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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 the Pith in p53 Variants of HCT116 and Colon Cancer Stem Cells

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ABSTRACT: 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 the phenolic content (Folin–Ciocalteu assay), antioxidant activity (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay), and phytochemical composition (LC–MS) in both the 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 the dermal layer extract of sweet sorghum contains more of the 3-deoxyanthocyanidins apigeninidin and luteolinidin than the pith, and this is associated with more anticancer activity. Furthermore, luteolinidin suppressed CCSC proliferation more than apigeninidin. In addition to being renewable biofuel, sweet sorghum may also serve as a source of health-promoting compounds.

KEYWORDS: sweet sorghum, *Sorghum bicolor*, biorefinery, biofuel, total phenolics, antioxidants, LC–MS, proliferation, apoptosis, HCT116, cancer stem cell, p53, apigeninidin, luteolinidin

INTRODUCTION

Colorectal cancer is the third most common cancer in males and females in the United States,¹ and it has been estimated that 80% of colon cancer cases are caused by diet.² 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.² This recent understanding has increased the demand for natural sources of plant phytochemicals with chemopreventive and chemo-protective 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.³

The progression from normal epithelium to colon cancer is a multistep process whereby a normal cell accumulates successive genetic alterations and establishes clones.⁴ The tumor suppressor gene p53 is responsible for growth and apoptosis, therefore preventing the accumulation of mutated genomes and the progression of cancer.⁵ In colorectal cancers, p53 is estimated to be abnormal in 50–75% of cases and marks the transition from preinvasive to invasive disease.⁴ 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.⁶ Cancer stem cells are known to be chemotherapy- and radiation-resistant, making them resilient to current standard of care therapies,⁷ 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 show efficacy not only 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.⁸ We have previously shown that combination of grape seed extract with resveratrol resulted in p53-dependent and -independent apoptosis of colon cancer HCT116 cells.⁹

Worldwide, sorghum is the fifth most cultivated cereal crop, after rice, wheat, corn, and barley.¹⁰ Sweet sorghums are varieties of *Sorghum bicolor* which concentrate simple sugars in

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the pith cells of the stalk ranging from 10% to 25%, with glucose and fructose as the main reducing sugars.¹¹ As a result, they have a smaller grain yield and thicker, taller stalks when compared to grain sorghums.¹¹ 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.¹⁰ Sweet sorghum can grow in marginal lands and therefore does not have to compete with food production.^{10,12} This is an attractive element to sweet sorghum, as biofuel production from sweet sorghum can add to the food supply instead of competing with it.

Biofuel production of sweet sorghum results in the generation of a large amount of biomass¹³ 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.¹⁴ Having been reported to be high in phenolic acids and other compounds,¹⁵ it has been shown that sorghums contain high levels of small-molecule bioactive compounds in the stalk, leaf, leaf sheath, glumes, and seed^{16–19} with reported bioactive properties.^{20–22} Apigeninidin and luteolinidin belong to the 3-deoxyanthocyanidin subclass of flavonoids (Figure 1) and are reported to be expressed in the

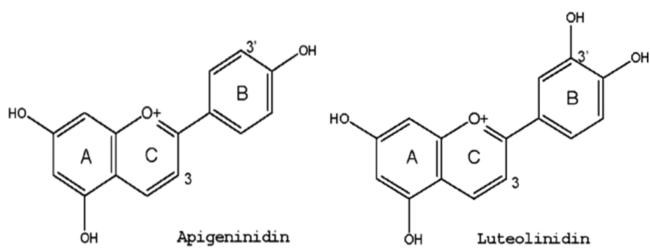


Figure 1. Structures of 3-deoxyanthocyanidins reported in this study.³⁰

glume, grain, leaf, and leaf sheath^{16–18} in *S. bicolor*. Furthermore, these compounds have been shown to be potent inhibitors of the HT-29 colon cancer cell line.²³

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.²⁴ The aims of this study are to (1) determine the content and composition of the pith and dermal layer from the 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, and (4) determine if p53, a critical tumor suppressor protein, is necessary for in vitro anticancer activity.

MATERIALS AND METHODS

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), and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) 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, apigeninidin and luteolinidin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Indofine Chemical Co. (Hillsborough, NJ), respectively, McCoy's medium and colon cancer stem cell medium were obtained from Sigma and Celprogen (San Pedro, CA), respectively, and fetal bovine serum and streptomycin/penicillin mixture were procured from Fisher Scientific.

Sweet Sorghum Materials and Extraction Preparation. Sweet sorghum materials from the 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 the 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 the 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 brought to 100 mL. The phenolic extracts were then stored at -20 °C until analysis for total phenolics and antioxidant activity and by 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 redissolved in 80% ethanol, aliquoted, and stored at -80 °C. Extracts prepared with 80% ethanol were concentrated under a nitrogen flow in a 35 °C water bath for 10 h. The extracts were then aliquoted and stored at -80 °C.

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 mass due to water loss. The results are expressed as percent dry matter.

For quantification of total phenolics, the Folin-Ciocalteu assay was used as described in Singleton's work²⁵ 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 milligrams of gallic acid equivalents per gram of sweet sorghum sample (mg of GAE/g) ± standard error.

Antioxidant activity was measured by the ABTS assay as previously described^{26,27} with minor modifications. In brief, 3 mM ABTS radical and 8 mM potassium persulfate were mixed in equal parts 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 μ L of the working solution was added to 10 μ L 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 ($n = 3$) milligrams of Trolox equivalents per gram of sweet sorghum sample (mg of TE/g) \pm standard error.

LC–MS Analysis. For LC–MS analysis, ethanolic extracts prepared from the pith and dermal layer of the Dale variety were concentrated 10-fold in triplicate before 1 μ L of sample was injected into a Waters Acquity UPLC system fitted with a Waters Acquity UPLCT3 column (1.8 μ M, 1.0 \times 100 mm). The sample was injected into 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 μ L/min for the complete run. The column temperature was 50 °C, and the samples were held at 5 °C.

Eluent from the UPLC system 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 1200 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 mode and 15–30 V for MS^E mode. The capillary voltage was set to 2200 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 a 2 μ g/mL concentration each of caffeine, reserpine, sulfadimethoxine, and terfenadine in 50% methanol. The performance was evaluated using the final three injections, and acceptable performance is indicated by a retention time error of ± 0.05 min, peak area RSD of <25%, and mass accuracy of <3 ppm 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 the 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 the compounds.

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, CD44, and CD133 were purchased from Celprogen. All cells were maintained at a temperature of 37 °C and 5% CO₂. HCT116 cells were maintained in McCoy's medium with 5% FBS. For experiments, HCT116 cells were treated in 10% charcoal stripped serum McCoy's medium. CCSCs were maintained in colon cancer stem cell medium. For experiments, CCSCs were treated in serum-free colon cancer stem cell medium (Celprogen). Concentrated extracts were dosed in medium normalized to total phenolic content as assessed by Folin–Ciocalteu assay. We used 80% ethanol and 5-FU as negative and positive controls, respectively.

Lentiviral shRNA Mediated Knockdown of p53 in CCSCs. CCSCs were infected with lentiviral particles encoding shRNA targeting p53 (Santa Cruz Biotechnology) according to the supplier's protocol. Briefly, CCSCs were infected at a multiplicity of infection of 10 in CCSC growth medium containing 5 μ g/mL Polybrene at 37 °C and 5% CO₂. After 24 h, the medium was replaced, and the cells were cultured for 2 d. Infected cells were then selected in the presence of puromycin (7.5 μ g/mL) for an additional 5 d.

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 \times 10⁴ cells per well. After 24 h, the medium was aspirated, and the cells were treated with controls and sweet sorghum extracts. The treatments were added in triplicate at a volume of 150 μ L per well and then allowed to incubate for 20 h at 37 °C. At this point, BrdU was added at a concentration of 10 μ M per well, and the treatments were incubated at 37 °C for an additional 4 h to allow incorporation of BrdU into cellular DNA (24 h total). The medium was removed, and the BrdU assay was performed according to the manufacturer's protocol. For cell counting, cells were seeded at 1 \times 10⁵ 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, the cells were removed from the plates and counted according to the 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 μ L 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 were normalized to cell count and are expressed as percentages of the solvent control. For TUNEL, cells were seeded into four-well chambered glass slides at a density of 1 \times 10⁵ cells per chamber. After the treatments, the slides were prepared and analyzed for TUNEL staining according to the manufacturer's protocols (Roche Diagnostics, Indianapolis, IN). For analysis, 10 images from each well were taken, containing at least 50 cells per image. The cells were counted, and the data are expressed as the percentage of TUNEL positive cells to total cells (percent apoptosis).

Colony Formation Assay. The colony formation assay was performed as described²⁸ with slight modifications. Colon cancer stem cells were seeded at 1.5 \times 10⁵ 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.²⁹

Statistical Analysis. Data are presented as means \pm standard error. Statistical significance was determined by one-way ANOVA, followed by the posthoc Fisher least significant difference test for multiple means comparisons using IBM's SPSS version 21. Means not sharing the same letter are statistically significant ($P < 0.05$).

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 layer and pith of two varieties of sweet sorghum were grown, processed, and

Table 1. Determination of TP, ABTS, and Dry Matter of the Pith and Dermal Layer from the Dale and M81E Sweet Sorghum Varieties^a

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

^aValues are presented as means ($n = 3$) \pm standard error. Different online Roman letters within a row indicate significant differences ($P < 0.05$) among the entries. ^bTotal phenolics analyzed by the Folin–Ciocalteu method, expressed as milligrams of gallic acid equivalents per 100 g of fresh mass. ^cAntioxidant activity as analyzed by ABTS assay, expressed as milligrams of Trolox equivalents per 100 g.

Table 2. Sweet Sorghum Compounds Identified by LC-MS Analysis

identity	formula ^a	t _R (min)	obsd mass	exact mass ^b	Δppm	intensity, ^d dermal layer	intensity, ^d pith	ref ^c
vanillic acid	C ₈ H ₈ O ₄	2.79	169.050	169.0495	2	100.9 ± 3.8	68.2 ± 7.4 ^f	16
p-coumaric acid	C ₉ H ₈ O ₃	0.85	165.055	165.0546	2	534.9 ± 14.6	492.0 ± 23.8	16
ferulic acid	C ₁₀ H ₁₀ O ₄	3.12	195.067	195.0652	9	27.6 ± 1.8	14.3 ± 1.5 ^f	16
caffeic acid	C ₉ H ₈ O ₄	3.16	181.050	181.0495	2	152.4 ± 1.3	26.5 ± 4.5 ^f	16
apigeninidin	C ₁₅ H ₁₁ O ₄ ⁺	4.29	255.066	255.0660 ^c	0	96.5 ± 20.2	7.9 ± 0.7 ^f	16–18
luteolinidin	C ₁₅ H ₁₁ O ₅ ⁺	5.97	271.061	271.0607 ^c	2	151.4 ± 30.8	7.8 ± 2.0 ^f	16–18
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	17
apigenin	C ₁₅ H ₁₀ O ₅	4.04	271.061	271.0607	3	71.2 ± 53.8	8.6 ± 1.2	16
luteolin	C ₁₅ H ₁₀ O ₆	5.48	287.056	287.0556	3	47.3 ± 54.2	4.6 ± 3.0	16,31
trans-resveratrol	C ₁₄ H ₁₂ O ₃	8.48	229.087	229.0859	4	28.8 ± 3.3	37.6 ± 2.9	16
luteoferon	C ₁₅ H ₁₄ O ₆	4.26	291.087	291.0863	2	16.3 ± 0.8	6.4 ± 0.3 ^f	

^aFormulas are based on [M]. ^bExact masses are based on [M + H]⁺ unless otherwise indicated. ^cMass based on [M]. ^dNormalized peak intensities based on peak areas normalized to the total ion signal in R. ^eReferences which have previously identified the compound listed. ^fSignificant differences ($P < 0.05$) observed among dermal and pith extracts.

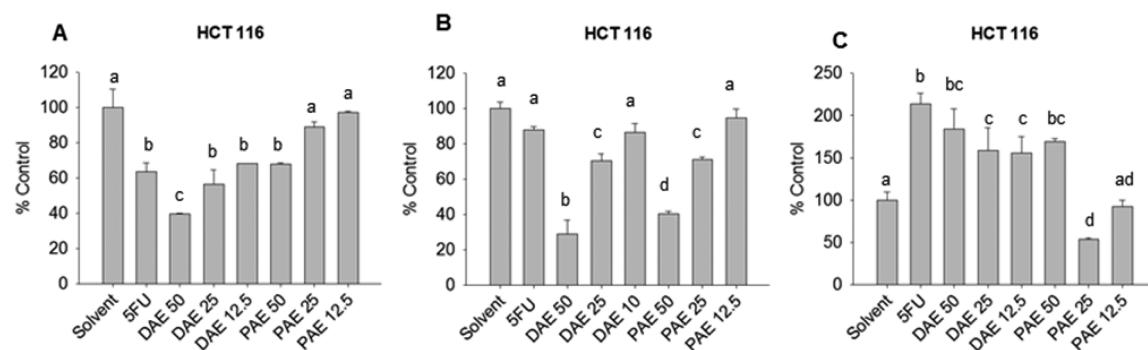


Figure 2. Antiproliferative proapoptotic activities of Dale dermal and pith phenolics measured by (A) cell count, (B) BrdU assay, and (C) Caspase 3/7 Glo assay. HCT116 cells were treated for 24 h with acetone extracts (DAE and PAE) at 10, 12.5, 25, and 50 µg of GAE/mL before estimation of proliferation and apoptosis. Values are presented as percentages 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 5-FU = 5-fluorouracil (18 µg/mL).

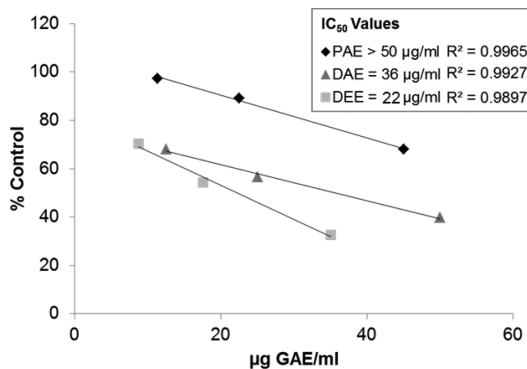


Figure 3. Dose-response curves for HCT116 cells. Cells were treated for 24 h with pith and dermal phenolic-rich extracts prepared by extraction with either acetone (AE) or ethanol (EE). The response is based on cell proliferation as estimated by cell counting. Simple linear regression was used to estimate IC₅₀ values. Values are presented as percentages of the solvent control (y axis) at a specific dose of the respective phenolic-rich extract in micrograms of GAE per milliliter (x axis).

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 micrograms of

gallic acid equivalents per milliliter of medium (µg of GAE/mL), which was determined by the Folin-Ciocalteu assay.

Dry Matter, Total Phenolics, Antioxidant Activity, and LC-MS Analysis. For dry matter content, we observed similar levels of dry matter in pith and dermal samples across both the Dale and M81E varieties. The dry matter content is summarized in Table 1. The dry matter of the pith from Dale and M81E was 22%. The dry matter content of the dermal layer was 39% and 42% for Dale and M81E, respectively (Table 1). These findings are in agreement with previously published data reporting dry matter content for the pith of sweet sorghum stalks of 30%,¹⁵ 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.

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 1). The total phenolic content of the dermal layer was higher than that of the pith in both varieties by 35% and 155% in Dale and M81E, respectively. A similar relationship was also observed for the antioxidant activity. Among the two varieties, Dale dermal extracts yielded more Trolox equivalents, yet fewer gallic acid equivalents as assessed by ABTS and Folin-Ciocalteu assays,

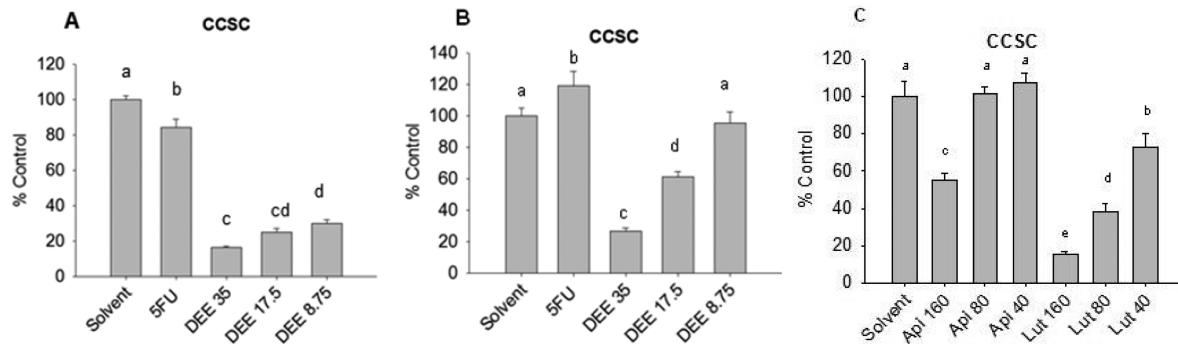


Figure 4. Antiproliferative activities of Dale dermal phenolics measured by (A) cell count and (B) BrdU assay. (C) Antiproliferative activities of apigeninidin and luteolinidin measured by BrdU. Colon cancer stem cells (CCSCs) were treated for 24 h with dermal phenolic-rich ethanol extracts of the Dale variety (DEE) at 8.75, 17.5, and 35 µg of GAE/mL before estimation of proliferation. Values are presented as percentages of the solvent control. For cell count, means ($n = 2$) \pm standard deviation are presented. For BrdU, means ($n = 3$) \pm standard error are presented. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control, and 5-FU = 5-fluorouracil (18 µg/mL).

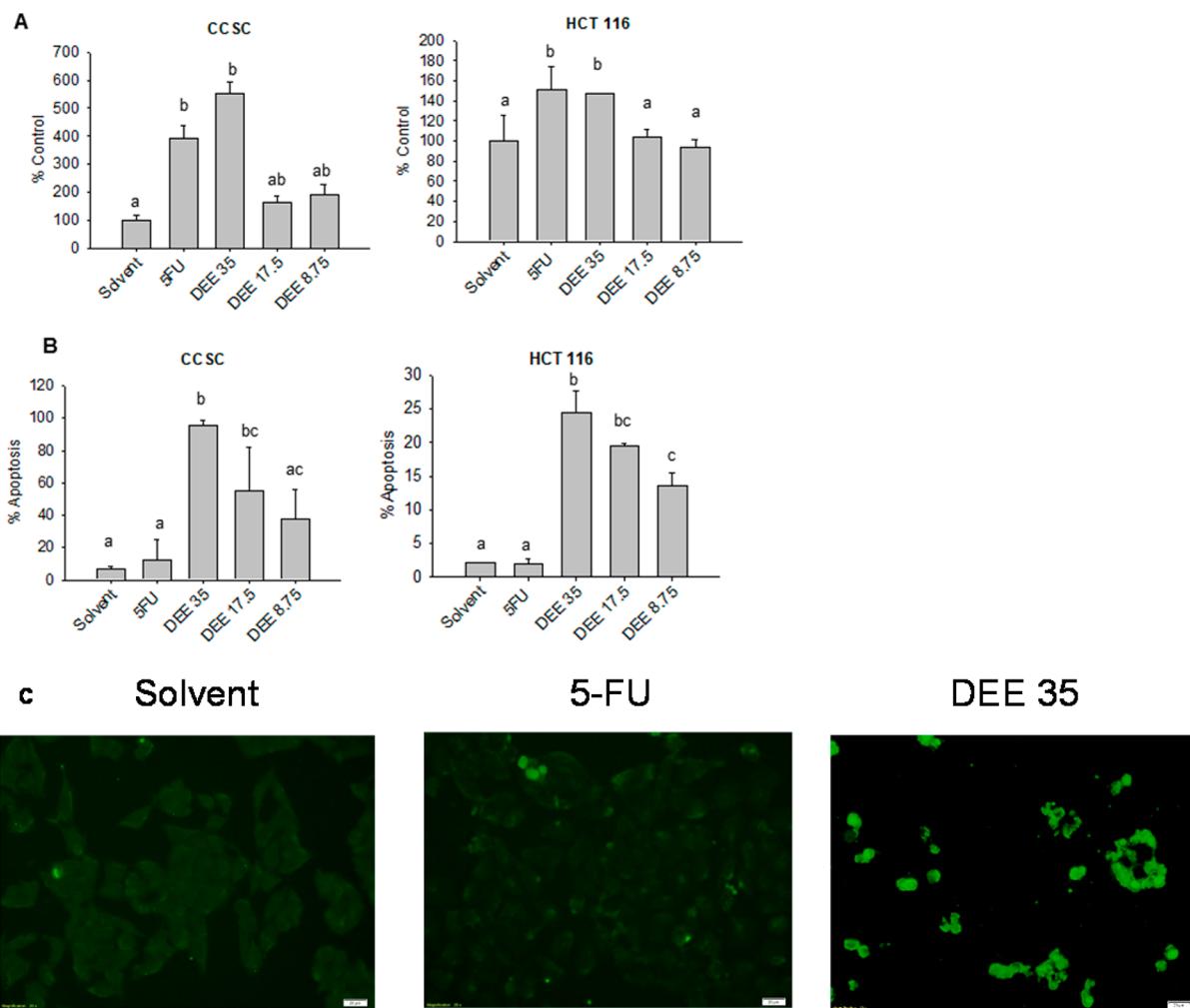


Figure 5. Proapoptotic activities of Dale dermal phenolics measured by (A) Caspase 3/7 Glo assay and (B) TUNEL assay. Colon cancer stem cells (CCSCs) and HCT116 cells were treated for 24 h with dermal phenolic-rich ethanol extracts of the Dale variety (DEE) at 8.75, 17.5, and 35 µg of GAE/mL before being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

respectively ($P < 0.05$). When the pith and dermal layer are combined, there is no difference between the gallic acid equivalents of Dale and M81E, but there are significantly more Trolox equivalents. This could be a result of a difference in the

compositions of the phenolics, 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 phenolic readings.

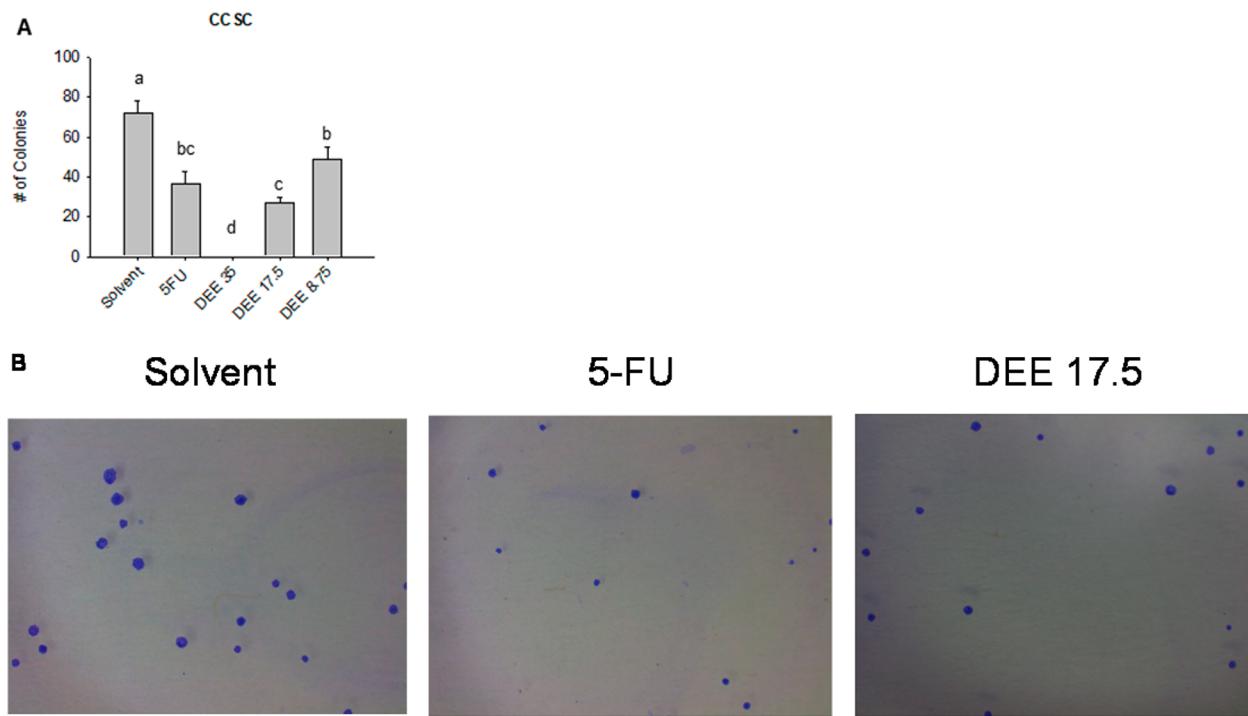


Figure 6. Effects of Dale dermal phenolics on colony formation. (A) Colon cancer stem cells (CCSCs) were treated for 24 h with dermal phenolic-rich ethanol extracts of the Dale variety (DEE) at 8.75, 17.5, and 35 μ g of GAE/mL before estimation of colony formation by colony forming assay. (B) Representative images taken from the colony forming assay. Values are presented as the mean ($n = 3$) number of colonies formed containing at least 50 cells \pm standard error. Different letters indicate significant differences ($P < 0.05$) among treatments. Solvent = solvent control, and 5-FU = 5-fluorouracil (18 μ g/mL).

Reducing sugars are known to be approximately twice as concentrated in the pith as in other components of the stalk,¹⁵ 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 the sweet sorghum pith and dermal layer, which are summarized in Table 2. 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 the leaf sheaths and leaves of sorghum.^{17,18} These compounds have been reported to have anticancer activities toward a number of cancer cell lines, including liver, leukemia, and colon.^{22,23,30} Previous studies have identified primarily two 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,^{17–19,31} 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 layer. 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.

Anticancer Activities of Sweet Sorghum Pith and Dermal Extracts.

Early in the pathogenesis of colorectal cancer, hyperproliferation 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.^{32,33} 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.³⁴ Today's therapies are ineffective at targeting cancer stem cells, only targeting the bulk of the rapidly dividing tumor cells.⁶ 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 as evaluated by activated caspase 3/7 activity and subsequently confirmed by TUNEL assay, and (3) self-renewal capability in CCSCs as analyzed by colony forming assay.

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

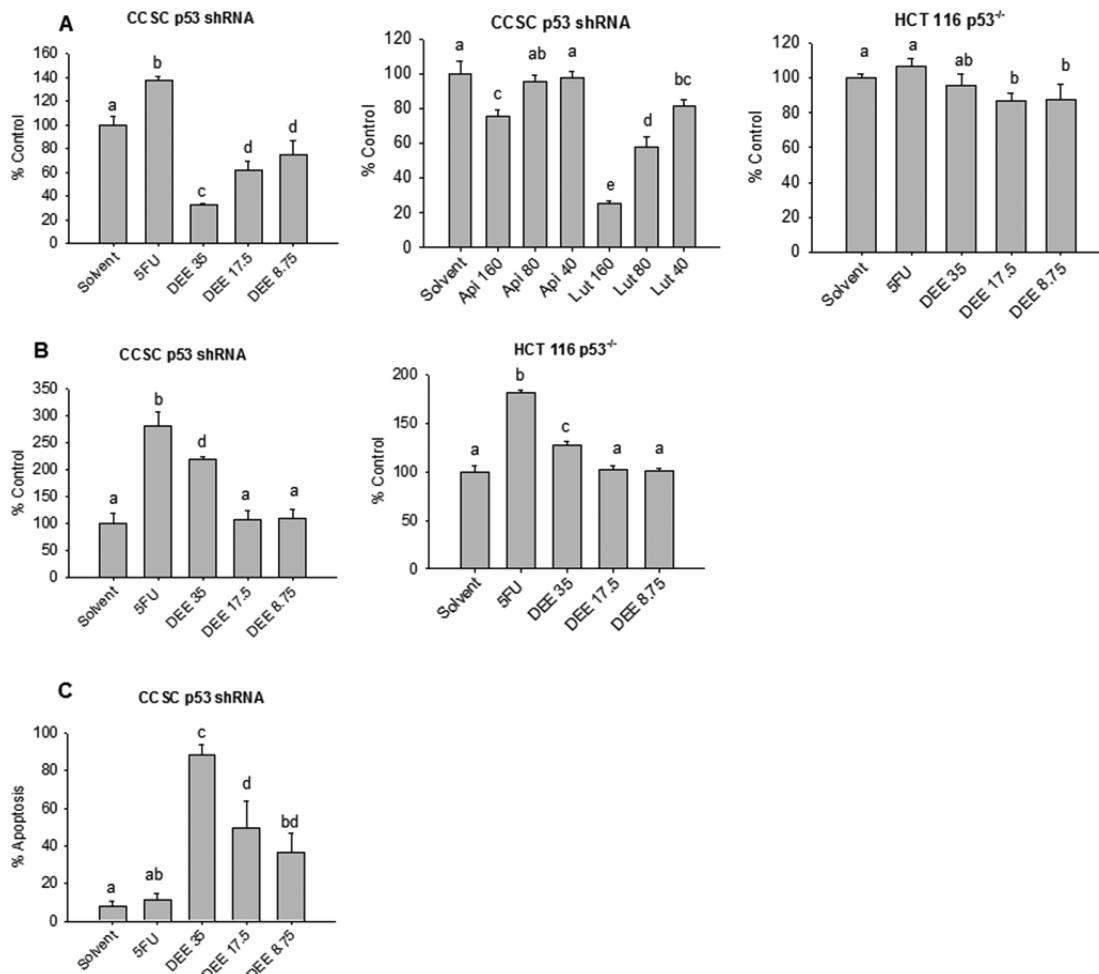


Figure 7. Antiproliferative and proapoptotic activities of Dale dermal phenolics in a p53-independent manner as measured by (A) BrdU assay, (B) Caspase 3/7 Glo assay, and (C) TUNEL assay. The antiproliferative effects of apigeninidin and luteolinidin were also measured by BrdU assay. Colon cancer stem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

(DAE) for 24 h as shown in Figure 2. 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 of GAE/mL, DAE suppressed the cell count 43% compared to only an 11% reduction for PAE. IC₅₀ values were calculated for PAE and DAE. The IC₅₀ for PAE was calculated to be above 50 µg of GAE/mL, while DAE was calculated to be 36 µg of GAE/mL. DAE was also able to induce apoptosis at lower concentrations than the pith as measured by caspase activity. At concentrations of 12.5, 25, and 50 µg of GAE/mL, DAE was able to increase active caspase levels to 155%, 159%, and 184% of that of a solvent control, respectively. Only 50 µg of 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.³⁵ Interestingly, our LC-MS analysis showed marked and significant differences between extracts from the dermal layer and pith (Table 2). 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 on the basis of relative peak intensities. This may explain the increased potency observed in dermal extracts.

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 colleagues³⁶ 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-fold when compared to 100% ethanol. Furthermore, 80% ethanol yielded approximately 60% of the phenolics as compared to 80% acetone. On this basis, we hypothesized that ethanol, a GRAS organic solvent, with water could be used to extract phenolics from the sweet sorghum dermal layer with effectiveness similar to that of aqueous acetone. Similar to Turkmen's findings, 80% ethanol yielded approximately 84% of the phenolics 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 that of DAE. As demonstrated by Figure 3, DEE had a superior IC_{50} value of 22 μ g of GAE/mL compared to the IC_{50} of 36 μ g of 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 the solvent control, DEE at all concentrations significantly increased apoptosis. Furthermore, this increase in apoptosis was more than that for the positive control, 5-FU. Our findings highlight the important aspect of solvent selection when extracting and analyzing *in vitro* bioactivities of plant-based extracts.

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.⁷ There is a need for standardized bioactive compounds and extracts that can effectively eliminate these cells, as current anticancer drugs often fail.⁷ 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 μ g of GAE/mL and 5-FU (18 μ g/mL, 100 μ M) for 24 h and measured proliferation and apoptosis (Figures 4 and 5). DEE potently suppressed proliferation to 73% of the control, as measured by BrdU, and increased the percentage of TUNEL positive cells (Figure 5B) from 7% to 95% of total cells ($P < 0.05$). Imaging of TUNEL stained cells (Figure 5C) 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} values were calculated as demonstrated in Figure 4C. Luteolinidin suppressed CCSC proliferation more than apigeninidin, as demonstrated by a lower IC_{50} value of 76 μ g/mL as compared to >160 μ g/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 6). We treated CCSCs with DEE and found a dose-dependent suppression in colony formation. Figure 6B 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.

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.²³ 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 μ g/mL for crude extracts and from approximately 13 to 20 μ g/mL 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.

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.³ It is estimated that half of all tumors carry some form of mutated p53 and the synergy of phytochemicals may provide p53-dependent and -independent mechanisms to compensate.^{37,38} 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 7). 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 μ g of 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 (CCSC 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 the control and elevated apoptosis of CCSC p53 shRNA cells in a dose-dependent fashion as measured by BrdU and TUNEL assays (Figure 7). We further tested the antiproliferative effects of apigeninidin and luteolinidin in CCSC p53 shRNA cells with BrdU (Figure 7). We show that both of the 3-deoxyanthocyanidins suppress proliferation in p53 knockdown cells with IC_{50} values of >160 and 103 μ g/mL for apigeninidin and luteolinidin, respectively. In agreement with previous results, the antiproliferative effect 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 it has been shown to have a 3-fold increase in antioxidant activity when compared to apigeninidin.³⁹ Our findings agree with

those of Marko et al.,⁴⁰ 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.⁴¹ 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 compared to SW480 cells. HCT116 and HT29 cells are 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 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 conclude the dermal extract's mechanisms of action.

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 the pith, and we believe this to be due to the different compositions of phenolics as illustrated in Table 2. 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 a 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*.

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Author Contributions

A.R.M. conducted research, writing of the manuscript, and contributed to the study's design. L.R. provided intellectual contributions, study design, and assisted in research. J.V. provided intellectual contributions, study design, and review of the manuscript.

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Notes

The authors declare the following competing financial interest(s): patent pending. Brian M. Pellens and D. Edward Settle, the Managing Principals of Great Valley Energy LLC, approached the authors with the idea of studying the bioactive and antioxidant properties of sweet sorghum fractions. They have applied for a patent for sorghum extract and its

therapeutic uses. In recognition of the authors' contribution, they have invited the authors to be named as co-inventors.

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