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

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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 GS-DDG lipids (p < 0.05). 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 control were not significantly different from the GS-DDG lipid treated cells, but dead cell counts increased by ~10% 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 GS-DDG lipids. Physiochemical characterization of the GS-DDG lipids used for the antiproliferation study showed the presence of vitamin E (predominantly γ-tocopherol), triacylglycerides (predominantly linoleic acid), policosanols, aldehydes, and sterols (predominantly campesterol and stigmasterol), each of which or as synergistic/additive group of constituents may be responsible for the antiproliferative effect.

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 camp-esterol 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.

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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 source for the niche but fast-growing functional food and nutraceutical (FNFs) 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-DDGs 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% t-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.

Cytotoxicity Assay.

Cytotoxicity assay results are reported as the mean ± standard deviation from four replicates (three to four analyses per replicate).

Total and percent dead cell counts 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 combined with the cells, and the total numbers of cells and of dead cells (stained) were counted on a Neubauer hemocytometer (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).

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 500, 600, or 700 μL/mL of solubilized GS-DDG lipid, medium + micelles (control), or fresh medium only, medium + micelles (blank). After 24 h, 100 μL/mL 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 FLOStar 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 deactivated 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 of the 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 followed by deactivation with 15 μL of 1 N HCl. The absorbance was monitored at 485 and 680 nm with a FLOStar 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 lysate)). Results are reported as the mean percent LDH ± 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 fluorescein-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 (Becton Dickinson FACScan, Franklin Lakes, NJ) and monitored at an excitation of 488 nm and an emission wavelength of 525 nm for the fluorescein 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 replicate (two 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, α,ω-tocopherol acetate, and three polyoxyethylene sorbitan monoleate, octacosanol, monoacylglycerides of oleic acid, 1,2-diacylglycerols of oleic acid, 1,3-diacylglycerides of oleic acid, and tricylglycerides 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 g general purpose,
$20 \times 20$ cm, 250 $\mu$m, Maidstone U.K.) using 10 and 5 $\mu$L aliquots. The standards/resolutions were dissolved with hexane, diethyl ether, and acetone/acetic acid at 1:2(v/v/v) in hexane, respectively. The lipids were then resolved by submerging the plate in 10% cupric sulfate in 8% phosphoric acid solution and charring at 165 $^\circ$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 $\mu$m 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 $^\circ$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 99.6% hexane, 0.2% acetic acid, and 0.02% methanol tert-butyl ether (mobile phase B) served as the mobile phase, which was set at a flow rate of 0.5 mL/min. Analyses were detected at 210 nm with a Beckman 5000 sealed tube detector. The injection volumes were 20 $\mu$L and 10 $\mu$L for quantitative determination of most fatty acids, respectively. The lipids were visualized by submerging the plate in 20% cupric sulfate in 8% phosphoric acid solution and charring at 165 $^\circ$C. The lipids were identified and quantified by comparison with internal standards. Results are expressed as the mean ± SD of triplicate analyses.

The ability of GS-DDG lipids to inhibit cancer proliferation was evaluated using the human colon carcinoma cell line (Caco-2) as colon cancer is the second and fourth leading cause of cancer death in North America/Europe and worldwide, respectively (23–25). Moreover, grain sorghum consumption has been shown to be associated with reduced risks for gastrointestinal-based cancers (10–12). The antiproliferation properties of GS-DDG lipids (100–1000 $\mu$g/mL in 100 $\mu$L/mL increments) were initially evaluated by monitoring the mitochondrial activity of GS-DDG lipid treated Caco-2 cells using the MTT assay. Because the GS-DDG samples were insoluble in the DMEM medium, micelles prepared with taurocholic acid sodium salt, lecithin, and cholesterol were able to solubilize the GS-DDG lipid at all treatment levels tested. While micelles have been used for delivering cholesterol and other sterols to mammalian cells (26), they are unique to a complex lipid extract. The data were thus normalized to the control (cells + micelles) as cell proliferation was affected by the micelle delivery system resulting in significantly lower percent cell viability compared to the blank (cells – micelles) (data not shown). As shown in Figure 1, percent cell viability decreased in a dose-dependent manner with significant differences (25–50% reduction) occurring at treatment levels ≥400 $\mu$g/mL. Percent viability was not significantly different for cells treated with 400–1000 $\mu$g/mL GS-DDG lipids, indicating a limiting dosage of 400 $\mu$g/mL. The effective antiproliferative treatment levels and/or exposure time (24 h) used for this study were lower than reported for several other types of natural extracts and/or pure components. For example, Yang et al. (27) demonstrated that the polar extracts from different varieties of onion reduced Caco-2 proliferation at EC$_{50}$ values between 30.5 and 141.1 $\mu$g/mL. In addition, Kuntz et al. (28) reported that pure phenolic compounds, including different types of flavones, flavonols, flavanones, and isoflavones, reduced Caco-2 proliferation at EC$_{50}$ values of 40–200 $\mu$M and exposure times of 72 h. Considering that a complex lipid extract is not widely recognized as an anticancer agent, particularly with respect to polar-based extracts or pure phenolics compounds, these results indicate that the GS-DDG lipids have effective antiproliferation properties.
decreased again with higher treatment doses (18, 29–31). Darbon and Casagrande (31) attributed their results to the ability of biological systems to compensate for low-level toxicity of a bioactive agent but are overcome at higher treatment doses. More studies are needed to understand this low-dose response as these results may have important clinical implications. For the purpose of this study, higher GS-DDG lipid dosage levels that adversely affected proliferation (400–600 μg/mL) were used for the remaining experiments.

Colon cancer usually presents as hyperproliferation of the epithelial lining due to deregulated cell cycle arrest and/or the suppression of programmed cell death (apoptosis) (24, 31). Dietary components have in turn been shown to control such abnormal growth by causing tumor cell death and/or by regulating the cell cycle (8, 17, 18, 27–34). To understand in part the chemoprotective properties of GS-DDG lipids against Caco-2 proliferation, overall cell growth was determined by monitoring cellular protein levels prior to and after the 24 h treatment period (Table 1). Protein levels of the control and treated cells were significantly higher post-treatment compared to the control pretreatment, showing that the cells continued to grow. Considering that the control and the treated cells had comparable protein levels, the results suggest that GS-DDG did not impact proliferation by inhibiting cell growth after 24 h of treatment.

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 with grain sorghum dry distiller’s lipids (GS-DDG) (in μg/mL) as compared to the control (0 μg/mL GS-DDG). Results that are significantly different (p < 0.05) are designated with different letters from each other, whereas + represents results that are not significantly different (p > 0.05) from the control.

![Figure 1. Percent cell viability based on the tetrazolium salt 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay of Caco-2 cells treated with grain sorghum dry distiller’s lipids (GS-DDG) (in μg/mL) as compared to the control (0 μg/mL GS-DDG). Results that are significantly different (p < 0.05) are designated with different letters from each other, whereas + represents results that are not significantly different (p > 0.05) from the control.](image)
the control (by 12–40%), but the cells treated 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 (A+/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 phagocytotic 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 antiproliferation effect.

Several major simple lipid classes, including triacylglycerides, free fatty acids, policosanols, free sterols, and steryl 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 (866.0 ± 23.3) followed by free fatty acids (71.0 ± 13) (Table 4). Fatty acid compositional analysis showed that these groups (in combination the steryl 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...
particularly β demonstrated that other GS-DDG lipid extracts contained carotenoids, effective doses reported in the literature. were each <0.5 nM, that is, again substantially lower than the of R (concentration of 0.39

Table 5. Composition of Plant Sterols in Grain Sorghum Dry Distiller’s Grain Lipids

<table>
<thead>
<tr>
<th>isomer</th>
<th>amount* (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sitosterol</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>stigmasterol</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>campesterol</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>total</td>
<td>9.9 ± 1.3</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean ± standard deviation of mg sterol/g of grain sorghum distiller’s grain lipids.

Figure 4. Percent abundance of fatty acids present in the grain sorghum dry distiller’s grain lipids. The fatty acid profiles were determined using gas chromatography–flame ionization, and the relative peak area of each fatty acid was obtained. Values represent the mean ± standard deviation of percent relative abundance of each fatty 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 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-16, 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 higher dead 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. (41) 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 Rf 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 (41). 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 physiochemical 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.

LITERATURE CITED


