Antiproliferation Properties of Grain Sorghum Dry Distiller’s Grain Lipids in Caco-2 Cells

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Antiproliferation Properties of Grain Sorghum
Dry Distiller’s Grain Lipids in Caco-2 Cells

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Abstract
Antiproliferative properties of lipids extracted from grain sorghum (GS) dry distiller’s grain (DDG) were analyzed to determine the feasibility of developing GS coproducts as a source for human health dietary ingredients. The lipid extract of GS-DDG was delivered to human colon carcinoma (Caco-2) cells by solubilizing 0–1000 μg/mL of GS-DDG lipids in 100 μg/mL increments with micelles. A significant reduction in cell viability (25–50%) resulted at treatment levels of 400–1000 μg/mL. Alternatively, total protein levels of cells treated with 400, 500, and 600 μg/mL of GS-DDG lipid were not significantly different from the control, indicating cell growth during the treatment period. Total cell counts for the latter sample with a concomitant increase of the intercellular protein lactate dehydrogenase leakage (30–40%) in the medium. Preliminary analysis by the fluorescence-activated cell method (FACs) demonstrated that nonviable cells were in either the early apoptotic, late apoptotic, or necrotic stage post-treatment with 400, 500, and 600 μg/mL of GS-DDG lipid. A significant antiproliferation effect on two of the four immortal colon cancer lines studied. Evidence linking dietary fatty acids to anticancer properties has also been reported. Rats induced with mammary cancer but fed a diet high in olive oil had a 10% reduction in tumors compared to a corn oil based diet (9). These results were attributed to the high levels of n-3 polyunsaturated fatty acids in olive relative to corn oil, which contains higher levels of n-6 polyunsaturated fatty acids.

Keywords: grain sorghum, antiproliferation, ethanol, dry distiller’s grain, Caco-2 cells

Introduction

The United States dominates sorghum production and distribution, accounting for 50% of the world’s exports but most of our own supply is used as animal feed (1). In addition, >1.3 million metric tons of grain sorghum serve as raw material for ethanol per year, yielding 8.2 kg of distiller’s dried grains with solubles (DDG) per 25 kg of grain sorghum (GS) (2). These statistics are expected to increase throughout the next decade to fulfill the 36 billion gallons per year renewable fuel mandate specified in the Renewable Fuels Standard by 2022 (3). Considering that the coproducts of dry-grind ethanol production are also distributed solely as animal feed, alternative applications for GS and its coproducts are needed to add value to this currently underutilized cereal.

Recent studies have shown that GS-DDG retains potentially valuable lipids at levels approximately 2-fold higher compared to their whole kernel counterpart (4). Composition analysis has further shown that the crude GS-DDG lipid fraction contains policosanols, aldehydes, triacylglycerides, hydrocarbons, tocopherols, phytosterols, free fatty acids, diacylglycerides, and vitamin E (2, 4, 5). Many of these lipids are garnering attention due to their potential anticancer properties. For example, Awad et al. (6) demonstrated that the phytosterol, β-sitosterol, decreased in vitro breast cancer growth by 70%, whereas campesterol had a weaker effect, reducing cell growth by 6%. Epidemiology studies completed in Uruguay showed an inverse correlation between stomach cancer and phytosterol consumption (7). Campbell et al. (8) showed that the vitamin E isomer, γ-tocopherol, exerted a significant antiproliferation effect on two of the four immortal colon cancer lines studied. Evidence linking dietary fatty acids to anticancer properties has also been reported. Rats induced with mammary cancer but fed a diet high in olive oil had a 10% reduction in tumors compared to a corn oil based diet (9). These results were attributed to the high levels of n-3 polyunsaturated fatty acids in olive relative to corn oil, which contains higher levels of n-6 polyunsaturated fatty acids.
Studies have yet to be completed that directly link GS-DDG lipids to anticancer benefits, but epidemiological data have shown reduced risks for gastrointestinal tract based cancers with GS consumption (10-12). If anticancer agents are also present in the GS-DDG lipid extract, it is reasonable to expect that this coproduct can be developed as an ingredient for health foods and nutraceutical (FNNS) market. Consumption of such products is increasing to unprecedented levels, particularly in Japan, Europe, and the United States due to rising health care costs and aging populations (13, 14). The objective of this study was to characterize the antiproliferation effects on the human cancer colon cell line (Caco-2) in response to GS-DDG crude lipid extract. Compositional analysis of the crude lipid extract used for this study was also completed to provide preliminary data on the possible agent(s) responsible for eliciting the health-benefiting responses.

Materials and Methods

Preparation of GS-DDG Lipid Extracts. A sample of GS-DDG was obtained from the U.S. Energy Partners facility located in Russell, KS. Lipids were extracted by recirculating 300 mL of hexane heated to 55 °C over 200 g of GS-DDG as described by Wang et al. (15). After an extraction period of 6 h, the solvent was evaporated under vacuum, and the resulting residue was stored at 4–10 °C until needed.

Lipid Solubilization. Micelles were prepared by combining 221 mg/mL lecithin with 161 mg/mL cholesterol and transferred in 0.041–0.048 mL aliquots into vials. GS-DDG lipid samples were dissolved in hexane at 40 mg/mL and transferred in 1–10 mg aliquots into the lecithin/cholesterol mixture. The samples were dried under nitrogen while heating at 50 °C for at least 5 min. Taurocholic acid sodium salt (5.4 mg) was added to each vial followed by 10 mL of the growth medium, Delbecco’s Modified Eagle Medium (DMEM). The samples were sonicated with an S-450D Branson digital sonifier (Danbury, CT) at 30% amplitude for 2–3 min. The solubilized lipids were stored at refrigerated temperatures for a maximum of 3 days.

Culture Preparation. Human colon carcinoma cells (Caco-2) (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 20% fetal bovine serum, 1% nonessential amino acids, 1% l-glutamine, and 50 μg/mL penicillin and streptomycin. The cells were cultured in 75 cm² collagen-coated culture flasks at 37 °C and 5.0% CO₂. The cells were passaged with 3 mL of trypsin for 5 min after reaching 80% confluency. Tests were conducted at cell passage numbers of 30–50.

Cellular Proliferation. Cell proliferation studies were initially completed with 10 treatments of 100–1000 μg/mL solubilized GS-DDG lipid extracts at 100 μg/mL increments. The cells were harvested by release with trypsin, counted with a Neubauer hemocytometer (VWR Scientifics, West Chester, PA) under a Microscopix IV900 series microscope (VWR Scientifics), and centrifuged at 2900 rpm for 5 min. The cells were resuspended in DMEM and dispensed in 96-well plates at 5 × 10³–1 × 10⁴ cells/well. After allowing 24 h for cell recovery at 37 °C in 5.0% CO₂, the medium was gently removed from each well and the lipid treatments were added. The plates were incubated for another 24 h, and percent cell viability was assessed with the tetrazolium salt 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay according to the manufacturer’s instructions. The MTT reagent (10 μL) was added to each well, and the plates were incubated at 37 °C and 5.0% CO₂ for another 2 h. The cells were then lysed with 100 μL/well of lysis buffer supplied with the MTT kit, followed by an additional 4 h of incubation. The absorbance (abs) was monitored at 580 and 620 nm with a FLOUstar Optima ELISA plate reader (BMG Labtech, Offenburg, Germany). Cell proliferation was monitored at 580 and 620 nm with a FLOUstar Optima ELISA plate reader. Percent LDH leakage was calculated as % viability = (abs sample/abs control) × 100. Results are reported as the mean ± standard deviation (SD) of percent viabilities of four replicates (three to four analyses per replicate).

A lactate dehydrogenase (LDH) kit was used to determine cellular membrane damage in response to lipid treatments as outlined by the manufacturer with minor modifications. Cells were transferred to 12.5 cm² culture flasks and allowed to recover in untreated DMEM for 24 h during incubation at 37 °C and 5.0% carbon dioxide in the atmosphere. After recovery, the medium was aspirated from the cells and replaced with 400, 500, or 600 μg/mL of solubilized GS-DDG lipid, medium + micelles (control), or fresh medium only, medium – micelles (blank). After 24 h, 100 μL of medium from each treatment group, control, and blank was transferred to a 96-well plate. The LDH reagent was mixed with equal volumes of dye solution, substrate, and enzyme preparation and added at a volume of 50 μL to each well. The plate was incubated at room temperature for 20 min, and the reaction was stopped with 15 μL of 1 N HCl. The absorbance was monitored at 485 and 680 nm with a FLOUstar Optima ELISA plate reader. To determine the total amount of intracellular LDH, the cells from the previous test were harvested from 12.5 cm² flasks with trypsin and de-activated by DMEM. A sample (500 μL) from each of the treatment groups, control, and blank was combined with 50 μL of the TOX-7 lysis buffer and incubated at 37 °C with 5.0% carbon dioxide for 45 min. Aliquots (100 μL) from each set of cells were plated onto a 96-well plate in triplicate. The LDH reagent was mixed with equal volumes of the dye solution, substrate, and enzyme preparation, which was added at a volume of 50 μL to each well. The plate was incubated at room temperature for 20 min after deactivation with 15 μL of 1 N HCl. The absorbance was monitored at 485 and 680 nm with a FLOUstar Optima ELISA plate reader. Percent LDH leakage was calculated by determining the net absorbance of 680 nm from 485 nm obtained from both the supernatant and cell lysis data and applying the following relationship: % LDH = (net abs of supernatant/ (net abs of supernatant + net abs of cell lysis)). Results are reported as the mean ± SEM of four replicates (three analyses per replicate).

Cellular necrosis and apoptosis in response to lipid treatments of 400, 500, and 600 μg/mL GS-DDG lipid were assessed by using the fluorescence-activated cell (FACS) method (17, 18). After treatment, cells were harvested and incubated with propidium iodide and fluorescent isothiocyanate–annexin V for 20–30 min at room temperature. The cells were loaded into the cuvette of a flow cytometer (Beckton Dickinson FACScan, Franklin Lakes, NJ) and monitored at an excitation of 488 nm and an emission wavelength of 525 nm for the fluorescence isothiocyanate–annexin V or 575 nm for the propidium iodide. Results are reported as the mean ± standard deviation (SD) of percent gated cells for one replication of each treatment. Percent dead cells were determined with the Trypan Blue exclusion dye assay as described by Wijeratne et al. with minor modifications (16). A 100 μL aliquot of the cells from the 12.5 cm² flask treated with 400, 500, or 600 μg/mL of micellized GS-DDG lipids was collected after harvesting with trypsin. Trypan Blue dye (100 μL) was then mixed with the cells, and the total numbers of cells and of dead cells (stained) were counted on a Neubauer hemocytometer (VWR Scientifics) under a Microscopix IV900 series microscope (VWR Scientifics). Percent dead cells was calculated as (stained cell counts/the total cell count) × 100. Total cell counts and percent dead cell counts are reported as the mean ± SEM of three replicates (three to four analyses per replicate).

GS-DDG Lipid Characterization. Simple lipid classes present in the extract were initially identified and quantitated using thin layer chromatography (TLC). A cocktail of standards containing cholesterol, fatty acid methyl esters, d-a-tocopherol acetate, and three polyoxyethylene-ethersorbitan monooleate, octacosanol, monoacylglycerides of oleic acid, 1,2-diacylglycerols of oleic acid, 1,3-diacylglycerides of oleic acid, and other 24 h, and the medium was carefully removed. Each well was washed with 50 μL of phosphate-buffered saline (PBS) followed by another 50 μL of PBS and 100 μL of the MTT lysis buffer. After 1 h of incubation at 37 °C, 50 μL of lysate from each well was transferred to a clean well containing 150 μL of PBS. BCA (150 μL) was added to each well followed by incubation at 37 °C for 2 h. The absorbance was measured at 544 nm, and protein levels were determined against a calibration curve generated with bovine serum albumin. Results are reported as the mean ± SEM of four replicates (three to four analyses per replicate).
triacylglycerides of oleic acid was prepared. The GS-DDG lipid sample was suspended in hexane at a concentration of 25−30 mg/mL. The samples/standards were spotted onto a Whatman silica 60 A TLC plate (60 general purpose, 20 × 20 cm, 250 μm, Maidsitone U.K.) using 10 and 5 μL aliquots. The samples/standards were resolved with hexane, di-ethyl ether and acetic acid [85:15:2 (v/v/v)]. The lipids were visualized by submerging the plate in 10% cupric sulfate in 8% phosphoric acid solution and charring at 165 °C for 10 min. The developed plate was scanned with a Kodak Gel Logic 440 Imaging System interfaced to Kodak ID Image Analysis software (Carestream Health, New Haven, CT).

A HPLC (Waters 510; Waters Corp., Milford, MA) equipped with a Luna 5 μm silica column (250 mm length, 4.6 mm id; Phenomenex, Torrance, CA) was also used to identify and quantify the several simple lipid classes. Detection was accomplished with an Alltech 800 evaporative light scattering detector (Deerfield, IL) operated at 50 °C. The nebulizer gas was nitrogen of industry grade, which was set at a flow pressure of 5.0 bar. Solvents consisting of 100% hexane (mobile phase A) and 99.8% hexane, 0.2% acetic acid, and 0.02% methyl tert-butyl ether (mobile phase B) set at a flow rate of 1 mL/min were used to separate the lipids using the following linear gradient: 0 min, 100% A; 2 min, 100% A; 3 min, 95% A; 10 min, 95% A; 14 min, 55% A; 15 min, 55% B; 23 min, 0% A; 26 min, 0% A. The column was heated to 38−40 °C; other exposed lines were maintained at 38−40 °C with heating tape. Chromatographic bands were identified and quantified with external standards. Results are expressed as the mean ± SD of a resolved lipid component or total GS-DDG lipid (mg/g) of triplicate analyses.

Compositional analysis of the tocopherols was performed with a HPLC method described by Bruni et al. (19). In brief, the sample was dissolved in hexane at a concentration of 30−40 mg/mL and injected onto a silica gel Lichrosorb Si-60 column (Agilent Technologies, Santa Clara, CA) interfaced to a Beckman System Gold 127 NM Solvent Module HPLC (Beckman Coulter Inc., Fullerton, CA) and heated to 30−32 °C. Isopropanol (0.05%) in hexane served as the mobile phase, which was set at a flow rate of 1 mL/min. Analytes were detected at 295 nm with a Beckman System Gold 166 UV−vis detector. The tocopherol peaks were identified and quantified by comparison with α-, γ-, and δ-tocopherol external standards. Results are expressed as the mean ± SD of tocopherols/total GS-DDG lipid (mg/g) of triplicate analyses.

A carotenoid assay was performed with a HPLC method described by Craft (20). GS-DDG lipids (2−3 mg) were dissolved in 0.25 mL of hexane and then combined with 0.25 mL of methanol. After continual shaking for ~1 h, the methanol phase was analyzed with a Waters 600 S HPLC (Milford, MA) system interfaced to a Waters Millennium 32 Chromatography Manager workstation. The samples were resolved using an Agilent Zorbax SB C-18 (Santa Clara, CA) column and a mobile phase of methanol, acetonitrile, and triethylamine [90:10:1 (v/v/v)] under isocratic conditions maintained at a flow rate of 1 mL/min. A Waters 996 photodiode array set at 450 nm was used as the detection mode. Results are not provided as carotenoids were not detected in triplicate analyses of the sample.

Quantification of sterols (free and esterified) was achieved with gas chromatography (GC) and flame ionization detection. GS-DDG lipids (10−20 mg) were prepared and analyzed according to the method of Schnieder et al. (21). The samples were injected (1 μL) onto an HP 5890 Series II GC with a DB-1 column (15 m × 0.25 mm) (J&W Scientific, Folsom, CA) under the following conditions: initial temperature, 190 °C for 1 min, increased to 220 at 3 °C/min; injector temperature, 270 °C; flame ionization detector temperature, 300 °C; helium carrier gas; and split ratio, 20:1. External standards of stigmastanol, campesterol, and β-sitosterol were used to identify and quantify the peaks. Results are expressed as the mean ± SD of a sterol/GS-DDG lipid (mg/g) of triplicate analyses.

Fatty acid profiles were determined by adapting the American Oil Chemists’ Society method. Cell-89-94). The lowest level of GS-DDG lipid extract (100 μg/mL) administered in this study resulted in significantly lower cell viability, whereas cells exposed to dosage levels of 200−300 μg/mL were comparable to the control (Figure 1). Other researchers have reported similiar but atypical responses for cells exposed to low doses of pure natural agents, that is, cell viability initially increased and then decreased or increased, increased and decreased again with higher treatment concentrations.
Acute cytotoxic effects of the GS-DDG lipids were assessed by measuring percent dead cell counts relative to total cells counts (Table 2). Total cell counts were not significantly different between the control and any of the lipid treatments tested (400, 500, and 600 μg/mL of GS-DDG lipids), again indicating that the treated cells grew during the exposure period. However, the percent dead cells were higher for each treatment group compared to the control but were similar between GS-DDG lipid dosage levels. A loss of cellular membrane integrity in response to each GS-DDG lipid was confirmed with the LDH assay. As shown in Figure 2, a significant increase of the cellular protein, LDH, was detected in the medium for samples treated with the GS-DDG lipids, but the LDH level for the 400 μg/mL treated cells was significantly lower compared to the 500 and 600 μg/mL doses, which may signify greater protection with higher GS-DDG doses. In addition, LDH levels were not significantly different between the blank and the control, indicating that the micelles were not acutely cytotoxic to the cells (Figure 2). These results are supported by the cell count data (Table 2) as significantly higher total cell counts occurred for the blank (cells – micelles) compared to the control (cells + micelles) but the percentages of dead cells were similar between the two samples. Because the dead cell counts accounted for only 10% of total cell count (Table 2), but overall viability was reduced by 25–50% as evidenced by the MTT results (Figure 1), the presence of apoptotic cells was determined by flow cytometry after staining with fluorescent isothiocyanate–annexin V (AV) and/or propidium iodide (PI). Cells were considered viable, early apoptotic, late apoptotic + necrotic, or necrotic for AV−/PI−, and AV+/PI−, AV+/PI+, and AV−/PI+ counts, respectively. Viable cell count decreased significantly for each dosage level compared to the control (by 12–40%), but the cells treated

Table 1. Total Protein Levels of Caco-2 Cells before and after 24 h of Treatment with Grain Sorghum Dry Distiller’s Grain Lipids

<table>
<thead>
<tr>
<th>lipid treatment (μg/mL)</th>
<th>total protein levelsa (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)b</td>
<td>107.5 ± 5.8 a</td>
</tr>
<tr>
<td>0 (control)c</td>
<td>151.1 ± 5.0 b</td>
</tr>
<tr>
<td>400</td>
<td>154.2 ± 8.0 b</td>
</tr>
<tr>
<td>500</td>
<td>145.7 ± 7.2 b</td>
</tr>
<tr>
<td>600</td>
<td>145.6 ± 7.4 b</td>
</tr>
</tbody>
</table>

a. Results are expressed as the mean ± standard error of the mean of total cellular protein (in μg/mL). Different letters represent results that are significantly different (p < 0.05).
b. Represents protein levels of Caco-2 cells after 24 h treatment.
c. Represents protein levels of Caco-2 cells before 24 h treatment.

Table 2. Total and Percent Dead Cell Counts of Caco-2 Cells after 24 h Treatment with Grain Sorghum Dry Distiller’s (GS-DDG) Grain Lipids

<table>
<thead>
<tr>
<th>GS-DDG lipid (μg/mL)</th>
<th>total countsa (cells/mL)</th>
<th>dead cell countsb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>2.27 ± 0.38 × 10^5 a</td>
<td>6.58 ± 0.61 a</td>
</tr>
<tr>
<td>0 (control)</td>
<td>1.14 ± 0.21 × 10^5 b</td>
<td>16.42 ± 4.89 a</td>
</tr>
<tr>
<td>400</td>
<td>9.93 ± 2.05 × 10^4 b</td>
<td>26.43 ± 4.13 b</td>
</tr>
<tr>
<td>500</td>
<td>7.80 ± 1.29 × 10^4 b</td>
<td>25.62 ± 8.99 b</td>
</tr>
<tr>
<td>600</td>
<td>9.35 ± 2.13 × 10^4 b</td>
<td>26.77 ± 4.35 b</td>
</tr>
</tbody>
</table>

a. Results are expressed as the mean ± standard error of the mean (SEM) of total cell counts per mL of medium after staining with Trypan Blue. Different letters in the column represent results that are significantly different (p < 0.05).
b. Results are expressed as the mean ± SEM of percent dead cell counts after staining with Trypan Blue. Different letters in the column represent results that are significantly different (p < 0.05).
Fluorescence-activated cell results of Caco-2 cells after 24 h
Percent of total lactate dehydrogenase (LDH) released from surface molecules that target the apoptotic cells for phagocytosis by including maintenance of intact plasma membrane and activation of 2–7 times more cells in either early apoptotic (AV+/PI−) or necrotic (Figure 2), but the 500 and 600 μg/mL doses resulted in 4–5 times and cacious as higher GS-DDG lipid levels as evidenced by the LDH data
control with the notable exception of the 400 μg/mL treated cells (Table 3), a population apoptotic cells was also detected after GS-DDG (Figure 3), a population apoptotic cells was also detected after GS-DDG (Figure 4).

Although PI-stained cells were apparent by the FACs method (Figure 1) when accounting for variability inherent to both assays. Similar to the Trypan Blue and %LDH assays, staining of cells by PI is an anti proliferative properties of Grain Sorghum DDG lipids in Caco-2 cells

with 400 μg/mL GS-DDG lipids resulted in significantly higher viable counts with respect to the 500 and 600 μg/mL treatments (Figure 3 and Table 3). These preliminary results support the MTT data (Figure 1) when accounting for variability inherent to both assays. Similar to the Trypan Blue and %LDH assays, staining of cells by PI is an indicator of the loss of plasma membrane integrity or necrotic cells. Although PI-stained cells were apparent by the FACs method (Figure 3), a population apoptotic cells was also detected after GS-DDG lipid treatment at significantly higher numbers compared to the control with the notable exception of the 400 μg/mL treated cells (Table 3). These results suggest that the 400 μg/mL dose may not be as efficacious as higher GS-DDG lipid levels as evidenced by the LDH data (Figure 2), but the 500 and 600 μg/mL doses resulted in 4–5 times and 2–7 times more cells in either early apoptotic (AV+/PI−) or necrotic (AV+/PI+) stages, respectively.

Apoptotic cells exhibit several characteristic morphologic patterns, including maintenance of intact plasma membrane and activation of surface molecules that target the apoptotic cells for phagocytosis by neighboring bodies (35). In contrast, necrotic cells demonstrate cell swelling and loss of membrane integrity culminating in the eventual release of inflammatory cellular contents. As a result, cancer treatment options triggering cell death by apoptosis are preferable to those that induce necrosis. It is unclear at this point whether the GS-DDG lipid directly induced necrosis or whether apoptotic cells proceeded to secondary necrosis due to the absence of a phagocytic response in pure cultures. Nonetheless, the cytotoxic effect (caused by a necrotic and/or an apoptotic response) was probably exhibited late in the 24 h treatment period before the cellular growth rate could be affected. Additional time course studies are needed to test this hypothesis.

**GS-DDG Lipid Characterization Study.** Physiochemical characterization of natural systems intended as FFN ingredient sources is needed to identify the constituents responsible for their health-promoting properties. Although other studies have reported the lipid composition of GS-DDGs (2, 4, 5), bioactive agents can be affected by extraction conditions, sampling handling, and extended storage periods and are thus prone to lot-to-lot variability. Therefore, the crude lipid extract used for the previous study was characterized in our laboratory to determine potential bioactive agents responsible for the antiproliferative effect.

Several major simple lipid classes, including triacylglycerides, free fatty acids, policosanols, free sterols, and stereryl esters, were initially detected with TLC, which were confirmed by HPLC-ELSD (data not shown). On the basis of the latter test, the most abundant lipid class was triacylglycerides (286.0 ± 23.3) followed by free fatty acids (71.0 ± 13) (Table 4). Fatty acid compositional analysis showed that these groups (in combination the sterol esters) were composed primarily of linoleic acid (C18:2) (Figure 4). Despite its being a potential pro-inflammatory compound, Sasaki et al. (36) reported that linoleic acid (100–400 μg/mL) decreased the growth rate of colon cancer cell lines (Colo320 and MKN28) by 22–98%. These results correlated with a reduction in tumor necrosis factor-α (Figure 4). The fatty acid composition was further confirmed by gas chromatography with flame ionization detection. The preliminary results support the MTT data (Figure 3).

### Table 3. Fluorescence-Activated Cell Results of Caco-2 Cells after 24 h Treatment with Grain Sorghum Dry Distiller’s Grain Lipids

<table>
<thead>
<tr>
<th>Lipid Treatment (μg/mL)</th>
<th>AV−/PI− (%)</th>
<th>AV+/PI− (%)</th>
<th>AV+/PI+ (%)</th>
<th>AV−/PI+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>84.8 ± 1.4 a</td>
<td>1.7 ± 1.4 a</td>
<td>6.8 ± 1.2 a</td>
<td>6.6 ± 0.2 a</td>
</tr>
<tr>
<td>400</td>
<td>74.4 ± 1.7 b</td>
<td>2.0 ± 0.6 ab</td>
<td>11.7 ± 2.1 ab</td>
<td>12.1 ± 0.7 a</td>
</tr>
<tr>
<td>500</td>
<td>50.2 ± 1.1 c</td>
<td>4.9 ± 0.8 b</td>
<td>28.7 ± 5.0 b</td>
<td>16.1 ± 4.7 a</td>
</tr>
<tr>
<td>600</td>
<td>57.1 ± 4.2 c</td>
<td>10.4 ± 1.8 b</td>
<td>30.8 ± 3.1 b</td>
<td>1.65 ± 0.5 a</td>
</tr>
</tbody>
</table>

a. Results expressed as the mean ± standard deviation of percent gated events of cells that stained as annexin V (AV−)/propidium (PI)− (viable cells), AV+/PI− (early apoptotic cells), AV+/PI+ (late apoptotic + necrotic cells), or AV−/PI+ (necrotic cells). Different letters in a column represent results that are significantly different (p < 0.05).

### Table 4. Levels of Simple Lipid Classes in Grain Sorghum Dry Distiller’s Grain Lipids

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Amount (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacylglycerides</td>
<td>Detectedb</td>
</tr>
<tr>
<td>Sterols (free and esterified)</td>
<td>9.9 ± 1.3</td>
</tr>
<tr>
<td>Tocopherols</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>Aldehydes and wax esters</td>
<td>16.0 ± 11.0</td>
</tr>
<tr>
<td>Free fatty acidsd</td>
<td>71.0 ± 13.0</td>
</tr>
<tr>
<td>Policosanolsd</td>
<td>39.0 ± 7.7</td>
</tr>
<tr>
<td>Triacylglyceridesd</td>
<td>866.0 ± 23.3</td>
</tr>
<tr>
<td>Hydrocarbonsd</td>
<td>Detectedd</td>
</tr>
</tbody>
</table>

a. Results expressed as the mean ± standard deviation of mg of lipid per gram of lipid.
b. Thin layer chromatography and densitometry.
c. High-performance liquid chromatography with ultraviolet detection.
d. Gas chromatography with a flame ionization detection.
e. High-performance liquid chromatography with evaporative light scattering detection.
f. Limit of quantitation (0.5 mg/g).
f. Limit of quantitation (0.8 mg/g).
development when treated and untreated Colo320 and MKN28 cells were injected into the peritoneal cavity of BALB/c nu/nu athymic mice. The GS-DDG lipid extract also contained relatively high levels of oleic acid (C18:1) (Figure 4), which has been shown to be a potential antiproliferative agent, albeit against breast cancer (37).

Moreover, GC analysis showed that the phytosterols (free and esterified) accounted for 9.9 ± 1.3 mg/g of the lipid extract (Table 4) with β-sitosterol and stigmasterol present at equivalent levels followed by campesterol (Table 5). Awad and Fink (38) reported that β-sitosterol, and possibly stigmasterol and campesterol, increased apoptotic activity in cancer cells. On the basis of the results cited (Table 5) and the GS-DDG lipid treatment levels used for this study, the β-sitosterol doses delivered to the cells were significantly lower (1–10 nM β-sitosterol) than the 30–60 μM 7-hydroxysitosterol required to induce apoptosis in Caco-2 cells as reported by Roussi et al. (39).

As previously discussed, Campbell et al. (8) studied the antiproliferation effects of tocopherols against several colon cancer cell lines (SW480, HCT-15, HCT-116, and HT-29) at levels representing dietary and supplementation levels of 25–200 μM, respectively. α-Tocopherol did not significantly affect cell proliferation, whereas the lowest dosage of γ-tocopherol resulted in the highest cell counts of SW480 and HCT-116 cells. Analysis of the GS-DDG lipid extract did show the presence of α-tocopherol (0.20 ± 0.00 mg/g) and γ-tocopherol (0.19 ± 0.01 mg/g) at a total concentration of 0.39 ± 0.01 mg/g (Table 4). Therefore, the levels of α- and γ-tocopherol in the 1000 μg/mL lipid treatment group were each <0.5 nM, that is, again substantially lower than the effective doses reported in the literature.

Because preliminary data obtained in our laboratory demonstrated that other GS-DDG lipid extracts contained carotenoids, particularly β-carotene (data not shown), the sample used for this study was tested accordingly. As potent antioxidants, carotenoids can deactivate free radicals that damage the basic structure of cells and thus lead to cancer (40). However, carotenoids were not detected in the GS-DDG lipid sample under the conditions of the test and thus could not account for the cited antiproliferation results. These compounds may have been lost during processing or may not be present in the GS cultivar that served as the raw material.

Other lipid components were detected in the GS-DDG lipid sample that to our knowledge have yet to be evaluated for their anticancer properties. For example, total levels of policosanols accounted for 39.0 ± 7.7 mg/g of the extract (Table 4). Compositional analysis of their chain lengths was not completed in our laboratory, but Hwang et al. (4) confirmed that the wax fraction of GS-DDG was composed of C28 and C30 chain policosanols, which is most likely the compositional profile of the sample used in this study. In addition, two unknown bands were detected via the TLC method. Hwang et al. (41) reported similar TLC banding patterns for lipids extracted from the GS whole kernel and confirmed that a band with the same R value as one of the unknown GS-DDG lipid groups was due to long-chain aldehydes. Moreover, Hwang et al. reported that long-chain aldehydes accounted for 23% of the wax fraction isolated from GS-DDG samples (4). Additionally, in-house analysis with HPLC-ELSD showed an intense band at the same retention time as the external aldehyde standard, albeit wax/steryl esters also coeluted with the former lipid group (data not shown). More tests are needed to definitely identify the suspect TLC band, but the combined information suggests that it may be due to long-chain aldehydes. The other unknown TLC band has yet to be identified by our laboratory or by any other published reports. Diacylglycerides and hydrocarbons were further detected in the extract but at levels lower than the quantitation capability of the assay (Table 4).

In summary, the physicochemical characterization study confirmed that the sample contained lipid-based constituents that have been linked to potent anticancer properties, but many were significantly below the effective concentrations reported in the literature with the notable exception of linolenic and oleic acid. The antiproliferation benefits of the GS-DDG extracts could be caused by synergistic and/or additive effects of the components as has been reported for many types of natural systems (42-44). The GS-DDG lipid extract also contains other types of lipids that have not been studied for their antiproliferation properties but may indeed be potent cancer prevention agents. Nonetheless, this project is significant as it is the first to report the chemoprotective potential of the GS-DDG lipid extract. Additional studies are needed to further elucidate the mechanisms by which GS-DDG lipids exert their antiproliferation properties against multiple types of cancers and to identify the specific component or combination of components responsible in order to develop this currently underutilized cereal and its coproducts as safe and effective source of ingredients for functional foods.

References


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