THESIS

SWEET SORGHUM (*SORGHUM BICOLOR*) BIOMASS, GENERATED FROM BIOFUEL PRODUCTION, AS A RESERVOIR OF BIOACTIVE COMPOUNDS FOR HUMAN HEALTH

Submitted by

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ABSTRACT

SWEET SORGHUM (*SORGHUM BICOLOR*) BIOMASS, GENERATED FROM BIOFUEL PRODUCTION, AS A RESERVOIR OF BIOACTIVE COMPOUNDS FOR HUMAN HEALTH

Sorghum bicolor is the fifth most cultivated cereal crop worldwide[1]. Varieties of *S bicolor*, known as grain sorghum, are cultivated for human food and animal feed. Certain varieties, known as sweet sorghum, concentrate sugar in the pith cells of the stalk and are cultivated for sugars and syrups. Recently, interest has grown in the use of sweet sorghum as a feedstock for biofuel production. This is due to reduced water requirements, shorter growing periods and reduced cultivation cost when compared to other feedstocks[1]. Biofuel production of sweet sorghum generates large masses of biological wastes, comprised of stalks, leaves, leaf sheaths and seed heads[2]. This biomass could serve as a potential reservoir for bioactive compounds for human health. Rich in phenolic acids and flavonoids, these parts of the sorghum plant have been used as a traditional medicine in African and Asian cultures for the treatment of various disease states, including cancer[3]. Furthermore, recent studies have elucidated *in vitro* and *in vivo* antiinflammatory, antioxidant, and anticancer activities of the seed head, leaf and leaf sheath[4–11]. Much less is known about the *in vitro* and *in vivo* bioactivities of the stalk components of sweet sorghum varieties. Here, the *in vitro* anticancer activity, and the *in vivo* antiinflammatory and antioxidant activity of sweet sorghum with a focus on the dermal layer, a component of sweet sorghum stalk, were investigated. It was hypothesized that the dermal layer contains phenolic compounds with antiproliferative, proapoptotic and

antiinflammatory/antioxidant properties. Given the high rates of cancer incidence and mortality, as well as the close connection to a Western diet, *in vitro* colon cancer cell models and *in vivo* Western diet induced obesity and oxidative stress models were utilized for the purposes of this study.

For *in vitro* studies, the colon cancer HCT116 cell line and colon cancer stem cells (CCSCs), and their p53 variants HCT116 $p53^{-/-}$ and CCSCs p53 shRNA, were treated with different doses of phenolic rich extracts from the stalk components (pith and dermal layer), the leaves, and the seed head. Phenolic compounds are ubiquitous throughout the plant kingdom, consistently display bioactivity, and their antioxidant activity has been linked to the anticancer activity of phenolics. Total phenolics and antioxidant activities were determined by the Folin-Ciocalteu and 2,2`-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays, respectively. Individual phenolic compounds were identified by LC-MS analysis. Increased proliferation and suppressed apoptosis are two important hallmarks of cancer and are commonly targets of anticancer therapies. Given this, cancer cell proliferation was assessed with cell counting and BrdU assay, and apoptosis was assessed by Caspase 3/7 Glo, PARP cleavage and TUNEL assays. The colony formation assay was used as a measure of cell stemness in CCSCs. Western blotting was used to determine protein levels associated with the β-catenin signaling. We first show that extracts from sweet sorghum dermal layer stimulated apoptosis and suppressed cellular proliferation in CCSCs and HCT116 cells, more so than the pith. Furthermore, the dermal's activity was diminished, but not abrogated in p53 variants of colon cancer cells indicating partial p53-dependency. The dermal layer reduced cancer cell stemness by decreasing colony formation of CCSCs, indicating anticancer activities beyond suppressing proliferation and inducing apoptosis. Interestingly, the dermal layer had more 3-

deoxyanthocyanidins than pith and this was associated with increased activity. These compounds are reported to have potent anticancer and phase II enzyme inducing activities[8]. Further investigations were carried out in the dermal layer, leaf and seed head to directly compare anticancer activities from these extracts. The molecular mechanisms for apoptosis were also investigated in the dermal layer and seed head, the most potent proapoptotic sweet sorghum components. Western blotting revealed that elevation in apoptosis was correlated with concurrent suppression of β-catenin ($r = -0.920$; $P < 0.01$), β-catenin's down-stream prosurvival targets c-Myc ($r = -0.922$; $P < 0.01$) and Survivin ($r = -0.519$; $P < 0.01$). Together, our data suggests that apoptosis was achieved via favorable modulation of aberrant β-catenin signaling.

For *in vivo* studies of inflammation and oxidative stress, the murine Western/high-fat diet (HFD; 40% kcal fat) induced obesity and oxidative stress model was utilized. Male and female A/J mice were provided with HFD and low-fat diet control (LFD) for 10 weeks with and without 1% sweet sorghum stalk (dermal layer) extract (SS). During the treatment period, weekly measures of bodyweight, feed intake and water intake were collected. At the end of the study, dual energy X-ray absorptiometry (DEXA) was performed to assess adiposity. After treatment period, mice were sacrificed, vital organs weighed, and tissues and plasma collected for further analysis. The inflammatory markers NF-κB, tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and IL-1β, the macrophage marker F4/80, and monocyte chemotactant protein-1 (MCP-1) were assessed in the colonic mucosa by qPCR. Barrier function of the colon was assessed by measuring the tight junction proteins, occludin and zona occluden-1 (ZO-1), by qPCR. Oxidative stress was assessed in the plasma by levels of 8-isoprostane (8IP). Feeding of SS extract in both LFD and HFD consuming groups did not negatively influence bodyweight, feed intake, water intake, and vital organ weights, indicating that SS extract was well-tolerated in male and female

A/J mice consuming HFD and LFD at 1% of diet. SS extract also reduced the systemic levels of oxidative stress as measured by plasma 8-isoprostane levels. SS extract did not improve adiposity in HFD consuming mice.

It was demonstrated that the stalk (pith and dermal layer), leaf, and seed head contain phenolic and antioxidant compounds conferring anticancer properties in colon cancer stem cells, supporting the hypothesis. This activity was most prominent in the dermal layer and seed head. Furthermore these extracts were shown to act via decreasing β-catenin and β-catenin's prosurvival target genes. Additionally, we have demonstrated phenolic rich extract from dermal has *in vivo* antioxidant activities, and was well-tolerated in a high-fat diet mouse model of obesity and oxidative stress. More research is still needed to further elucidate the mechanisms through which sweet sorghum derived bioactive compounds favorably alter β-catenin levels *in vitro* and oxidative stress *in vivo.* Together this data suggests that sweet sorghum, an attractive source of fermentable sugars for biofuel production producing large quantities of biomass, is also an attractive reservoir of bioactive phenolic compounds with human health benefiting properties.

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I would like to thank Edward Settle of Great Valley Energy for his support in this project. He was a driving force of inquisitive enthusiasm and was a source of inspiration. He and Great Valley Energy supplied processed sweet sorghum materials and funding for this study, thus making it possible, and more importantly, enjoyable.

I must thank all the many members of the Vanamala lab who have helped me along the way. Your help is immeasurable. Without such assistance, this work would surely be weakened. I wish I had the space to include all the ways in which you assisted my efforts. I shall hope that listing your names will suffice. In alphabetical order by last name; Fauzi Amer, Amulya Balumuri, Maria Kasdagly, Guarav Madiwale, Laura Markham, Orlando Perez, Emily Poole, Sridhar Radhakrishnan, Venkata Rohit, Dr Ramakrishna Vadde, and Xia Xiao.

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Finally, I would like to thank the scientific giants who lent me their shoulders to stand on as I have taken on this scientific endeavor.

DEDICATION

This work is dedicated to my best friend and wife, Saja Hindi; to my parents, Janet Taylor and Charles Massey; to my siblings, Chris Massey, Derrick Hudson, Heather Brink, Tim Massey, and Steve Massey; and to my in-laws, Iyad Hindi, Samar Shawa, Abdulrahman Hindi, and Doha Hindi

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CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction.

Sorghum bicolor is the fifth most cultivated cereal crop worldwide[1]. Varieties of *S bicolor*, known as grain sorghum, are cultivated for human food and animal feed. Other varieties, known as sweet sorghum, concentrate sugar in the pith cells of the stalk. As a consequence, sweet sorghums have thicker taller stalks with smaller seed heads[12]. Recently, interest has grown in the use of sweet sorghum as a feedstock for biofuel production due to reduced water requirements, shorter growing periods and reduced cultivation cost when compared to other feedstocks[1]**.** Furthermore, sweet sorghum can grow in marginal lands, and therefore does not have to compete with current food production, as is the case with corn and sugar cane[1,13]. Production of biofuel from sweet sorghum can be made more attractive when producers utilize the biorefinery approach[14]. In this approach, the entire above ground portion of the plant is utilized in the creation of energy and value-added products, minimizing waste, and thereby reducing the costs associated with the generation of biofuels[15]. Such products could include bioactive extracts prepared for the purposes of human health.

Biofuel production of sweet sorghum generates large masses of biological wastes comprised of stalks, leaves, leaf sheaths and immature seed heads[2]. Establishing biorefinery processes for sweet sorghum and other sources of biomass has been the center of recent investigations and reviews[14–16]. These publications have focused on: Creation of ethanol; Generation of steam power from combustion; Generation of butanol; Creation of fiber products and wood plastic composites; Production of nanomaterials; etc[14,15]. Recently, there has been an increasing interest in the use of biomass as a source of bioactive plant metabolites for human

health and other purposes[17]. These bioactive metabolites may include phenolics, carotenoids, glucosinolates and others depending on the source of bioactive compounds. Phenolics, a broad class of plant compounds that include one or multiple phenolic ring structures, are broadly spread throughout the plant kingdom[18]. It has been well reported that stalks, leafs and seed heads of sorghum contain phenolics[4–7] with bioactivity[15–17]. However, most of the information pertains to grain sorghum varieties, not sweet sorghum varieties. In addition, these products, particularly the leaves and leaf sheaths, have been used by traditional healers in Africa and India for anemia, epilepsy, stomach ache, and interestingly, cancer[3].

Recent evidence suggests that bioactive compounds (e.g. phenolic acids, flavonoids, stilbenoids, carotenoids) confer the protective and preventive effects against chronic diseases, such as cancer, associated with a plant-based diet[19]. Colorectal cancer is one such disease and is estimated to be the second most common cancer in females and third most common in males in the US[20]. Furthermore, it has been estimated that up to 80% of colorectal cancers are caused by diet and dietary factors[19]. This understanding has increased the demand for information regarding the role of dietary constituents in the prevention and treatment of diseases, such as colorectal cancers. In fact, there are multiple clinical trials investigating the preventive and therapeutic roles of curcumin, resveratrol, and n-3 polyunsaturated fatty acids for colon cancer[21].

The process by which normal colonic epithelium progresses to colon cancer is multi-step, whereby cells accumulate successive genetic alterations, establish clones and gain a proliferative advantage[22]. The traditional pathway to colorectal cancer accounts for 70% to 85% of all cancers and starts with loss of APC function[22], which results in accumulation of β-catenin and aberrant WNT/β-catenin signaling. This aberrant β-catenin signaling occurs in almost all cases and drives colorectal carcinogenesis[23,24]. Loss of APC is then followed by mutation of K-ras, loss of 18q,

and finally loss of p53 via 17p loss[22]. p53 is responsible for cell cycle arrest and apoptosis[25]. Therefore, it prevents the build-up of mutated genomes and the advancement of cancer. p53 is abnormal in 50% to 75% of all colorectal cancers, and this marks the transition from noninvasive to invasive disease[22]. Furthermore, loss of p53 disrupts normal tumor responses to conventional therapies, such as 5-Fluorouracil, thus making it difficult to treat these advanced tumors with conventional therapies[26]. Bioactive compounds are often found in complex mixtures in nature[27], and, unlike a single therapeutic agent, contain the ability to influence multiple cellular pathways. Mixtures such as green tea extract and grape seed extract with resveratrol have demonstrated anticancer activity that act in both p53-dependent and p53-independent mechanisms, pointing to the ability of these mixtures to influence multiple pathways[28,29]. These findings warrant further investigations into uncovering natural sources of potential chemotherapeutic and chemopreventive agents.

Inflammation and inflammatory conditions of the bowels have been strongly linked to colon cancer[30–32]. In all patients with inflammatory bowel disease (IBD), 20% will develop what is known as colitis-associated cancers[33]. These cancers are a subset of colorectal cancers associated with IBD with a similar carcinogenesis to that of sporadic colorectal cancers[33]. Non-colitis-associated colorectal cancers (i.e. sporadic colorectal cancer) have much in common with colitis-associated cancer, including increased inflammatory cytokine expression and immune cell infiltrates[33]. Inflammation promotes the production of reactive oxygen species, which cause DNA oxidative damage[34], and this may be a source of somatic mutations promoting tumor progression. Furthermore, inflammatory signaling can be mitogenic, promoting the growth of pre-cancerous and cancerous cells[34,35]. Obesity is a condition associated with chronic inflammation and oxidative stress, and this may be important in the promotion of obesity-related

cancer[36]. The prevalence of obesity is high and it is estimated that 20% of cancer cases are attributable to obesity[37,38].

Intriguingly, there many studies documenting the antiinflammatory and antioxidant properties of bioactive compounds. For example isothiocyanates and curcumin decreased NF-κB signaling[39]. Grapefruit bioactives suppressed COX-2 expression and colon carcinogenesis in an azoxymethane model of colorectal cancer[40]. Whole plant foods, such as berries, broccoli, and grape fruit, are reported to have beneficial properties for obesity-enhanced cancers[36]. Luteolin, a flavonoid found in *S bicolor* decreased COX-2 expression in a rodent model of skin cancer[41].

Developing new and sustainable sources of bioactive compounds with therapeutic value will be important in the future treatment and prevention of chronic disease. Given the need for non-conventional therapies in the treatment of colorectal cancer, the prevalence of high-fat diet and obesity in the general public, and the general lack of information with respect to sweet sorghum bioactives, the anticancer properties of sweet sorghum were investigated in *in vitro* models of colorectal cancer, and the antiinflammatory and antioxidant properties in an *in vivo* murine model of high-fat diet induced obesity and colonic inflammation.

1.2 Literature Review.

1.2.1 The Sweet Sorghum Plant.

Placing fifth worldwide in cereal production[1], *Sorghum bicolor* is a cereal of high genetic variability[42], resulting in high morphological and phenotypic variation[43]. This variation results in varieties of *Sorghum bicolor*, called sweet sorghum, which concentrate simple sugars (glucose, fructose and sucrose[12]) in the pith cells of the stalk[1,43]. As a result of this sugar storage, sweet sorghum varieties typically have taller, thicker stalks and smaller seed heads[12]. This can be seen in Figure 1. Given these unique characteristics, it is no surprise that sweet sorghums are used to

produce a myriad of products, including syrups, sugars, alcohol, paper, fencing, roofing, chewing, and forage[12,16]. Sweet sorghums, and other *S. bicolor* ssp., are C4 plants characterized by high photosynthetic efficiency, producing high quantities of biomass[44]. Biomass of sweet sorghum includes stalk (pith and dermal layer), leaves, leaf sheaths, and seed head.

Figure 1. A cross-section of sweet sorghum stalk and a crop of sweet sorghum. (Left) A cross-section of a sorghum stalk illustrating the outer dermal layer and inner pith section. Adapted from[45]. A crop of grain sorghum next to a crop of sweet sorghum (Right).

In recent decades, interest in the use of sweet sorghum as a feedstock for biofuel has increased[16,46,47]. Sweet sorghum is has been recommended as an alternative biofuel, as it can be advantageous to other feedstocks depending on location and grower's needs (Table 1)[1]. Sweet sorghum has a shorter growing period of 4 months and can be harvested multiple times in one growing season[1]. Sweet sorghum can grow on marginal lands not used for food production[48]. Sweet sorghum also has reduced water requirements, reducing the cost of irrigation when compared to corn and cane sugar[1]. This is in contrast to corn ethanol, as the edible starches are converted to the fermentable sugars needed for ethanol production. Additionally, sorghum directly produces fermentable sugars, bypassing unnecessary enzymatic conversions of starch.

These characteristics result in a feedstock for biofuel production requiring less water and financial cost to produce[1]. This combination may prove sweet sorghum as an attractive alternative to other biofuel feedstocks.

Table 1. Comparison of sweet sorghum biofuel production to other feedstocks.

parameter	sweet sorghum	sugarcane	maize
crop duration (months)	4	12	4
water needs (m^3/ha)	4000	36000	8000
grain yield (t/ha)	2.0	--	3.5
green stalk yield (t/ha)	35	75	45
cultivation cost with irrigation ($/ha)	238	995	287
total ethanol (L/ha)	3160	8925	3216
ethanol cost ($/kL)	75.3	111.5	89.2

Adapted from and further reviewed in[1].

The biorefinery approach to biofuel production is a concept, which mimics the traditional oil refinery, makes use of the entire above ground portion of the plant to produce energy and high value products, reducing the cost of biofuel production[15]. With respect to sweet sorghum, investigations into applying the biorefinery approach to sweet sorghum have focused on: 1) The creation of ethanol; 2) The generation of steam power from combustion; 3) generation of butanol; and 4) creation of fiber products and wood plastic composites[14,15]. Figure 2 illustrates one such example from[14]. Recently, there has been an increasing interest in the use of plant secondary metabolites, which have inherent health benefits, as valuable biofuel co-products[17]. For example, phenolics are a broad class of plant secondary compounds that include a single or multiple phenolic ring structures, and are found throughout the plant kingdom[18]. Furthermore,

these phenolics compounds have been reported to be present in various sources of sorghum biomass (stalk, leaves, leaf sheaths, grain, glume, etc).

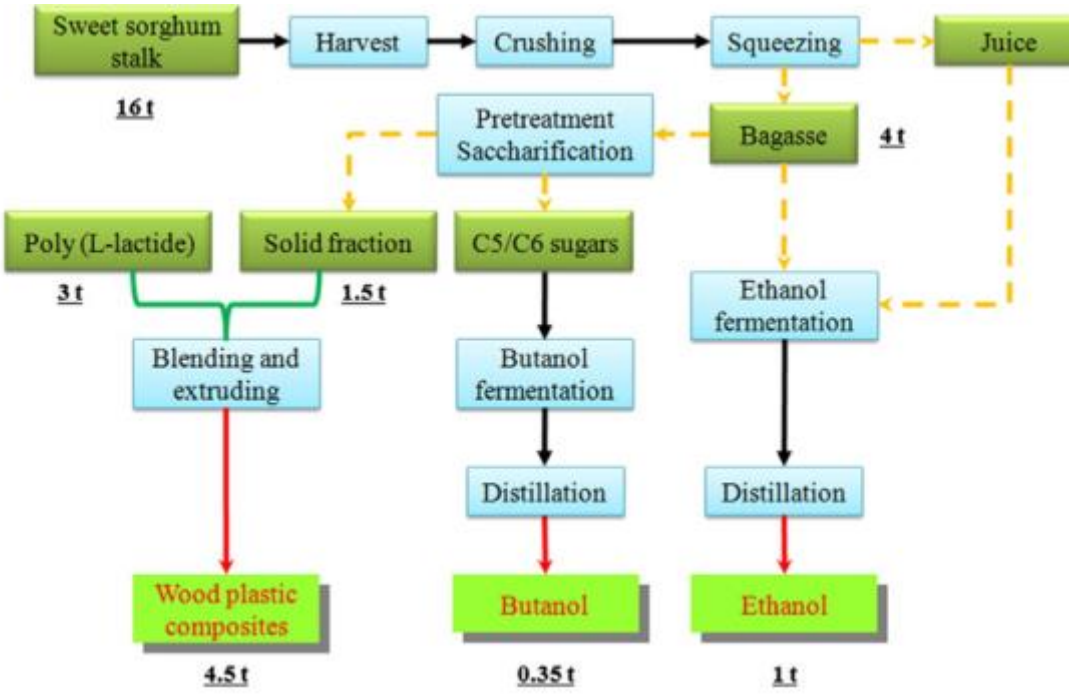


Figure 2. A schematic drawing of the biorefining sweet sorghum stalk. Adapted from[14].

In a process developed by Great Valley Energy LLC, the stalk of sweet sorghum is cut into approximately 30 cm segments, called billets. These billets are further processed into sugary pith cells from the dermal layer as illustrated in Figure 3. The simple fermentable sugars are then separated from the pith cells and are fermented into ethanol. This leaves large amounts of dermal layer from the stalk of sweet sorghum, which has been proposed to be used for creating wax products, pulp, paper, and other products (Figure 4). Currently, there is relatively little known with respect to the *in vitro* and *in vivo* bioactive compounds and bioactivity properties of the stalk of sweet sorghum. Given the unique opportunity for this important component to serve as a source of bioactive compounds for human health, more research is needed to assess these aspects of the sweet sorghum plant.

Figure 3. Dermal layer (left) and pith (right) of sweet sorghum stalk after processing.

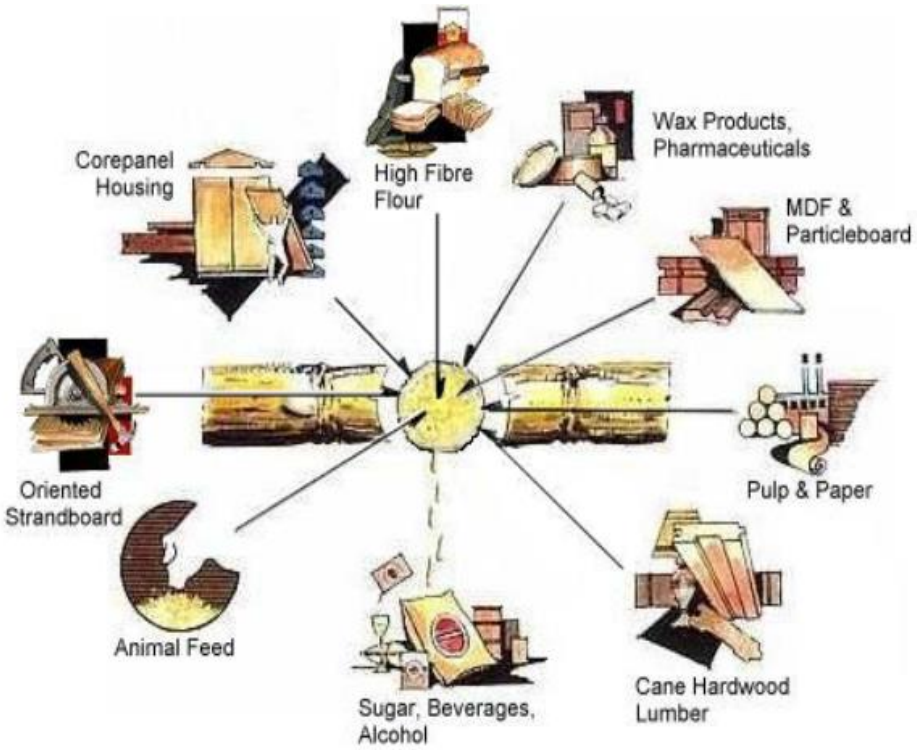


Figure 4. Proposed uses for sorghum stalk by Great Valley Energy, LLC.

1.2.2 Sorghum Phenolics and Bioactivity.

Phenolics represents a broad class of compounds containing one or more aromatic ring structures with one or more hydroxyl substitutions[18]. *Sorghum bicolor* contains phenolic compounds with antioxidant activity, which confer protective effects to the sorghum plant[49]. When consumed by humans, these same phenolic compounds confer health benefits, referred to here as bioactivity[50]. Phenolic compounds have the ability to reduce free radicals, quenching free radical species and terminating free radical chain reactions, which can promote, among many things, damage of genomic DNA[18,51]. Total phenolics (TP) and antioxidant activity (AOA) can therefore be measured in a phenolic rich extract by the ability of the extract to reduce a substance by donating protons or electrons.

The Folin-Ciocalteu assay is used to assess the level of TP. It is based on the ability of a source of phenolics to participate in a hydrogen atom transfer to reduce the Folin-Ciocalteu reagent comprised of phosphomolybdate and phosphotungstate creating a blue color with a maximum absorbance at 765 nm[52]. Gallic acid is by far the most common standard phenolic compound used for quantification purposes in the Folin-Ciocalteu assay. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) are stable free radical pigments, which are reduced in the presence of antioxidants to form a colorless product[53]. The disappearance of free radical can therefore be measured by a spectrophotometer. Assays based on the quantification of the disappearance of the free radical species can quantify the AOA of an unknown to that of a standard solution. Trolox is most commonly used in these assays for the quantification of antioxidant activity. The oxygen radical absorbance capacity (ORAC) assay is another method for measuring AOA of a substance. It is based on the ability of a substance to prevent the oxidative degeneration of a fluorescent

molecule, typically fluorescein. Like DPPH and ABTS, Trolox is used as a standard antioxidant and used for quantification purposes.

Table 2 summarizes previous efforts to quantify TP and AOA from different components of *S. bicolor* separated by the type of sorghum used in the study. The levels of TP and AOA in sorghum range widely and depend on the component of sorghum extracted, as well as genotype. The grain has been the most studied with respect to TP and AOA. TP and AOA values have been reported to range from 2 to 9 mg GAE/g (Folin-Ciocalteu assay)[54] and from 5 to 85 μmol TE/g (DPPH assay)[6,55] in the grain of grain sorghum, respectively. The grain of dye sorghum contains much higher TP and AOA with reported values as high as 23 mg GAE/g and 147 μmol TE/g (DPPH), respectively. The presence of concentrated polyphenolic pigments, known as anthocyanins, may be responsible for these observations, as they can contribute to TP and AOA. Furthermore, antioxidants seem to be more concentrated in the bran, which makes up the hard outer layer of the grain, containing up to 782 μmol TE/g (ABTS)[6]. Other parts of the sorghum plant, including the leaves and stalk of sorghum have been reported to contain 11.7 and 1.0 mg GAE/g of total phenolics, respectively[56]. The leaf sheaths of dye sorghum have been reported to contain as much as 135 mg GAE/g[4]. This is also due to the presence of anthocyanins, which are concentrated in the leaf sheaths of these varieties of sorghums. These data reflect the high variation of TP and AOA inherent in the different varieties and components of sorghum, which may affect related bioactivities.

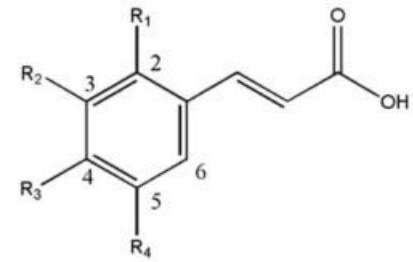
Phenolic acids represent the simplest phenolic compounds, containing a single aromatic ring with hydroxyl and methoxyl substituted groups, and are derivatives of cinnamic acid and hydroxybenzoic acid[49]. They are spread broadly throughout the plant kingdom and estimated to contribute to one third of the total phenolic compounds consumed in the diet[57]. The major

Table 2. Comparisons of reported *Sorghum bicolor* total phenolic and antioxidant activity.

sorghum	source	method	content[a]
grain sorghums	grain	ABTS	10-170 µmol TE/g[54]
			125-562 µmol TE/g[55]
		DPPH	5-85 µmol TE/g[54]
			15-43 µmol TE/g[55]
		Folin-Ciocalteu	2-9 mg GAE/g[54]
			8-30 mg GAE/g[55]
		ORAC	81-236 µmol TE/g[55]
	leaves	Folin-Ciocalteu	3.4-11.7 mg GAE/g[56]
	stalk	Folin-Ciocalteu	0.4-1.0 mg GAE/g[56]
dye sorghums	grain	DPPH	83-147 µmol TE/g[5]
		Folin-Ciocalteu	9-23 mg GAE/g[5]
			10.6-17.5 mg TAE/g[58]
	leaf sheath	ABTS	3760-5580 µmol TE/g DM[4]
		Folin-Ciocalteu	65-135 mg GAE/g[4]
specialty sorghums	bran	ABTS	28-786 µmol TE/g[6]
		DPPH	21-716 µmol TE/g[6]
	grain	ABTS	6-226 µmol TE/g[6]
		DPPH	6-202 µmol TE/g[6]

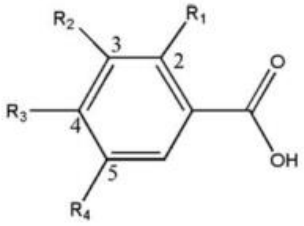
[a]Superscripted numbers indicate references. DM = dry matter; TE = Trolox equivalents; GAE = gallic acid equivalents.

phenolic acids identified in sorghum grain include protocatechuic, gentisic, caffeic, cinnamic, ferulic, sinapic, salicylic and *p*-coumaric[59] and are illustrated in Figure 5. Vanillic, *p*-hyrdroxybenzoic and gallic acids have also been identified in the grain of sorghum[5]. *p*-coumaric, *o*-coumaric, and *p*-hydroxybenzoic acids have been identified in the leaf sheaths of sorghum varieties[4]. In the leaves, *p*-hydroxybenzoic, *o*-coumaric, *p*-coumaric, caffeic, ferulic, and gentisic acids have been reported to inhibit *Locusta migratoria* feeding[60], indicating the importance of these compounds to plant defense. *p*-coumaric and ferulic acids have been reported in the stalks of sorghum[44]. Table 3 summarizes efforts to identify and quantify phenolic acids and illustrates the variability in expression of these compounds in the parts of the sorghum plant. For example, there is nearly a 4-fold increase in the levels of *p*-coumaric acid from the grain to the leaf sheath.



Cinnamic acids (**17-21**)

Caffeic acid (**17**): $R_1 = R_4 = H$, $R_2 = R_3 = OH$
Ferulic acid (**18**): $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$
o-coumaric acid (**19**): $R_1 = OH$, $R_2 = R_3 = R_4 = H$
p-coumaric acid (**20**): $R_1 = R_2 = R_4 = H$, $R_3 = OH$
Sinapic (**21**): $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$



Benzoic acids (**11-16**)

Gallic acid (**11**): $R_1 = H$, $R_2 = R_3 = R_4 = OH$
Gentisic acid (**12**): $R_1 = R_4 = OH$, $R_2 = R_3 = H$
Salicylic acid (**13**): $R_1 = OH$, $R_2 = R_3 = R_4 = H$
p-hydroxybenzoic acid (**14**): $R_1 = R_2 = R_4 = H$, $R_3 = OH$
Syringic (**15**): $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$
Protocatechuic(**16**): $R_1 = R_4 = H$, $R_2 = R_3 = OH$

Figure 5. Phenolic acids identified in *Sorghum bicolor*. Adapted from[61].

Table 3. Reported phenolic acids in *Sorghum bicolor.*

class	compound	source	concentration[a]
phenolic acids	protocatechuic acid	grain	7-141 mg/kg[5]
	p-hydroxybenzoic acid	grain	15-34 mg/kg[5]
		leaf	reported[60]
	vanillic acid	grain	8-51 mg/kg[5]
		leaf	reported[60]
	p-coumaric acid	grain	86-232 mg/kg[5]
		leaf sheath	512-834 mg/kg[4]
		leaf	reported[60]
		pith	13907 g/kg CWR[44]
		bark	19893 g/kg CWR[44]
	o-coumaric acid	leaf	reported[60]
	ferulic acid	grain	105-343 mg/kg[5]
		leaf	reported[60]
		pith	6466 g/kg CWR[(b)44]
		bark	8446 g/kg CWR[(b)44]
	gallic acid	grain	20-46 mg/kg[5]
	gentisic	leaf	reported[60]
	caffeic acid	grain	26-52 mg/kg[5]
	cinnamic acid	grain	5-20 mg/kg[5]
	hydroxybenzoic acid	leaf sheath	381-1555 mg/kg[4]
	salicylic	grain	reported[59]
	syringic	grain	reported[59]
	sinapic	grain	reported[59]

[a]Superscripted numbers indicate references. Reported, references which only reported presence of compound.
[b]CWR, cell wall residue.

The bioactivity of phenolic acids in cancer cells have been well reported and are summarized here in Table 4. Phenolic acids are able to alter many steps of the carcinogenesis process. DNA damage as a result of oxidative damage contributes the formation of somatic mutations which contribute to carcinogenesis[51]. Certain phenolic acids, such as ferulic and cinnamic acid, have been shown to inhibit the promotion stage of cancer by decreasing DNA damage[62]. This is further supported by cinnamic acid's effect on increasing glutathione S transferase activity[63], an important enzyme in antioxidant defense, which helps in decreasing oxidative stress[63]. Hyper-proliferation and protection from apoptosis are important hallmarks to cancer[64]. Phenolic acids such as *p*-coumaric acid[62] and vanillic acid[65] have been shown to decrease proliferation and increase apoptosis in a number of cancer cell lines, including colon cancer cells. COX-2 contributes to inflammation, proliferation, and reduced apoptosis and Inhibitors of COX-2 activity have long been associated with decreased rates colorectal cancer[66]. Ferulic acid was demonstrated to reduce colon cancer HT29 cell proliferation through altering COX-2[62]. Interestingly, these phenolic acids have been identified in the grain, leaf, leaf sheath and stalk of sorghum varieties.

Table 4. Bioactivity of phenolic acid compounds identified in *Sorghum bicolor.*

class	compound	model	biological activity[a]
phenolic acids	protocatechuic acid	HepG2 liver cancer cells	↑ apoptosis through JNK & p38 stimulation[67]
		T47D breast cancer cells	↓ proliferation; ↑ apoptosis[68]
	vanillic acid	HT-29 colon cancer cells	↓ proliferation; ↑apoptosis[65]
		NIH/3T3	↓ proliferation; ↑apoptosis[65]
	p-coumaric acid	HT-29 colon cancer cells	↓ proliferation[62]
		EMT-6 breast cancer cells	↓ proliferation[62]
		SW-620 colon cancer cells	↓ proliferation[62]
		LOVO colon cancer cells	↓ proliferation[62]
		HCT-8 colon cancer cells	↓ proliferation[62]
	ferulic acid	HT-29 colon cancer cells	↓proliferation; ↓ genotoxicity; ↓ COX-2[62]
		T47D breast cancer cells	↓ proliferation; ↑ apoptosis[68]
	caffeic acid	T47D breast cancer cells	↓ proliferation; ↑ apoptosis[68]
	cinnamic acid	F344 rats colonic mucosa	↓ genotoxicity; ↑ glutathione-S-transferase[63]

[a]Superscripted numbers indicate references.

Flavonoids are the second major class of sorghum phenolics (Figure 6) and represent the largest class of phenolics in the plant kingdom, and can be divided into six major subclasses based on C-ring substitutions; flavanones, flavonols, flavones, catechins, anthocyanidins, and isoflavones[69]. Table 5 summarizes previous efforts to identify and quantify sorghum flavonoids. In sorghum, anthocyanidins, flavones, flavanones, and flavonols have been previously identified. The anthocyanidins apigeninidin and luteolinidin, and their methoxylated counterparts, 7-methoxyapigeninidin and 5-methoxyluteolinidin, are seemingly ubiquitous in the sorghum plant and have been identified in sorghum glume, grain, leaf and leaf sheath[5,70]. These compounds lack a hydroxyl group on the 3-carbon and are collectively known as 3-deoxyanthocyanidins. When compared to their 3-hydroxylated counterparts, these compounds are more stable in acidic solutions and have been shown to be more cytotoxic to cancer cells[4,71]. These compounds also induce detoxifying phase II enzymes[8]. Sorghum is the only plant known to contain significant quantities of these 3-deoxyanthocyanidins suggesting it to be a unique reservoir of health benefiting compounds[4].

Apigenin, luteolin, and tricin are flavones identified in the grain and the stem of sorghum[5,7,72]. Apigenin has demonstrated anticancer activity in HeLa cells and Hep3B cells[11,73], and luteolin has demonstrated anticancer activity *in vitro* and *in vivo*[41]. While certain flavonoids are seemingly ubiquitous throughout the sorghum plant, the flavanones naringenin and eriodictyol have only been identified in the grain of sorghum[74] and is currently unknown if it is expressed in measurable quantities in other parts of the sorghum plant. Naringenin decreases tumor growth in ddY mice implanted with S-180 sarcoma cells[9], and Eriodictyol inhibits EGF induced JB6 Cl41 cell transformation *in vitro*[75]. Kaempferol 3-rutinoside-7-glucuronide[76], taxifolin and related glycosides[77], quercetin glycosides[7], catechins[78], epicatecin[78] and

procyanidins[78] have also been reported in sorghum grain and each demonstrating unique anticancer properties.

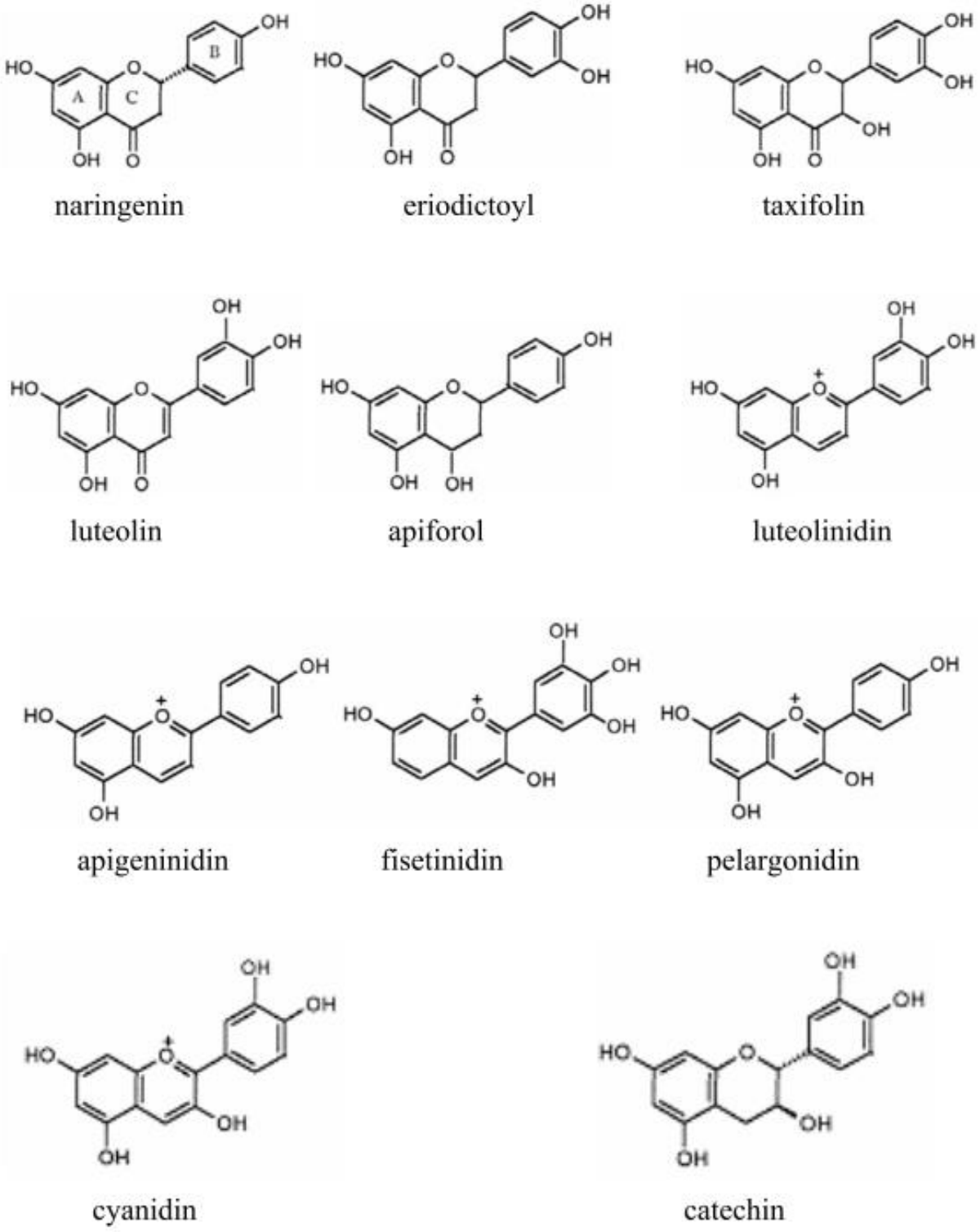


Figure 6. Common flavonoids identified in *Sorghum bicolor*. Adapted from[49].

Table 5. Reported flavonoids in *Sorghum bicolor.*

class	compound	source	concentration[a]
anthocyanidins	apigeninidin	glume	~100-~7000 mg/kg[70]
		grain	300-1000 mg/kg[5]
		leaf	0-~1300 mg/kg[70]
		leaf sheath	14720-45760 mg/kg[4]
	luteolinidin	glume	0-~1000 mg/kg[70]
		grain	trace-1500 mg/kg[5]
		leaf	0-~500 mg/kg[70]
		leaf sheath	430-1660 mg/kg[4]
	7-methoxyapigenindin	glume	0-~2000 mg/kg[70]
		grain	0.4-137.4 mg/kg[5]
		leaf	0-~350 mg/kg[70]
		leaf sheath	0-~2500 mg/kg[70]
	5-methoxyluteolinidin	glume	0-~300 mg/kg[70]
		grain	0.3-153.5 mg/kg[5]
		leaf	0-~200 mg/kg[70]
		leaf sheath	0-~4500 mg/kg[70]
	malvidin	leaf sheath	570-1030 mg/kg[4]
flavones	apigenin	grain	2.8-203.7 mg/kg[74]
		stem	reported[72]
	luteolin	grain	2.6-182.2 mg/kg[74]
		stem	reported[72]
	tricin	stem	reported[7]
flavanones	naringenin	grain	5.6-48.4 mg/kg[74]
	eriodictyol	grain	5.6-12.9 mg/kg[74]
	eriodictyol 5-glucoside	grain	reported[79]
flavonols	kaempferol 3-rutinoside-7-glucuronide	grain	reported[76]
	quercetin 3,4'-dimethyl ether	stem	reported[7]
Dihydroflavonols[b]	taxifolin	grain	reported[77]
	taxifolin 7-glucoside	grain	reported[77]
flavan-3-ols[b]	catechin	grain	10-180 mg/kg[78]
	epicatechin	grain	10-180 mg/kg[78]
	procyanidins	grain	1300 – 22000 mg/kg[78]
stilbenes	*trans*-resveratrol	grain	reported[5]
	trans-piceid	grain	reported[5]

[a]Superscripted numbers indicate references. Reported, references which only reported presence of compound.
[b]Sub-class of flavonols.

Echoing the wide range in TP and AOA, flavonoid content is highly variable and ranges widely depending on part of the plant under investigation. For example, there is a 45-fold difference in the highest reported levels of apigeninidin in the grain and leaf sheath of sorghum. Therefore, the large variation in quantity and composition of phenolics from these different parts of the sorghum plant is expected to create a large variation in the bioactivity associated with the

phenolics from each part of the plant. This warrants further investigations into the bioactivity of the different parts of the sorghum plant in relation to their bioactive content.

Apart from phenolic acids and flavonoids, other phenolic compounds have been reported in sorghum grain. The stilbenoids piceid and resveratrol have been reported in the grain of red sorghum[5]. These compounds are known contain high antioxidant activity and have demonstrated strong bioactivities in many studies. For example, resveratrol decreased the number of high multiplicity aberrant crypt foci, a biomarker for colorectal cancer, in rats[80]. Additionally, resveratrol induced apoptosis in the HCT116 colon cancer cell line[81].

Table 6. Bioactivity of flavonoid compounds identified in *Sorghum bicolor.*

class	compound	model	biological activity[a]
anthocyan-idins	3-DXA rich sorghum extract	Hepa 1c1c7	↑ phase II enzymes[8]
		HT-29 colon cancer cells	↓ proliferation[8]
	luteolinidin	HL-60; HepG2	↓ proliferation[10]
	apigeninidin	HL-60 leukemia cells	↑ apoptosis; ↑Bak; ↑Bax[82]
flavones	apigenin	Hep3B liver cancer cells	↓ VEGF expression[11];
		HeLa cervical cancer cells	G1 cell cycle arrest; ↑ apoptosis[73]
	luteolin	JB6 P+ mouse epidermal cells	↓ Nf-κB, ↓ Src kinase, ↓ PKCε, ↓ COX-2[41]
		SKH-1 hairless mouse	↓ UVB induced skin cancer[41]
flavanones	naringenin	ddY mice implanted with S-180 Sarcoma	↓ tumor growth[9]
	eriodictyol	JB6 Cl41 cells	↓ RSK2; ↓ EGF-induced cell transformation[75]
flavonols	kaempferol	A549 lung cancer cells	↓ proliferation; ↑ apoptosis via MEK-MAPK[83]
	quercetin	HCC1937 breast cancer cells	↓ proliferation; ↓ PI3K-Akt/PKB pathway[84]
	taxifolin	HCT116 colon cancer cells	↑ detoxifying enzymes by activating antioxidant response element[85]
flavan-3-ols	catechin	PANC-1 pancreatic cancer cells	↑ apoptosis[86]
		MIAPACA pancreatic cancer cells	↑ apoptosis[86]
stilbenes	*trans*-resveratrol	F344 rats	↓ HMACF[80]
		C57BL/6NIA mice	↓ IGF-1; ↓ insulin[87]
		HCT116 colon cancer cells	↑ apoptosis; ↑ caspases 2,9,8, & 3[81]

[a]Superscripted numbers indicate references.

Collectively, this data demonstrates that the sorghum plant contains a wide variety of phenolic compounds including phenolic acids, a myriad of flavonoids and stilbenoids. These

compounds are widely distributed throughout the plant with a wide range in content, which is dependent on the component and variety under investigation. These compounds, in isolation and as part of complex bioactive extracts, have demonstrated anticancer and antiinflammatory bioactivities in multiple *in vitro* and *in vivo* models.

1.2.3 Anticancer Mechanisms of Bioactive Compounds

There is a growing literature reporting the anticancer activity associated with sweet sorghum and related bioactive compounds. For example 3-deoxyanthocyanidin rich extracts were reported to induce phase II detoxifying enzymes[4], apigeninidin and luteolinidin suppressed proliferation[10], and yet other compounds induced apoptosis or favorably altered some host genes to elicit health-promoting responses, disfavoring cancer survival. There are multiple review articles in the literature addressing this enigma related to anticancer mechanisms of bioactive compounds. Although it would be out of the scope of this literature review to go in depth into all known altered pathways related to the effects of bioactive compounds on inflammation, proliferation, apoptosis, angiogenesis, metastasis, etc., it warrants sufficient discussion on the topic of the fundamental molecular mechanisms of bioactive compounds responsible for the multiplicity of observed effects they elicit.

Reactive oxygen species (ROS), such as oxygen radicals (e.g. superoxide) or non-radical oxygen compounds (e.g. hydrogen peroxide), which are easily converted into radicals, are highly reactive and promote oxidative stress[88,89]. ROS and other reactive species are a natural consequence of aerobic metabolism and are typically buffered by inherent enzymatic and non-enzymatic cellular antioxidant systems (e.g. antioxidant vitamins, catalase, superoxide dismutase, etc)[90]. When natural oxidative stress defenses are overwhelmed, reactive species can damage the DNA, proteins and lipids implicated in many chronic disease states[89,90]. This is

collectively referred to as oxidative stress. Oxidative stress is directly involved in the initiation, promotion and progression stages of carcinogenesis through direct DNA damage, altered gene expression and signaling pathways[88]. In this way, bioactive compounds, acting as direct antioxidants, can interfere with all three of these steps in carcinogenesis. Furthermore, oxidative stress incites inflammatory processes through activating proinflammatory transcription factors (e.g. NF-κB, AP-1, HIF-1α, PPAR-γ, Nrf2, etc)[88]. This altered inflammatory/oxidative stress axis also promotes cancer through increasing radio-resistance, chemo-resistance, cellular proliferation, angiogenesis, and metastasis, and by decreasing apoptosis[88]. Intriguingly, bioactive compounds have demonstrated numerous antiinflammatory responses involving multiple pathways. For example, resveratrol was shown to decrease lipopolysaccharide induced NF-κB activation in colorectal cancer cells[91]. Apigenin has been reported to inhibit NF-κB, COX-2, VEGF, and HIF1α[34]. Grapefruit bioactive compounds were shown to inhibit iNOS and COX-2 expression in the rodent azoxymethane-induced colorectal cancer model[40].

Oxidative stress is also associated with key cancer-related pathways regulating cellular proliferation, apoptosis and metastasis. Generation of ROS is often observed in cells undergoing apoptosis caused by anticancer agents and may be a common mediator of apoptosis across a wide range of proapoptotic agents[92]. Oxidative damage can act as a direct trigger of apoptosis and cell cycle arrest by damaging DNA and stabilizing p53[92]. This can induce cell cycle arrest through p53 induced p21 expression, and subsequently apoptosis when DNA is beyond repair[92]. Beyond DNA damage, ROS has been shown to regulate major cell signaling pathways including MAPK pathway, which lead to the activation of redox sensitive transcription factors NF-κB and activator protein-1[93]. Furthermore, oxidative stress directly alters and inhibits the phosphatase catalytic site of PTEN, causing an increase in IP3 levels and thus promotes Akt signaling[94].

Interestingly, there are many examples in the literature of bioactive compounds influencing redox sensitive cancer pathways. For example, quercetin inhibited breast cancer cell proliferation through suppressed Akt in cells with and without PTEN knockout[84]. Resveratrol and mulberry fruit induced apoptosis in a HT-29 and A172 cancer cells via ROS-dependent mitochondria pathways, respectively[95,96]. Curcumin caused G2/M cycle arrest and apoptosis in lung cancer cells due to in part by DNA damage inducing p53 stabilization[97].

Ironically, the anticancer activity of bioactive compounds hinge on this balance between ROS and antioxidant defense. For example, quercetin, at lower concentrations (1-40 μM) may prevent cancer initiation by acting directly as an antioxidant neutralizing ROS and other radicals and inhibiting DNA damage[98]. However, after tumor formation quercetin and similar compounds continue to have beneficial effects at higher concentrations (>40 μM for quercetin)[98]. At these concentrations, bioactive compounds, such as quercetin, elevate ROS to levels that signal apoptotic responses via DNA damage stabilization of p53[98]. However, many cancers circumvent this protective mechanism by inactivating p53[22,99]. This might imply that quercetin and similar compounds are unsafe to non-cancerous cells at such levels. However, numerous accounts report that bioactive compounds are selective and generally induce cytotoxic effects only in cancerous cells[100–103]. This is disquieting nonetheless, as it would be easy to imagine scenarios where such mechanisms might fail. For example, the generation of excess ROS in normal cells would contribute to genetic mutations. Alternatively bioactives, by acting as an antioxidant in cancerous cells to suppress ROS, would prevent the induction of important tumor suppressors, such as p53. This may be the case as to why clinical trials utilizing single bioactive agents fail to produce consistent results[27,104]. Furthermore, it was recently shown that single antioxidant compounds accelerated lung cancer progression via ROS-p53 axis disruption, supporting this notion[105].

Given this dichotomy of antioxidant/prooxidant and tumor-promoting/tumor-initiating responses, it may be that another main influence of bioactive compounds are through additional molecular mechanisms responsible for their preventive and protective effects. In addition to prooxidant and antioxidant mechanisms of individual or multiple compounds, recent evidence suggests that multiple compounds act synergistically/additively to directly interact with proteins of interest, altering cellular responses. For example, resveratrol was shown to directly inhibit COX-2 in cell and non-cell based assays[106]. Quercetin and myrecitin were shown to dock with the opening of allosteric inhibitory pocket of MEK1[107]. In fact, quercetin was shown to out-perform the MEK1 inhibitor PD098059[107]. Ultraviolet B light (UVB) promotes skin cancer partly by inducing COX-2 expression through Fyn kinase[108]. Caffeic acid was reported to inhibit UVB-induced skin carcinogenesis by direct inhibiting Fyn kinase activity[108].

These and similar studies point to anticancer mechanisms by which bioactive compounds directly modulate cell signaling pathways through physical interactions with proteins. This may partly explain why simple changes to biochemical structures (hydroxylation, methylation, etc) have been reported to alter the bioactivity of structurally related compounds. For example, B-ring substation patterns were shown to change depending on location and number of hydroxyl and methoxyl groups[109]. If direct interaction with proteins is a major anticancer mechanism of bioactive compounds, then it is conceivable that these compounds can influence, to some degree, every aspect of the Hallmarks of Cancer as described by Hanahan and Weinberg[64] through interactions with cell signaling circuitry, transcription factors controlling gene expression, cellular metabolism, as well as replication, transduction, and translation machinery. It may also explain, why in many cases single compounds do not elicit response in the complex heterogeneous tumor environment, as multiple pathways related to apoptosis, cell cycle,

inflammation, metabolism, etc. are unfavorably altered and would require multiple compounds disrupting multiple pathways to elicit an anticancer effect observable at a phenotypic level.

When taken together, the evidence presented points to a highly complex and sophisticated means by which multiple bioactive compounds elicit anticancer responses. Furthermore, these responses may not be mutually exclusive and depend on the presence of multiple compounds (similar to what is found in fruits and vegetables) before the full scope of anticancer effects are observed. These effects are multi-layered and most probably involve multiple mechanisms, including antioxidant effects, prooxidant effects, antiinflammatory effects, direct protein interactions, and other yet defined mechanisms.

1.2.4 Colorectal Cancer and Natural Chemoprevention and Chemotherapy.

1.2.4.1 The Colon and Colorectal Cancer.

The colon is the terminal segment of the gastrointestinal tract, measuring 1.0 to 1.5 m in length[110]. Depicted in Figure 7, the colon starts at the cecum, following the periphery of the peritoneal cavity ending at the rectum, which extends into the pelvis terminating at the anal canal[110]. The colon can be divided into left and right colon and further subdivided into ascending colon forming the right colon, and the transverse and descending colon, which together forms the left colon[110]. The basic functions of the colon are to transport digested matter, absorb water, minerals and some vitamins, and provide conditions for bacterial fermentation[110]. When observing a cross section of the colon, one sees the serosa, the circular and longitudinal muscle layers, the submucosa, the muscularis mucosae, and the mucosa[111]. The latter is formed by lamina propria, which contains ~80% of the antibody secreting cells of the body, and the crypts of the colon, which is lined by colonic epithelial cells[110].

At the base of the colon crypt (depicted in Figure 8) lies a population of 4 - 6 stem cells[112]. Upon signal, the colon stem cell, residing at the base of the crypt, divides asymmetrically, giving rise to one progenitor cell and one stem cell[112]. The progenitor cell moves up the crypt toward the lumen of the colon and continues to divide for the next one third of the crypt, called the proliferation zone[113]. The progenitor cell then differentiates in the final one third of the crypt and is sloughed off into the lumen every 4-5 d[112].

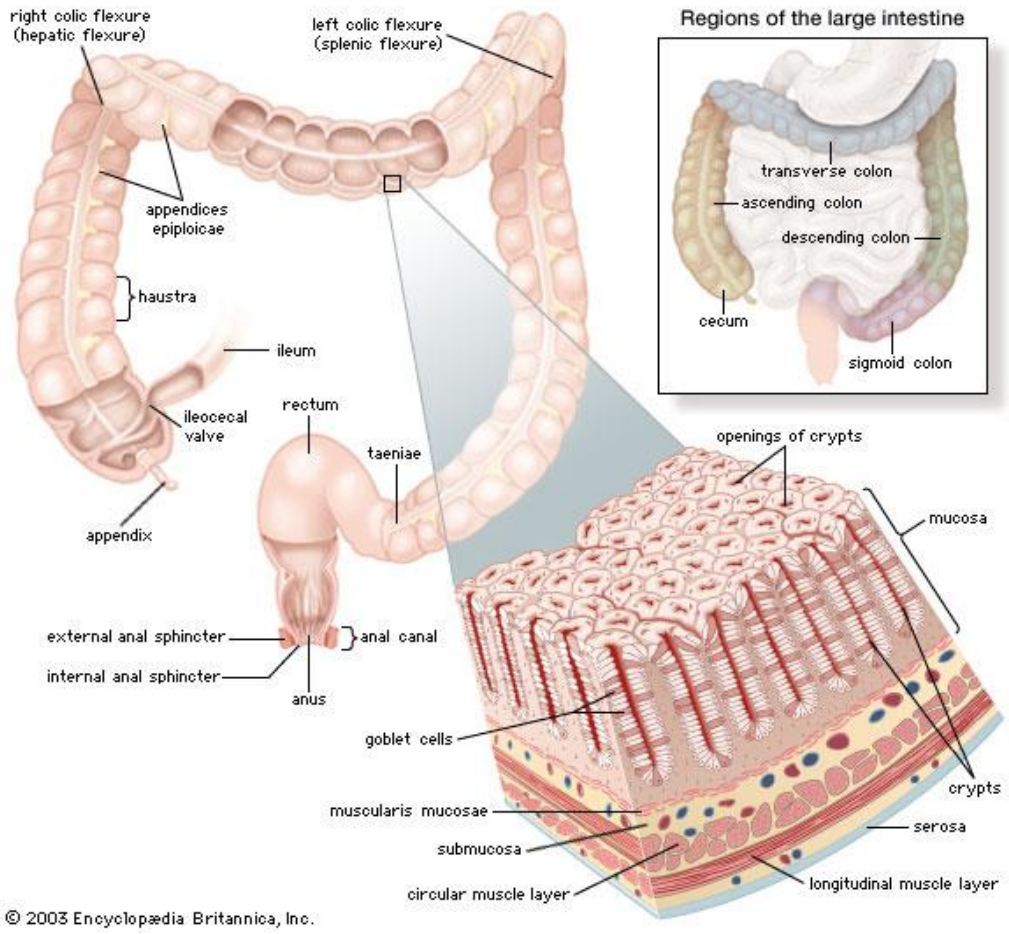


Figure 7. Physiology of the large intestine. Adapted from[111].

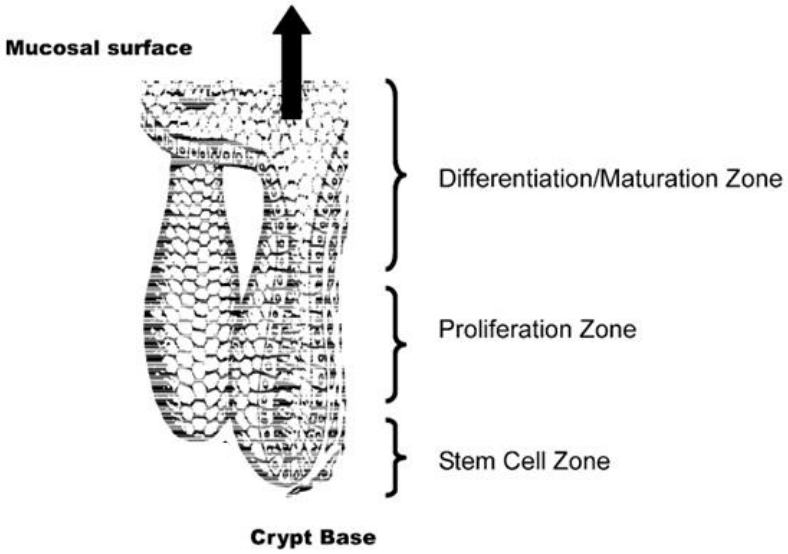


Figure 8. Organization of the colon crypt. Adapted from[113].

Colorectal cancer is estimated to be the second most common cancer in females and third most common in males in the US[20]. Worldwide colorectal cancers impact one million people every year, resulting in over half a million cancer deaths[114]. Numerous epidemiological studies indicate an increase in the consumption of fruits and vegetables quantity and variety is associated with decreased incidence of cancer, cardiovascular disease, and stroke[115–118]. In accordance with this, it has been estimated that 80% of colorectal cancers can be attributed to dietary factors[19]. To understand the role between diet and colorectal cancer it is important to have a greater understanding of the carcinogenesis of cancer.

The process by which normal colonic epithelium progresses to colon cancer is multi-step, whereby cells accumulate successive genetic alterations, establish clones and gain a proliferative advantage[22]. The traditional pathway to colorectal cancer accounts for 70% to 85% of all cancers and starts with loss of APC function[22], which results in accumulation of β-catenin and aberrant WNT/β-catenin. This aberrant β-catenin signaling occurs in almost all cases and drives colorectal carcinogenesis[23,24]. Loss of APC is then followed by mutation of K-ras, loss of 18q, and finally

loss of p53 via 17p loss[22]. p53 is responsible for cell cycle arrest and apoptosis[25]. Therefore, it prevents the build-up of mutated genomes and the advancement of cancer. p53 is abnormal in 50% to 75% of all colorectal cancers, and this marks the transition from noninvasive to invasive disease[22]. Furthermore, loss of p53 disrupts normal tumor responses to conventional therapies, such as 5-Fluorouracil (5-FU), thus making it difficult to treat these advanced tumors with conventional therapies[26]. 5-FU induces p53 expression through inhibiting thymidylate synthase (TS) leading to decreased incorporation of TS metabolites into DNA and RNA[26]. This causes damage that, among other responses, causes apoptosis induced partly by p53[26]. In this regard, 5-FU acts analogously to the prooxidant anticancer mechanism of bioactive compounds. However, bioactive compounds are often found in complex mixtures in nature[27], and, unlike a single therapeutic agent, contain the ability to influence multiple cellular pathways. Mixtures such as green tea extract and grape seed extract with resveratrol have demonstrated anticancer activity that act in both p53-dependent and p53-independent mechanisms pointing, to the ability of these mixtures to influence multiple pathways[28,29]. These findings warrant further investigations into uncovering natural sources of potential chemotherapeutic and chemopreventive agents.

1.2.4.2 Colorectal Cancer and the Stem Cell Hypothesis.

Embryonic stem cells are cells of the inner cell mass of the blastocyte, an embryonic structure formed during early stages of embryogenesis[119]. Embryonic stem cells are defined by their ability for self-renewal and their ability to differentiate into any cell lineage, including adult stem cells, a feature known as totipotency or pluripotency[120]. Adult stem cells have limited differentiation power (multipotency), and these cells differentiate into cells of their respective organs[120]. These cells occur in numerous somatic tissues including liver, blood, brain, colon, mammary, etc. (Figure 9)[121]. Adult stem cells are spread throughout the body in small numbers

and are typically maintained in a specialized region called the stem cell niche (Figure 10)[122]. Niches are composed of specialized cells that provide an anchor, secrete regulator signals, and control the proliferative behavior of adult stem cells[122].

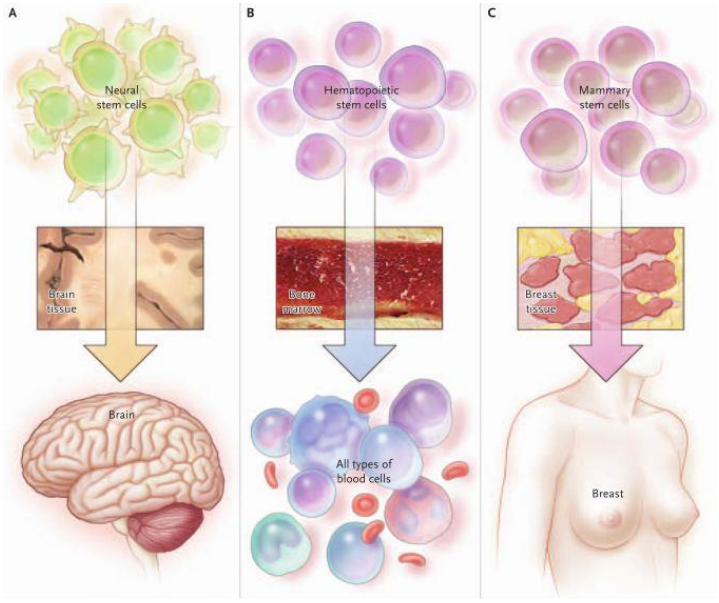


Figure 9. Examples of stem cells found in adult somatic tissues. Adapted from[121].

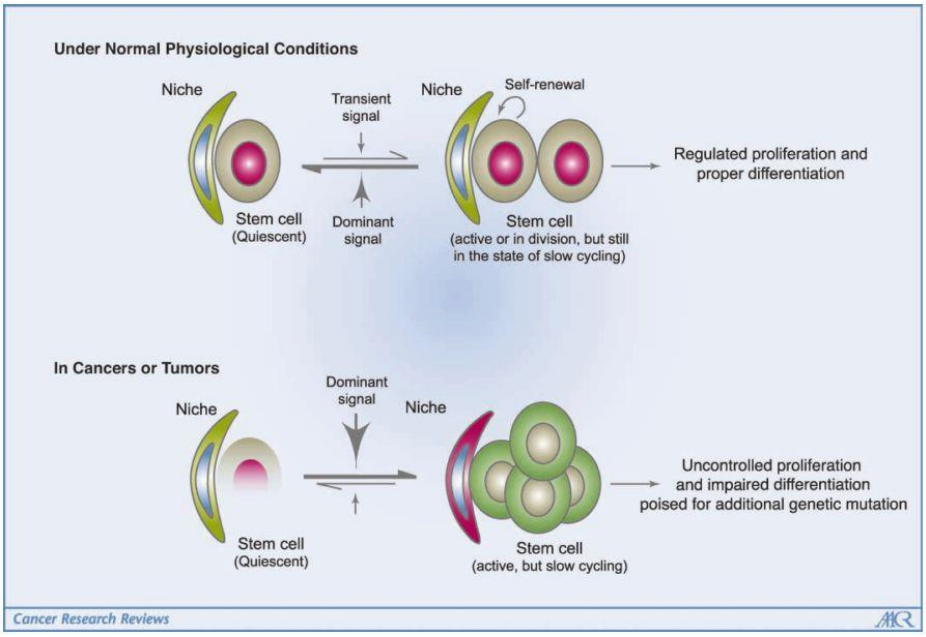


Figure 10. Stem cell niches in normal and cancerous conditions. Adapted from[122].

Increased understanding in the biology of stem cells has influenced our understanding tumorigenesis[121]. The cancer stem cell theory suggests that at the heart of most tumors exist a small number of cancer stem cells that drive the proliferation, renewal, and metastasis of cancers[121,122]. Cancer stem cells are aberrant forms of stem cells that are characterized the ability to develop into any cell in the overall tumor population due to self-renewal, symmetric and asymmetric division, multipotency, and the proliferative ability to drive continued expansion of the population of malignant cells[121]. Interestingly, these are characteristics shared with normal stem cells[121]. However, in cancer, the stem cell has acquired mutations that allow for malignant growth that is independent of the stem cell niche (Figure 10), highlighting the importance of niche-related regulation of proliferation and differentiation[122].

Wnt refers to wingless, a term coined in its discovery in drosophila development[123]. Wnt signals bind to G-protein coupled receptors (Frizzled) and result in disruption of a multi-protein complex containing APC, Axin, GSK-3β and β-catenin[124]. Normally, APC facilitates the binding of GSK-3β to β-catenin, resulting in the phosphorylation and degradation of β-catenin[124]. Wnt signals therefore disrupt this complex allowing for the accumulation of cytosolic β-catenin, and subsequent translocation to the nucleus[124]. Here it associates with members of the TCF/LEF family of transcription factors thus activating specific Wnt targets[125]. Under physiological conditions, this signaling pathway plays a critical role in regulating stem cell and crypt homeostasis[125]. As previously mentioned, loss of APC is an early event in colorectal cancer and results in similar transactivation of Wnt targets. Furthermore, established colorectal cancers depend on Wnt signaling[125]. However, colorectal cancers with established APC mutations show heterogeneous staining patterns for nuclear β-catenin, lending support to the cancer stem cell hypothesis[125].

Interestingly, bioactive compounds have shown efficacy in positively altering Wnt signaling in cancer cells. For example, genistein, an isoflavones found in soy, was shown to inhibit Wnt signaling through increased expression of GSK-3β[126]. Genistein treatment increased binding of β-catenin to GSK-3β, resulting in its subsequent phosphorylation and degradation[126]. Curcumin, a bioactive compound found in turmeric, inhibited Wnt signals resulting in decreased breast tumorosphere formation[126]. Curcumin has also diminished β-catenin signaling in HCT116 cells[126]. These and similar findings point to a larger role of dietary bioactive compounds in the prevention and treatment of colon cancer, and the lack of dietary bioactive compounds is possibly a major reason for the high percentage of colorectal cancers attributed to diet.

1.2.4.3 Inflammation and Colorectal Cancer.

Although incidence and mortality is high, evidence suggests that a majority of colorectal cancers are promoted by lifestyle factors[127]. Obesity, a known risk factor for colorectal cancer and lifestyle-related condition, is characterized by elevated systemic levels of insulin and inflammation, which may act in concert to accelerate colorectal carcinogenesis[36,127]. Increased adiposity causes chronic systemic inflammation as a result from the recruitment of macrophages into the adipose tissue and decreased secretion of the antiinflammatory adipokine adiponectin[127]. Decreased levels of adiponectin results in a decrease in the expression of tumor suppressors like p53, and an increase in the levels of proinflammatory cytokines, which increases the levels of ROS, which induces the AMP-activated protein kinase signaling pathway[127].

Inflammation and inflammatory conditions of the bowels have been strongly linked to colon cancer[30–32]. In all patients with inflammatory bowel disease (IBD), 20% will develop what is known as colitis-associated colorectal cancers[33]. These cancers are a subset of colorectal cancers associated with IBD with a similar carcinogenesis to that of sporadic colorectal

cancers[33]. Colorectal cancers not associated with IBD have increased inflammatory cytokine expression and immune cell infiltrates[33]. Inflammation promotes the production of reactive oxygen species, which cause DNA oxidative damage[34], and this may be a source of somatic mutations promoting tumor progression. Furthermore, inflammatory signaling can be mitogenic, promoting the growth of pre-cancerous and cancerous cells[34,35]. Obesity is a condition associated with chronic inflammation and this may be important in the promotion of cancer. The prevalence of obesity is high and it is estimated that 20% of cancer cases are attributable to this chronic disease state[37,38].

The use rodent models of high-fat diet induced obesity have been reported in numerous studies investigating metabolic disorders associated with obesity. These studies have revealed a predictable progression of metabolic disease, which starts with changes in gut microbiota, increased intestinal permeability, and metabolic endotoxemia[128]. Feeding a high-fat diet to mice has been revealed to increase lipopolysaccharide containing microbiota and serum LPS concentrations[128]. HFD feeding has also been shown to increase intestinal inflammation and promotes oxidative stress through increased TLR-4 signaling and NF-κB activation[129,130]. HFD was also associated with increases in colonic IL-6, an inflammatory cytokine, and this was associated with increases in the development of pre-cancerous lesions in azoxymethane injected mice[131]. Oxidative stress can cause genomic damage resulting in somatic mutations and this can contribute to carcinogenesis. It has been shown that bioactive compounds can attenuate high-fat diet induced inflammation, oxidative stress and colon carcinogenesis. For example, red wine polyphenols were shown to decrease DNA oxidative damage in animals fed high-fat diet[130]. Furthermore, this study investigated carcinogen-induced colon cancer in mice fed a high-fat diet

and reported a decrease in colon tumor formation and changes in inflammatory gene expression[130].

There are many studies documenting the antiinflammatory properties of bioactive compounds. For example isothiocyanates and curcumin decreased NF-κB signaling[39]. Grapefruit bioactives suppressed COX-2 expression and colon carcinogenesis in an azoxymethane model of colorectal cancer[40]. Whole plant foods, such as berries, broccoli, and grape fruit, are reported to have beneficial properties for obesity-enhanced cancers (reviewed in[36]). These and similar findings suggests a protective antiinflammatory role for dietary bioactive compounds.

1.3 Summary.

It is now understood that cancer is a heterogeneous disease characterized by mutations of normal genes causing uncontrolled cell growth. Cancer stem cells are aberrant forms of normal adult stem cells[120]. Understanding the role of stem cells in carcinogenesis is providing new insights and opportunities in the targeted treatment and prevention of colon cancer[120,132,133]. Indeed, several studies investigating targeted removal of cancer stem cells through molecular targets have yielded promising results[134–138]. Recent research into the role of bioactive compounds in targeting this elite class of cancer cell is promising[133].

Research into the bioactive constituents of foods has revealed that plants contain a plethora of secondary metabolites. There are over 100,000 bioactive compounds in plants and are broadly classified by structure (e.g. phenolics, carotenoids, glucosinolates, etc.). These compounds interact with many molecular pathways in promoting health and preventing disease states *in vitro* and *in vivo*[139]. Bioactive compounds have demonstrated antioxidant, antiinflammatory, and anticancer activities across cell models, animal models, and, most importantly, human models[18,140,141]. Moreover, these compounds seem to have synergistic health

promoting effects when consumed in combinations or as whole foods[27,142,143]. This suggests that consuming whole foods and a variety of bioactive compounds are important for the prevention of diseases including cancer. Numerous studies investigating anticancer properties in disease models have elucidated that bioactive compounds interact with multiple molecular pathways influencing cell behavior.

Sorghum bicolor, known simply as sorghum, is grown primarily for grain production in arid parts of the world due to its low water requirements. Sorghums have been reported to be rich in phenolics and antioxidants with bioactivity in numerous *in vitro* and *in vivo* models. This activity is not exclusive to the commonly consumed grain, but also true of the leaves, leaf and leaf sheath. In fact, the leaves and leaf sheaths have been used in traditional medicines for the treatment of diseases, including cancers. Much less is known about the *in vitro* and *in vivo* bioactivity the stalk of *S. bicolor*, nor is there much known with regards to sweet sorghum varieties of *S. bicolor*. Sweet sorghums are varieties of S. bicolor which concentrate sugars in the stalk and are grown for making sugars and syrups. Interest has grown in the use of sweet sorghum as a biofuel in the biorefinery approach to biofuel production. This generates large volumes of biomass, which may serve as reservoirs of human health–promoting bioactive compounds. Great Valley Energy LLC has patented technology for separating pith cells from the dermal layer of the stalk after separating the leaves and seed head. The main goal of this research is to investigate the *in vitro* and *in vivo* bioactivity in extracts from the stalk (pith and dermal layer) of different varieties of sweet sorghum against colon cancer cells and colon cancer stem cells. We also aim to investigate molecular mechanisms responsible for any observed activities and to identify phenolic bioactive compounds contained within these extracts. Given the predisposition of inflammatory conditions and dietary/lifestyle factors in the promotion of

colorectal cancer, we also aim to assess the *in vivo* antiinflammatory and antioxidant bioactivity of dermal extract in a murine high-fat diet induced obesity and inflammation model.

CHAPTER 2: THE DERMAL LAYER OF SWEET SORGHUM (*SORGHUM BICOLOR*) STALK, A BYPRODUCT OF BIOFUEL PRODUCTION AND SOURCE OF UNIQUE 3-DEOXYANTHOCYANIDINS, HAS MORE ANTIPROLIFERATIVE AND PROAPOPTOTIC ACTIVITY THAN PITH IN P53 VARIANTS OF HCT116 AND COLON CANCER STEM CELLS

2.1 Overview.

There is a growing interest in the utilization of sweet sorghum as a renewable resource for biofuels. During the biofuel production process, large quantities of biomass are generated creating a rich source of bioactive compounds. However, knowledge of sweet sorghum stalk is lacking. We measured phenolic content (Folin-Ciocalteu assay), antioxidant activity (2,2`-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay) and phytochemical composition (LC-MS) in both pith and dermal layer of the stalk. We further tested the antiproliferative (5-bromo-2`-deoxyuridine assay) and proapoptotic (terminal deoxynucleotidyl transferase dUTP nick end labeling assay) activities of these extracts using HCT116 cells and colon cancer stem cells (CCSCs) with and without the tumor suppressor gene p53. For the first time, we show that dermal layer extract of sweet sorghum contains more of the 3-deoxyanthocyanidins apigeninidin and luteolinidin than pith, and this was associated with more anticancer activity. Furthermore, luteolinidin suppressed CCSCs proliferation more than apigeninidin. In addition to being renewable biofuel, sweet sorghum may also serve as source of health-promoting compounds.

Keywords: *sweet sorghum, Sorghum bicolor, biorefinery, biofuel, total phenolics, antioxidants, LC−MS, proliferation, apoptosis*

2.2 Introduction.

Colorectal cancer is the third most common cancer in males and females in the United States[20], and it has been estimated that 80% of colon cancer cases are caused by diet[19]. Evidence suggests that small molecule bioactive compounds confer the protective and preventive effects against chronic diseases, such as cancer, associated with a plant-based diet[19]. This recent understanding has increased the demand for natural sources of plant phytochemicals with chemopreventive and chemoprotective properties. In fact, there are multiple clinical trials investigating the preventive and therapeutic roles of curcumin, resveratrol, and n-3 polyunsaturated fatty acids for colon cancer[21].

The progression from normal epithelium to colon cancer is a multistep process whereby a normal cell accumulates successive genetic alterations and establishes clones[22]. The tumor suppressor gene p53 is responsible for growth and apoptosis, therefore preventing the accumulation of mutated genomes and the progression of cancer[144]. In colorectal cancers, p53 is estimated to be abnormal in 50-75% of cases and marks the transition from preinvasive to invasive disease[22]. It is believed that colon cancer is a disease of aberrant stem cell populations, as stem cells have the ability to self-renew for many generations, making them long-lived enough to acquire the mutations necessary to manifest the disease[145]. Cancer stem cells are known to be chemotherapy- and radiation-resistant, making them resilient to current standard of care therapies[133], which often reduce the tumor mass but do not eradicate the disease. Therefore, for a treatment to be considered effective against colon cancer, it needs to not only show efficacy in colon cancer cells, representing the bulk of the tumor mass, but also against the cancer stem cells and cells with loss of p53. There is already *in vitro* and *in vivo* evidence supporting the efficacy of small molecule bioactive compounds in the selective removal of colon cancer stem

cells and cells with abnormal p53. Lin *et al.* demonstrated that curcumin and a curcumin analogue successfully targeted stem cells derived from colon cancer cell lines *in vitro*[137]. We have previously shown that combination of grape seed extract with resveratrol resulted in p53-dependent and -independent apoptosis of colon cancer HCT116 cells[29].

Worldwide, sorghum is the fifth most cultivated cereal crop, after rice, wheat, corn, and barley[1]. Sweet sorghums are varieties of *Sorghum bicolor* which concentrate simple sugars in the pith cells of the stalk ranging from 10% to 25%, with glucose and fructose as the main reducing sugars[12]. As a result, they have smaller grain yield and thicker taller stalks when compared to grain sorghums[12]. There is a growing interest worldwide in using sweet sorghum as a replacement crop for sugar cane in the production of biofuels. Advantages of sweet sorghum over cane sugar include reduced water requirements, shorter growing periods, and reduced cultivation cost[1]. Sweet sorghum can grow in marginal lands and therefore does not have to compete with food production[1,13].

Biofuel production of sweet sorghum results in the generation of a large amount of biomass[2] comprised of stalks, leaves, and leaf sheaths, which is commonly treated as waste or utilized as a fodder. In addition, these products, particularly the leaves and leaf sheaths, have been used by traditional healers in Africa and India for anemia, epilepsy, stomach ache, and, interestingly, cancer.[3] Having been reported to be high in phenolic acids and other compounds,[44] it has been shown that sorghums contains high levels of small molecule bioactive compounds in the stalk, leaf, leaf sheath, glumes and seed[4,5,7,70] with reported bioactive properties[62,67,82]. Apigeninidin and luteolinidin belong to the 3-deoxyanthocyanidin subclass of flavonoids (Figure 11) and are reported to be expressed in the glume, grain, leaf and leaf sheath[4,5,70], in *S. bicolor*.

Furthermore, these compounds have been shown to be potent inhibitors of the HT-29 colon cancer cell line[8].

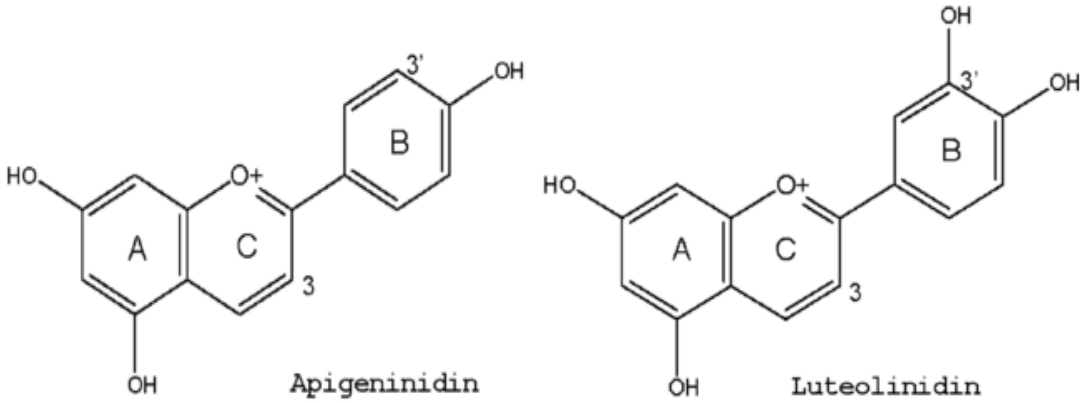


Figure 11: Structures of 3-deoxyanthocyanidins reported in this study[10].

This combination of biomass and bioactive compounds makes an attractive combination for generating large quantities of bioactive compounds for human health. In a process developed by Great Valley Energy LLC, processing of sweet sorghum strips the inner sugary cells of the pith from the outer dermal layer resulting in two fractions, the pith and dermal layer. Currently, it is unknown if these fractions contain 3-deoxyanthocyanidins, or the contribution of these compounds to its bioactivity. The pith is then further processed into biofuel while the dermal layer can be processed to make other byproducts in a "biorefinery" approach to making biofuels. In this approach, the entire above ground sweet sorghum plant is utilized in creating products, which adds value to the process reducing the costs of generating biofuels.[15] The aims of this paper are to (1) determine the content and composition of the pith and dermal layer from Dale and M81E varieties, (2) characterize the antiproliferative and proapoptotic activities of these extracts in human colon cancer HCT116 cells and colon cancer stem cells, (3) assess the bioactivity of apigeninidin and luteolinidin, (4) determine if p53, a critical tumor suppressor protein, is necessary for in vitro anticancer activity.

2.3 Materials and Methods.

2.3.1 Chemicals and Reagents.

For quantification of total phenolic and antioxidant activity: Sodium bicarbonate, monobasic sodium phosphate, dibasic sodium phosphate, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA); potassium persulfate was purchased from Mallinckrodt chemicals (Hazelwood, MO); 2,2`-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reagent, Folin-Ciocalteu reagent, Trolox, and gallic acid were purchased from Sigma (St. Louis, MO).

For cell culture and experiments, 5-fluorouracil (5-FU) was purchased from Sigma (St. Louis, MO). Apigeninidin and luteolinidin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Indofine Chemical Company (Hillsborough, NJ), respectively. McCoy's media and colon cancer stem cell media were obtained from Sigma (St. Louis, MO) and Celprogen (San Pedro, CA), respectively, and fetal bovine serum and streptomycin/penicillin mixture were procured from Fisher Scientific (Pittsburgh, PA).

2.3.2 Sweet Sorghum Materials and Extraction Preparation.

Sweet sorghum materials from Dale and M81E varieties used in this study were grown in Bakersfield, CA, and generously provided by Great Valley Energy LLC (Bakersfield, CA). Two extraction solvents were utilized for the study. Acetone (80%) was identified in the literature and empirically as a superior extraction solvent for sweet sorghum dermal and pith layers. Ethanol (80%) was used as a GRAS alternative. Fresh sweet sorghum sample (2 g) was extracted with 20 mL of extraction solvent chilled to -20 °C. This was to prevent excessive heating during the homogenization of sample. This mixture was then homogenized for 2 min using an IKA T25 digital ultra TURRAX at 10 000 rpm. The tubes were then vortexed and the resulting mixture was then centrifuged at 4000 rpm for 5 min. The supernatant was filtered using a 0.45 µm PTFE

syringe filter (Tisch Scientific; North Bend, OH). The resulting pellet was then resuspended in 20 mL of extraction solvent by vortexing for 1 min and centrifuged, and supernatant was collected and filtered. This was repeated for a third round of extraction, after which the filtrates were combined and the final volume was made to 100 mL. The phenolic extracts were then stored at -20 °C until analyzed for total phenolics, antioxidant activity and LC-MS.

Phenolic enriched extracts were prepared by extracting 2 g of sample in 20 mL of extraction solvent chilled to -20 °C. This mixture was homogenized for 2 min with an IKA T25 Digital Ultra TURRAX set to 10 000 rpm. The homogenate was then placed on a reciprocal shaker overnight in the dark at 4 °C to allow for diffusion of phenolics to create an enriched extract. The mixture was then centrifuged and the supernatant was filtered with 0.45 µm PTFE filters (Tisch Scientific). Extracts prepared with 80% acetone were dried in a Buchi rotavapor (Flawil, Switzerland) to remove the acetone. The aqueous portion was then lyophilized, which yielded a brown powder. This was re-dissolved in 80% ethanol, aliquoted and stored at -80 °C. Extracts prepared with 80% ethanol were concentrated under nitrogen flow in a 35 °C water bath for 10 h. Extracts were then aliquoted and stored at -80 °C.

2.3.3 Quantification of Dry Matter, Total Phenolics and Antioxidant Activity.

Dry matter quantification was determined by freeze drying 2 g samples in triplicate and measuring changes in weight due to water loss. Results were expressed as percent dry matter.

For quantification of total phenolics, the Folin-Ciocalteu assay was used as described in Singleton's work[52] with minor modifications. In triplicate, 35 µL of extract, standard, or solvent was added to individual wells of a 96-well plate followed by the addition of 150 µL of 1 N Folin-Ciocalteu reagent. The contents were mixed and allowed to react for 5 min under dark conditions. After 5 min, 115 µL of 7.5% sodium bicarbonate was added, and the contents were

mixed and incubated in the dark at 45 °C for 30 min. The plate was then cooled for 1 h in the dark at room temperature. Finally, the absorbance was measured at 765 nm with a Biotek Synergy 2 plate reader (Winooski, VT). Gallic acid was used as a standard, and equivalency was calculated from a linear regression of standard concentrations. Data are reported as mean milligram gallic acid equivalents per gram of sweet sorghum sample (mg GAE/g) ± standard error.

Antioxidant activity was measured by the ABTS assay as previously described[6,146] with minor modifications. In brief, 3 mM ABTS radical and 8 mM potassium persulfate were mixed and reacted for at least 12 h at room temperature in the dark. Then this solution was mixed 1:30 with pH 7.4 phosphate buffer to create a working ABTS solution. In triplicate, 290 µL of the working solution was added to 10 µL of sample, standard, or solvent in a 96-well plate. This was allowed to react for 30 min at room temperature under dark conditions, after which the absorbance was measured at 734 nm using the aforementioned plate reader. The antioxidant activity is expressed as mean (n = 3) milligram Trolox equivalents per gram of sweet sorghum sample (mg TE/g) ± standard error.

2.3.4 LC-MS Analysis.

For LC-MS analysis, ethanolic extracts prepared from the pith and dermal of Dale variety were concentrated 10-fold before one µL of sample was injected into a Waters Acquity UPLC system fitted with a Waters Acquity UPLCT3 column (1.8 µM, 1.0 X 100 mm). The sample was injected in 100% solvent A (water, 0.1% formic acid) and this was held for 1 min. This was followed by a 12 min linear gradient to 95% solvent B (acetonitrile, 0.1% formic acid). The flow rate was held constant at 200 µL/min for the complete run. The column temperature was 50 °C and samples were held at 5 °C.

Eluent from the UPLC was infused into a Waters Xevo G2 Q-Tof MS instrument fitted with an electrospray source. Data were collected in the positive ion mode, scanning from 50 to 1,200 at a rate of 0.2 s per scan, alternating between MS and MS^E mode. The collision energy was set to 6 V for MS and 15-30 V for MS^E mode. The capillary voltage was set to 2 200 V, the source temperature was 150 °C, the desolvation temperature was 350 °C, and the desolvation gas flow rate was 800 L/h of nitrogen. Waters raw data files were converted to .cdf format using Databridge software (Waters), and feature detection was performed using XCMS. Raw peaks were normalized to total ion signal in R. The UPLC-MS system was quality checked by making five injections of a standard solution containing 2 µg/mL each of caffeine, reserpine, sulfadimethoxine, and terfenadine in 50% methanol. The performance is evaluated using the final three injections, and acceptable performance is indicates by a retention time +/- 0.05 minutes, peak area RSD of < 25%, and mass accuracy of < 3ppm error for all four compounds. Calibration was performed prior to analysis via sodium formate, with mass accuracy within 1 ppm. Compounds were identified by comparing MS spectra obtained from LC-MS analysis to NIST, METLIN and MassBank databases using 10 ppm as the upper limit for accuracy. In cases where spectra were not obtainable, accurate mass was used to identify compounds.

2.3.5 Cell Lines and Culturing Procedures.

HCT116 and HCT116 $p53^{-/-}$ cells were generously provided by Dr. Bert Vogelstein (School of Medicine, Johns Hopkins University, Baltimore, MD). Colon cancer stem cells (CCSCs) positive for CD34, CD 44 and CD133 were purchased from Celprogen. All cells were maintained at a temperature of 37 °C and 5% CO_2. HCT116 cells were maintained in McCoy's media with 5% FBS. For experiments, HCT116 cells were treated in 10% charcoal stripped serum McCoy's media. CCSCs were maintained in colon cancer stem cell media (Celprogen).

For experiments, CCSCs were treated in serum free colon cancer stem cell media (Celprogen). Concentrated extracts were dosed in media normalized to total phenolics content as assessed by Folin-Ciocalteu assay. We used 80% ethanol and 5-FU as negative and positive controls, respectively.

2.3.6 Lentiviral shRNA Mediated Knockdown of p53 in CCSCs.

CCSCs were transfected with lentiviral particles encoding shRNA targeting p53 (Santa Cruz Biotechnology Inc. Santa Cruz, CA) according to the supplier's protocol. Briefly, CCSCs were infected at a multiplicity of infection of 10 in CCSCs growth medium containing 5 µg/mL of polybrene at 37 °C and 5% CO_2. After 24 hours, media was replaced and the cells were cultured for 2 d. Transfected cells were then selected in the presence of puromycin (7.5 µg/mL) for an additional 5 d, and p53 suppression confirmed by Western blotting (Figure 12).

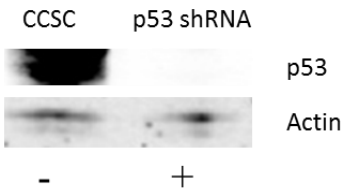


Figure 12. p53 expression in CCSCs with and without p53 shRNA.

2.3.7 Proliferation and Apoptosis.

Cell proliferation was assessed via 5-bromo-2`- deoxyuridine (BrdU) assay (Cell Signaling Technologies; Beverly, MA) and cell counting using an automated cell counter (Nexcelom Bioscience, Lawrence, MA). Briefly, HCT116 cells and CCSCs were grown in 96-well plates at 2 X 10^4 cells per well. After 24 h, the media was aspirated, and the cells were treated with controls and sweet sorghum extracts. The treatments were added in triplicate at the volume of 150 µl per well and then allowed to incubate for 20 h at 37 °C. At this point, BrdU was added at a concentration of 10 µM per well and incubated at 37 °C for an additional 4 h to allow incorporation of BrdU into cellular DNA (24 h total). The media was removed and the

BrdU assay was performed according to manufacturer's protocol. For cell counting, cells were seeded at 1 X 10^5 cells per well in 12-well plates. After 24 h, control compounds and sweet sorghum extracts were added at a volume of 1 mL per well. After 24 h, cells were removed from plates and cells counted according to manufacturer's protocol.

Apoptosis was analyzed by measuring active caspase activity with a Caspase-Glo 3/7 assay kit (Promega Corp.; Madison, WI) and confirmed with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. For measuring caspase activity, cells were seeded and treated with control compounds and test extracts the same as in the cell counting experiments. After 24 h of incubation, 100 µL of HCT116 cells and CCSCs were transferred into a white 96-well plate and the Caspase-Glo 3/7 assay was performed according to the company's protocol. The data was normalized to cell count and expressed as percent of the solvent control. For TUNEL, cells were seeded into 4 well chambered glass slides at a density of 1 X 10^5 cells per chamber. After treatments, the slides were prepared and analyzed for TUNEL staining according to manufacturer's protocols (Roche Diagnostics; Indianapolis, IN). For analysis, 10 images from each well containing were taken containing at least 50 cells per image. Cells were counted and data expressed as the percentage of TUNEL positive cells to total cells (percent apoptosis).

2.3.8 Colony Formation Assay.

The colony formation assay was performed as described[147] with slight modifications. Colon cancer stem cells were seeded at 1.5 X 10^5 cells per well in a six-well tissue culture plate (TPP; St Louis, MO). After adhesion (24 h), the cells were treated with controls and test compounds for 24 h. The cells were then replated at a density of 100 cells per well in another six-well tissue culture plate and colony formation was measured as described[148].

2.3.9 Statistical Analysis.

Data are presented as means ± standard error. Statistical significance was determined by one way ANOVA, followed by the post-hoc Fisher least significant difference test for multiple means comparisons using IBM's SPSS v21. Means not sharing the same letter were statistically significant ($P < 0.05$).

2.4 Results and Discussion.

Sweet sorghum processing generates significant amounts of biomass containing bioactive phytochemicals and represents a promising source of human health-promoting bioactive compounds. In this study, the dermal and pith of two varieties of sweet sorghum were grown, processed, and analyzed for total phenolics and antioxidant activity. The effects of sweet sorghum pith and dermal layer extracts on colon cancer stem cell proliferation, apoptosis, and stemness were also studied. For cell based experiments, cells were dosed with sweet sorghum phenolic rich extracts based on microgram gallic acid equivalents per milliliter media (µg GAE/mL), which was determined by the Folin-Ciocalteu assay.

2.4.1 Dry Matter, Total Phenolics, Antioxidant Activity, and LC-MS Analysis.

For dry matter content, we observed similar levels of dry matter of pith and dermal samples across both Dale and M81E varieties. The dry matter content is summarized in Table 7. The dry matter of Pith from Dale and M81E was 22%. The dry matter content of dermal layer was 39% and 42% for Dale and M81E, respectively (Table 7). These findings are in agreement with previously published data reporting dry matter content for pith of sweet sorghum stalks to contain 30% dry matter[44], reflecting the high moisture content necessary to store and concentrate sugars. To the best of our knowledge, this is the first study reporting the difference between the moisture content in pith cells and dermal cells of sweet sorghum.

Table 7. Determination of TP, ABTS and dry matter of pith and dermal from Dale and M81E sweet sorghum varieties.

	Dale		M81E	
	dermal	pith	dermal	pith
TP[b]	172 ± 6[a]	127 ± 5[b]	225 ± 11[c]	88 ± 3[d]
ABTS[c]	570 ± 54[a]	248 ± 12[b]	365 ± 12[c]	128 ± 12[d]
dry matter	39.0 ± 0.7[a]	21.6 ± 0.2[b]	42.2 ± 0.8[c]	21.5 ± 0.3[b]

Values are presented as means (n = 3) ± standard error. Different online Roman letters within a row indicate significant differences ($P < 0.05$) among the entries. [b]TP, total phenolics analyzed by Folin-Ciocalteu method, expressed as milligram of gallic acid equivalents per 100 g fresh mass. [c]antioxidant activity as analyzed by ABTS assay, expressed as milligram Trolox Equivalents per 100g.

We next sought to characterize the total phenolics and antioxidant activities of sweet sorghum stalk components. Acetone (80%) extracts were used to determine total phenolics and antioxidant activities of sorghum samples by Folin-Ciocalteu and ABTS, respectively (Table 7). The total phenolics content of the dermal layer was higher than that of pith in both varieties by 35% and 155% in Dale and M81E, respectively. A similar relationship was also observed in the antioxidant activity. Amongst the two varieties, Dale dermal extracts yielded more Trolox equivalents, yet fewer gallic acid equivalents as assessed by ABTS and Folin-Ciocalteu assays, respectively ($P < 0.05$). When pith and dermal are combined there is no difference between the gallic acid equivalents of Dale and M81E, but there is significantly more Trolox equivalents. This could be a resultant of a difference in the compositions of phenolic, antioxidant, and other reducing substances contained in the two varieties. Reducing sugars such as sucrose, fructose and glucose are concentrated in the pith of sweet sorghum and contribute to total phenolics readings. Reducing sugars are known to be approximately twice as concentrated in the pith as in other components of the stalk[44] and differences in reducing sugars could explain this discrepancy.

We next sought to analyze bioactive compounds from the pith and dermal components of sweet sorghum stalk by LC-MS analysis. We were able to identify phenolic acids, flavonoids and

stilbenoids from the phenolic extracts of sweet sorghum pith and dermal layer, which are summarized in Table 8. These compounds have been identified previously in sorghum and have also been reported to have potent *in vitro* and *in vivo* anticancer activities. For example, we were able to identify luteolinidin and apigenidin, which have been previously reported in leaf sheaths and leaves of sorghum[4,70]. These compounds have been reported to have anticancer activities toward a number of cancer cell lines including liver, leukemia and colon[8,10,82].

Table 8. Compounds identified in sweet sorghum by LC-MS analysis.

identity	formula[a]	t_R (min)	obsd mass	exact mass[b]	Δppm	intensity,[d] dermal	intensity,[d] pith	ref[e]
vanillic acid	$C_8H_8O_4$	2.79	169.050	169.0495	2	100.9 ± 3.8	68.2 ± 7.4[f]	5
p-coumaric acid	$C_9H_8O_3$	0.85	165.055	165.0546	2	534.9 ± 14.6	492.0 ± 23.8	5
ferulic acid	$C_{10}H_{10}O_4$	3.12	195.067	195.0652	9	27.6 ± 1.8	14.3 ± 1.5[f]	5
caffeic acid	$C_9H_8O_4$	3.16	181.050	181.0495	2	152.4 ± 1.3	26.5 ± 4.5[f]	5
apigeninidin	$C_{15}H_{11}O_4^+$	4.29	255.066	255.0660[c]	0	96.5 ± 20.2	7.9 ± 0.7[f]	4,5,70
luteolinidin	$C_{15}H_{11}O_5^+$	5.97	271.061	271.0607[c]	2	151.4 ± 30.8	7.8 ± 2.0[f]	4,5,70
malvidin 3-*O*-glucoside	$C_{23}H_{25}O_{12}^+$	4.76	493.134	493.1314[c]	3	168.6 ± 5.7	12.7 ± 1.4[f]	4
apigenin	$C_{15}H_{10}O_5$	4.04	271.061	271.0607	3	71.2 ± 53.8	8.6 ± 1.2	5
luteolin	$C_{15}H_{10}O_6$	5.48	287.056	287.0556	3	47.3 ± 54.2	4.6 ± 3.0	5,72
trans-resveratrol	$C_{14}H_{12}O_3$	8.48	229.087	229.0859	4	28.8 ± 3.3	37.6 ± 2.9	5
luteoferol	$C_{15}H_{14}O_6$	4.26	291.087	291.0863	2	16.3 ± 0.8	6.4 ± 0.3*	

[a]Formulas are based on [M]. [b]Exact masses are based on $[M+H]^+$ unless otherwise indicated. [c]Mass based on [M]. [d]Normalized peak intensities based on peak areas normalized to total ion signal in R. [e]References which have previously identified the compound listed. [f]Significant differences ($P < 0.05$) observed among dermal and pith.

Previous studies have identified primarily 2 major classes of bioactive phenolic compounds, phenolic acids and flavonoids, in sorghums and sweet sorghums. These investigations primarily focused on the grains or seed heads from grain sorghum for its relevance as a staple in human diets. Other investigations have focused on the leaf, leaf sheath, infected sorghum seedlings, and glume[4,7,70,72], and these parts of the plants are pertinent to traditional medicine. These studies have identified multiple phenolic compounds with potent *in vitro* and *in vivo* activities. These include phenolic acids, anthocyanidins, and flavones. Few studies have

investigated sweet sorghum with respect to its bioactive content, especially the stalk components, pith and dermal. On the basis of these observations, we hypothesized that extracts from the sweet sorghum stalk components pith and dermal layer, containing multiple bioactives would suppress proliferation and induce apoptosis in colon cancer cells.

2.4.2 Anticancer Activities of Sweet Sorghum Pith and Dermal Extracts.

Early in the pathogenesis of colorectal cancer, hyper-proliferation of colonocytes resulting from a deregulation of cellular proliferation and a suppression of apoptosis results in the formation of aberrant crypt foci and, subsequently, adenomas. Colon stem cells, residing in the base of the crypt, are responsible for renewing the colonic epithelium during one's lifetime[149]. Furthermore, they are long-lived, self-renewing cells and have a high capacity for proliferation, a combination that makes them a likely candidate to acquire the mutations necessary to give rise to colon cancers[122]. Today's therapies are ineffective at targeting cancer stem cells, only targeting the bulk of the rapidly dividing tumor cells[145]. Therefore, effective treatment strategies for colon cancer should suppress proliferation and induce apoptosis in the rapidly dividing tumor mass as well as the cancer stem cells. This would rid the tumor mass of the tumor promoting stem cells as well as remove the bulk of the tumor. We assessed the pith's and dermal layer's bioactivity by measuring (1) antiproliferative effects in colon cancer HCT116 cells and CCSCs as evaluated by cell count and BrdU assay, (2) proapoptotic effects in HCT116 cells and CCSCs cells as evaluated by activated caspase 3/7 activity and subsequently confirmed by TUNEL assay, and (3) self-renewal in CCSCs as analyzed by colony forming assay.

2.4.3 Differential Activities of Pith and Dermal Extracts.

To investigate the differences in the anticancer effects between pith and dermal extracts, colon cancer HCT116 cells were treated with phenolic rich extracts prepared from Dale pith 80%

acetone extract (PAE) and Dale dermal 80% acetone extract (DAE) for 24 h as shown in Figure 13. Extract from DAE suppressed cell proliferation more than PAE, as shown by cell count and BrdU assays. This may be due to higher levels of reducing sugars in the pith. At 25 µg GAE/mL, DAE suppressed cell count 43% compared to only an 11% reduction for PAE. IC_{50} values were calculated for PAE and DAE. The IC_{50} for PAE was calculated to be above 50 µg GAE/mL, while DAE was calculated to be 36 µg GAE/mL. DAE was also able to induce apoptosis at lower concentrations than pith as measured by caspase activity. At concentrations of 12.5, 25, and 50 µg GAE/mL, DAE was able to increase active caspase levels to 155%, 159%, and 184% to that of a solvent control, respectively. Only 50 µg GAE/mL of PAE was able to increase active caspases 3 and 7 in HCT116 cancer cells (169%, $P < 0.05$). These data implicated DAE as a more effective antiproliferative and proapoptotic extract than PAE, and therefore further experiments focused solely on extracts from the dermal layer of the Dale variety. These differences in bioactivities between the pith and dermal layer of the Dale variety stalk may be explained by the differences in their composition and content of bioactive compounds. These results are analogous to differences observed in the bioactivities of different parts of edible fruits. For example, only the ethanolic extracts from the peel of mango, but not the flesh, induced apoptosis in cervical cancer HeLa cells[150]. Interestingly, our LC-MS analysis of pith and dermal extracts showed marked and significant differences between extracts from dermal and pith (Table 8). For example, the ethanolic pith extract contained only 8% of the apigeninidin and 5% of the luteolinidin as compared to the levels of these compounds in the ethanolic dermal extract based on relative peak intensities. This may explain the increased potency observed in dermal extracts.

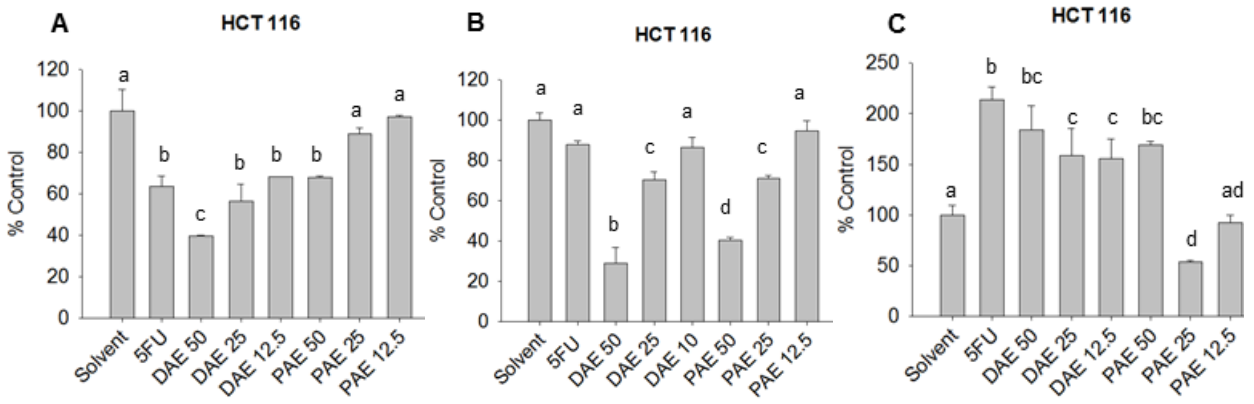


Figure 13. Antiproliferative proapoptotic activities of Dale dermal and pith phenolics measured by **(A)** cell count and **(B)** BrdU assay, and **(C)** Caspase 3/7 Glo assay. HCT116 cells were treated for 24 h with acetone extracts (DAE and PAE) at 10, 12.5, 25, and 50 µg GAE/mL before estimation of proliferation and apoptosis. Values are presented as percent of the solvent control. For cell count and Caspase 3/7 Glo, means (n = 2) ± standard deviation are presented. For BrdU, means (n = 3) ± standard error are presented. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control; and 5FU = 5-fluorouracil (18 µg/mL).

2.4.4 Solvent-dependent Activities of Dermal Extracts.

Previous quantitative and *in vitro* experiments demonstrated that aqueous acetone makes a good extraction solvent for dermal phenolic compounds with antioxidant and anticancer activity. However, acetone is not an environmentally safe solvent for extraction of bioactive compounds, especially for extracts intended for human consumption. Turkmen and colleagues[151] tested the ability of different organic solvents with varying percentages of water to extract phenolics from mate and black tea. They found that while 100% ethanol was ineffective at extracting tea polyphenols, addition of 20% water increased polyphenol extraction by approximately 27 times when compared to 100% ethanol. Furthermore, 80% ethanol yielded approximately 60% of the phenolics as that of 80% acetone. On this basis, we hypothesized that ethanol, a GRAS organic solvent, with water could be used to extract phenolics from sweet sorghum dermal layer with similar effectiveness as aqueous acetone. Similar to Turkmen's findings, 80% ethanol yielded approximately 84% compared to 80% acetone (data not shown).

We next tested the bioactivity of the phenolic rich dermal extract prepared from 80% ethanol (DEE) in HCT116 and compared it to DAE. As demonstrated by Figure 14, DEE had a superior IC_{50} value of 22 compared to the IC_{50} of 36 µg GAE/mL for DAE. Given the GRAS status of ethanol and the greater *in vitro* bioactivity against HCT116 cells, further experiments utilized DEE. We further tested DEE for proapoptotic activities by the Caspase 3/7 Glo assay and TUNEL assay. When compared to solvent control, DEE at all concentrations significantly increased apoptosis. Furthermore, this increase in apoptosis was more than the positive control 5-FU, which was used in this study as a comparison of sweet sorghum extracts to a standard chemotherapeutic agent. Our findings highlight the important aspect of solvent selection when extracting and analyzing *in vitro* bioactivities of plant-based extracts.

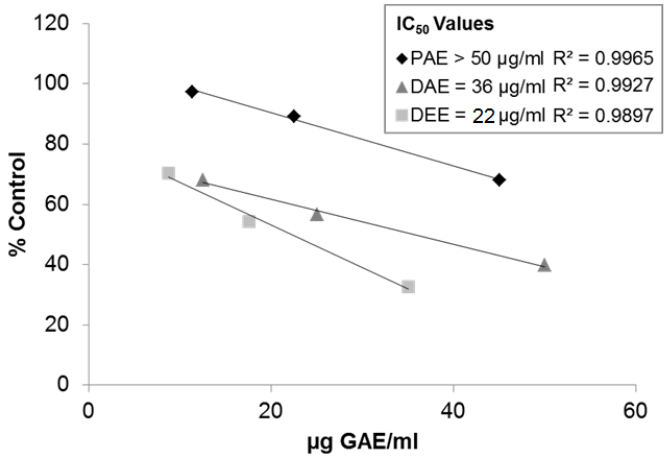


Figure 14. Dose-response curves for HCT116 cells. Cells were treated for 24 h with pith and dermal phenolic rich extracts prepared by extraction with either acetone (AE) or ethanol (EE). The response is based on cell proliferation as estimated by cell counting. Simple linear regression was used to estimate IC_{50} values. Values are presented as percentages of the solvent control (% Control) (y axis) at a specific dose of the respective phenolic-rich extract in micrograms of GAE per milliliter (µg GAE/mL) (x axis).

2.4.5 DEE Effectively Eliminates Colon Cancer Stem Cells.

There is growing evidence that cancers arise from a small population of adult cancer stem cells. They are also resistant to many standard of care therapies (e.g. chemotherapy and

radiotherapy), and the inability to rid the body of these persistent cells is thought to be a primary cause of colon cancer recurrence[133]. There is a need for standardized bioactive compounds and extracts that can effectively eliminate these cells, as current anticancer drugs often fail[133]. We tested DEE against colon cancer stem cells and compared it to 5-FU, a common chemotherapeutic for the treatment of colon cancer. We treated colon cancer stem cells with DEE at 8.75, 17.5 and 35 µg GAE/mL, and 5-FU (18 µg/mL, 100 µM) for 24 h and measured proliferation, and apoptosis (Figure 15 and 16). DEE potently suppressed proliferation to73% of control, as measured by BrdU, and increased the percentage of TUNEL positive cells from 7% to 95% of total cells ($P < 0.05$). Imaging of TUNEL stained cells (Figure 16C) shows increases in DNA fragmentation (increased fluorescence) and changes in cell morphology consistent with apoptosis. We further tested the antiproliferative activity of apigeninidin and luteolinidin against CCSCs with BrdU assay and IC_{50} were calculated as demonstrated in Figure 15C. Luteolinidin suppressed CCSC proliferation more than apigeninidin, as demonstrated by a lower IC_{50} value of 76 µg/ml as compared to > 160 µg/mL.

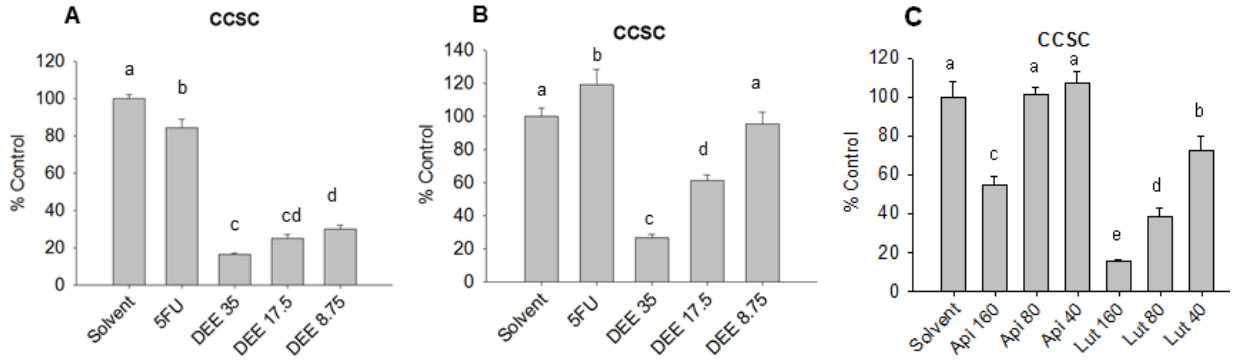


Figure 15. Antiproliferative activities of Dale dermal phenolics measured by **(A)** cell count, **B)** BrdU assay, and **(C)** Antiproliferative activities of apigeninidin (Api) and luteolinidin (Lut) measured by BrdU. Colon cancer stem cells (CCSCs) were treated for 24 h with dermal phenolic rich ethanol extracts of Dale variety (DEE) at 8.75, 17.5, and 35 µg GAE/mL before estimation of proliferation. Values are presented as percent of the solvent control (% Control). For cell count, means (n = 2) ± standard deviation are presented. For BrdU, means (n = 3) ± standard error are presented. Different letters indicate significant differences ($P < 0.05$). Solvent = solvent control; and 5FU = 5-fluorouracil (18 µg/mL).

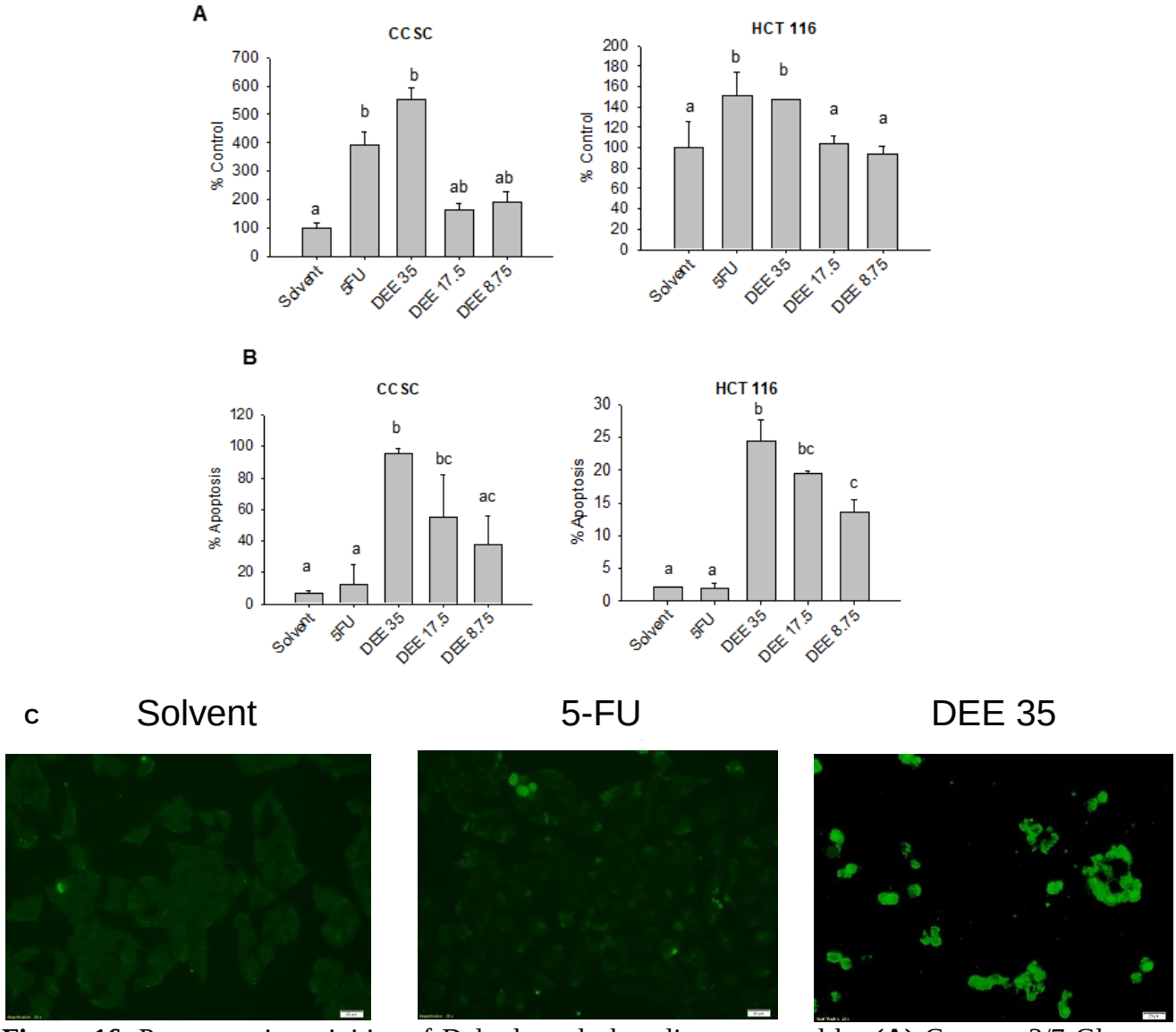


Figure 16. Proapoptotic activities of Dale dermal phenolics measured by **(A)** Caspase 3/7 Glo assay, and **(B)** TUNEL assay. Colon cancer stem cells (CCSCs) and HCT116 cells were treated for 24 h with dermal phenolic rich ethanol extracts of Dale variety (DEE) at 8.75, 17.5, and 35 µg GAE/mL before being tested. Values are presented as mean (n = 2) percentages of the solvent control ± standard deviation. **(C)** Representative images of CCSCs from TUNEL assay. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control; and 5FU = 5-fluorouracil (18 µg/mL).

To further assess DEE's ability to target CCSC properties other than proliferation and apoptosis, the self-renewal capability of CCSCs' self-renewal was measured by colony forming assay (Figure 17). We treated CCSCs with DEE and found a dose-dependent suppression in

colony formation. Figure 17B shows representative images collected from the colony forming assay and demonstrates the decreased colony number and size associated with treatment in comparison to the control. At the highest concentration, DEE completely suppressed CCSC colony formation, and this activity was greater than that of 5-FU treatment. This demonstrates that, in addition to the antiproliferative and proapoptotic activities, DEE alters the stemlike properties by inhibiting colon cancer stem cell self-renewal than 5-FU.

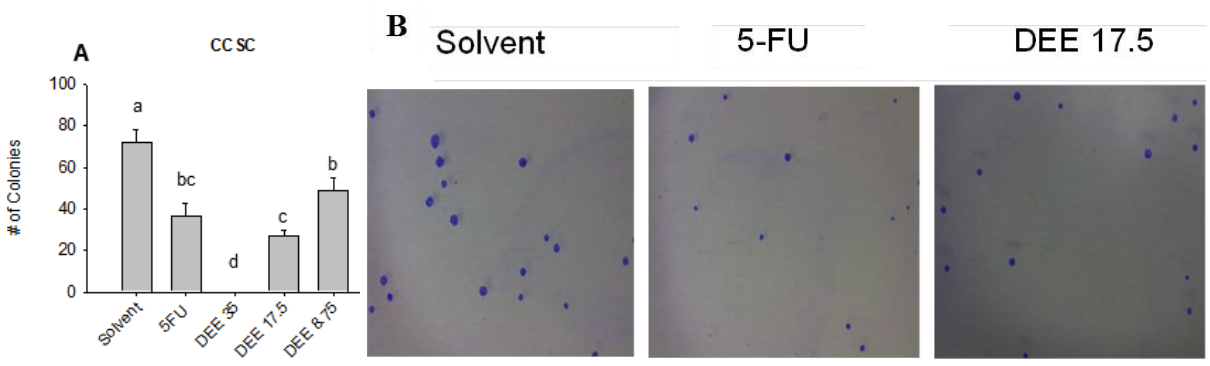


Figure 17. Effects of Dale dermal phenolics on colony formation. **(A)** Colon cancer stem cells (CCSCs) were treated for 24 h with dermal phenolic rich ethanol extracts of Dale variety (DEE) at 8.75, 17.5, and 35 µg GAE/mL before estimation of colony formation by colony forming assay. **(B)** Representative images taken from the colony forming assay. Values are presented as the mean (n = 3) number of colonies formed containing at least 50 cells ± standard error. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control; and 5FU = 5-fluorouracil (18 µg/mL).

There has been little research done on the *in vitro* anticancer activities of sorghum stalk, especially sweet sorghum, on cancer cells and advanced cancer stem cells. Research from Yang *et al.*[8] demonstrated that grain extracts from pigmented grain sorghums contained high levels of 3-deoxyanthocyanins and had strong antiproliferative activities *in vitro* against colon cancer HT-29 cells. Furthermore, they tested the antiproliferative effects of individual 3-deoxyanthocyanidins. They reported IC_{50} values ranging from 180 to 557 µg/mL for crude extracts and approximately 13 to 20 µg/mL values for 3-deoxyanthocyanidins. In agreement with Yang's findings, we too have demonstrated that phenolic rich extract from the dermal layer of

sweet sorghum stalks contains 3-deoxyanthocyanidins and has potent antiproliferative and proapoptotic activities. The fact that dermal phenolic rich extract has activities in colon cancer cells and colon cancer stem cells highlights a need for further research of sweet sorghum's anticancer activity *in vivo* for its potential as a chemotherapeutic agent against colon cancer.

2.4.6 Effects of p53 on the Anticancer Activity of DEE.

The p53 tumor suppressor gene is an integral regulator of cell cycle control, DNA repair and apoptosis[21]. It is estimated that half of all tumors carry some form of mutated p53[25]. Effective preventive and therapeutic colon cancer strategies should successfully target p53-dependent and -independent pathways. To test the p53-dependency of DEE's anticancer activity, we treated HCT116 $p53^{-/-}$ cells carrying p53 deletions in both alleles of the p53 gene with DEE for 24 h and measured changes in proliferation and caspase activity (Figure 18). We observed no significant changes between HCT116 $p53^{-/-}$ cells treated with DEE and solvent controls in cellular proliferation as measured by the BrdU assay, indicating that DEE's ability to suppress cellular proliferation is p53-dependent. However, we did observe a significant increase in active caspase activity at the highest dose of DEE. This indicates that DEE may act through p53-independent pathways of apoptosis only at concentrations at and above 35 µg GAE/mL and this may also be true of DEE's antiproliferative effects.

We further tested p53-dependency in CCSCs by knocking down p53 expression with lentiviral infection containing p53 shRNA (CCSCs p53 shRNA). The shRNA is constitutively expressed due to the presence of a strong promoter binding to p53 mRNA preventing its translation. In this model of p53 knockdown, DEE suppressed proliferation up to 33% of control and elevated apoptosis of CCSCs p53 shRNA cells in a dose-dependent fashion as measured by

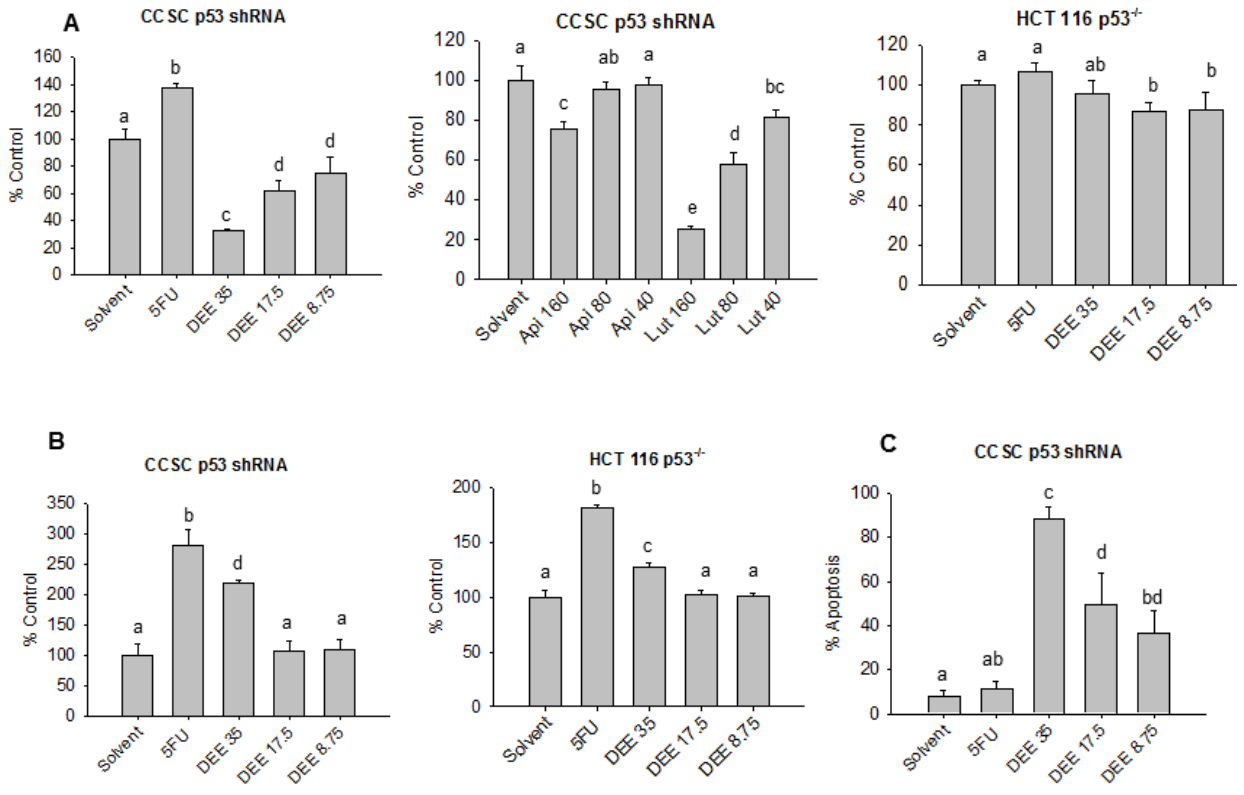


Figure 18. Antiproliferative and proapoptotic activities of Dale dermal phenolics in p53 variants of CCSCs and HCT116 cells measured in a p53-independent manner as measured by **(A)** BrdU assay, **(B)** Caspase 3/7 Glo assay, and **(C)** TUNEL assay. The antiproliferative effects of apigeninidin and luteolinidin were also measured by BrdU assay. Colon cancer stem cells (CCSCs) with p53 shRNA and HCT116 $p53^{-/-}$ cells were treated for 24 h with dermal phenolic rich ethanol extracts of Dale variety (DEE) at 8.75, 17.5, and 35 µg GAE/mL before being tested. For BrdU assay, values are presented as mean (n = 3) percentages of the solvent control ± standard error. For Caspase 3/7 Glo and TUNEL assays, values are presented as mean percentages (n = 2) of the solvent control ± standard deviation. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control; and 5-FU = 5-fluorouracil (18 µg/mL).

BrdU and TUNEL assays (Figure 18). We further tested the antiproliferative effects of apigeninidin and luteolinidin in CCSCs p53 shRNA cells with BrdU (Figure 18). We show thatoth of the 3-deoxyanthocyanidins suppress proliferation in p53 knockdown cells with IC_{50} values of > 160 and 103 µg/mL for apigeninidin and luteolinidin, respectively. In agreement with previous results, the antiproliferative effects of luteolinidin surpassed that of apigeninidin. Our findings may be due to the slight variation in structure, as luteolinidin contains one extra hydroxyl group on the 3` carbon of the B-ring of the flavonoid structure, and has been shown to

have a 3-fold increase in antioxidant activity when compared to apigeninidin[71]. Our findings agree with Marko *et al.*,[109] who reported increased activity of anthocyanidins with a higher degree of B-ring hydroxylation.

In this study, we demonstrated differential activity observed between CCSCs and the early stage HCT116 cell lines. This differential activity is thought to be a result of the selective toxicity of complex bioactive extracts against cancer cells as compared to normal epithelial cells. Earlier staged and more differentiated cancer cell lines more resemble the normal differentiated epithelial cells than do more advanced cancer cell lines. This was demonstrated in work by Seeram *et al.*[152] with total cranberry extract in four colon cancer cell types (SW480, SW620, HCT116 and HT29). SW480 and SW620 represent two cell lines from the same patient. The former representing the earlier staged primary tumor and the latter derived from a metastasis. Total cranberry extract showed 35% more antiproliferative activity against the advanced metastatic SW620 cell line when compared to SW480 cells. HCT116 and HT29 cells are both colon cancer cell lines representing early and late stage disease, respectively. Total cranberry extract showed greater proliferation inhibition activity in the advanced HT29 cell line when compared to HCT116 cells. These reports corroborate our findings that sweet sorghum extract was more efficacious against CCSCs as compared to HCT116 cells. While our results point to p53-independent activity, more work is needed to determine the mechanisms of DEE.

In the present study, we have shown for the first time that the dermal layer of sweet sorghum is rich in total phenolics and antioxidants including 3-deoxyanthocyanidins with potent *in vitro* anticancer activity against colon cancer HCT116 cells and CCSCs. This activity was greater than that of pith and we believe this to be due to the different compositions of phenolics as illustrated in Table 8. Furthermore, we demonstrated that the ethanolic extract was more

potent in suppressing the proliferation of colon cancer cells highlighting the importance of solvent selection in developing natural based phenolic rich extracts. In addition to being a source of renewable biofuel, sweet sorghum may also serve as source of health-promoting compounds for the prevention and treatment of inflammatory diseases such as colon cancer. For this to be a reality, further research is needed to determine potential toxicities and the bioactivities of sweet sorghum dermal extracts *in vivo*.

CHAPTER 3: EXTRACTS FROM SWEET SORGHUM (*SORGHUM BICOLOR*), A FEEDSTOCK FOR BIOREFINING, SUPPRESSED PROLIFERATION AND ELEVATED APOPTOSIS IN HUMAN COLON CANCER STEM CELLS VIA SUPPRESSION OF WNT/B-CATENIN SIGNALING PATHWAY

3.1 Overview.

In colorectal cancer, p53 is commonly inactivated, associated with chemotherapy-resistance, and marks the transition from non-invasive to invasive disease. Cancers, including colorectal cancer, are thought to be a disease of aberrant stem cell populations, as stem cells are able to self-renew, making them long-lived enough to acquire mutations necessary to manifest the disease. We have shown that extracts from sweet sorghum stalk components kill colon cancer stem cells (CCSCs) in a p53-independent fashion. However, the underlying mechanisms are unknown. In the present study, CCSCs were transfected with short hairpin-RNA against p53 (CCSCs p53 shRNA) and treated with sweet sorghum phenolics extracted from different plant components (dermal layer, leaf, seed head and whole plant). While all components demonstrated antiproliferative and proapoptotic effects in CCSCs, phenolics extracted from the dermal layer and seed head effectively killed CCSCs by increasing effector caspase activity, PARP cleavage, and DNA fragmentation in a p53-dependent and partial p53-dependent fashion, respectively. Further investigations revealed the antiproliferative and proapoptotic effects were associated with decreases in β-catenin protein levels, and β-catenin targets Cyclin D1, cMyc, and Survivin. These results suggest that the antiproliferative and proapoptotic effects of sweet sorghum extracts in CCSCs work via decreasing β-catenin prosurvival signaling in a p53-dependent (dermal layer) and p53-independent (seed head) fashion.

Keywords: *Dale, M81E, proliferation, apoptosis, cMyc, cyclin D1, survivin, LC-MS*

3.2 Introduction.

Colorectal cancer is the third most common cancer in males and the second most common in females[153], and up to 80% of colon cancer cases are thought to be caused by diet[19]. Recent evidence implicates that bioactive compounds (e.g. phenolic acids, flavonoids, stilbenoids, carotenoids) are responsible for imparting protective and preventive properties against chronic diseases[19]. This understanding has increased the demand for natural products meant to protect and prevent against diseases such as colorectal cancer.

The process by which a normal epithelium progresses to colon cancer is multi-step, whereby normal cells accumulate successive genetic alterations and establish clones[22]. In colorectal cancers, aberrant WNT/β-catenin signaling occurs in almost all cases and drives carcinogenesis[23,24]. However, further genetic events must occur to fully develop disease malignancy[24]. The traditional pathway to colorectal cancer accounts for 70% to 85% of all cancers[22]. This pathway starts with loss or mutation of APC resulting in β-catenin accumulation, followed by mutation in K-ras, loss of 18q, and finally loss of p53 via loss of 17p[22]. p53 is responsible for cell growth and apoptosis, therefore it prevents the build-up of mutated genomes and the advancement of cancer. It is estimated that p53 is abnormal in 50% to 75% of colorectal cancer cases, and this marks the transition from noninvasive to invasive disease[22]. Disruption of p53 is known to alter tumor responses to 5-Fluorouracil based therapies making it more difficult to effectively eliminate colorectal tumors by conventional chemotherapies[26]. Situations such as these create a need for new therapies which can target these drug resistant tumors.

β-catenin accumulation is understood to be an important driver in the carcinogenesis of colorectal cancers. It is present in both familial and sporadic forms of the disease. Excess β-catenin accumulation promotes transcriptionally active p53[154]. In addition, active p53 results in

the degradation of β-catenin[155]. β-catenin is further regulated by GSK-3β, which phosphorylates β-catenin at the N-terminus of the protein[156]. Binding of APC and Axin to GSK-3β and β-catenin facilitates this event and results in the ubiquitination and degradation of β-catenin[156]. In the absence of such regulation, β-catenin accumulates in the cytosol and translocates to the nucleus where it interacts with members of the Tcf/Lef family of transcription factors resulting in transactivation of prosurvival target proteins, such as cMyc, Cyclin D1, and Survivin[123]. GSK-3β itself is regulated by several phosphorylation events, impacting its ability to control β-catenin levels. The phosphorylation of serine residues (e.g. S9) results in decreased GSK-3β activity, while phosphorylation of tyrosine residues (e.g. Y219) results in its activation[157].

Currently, it is believed that aberrant stem cell populations are the root cause of colorectal cancers, as stem cells are able to self-renew, making them long-lived enough to acquire mutations necessary to manifest the disease[145]. Interestingly, it has been shown that Wnt/β-catenin signaling defines colon cancer stem cells in the tumor situation[125]. Furthermore, they are known to be chemo- and radiation-resistant making it difficult to treat with current standard of care practices[133], which often fail to fully eradicate the disease. It is imperative, therefore, that a cancer treatment show efficacy in the stem cell population and populations with aberrant β-catenin signaling and loss of p53. There is evidence for the efficacy of bioactive compounds in the selective removal of cancer stem cells and cancer cells with abnormal p53. Resveratrol was shown to inhibit prostate cancer stem-like cells by targeting fatty acid synthesis[158]. Curcumin and a curcumin analogue successfully targeted stem cells derived from colon cancer cell lines[137]. We have shown the combination of grape seed extract with resveratrol resulted in p53-dependent and p53-independent apoptosis in colon cancer HCT116 cells[29]. This

evidence warrants further investigation into the development of novel bioactive compound treatments for colorectal cancer.

Sorghum is an important food source worldwide, placing fifth in cereal crop cultivation[1]. Sweet sorghums are varieties of *Sorghum bicolor*, which concentrate simple sugars in the stalks and have relatively smaller seed heads. They have been used traditionally for sugar and syrup production. Sweet sorghums has been identified as an ideal source for the sugars necessary for biofuel conversion. Sweet sorghum can be advantageous over cane sugar and corn because of reduced water requirements, shorter growing periods and reduced cultivation cost[1]**.** Sweet sorghum can grow in marginal lands, and therefore does not have to compete with land used for food production, as is the case with corn and sugar cane[1,13]. Sweet sorghum and other crops can be made more attractive for biofuel conversion when producers utilize the biorefinery approach. In this approach, the entire above ground portion of the plant is utilized in the creation of energy, reduction of waste streams, and production of value-added byproducts[14,15], thereby reducing the costs associated with the generation of biofuels. Value-added by products could include bioactive extracts prepared for the purposes of human health.

Our previous work on the bioactivity of sweet sorghum stalk components showed that dermal extracts were more potent *in vitro* against colon cancer stem cells, as compared to pith[159]. However, as the whole above ground portion of the plant will be available for the biorefinery approach, it is important to evaluate the bioactivity of extracts from different parts of the sweet sorghum plant in direct comparison with one another. Such information is currently understudied in sweet sorghums. In this study, the *in vitro* anticancer activities of pith, dermal, leaf and seed head from Dale and M81E sweet sorghum varieties harvested at four different stages during plant maturity were compared to understand the effects of time of harvest. Finally, the extent to

which sweet sorghum extract bioactivity is dependent on p53 status, and its capacity to suppress β-catenin and β-catenin's downstream targets, cMyc, Survivin, and Cyclin D were investigated.

3.3 Materials and Methods.

3.3.1 Chemicals and Reagents.

Sodium bicarbonate, monobasic sodium phosphate, dibasic sodium phosphate, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA); potassium persulfate was purchased from Mallinckrodt chemicals (Hazelwood, MO); ABTS reagent, Folin-Ciocalteu reagent, Trolox, and gallic acid were purchased from Sigma (St. Louis, MO) for quantification of total phenolic and antioxidant activity.

For cell culture experiments, 5-Fluorouracil (5-FU) was used as a positive control and procured from Sigma (St. Louis, MO); colon cancer stem cell media was obtained from Celprogen (San Pedro, CA).

3.3.2 Sweet Sorghum Extraction.

The sweet sorghum used in this study was generously provided by Great Valley Energy LLC (Bakersfield, CA). Dale and M81E were grown in Bakersfield, CA and harvested at 117, 125, 138 and 152 d for dermal and 131, 145, 155, and 160 d for seed head. Later time points were chosen for seed head as maturation of this part of the plant occurs after stalk, based on sugar concentration. Samples included pith, dermal, leaf, seed head, and whole plant were mechanically separated and immediately frozen. Samples were shipped frozen overnight to Fort Collins, CO and then stored in -20 °C until needed. Two g of fresh sweet sorghum sample was extracted with 20 mL of 80% ethanol chilled to -20 °C to prevent excessive heating during the homogenization of sample. The mixture was then homogenized for 2 min using an IKA T25 digital ultra TURRAX at 10 000 rpm. The extraction tubes were then vortexed for 1 min and the

resulting mixture was centrifuged at 4000 rpm for 5 min. The resulting pellet was then re-extracted twice with 20 mL of extraction solvent. The filtrates were combined, filtered through 0.45 micron hydrophilic PTFE syringe filters, and the final volume was made to 100 mL. Samples were then aliquoted and placed in -20 °C freezer until further analyzed.

Phenolic enriched extracts were prepared by extracting two g sample in 20 mL of 80% ethanol chilled to -20 °C. Sample was homogenized with an IKA T25 Digital Ultra TURRAX set to 10 000 rpm for 2 min. The slurry was then mixed overnight on a reciprocal shaker under dark conditions and held at 4 °C. The mixture was then centrifuged and the supernatant was filtered with 0.45 µm PTFE filters (Tisch Scientific, North Bend, OH) into glass test tubes. Extracts were then further concentrated using nitrogen flow for approximately 10 h in a 35 °C water bath. The extracts were then quantified for total phenolics, aliquoted and stored in -80 °C until needed. For cell culture experiments, cells were dosed based on µg gallic acid equivalent per mL (µg GAE/mL).

3.3.3 Quantification of Total Phenolics and Antioxidant Activity.

Total phenolics were quantified by the Folin-Ciocalteu assay as described in Singleton's work[52] with minor modifications. Thirty-five µL of extract, standard or solvent was mixed with 150 µL of 0.2 N Folin-Ciocalteu reagent in a 96-well plate. After mixing and incubating for 5 min, 115 µL of 7.5% sodium bicarbonate was added, the contents mixed, and the plate was then incubated at 45 C for 30 min. The plate was allowed to cool for 1 h in dark conditions before absorbance at 765 nm was measured with a Biotek Synergy 2 plate reader (Winooski, VT). Data were reported as mean mg gallic acid equivalents per g of sweet sorghum sample (mg GAE/g) ± standard error.

For quantification of antioxidant activity, the 2,2`-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was performed as previously described[6,146] with minor modifications. Briefly, ABTS working solution was prepared fresh by mixing 3 mM ABTS radical with 8 mM potassium persulfate and allowing this solution to react for at least 12 h in the dark before mixing with a pH 7.4 phosphate buffer to form the final solution. In triplicate, 10 µL of sample, standard, or solvent was added to individual wells of a 96-well plate followed by the addition of 290 µL of working ABTS solution. The resulting mixture was then mixed and allowed to react for 30 min before absorbance at 734 nm was measured using the aforementioned plate reader. The antioxidant activity was expressed as mean (n = 3) mg Trolox equivalents per g of sweet sorghum sample (mg TE/g) ± standard error.

3.3.4 LC-MS Analysis.

A Waters Acquity UPLC system fitted with a Waters Acquity UPLCT3 column (1.8 µM, 1.0 X 100 mm) was used to achieve UPLC separation and a Waters Xevo G2 Q-Tof MS fitted with an electrospray source was used for accurate mass analysis. One µL of sample held at 5 °C was injected into the UPLC with 100% solvent A (water, 0.1% formic acid) and this was held for 1 min. Next, a 12 min linear gradient to 95% solvent B (acetonitrile, 0.1% formic acid) was applied. The flow rate was maintained at a constant 200 µL/min and the column was maintained at a constant 50 °C for the entirety of the run.

Eluent from the UPLC was then infused into MS running in the positive ion mode, scanning from 50-1200 at a rate of 0.2 s/scan, alternating between MS and MS^E mode. The collision energy was set to 6 V and 15-30 V for MS and MS^E modes, respectively. Sodium formate was used to calibrate the equipment with mass accuracy within 1 ppm prior to the run. The capillary voltage was set to 2200 V, the source and the desolvation temperatures were set to

150 °C and 350 °C, respectively. The desolvation gas flow rate was 800 L/h of nitrogen. Waters raw data files were converted to .cdf format using Databridge software (Waters), and feature detection was performed using XCMS. Raw peaks were normalized to total ion signal in R.

3.3.5 Cell Lines and Culturing Procedures.

Colon cancer stem cells (CCSCs) were purchased from Celprogen (San Pedro, CA) and were maintained at a temperature of 37 °C and 5% CO_2. CCSCs were maintained in colon cancer stem cell media (Celprogen) and passaged 1:10 to 1:20 when cells were 80% confluent. For experiments, CCSCs were treated in serum free colon cancer stem cell media (Celprogen) with sweet sorghum extracts based on µg GAE/mL in accordance with our previously published protocols[160,161].

3.3.6 Lentiviral shRNA Mediated Knockdown of p53 in CCSCs.

Lentiviral particles encoding shRNA targeting p53 (Santa Cruz Biotechnology Inc; Santa Cruz, CA) were used to mediate p53 suppression in CCSCs at a multiplicity of 10 in growth medium containing 5 µg/mL polybrene. After 24 h of transfection, media was replaced and the cells were cultured for 2 d. Transfected cells were then selected in the presence of puromycin (7.5 µg/mL) for an additional 5 d. Western blots were then performed on transfected whole cell lysates to confirm the suppression of p53.

3.3.7 Proliferation and Apoptosis.

Cell proliferation was assessed via 5-bromo-2'-deoxyuridine (BrdU) assay (Cell Signaling Technology, Beverly, MA). Briefly, CCSCs were seeded in 96-well plates at 2 X 10^4 cells per well. After 24 h incubation, the media was aspirated and the cells were treated with sweet sorghum extracts in triplicate with serum free media for 20 h at 37 °C. At the end of the treatment period, BrdU was added at a concentration of 10 µM per well to each well and

incubated at 37 °C for 4 h to allow incorporation of BrdU into cellular DNA resulting in 24 h total incubation. Media was then aspirated and the BrdU assay was performed according to manufacturer's protocol. IC_{50} values were calculated and used to standardize dosage of dermal and seed head phenolics in subsequent studies of apoptosis. The annotations ½IC_{50}, IC_{50} and 2IC_{50} are used in Figures 21 and 22. This refers to ½, 1 and 2 times the IC_{50} value for each respective treatment in each respective cell lines. This equates to treatments of 5, 10 and 20 µg GAE/mL for a treatment with an IC_{50} of 10 µg GAE/mL in a particular cell line.

Apoptosis was analyzed by measuring active caspases 3 and 7 with a Caspase-Glo 3/7 assay kit (PromegaCorp., Madison, WI) and by assessing DNA fragmentation with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. For measuring caspase activity, cells were treated for 12 or 24 h with treatments. Then cells were trypsinized and 200 µL of media containing CCSCs or CCSCs p53 shRNA were transferred into a white 96-well plate and caspase activity was measured according to manufacturer's protocol. Data were normalized to cell count and expressed as percent of solvent control. For TUNEL assay, cells were seeded into four-well chambered glass slides at a density of 1 X 10^5 cells per chamber. After treatments with control compounds and sweet sorghum extracts, the slides were prepared and analyzed for TUNEL staining according to manufacturer's protocols (In Situ Cell Detection Kit, FL: Roche Diagnostics; Mannheim, Germany).

3.3.8 Western Blotting.

Cells were plated in six-well plates at a concentration of 3.0 X 10^5 cells per well in human colon cancer stem cell medium. After 24 h, cells were treated in serum free medium for 18 h. Cells were then mixed in 150 µL/well high salt buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 % Glycerol, 1 % Triton-X, pH 7.5) containing protease inhibitors

(Thermo Scientific; Rockford, IL) and lysed using sonication. Protein (40 µg) was then subjected to gel electrophoresis on Criterion XT Bis-Tris 4 - 12 % Gel (Biorad; Hercules, CA) and transferred to PVDC membrane. The membrane was blocked using Superblock (Thermo Scientific; Rockford, IL) overnight at 4 °C. The antibody against PARP (9542p) was purchased from Cell Signaling Technologies (San Diego, CA). Antibodies against β-actin (sc-1616), p53 (sc-6243), β-catenin (sc-7963), survivin (sc-17779), cyclin D1 (sc-20044), cMyc (sc-40), GSK-3β (sc-9166), pGSK-3β^{S9} (sc-11757), and pGSK-3β^{Y219} (sc-135653) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All primary antibodies were incubated overnight at 4 °C at a dilution of 1:500. IRDye 800 Donkey anti-goat, IRDye 680 goat anti-mouse, IRDye 800 goat anti-mouse, IRDye 680 goat anti-rabbit, and IRDye 800 goat anti-rabbit secondary antibodies were purchased from LI-COR Bioscience (Lincoln, NE) and incubated for 2 h at room temperature at a dilution of 1:10000. Blots were imaged and quantified using the Odyssey Infrared Imaging System and software (LI-COR Biotechnology; Lincoln, NE).

3.3.9 Statistical Analysis.

Statistical analysis was done using SAS version 9.3 and significance was determined by one way ANOVA, followed by the post-hoc Fisher least significant difference (LSD) test for multiple means comparisons. For Western blotting, data were transformed by natural log before being subjected to one way ANOVA and LSD statistical analyses. Pearson's correlations were performed using IBM's SPSS version 21. Values not sharing the same letter were statistically significant ($P < 0.05$).

3.4 Results and Discussion.

3.4.1 Component Analysis Reveals Seed Head and Dermal as Most Potent Sources of Anticancer Phenolics from Sweet Sorghum.

Data for the TP and ABTS values of pith, dermal, leaf, seed head and whole plant components from the sweet sorghum variety M81E are summarized in Figure 19A. We observed that the leaf and seed head had significantly higher TP with values of 4.45 and 4.37 mg GAE/g, respectively ($P < 0.05$). Dermal and the whole plant had similar levels of TP, 1.07 and 1.17 mg GAE/g, respectively. However, this was not significantly different than pith (0.14 mg GAE/g) at the 5% level. Similar findings were found for the ABTS values of sweet sorghum components. ABTS values for leaf and seed head were the highest at 16.98 and 19.18 mg TE/g, respectively. The dermal (7.31 mg TE/g) and the whole plant (7.64 mg TE/g) had significantly higher ABTS than the pith at 0.35 mg TE/g ($P < 0.05$). Our findings are similar to the findings of Awika *et al*. who reported TP and ABTS values for sorghum grains to range from 1 - 13 mg GAE/g and 1.5 - 27 mg TE/g (6 - 108 µMole TE/g) on a dry matter basis, respectively[6]. Ring *et al*. investigated the levels of phenolics in different grain sorghum tissues during maturation and reported similar TP values for the leaves (3.4 – 11.7 mg GAE/g) and stalk (0.6 – 1.0 mg GAE/g)[56]. This indicates there is a similarity in the quantities of TP and other reducing agents expressed in sweet sorghums and grain sorghums, despite the high morphologic and phenotypic variation.

The antiproliferative and proapoptotic properties of these extracts *in vitro* against CCSCs were investigated, and the data are summarized in Figures 19B-D. Due to the low concentration of phenolics and our previous findings suggesting lack of *in vitro* bioactivity[159], pith extract was excluded from this analysis. Dermal and seed head extract suppressed proliferation at 35 µg GAE/mL to 55% and 38% of control, respectively, as measured by BrdU incorporation. This was

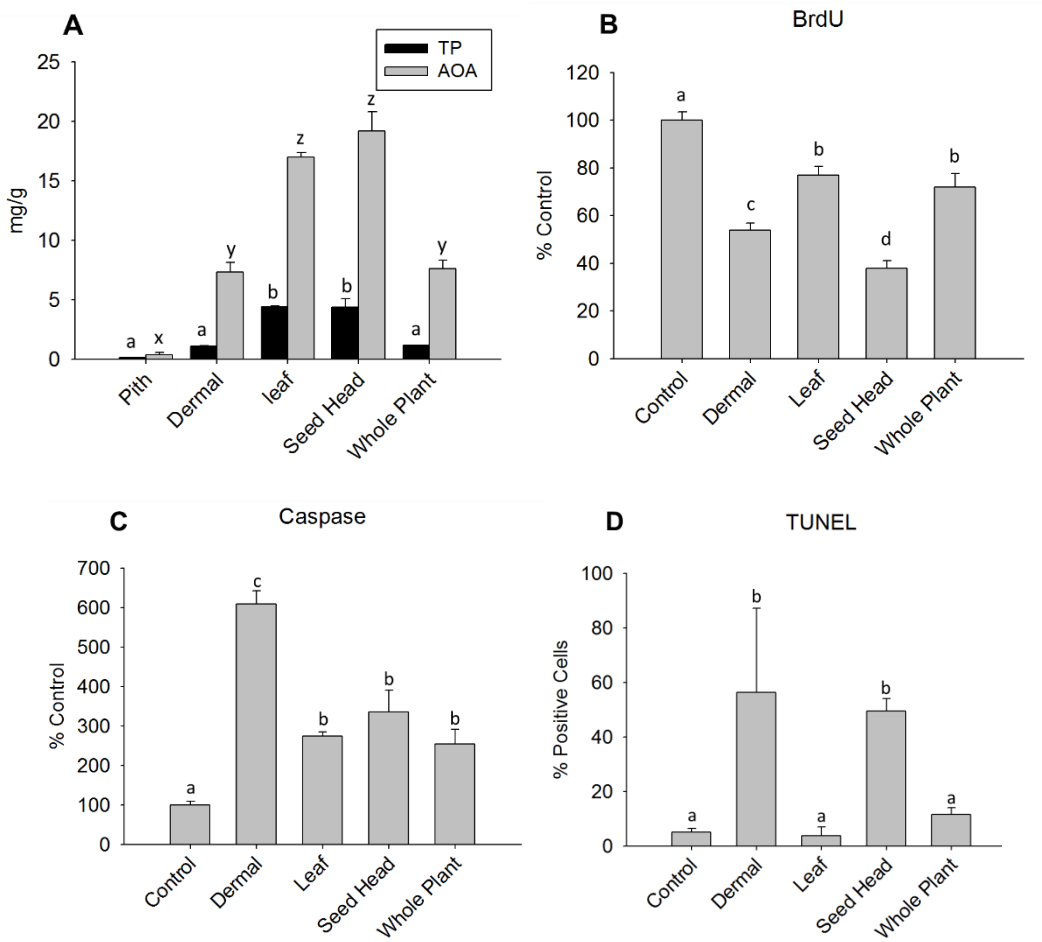


Figure 19. Extracts prepared from sweet sorghum variety M81E components (pith, dermal, leaf, seed head, and whole plant) contain total phenolics and antioxidants with antiproliferative and proapoptotic activity. **(A)** The total phenolics and ABTS values of sweet sorghum components as measured by the Folin-Ciocalteu and ABTS assay, respectively. Values are presented in mg gallic acid equivalents per g of tissue and mg Trolox equivalents per g of tissue (collectively referred as mg/g) for total phenolics and ABTS, respectively. Mean mg/g values (n = 3) ± standard error are represented. **(B)** The antiproliferative activity of sweet sorghum component extracts after 24 h treatment with 35 µg GAE/mL, as measured by the BrdU assay. Data are presented as percent of control (% control) as means (n = 3) ± standard error. Sweet sorghum component extracts induce effector caspase activation and DNA fragmentation after 24 h treatment with 35 µg GAE/mL, as measured by **(C)** Caspase 3/7 Glo assay and **(D)** TUNEL assay. Data for Caspase 3/7 Glo assay are presented as percent of control as means (n = 2) ± standard deviation. Data for TUNEL assay are presented as mean (n = 2) apoptotic index (percent positive cells) ± standard deviation. Different letters indicate significant differences ($P < 0.05$) among treatments. Control = 80% ethanol used as negative control.

significantly more than leaf (78%) or whole plant extract (73%) ($P < 0.05$). Proapoptotic activity of the extracts was measured by the Caspase 3/7 Glo assay and TUNEL assay. Dermal extracts were more potent in activating caspases (610%), as compared to other components and whole

plant (Figure 19C). Leaf, seed head, and whole plant extracts all induced caspase activation to similar levels in comparison to control (274%, 336%, and 255%, respectively). Interestingly, only extracts from dermal and seed head increased the percentage of apoptotic cells as measured by the TUNEL assay to 57% and 49%, respectively ($P < 0.05$). Based on the observed difference in the proapoptotic effects of dermal and seed extracts in comparison to other components, these components were selected for further investigations. Despite high TP and ABTS values, leaf extract only marginally decreased CCSCs proliferation and failed to increase apoptosis as measure by TUNEL positive staining. It is important to note that these extracts did increase activated caspases 3 and 7 after 12 h and likely that with time would have resulted in DNA cleavage observable by TUNEL staining.

3.4.2 Effects of Time of Harvest on the Bioactivity of Sweet Sorghum Components.

The time of harvest has been shown to influence levels of bioactive compounds in sorghum[162,163]. We wanted to know if this variable had an influence on the levels and bioactivity of sweet sorghum. Table 9 shows TP, ABTS and BrdU values from the dermal and seed head of Dale and M81E sweet sorghum varieties at four times of harvest to assess the impact of plant development on the quantities and activity of dermal and seed head phenolic rich extracts. The TP and ABTS values for both dermal and seed head were similar in all varieties. For dermal, TP and ABTS values ranged from 0.84 – 2.14 mg GAE/g and from 2.32 – 6.48 mg TE/g for dermal across all varieties, respectively. In agreement with the component analysis, the seed head had higher TP and ABTS values than did dermal, indicating that the seed head of sweet sorghum is a more concentrated source of phenolics. Seed head TP and ABTS values ranged from 2.28 – 8.88 mg GAE/g and from 12.64 – 28.65 mg TE/g across all varieties, respectively. As expected, and

Table 9. Determination of TP, ABTS and BrdU in dermal and seed head extracts from Dale and M81E varieties of sweet sorghum at different times of harvest.

	dermal				seed head			
variety	**DAP [a]**	**TP[b]**	**ABTS[c]**	**BrdU[d]**	**DAP**	**TP**	**ABTS**	**BrdU**
Dale	117*	0.84 ± 0.04	2.32 ± 0.17	59 ± 3.5	131**	2.30 ± 0.23	13.03 ± 0.94	32 ± 3.0
	125*	1.10 ± 0.01	3.18 ± 0.09	120 ± 2.8	145**	2.46 ± 0.11	13.93 ± 0.35	26 ± 2.3
	138*	1.42 ± 0.09	3.85 ± 0.32	109 ± 1.3	153**	2.28 ± 0.03	12.64 ± 0.58	49 ± 2.3
	152*	1.48 ± 0.14	4.11 ± 0.59	109 ± 1.6	160**	2.82 ± 0.25	14.97 ± 0.72	79 ± 1.0
M81E	117*	1.52 ± 0.06	4.68 ± 0.31	72 ± 1.2	131**	6.01 ± 0.38	25.58 ± 1.23	15 ± 0.5
	125*	1.86 ± 0.08	5.32 ± 0.16	54 ± 2.1	145**	5.00 ± 0.32	21.57 ± 1.22	13 ± 0.4
	138*	2.14 ± 0.14	6.38 ± 0.55	33 ± 4.7	153**	8.02 ± 0.23	28.98 ± 0.32	2 ± 0.3
	152*	2.10 ± 0.04	6.48 ± 0.28	91 ± 0.9	160**	8.88 ± 0.46	28.65 ± 0.07	5 ± 0.6

[a] DAP = Days after planting. Sweet sorghums were harvested at different times represented as d after planting. * Were planted on 05/10/2012. ** Were planted on 06/13/2012. [b]Total phenolics (TP) were determined by the Folin-Ciocalteu assay and data are presented as mg gallic acid equivalents per g sample (mg GAE/g). [c]ABTS values were determined by the ABTS method and data are presented as mg Trolox equivalents per g sample (mg GAE/g). [d]CCSCs were treated for 24 h with dermal or seed head extracts at 35 µg GAE/mL concentration. Values were determined from the BrdU assay and expressed as percent of control. Low values indicate proliferation suppression. All data are presented as means (n = 3) ± standard error. Highlighted cells were selected for subsequent investigations.

in agreement with previous works on sorghum[6,54], there was a high correlation between sweet sorghum TP and ABTS values of both dermal ($r = 0.966$, $P < 0.0005$) and seed head ($r = 0.974$, $P < 0.0005$). Furthermore, time of harvest correlated with dermal TP ($r = 0.476$, $P = 0.019$) and ABTS values ($r = 0.531$, $P = 0.008$). This indicates that as the sweet sorghum plant matures, it concentrates phenolics and antioxidants in the dermal layer of the stem.

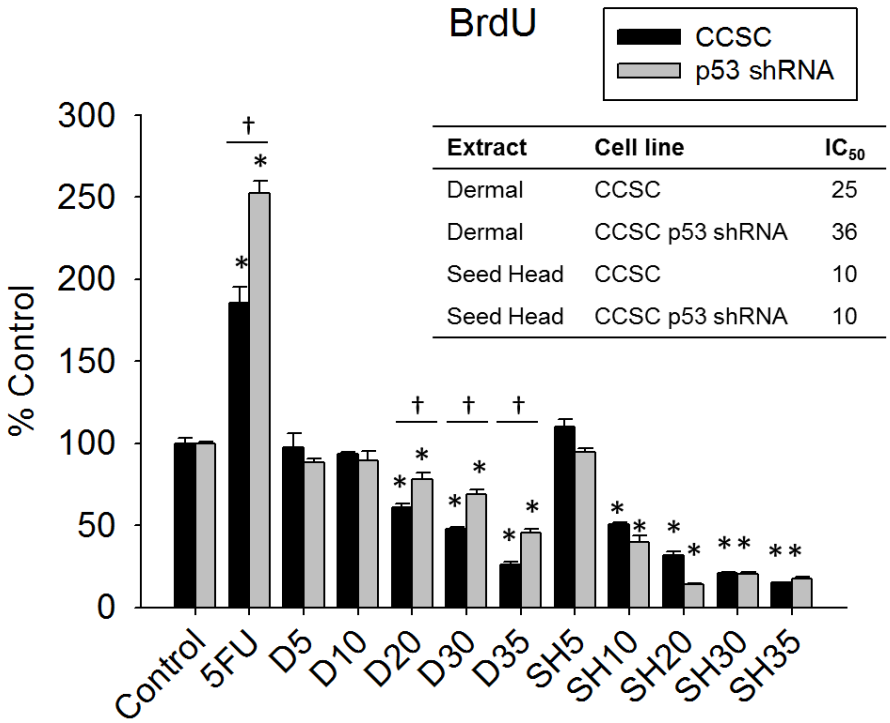
One of the most important roles of phenolics in plants is to defend the plant from pathogenic infection and to deter herbivorous predation[50]. Therefore it is logical for sweet sorghum to concentrate more phenolics in the seed head than dermal layer as a means of preserving its genome. Indeed, certain bird resistant varieties of grain sorghum have been identified, and these varieties differ based on the presence of tannins, a broad class of astringent polyphenolics, which deter feeding[164,165]. As sweet sorghums mature, sugars are concentrated in the stalk and this puts the plant at risk for boring insects. Thus, it is logical for the plant to

concentrate phenolics during plant development to prevent and protect the plant from attack. The antiproliferative activity varied widely amongst varieties and time of harvest in sweet sorghum dermal and seed head components. We found significant negative correlations between seed head BrdU antiproliferative values and seed head TP ($r = -0.716$, $P < 0.0005$) and ABTS ($r = -0.726$, $P < 0.0005$) values. This may reflect changing bioactive profiles in the seed head and warrants further investigation. For dermal extracts, BrdU antiproliferative activity did not correlate with time of harvest, TP values, nor ABTS values, implying that the antiproliferative activity of these extracts cannot be explained by quantity of the phenolics in the plant and may be better explained by the composition of the phenolics at the time of harvest. One confounding factor may be differences in non-bioactive reducing, as these can contribute to TP and ABTS readings without contributing to antiproliferative activity of the extract. Nonetheless, our analysis shows extracts from multiple varieties of sweet sorghum dermal and seed head, harvested at various stages of maturity, contain strong antiproliferative constituents. This supports the use of dermal and seed head as a source of bioactive compounds for human health in the biorefinery approach to biofuel conversion from sweet sorghum. This is important as variety and time of harvest may vary between seasons and between producers.

3.4.3 The Anticancer Effects of Dermal and Seed Head Phenolics

To better understand the antiproliferative and proapoptotic effects of sweet sorghum phenolics, and to determine the extent to which these effects are p53-dependent, successive studies were performed. The highlighted rows in Table 9 indicate the source of phenolic rich extracts to be used in subsequent studies of proliferation and apoptosis. These materials were selected based primarily on the ability of the extract to decrease proliferation as measured by BrdU assay. We then compared observed bioactivities of sweet sorghum extracts with the

standard chemotherapeutic drug 5-FU. As shown in Figure 20, IC_{50} values were calculated by BrdU assay after 24 h treatment in CCSCs and CCSCs p53 shRNA. Both dermal and seed head dose-dependently decreased cellular proliferation in both cell lines. Dermal extract had IC_{50} values of 25 and 36 µg GAE/mL in CCSCs and CCSCs p53 shRNA, respectively. Seed head extracts were shown to be more potent than dermal and independent of p53 as treatment resulted in IC_{50} values of 10 µg GAE/mL in both cell lines with no statistical differences observed between cell lines. However, in CCSCs p53 shRNA, seed head extracts suppressed proliferation in a p53 independent fashion, whereas the dermal extract was not as effective in the p53 shRNA cell line and this was reflected by a higher IC_{50} for dermal extract. Interestingly, we observed 5-FU induced increases in rates of proliferation in CCSCs and CCSCs p53 shRNA. This is in



Extract	Cell line	IC_{50}
Dermal	CCSC	25
Dermal	CCSC p53 shRNA	36
Seed Head	CCSC	10
Seed Head	CCSC p53 shRNA	10

Figure 20. The antiproliferative activity of select dermal and seed head extracts and calculated IC_{50} values were determined by BrdU assay after 24 h treatment with extracts. Data are presented as percent of control as means (n = 3) ± standard error. * Significant difference from control ($P < 0.05$). † Significant differences between CCSCs and CCSCs p53 shRNA of the same treatment ($P < 0.05$). Control = 80% ethanol used for solvent control; D = Dermal treatments at concentrations indicated by number in µg GAE/mL; SH = Seed head extract treatments at concentrations indicated by number in µg GAE/mL; 5FU = 5-fluorouracil (18 µg/mL).

contrast to previous studies reporting decreases in the proliferation of multiple colon cancer cell lines[166,167]. However, it has been reported that ATP synthase inhibitors in conjunction with 5-FU treatment increased colon cancer cell proliferation[168].

The proapopototic activity of sweet sorghum extracts are summarized in Figure 21 and assessed by activation of effector caspases with Caspase 3/7 Glo assay, PARP cleavage by Western blotting, and apoptotic index with TUNEL staining (% TUNEL positive staining). Dermal extracts, only after 18 h, significantly increase the levels of cleaved PARP by 7.8-fold and 2.1-fold in CCSCs and CCSCs p53 shRNA, respectively. Furthermore, dermal extract at 2 times the IC_{50} concentration increased the percentage of TUNEL positive CCSCs and CCSCs p53 shRNA to 82% and 45%, respectively. Therefore, the proapoptotic effects observed in dermal treated cells was most probably mediated via activated caspases as activated effector caspases are responsible for PARP cleavage. Given the increase in caspase activity observed in dermal treated cells after 24 h (Figure 19C), it may be that activation of effector caspases comes after the 12 h time point we measured for this analysis. Our results further show the activity of dermal extract was markedly and significantly diminished by suppression of the tumor suppressor p53 in CCSCs p53 shRNA, indicating a partial dependence on p53 for the mediation of dermal extract's activity. This was not observed in cells treated with seed head extract. After 12 h of seed head treatment, caspase activation was increased from 100% to 5118% control and from 100% to 4503% of control in CCSCs and CCSCs p53 shRNA, respectively. There was no difference between cell lines in caspase activation, indicating a p53-independence. Interestingly, this surpassed caspase activation induced by 5-FU. Seed head treatment resulted in 4.7-fold and 1.3-fold decreases in full length PARP and 21-fold and 38-fold increases in the 82 kDa PARP cleavage product in CCSCs and CCSCs p53 shRNA, respectively. TUNEL staining also

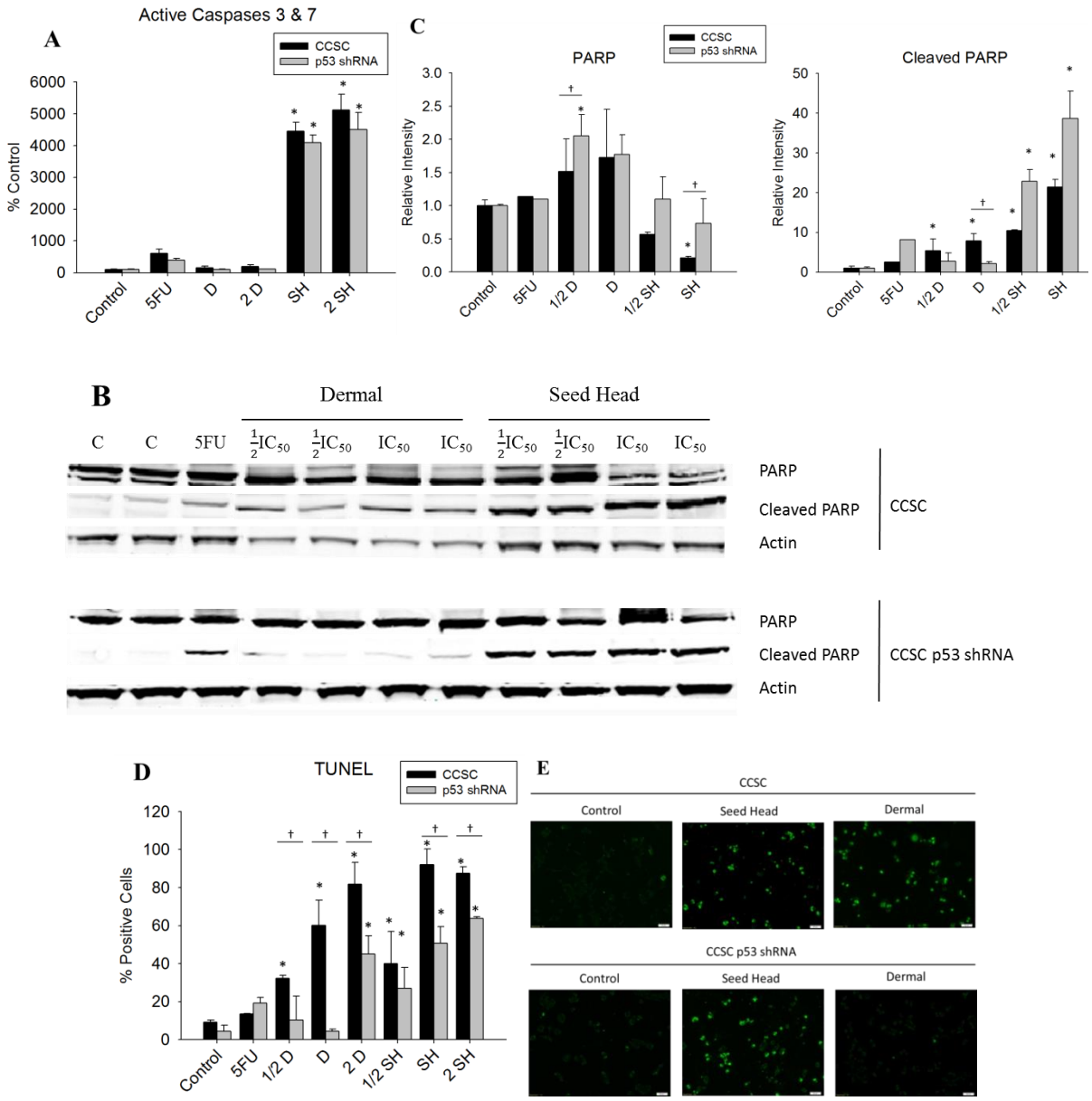


Figure 21. Extracts from sweet sorghum dermal and seed head induce effector caspases-3 and -7 activity, PARP cleavage, and DNA fragmentation, as measured by Caspase 3/7 Glo assay, Western blotting, and TUNEL assay in Colon Cancer Stem Cells (CCSCs). **(A)** Data from Caspase 3/7 Glo assay are presented as percent of the solvent control (% Control) as means (n = 2) ± standard deviation. **(B)** Images from Western blots probed with antibodies against PARP and β-actin. **(C)** Levels of PARP and cleaved PARP were quantified by densitometry relative to β-actin with control lanes set to 1. **(D)** Results from TUNEL assay experiments are presented at apoptotic index (% Postive Cells) as means (n = 2) ± standard deviation. **(E)** Representative images of TUNEL staining in cells treated with control, dermal or seed head treatments at their respective IC_{50} values. Images from top row are from CCSCs and bottom row are from CCSCs p53 shRNA. * Indicates significant difference from control ($P < 0.05$). † Indicates significant differences between CCSCs and CCSCs p53 shRNA of the same treatment ($P < 0.05$). The coefficient preceding the treatment represents dose (i.e. 1/2 is equivalent to 1/2 of the IC_{50} value) Control = 80% ethanol used for solvent control; D = Dermal extract; SH = Seed head extract; 5FU = 5-fluorouracil (18 µg/mL).

indicated apoptosis after 18 h of seed head treatment, as seed head increased the percentage of TUNEL positive CCSCs to 88% and to 64% in CCSCs p53 shRNA at a concentration of 2 times the IC_{50}.

3.4.4 Dermal and Seed head Suppresses β-Catenin Levels and Downstream Targets.

Having shown differences in the dependence of p53 status for the activity of dermal and seed head extracts, successive investigations were carried out to assess the molecular mechanisms responsible for the activities observed in sweet sorghum phenolics. Given the prominence of aberrant Wnt/β-catenin in colon cancer, we treated CCSCs and CCSCs p53 shRNA with dermal and seed head phenolics for 18 h and Western blotting was performed to measure protein expression. Figure 22 demonstrates treatment with seed head decreased β-catenin in a dose-dependent manner in both CCSCs and CCSCs p53 shRNA. However, this activity was slightly diminished in the p53 shRNA cell line, indicating a need of p53 for full activity. Dermal phenolics also decreased β-catenin levels in CCSCs. However, this activity was completely absent in p53 shRNA cells, indicating p53-dependency.

β-catenin's prosurvival effects are partly mediated via the transactivation of target genes, such as cMyc, Cyclin D1, and Survivin[123]. In accordance with our observations, seed head treatment of both cell types resulted in concurrent decreases in cMyc and Survivin in a partly p53-independent manner. Dermal treatment also resulted in the decrease of these β-catenin targets in CCSCs. However, dermal activity was diminished in CCSCs p53 shRNA and determined to be p53-dependent. The only exception to this was Cyclin D1. Dermal significantly and dose dependently suppressed Cyclin D1 levels in both cell lines, indicating that Dermal's effects on Cyclin D1 are p53-independent. Furthermore, this indicates that suppression of Cyclin D1 may be achieved through other pathways. Significant correlation was observed between

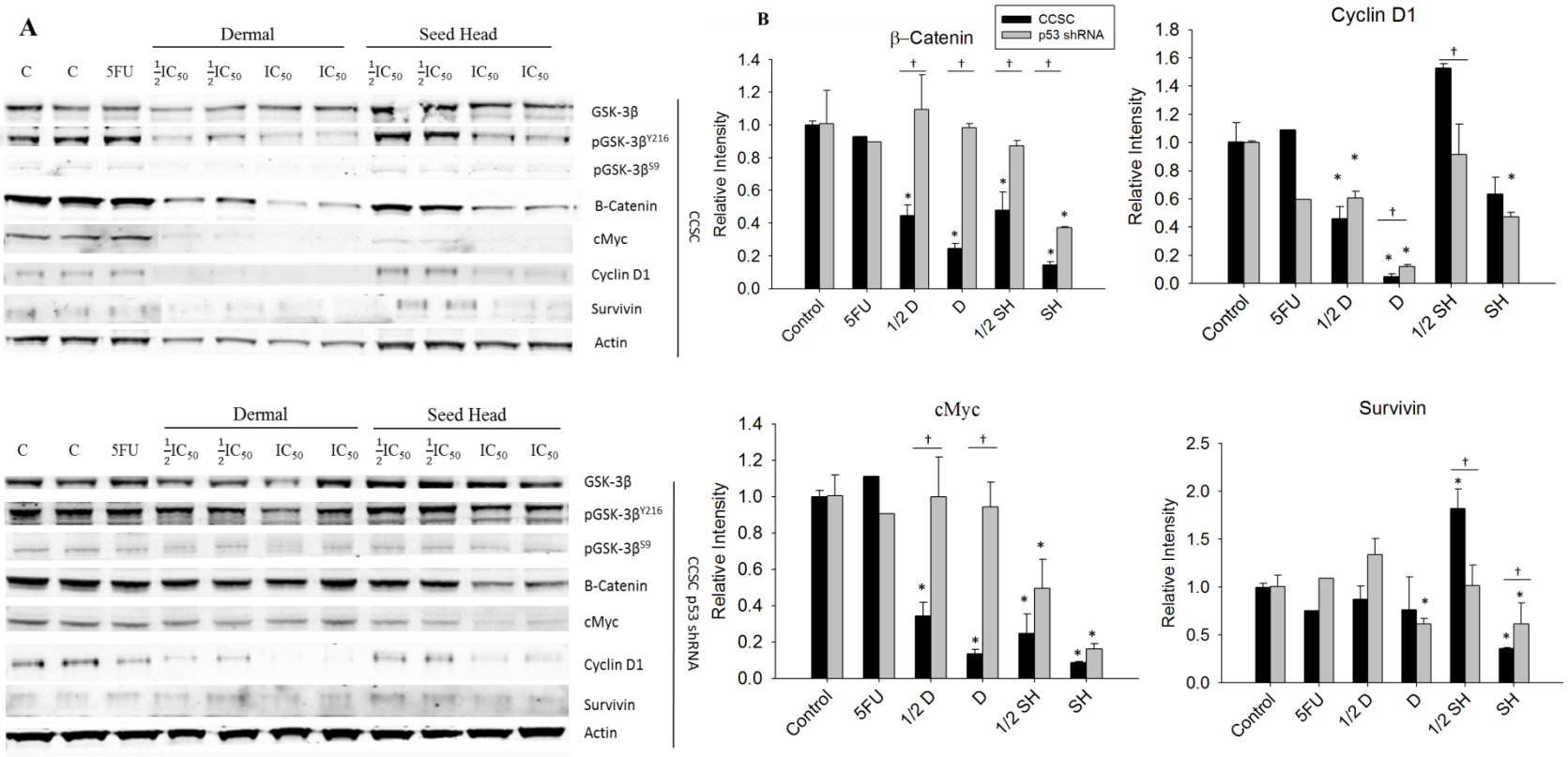


Figure 22. Extracts from sweet sorghum dermal and seed head decrease levels of β-catenin and β-catenin targets cMyc, Cyclin D1, and Survivin in a p53-dependent manner for dermal treated cells and a partial p53-dependent manner for seed head treated Colon Cancer Stem Cells (CCSCs). **(A)** CCSCs and CCSCs p53 shRNA were treated with dermal or seed head extracts for 18 h and whole cell lysates were subjected to Western blotting and probed with antibodies against GSK-3β, pGSK-3β^{S9}, pGSK-3β^{Y216}, β-catenin, cMyc, Cyclin D1, Survivin, and β-actin. **(B)** Levels of β-catenin, cMyc, Cyclin D1, and Survivin were quantified by densitometry relative to β-actin with control lanes set to1. Data are presented as means (n = 2) ± standard deviation. * Indicates significant difference from control ($P < 0.05$). † Indicates significant differences between CCSCs and CCSCs p53 shRNA of the same treatment ($P < 0.05$). Control (C) = 80% ethanol used for solvent control; D = Dermal extract treatments; SH = Seed head extract treatments. Dermal and seed head treatments were dosed based on IC_{50} values in µg GAE/mL. The coefficient preceding the treatment represents dose (i.e. 1/2 is equivalent to 1/2 of the IC_{50} value). 5FU = 5-fluorouracil (18 µg/mL).

apoptosis (TUNEL staining) and β-catenin ($r = -0.920$, $P < 0.01$), cMyc ($r = -0.922$, $P < 0.01$), and Survivin ($r = -0.519$, $P < 0.01$). We did not find significant correlations between apoptosis and Cyclin D1, which is to be expected as it functions to suppress cell cycle progression and is not thought to influence apoptosis.

GSK-3β, pGSK-3β^{S9} and pGSK-3β^{Y219} were probed with Western blotting to determine if β-catenin decreases in response to treatment were a result of altered GSK-3β activity. Figure 22a shows that while GSK-3β, and pGSK-3β^{S9} levels remained unaltered, levels of pGSK-3β^{Y219}decreased in CCSCs. This is contrary to expectation, as decreased levels of active pGSK-3β^{Y219}would seemingly increase β-catenin. However, decreases in β-catenin levels could be explained by enhanced binding of GSK-3β to β-catenin through interaction with APC. As this was not measured in the current study, this will remain purely as speculation. Nonetheless, we show for the first time that sweet sorghum extracts dose-dependently decreased β-catenin levels, and this was concurrently associated with decreased cellular proliferation, increased apoptosis, and decreased expression of cMyc, Cyclin D1 and Survivin. This was found to be partly p53-independent in seed head extract and p53-dependent in dermal extract.

Differences observed in LC-MS anlaysis may explain differences in proapoptotic and antiproliferative activity observed between dermal and seed head, demonstrating that the composition of phenolics as determined by source is an important determinant of an extract's effectiveness against cancer stem cells and dependence on p53 for activity. These bioactive compounds contained within the seed head extract and dermal extract of sweet sorghum possess antiproliferative and proapoptotic activity against colon cancer stem cells in a partial p53-dependent manner for seed head extracts and p53-dependent for dermal extracts. Apoptosis was

mediated via activation of effector caspases, resulting in activation of PARP and subsequent DNA fragmentation.

To better understand the differences observed in dermal's and seed head's *in vitro* activity, we next performed LC-MS analysis to assess differences in polyphenolics. Analysis revealed 842 different features that differed by at least 2-fold (Figure 23) ($P < 0.05$). We were also able to identify differences in multiple phenolic compounds previously reported in sweet sorghum and reported to have bioactivity (Table 10). In total, we identified 10 compounds; apigeninidin, luteolinidin, malvidin glucoside, apigenin, luteolin, naringenin, naringenin glucoside, eriodictyol glucoside, taxifolin, and catechin. Based on relative abundance, seed head contained 9813% more apigeninidin and 650% more luteolininidin, a class of 3-deoxyanthocyanidins with potent phase II enzyme inducing and anticancer activity[8]. Seed head also contained significantly more luteolin, apigenin, naringenin glucoside, and Eriodictyol glucoside.

Table 10. Phenolic compounds detected by LC-MS in dermal and seed head extracts.

identity	formula[a]	Rt (min)	observed mass	exact mass[b]	Δppm	intensity[c] dermal	intensity[c] seed head	ref[d]
apigeninidin	$C_{15}H_{11}O_4^+$	4.26	255.066	255.0660[M]	0	29	2875*	4,5,70
luteolinidin	$C_{15}H_{11}O_5^+$	5.97	271.061	271.0607[M]	1	6	45*	4,5,70
malvidin 3-*O*-glucoside	$C_{23}H_{25}O_{12}^+$	4.83	493.134	493.1314[M]	5	221*	91	4
apigenin	$C_{15}H_{10}O_5$	4.02	271.061	271.0607	1	41	5934*	5
luteolin	$C_{15}H_{10}O_6$	3.37	287.056	287.0556	1	12	162*	5,72
naringenin	$C_{15}H_{12}O_5$	5.83	273.076	273.0764	2	25*	3	5
naringenin 7 O glucoside	$C_{21}H_{22}O_{10}$	4.23	435.129	435.1292	1	6	84*	169
eriodictyol 5-glucoside	$C_{21}H_{22}O_{11}$	4.28	451.123	451.124	2	10	464*	79
taxifolin	$C_{15}H_{12}O_7$	3.37	305.066	305.0662	1	188*	34	5
catechin	$C_{15}H_{14}O_6$	4.07	291.086	291.0869	3	7	86*	5

[a]Formulas are based on [M]. [b]Exact masses are based on $[M+H]^+$ unless otherwise indicated. [M]Mass based on $[M]^+$. [c]Normalized peak intensities based on peak areas normalized to total ion signal in R. [d]References which have previously identified the compound listed. * Significant differences ($P < 0.05$) observed between dermal and seed head extracts.

Interestingly, luteolin and apigenin have demonstrated proapoptotic activity in the p53 mutated colon cancer HT29 cell line[170,171]. This supports our observations of p53-independent apoptosis in seed head treated CCSCs. On the other hand, the dermal layer contained more of the malvidin glucoside, naringenin and taxifolin as compared to seed head. These differences observed in LC-MS anlaysis may explain differences in proapoptotic and antiproliferative activity observed between dermal and seed head, demonstrating that the composition of phenolics as determined by source is an important determinant of an extract's effectiveness against cancer stem cells and dependence on p53 for activity.

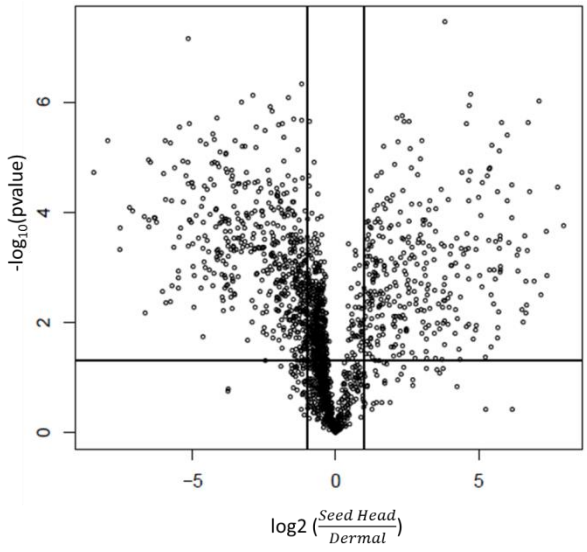


Figure 23. Extracts from the dermal and seed head extract differed in metabolomics profiling. Volcano plot illustrating different features between dermal and seed head extracts. $\log_2$ fold change (seed head/dermal) is plotted on X-axis against the $-\log_{10}$ Pvalue. Features greater than 2-fold change between seed head and dermal are represented by points that fall outside of the vertical bars. Significantly different features ($P < 0.05$) are those features that fall above the horizontal bar.

The cumulative results from this study suggest that the phenolics of sweet sorghum component dermal and seed head have antiproliferative and proapoptotic activity. Seed head and dermal extracts were the most potent, while leaf and stalk pith were least effective. Antiproliferative effects of seed head and dermal were p53-independent and partially p53-dependent, respectively. Proapoptotic effects were partially p53-dependent for both extracts.

Proapoptotic activity tracked closely with altered β-catenin levels, which resulted in decreased proliferative and survival signaling by way of decreased cMyc, cyclin D1, and survivin expression (Figure 22). Differences in composition of phenolic compounds contained in the dermal and seed head may help to explain differences in anticancer effects. A complete understanding of the mechanism(s) of action is still unknown and further research is needed to elucidate sweet sorghum's anticancer properties and potential uses as a chemopreventive and chemotherapeutic agent in vivo. Furthermore, research is needed to understand the mechanisms through which these extracts suppress β-catenin. While serving as a feedstock for biofuel production, sweet sorghum biomass generated by biorefining may also serve as a reservoir for bioactive compounds for human health

CHAPTER 4: PHENOLIC RICH EXTRACTS FROM THE DERMAL LAYER OF SWEET SORGHUM (*SORGHUM BICOLOR)* STALK, A BYPRODUCT OF BIOFUEL PRODUCTION, REDUCES SYSTEMIC OXIDATIVE STRESS IN A MURINE MODEL OF HIGH-FAT DIET INDUCED OBESITY

4.1 Overview.

Obesity is now a major global health problem and is often associated with chronic, sub-acute inflammation and increased oxidative stress. *Sorghum bicolor* is an important agricultural crop and the grain, bran, leaves, and leaf sheaths contain phenolic acids, anthocyanidins, anthocyanins, and other flavonoids with antiinflammatory and antioxidant activities. However, less has been documented on the *in vivo* safety and bioactivity of *S. bicolor* stalk. The protective effects of an ethanolic extract from the dermal layer of the stalk (SS) were examined in male and female mice consuming low-fat diet (LFD) and high-fat diet (HFD). Extract was supplied as a lyophilized powder in the diets at 1% w/w. During the last week, mice were subjected to dual energy x-ray absorptiometry and glucose tolerance test to assess adiposity and metabolic disorder, respectively. After 10 wk of feeding, mice were sacrificed, vital organs weighed, and tissues and plasma collected for further analysis. The inflammatory markers, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-1β, the macrophage marker, F4/80, and monocyte chemotactant protein 1 (MCP-1), and the tight junction proteins zona occluden 1 (ZO-1) and occludin were assessed in the colonic mucosa by qPCR. SS was well-tolerated by mice consuming LFD and HFD, as indicated by normal body weight, vital organ weights, feed intake, and water intake. Addition of SS had no effects on adiposity. SS did suppress oxidative stress as indicated by reduced plasma 8-isoprostane levels. These results support *S. bicolor* stalk as a safe source of bioactive compounds with *in vivo* antioxidant activity.

Keywords: *obesity, high-fat diet, oxidative stress, Sorghum bicolor, sweet sorghum, stalk*

4.2 Introduction.

Obesity is now a major global health problem[172]. Obesity is often associated with chronic, sub-acute inflammation and increased oxidative stress[173]. Indeed, inflammation and oxidative stress is now recognized as an emerging feature of the metabolic syndrome[174] and is thought to play a causal role in insulin resistance[175]. Furthermore, obesity is linked to an increased risk for many chronic diseases such as cardiovascular disease and cancer[36,176]. Obesity has been linked to colon cancer, which is promoted by altered inflammation, oxidative stress, and endocrine signaling that accompanies obesity[36].

In rodent models of high-fat diet induced obesity, bioactive compounds have demonstrated antiinflammatory and antioxidant properties[177,178]. *Sorghum bicolor* is an agriculturally important cereal crop, placing fifth in worldwide cereal production[1]. Furthermore, it has been used in traditional medicines for the treatment of many disease states[3]. Recent investigations have elucidated some antiinflammatory and antioxidant activities of *S. bicolor* extracts *in vitro* and *in vivo*[179–181]. Sweet sorghums are varieties of *S. bicolor*, which concentrate simple sugars in the stalk, and as a result, have larger thicker stalks with smaller seed heads. *S. bicolor* grains, bran, leaves, and leaf sheaths have also been shown to contain phenolic acids, anthocyanidins, anthocyanins, and other flavonoids with bioactivity[4,5,7,62,67,70,82]. These have been reported in the grain, leaves, and leaf sheathes (also known as leaf base). Sorghum leaf base extracts have demonstrated *in vitro* antiinflammatory[181] and *in vivo* antioxidant activity[182]. However, antioxidant and antiinflammatory properties of the stalk have not been well characterized. In this study, we used a high-fat diet to induce obesity in male and female mice and investigated the antioxidant and antiinflammatory effects of oral administration of sweet sorghum stalk extracts.

4.3 Materials and Methods.

4.3.1 Sweet Sorghum Extraction.

Sweet sorghum dermal raw material was generously provided by Great Valley Energy LLC (Bakersfield, CA). Extract from this material was prepared by homogenizing approximately 1 kg dermal material in 80% ethanol (1:10) chilled to -20 °C to prevent excess heating with an IKA T25 Digital Ultra TURRAX set to 10 000 rpm. The resulting slurry was then gravity filtered with coffee filters to remove particulates in dark conditions. The resulting extract was dried under nitrogen flow at 35 °C for 24 h to remove ethanol. The aqueous portion was then frozen and lyophilized (Virtis Genesis 25LL; Gardiner, NY) into a powder.

4.3.2 Animal Care and Treatment.

Male and Female A/J mice from Jackson Laboratory (Bar Harbor, Maine) were housed 4 or 5 animals per cage in the controlled conditions of a constant 22 °C temperature, 12 h light/dark cycles, and free access to food and water. Seventeen male and seventeen female three-week-old mice were randomly assigned by gender and weight into one of five dietary treatment groups. These treatments were low-fat diet (LFD: n = 8; 4 males and 4 females), low-fat diet plus 1% sweet sorghum dermal layer extract (LFD + SS: n = 9; 4 males and 5 females), high-fat diet (HFD: n = 8; 4 males and 4 females), and HFD plus 1% SS (HFD + SS: n = 9; 5 males and 4 females). LFD was prepared entirely of the standard AIN-93G rodent chow. HFD was a westernized diet consisting of 40% kcal from dietary fat. Diets containing 1% SS had equivalent amounts of corn starch displaced in the diets by presence of dried extracts on a per weight basis. The 1% diet was chosen based on toxicity studies of rodents consuming extracts of sorghum leaf base[183]. At this level, a mouse weighing 25 g and consuming 5 g diet per d would not receive more than 2000 mg/kg/d of diet. All diets were prepared by Harlan Tekland (Madison, WI) and

are outlined in Table 11. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Colorado State University.

Table 11. Composition of control and experimental diets.

ingredients (%)	**LFD**	**LFD + SS**	**HFD**	**HFD + SS**
sweet sorghum dermal extract	0.0	1.0	0.0	1.0
casein	20.0	20.0	19.5	19.5
L-cysteine	0.3	0.3	0.3	0.3
sucrose	10.0	10.0	26.1	26.1
maltodextrin	13.2	13.2	7.5	7.5
corn starch	39.8	38.5	21.1	20.1
cellulose	5.0	5.0	2.0	2.0
soybean oil	7.0	7.0	0.0	0.0
anhydrous milkfat	0.0	0.0	5.5	5.5
lard	0.0	0.0	2.0	2.0
beef tallow	0.0	0.0	3.5	3.5
corn oil	0.0	0.0	5.5	5.5
peanut oil	0.0	0.0	1.0	1.0
hydrogenated vegetable shortening	0.0	0.0	2.5	2.5
vitamin and mineral mix (low Ca, high P)	4.5	4.5	3.5	3.5
fatty acid composition (%)	**LFD**	**LFD + SS**	**HFD**	**HFD + SS**
PUFA	3.78	3.78	4.44	4.44
MUFA	1.54	1.54	7.68	7.68
SFA	1.05	1.05	8.08	8.08

LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with sweet sorghum dermal layer extract; HFD + SS = HFD with sweet sorghum dermal layer extract; PUFA = Polyunsaturated fatty acids; MUFA = Monounsaturated fatty acids; SFA = Saturated fatty acids.

4.3.3 Glucose Tolerance Test.

The glucose tolerance test were performed using recommendations from[184]. Mice were fasted for 6 h before a solution of 50% glucose was injected intraperitoneally at 2 g/kg bodyweight. Blood samples were collected from the tail vein and measured with glucometer at 0, 15, 30, 60 and 120 min. Blood glucose measurements were then plotted and the area under the curve (AUC) was calculated in Microsoft Excel. Data are expressed as mean AUC ± standard error.

4.3.4 Body Weight and Dual Energy X-Ray Absorptiometry.

Body weights were measured weekly. Percentage body fat was assessed by dual energy x-ray absorptiometry (DEXA) scan at wk 9 of the study. Mice were anesthetized under

isoflurane during the scanning procedure. Each animal was scanned in triplicate and data presented as mean percentages body fat ± standard error. Fat mass was calculated by multiplying the bodyweight of animal (g) to the percentage body fat and is presented as mean fat mass ± standard error.

4.3.5 RNA Extraction and Quantitative Real Time PCR.

RNA was isolated using the TRIzol method (Invitrogen, Carlsbad CA). cDNA was generated using qScript cDNA Supermix (Quanta Biosciences; Gaithersburg, MD) according to manufacturer's protocol. Real-time qPCR was performed on the Eco Real Time PCR System (Illumina; San Diego, CA). All primers were purchased from Fisher Scientific (Pittsburg, PA) and the sequences are outlined in Table 12. The relative expression of each gene was normalized to β-actin. A mixture of the cDNA from all samples was used as the calibrator to which all other groups were compared using the ΔΔCq method. All values are expressed as mean fold change ± standard error compared to LFD control.

Table 12. Primer sequences used for qPCR experiments.

gene	forward sequence 5'to 3'	reverse sequence 5'to 3'	amplicon length
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	154
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	61
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG	111
TLR4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA	129
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	89
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	76
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT	121
F4/80	TGACTCACCTTGTGGTCCTAA	CTTCCCAGAATCCAGTCTTTCC	111
Occludin	TTGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG	129
ZO-1	GCCGCTAAGAGCACAGCAA	TCCCCACTCTGAAAATGAGGA	134

4.3.6 Sample Collection.

Animals were euthanized by cardiac puncture and cervical dislocation under isoflurane anesthesia. Blood was collected in heparin laden tubes and immediately centrifuged at 4 °C to collect plasma. Plasma samples were then aliquoted and frozen to -80 °C. Plasma 8-Isoprostane

levels were measured using the 8-Isoprostane EIA kit according to manufacturer's protocol (Cayman Chemicals; Ann Arbor, MI). The colon was resected, cleaned with phosphate buffered saline, and the distal and proximal colonic mucosa was scrapped, collected, and immediately frozen in liquid nitrogen and stored at -80 °C for further analysis. Vital organs were removed, trimmed of excess fat, and weighed.

4.3.7 Statistical Analysis.

Data are presented as means ± standard error. Statistical analysis was done using SAS version 9.3 and significance was determined by one-way ANOVA, followed by the post-hoc Fisher least significant difference (LSD) test for multiple means comparisons. Values not sharing the same letter were statistically significant ($P < 0.05$). * Indicates significant difference from LFD control ($P < 0.05$). † Indicates trend from LFD control ($P < 0.10$). ‡ Indicates significant difference from HFD control ($P < 0.05$).

4.4 Results and Discussion.

4.4.1 Sweet Sorghum is Well-tolerated in A/J Mice Fed Low-fat and High-fat Diets.

There was no unexpected mortality in animals consuming LFD, HFD, or diets containing SS extract at 1% diet for 10 wk. Furthermore, there were no observable adverse events due to treatment over the study period. Decreases in feed intake and water intake could be a result of toxicity associated with toxic compounds in the diet. As seen in Figure 24, there was no loss of feed intake or water intake observed in mice fed SS containing diets when compared to LFD and HFD controls. Generally, mice fed HFD + SS diet ate less on average than HFD control animals. However, this was not sufficient to cause any detrimental reductions in body weight (Figure 25). The body weights of mice in all groups tracked very closely with no significant differences

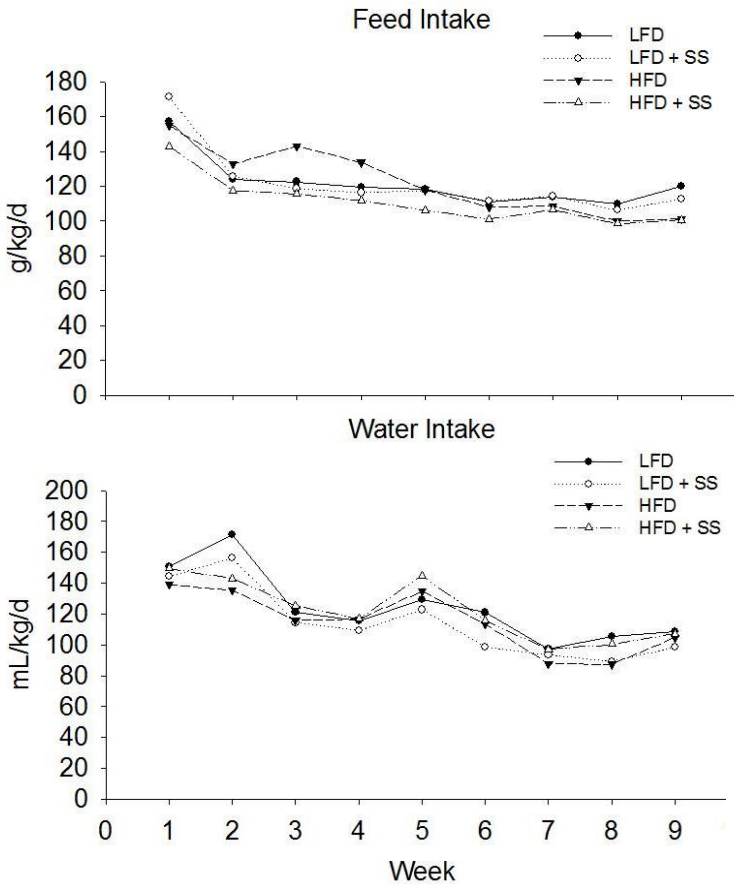


Figure 24. Feed and water intakes for A/J mice. Weekly measures were taken through the study. Feed intake is presented as mean g of feed intake per kg of animal body weight per day (g/kg/d). Water intake is presented as mean mL of water intake per kg of animal body weight per day (mL/kg/d). LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS.

between groups after 9 wk of diet. These results mirror those of Nwinyi's group, who found that oral administration of sorghum leaf sheath at 400 mg/kg caused no significant changes in mean feed and water intakes, and body weights in rats after 4 wk[185].

The results of organ weights are summarized in Table 13. Except for the uterus, there were no significant differences observed in any of the vital and reproductive organs measured between diets containing SS and their respective control diets. Our results are in accordance with previous work reporting no differences in the liver, kidney, heart, spleen, and lungs in rats given

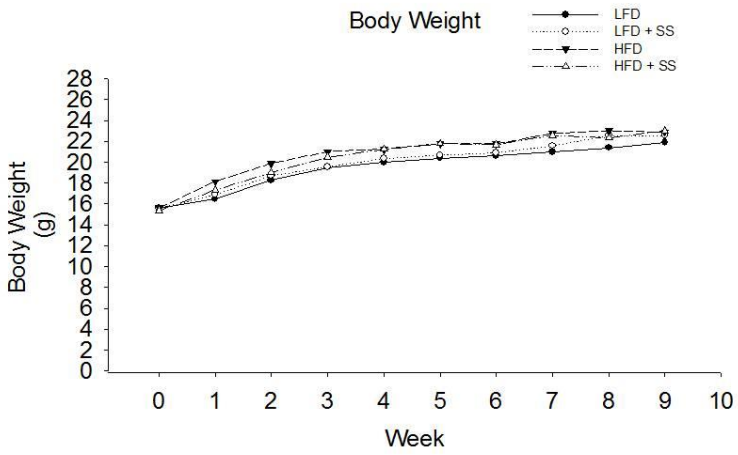


Figure 25. Body weight for A/J mice. Weekly measures of bodyweight were taken throughout the study and are presented in mean g of bodyweight. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS.

oral sorghum leaf sheath at 400 mg/kg for 4 wk[185]. A similar study reported no changes in the weights of the liver, spleen, kidneys, and lungs of rats given oral sorghum bran extract at a concentration of 5000 mg/kg for 14 d[186]. There were no effects of HFD or HFD + SS in organ weights of male mice ($P < 0.05$). In female mice, HFD did increase the weight of the liver. This may have been a result of liver steatosis which often accompanies HFD feeding in mice. The weight of uterus differed significantly between the highly variable HFD (197 ± 107 mg) and HFD + SS (64 ± 8 mg). However, neither of these groups differed from LFD fed control animals ($P < 0.05$). A HFD has been demonstrated to increase the incidence of sporadic uterine adenocarcinomas in female Donryu rat to 18% while LFD incidence was 0%[187]. Furthermore, this was associated with changes in the estrogen to progesterone ratio and earlier histological changes in the uterus as compared to basal diets[187]. It is therefore plausible uterine weight in HFD fed animals is a result of the cancer promoting effects of a HFD. This would explain the increased uterine weight in these animals along with the increase in variation, as not all animals would develop uterine tumors (only an 18% tumor incidence rate reported[187]). If this is true, it

would then allude to a protective effect of SS in the HFD + SS group, as these animals did not show increased uterine weight. However, as the uterus was not a focus of the present study, these comments will remain purely the authors' speculation, and will need to be addressed in future studies. When the results from feed intake, water intake, body weight and organ weight are taken together, our observations support that consumption of SS was well-tolerated by mice.

Table 13. Absolute organ weights of male and female A/J mice fed control and test diets.

	Males			
organ	**LFD**	**LFD + SS**	**HFD**	**HFD + SS**
colon length (cm)	10.3 ± 0.7	10.1 ± 0.4	9.5 ± 0.3	9.7 ± 0.6
liver	919 ± 57.8	938 ± 59.6	990 ± 108.2	985 ± 60.4
kidneys	307 ± 14.8	319 ± 20.6	311 ± 35.9	293 ± 11.8
lungs	189 ± 8.9	166 ± 10.2	181 ± 19.6	175 ± 12.0
spleen	64 ± 2.3	70 ± 1.7	63 ± 3.8	65 ± 3.7
pancreas	137 ± 14.1	130 ± 15.0	137 ± 31.3	129 ± 7.8
brain	399 ± 1.8	408 ± 3.0	398 ± 17.6	391 ± 5.0
testes & epididymides	273 ± 8.7	246 ± 20.7	276 ± 8.7	248 ± 4.8
prostate & seminal vesicles	251 ± 23.1	249 ± 19.6	295 ± 32.2	254 ± 2.3
	Females			
organ	**LFD**	**LFD + SS**	**HFD**	**HFD + SS**
colon length (cm)	9.3 ± 0.3	9.2 ± 0.5	9.5 ± 0.3	9.3 ± 0.2
liver	794 ± 39.5^{a}	849 ± 35.4^{ab}	894 ± 28.6^{b}	924 ± 41.2^{b}
kidneys	241 ± 13.9	266 ± 12.6	259 ± 12.9	271 ± 19.1
lungs	164 ± 14.0	168 ± 12.1	165 ± 10.6	154 ± 2.7
spleen	68 ± 2.4	70 ± 4.0	71 ± 3.6	70 ± 13.4
pancreas	119 ± 5.8	123 ± 7.4	112 ± 8.2	129 ± 7.8
brain	396 ± 11.0	403 ± 10.1	399 ± 4.1	389 ± 7.6
uterus	89 ± 8.4^{ab}	82 ± 5.9^{ab}	197 ± 107.2^{a}	64 ± 7.9^{b}
ovaries	13 ± 4.0	21 ± 7.6	12 ± 1.8	15 ± 4.5

LFD = low-fat diet; HFD = high-fat diet; SS = diet containing 1% w/w sweet sorghum dermal layer extract. Data are presented as mean weight ± standard error, except for colon, which is presented as mean length ± standard error. Different letters indicate significant differences ($P < 0.05$).

4.4.2 Effects of a High-fat Diet on Body Composition and Glucose Tolerance in A/J Mice.

After 9 wk of feeding, A/J mice were subjected to Dual Energy X-ray Absorptiometry (DEXA) to assess the effects of HFD on body composition. While there were no significant differences in animal weights after 9 wk feeding (Figure 25), DEXA scans (Figure 26) revealed that HFD fed mice were significantly fatter than their LFD counterparts. The average body fat

percentage of mice fed HFD increased from 20.9% to 27.3% ($P < 0.05$) and there was a trend for an increase in average body fat mass from 4.7 g to 6.4 g fat ($P < 0.1$). We observed no significant differences in the percent body fat or fat mass in mice consuming HFD + SS from HFD, or in mice consuming LFD + SS from LFD, which indicates that SS extract is neither pro- nor anti-obesogenic.

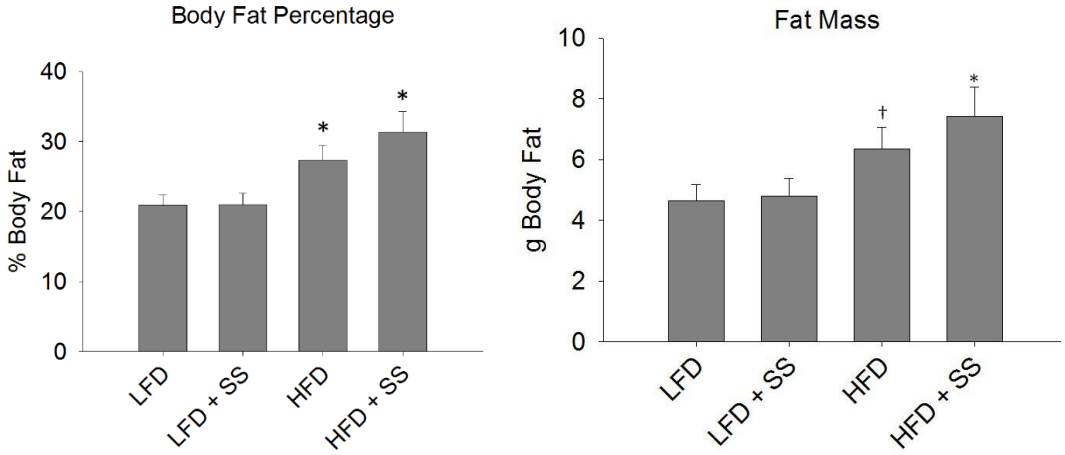


Figure 26. The fat composition of A/J mice after 9 wk of high and low-fat diets with and without test compounds as determined by Dual Energy X-ray Absorptiometry. Values are presented as mean body fat percentages (% Body Fat) ± standard error and mean grams of body fat (g Body Fat) ± standard error, respectively. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS. * Significantly different compared to LFD ($P < 0.05$). † Trend compared to LFD ($P < 0.05$)

Glucose tolerance tests (GTT) were also performed after 9 wk of feeding and results displayed in Figure 27. After 9 wk of feeding, the HFD group demonstrated higher AUC values, however, this was not significantly different from LFD control ($P = 0.12$). This lack of response in AUC values in the HFD group may be due to the presence of equal numbers of females in the each group. Recent investigations into the role of gender in HFD induced obesity and metabolic disorder have revealed that female mice are protected from HFD induced obesity and metabolic disorder when compared to their male counterparts[188–190]. While the mechanism for this is not completely understood, it seems that estrogen plays a key role, as ovariectomized mice respond

similarly to HFD induced obesity and metabolic disorder as male mice[190]. HFD + SS group had significantly higher AUC values than LFD control. However, this was not different than HFD.

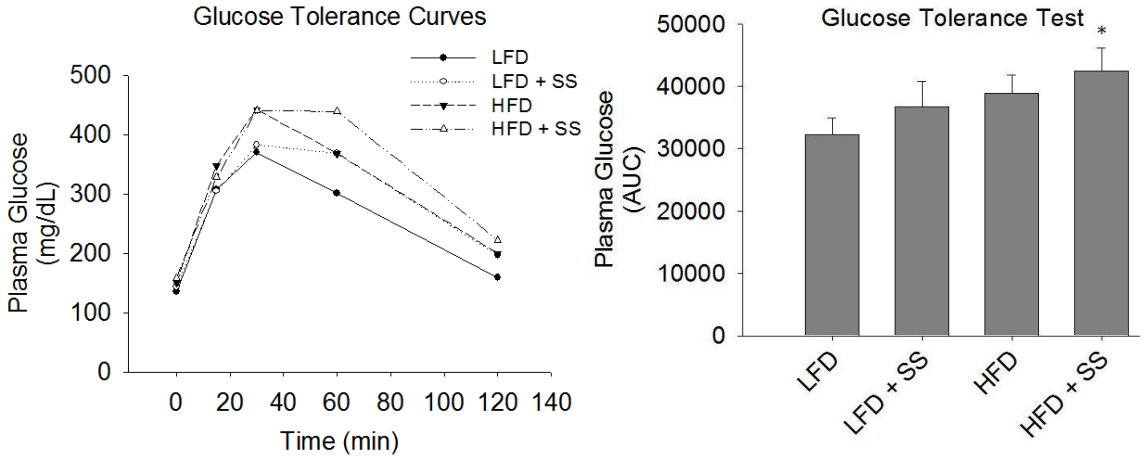


Figure 27. Glucose tolerance tests in A/J mice. Glucose tolerance test was performed in A/J mice at wk 9 of the study after a 6 hr fast. Blood glucose (mg/dL) was measured at 0, 15, 30, 60 and 120 min after initial injection. Area under the curve (AUC) was calculated for each group and presented as mean AUC ± standard error. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS. * Significantly different compared to LFD ($P < 0.05$).

4.4.3 High-fat Diet did not Alter Colonic Inflammation and Barrier Function.

HFD induced obesity has been reported to increase intestinal inflammation through altering intestinal microbiota, which leads to increased intestinal permeability, resulting in activation of toll-like receptors, inducing inflammation in the intestinal mucosa[128,191,192]. To understand the inflammatory changes occurring in the colon of HFD animals, real-time quantitative PCR was used to assess the mRNA expression levels for TLR-4, TNF-α, NF-κB, IL-1β, and IL-6 in the colonic mucosa (Figure 28). There were no differences observed in any of the inflammatory markers when comparing HFD to LFD. Our data indicates that HFD feeding does not increase colonic inflammation in A/J mice after 10 wk.

Recent studies have suggested that intestinal macrophages play important roles in maintaining homeostasis by eliminating intestinal pathogens while negatively regulating excess immune responses to commensal bacteria[193]. Lamina propria macrophages spontaneously

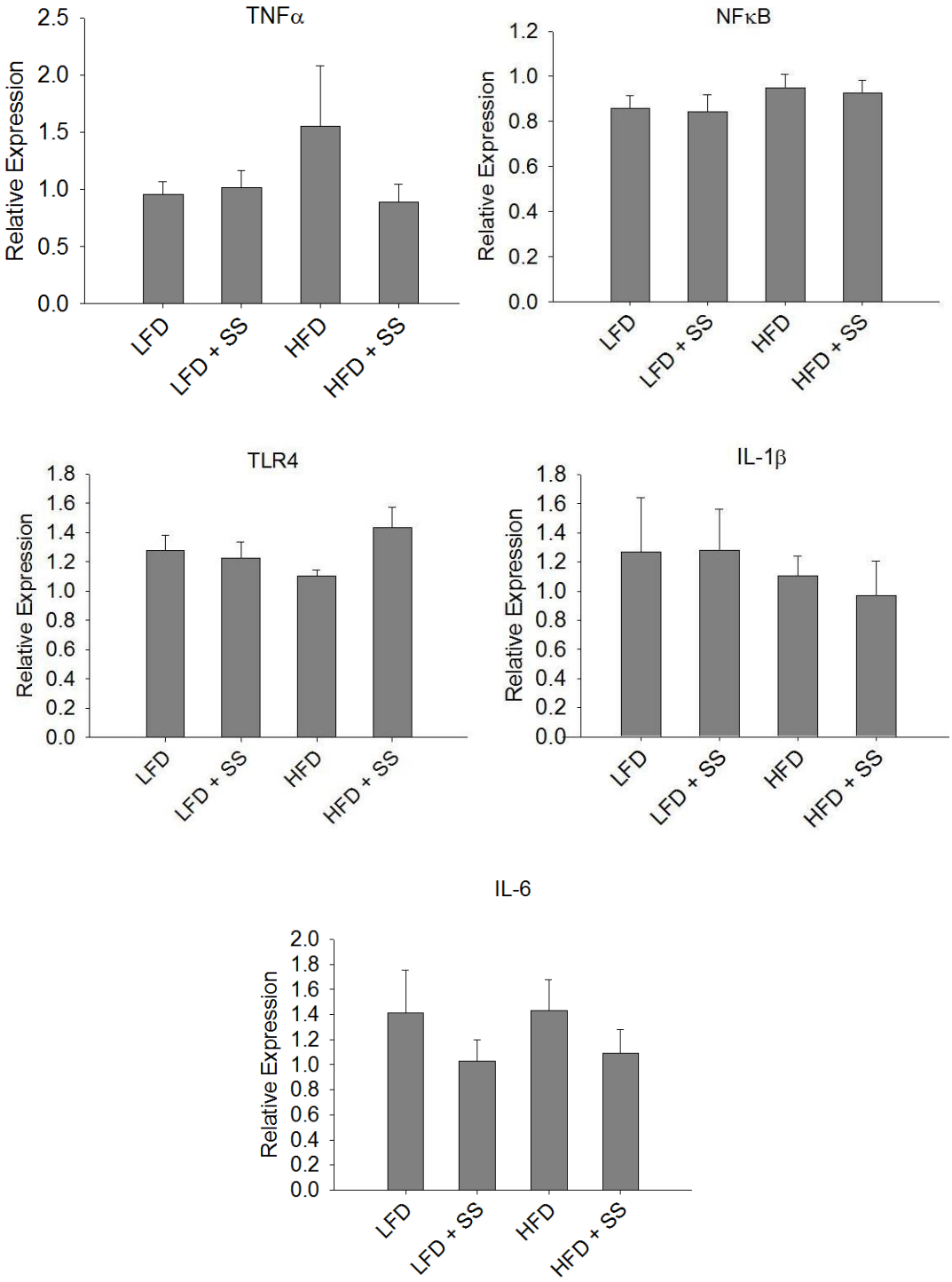


Figure 28. Relative mRNA expression levels of TNFα, TLR4, NF-κB, IL-1β, and IL-6 in the distal colonic mucosa of A/J mice. The relative expression of each gene was normalized to β-actin. A mixture of the cDNA from all samples was used as the calibrator to which all other groups were compared using the ΔΔCq method. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS.

produce large amounts of antiinflammatory IL-10 and play a central role in regulation of immune responses against commensal bacteria[194]. In addition, these macrophages have been shown to produce large amounts of MCP-1, and MCP-1 deficiency caused impaired IL-10 production, which exacerbated intestinal inflammation[194]. Furthermore, it has been shown that these populations change during chemically induced colitis[194]. To assess changes in intestinal macrophage populations in HFD fed A/J mice, we used real time quantitative PCR to measure mRNA expression of the macrophage marker F4/80 and MCP-1 (Figure 29). Only the mice fed HFD + SS differed significantly in F4/80 gene expression from LFD. However, this did not

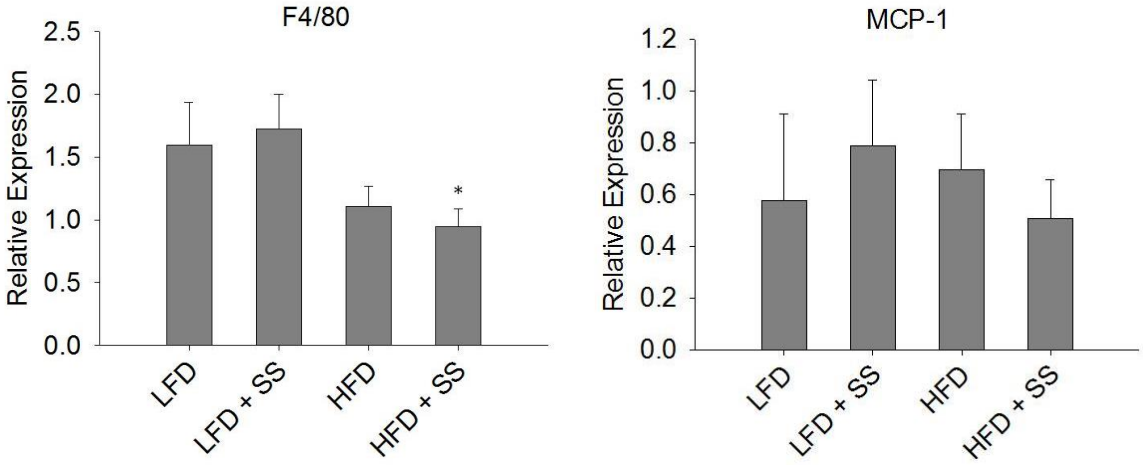


Figure 29. Relative mRNA expression levels of the macrophage marker F4/80 and MCP-1 in the distal colonic mucosa of A/J mice. A mixture of the cDNA from all samples was used as the calibrator and normalized to β-actin to which all other groups were compared using the ΔΔCq method. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS. * Significant differences from LFD control ($P < 0.05$).

seem to alter colonic inflammation as there was no change in colonic inflammatory marker gene expression. MCP-1 expression levels were unchanged across all treatment groups. Our data suggest that 9 wk of HFD does not alter lamina propria macrophages.

We investigated the impact of HFD induced colitis on colonic barrier function by measuring occludin and ZO-1 mRNA expression levels by qPCR in A/J mice consuming LFD

and HFD. HFD has been previously shown to increase intestinal permeability via decreasing expression of tight junction proteins[128]. HFD has been demonstrated to induce changes in gut microbiota, leading to an increase in systemic lipopolysaccharide levels, activating toll-like receptors in epithelial and immunological cells of the mucosa[195]. This leads to altered barrier function causing inflammation in the lamina propria, which may lead to mesenteric fat inflammation[196], and ultimately, systemic inflammation as observed by inflammation in liver and other organs[195]. Occludin and ZO-1 are proteins which help to form tight junctions between the intestinal epithelial cells. Figure 30 shows the relative mRNA expression for occludin and ZO-1. Occludin and ZO-1 levels were not changed by HFD or by the addition of SS to the diets of mice. Our data indicate that 9 wk of HFD with or without SS, does not alter colonic barrier function.

Our results from the qPCR analysis of inflammatory markers, macrophage markers, and junction proteins demonstrate that a HFD in A/J mice does not alter inflammation in the colon after 10 wk of diet. This evidence supports recent investigations, which have indicated that a HFD in mice induces intestinal inflammation in the small intestine, but not the colon by increasing TNFα levels[191].

4.4.4 Addition of Sweet Sorghum Ameliorates High-fat Diet Induced Increases in Systemic Oxidative Stress.

Models of high-fat diet induced obesity, diabetes and hypertension have been shown to increase oxidative stress[197,198]. Isoprostanes are produced non-enzymatically by the sporadic oxidation of arachidonic acid in tissue phospholipid[199]. Therefore, isoprostanes are elevated during periods of oxidative stress. 8-isoprostane (8IP) is an isoprostane known to cause

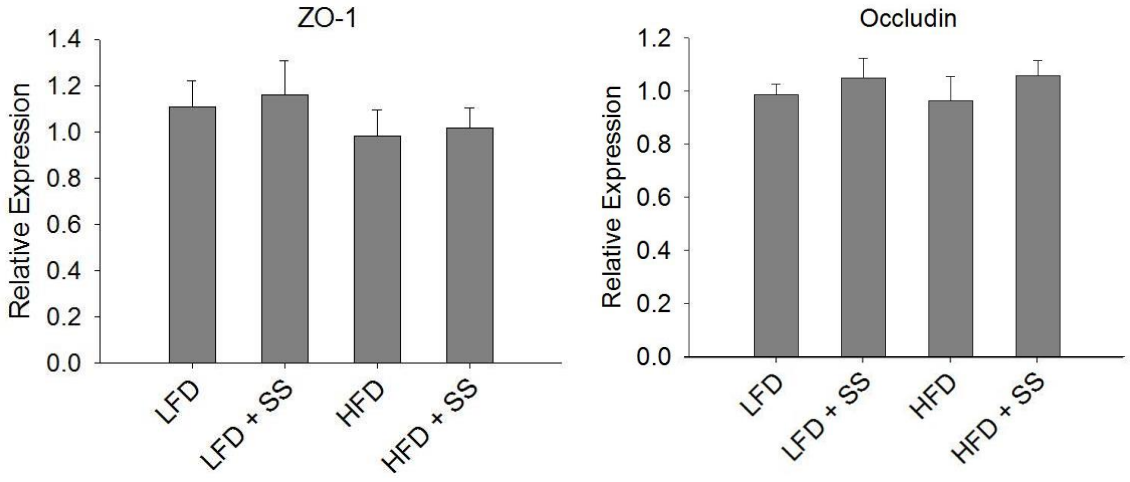


Figure 30. Relative mRNA expression levels of the genes encoding the tight junction proteins occludin and ZO-1 in the distal colonic mucosa of A/J mice. The relative expression of each gene was normalized to β-actin. A mixture of the cDNA from all samples was used as the calibrator to which all other groups were compared using the ΔΔCq method. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS.

pulmonary and renal vasoconstriction[199]. 8IP is also known to be increased in HFD models of obesity[197,198] and increasing consumption of phenolic compounds have been shown to decrease oxidative stress as measured by 8IP[200,201]. We next hypothesized that HFD would cause systemic increases in oxidative stress of mice consuming HFD and that SS phenolics would ameliorate this increase. Oxidative stress was estimated by the presence of 8IP in mice plasma collected at the termination of the study. To our knowledge, this is the first study reporting 8IP plasma levels in the A/J background fed LFD and HFD. Figure 31 demonstrates the levels of plasma 8IP in A/J mice after 10 wk of study. In the LFD control fed animals, plasma 8-IP was approximately 200 pg/ml in A/J mice. This is similar to plasma 8-IP levels reported in C57BL/KsJ wild type and diabetic *db/db* of approximately 200 pg/mL and 300 pg/mL, respectively[202]. HFD fed male and female A/J mice had approximately a 2.5-fold increase in plasma 8IP from control from 208 to 505 pg/mL, which was significantly different from LFD control. This is in agreement with a study reporting that the levels of urinary 8-IP was about 3-fold higher in HFD fed rats after 16

wk feeding[198]. In comparison, mice fed HFD + SS had only a 1.4-fold increase in plasma 8IP levels (294 pg/mL), which was not statistically different from control (P = 0.351). Similar to those of Olayinka's group[182], who demonstrated sorghum leaf sheath extract dose-dependently decreased hepatic lipid peroxidation in rats with cadmium-induced oxidative stress, our findings support the use of SS as a source of dietary antioxidant with *in vivo* efficacy.

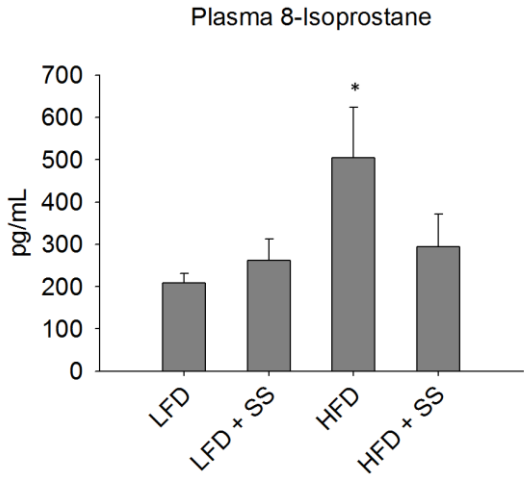


Figure 31. Sweet sorghum extract ameliorates high-fat diet induced oxidative stress. Oxidative stress levels, as assessed by 8-Isoprostane (8-IP), were measured in the plasma collected from A/J mice at time of animal sacrifice. The levels of 8-IP were reported as pg of 8-IP per mL of plasma (pg/mL). Feeding of high-fat diet (HFD) elevated plasma 8-IP in mice from low-fat diet controls (LFD). Addition of 1% sweet sorghum dermal layer extract (SS) to HFD significantly improved 8-IP levels, indicating its efficacy as an *in vivo* antioxidant. LFD = Low-fat diet control; HFD = High-fat diet control; LFD + SS = LFD with 1% sweet sorghum dermal layer extract (SS); HFD + SS = HFD with 1% SS. * Significantly different compared to LFD (P < 0.05).

In conclusion, ethanolic extract from the stalk of *S. bicolor* was well-tolerated in mice consuming HFD and LFD, as shown by feed intake, water intake, bodyweight and vital organ weights. HFD feeding did increase adiposity as measured by DEXA scanning. Addition of SS to diets proved to be neither pro- nor anti-obesogenic. HFD feeding resulted in non-significant increases in glucose tolerance, as measured by glucose tolerance test, in A/J mice. Furthermore, HFD feeding in A/J mice, did not cause changes in colonic inflammation, lamina propria macrophages, or intestinal permeability in the colonic mucosa, as determined from qPCR

analysis of mRNA gene expression. SS extract was further demonstrated to have *in vivo* antioxidant activity by reducing systemic levels of oxidative stress, as measured by 8-isoprostane. This study supports the use of SS as a safe source of dietary antioxidant bioactive compounds.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In this work, we investigated the use of sweet sorghum (*Sorghum bicolor*) biomass as a reservoir of human health-benefiting bioactive compounds in the context of the biorefinery approach to biofuel production. We explored the *in vitro* anticancer properties in colorectal cancer cell lines. While the use of aqueous acetone was the most effective extraction solvent for sweet sorghum bioactive compounds tested, aqueous ethanol, a GRAS alternative, proved to be nearly as efficient an extraction solvent. Furthermore, extracts from aqueous ethanol demonstrated greater antiproliferative activities in colon cancer cells, indicating that solvent selection is an important factor when extracting anticancer bioactive compounds. These investigations further elucidated strong antiproliferative and proapoptotic activities of sweet sorghum component with pith < leaf < dermal < seed head. Investigations into molecular mechanisms revealed dermal and seed head extracts to function partly through altering β-catenin and β-catenin's down-stream prosurvival targets. This activity was deemed dependent upon functional p53 presence for dermal extract and partially p53-dependent for seed head extract.

We also assessed *in vivo* bioactivity of the less consumed sweet sorghum stalk dermal layer component in a murine western style diet model of obesity and oxidative stress. Sweet sorghum dermal layer was well-tolerated in mice fed both high-fat and low-fat diets with extract at 1% w/w. This was indicated by normal levels of feed intake, water intake, body weight and organ weights in mice consuming dermal layer extract. Results further show that sweet sorghum dermal layer demonstrated antioxidant properties in mice fed high-fat diets for 10 weeks.

Having demonstrated a wide range of bioactivity *in vitro* and *in vivo* in sweet sorghum biomass, further research is now needed to assess the safety and anticancer activities of these extracts in pre-clinical models of colorectal cancer. Two such models are the azoxymethane-

induced sporadic colon cancer rodent model and the western diet induced sporadic colon cancer rodent model. With these and other models, it may be possible to elucidate *in vivo* anticancer activity of sweet sorghum extracts. They could further assess the molecular mechanisms responsible for the observed activities, such as the β-catenin signaling pathway, which we observed to be positively altered and associated with anticancer activity of sweet sorghum extracts *in vitro*. If proven successful, clinical trials would then be required. Further research is also needed for the scaling up and validation of extraction methods to be implemented in large scale production facilities to ensure minimal losses in bioactivity of sweet sorghum extracts. Such research would also provide much needed insights into the economical and cost-benefit assessments for sweet sorghum extract producers.

In conclusion, sweet sorghum is an important agricultural crop with great promise for sustainable biofuel production. When implementing the biorefinery approach to biofuel production, the biomass from sweet sorghum may also serve as an important reservoir for bioactive compound for human health with anticancer and antioxidant properties.

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