibited the growth of *Legionella pneumophila* (Gilchrist et al., 2020).

In vitro, sorghum was shown to have an anti-aggregation effect in human blood samples. The effect of black sorghum polyphenolic extract on the activation and aggregation of platelets as well as the release of platelet microparticles was investigated. Human blood samples were treated with various concentration of the extract and found that the extract reduced aggregation of platelets as well as the release of platelet microparticles but did not have a significant effect on platelet activation (Nignpense, Chinkwo, Blanchard, & Santhakumar, 2020).

Sorghum polyphenols can also have an impact on the microbiome of the intestinal tract. The impact of sorghum polyphenols on the microbiome of normal weight and obese individuals was investigated using fecal samples Samples treated with the sorghum extracts mixed with fructooligosaccharides (FOS) significantly increased levels of *Bifidobacterium*, *Lactobacillus*,

Prevotella and *Roseburia* , bacteria genera that are commonly found in the intestines (Ashley et al., 2019). In another study sorghum and other cereals including wheat, rice and oats were used to treat infant fecal samples. The addition of cereals such as sorghum increased the growth of beneficial bacteria digesting plant polysaccharides as well as the production of short chain fatty acids (Gamage et al., 2017). Rats fed extruded sorghum showed increased levels of beneficial *Bacterioides* bacteria and decreased *Firmicutes* bacteria, which have been correlated with intestinal dysbiosis (de Sousa et al., 2019). Bacterial richness also increased significantly in rats fed sumac and high tannin black sorghum with a potential impact in reducing colitis (Ritchie et al., 2015).

6 Anti-nutritional effects of sorghum polyphenols

While polyphenols found in sorghum have many benefits, they can also have anti-nutritional effects. Among these negative effects is interference in the absorption of minerals. In rabbits fed low or high tannin sorghum diets, rabbits consuming high tannin sorghum had decreased body weight and inhibited lipase, trypsin, and a-amylase activity along with a decrease in calcium absorption (Al-Mamary, Molham, Abdulwali, & Al-Obeidi, 2001). Comparing tannin, phytate and mineral levels in whole sorghum grain and in processed breakfast cereal, breakfast cereals with lower tannin and phytate content had higher availability of calcium, iron and zinc, suggesting that tannins reduce mineral availability (G. Wu, Ashton, Simic, Fang, & Johnson, 2018). Rats fed red and white sorghum had lower calcium absorbance than control diet rats (Galán, Weisstaub, Zuleta, & Drago, 2020). Comparing the use of corn, wheat and sorghum-based diets for broiler chickens, it was found that sorghum-fed chickens had the lowest growth and energy utilization due to higher levels of phenolic compounds and kafirin. Chickens fed red

sorghum had higher metabolizable energy (the energy left for functional use after excretion loss) and higher starch digestibility than white sorghum (Moss et al., 2020). It has been found that when sorghum was fermented as gruel and had the enzymes wheat phytase and mushroom polyphenol oxidase added, polyphenolic content decreased while iron accessibility increased (Towo, Matuschek, & Svanberg, 2006). A diet mixing sorghum and rice found that sorghum reduced protein digestibility and energy metabolization compared to exclusively rice diets (Teixeira, Pinto, de Mello Kessler, & Trevizan, 2019). Chicks fed a high tannin sorghum diet had significantly reduced calcium, phosphorous, iron, potassium, magnesium and cobalt, as well as reduced weight gain (Hassan, Elzubeir, & El Tinay, 2003).

Polyphenols can also have a negative impact on the digestibility of starch and protein. It has been found that sorghum with high tannins increased resistant starch levels and decreased starch digestibility (Barros, Awika, & Rooney, 2012). When porridges were mixed with sorghum extracts it was found that high phenolic sorghum extracts increased resistant starch (Lemlioglu-Austin, Turner, McDonough, & Rooney, 2012). When replacing up to 50% of the starch weight with purified sorghum tannins, resistant starch levels increased, probably due to tannins binding amylose, while slowly digestible starch (SDS) levels decreased (Mkandawire et al., 2013). White and pigmented sorghum were cooked to measure the impact of polyphenols in pigmented sorghum flour on the digestibility of starch. Cooked white sorghum flour was found to have less resistant starch than cooked pigmented sorghum flour, which was higher in total phenols. The cooked pigmented sorghum flour also had lower rapidly digestible starch than the cooked white sorghum flour (Rocchetti et al., 2020).

When assessing the effect of nixtamalization on the bioaccessibility of protein in red and white sorghum flour during *in vitro* digestion, sorghum that underwent nixtamalization, a process

where grain is soaked and cooked in an alkaline solution prior to being hulled, and cooking processes were found to have higher protein bioaccessibility due to a breakdown in tannin-protein complexes (Cabrera-Ramírez et al., 2020). Pigs fed a sorghum diet had lower nitrogen digestion and excreted higher levels of nitrogen in their feces, indicating poor protein absorption (Pan & An, 2020). Mixed sorghum and quinoa have been assessed for protein digestibility and antioxidant levels. Sorghum and quinoa flours showed no significant change in protein, lipid, fiber or antioxidant composition after heat treatment, and the sorghum quinoa flour mixture had the benefits of increased protein quality from the quinoa flour, and increased antioxidant capacity due to the sorghum flour (Medina Martinez et al., 2020).

7 Food processing effects on sorghum polyphenols

Preparation methods can affect the availability of micronutrients and bioactive compounds in sorghum. Three sorghum genotypes were cooked via either dry heat in a conventional oven or through extrusion. Analysis of phenolic content after the cooking process revealed that extrusion resulted in a less phenolic content compared to dry heat cooking (Cardoso et al., 2015). However, extruded sorghum still had a higher phenolic content than unprocessed sorghum bran as well as greater antioxidant activity (Salazar Lopez et al., 2016). Another study found that extrusion processing increased total phenol content of sorghum bran by up to 14.1% under optimal conditions compared to non-extruded sorghum bran (Ortiz-Cruz et al., 2020). Catechin levels were found to be higher in pigs fed a sorghum extruded mix whilst procyanindins were reduced, due to depolymerization caused by the extrusion process (Gu, House, Rooney, & Prior, 2008). In contrast total polyphenolic content levels were found to decrease significantly via cooking, particularly when microwaved. However, total flavonoid content was only significantly effected in terms of bioavailability by microwave cooking (Hithamani & Srinivasan, 2014).

Cooking and nixtamalization together were shown to increase the bioavailability of total phenols in sorghum, while nixtamalization decreased condensed tannin levels (Luzardo-Ocampo et al., 2020). Total phenolic content was reduced via metabolization by bacteria during fermentation (Svensson, Sekwati-Monang, Lutz, Schieber, & Gänzle, 2010).

Sorghum tea is another way sorghum is consumed. The sorghum tea-making process was found to increase total polyphenol, flavonoid and condensed tannin content without reducing antioxidant activity (Xiong, Zhang, Luo, Johnson, & Fang, 2019). Soaking, steaming and roasting steps each had different effects on the total polyphenolic content of sorghum tea. Steaming increased ferulic and p-coumaric acid levels, while roasting significantly increased total phenolic content, total flavonoid content, and proanthocyanidin content compared to raw sorghum grain (L. Wu, Huang, Qin, & Ren, 2013). In fermented sorghum grain tea, total polyphenols were significantly reduced by soaking, steaming and fermenting. However, roasting the grain caused an increase in total phenolic content (Sun, Wang, Zhang, Ajlouni, & Fang, 2020).

Fermentation is also used to produce sorghum foods. It has been found that fermentation and cooking sorghum increase total polyphenol content and antioxidant activity compared to unfermented sorghum grain (Salih, Ahmed, Ezzdeen, & Hamza, 2020). When comparing fermented and unfermented red sorghum, total phenolic content was increased by fermentation (Svensson et al., 2010). Breakfast cereal made from fermented flour had increased protein content and minerals such as magnesium, calcium and iron, but decreased tannin and phenolic levels (Kapoor, Abraham, & Jain). Malting was found to decrease phenolic content. While flavonoid content decreased during malting, anthocyanin content significantly increased (Khoddami, Mohammadrezaei, & Roberts, 2017). Inhibitory abilities of the polyphenols against

enzymes also decreased as roasting temperature increased (Irondi et al., 2019). When sorghum is mixed as part of a powdered chocolate beverage, it is possible to increase the bioactive compounds of the beverage without damaging the sensory aspects of the product (Queiroz et al., 2018).

8 Sorghum as a healthy gluten free food

Sorghum is being considered as an alternative source of grain for those who are unable to consume gluten such as celiac disease patients. Results indicated that gliadin -like peptides were not present in any of the sorghum samples, suggesting sorghum is safe for consumption for individuals with celiac disease (Pontieri et al., 2013). When the cornstarch to sorghum flour ratio was increased while evaluating the use of white sorghum flour in making bread, the specific volume of the bread increased as well as the crumb grain score. Optimal ingredient ratios were found to be 0.55 cornstarch to sorghum flour, 90% water added and 3% hydroxypropyl methylcellulose, which is used to increase viscosity of dough (Velázquez, Sánchez, Osella, & Santiago, 2012). An evaluation of the most beneficial bacterial cultures for use in producing sourdough bread made with sorghum flour revealed that of 103 lactic acid bacteria (LAB) strains and 20 yeast strains isolated from Nigerian sorghum flour, 3 specific LAB, *Pediococcus pentosaceus* SA8, *Weisella confusa* SD8, *P Pediococcus pentosaceus* LD7, and 1 yeast, *Saccharomyces cerevisiae* YC1 met the requirements necessary to serve as starter cultures. Qualities necessary for yeast selection were high CO2 production a high tolerance for low pH and acetic acid, while LAB qualities included proteolytic activity, acidification and high Exopolysaccharide production (Ogunsakin et al., 2017).

Pasta made with sorghum flour had high protein and dietary fiber content but had slower starch digestion and lower estimated glycemic index when compared to other gluten-free pasta samples. The bioavailability of the polyphenols in the sorghum flour pasta was significantly higher than other types of pasta (Palavecino, Ribotta, León, & Bustos, 2019). When sorghum flour was added to durum wheat semolina pasta, resistant starch and total polyphenolic content increased. However, the cooking process reduced the total phenolic content and antioxidant activity overall, by reducing free phenolic acids while increasing bound phenolic acids (Khan, Yousif, Johnson, & Gamlath, 2013). The development of gluten free cookies and savory pies using high tannin sorghum had significantly higher resistant and slowly digestible starches (Soares et al., 2019).

There have also been efforts to improve the health benefits of gluten free food. The addition of phytase to sorghum flour increased the bioavailability of minerals such as iron, calcium and zinc by reducing *myo*-inositol phosphates, particularly the inositol hexaphosphate (IP6) fraction. The IP6 fraction and IP5 fraction of *myo*-inositol phosphates are known to inhibit mineral absorption (Rebellato, Orlando, Thedoropoulos, Greiner, & Pallone, 2020). Sorghum was prepared using ultrasonication to explore potential impacts on kafirin. The ultrasonication process modified the secondary structure of kafirin, which allowed hydrolysis by pepsin-pancreatin hydrolysis and increased antioxidant activity and digestibility (Sullivan, Pangloli, & Dia, 2018a).

9 Conclusions and future trends

Efforts are being made to breed varieties of sorghum with potential health benefits. In evaluating the genetic diversity of local sorghum grown in Ghana, 41 varieties were sorted into 7 genetically similar clusters. They found that there were three distinct genotypes that stood out

that could be used to improve the breeding programs in Ghana (Danquah, Galyuon, Otwe, & Asante, 2019). Ideal environmental characteristics are also being investigated to improve crop yield and growth. A study of sorghum grown in Australia found that sorghum hybrids with multiple tillers make sorghum more sensitive to the environment but may increase yield stability (Clarke et al., 2019). Several varieties also showed chill tolerance and early maturity, which indicated they could be used to breed sorghum in more temperate climates (Schaffasz, Windpassinger, Friedt, Snowdon, & Wittkop, 2019). Six sorghum varieties were grown under controlled conditions in two growth chambers at with high temperatures of 32C or 38C. Phenolic content and antioxidant content were significantly influenced by genotype while temperature had no significant impact in most cases (G. Wu et al., 2016). 60 sorghum varieties grown in India suggested that genotype had a stronger influence on total phenolic content (Aruna et al., 2020).

Sorghum is an underrated food with a diverse variety of micronutrients and bioactive compounds. With polyphenols showing promise in potential anti-cancer and anti-inflammatory properties, sorghum is becoming an increasingly attractive food source. While it does possess some limitations regarding cooking and preparation methods as well as anti-nutritional properties, methods are being explored to overcome these issues. Efforts are also being made to breed new sorghum types to increase polyphenol content, as well to improve other aspects of the crop. However, while studies suggest that there may be many health benefits to consuming sorghum, more research is necessary to identify and support these findings.

Table1.1 - Benefits of grains other than sorghum

Grain	Polyphenols discussed	Effects	Reference
Tunisian Durum Wheat	Ferulic Acid	Wide variety of polyphenols	[9]
Barley	p-hydroxybenzoic acid, gallic acid, vanillic acid	Significant differences of phytonutrients between outer layers and endosperm	[11]
Rice	Anthocyanins	Wide variety of polyphenols between samples	[13]
Oats	Ferulic acid	Unique phenol – avenanthramide; wide range of phenol quantities	[14]
Quinoa Flour	Polyphenols	Significantly Higher polyphenolic and antioxidant levels than wheat	[15]
Purple corn	Polyphenols	Natural source of pesticide	[16]
Purple Corn	Polyphenols	Natural source of pesticide	[17]
wheat, sorghum, chickpeas and green gram	Total Polyphenols, Tannins	Sorghum polyphenols had lowest bioavailability	[18]
Wheat flour	Hydroxybenzoic acid glucoside, tryptophan, 6-C-glucosyl-8-C-arabinosyl-apigenin and diferulic acids	Cooking increased bound polyphenols	[19]
Wheat	Ferulic acid	Ferulic acid content is high in wheat seed coats	[10]
Pigmented Rice	Total phenolic, Anthocyanidin, Proanthocyanidins	Obesity prevention, lipid accumulation reduction	[22]
Oats	Avenanthramides, polyphenols	Anti-inflammatory	[20]

Oats	Avenanthramides	Inhibit NF-kB activation, decrease endothelial proinflammatory cytokine production	[21]
Black highland Barley	Ferulic acid, p-Coumaric acid	Strong antioxidant ability	[23]
Colored Barley	Total phenolic acids	Significant variations in polyphenol levels among samples	[12]

Table 1.2 - Sorghum Studies

Use	Model	Sorghum Used	Effect	Reference
Anti-Cancer	SW480 Cancer cells	Shawaya short black 1, IS11316, QL33,QL33/QL36, B923296, QT12	Anti-proliferative/Apoptosis	[46]
	HepG2, Caco2 cancer cells	PI574081 extract	Anti-proliferative/ Apoptosis/ Cell cycle arrest	[47]
	MCF-7 Cancer Cells	Red sorghum from Tamil Nadu, India	Anti-Proliferative/	[119]
	MDA-MB 231, MCF-7 Cancer cells	Hwanggeumchal	Tumor suppression, Migration inhibition, Anti-metastatic	[49]
	Mouse Model	PI570481, SC84 and Sumac bran extract	Tumor size and load reduction	[120]

	PC3M Prostate Cancer cells	Donganme Extract	Antiproliferative/Apoptotic /Reduced metastases	[50]
	HepG2, HCT-15 Cancer cells	TX430, Commercial Sumac sorghum, PI570481	Anti-proliferative	[51]
	Colon cancer cells/ Wistar rats	Red and white sorghum	Anti-proliferative	[52]
	Porcine Pancreatic alpha-amylase	Sumac sorghum, Black sorghum	Alpha amylase and aromatase inhibition	[121]
	MCF-7 cancer cells, YAMC	ATX631 x RTX436, TX2911, TX430	Estrogenic effects/ Apoptosis	[54]
	YAMC		Estrogenic Effects	[55]
	HEPG2 cells	Ultrasound assisted extracted red sorghum	Antiproliferative	[56]
Anti-Inflammation	THP-1 Human Macrophages	Kafirin Extract from sorghum	Inflammation inhibition / Reduced cytokine production/ Reduction of ROS	[57]
	Clinical trial	Extruded sorghum	Reduced inflammation/ oxidative stress	[58]

	Clinical trial	Red sorghum flour	Higher antioxidant activity	[59]
	Mouse and cancer cells	Black sorghum bran extract	Reduced inflammation/ reduced production of TNF-a, IL-1B	[122]
	HUVEC cells	Shawaya Short black 1	Reduced oxidative stress / reduced inflammation	[61]
	Wistar Rat Model	BRS 305, BRS 309, BRS 310	Reduced inflammation/ oxidative stress	[62]
Anti-Obesity	Wistar Rat Model	Extruded Sorghum Flour	Decreased lipogenesis	[63]
	Wistar Rat Model	Extruded or raw sorghum bran UDG110	Reduced fat storage/ reduced glucose and insulin levels	[64]
	Rat Model	Sorghum flour factions	Decreased liver fat, increased glucose tolerance and insulin resistance	[65]
	Wistar Rat Model	Extruded Sorghum flour	Decreased fat/ increased glucose tolerance	[66]
	Clinical Trial	Extruded sorghum	Reduced body fat	[67]
Microbiome Effects	Human Fecal samples	Black or sumac sorghum extract	Selectively increased/ modified bacterial levels	[76]
	Human Fecal samples	Sorghum	Increased in select bacteria growth, increased short chain fatty acids	[77]

	Rat model	Extruded sorghum flour	Modified bacterial levels	[78]
	Rat model	Sumac and high tannin black sorghum	Increased bacterial richness, modified bacterial levels	[79]
Other uses	Human polymorpho nuclear and mononuclear cells	Sorghum leaves	Increased natural killer cell activation, Decreased ROS formation and cell migration	[68]
	Rat model	Sorghum leaves	Anti-inflammatory effect	[69]
	Clinical trial	Sorghum leaves	Improved CD4 counts, increased hemoglobin	[70]
	Human foreskin fibroblasts, human aortic endothelial cells	Sorghum leaves	Inhibited T. gondii growth	[71]
		Sorghum leaves	High polyphenolic content	[72]
	Agar Medium with multiple microbe varieties	Algerian Sorghum samples: AS12, AS20, I27, H40	Inhibited microbial growth	[73]
	Mouse Macrophage s	PI570481 bran extract	Reduced Legionella replication, increased	[74]

			TNF and IL6 production	
	Human Blood samples	Shawaya short black 1 sorghum extract	Increased platelet aggregation and microparticles release	[75]

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Chapter 2 - Evaluation of ethanol-based extraction conditions of sorghum bran bioactive compounds with downstream anti-proliferative properties in human cancer cells

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This original research article was published in Heliyon Chemistry Special Issue.

https://doi.org/10.1016/j.heliyon.2019.e01589

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Abstract

Certain foods such as turmeric and green tea have been extensively for anticancer properties, while high polyphenol sorghum has not received the same attention. Some bioactive compounds in *Sorghum bicolor* with anticancer activity have been identified, indicating the further need for research and screening methods of high polyphenol sorghum varieties. This study was aimed at improving the extraction of sorghum bioactive compounds by using food-grade solvents using ethanol and citric acid. We used three sorghum varieties and green tea (GT) as a control. The extraction methods were screened for anticancer properties in HepG2 and HCT-15 cancer cell lines, using a cell viability assay. Extraction conditions were optimized for anticancer compounds from a high-phenolic sorghum variety (HP), sumac sorghum (CS), and GT. HP was more effective at inhibiting cell viability than CB, CS, and GT. The results demonstrate an efficient method for extracting sorghum bioactive compounds for future anticancer research.

Keywords

Sorghum, Polyphenols, Cancer, Green tea, Cell viability, Extraction

Abbreviations:

HP: High phenolic black sorghum bran PI570481
CB: Commercial black sorghum bran
CS: Commercial sumac sorghum bran
GT: Matcha green tea powder

Introduction

Cancer is one of the leading causes of death in the United States and the rest of the world, and the possibility of cancer prevention through dietary interventions, especially with high-

polyphenol foods, has become an increasingly attractive research area (Duthie, Duthie, & Kyle, 2000). Sorghum is a genetically diverse crop and several varieties (germplasms) exist with extremely high amounts of polyphenols (Harrison, 2015). Previous research suggests that high-polyphenol sorghum may have components with a strong anticancer activity(Joseph M. Awika & Rooney, 2004; Smolensky et al., 2018; Yang, Browning, & Awika, 2009). To further evaluate sorghum polyphenols as anticancer agents and potentially market sorghum and/or its extracts as health-promoting supplements, the identification of both the optimal extraction methods and specific anticancer molecules are crucial. In many previous studies, methanol, acetone, and/or hydrochloric acid have been used to extract sorghum polyphenols (Joseph M Awika, Rooney, & Waniska, 2005; Devi, Kumar, & Das, 2011). However, these extraction methods present problems when evaluating sorghum polyphenols for future in vivo studies and the potential marketing of the extracts. Methanol is not an approved food ingredient and both acetone and hydrochloric acid are highly regulated and their application is limited to very specific food processes with only very small residual amounts allowed. On the other hand, both ethanol and citric acid are generally recognized as safe (GRAS) (FDA, 2018). Both ethanol and citric acid have been successfully used in the extraction of polyphenols from green tea (Rusak, Komes, Likić, Horžić, & Kovač, 2008). Furthermore, previous research has suggested that ethanol-based extractions of sorghum polyphenols are absorbable in the intestinal tract, while no such data exist for other extraction methods (Jimenez-Ramsey, Rogler, Housley, Butler, & Elkin, 1994). Sorghum bioactive compounds extracted using 50% v/v ethanol were previously used in cell culture studies (Burdette et al., 2010). Our laboratory previously used 50% v/v ethanol extracts of high-polyphenol sorghum bran to evaluate potential anticancer effects of sorghum in HepG2 and Caco2 cell lines (Smolensky et al., 2018). While extraction with 50% v/v ethanol provided positive results, the extraction method used should be optimized for further research. Previous research has suggested that the addition of citric acid and/or an increase in the extraction temperature can enhance the extraction of healthy polyphenols from plants such as green tea and turmeric (Paulucci, Couto, Teixeira, & Freitas, 2013; Rusak et al., 2008; Zimmermann & Gleichenhagen, 2011).

In order to develop the optimum extraction procedure, which could be further used in anticancer research including in vivo studies, we investigated various phenolic extraction procedures by adjusting the ethanol content, adding citric acid, and increasing heat during the extraction.

Studies optimizing extraction methods of bioactive compounds from plant tissue tend to rely on chemical assays exclusively. However, to our knowledge, assessments of the biological effects of these extraction conditions have not been conducted. Although chemical assays provide some useful information regarding the content of the extracts, measurement of the further downstream effects of various extraction conditions is also important. In addition, the results of chemical assays may show little relevance for the further downstream biological effects when the complete chemical makeup of the crude extracts is unknown, unless detailed liquid chromatography with tandem mass spectroscopy studies are done. In order to test the biological effects of the extraction conditions, the anticancer effects related to the extraction conditions were measured using the MTS cell viability assay with two cancer cell lines, HCT-15 and HepG2. This research will also provide better material for compound identification by identifying the most effective crude extraction method using food-grade solvents.

Materials and Methods

Reagents

The chemicals used were purchased from Fisher Scientific (Pittsburgh, PA, USA), unless otherwise stated.

Plant Material

Three types of sorghum bran were used to evaluate extraction procedures. Two of these were commercial sorghum varieties grown in western Kansas, namely, commercial black sorghum (TX430) bran (CB) and commercial sumac sorghum bran (CS). The third type was the novel high-polyphenol black sorghum (HP; accession number, PI570481), which has been previously used in our studies (Smolensky et al., 2018); this variety was grown in Puerto Vallarta, Mexico, during the 2014 winter nursery season and the bran was decorticated in house. Organically grown matcha green tea (Jade Leaf brand; GT) was purchased commercially.

Total Phenolic Extraction

The dry material was combined with solvents A-F (10% w/v; Table 1), and the samples were allowed to mix on a shaker for 2 h at 20°C and stored at -20°C overnight. The samples were then centrifuged at 3000 x *g* for 10 min, and the solid pellet was discarded. The supernatant was used as the total phenolic extract.

In order to test the effects of increasing temperature on total phenolic extraction, Solvent E (70% v/v ethanol plus 5% w/v citric acid) was chosen as a solvent due to its efficacy, and total phenolic extraction was performed using the protocol stated above, with the temperature during the 2-h shaking period being adjusted to 20°C, 40°C, or 60°C.

Table 2.1 - Composition of the solvents used to extract bioactive compounds from sorghum bran.

Solvent	Ethanol % v/v	Citric acid % w/v
A	50.00%	0.00%
B	70.00%	0.00%
C	90.00%	0.00%
D	50.00%	5.00%
E	70.00%	5.00%
F	90.00%	5.00%

Measurement of Total Phenolic Content

The previously published Folin-Ciocalteu (FC) assay was used to measure the total phenolic content in the total phenolic extracts (Herald, Gadgil, & Tilley, 2012). Gallic acid 0-800 µg/L was used as the standard to determine the gallic acid equivalent per gram (GAE/g) levels of the dry material. The diluted sample (25 µL) was combined with 75 µL of distilled water and 25 µL of FC reagent and incubated for 6 min at 20°C. Next, 100 µL of 7.5% w/v Na_2CO_3 was added to each well, and the plate was incubated at 20°C in the dark for 90 min. After the incubation period, absorbance was measured using a Biotek H4 Plate Reader (Winooski, VT, USA) at 765 nm.

Cell Culture

Human colorectal adenocarcinoma (HCT-15) and human hepatocellular carcinoma (HEPG2) cells were purchased from American Type Culture Collection (Manassas, VA). HCT-15 cells were grown in RPMI 1640 medium, and HepG2 cells were grown in minimum essential medium

(MEM). The media were supplemented with 10% fetal bovine serum and 1X anti-biotic anti-mycotic solution. The cells were grown and treated at 37°C and 5% CO_2.

Cell Viability Assay

Cell viability was measured using the Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Madison, WI) in accordance with the kit instructions. In brief, 5×10^3 cells were plated in a 96-well tissue culture plate and allowed to attach for 24 h. The cells were then treated with either the extract or the specific solvent used in the extraction vehicle control, after which they were allowed to grow for an additional 48 h. The cells were then washed with phosphate-buffered saline (PBS), and 100 µL of fresh media containing 20% v/v MTS reagent was added to the wells. The plates were incubated for 45 min, and absorbance was measured at 490 nm on a Biotek H4 Plate Reader (Winooski, VT, USA). All data (minus blank) were normalized to the values for the specific solvent (vehicle) treatment.

Statistical Analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons in GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, USA: www.graphpad.com).

Results

Solvent composition influences both the total phenolic content extracted from sorghum bran and the efficacy of the bran extracts in inhibiting cancer cell proliferation

The total phenolic content varied with different solvents (Fig. 2.1A). For HP, the total phenolic content ranged from 49.6 mg gallic acid equivalent (GAE)/g (solvent E) to 66.3 mg GAE/g (solvent D). For CB, the total phenolic content ranged from 17.0 mg GAE/g (solvent C) to 26.6 mg GAE/g (solvent D), whereas for CS, it ranged from 12.6 mg GAE/g (solvent F) to 20.2 mg GAE/g (solvent E). The only significant difference in the total phenolic content was observed in HP extractions with solvent E (49.6 mg GAE/g) in comparison with those with solvents C and D (64.5 mg GAE/g and 66.3 mg GAE/g, respectively). There was no significant difference in the total phenolic content extracted from CB and CS.

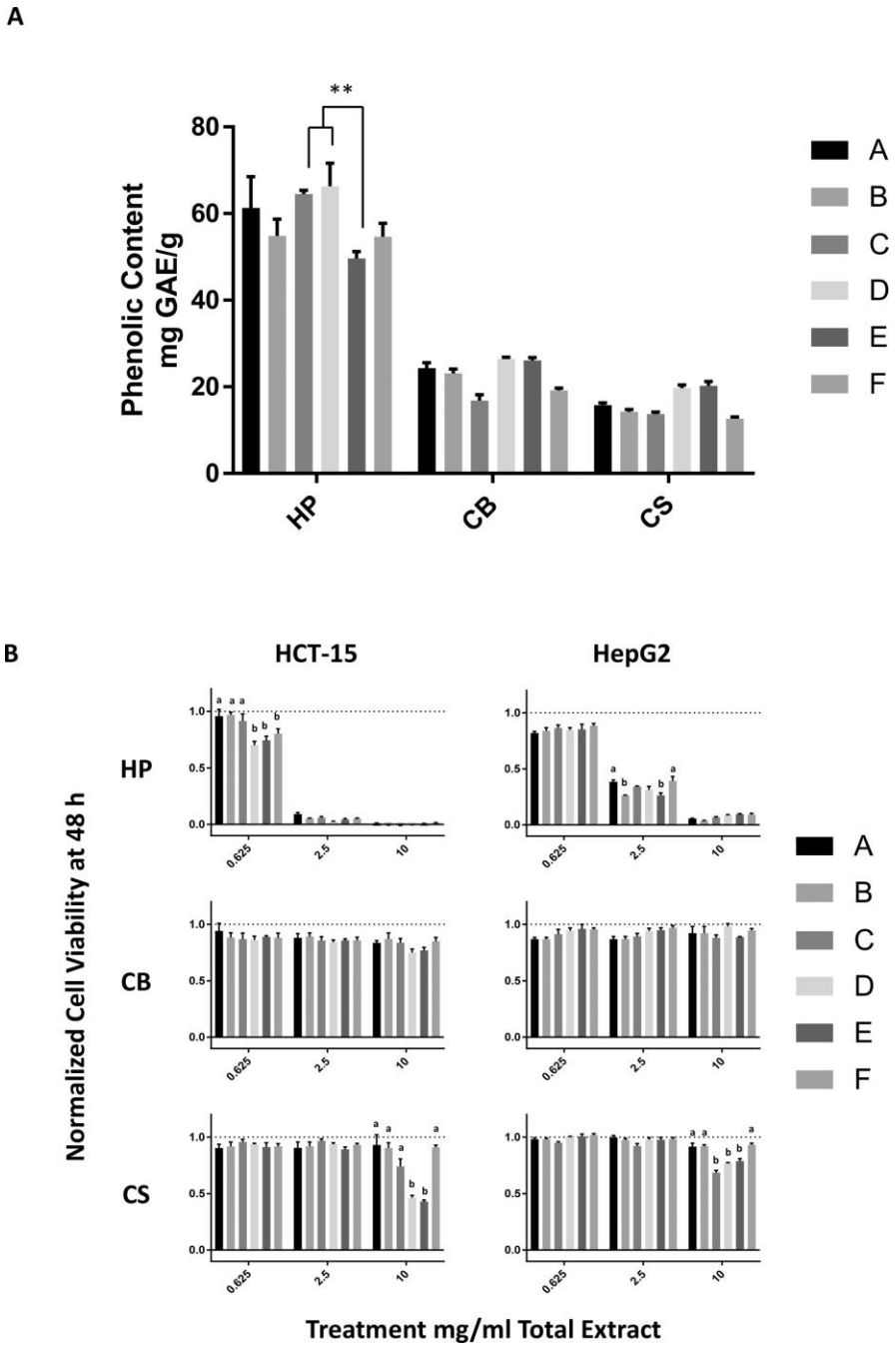

Figure 2.2.1 - Effects of ethanol content and citric acid on extraction of sorghum bioactive compounds. A) Total phenolic content, extracted using solvents A-F, of sorghum bran extracts represented as mg gallic acid equivalents obtained from 1 g of dry bran (mg GAE/g). Data represent the averages of three separate extractions ± SEM **P ≤ 0.01. B) Effect of the three sorghum bran extracts, obtained using solvents A-F, on cancer (HCT-15 left and HepG2 right) cell viability after 48 h of treatment. The data represent the average of three separate experiments using extracts of three separate extractions ± SEM. Different letters represent a significant difference between solvents used with the same bran and at the same dose with P ≤ 0.05.

The bioactivity of compounds obtained using various solvents was assessed with the MTS assay, which measured the inhibition of cancer cell proliferation/viability in two different cancer cell lines, HCT-15 and HepG2 (Fig. 2.1B). For extracts obtained using HP, both 2.5 mg/mL and 10 mg/mL doses resulted in almost complete reduction of viability in HCT-15 cells and therefore did not show significant differences among solvents. However, at a dose of 0.625 mg/mL, HP had varying effects on cell viability in HCT-15 cells; the extracts obtained using solvents D and E reduced HCT-15 cell viability to a significantly greater degree than those obtained using solvents A-C. In assessments with HepG2 cells and HP extracts, the 0.625 mg/mL dose resulted in a modest reduction in cell viability with no differences observed between extracts, while the 10 mg/mL dose resulted in almost complete reduction in cell viability with no significant differences between extracts either. However, at a dose of 2.5 mg/mL, HP extracts had varying effects on cell viability, with the extracts obtained using solvents B and E showing a significantly greater anti-cell viability effect than those shown by the extracts obtained using solvents A and F.

Treatments performed using CB extracts showed only a modest reduction in cell viability at all doses for both HCT-15 and HepG2 cells, with no significant differences in the cell viability-reducing ability between extracts obtained with different solvents. In contrast, the CS extracts did not show significant cell viability-reducing ability at a dose of 0.625 mg/mL or 2.5 mg/mL in both HCT-15 and HepG2 cells. However, at the 10 mg/mL dose, the CS extracts showed significant anti-cell viability effects that differed according to the solvents used: CS extracts obtained using solvents D and E showed a significantly greater anti-proliferative effect than that shown by the extracts obtained using solvents A, B, C, and F in HCT-15 cells while extracts obtained using solvents C, D, and E showed a significantly greater anti-proliferative effect than that shown by the extracts obtained using solvents A, B, and F in HepG2 cells. Overall, solvent E (70% v/v ethanol with 5% w/v citric acid) was the most effective in extracting bioactive compounds with anti-proliferative effects on both cancer cell lines for both HP and CS. Therefore, solvent E was chosen to evaluate the effects of increasing temperature on the extraction of bioactive compounds with anti-proliferative effects on cancer cells.

Increasing temperature during extraction of bioactive compounds from sorghum bran does not increase phenolic content and reduces efficacy against cancer cell proliferation

Temperature did not have a significant effect on the extraction levels of total phenolic content as measured by the FC assay (Fig. 2.2A). However, an increase in temperature did cause variations in the anti-cell viability effects of both HP and CS extracts (Fig. 2.2B). An increase in the extraction temperature from 20°C to either 40°C or 60°C significantly reduced the efficacy of the HP extract in reducing HCT-15 cell viability at a dose of 0.625 mg/mL. In contrast, while CB extracts showed no temperature-related differences in their effect on HCT-15 cell viability, CS extracts obtained at 60°C showed significantly reduced anti-proliferative effects on HCT-15 cells in comparison with those of CS extracts obtained at 20°C. Since the increasing temperature did not improve the extraction of the total phenolic content and in fact adversely affected the ability of the extracts to reduce the viability of HCT-15 cells, extractions at higher temperatures were not evaluated further.

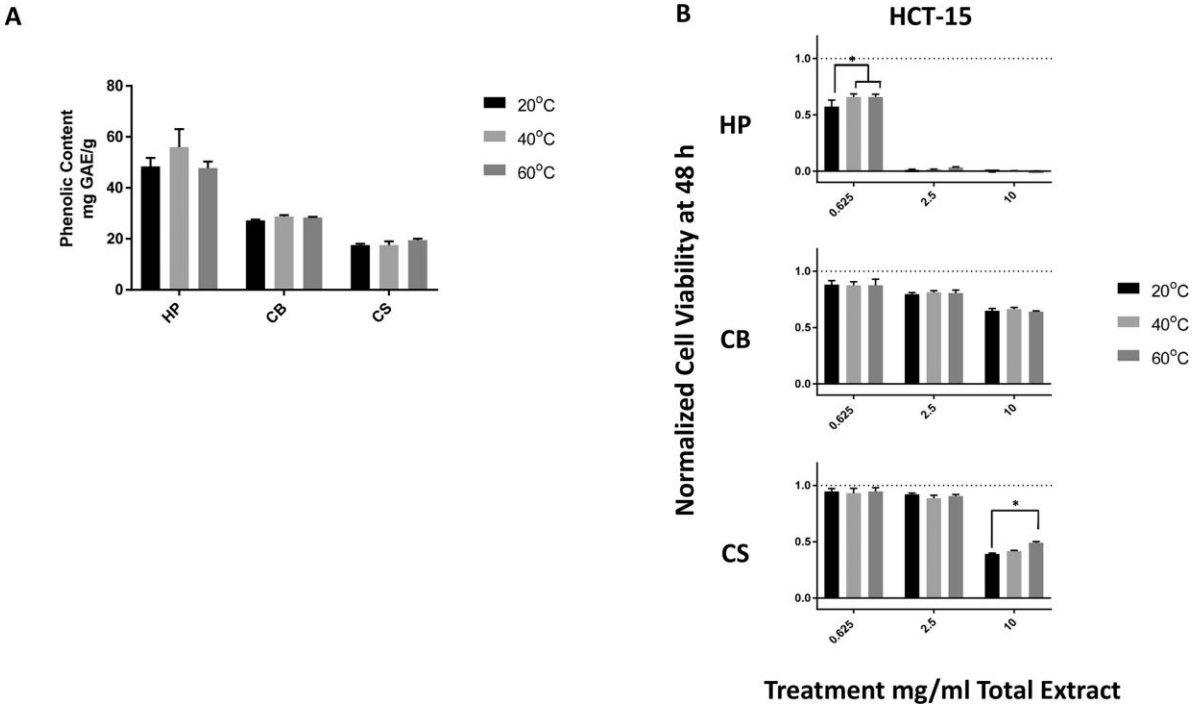

Figure 2.2 - Effects of increasing temperature on extraction of sorghum bioactive compounds by using solvent E A) Total phenolic content, extracted using three temperatures, of sorghum bran extracts represented as mg gallic acid equivalents obtained from 1 g of dry bran (mg GAE/g). B) Effect of the three sorghum bran extracts, extracted

at 20 °C, 40 °C, and 60 °C on HCT-15 cancer cell viability after 48 h of treatment, normalized to solvent only control. The data represent the anti-proliferative effects of three separate extracts ± SEM. Different letters represent significant differences between extraction temperatures used with the same bran and at the same dose with *P ≤ 0.05.

HP extracts show greater anti-proliferative effects than those shown by green tea extracts

Green tea has been extensively studied both in vitro and in vivo for its anticancer effects (Hayakawa et al., 2016; Ullah et al., 2016). Therefore, we compared the effects of various extraction conditions on the anti-proliferative effects of green tea extracts on both HepG2 and HCT-15 cancer cells. GT is considered to be particularly high in polyphenol content and was chosen because it is available in powdered form and can be easily incorporated into foods, similar to sorghum bran (Phongnarisorn, Orfila, Holmes, & Marshall, 2018).

We used the six solvent conditions A-F to extract GT bioactive compounds and measured the total phenolic levels and the extracts' ability to inhibit cancer cell proliferation. While the different solvents did not yield significant differences in total phenolic levels (Fig. 2.3A), similar to the findings for sorghum extracts, the extracts obtained with solvent E (70% v/v ethanol with 5% w/v citric acid) were the most effective at inhibiting cancer cell proliferation in HepG2 and HCT-15 cells (Fig. 2.3B). The effects of different extraction temperatures were also tested on the GT extracts by using solvent E, and no significant differences were observed in the total phenolic content extracted or the efficacy against cancer cells (Fig. 2.2).

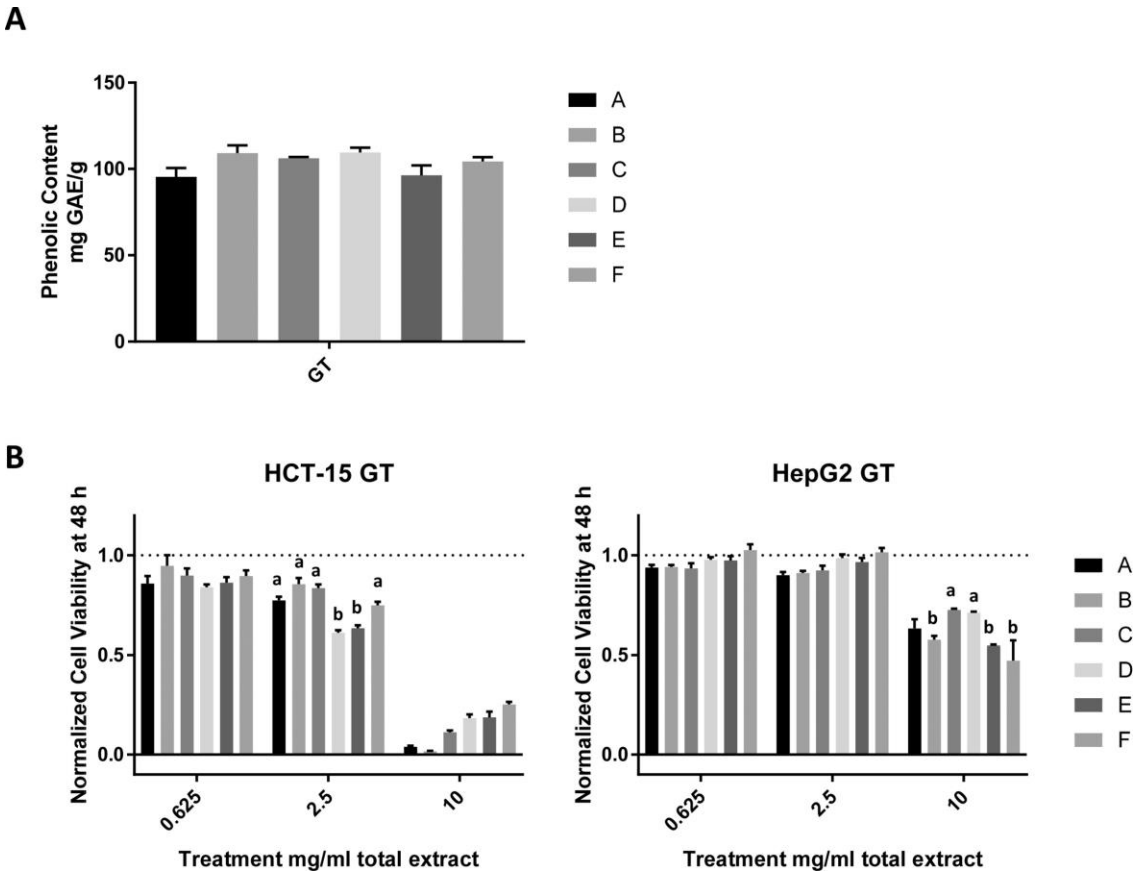

Figure 2.3 - Effects of ethanol content and citric acid on extraction of green tea bioactive compounds. A) Total phenolic content of matcha green tea extracts obtained using solvents A-F, represented as mg gallic acid equivalents extracted from 1 g of matcha green tea powder (mg GAE/g). B) Cell viability of HCT-15 cells (left) and HepG2 cells (right) after 48 h of treatment. The data represent the anti-proliferative effects of three separate extracts ± SEM. Different letters represent significant differences between extraction temperatures used with the same bran and at the same dose with *P ≤ 0.05. Note: No viable HCT-15 cells were visible under the microscope at 10 mg/mL treatments with all solvents and color changes in media were observed when citric acid was present, indicating that the results may not represent actual cell viability of HCT-15 cells for that high dose.

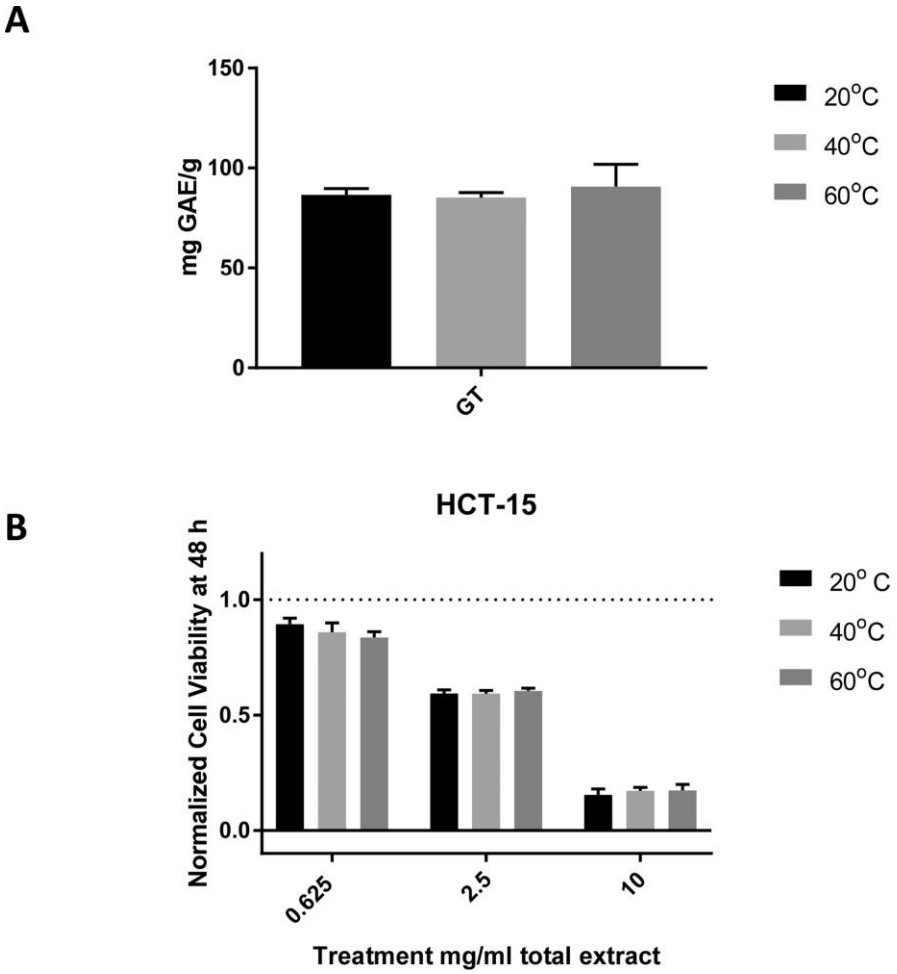

Figure 2.4 - **A) Effects of increasing temperature on extraction of green tea bioactive compounds** obtained using solvent E, represented as mg gallic acid equivalents extracted from 1 g of matcha green tea (mg GAE/g). B) Cell viability of HCT-15 cells after 48 h of treatment using green tea solvents extracted with solvent E at 20 °C, 40 °C, and 60 °C. No significant differences in phenolic contents or effects on HCT-15 cell viability were observed. The data represent the average of three separate extractions ± SEM.

In a direct comparison of the anti-proliferative effects of the three sorghum bran extracts against the GT extract obtained using the same extraction conditions (solvent E, 20°C), the HP extract was significantly more effective in inhibiting HCT-15 and HepG2 cancer cell proliferation than were the extracts of the other two commercial varieties of sorghum and commercial green tea, at all three doses tested (Fig.2.3). The GT extract inhibited cancer cell proliferation more effectively than both the CB and CS extracts at doses of 2.5 mg/mL and 10 mg/mL for HCT-15 cells and at the dose of 10 mg/mL for HepG2 cells. The CS extract was

significantly more effective than the CB extract at inhibiting the proliferation of both HCT-15 and HepG2 cells at a dose of 10 mg/mL.

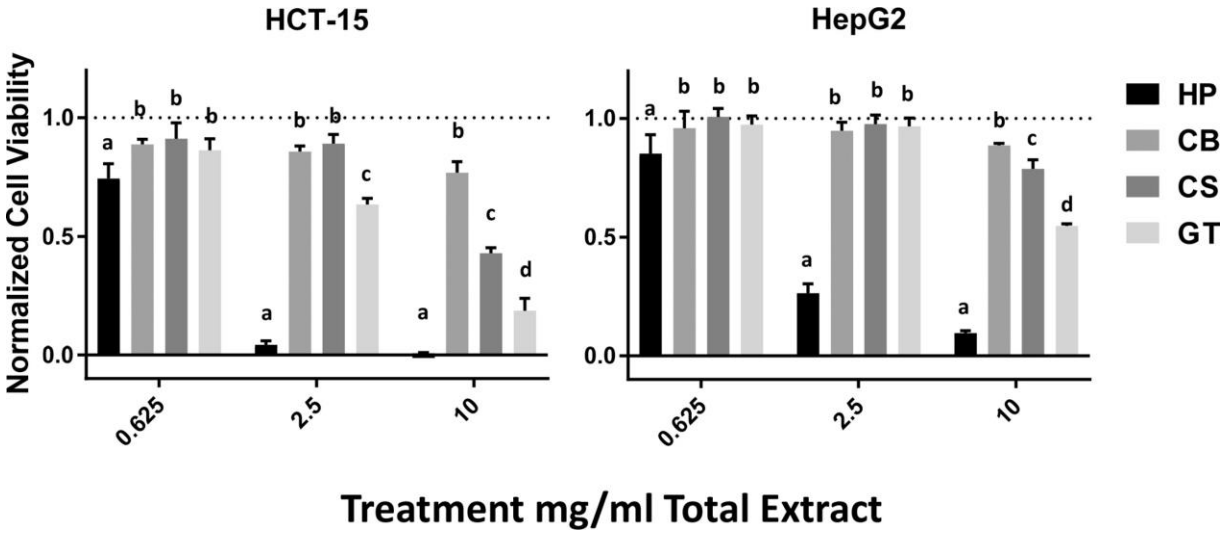

Figure 2.5 - Direct comparison of the anti-proliferative effects of sorghum bran and green tea bioactive compounds extracted under identical conditions (solvent E at 20 °C). HCT-15 (left) and HepG2 (right) cells. The data represent the average of three separate extracts ± SEM. Different letters represent a significant difference between HP, CB, CS, and GT at the same treatment dose, $P \leq 0.05$. t

Discussion

The objectives of this study were to optimize the extraction conditions of sorghum bioactive compounds with potential antiproliferative properties against cancer cells and to compare the antiproliferative effects of HP, CS, CB, and commercial GT, in vitro. Our results showed that varying the concentration of ethanol and adding citric acid produced extracts with similar amounts of polyphenols. There was no significant difference observed between the phenolic content of the extracts with the exception of less phenolic content in HP extracts obtained using solvent E when compared to that with solvent C and D. Interestingly, HP and CS extracts obtained using solvents D and E had the greatest anti-proliferative effects on both cancer cells lines. While extracts obtained using solvents D and E showed similar results in the cell viability assay, those obtained using solvent E has a significantly lower polyphenol content than that in the extracts obtained using solvent D, indicating the polyphenols obtained with solvent E were more bioactive. These results raise three possibilities. Solvent E aids in the extraction of compounds with more potential antiproliferative bioactivity by 1) facilitating the extraction of specific molecules that have a greater anti-proliferative effect on cancer cells, 2) facilitating the

extraction of the same bioactive compounds as those obtained using other solvents, but allowing them for to be more readily taken up by cells due to a cleaner extraction, and 3) chemically modifying the bioactive compounds to have a greater anti-proliferative effect on cancer cells. Sorghum has been shown to contain several specific compounds with potential anticancer properties. 3-Deoxyanthocyanins in black sorghum have been shown to possess both anticancer and antioxidant properties in vitro (Yang et al., 2009). Aside from 3-deoxyanthocyanins, sorghum contains flavones with estrogenic properties, which have an anticancer effect in vitro(Yang, Allred, Geera, Allred, & Awika, 2012). Differences were observed between the effects of solvents on two different cancer cell lines. For example, 90% ethanol extract of CS was effective in reducing the viability of HepG2 cells but not HCT-15 cells, compared to the extracts obtained using other solvents. This further demonstrates the need for screening extraction methods using biological systems of interest.

In conclusion, the results indicate that using solvent E (70% v/v ethanol, 5% w/v citric acid) was more effective for the extraction of bioactive compounds with potential antiproliferative effects against cancer cells from HP, CS, and commercial GT than was solvent A (50% v/v ethanol), as published previously. Our findings stress that chemical assays, which estimate the total amount of phenolic content, may not correlate with the biological effects of crude extracts from sorghum and other plant material. This is especially true when the exact composition of the crude extract is unknown. Because biological effects can vary between different sorghum varieties, screening methods for studying plant extracts should not only involve chemical assays but evaluations of biological effects of interest as well. Future research should focus on identifying the specific antiproliferative compounds in sorghum bran that can be screened against existing cancer models both in vitro and in vivo. The ability to extract these compounds more efficiently in a solvent containing food approved ingredients will greatly contribute to future studies.

Declarations

Author contribution statement

Sarah Cox: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Leela Noronha, Weiqun Wang, Seong-Ho Lee: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Thomas Herald, Scott Bean: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ramasamy Perumal: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dmitriy Smolensky: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by the United States Department of Agri-culture, Agricultural Research Service. This paper is Contribution No.19-257-J from the Kansas Agricultural Experiment Station.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper

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Chapter 3 - Effects of sorghum polyphenols on RAW 264.7 inflammation and cell death

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Contributions of Sarah Cox: Extractions, cell culture, Nitric oxide measurement, ELISA, microscopy, qPCR, Writing

Western blot was performed by Joaquin De Leon

Abstract

Sorghum grain has been gaining increasing attention for its potential health benefits, particularly due to its polyphenols. While polyphenol content ranges widely among sorghum varieties, a novel high polyphenol variety and a brown high polyphenol sorghum variety have shown promise in anti-proliferation of cancer cells. This study took the same two sorghum varieties and evaluated their effects on inflammation in RAW 264.7 cells. Nitric oxide production was not significantly changed in LPS activated cells. However, cell death was observed along with a change in cell morphology wherein vacuole like structures were formed. IL-6 and IL-10 showed a significant decrease in production in both polyphenolic extract treated cells. LC3 II expression increased as the sorghum extract concentration of the treatments increased, indicating the presence of autophagy.

Keywords: Sorghum, Polyphenols, RAW 264.7, Anti-inflammatory, STAT 3

Introduction

Sorghum bicolor is a drought resistant grain grown around the world as a dietary staple. In the United States sorghum is used primarily as livestock feed and in ethanol production. However, interest has been growing in the potential health benefits and bioactive properties of sorghum. Particular interest is being seen in the phenolic compounds of sorghum. Numerous studies have shown strong evidence of anticancer properties ("Evaluation of Antiproliferative Activity of Red Sorghum Bran Anthocyanin on a Human Breast Cancer Cell Line (MCF-7)," 2011; Smolensky et al., 2018). Other studies have shown positive results in reducing obesity (Anunciação et al., 2019; de Sousa et al., 2018). The anti-inflammatory properties of sorghum polyphenols are another characteristic that is being explored as a potential health benefit. Chronic inflammation is associated with a large number of autoimmune diseases. With an increasing public interest in utilizing food as a means of reducing chronic disease, different food sources are being evaluated for their potential in this area. Sorghum is among the foods that have shown some anti-inflammatory abilities. Purified kafirin extract from sorghum has been shown to reduce several proinflammatory cytokines when cells were cotreated with LPS in human macrophage cells (Sullivan, Pangloli, & Dia, 2018). Plum polyphenols have also been shown to have an anti- inflammatory effect in RAW 264.7 cells through a reduction in nitric oxide and Cyclooxygenase-2 (Hooshmand et al., 2015).

The goal of this study was to evaluate the effects of total polyphenolic extracts from two sorghum varieties on the anti-inflammatory functions on RAW 264.7 cells. Having observed significant results using these sorghum varieties in killing colon cancer cells (Smolensky et al., 2018), we decided to evaluate their effects on RAW 264.7 macrophage-like cells. Anti-inflammatory and inflammatory associated cytokines were measured, along with nitric oxide production. Light microscopy was used to observed physical changes to cells during the treatment process. Western blot analysis of genes linked to inflammation and

autophagy was also performed. Ultimately it was found that while nitric oxide production was not significantly changed, expression of anti-inflammatory cytokines IL-6 and IL-10 was reduced in activated raw 264.7 cells and LC3-II levels were increased.

Methods and Materials

Reagents

All chemicals and consumables used were from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

Plant Materials

Two varieties of sorghum were used in this study. A novel high polyphenol sorghum grain (PI570481) was procured from a sorghum breeder in Mexico. The second variety was a high polyphenol brown sorghum grown in Kansas. Samples were decorticated using a Tangential Abrasive Dehulling Device (Model #4E-110/220) (Venables Machine Works LTD., Saskatoon, Sask., Canada). The bran was then ground using a UDY mill Cyclone Sample Mill (Model # 3010-030) (UDY Corporation, Fort Collins, CO, USA) with a .25mm screen in order to obtain the finest possible bran particles to use for total phenol extraction.

Total phenol extractions

Sorghum bran samples were measured for total polyphenol content in previous studies (Cox et al., 2019). 1.0 g of either novel high polyphenol (HP) or high polyphenol brown sorghum (SC84) bran was mixed with 70% ethanol and 5% Citric acid. Samples were placed on a shaker for two hours before being stored overnight in -20^{o}C. Samples were then centrifuged and decanted. The supernatant was used as total polyphenol extract.

Cell Culture

RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA). They were grown in minimum essential media (MEM) with 10% fetal bovine serum and 1X anti-biotic anti-mycotic and incubated at 37^{o}C and 5.0% CO_2.

Nitric Oxide measurement

RAW 264.7 cells were plated at concentration of $2x10^4$ cells per well in a 24 well plate 24 hours prior to treatment. Treatments consisted of multiple concentrations of sorghum total polyphenol extracts, including 2.5, 1.25, and 0.625 mg/ GAE. The cells were also cotreated with 100 ng/ml LPS and 25 units per ml IFN γ. Cells were then incubated for 12 hours, at which point media was harvested and stored at -80^{o}C until analysis was performed. Nitric oxide levels were measured using the Nitric Oxide Assay Kit from Qiagen (Hilden, Germany). In short, the media samples were thawed. Nitrite standards of 0, 30, 60 and 100uM were mixed. 300uL of samples and standards were mixed with ZnSO4, vortexed, and then mixed with 16uL of NaOH and vortexed once more. Samples were centrifuged at 14,000rpm for 10 minutes after which 100ul of supernatant were transferred to clean 1.7mL tubes in duplicate. A working reagent was made according to kit instructions and 200uL of it was mixed with each sample and vortexed. Samples were then incubated for an hour at 37^{o}C, briefly centrifuged, and 250uL of each sample was plated on a 96-well plate. The plate was then read on a Biotek H4 Plate Reader (Winooski, VT, USA) at 540nm.

Anti-inflammatory Cytokine ELISA analysis

RAW 264.7 cells were plated 24 hours on a 24 well plate at a concentration of $2x10^4$ cells per well. They cotreated with either 2.5, 1.25, or 0.625 mg/ GAE with and without LPS and IFN γ. Media was collected 12 hours after treatment and the duplicates were pooled prior to storage

in -80^{o}C until use. The Multi-Analyte ELISArray Kit (MEM-004A) from Qiagen (Hilden, Germany) was used to measure 12 anti-inflammatory associated cytokines according to kit instructions. 650uL of each sample was centrifuged at 1000xg for 10 minutes. The plate was read at both 450nm and 570 nm on a Biotek H4 Plate Reader (Winooski, VT, US) and the results from the 570nm read subtracted from the 450nm results.

Cell Microscopy

RAW 264.7 cells were treated 1) vehicle, 2) extract at 0.625 mg/ml, 3) extract at 1.25 mg/ml, 4) extract at 2.5 mg/ml , 5) 100 ng/ml LPS and 25 units per ml IFN γ, 6) extract at 0.625 mg/ml, 100 ng/ml LPS and 25 units per ml IFN γ, 7) extract at 1.25 mg/ml, 100 ng/ml LPS and 25 units per ml IFN γ, 8) extract at 2.5 mg/ml, 100 ng/ml LPS and 25 units per ml IFN γ for both the HP and SC84 extracts. The cells were plated at a density of $2x10^4$ cells per well in a 24 well plate 24 hours prior to treatment. Cell morphology was then observed on a Light Microscope at 3, 6, 12, and 24 hours of incubation.

Quantitative Polymerase Chain Reaction (qPCR)

An RT2 Profiler PCR Array from Qiagen (Mouse Inflammatory Cytokines & Receptors Cat. no. 330231 PAMM-011ZA) was used to analyze the expression of genes involved in the inflammatory and anti-inflammatory responses. A concentration of $4x10^5$ RAW 264.7 cells was plated per well in a six well plate. Cells were treated with 1) vehicle, 2) extract at 0.625 mg/ml, 3) extract at 1.25 mg/ml, 4) 100 ng/ml LPS and 25 units per ml IFN γ, 5) extract at 0.625 mg/ml, 100 ng/ml LPS and 25 units per ml IFN γ, or 6) extract at 1.25 mg/ml, 100 ng/ml LPS and 25 units per ml IFN γ for both the HP and SC84 extracts. Sample RNA was collected at 12 hours using Trizol and was stored at -80^{o}C. RNA was thawed and converted to cDNA using a RT^2 First Strand Kit from Qiagen (Hilden, Germany) according to provided instructions. cDNA was then

prepared for real time PCR using the RT^2 SYBR Green qPCR mastermix kit and RT^2 Profiler PCR Array from Qiagen using provided instructions. The plate was then run on a Roche Light Cycler 96 (Basel, Switzerland). Collected data was then analyzed by inputting it into the Qiagen The GeneGlobe Data Analysis Center tools (https://geneglobe.qiagen.com/us/analyze).

Western Blot

RAW 264.7 cells were plated at a density of $4x10^5$ cells per well in 10 cm cell culture dishes. Cells with and without LPS and IFN γ activation were cotreated with 0.625 and 1.25 mg/mL HP polyphenol extract and Rapamycin. DNA was harvested using RIPA buffer with protease-phosphatase inhibitor and stored at -80°C until use. Samples were thawed and immediately centrifuged at 4°C at 12,700RPM and supernatant transferred to a new microtube. Protein concentration was determined using Pierce BCA Protein assay kit with bovine serum albumin (BSA) used to generate the standard curve. 40 µg of supernatant was loaded onto a 4-20% Mini-Protean TGX Gel with 10 µL of precision plus protein dual color as the standard. The gels were then subjected to electrophoresis of 75 volts for 45 minutes before increasing to 150 volts for 5 minutes.

Protein was then transferred onto a nitrocellulose membrane using Bio-Rad Trans Blot Turbo Transfer system and the membrane then washed using 1XTBST, and then exposed to 30 mL 1.5X Animal free blocking solution at room temperature for 5.5 hours. Membranes were then incubated with 10 mL of 1:1000 LC3A/B (D3U4C) (Cell Signaling #12741S) for shaking 11 hours at 4°C and then washed 3 times with 1XTBST. Anti-Rabbit IgG, HRP-Linked,10 mL, 1:30,000 (cell signaling # 7074P2) was then applied to the membrane which was then incubated shaking for 1.5 hours at room temperature. The membrane was then washed 3 times and then covered with 3 mL of Amersham ECL Prime Western Blotting Detection reagent for 3 minutes.

Azure biosystems c400 2-minute exposure was used to visualize the bands before washing the membrane 2 times. The membrane was then blocked for one more hour at room temperature before being incubated in 14 mL of β-actin (8H10D10) HRP conjugated 1:10,000 (Cell Signaling # 12262S) for 2 hours at room temperature. Membrane was then washed 3 times and imaged again using the same process. Image J software was used to analyze the western blots.

Statistical Analysis

Statistical analysis was done using Two Way Anova using Graphpad Prism Version 8 (GraphPad Software, La Jolla, CA, USA). qPCR data was analyzed using the Qiagen website qPCR The GeneGlobe Data Analysis Center tools (https://geneglobe.qiagen.com/us/analyze).

Results

Polyphenols had no statistical effect on Nitric Oxide

Sorghum polyphenolic extract treatments showed no significant changes in nitric oxide production in either sorghum variety. The LPS and IFN γ activated raw cells did produce significantly higher amounts of nitric oxide compared to the cells which did not receive the LPS and IFN γ treatments (Fig 3.1). However, when cells cotreated with the sorghum and the LPS and IFN γ were compared to LPS and IFN γ activated cells with no polyphenolic extract treatment, there was a decreasing trend that was not statistically significant after cotreatment HP or SC.

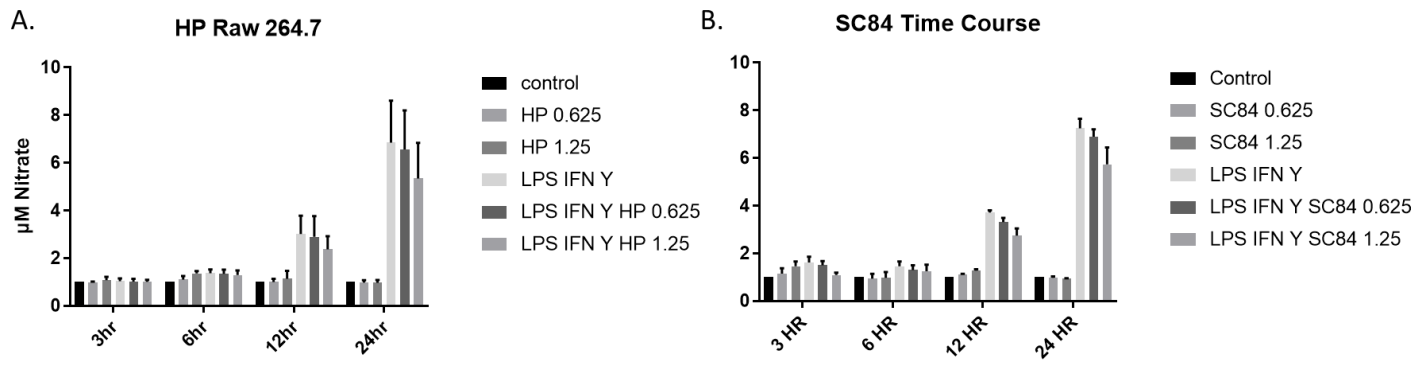
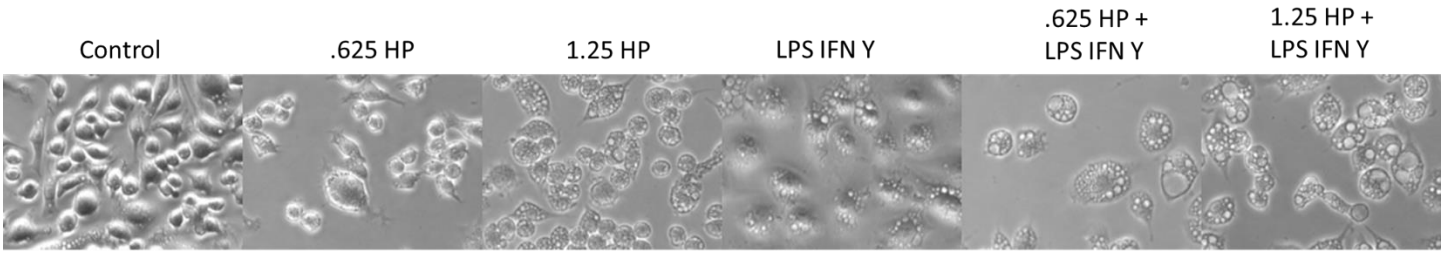


Figure 3.1 - Nitric oxide time course. RAW 264.7 cells were treated with vehicle, 0.625 and 1.25 mg/mL of polyphenol extract from either HP or SC84. This figure shows the Nitric Oxide levels present in RAW 264.7 cells harvested at multiple time points (3, 6, 12, and 24 hrs) after treatment for A) HP extract and B) SC84 Extract. $P<0.05$

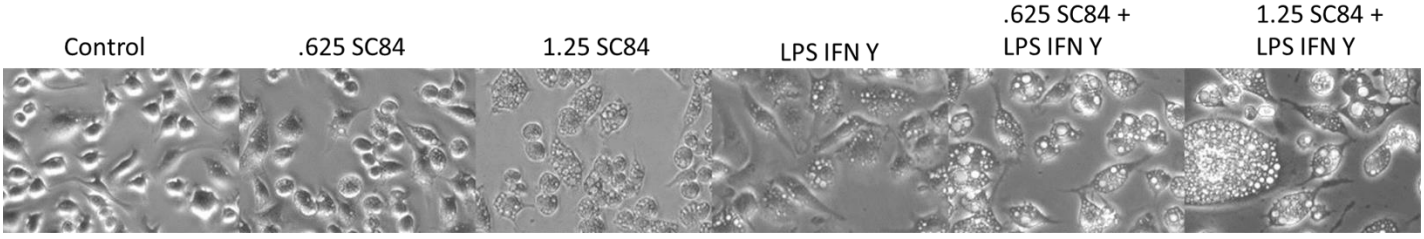
Total Polyphenol extract caused cell death and cell morphology changes

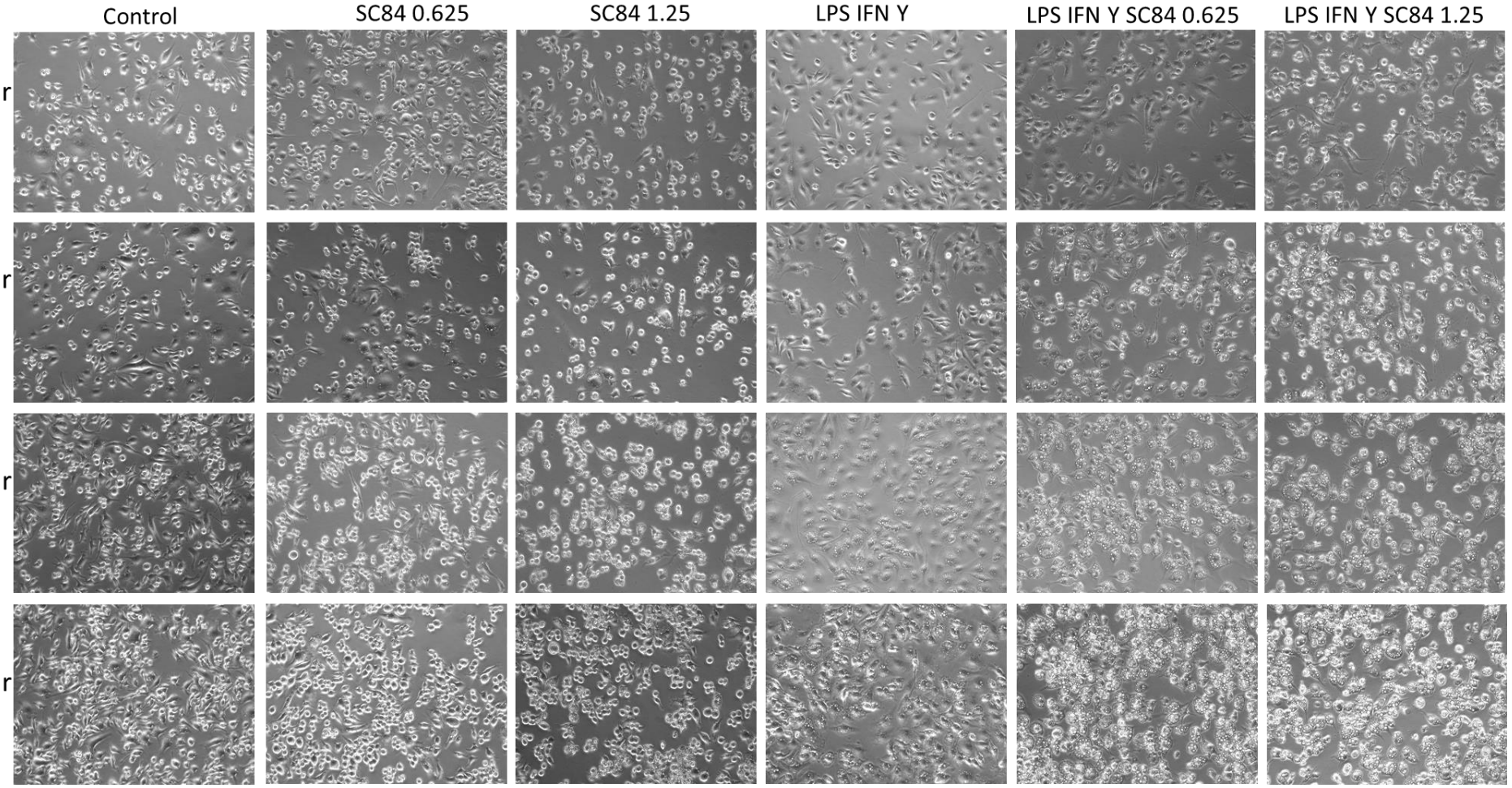
Microscopic observation of the raw cells at each time point revealed a time dependent change in morphology in those cells cotreated with the polyphenolic extract, LPS and IFN γ. Vacuole like structures were observed forming and a lower number of total cells was also observed. This reaction was seen in both the HP and SC84 varieties and increased over time.

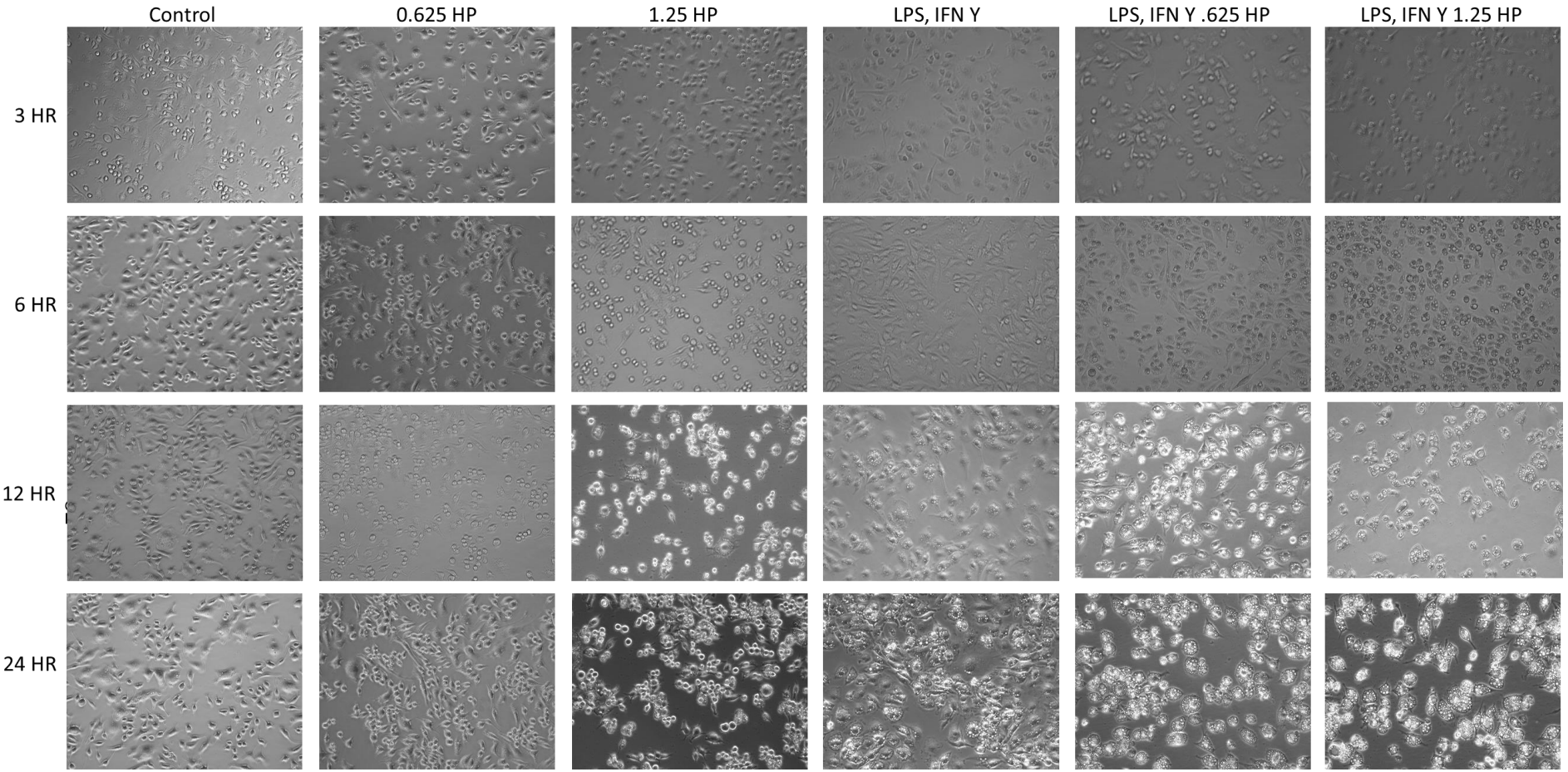
A. HP 12 hour Treatment



B. SC84 12 hour Treatment




Control
SC84 0.625
SC84 1.25
LPS IFN Y
LPS IFN Y SC84 0.625
LPS IFN Y SC84 1.25
3 Hr
6 Hr
12Hr
24Hr


Control
0.625 HP
1.25 HP
LPS, IFN Y
LPS, IFN Y .625 HP
LPS, IFN Y 1.25 HP
3 HR
6 HR
12 HR
24 HR

Morphological changes observed at 12 hour time point. RAW 264.7 cells were treated with 0.625 or 1.25 mg/mL of Sorghum extract as were LPS and IFNY activated cells. Microscopic observation and photos were taken at 3, 6, 12 and 24 hrs. This figure shows the 12 hour time point for A) HP and B) SC84 phenolic extracts. The morphology is visibly different between the activated cells treated with extract and those not activated. The vacuole like structures are distinctive in comparison. C) Sc84 and HP cells viewed over time.

LPS/ IFN γ and sorghum extract cotreatment modifies cytokine expression

12 cytokines associated with inflammation were measured via ELISA. LPS and IFN γ treatment significantly increased IL6 and IL-10 in the supernatant when compared to vehicle. Cotreatment of HP, LPS and IFN γ significantly reduced IL6 and IL10 compared to LPS and IFN γ treatment alone (fig. 3.3). There was also significant reduction in IFN γ, $P<0.05$, despite being treated with it. IL-2, IL-4, Il-12, and IL-17A all showed significant dose dependent increases in both the LPS and IFN γ activated cells and the cells that were not activated with LPS and IFN γ. GM-CSF showed no significant changes between the extract concentrations or the LPS and IFN γ activated compared to the non LPS IFN γ activated cells. The SC84 polyphenolic extract treatments yielded similar results (fig. 3.4). SC84 treated cells also showed a dose dependent increase in G-CSF production.

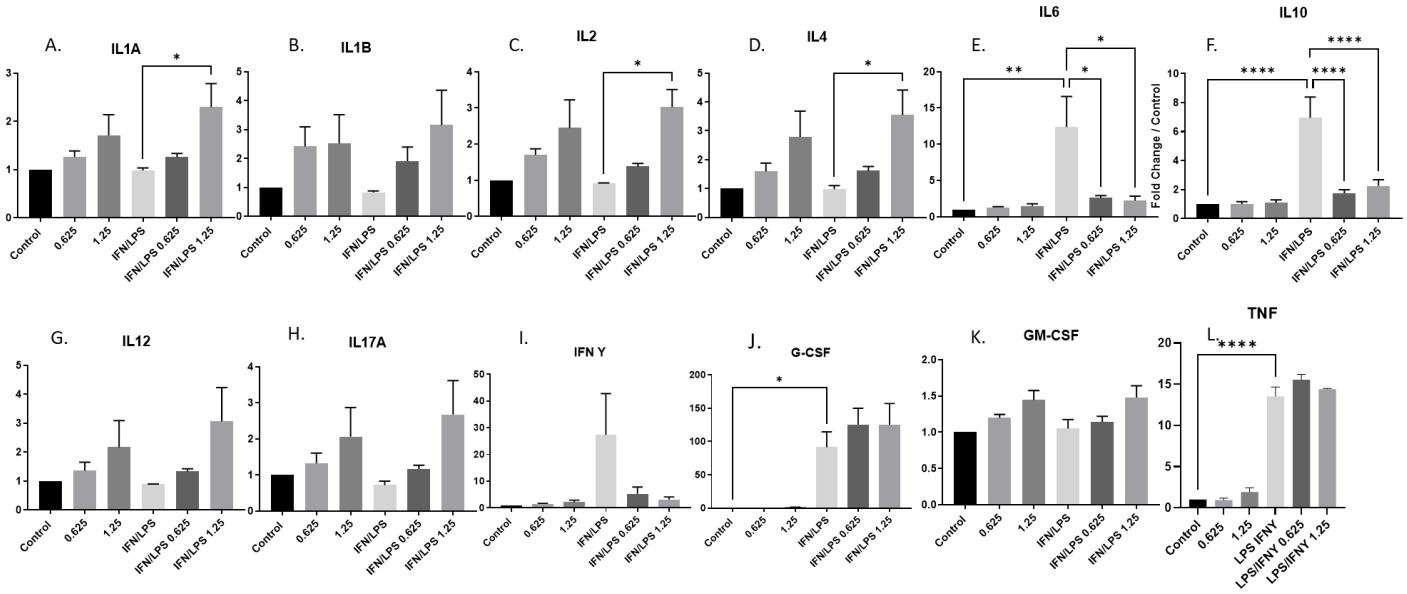


Figure 3.3 - HP ELISA Data -RAW 264.7 cells were treated with either 1.25 or 0.625 mg/ml HP polyphenolic extract. This figure shows significant increases in IL-1A, IL-1B, IL-2 and IL-4 and significant decreases in IL-6, IL-10 and IFN γ The data was normalized to vehicle treated control. P<0.05

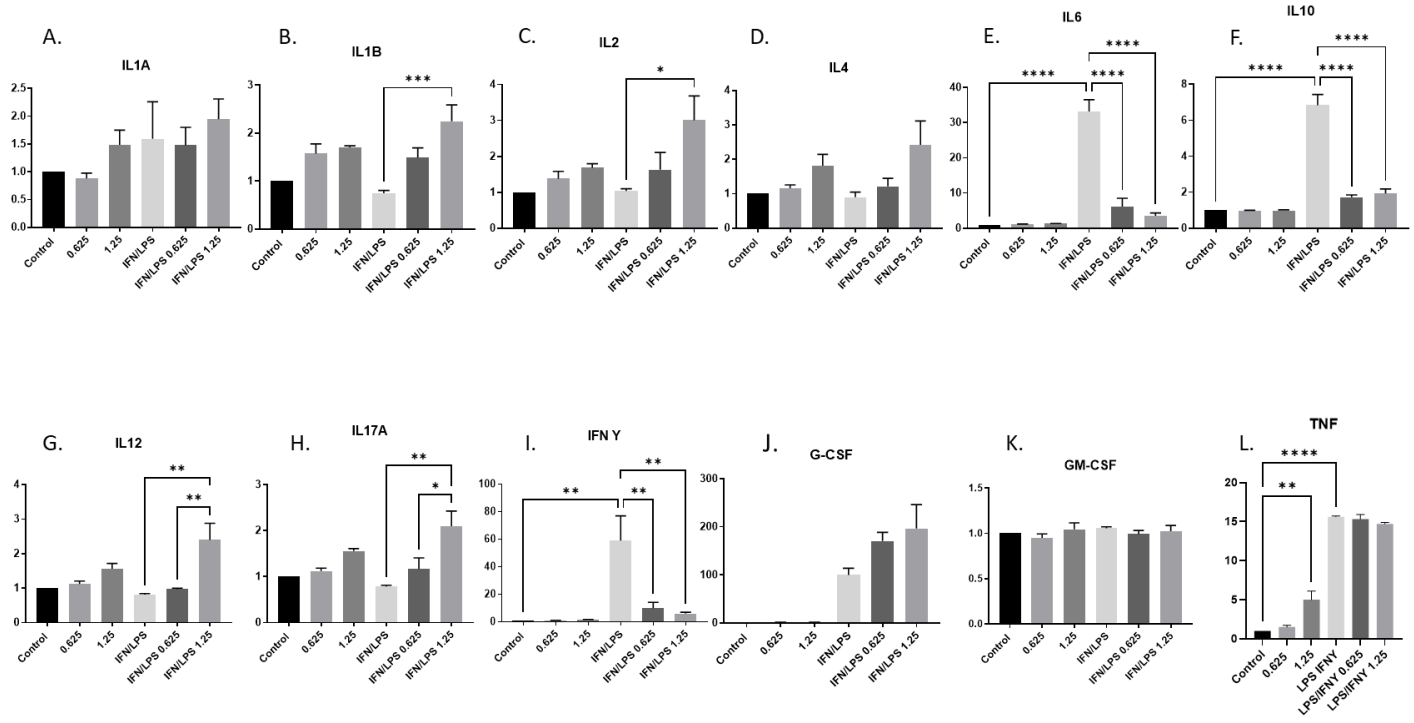


Figure 3.4 - SC84 ELISA Data - RAW 264.7 cells were treated with either 1.25 or 0.625 mg/ml SC84 polyphenolic extract. This figure shows significant increase in IL-1b,IL-2, IL-12, IL-17A and G-CSF and significant decrease in IL-6, IL-10 and IFN γ. The data was normalized to vehicle treated control. $P<0.05$

QPCR measured genes

In the HP cells, when compared to the control, there was significant upregulation in interleukin 1rn (IL1rn), interleukin 1a (IL1a), C-C Motif Chemokine Ligand 17 (Ccl7), C-C Motif Chemokine Receptor 1 (Ccr1), Tumor necrosis factor ligand superfamily member 13 (TNFsf13), Oncostatin M (OSm) and C-C Motif Chemokine Ligand 2 (Ccl2) (fig. 3.5). IL1rn was the most upregulated by 9.31 fold. Ltb and IL6ra were significantly downregulated. Similar results were seen between the sc84 and control, with IL1rn, Colony Stimulating Factor 2 (Csf2), IL1a, TNFsf13, and Osm increased, and Interleukin 6ra (Il6ra) and Secreted Phosphoprotein 1 (Spp1) decreased (fig. 3.6). IL1rn was also the most upregulated by the SC84 treatments by 8.42 fold.

IL6ra is down regulated in both the SC84 and HP treated cells. The IL1rn, IL1a, TNFsf13 and Osm genes are upregulated in both sorghum polyphenol treatments.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Aimp1	Bmp2	Ccl1	Ccl11	Ccl12	Ccl17	Ccl19	Ccl2	Ccl20	Ccl22	Ccl24	Ccl3
B	Ccl4	Ccl5	Ccl6	Ccl7	Ccl8	Ccl9	Ccr1	Ccr10	Ccr2	Ccr3	Ccr4	Ccr5
C	Ccr6	Ccr8	Ccl40lg	Csf1	Csf2	Csf3	Cx3cl1	Cxcl1	Cxcl10	Cxcl11	Cxcl12	Cxcl13
D	Cxcl15	Cxcl5	Cxcl9	Cxcr2	Cxcr3	Cxcr5	Fasl	Ifng	Il10ra	Il10rb	Il11	Il13
E	Il15	Il16	Il17a	Il17b	Il17F	Il1a	Il1b	Il1r1	Il1rn	Il21	Il27	Il2rb
F	Il2rg	Il3	Il33	Il4	Il5	Il5ra	Il6ra	Il6st	Il7	Lta	Ltb	Mif
G	Nampt	Osm	Pf4	Spp1	Tnf	Tnfrsf11b	Tnfsf10	Tnfsf11	Tnfsf13	Tnfsf13b	Tnfsf4	Vegfa
H	Actb	B2m	Gapdh	Gusb	Hsp90ab1	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 3.5 PCR plate

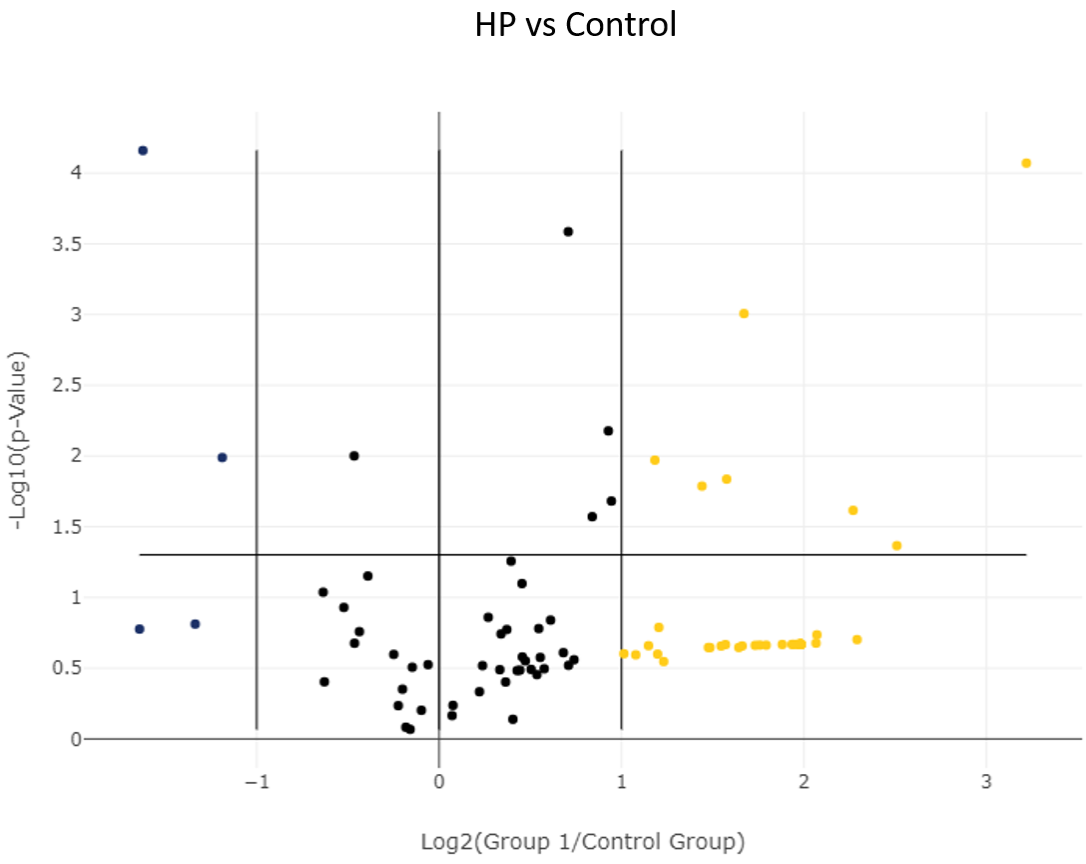


Figure 3.6 qPCR HP vs Control. Genes showing significant change between control and HP polyphenol extract treated cells.

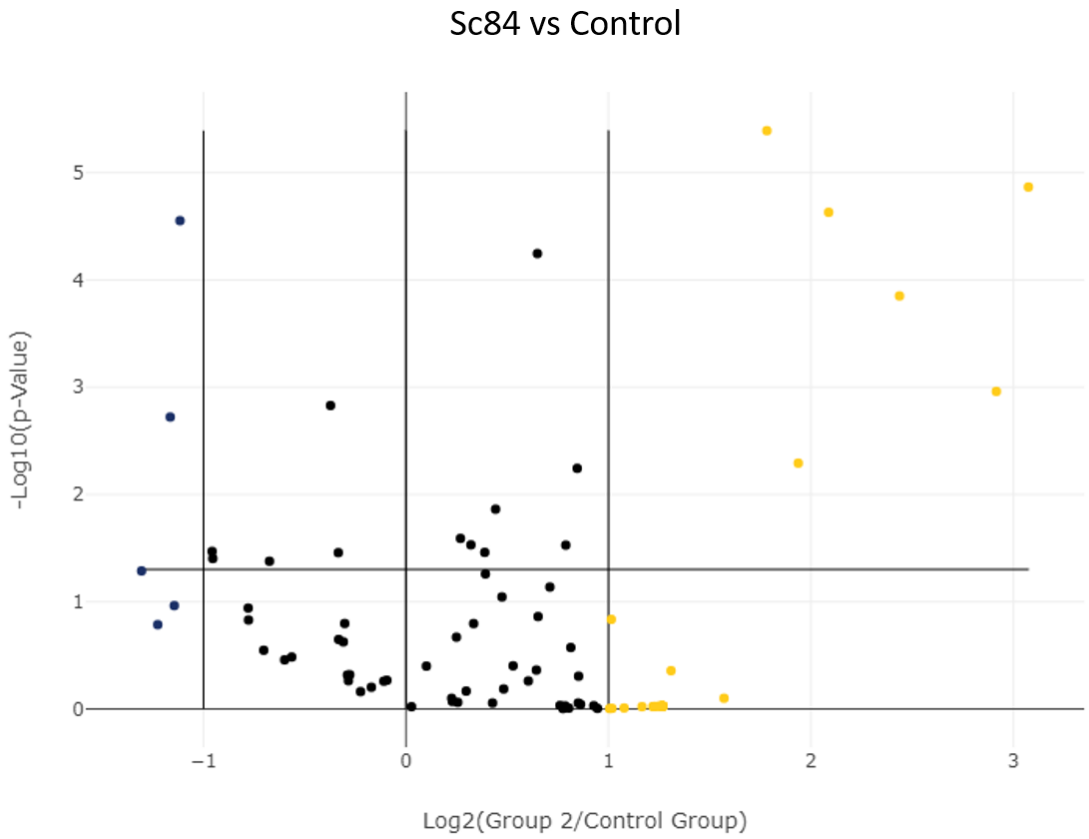


Figure 3.7 qPCR SC84 vs control. Data showing genes that showed significant change between the control and SC84 polyphenol extract treated RAW 264.7 cells.

There were 32 significantly upregulated cytokines when comparing LPS activated cells to the control (See table 3.1).

Table 3.1 qPCR results of LPS IFN γ activated cells compared to control

LPS IFN γ vs Control		
Gene	p-Value	Fold Change
Csf3	<0.000001	4451.27
Il1b	<0.000001	3198.84
Il1a	<0.000001	1473.47
Ccl12	0.002007	679.5
Ccl5	<0.000001	529.44
Cxcl9	0.000032	529.44
Il27	<0.000001	514.37
Il33	0.000001	458.25
Cxcl11	0.000020	223.63

Ccl7	<0.000001	188.27
Cxcl10	0.000046	155.6
Ccl8	0.000111	55.91
Ccl22	0.000005	48.62
Ccl6	0.000062	43.56
Il1rn	<0.000001	42.86
Csf1	<0.000001	37.4
Il15	<0.000001	14.77
Ccr1	0.000001	14.16
Ccl2	<0.000001	13.71
Tnf	0.000023	9.56
Ccl3	<0.000001	8.03
Vegfa	0.000051	5.18
Nampt	<0.000001	5.07
Il10ra	0.000009	5.05
Ccl4	<0.000001	5.02
Osm	0.000839	3.78
Il17f	0.009652	3.7
Tnfsf10	0.001035	3.51
Lta	0.020656	3
Ccr5	0.048055	2.92
Ccr3	0.018276	2.45
Il7	0.000951	2.06
Mif	0.000045	0.47
Aimp1	<0.000001	0.37
Spp1	0.000007	0.34
Il6ra	<0.000001	0.25
Pf4	0.000042	0.22
Tnfsf13	0.000046	0.22
Il16	0.000026	0.17
Tnfsf13b	0.000003	0.14
Ccr10	<0.000001	0.13
Cxcr3	0.000034	0.07

LPS and IFN γ activated cells treated with HP extract compared to LPS and IFN γ activated cells without the polyphenolic extract treatment had a greater number of down regulated genes than upregulated, with CXCL11 being the most decreased by 0.03 fold and Ccl17 was the most increased by 5.93 fold (see table 3.2). The SC84 LPS and IFN γ treated cells had a roughly equal amount of increased and decreased cytokines compared to the LPS and IFN γ treated cells. CCr5 was the most increased by 5.51 fold and CXCL11 was the most decreased again by 0.02 fold (see table 3.3). Both the SC84 and HP treated LPS and IFN y activated cells

had CCl17, Pf4, CCr5, OSM, TNFsnf13, TNF, Csf3, Ccl22, and Mif upregulated and Il1a, Csf1, Ccr1, Ccl5, Il15, Il27, Il6ra, Il1b, Cxcl9, Il33, Cxcl10, Il17f and Cxcl11 downregulated.

Table 3.2 - Activated HP cells compared to activated control

HP LPS IFN γ vs LPS IFN		
Gene	p-Value	Fold Change
Ccl17	0.026084	5.93
Pf4	0.024581	4.73
Ccr5	0.000279	4.22
Osm	0.008917	3.8
Tnfsf13	0.010019	3.44
Tnf	0.001635	2.9
Csf3	0.000643	2.72
Ccl22	0.001109	2.57
Mif	0.01329	2.24
Il1a	0.013685	0.38
Csf1	0.000761	0.34
Ccl8	0.029034	0.34
Ccr1	0.001548	0.32
Ccl5	0.000703	0.31
Il15	0.000026	0.29
Il27	0.000138	0.29
Il6ra	0.001516	0.28
Il1b	0.000622	0.21
Cxcl9	0.007616	0.15
Il33	0.000318	0.12
Cxcl10	0.004108	0.11
Il17f	0.005356	0.09
Cxcl11	0.001741	0.03

Table 3.3 - Activated SC84 treated cells compared to activated controls

SC84 LPS IFN VS LPS IFN		
Gene	p-Value	Fold Change
Ccr5	0.000649	5.51
Csf2	0.000102	4.91
Pf4	0.000293	4.01

Ccl17	0.017282	3.49
Ccr10	0.001943	3.48
Tnfsf13	0.002054	3.48
Tnfsf13b	0.001796	3.29
Tnf	0.00005	3.22
Csf3	0.000027	2.97
Mif	0.00133	2.83
Il5	0.025214	2.8
Ccl22	0.002133	2.74
Cxcr3	0.006587	2.12
Osm	0.012963	2.09
Ccl3	0.00322	2.06
Il10ra	0.003552	0.37
Ccl5	0.000887	0.34
Il16	0.001561	0.33
Ccr1	0.002173	0.33
Ccl6	0.019152	0.33
Il17f	0.015516	0.27
Il1a	0.000395	0.26
Il15	0.000014	0.21
Il27	0.000033	0.2
Csf1	0.000182	0.19
Il6ra	0.00017	0.18
Cxcl9	0.004854	0.17
Il1b	0.000049	0.15
Cxcl10	0.003865	0.1
Il33	0.000248	0.09
Cxcl11	0.001394	0.02

Western Blot results for autophagy

LC3 expression was measured in raw cells. There was a dose dependent trend in increasing expression of LC3 II as the concentration of the HP polyphenolic extract increased (fig 3.7). However, significance was only found in the highest concentration of 1.25 mg/mL of HP polyphenol extract when compared to the control and to those cells treated with only LPS and IFN γ.

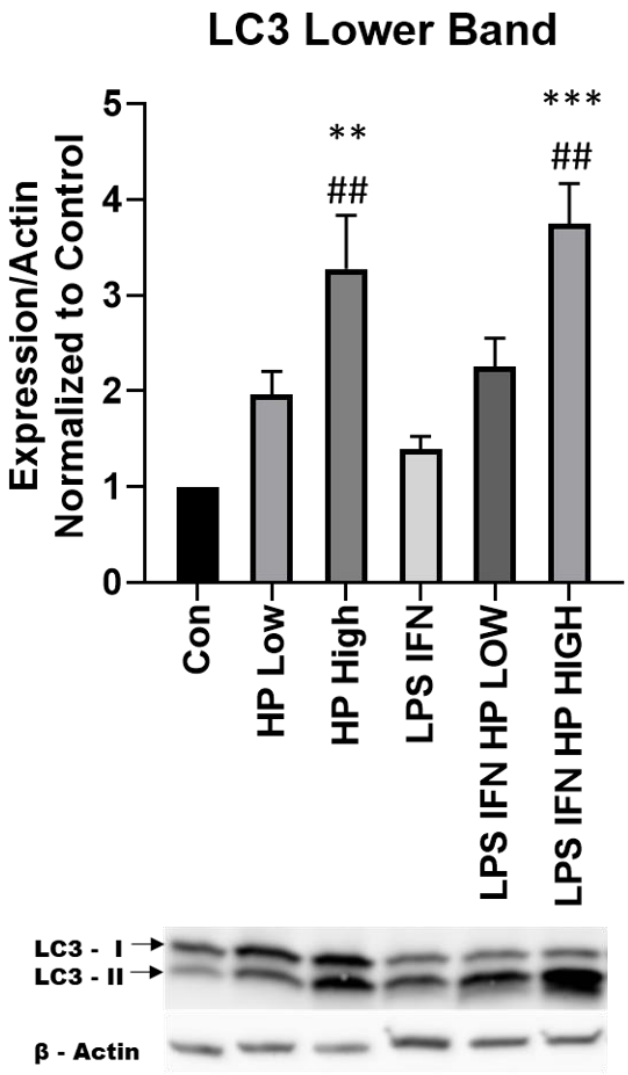


Figure 3.8 - LC3 expression in RAW 264.7 cells treated with 0.625 or 1.25 mg/mL phenolic extracted from Sorghum variety HP. Treating with high concentration extract results in greater presence of LC3 II when compared to control and LPS/IFNγ. This effect is even more pronounced in cells treated with the higher concentration of phenolic extract. ** P<0.01, ***P<0.001

* To control

to LPS/IFN

Discussion

The objective of this study was to evaluate the effect of two different sorghum varieties total polyphenolic extracts on inflammation using RAW 264.7 cells. Our results showed a decrease in cytokines Il6 and Il10 as well as a significant decrease in IFN γ despite exogenous

IFN γ being added to the cells. The reduction of IFN γ would suggest an anti-inflammatory effect of the sorghum polyphenols, as it activates macrophages and thus IFN γ reduction would reduce activation of macrophages and inflammation.

Il6 is a cytokine that is produced at sites of inflammation. The reduction of IL-6 observed in the cells treated with the sorghum polyphenols indicates an anti-inflammatory effect by the sorghum polyphenols through a reduction of STAT3 activation (Hodge, Hurt, & Farrar, 2005). Reduced STAT 3 activation is important because STAT 3 inhibits autophagy (You et al., 2015). A reduction of STAT 3 would also reduce its ability to inhibit autophagy. Il-10 reduction also indicates an anti-inflammatory effect as IL-10 plays a role in regulating the JAK-STAT signaling pathway and STAT3 is critical to the anti-inflammatory functions of IL-10 (Riley, Takeda, Akira, & Schreiber, 1999). Reduction of IL-10 signaling would result in a reduction in phosphorylation of STAT 3 which mediates expression of multiple pro-inflammatory cytokine genes such as TNF and IL-12B (Hutchins, Diez, & Miranda-Saavedra, 2013). However, it is important to note that IL-10 and IL-6 can also have pro-inflammatory effects in addition to anti-inflammatory effects (Luo & Zheng, 2016).For example, Il-10 can increase IFN γ expression in CD8+ T cells and IL-6 can reduce apoptosis, resulting in T-cell population increase (Mühl, 2013; Rochman, Paul, & Ben-Sasson, 2005). G-CSF also increases when treated by the extracts, indicating potential reduction in STAT3 activation, as the STAT3 activation is necessary for G-CSF induced cell proliferation and differentiation (McLemore et al., 2001).

The QPCR data shows that the mRNA expression for IL1a and IL1b were downregulated in the LPS and IFN γ activated cells treated with HP and SC84 polyphenolic extract compared to the activated cells treated with vehicle. In addition, the TNF gene was upregulated in both. IL1a

and IL1b were both shown to have a significant increase in production in LPS and IFN γ activated cells treated with HP sorghum extract, while in SC84 treated cells, only IL-1B had a significant increase. Il-1B is a known inducer of IL-6 secretion in monocytes, fibroblasts, endothelial cells, and keratinocytes, however, Il-6 levels were significantly reduced (Hodge et al., 2005) (Tosato & Jones, 1990).The down regulation of IL-1A and IL-1B expression may initially seem contradictory to the ELISA results which show increased levels of these two cytokines however this can be explained via the nature of Raw 264.7 cells. Raw 264.7 cells are a poor model for IL-1 production due to a dysfunctional inflammasome (Pelegrin, Barroso-Gutierrez, & Surprenant, 2008). The raw 264.7 cells do not have the apoptosis-associated speck-like protein containing a C-terminal caspase-activating recruiting domain (ASC) adaptor protein necessary for effectively activating the inflammasome.

LC3 II increased in expression as the HP polyphenol extract concentration increased. LC3 has a central role in autophagy, and an increase in the expression of LC3II would indicate that there is an increase in the presence of autophagosomes. LC3 II is the cleaved form of LC3 that has attached to the surface of the autophagosome. This suggests that the morphological changes observed in the raw cells using the light microscope most likely represented autophagic process, at least in part.

In conclusion, the results of this study indicate that the polyphenols present in the novel High polyphenol and SC84 sorghum grains modulate inflammation in LPS/IFN γ activated RAW 264.7 cells. Further research should be done to evaluate the impact of sorghum polyphenols on other inflammation associated pathways and transcription factors, such as the MAPK pathway and NF-KB. Impacts of sorghum polyphenols on cyclooxygenase-2 (COX-2) production should also be explored to further understand the effect of polyphenols on inflammation.

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Chapter 4 - Discussion and conclusions

The purpose of the research presented was to evaluate and optimize the sorghum polyphenol extraction process, and to evaluate the bioactivity and immunological effects of the polyphenolic compounds of a novel high polyphenol black sorghum and high polyphenol brown sorghum extracted using the optimized assay.

Extraction optimization

The evaluation of the extraction process assessed the impact of multiple variables within the assay on the efficiency of extracting the polyphenolic compounds as well as how the bioactivity and anti-proliferative effects of the compounds may be affected. Variables assessed included ethanol levels in the solvent, the presence of citric acid in the solvent and temperature of the bran and solvent mixture during the shaking process.

The HP sorghum extract had the highest levels of polyphenols, followed by the commercial black and the commercial sumac, respectively. However, while HP sorghum extract also had the strongest anti-proliferative effects, commercial sumac had stronger anti-proliferative effects than commercial black extract, despite having lower levels of total polyphenols. This indicates that the composition of polyphenolic compounds present varies significantly, and that those present in higher levels in the commercial sumac are have stronger anti-proliferative effects than those in the commercial black.

The effect of temperature in the extraction process was not discernable during the quantification of total polyphenolic compounds using the Folin-Ciocalteu assay; there was no significant differences in total polyphenol levels between samples prepared at the different temperatures. However, temperature did affect the bioactivity of the polyphenols. The HP and

commercial sumac extracts both showed significantly reduced anti-proliferative effects on the HCT15 cells when prepared at 60^{o} C compared to 20^{o}C. the HP extract anti-proliferation effect was also significantly reduced at 40^{o}C. As the increase in temperature proved diminish polyphenol's bioactivity *in vitro,* 20^{o}C was deemed the optimal extraction temperature.

The solvent composition played a critical role in the quantity of total polyphenols extracted. There was some variability of total phenolic content among the six solvents tested. However, significant consideration was given to the effect of the different solvents on the bioactivity of the polyphenols. Solvent E, 70% ETOH and 5% citric acid, was selected as the most efficient solvent; however, it did have significantly lower polyphenolic levels compared to the other solvents in the HP extracts. It was selected despite this as the extracts made using this solvent demonstrated the strongest anti-proliferative effects.

Green tea was included in this study to compare to the sorghum extracts. There has been a great deal of research into the anti-cancer effects of green tea and would provide strong context with which to compare the sorghum polyphenols. When using the optimized extraction solvent, the HP sorghum extract had a significantly stronger anti-proliferative effect on both the HEPG2 and HCT15 cells than the green tea extract. However, the green tea extract did inhibit the cell viability of the HCT15 and HEPG2 cells at the highest concentrated treatment more effectively than the commercial sorghum varieties but not the novel high phenolic variety of sorghum.

Immunological effects of polyphenolic extracts

Raw cells were chosen for this study as they are mouse cells that exhibit macrophage like behavior and are well-established in the study of inflammatory and anti-inflammatory responses using plant extracts (Koh, Cha, Ko, Park, & Choi, 2010; Li et al., 2018; E.-J. Yang, Yim, Song, Kim, & Hyun, 2009). The inflammatory effects of the novel HP and the SC84 extracts on RAW

264.7 cells were measured for exposure over time. Nitric oxide levels were not significantly affected in any concentration of extract treatment. As an increase in nitric oxide production would be an indication of increased inflammation, this data indicates that although the extracts do not demonstrate pro-inflammatory actions here, they also do not demonstrate anti-inflammatory effects either. Large vacuole-like structures were observed forming over a 12-hour period in RAW 264.7 cells treated with the HP and SC84 extracts. These structures were significantly more prominent in the cells cotreated with IFN-Y, than the cells that had been treated only with sorghum extracts.

The LPS.IFNY significantly increased both IL-6 and IL-10 but when the samples were cotreated with extracts and LPS/IFNY, the increase in IL-6 and IL-10 was attenuated. Both of these cytokines are known to have some pro-inflammatory but are primarily anti-inflammatory and play roles in initiating and regulating the JAK-STAT pathway (Heinrich et al., 2003; Kai et al., 2005; O'Farrell, Liu, Moore, & Mui, 1998). Il-6 can help tumors circumvent the immune system through STAT3 activation inside tumor cells, increasing anti-apoptotic genes as well as pro-proliferative and angiogenic genes (Wang et al., 2009; Yu, Kortylewski, & Pardoll, 2007). Similarly, IL-10 acts through STAT3 as well in tumor cells. Tumor associated macrophages (TAMs) produce high levels of IL-10, inhibiting the anti-tumor response of T cells (C. Yang et al., 2015). Increased STAT3, which is activated by IL-10, can desensitize tumors to chemotherapy and high levels of IL-10 are indicators of poor prognosis. The JAK-STAT pathway induces the production of more pro-inflammatory cytokines as well as increasing cell proliferation. The reduction of IL-6 and IL-10 in turn reduces the activation of the pathway. This could possibly reduce cell apoptosis and production of pro-inflammatory cytokines (Capiralla et al., 2012). However, there is also a significant increase in IL-12 at the highest dose of both

extracts. IL-12 is a pro-inflammatory cytokine associated with the activation and regulation of multiple innate immune system cells, such as T-cells and natural killer cells (Sun, He, Nair, Yeung, & Egwuagu, 2015).

The qPCR results covered an extensive range. IL-6ra was decreased in both the HP and SC84 treatments, which, together with the ELISA results, provides further evidence that IL-6 signaling was reduced. Additionally, qPCR showed that TNF expression is significantly increased in both HP and SC84 treated activated cells. However, IL-1a and IL-1b were both downregulated in HP and SC84 treated RAW 264.7 cells that were activated. Both of these cytokines are pro-inflammatory. However, increased levels of IL-1a and IL-1B were observed in the ELISA results. This can be explained by the fact that raw 264.7 cells are poor models to study IL1 responses in. Raw 264.7 cells are known to have dysfunctional inflammasomes due to a lack of the ASC adaptor protein (Pelegrin et al., 2008). Due to the dysfunctional status of the inflammasome, IL-1A and IL-1B are not able to be produced effectively in raw 264.7 cells, and thus are not a good model to measure these cytokines in.

The protein expression of LC3II was significantly increased when treated with 1.25 mg/ml HP phenolic extract, indicating a potential increase in autophagy. This increase may assist in explaining the extensive vacuole-like formation observed over time in the RAW 264.7 cells. Autophagy begins with the formation of vacuole-like autophagosomes, which may be part of the vacuolization observed in the RAW 264.7 cells (Khandia et al., 2019). However, because this measurement is from a single time point, it is possible that the conversion of LC3 I to LC3II is occurring but that autophagy is not occurring and thus LC3II is continuously increasing. In order to measure whether autophagy is truly taking place, LC3II levels must be measured over time using control cells treated with lysosomal inhibitors such as bafilomycin A. This would prevent

the breakdown of the autophagosome contents including LC3II. If differences occur between these results, autophagic flux is likely to be occurring (Yoshii & Mizushima, 2017).

Future Directions

There is a great deal of promise in the future of sorghum. As it has been bred less than many other types of cereal grains, it has significantly more diversity than other grains. Additionally, as there is a growing interest in the consumption functional foods for health purposes, the investigation into the potential benefits of sorghum has a growing audience. The development of sorghum lines with the intent to increase specific phytochemicals with regards to specific health benefits would certainly promote interest in sorghum and encourage economic investment in further research.

Another area of potential is the use of sorghum polyphenols as a vaccine adjuvant. Adjuvants are substances used to increase the immune response to antigens in order to defend against viruses and cancer. There are other types of polyphenols that have shown benefits as adjuvants. For example, Brazilian green propolis which demonstrated increased cellular and humoral responses in mice when used as an adjuvant in a SuHV-1 vaccine and compared to the same vaccine using aluminum hydroxide as a control (Fischer et al., 2010).

The novel high polyphenolic black sorghum line has shown significant promise in anti-proliferative and anti-inflammatory studies, as has the SC84 sorghum line. However, more research is required to characterize the polyphenolic compounds within these lines. To better understand the effects of sorghum polyphenols on inflammation, its effects on Nuclear Factor-κB (NF-κB) signaling should be evaluated as it is part of the pro-inflammatory response. Measuring the effects of sorghum polyphenols on STAT3 would also be interesting as it is downstream of

IL-6 and IL-10. An additional measurement of sorghum polyphenol inflammation effects would be to measure activity of cyclooxygenase-2 (COX-2) as it is a key mediator of inflammatory pathways.

Conclusions

A thorough evaluation of numerous variables within the total polyphenolic extraction process led to the determination that a 70% ethanol and 5% citric acid solvent prepared at 20^{o}C provides the most efficient extraction of the total phenolic content from sorghum bran, without causing an undesired reduction in anti-proliferative effects. Additionally, the HP, CB and CS varieties of sorghum all showed anti-proliferative properties, though HP showed significantly higher effects. When RAW 264.7 cells were treated with HP and SC84 polyphenolic extracts, there was reduced production of key pro-inflammatory cytokines IL-6 and IL-10, although there was no significant change in nitric oxide production. There was extensive vacuolization within the RAW 264.7 cells, and an increase in LC3 II expression indicating increased autophagy (Tanida, Ueno, & Kominami, 2008). The optimization of the food-grade extraction process may be beneficial for further sorghum polyphenol research requiring total polyphenol extraction, and the immunological effects of these polyphenols may be used to help develop treatments such as use as an adjuvant or dietary supplement.

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