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Sorghum 3-Deoxyanthocyanins Possess Strong Phase II Enzyme Inducer Activity and Cancer Cell Growth Inhibition Properties

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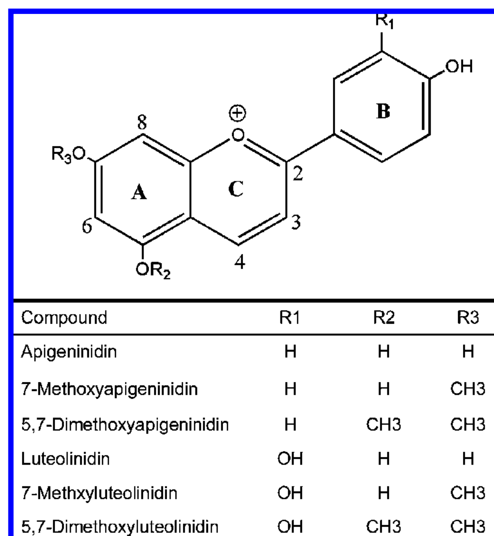
3-Deoxyanthoxyanins (3-DXA) possess unique chemical and biochemical properties and may be useful in helping reduce incidence of gastrointestinal cancer. This study tested sorghum extracts rich in 3-DXA as well as isolated and synthetic 3-DXA for potential to induce activity of phase II enzymes in murine hepatoma cells using the NAD(P)H:quinone oxidoreductase (NQO) assay and to inhibit proliferation of the HT-29 human colon cancer cells using MTT and PicoGreen assays. Crude black sorghum extract that contained high levels of methoxylated 3-DXA was a strong inducer of NQO activity (3.0 times at 50 μg/mL), compared to red or white sorghum extracts with low or no methoxylated 3-DXA (1.6 times at 200 μg/mL). All sorghum extracts had strong antiproliferative activity against HT-29 cells after 48 h of incubation (IC_{50} = 180−557 μg/mL). Among isolated fractions, nonmethoxylated 3-DXA were very effective against HT-29 cell growth (IC_{50} = 44−68 μM at 48 h), but were noninducers of NQO. On the other hand, the methoxylated 3-DXA had both strong antiproliferative activity (IC_{50} < 1.5−53 μM) and NQO inducer activity (2−3.7 times). Dimethoxylated 3-DXA were more potent than monomethoxylated analogues. Methoxylation of 3-DXA is essential for NQO activity and also enhances tumor cell growth inhibition.

KEYWORDS: Antioxidants; colon cancer; 3-deoxyanthocyanins; phase II enzymes; sorghum

INTRODUCTION

3-Deoxyanthocyanins (**Figure 1**) are a rare class of plant pigments with chemical properties that are very distinct from those of their anthocyanin analogues. For example, the 3-deoxyanthocyanins (3-DXA) are more stable to light, heat, and change in pH than anthocyanins (*1, 2*). Additionally, they are also more resistant to bleaching in the presence of common food additives such as ascorbic acid that readily degrade anthocyanins (*3, 4*). One of the theories used to explain the unusual stability of the 3-DXA is their high deprotonation constants relative to their hydration constants; this favors equilibrium toward the colored quinoidal bases (as opposed to the colorless carbinol species that dominate in the case of anthocyanins) in moderate−mildly acidic environments (*1, 5*). Currently, sorghum is the only known natural food source of the 3-DXA in significant quantities (*6*). The 3-DXA thus present good potential as stable natural food colorants.

The unique properties of 3-DXA may extend to their biochemical activity as well. For example, Shih et al. (*7*) recently demonstrated that the major sorghum 3-DXA aglycons, apigeninidin and luteolinidin, were more cytotoxic to human cancer cells than their anthocyanidin analogues, cyanidin and pelargonidin. Epidemiological evidence also demonstrates that



Compound	R1	R2	R3
Apigeninidin	H	H	H
7-Methoxyapigeninidin	H	H	CH3
5,7-Dimethoxyapigeninidin	H	CH3	CH3
Luteolinidin	OH	H	H
7-Methxyluteolinidin	OH	H	CH3
5,7-Dimethoxyluteolinidin	OH	CH3	CH3

Figure 1. 3-Deoxyanthocyanin aglycons tested in this study.

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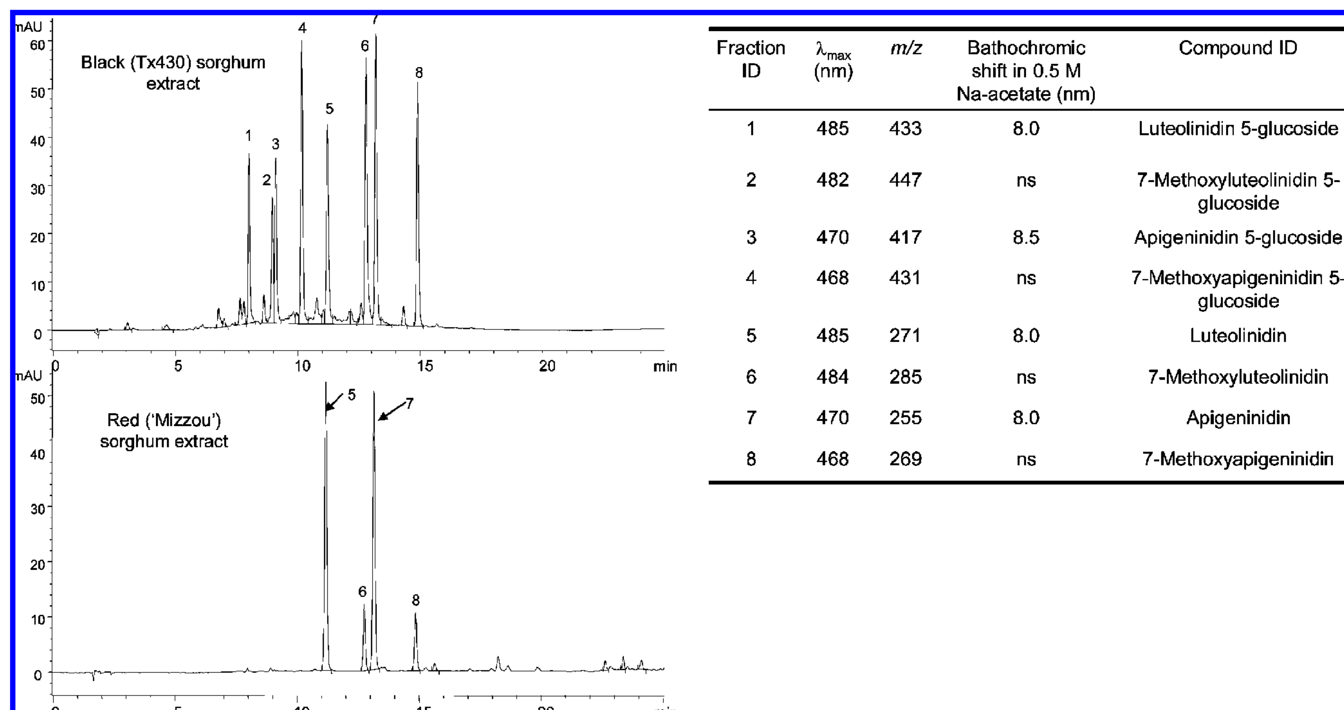

Fraction ID	λ_{max} (nm)	m/z	Bathochromic shift in 0.5 M Na-acetate (nm)	Compound ID
1	485	433	8.0	Luteolinidin 5-glucoside
2	482	447	ns	7-Methoxyluteolinidin 5-glucoside
3	470	417	8.5	Apigeninidin 5-glucoside
4	468	431	ns	7-Methoxyapigeninidin 5-glucoside
5	485	271	8.0	Luteolinidin
6	484	285	ns	7-Methoxyluteolinidin
7	470	255	8.0	Apigeninidin
8	468	269	ns	7-Methoxyapigeninidin

Figure 2. Analytical HPLC chromatograms of black and red sorghum extracts monitored at 480 nm and spectroscopic data used to aid in the identification of the major peaks. Bathochromic shift in sodium acetate was used to determine the position of the $-OCH_3$ group on the A-ring. ns, nonsignificant.

increased consumption of sorghum is linked to reduced risk of certain types of cancer of the gastrointestinal tract (GIT), especially esophageal cancer (*8−10*). To date, no evidence is available on which components of sorghum may contribute to its cancer chemoprevention. This information is critical if we are to move ahead in developing foods and food ingredients that aim to prevent the development of specific types of cancer in at-risk populations. The 3-DXA are likely candidates that deserve systematic investigation.

Modulation of levels of biotransformation enzymes that facilitate the elimination of endogenous and environmental carcinogens is widely recognized as one of the successful strategies to retard or block carcinogenesis (*11*). Induction of detoxification and antioxidant enzymes through the activation of intracellular signaling mediated by NF-E2-related factor 2 (Nrf2) is believed to be a central mechanism by which bioactive agents impart their chemopreventive activity (*11*). Hence, phase II enzyme activity induction is a good indicator of the protection of animal cells against carcinogens and oxidant toxicity (*12*). The enzyme NAD(P)H:quinone oxidoreductase (NQO) is a widely accepted marker for protective phase II enzyme induction (*13, 14*). Even though anthocyanins and other flavonoids without a 2,3 double bond in the C-ring were reported not to induce the chemoprotective enzymes (*14, 15*), we hypothesize that the 3-DXA may possibly induce these enzymes owing to their unique chemical properties. Hence, this work aims to determine if 3-deoxyanthocyanin-rich sorghum extracts can induce NQO activity in vitro and how the structures of isolated 3-DXA affect their inducer activity. We also establish how the identified 3-DXA inhibit proliferation of human colon cancer cells in vitro.

MATERIALS AND METHODS

Sorghum Extracts and Standards. Two sorghum varieties, a black (Tx430, grown in 2004 in College Station, TX) and a red (Mizzou, grown in 2006 in Columbia, MO), with different 3-DXA pigment compositions (based on preliminary screening) were selected for use in this study. In addition, we used a nonpigmented white sorghum (KARI-Mtama, from Egerton University, Kenya) as a control. These samples did not contain any condensed tannins. Samples were kept at −35 °C prior to use. Samples were ground in a cyclone mill (UDY, Boulder, CO) to pass through a 0.1 mm screen before extraction. Ground powder was defatted twice with excess amounts of hexanes and then dried overnight in a fume hood. The defatted powder was then extracted in 70% aqueous acetone acidified with 0.1% HCl for 30 min in a shaker. Extracts were then centrifuged (3100*g*) for 10 min at 4 °C, and the acetone was immediately removed from the supernatant under vacuum; the aqueous extracts were then freeze-dried as crude extract and stored at −35 °C until used. Commercial red cabbage pigment extract (Voigt Global Inc., Kansas City, MO) was used as a natural source of anthocyanins for comparison. 3-Deoxyanthocyanidin standards (≥95% pure) were obtained from AlsaChim (Strasbourg, France), whereas L-sulforaphane (≥95% pure) and genistein (≥98% pure) were purchased from Sigma (St. Louis, MO).

Phenolic Content and Antioxidant Assay of Crude Extracts. For 3-deoxyanthocyanin pigment content and Folin−Ciocalteu phenol, extracts were dissolved in 1% HCl acidified methanol. 3-Deoxyanthocyanin pigment content was measured as detailed by Awika et al. (*2*), whereas the Folin−Ciocalteu method as detailed by Kaluza et al. (*16*) was used to estimate phenols content. Extracts for antioxidant activity assays were dissolved in 70% aqueous acetone and analyzed for 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and oxygen radical absorbance capacity (ORAC) free radical scavenging properties as described by Awika et al. (*17*). A BioTek Synergy HT plate reader (BioTek, Winooski, VT) was used for the ORAC assay. Assays were repeated three separate times.

Analytical HPLC and MS. Freeze-dried extracts were dissolved in 10% methanol (in deionized water) containing 1% formic acid and filtered through a 0.45 μm membrane before analysis. A reversed phase 150 × 2.00 mm, 5 μm, C-18 column (Phenomenex, Torrance, CA) was used for separation. An Agilent 1100/1200 HPLC system with a diode array detector (Agilent Technologies, Santa Clara, CA) was used. Analytical conditions were as follows: autosampler temperature, 4 °C; column temperature, 35 °C; injection volume, 5.00 μL; detection, 200−700 nm; monitoring wavelength, 480 nm; flow rate, 0.25 mL/min. The mobile phase consisted of (A) 1% formic acid in water and (B) 1% formic acid and 50% acetonitrile in water. The 30 min elution gradient for B was as follows: 0−2 min, 10% isocratic; 2−15 min,

10−70%; 15−20 min, 70−100%; 20−23 min, 100% isocratic; 23−24 min, 100−10%; 25−30 min, 10% isocratic.

MS analysis was performed using a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer equipped with an API2 source, Performance Pack (with wider orifice in the skimmer and an extra turbo pump on the source), and an electrospray ionization (ESI) interface (ThermoFinnigan, San Jose, CA). The electrospray needle voltage was 4.5 kV, and the heated inlet capillary was equilibrated at 250 °C. All voltages were optimized to maximize ion transmission and minimize unwanted fragmentation. Spectra were recorded in positive ion mode between *m/z* 150 and 1000. The 3-DXA composition of the two pigmented sorghum varieties is shown in **Figure 2**.

Semipreparative HPLC. To fractionate individual compounds, the freeze-dried crude extracts were dissolved in 10% methanol adjusted to pH 2.0 with HCl, filtered through a 0.45 μm membrane, and directly fractionated on a semipreparative Luna 250 × 10 mm C-18(2) column (Phenomenex, Torrance, CA) with the aid of the Agilent system described above. The system was modified by attaching a 1400 μL injection loop to the autosampler, replacing the standard flow cell with a micro flow cell (3 mm path length) in the detector, and adding an Agilent 1100 fraction collector to the setup. Separation conditions were as follows: flow rate, 5 mL/min; injection volume, 800 μL; autosampler temperature, 4 °C; column temperature, 35 °C; monitoring wavelengths, 280, 320, and 480 nm with DAD spectra recorded from 190 to 700 nm. Solvents were 1% formic acid in water (A) and 1:49:50 formic acid/water/acetonitrile (B). Flow conditions were (%B) 0−2 min, 10−18%; 2−31 min, 18−45%; 31−33 min, 45−90%; 33−34 min, 90% (isocratic); 34−35 min, 90−10%; 35−41 min, 10%. The procedure was repeated and individual fractions were pooled until enough of the major compounds were obtained for analysis. Fraction purity was checked by injecting the pooled fractions and using the analytical HPLC procedure described above; separation was very effective, and all fractions were >95% pure. The pooled fractions were freeze-dried and kept at −35° until used.

Cell Culture. Murine hepatoma Hepa 1c1c7 and human colon carcinoma HT-29 cell lines purchased from ATCC (Manassas, VA) were used in this study. The Hepa 1c1c7 cell line was cultured in Gibco α-MEM medium supplemented with 10% fetal bovine serum; the HT-29 cell line was cultured in Gibco McCoy's 5A medium supplemented with 10% fetal bovine serum. Cell lines were subcultured every 3 or 4 days and incubated in a humidified incubator (5% CO_2, 37 °C).

NAD(P)H:Quinone Oxidoreductase (NQO) Inducer Capacity and Cytotoxicity Assay. The Hepa1c1c7 cell line was used for this assay; NQO inducer activity was measured as described by Prochaska and Santamaria (*18*). Briefly, cells were grown in 96-well microtiter plates; 10000 cells were introduced into each well, grown for 24 h, and then induced for 24 h with medium containing various concentrations of crude extracts (1−400 μg/mL) and isolated fractions (0.2−200 μM) (dissolved in dimethyl sulfoxide, DMSO) in duplicate plates. The final volume in each well was 200 μL, and DMSO concentration was maintained at 0.1%. Cells in one plate were lysed with digitonin, and then reaction mixture was added. After 5 min, reaction was stopped using 0.3 mM dicoumarol; NQO activity was measured optically on the basis of the formation of brown-blue reduced tetrazolium dye, at 610 nm. The second plate was used to measure cell density (indicator of cytotoxicity) by staining with crystal violet. Quantitative information on inducer potency was obtained as described by Prochaska and Santamaria (*18*); specific inducer ratio was calculated as enzyme activity per unit cell population in treated cells divided by enzyme activity per unit cell population in negative control (0.1% DMSO). Sulforaphane was used as a positive control; this compound is a known potent natural phase II enzyme inducer found in broccoli.

Viable Cell Population and Double-Stranded (ds) DNA Assay. The HT-29 cell line was used for these assays. For viable cell populations assay, 2000 cells/well plated into clear 96-well plates were incubated with different concentrations of test samples as indicated above (or 0.1% DMSO for control) for 48 h, after which the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma) was used to determine viable cell population using standard protocols. The PicoGreen method outlined by Ahn et al. (*19*) was used to quantify dsDNA. Cells (2000/well) were plated in 96-well black plates with clear bottoms and incubated for 48 h. A Quant-iT PicoGreen Assay Kit (Invitrogen Inc., Carlsbad, CA) was then used for dsDNA measurement following instructions by the manufacturer. Plates were read at 480 nm (excitation) and 520 nm (emission). Genistein was used as a positive control in both assays.

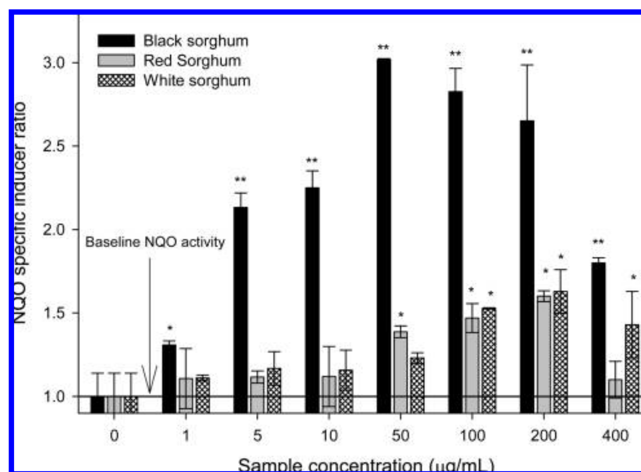

Figure 3. NAD(P)H:quinone oxidoreductase specific inducer capacity of sorghum extracts. Hepa1c1c7 murine hepatoma cell lines were used for the assay. Cells (10000/well) were induced with extracts for 24 h before assay. Error bars represent ± sd from three separate experiments. *, $P < 0.05$; **, $P < 0.001$, compared with control (Bonferroni multiple-comparison test).

A BioTek Synergy HT plate reader (BioTek Instruments) was used for assays described above. At least four wells were used for every sample concentration per analysis. All enzyme activity and proliferation assays were repeated on three separate occasions.

RESULTS AND DISCUSSION

Composition and NQO Inducer Activity of Crude Extracts. Composition of sorghum extracts was established on the basis of HPLC-MS data (elution time, UV−vis spectra, *m/z*) and comparison to authentic standards. However, because 5- or 7-methoxylated 3-DXA could not be distinguished from each other by these methods, we additionally used a sodium acetate test (*20*) to establish the position of the methoxyl group on the A-ring. A hydroxyl group at position 7 is required to produce a bathochromic shift in 0.5 M sodium acetate (**Figure 2**). Similar to previous findings (*2, 21*), sorghum pigments were exclusively 3-DXA and their derivatives. HPLC-MS analysis of the crude extracts showed that the red sorghum pigments were mostly luteolinidin (43%) and apigeninidin (41%), with their 7-methoxylated forms accounting for 7 and 6%, respectively, of the total pigments (**Figure 2**). The composition of black sorghum pigments, on the other hand, was more complex; 5-glycosides of both nonmethoxylated and 7-methoxylated forms of luteolinidin and apigeninidin were present (**Figure 2**), but these were not detected in the red sorghum extract. Another notable difference in composition was that in the black sorghum pigments, the 7-methoxylated derivatives of apigeninidin and luteolinidin constituted almost half (49%) of the total pigments, whereas they were only 13% of total pigments in the red sorghum extract.

The crude extracts of all three sorghum varieties showed NQO inducer activity (**Figure 3**). However, the black sorghum extract showed a much stronger NQO specific inducer capacity, doubling the enzyme activity at 5 μg/mL concentration and increasing the activity maximally by 3.0 times at a concentration of 50 μg/mL. Surprisingly, the red sorghum extract, which contained 29.2 mg/g 3-DXA (**Table 1**), had an NQO inducer

Table 1. Phenolic Content and Free Radical Scavenging Capacity of the Freeze-Dried Sorghum Extracts

extract	3-DXA pigment[a] (mg of LE/g)	Folin–Ciocalteu phenols[b] (mg of GAE/g)	ORAC[c] (μmol TE/g)	ABTS[d] (μmol TE/g)
black sorghum	75.0 ± 1.3	92.2 ± 4.3	2277 ± 207	2680 ± 170
red sorghum	29.2 ± 0.2	81.5 ± 3.2	2049 ± 198	1060 ± 72
white sorghum	0.86 ± 0.31	48.3 ± 0.3	1467 ± 97	411 ± 42

[a] 3-Deoxyanthocyanins, luteolinidin equivalents (mean ± sd from three separate experiments). [b] Gallic acid equivalents (mean ± sd from three separate experiments). [c] Oxygen radical absorbance capacity, Trolox equivalents (mean ± sd from three separate experiments). [d] 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) antioxidant assay, Trolox equivalents (mean ± sd from three separate experiments).

capacity nearly identical to that of the white sorghum extract (0.86 mg/g 3-DXA), maximally increasing the enzyme activity by 60% at 200 μg/mL. This led us to believe the high inducer capacity of the black sorghum was possibly due to its composition of 3-DXA (**Figure 2**) and not the content of 3-DXA per se (**Table 1**). It was also possible that other nonpigment components of the black sorghum produced the high inducer activity. However, when we washed the extract on a C-18 cartridge to remove most of the nonpigmented phenols, the high NQO inducer capacity of the pigment fraction was maintained. Also, the free radical scavenging capacity and phenol content (**Table 1**) could not account for the observed differences in inducer capacity. This suggests that the composition of the 3-DXA in the black sorghum extract was likely responsible for the high NQO inducer capacity.

Srivastava et al. (*15*) observed that natural anthocyanin extracts had no effect on NQO enzyme activity, and Uda et al. (*14*) proposed that a 2,3 double bond in the C-ring of flavonoids (see **Figure 1**) was essential for induction of NQO enzyme. This would make our findings somewhat unusual. The molecular orientation of the 3-DXA compounds in aqueous solution at the near-neutral pH used in this study may partly explain the results. Unlike anthocyanins that readily hydrate and primarily exist as hemiacetals (carbinol pseudobases) at these pH levels (*2*), the 3-DXA molecules generally have higher deprotonation constants (K_a) than hydration constants (K_h) and thus exist primarily in quinoidal forms at near-neutral pH (*1, 5*). Some of these quinoidal bases contain the 2,3 double bond in the C-ring (see ref *5*), which would satisfy the condition proposed by Uda et al. (*14*) as required for NQO activity. However, this cannot account for the fact that the red sorghum extract, which had a significant level of 3-DXA pigments, did not have any advantage over the nonpigmented white sorghum in terms of inducer capacity. This necessitated fractionation of the major 3-DXA compounds in the sorghum extracts to help gain insight on how structure affected NQO activity.

Effect of 3-DXA Structure on Their NQO Inducer Activity. A clear structure–activity relationship was obtained for the isolated 3-DXA molecules, with the important results represented in **Figures 4** and **5**. Interestingly, apigeninidin (**Figure 4**) and luteolinidin (**Figure 5**), the backbones of sorghum 3-DXA and the major components of the red sorghum pigments (**Figure 2**), did not show any significant NQO inducer activity. Their glycosides found in black sorghum similarly did not show any inducer activity (data not shown). This indicates that the quinoidal species, the primary form of these molecules at neutral pH, were not in themselves responsible for the apparent inducer activity observed in the crude extracts. The 7-methoxylated forms of these compounds, on the other hand, were strong NQO inducers (**Figures 4** and **5**). 7-Methoxyapigeninidin induced NQO maximally (2.4 times) at 50 μM (**Figure 4**), similar to 7-methoxyluteolinidin, which showed maximum NQO inducer activity (2.5 times) at 50 μM (**Figure 5**). Even though we have purified only limited quantities of the 7-methoxylated glycosides

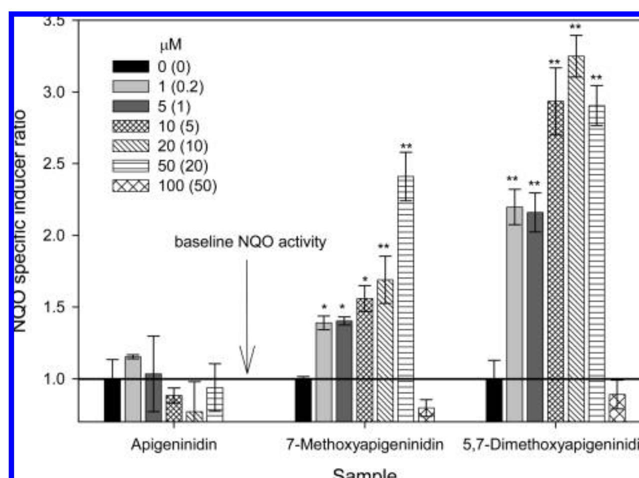

Figure 4. NAD(P)H:quinone oxidoreductase specific inducer capacity of apigeninidin and its methoxylated derivative found in black sorghum compared to synthetic dimethoxyapigeninidin. Hepa1c1c7 murine hepatoma cell lines were used for the assay. Cells (10000/well) were induced with extracts for 24 h before assay. Error bars represent ± sd from 3three separate experiments. *, $P < 0.05$; **,$P < 0.001$, compared with control (Bonferroni multiple-comparison test). Sample concentrations of the synthetic 5,7-dimethoxyapigeninidin are given in parentheses.

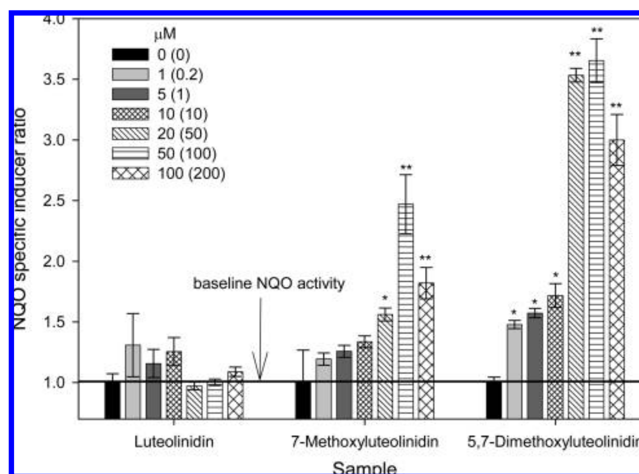

Figure 5. NAD(P)H:quinone oxidoreductase specific inducer capacity of luteolinidin and its methoxylated derivative found in black sorghum compared to synthetic dimethoxyluteolinidin. Hepa1c1c7 murine hepatoma cell lines were used for the assay. Cells (10000/well) were induced with extracts for 24 h before assay. Error bars represent ±sd from three separate experiments. *, $P < 0.05$; **, $P < 0.001$, compared with control (Bonferroni multiple-comparison test). Sample concentrations of the synthetic 5,7-dimethoxyluteolinidin are given in parentheses.

of luteolinidin and apigeninidin, preliminary evidence (not enough replications to report) showed that they also had significant NQO inducer activity. Our findings indicate that methoxylation on the A-ring is a prerequisite for 3-DXA inducer

activity. This is especially significant given that anthocyanins (e.g., malvidin), unlike the 3-DXA, are normally methoxylated on the B-ring. The results seem to explain the large difference in inducer capacity of the crude black sorghum extract compared to the red sorghum extract (**Figure 3**) and why the red sorghum extract had a nearly identical inducer capacity as the white sorghum extract. The active 7-methoxyl derivatives of 3-DXA comprised about 50% of black sorghum pigments, whereas they constituted only 13% of the red sorghum extract. The marginal NQO inducer capacity of the red and white sorghum extracts was likely due to other non-3-DXA components in the extracts. This finding may be important in the selection and breeding of sorghum varieties that produce larger quantities of the active methoxyl derivatives of the 3-DXA. It may also be important in selecting cultural practices that will maximize accumulation of desirable molecules as well as designing processing technologies that optimize their bioavailability and bioactivity.

To determine if methoxylation at other positions on the A-ring also produced inducer activity, we tested a synthetic apigeninidin standard methoxylated at position 5 (the natural sorghum 3-DXAs we isolated were methoxylated at only position 7). This molecule also showed NQO inducer activity nearly similar to that of the natural 7-methoxyapigeninidin (2.1 times at 50 μM), suggesting that the presence of the methoxyl group and not its position was critical to NQO inducer activity.

Because the presence of one methoxyl group on the 3-DXA molecules produced such a significant effect on NQO enzymes, we were further interested in whether double-methoxylation on the A-ring would result in enhanced activity. However, we did not detect any dimethoxylated forms of the 3-DXA in the sorghum extracts. Hence, we tested synthetic 5,7-dimethoxylated analogues of apigeninidin and luteolinidin (AlsaChim, Strasbourg, France). Both synthetic 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin showed stronger NQO inducer capacity (maximum capacity of 3.2 and 3.7 times, respectively) than their natural monomethoxylated derivatives (**Figures 4** and **5**). However, the dimethoxyapigeninidin was more potent, maximally inducing NQO activity at 5−20 μM, compared to dimethoxyluteolinidin, which maximally induced NQO at 50−100 μM. In comparison, sulforaphane, the well-studied powerful NQO inducer we used as a control, showed a maximum NQO inducer activity of 2.6 times at 2.0 μM under the conditions used in this study (data not shown).

Efficacy of natural flavonoids depends not only on how easily they can pass across cell membrane but also on whether they can access target sites within the cell and interact with relevant enzymes and other cellular components. Thus, structural conformation as well as partition coefficient (lipophilicity) of a molecule will affect its bioactivity (*22*). Our findings show a clear structure−activity relationship for the two families of 3-DXA, apigeninidin and luteolinidin. Substituting the −OH groups on the A-ring with $-OCH_3$ groups significantly increased inducer capacity of these compounds.

Structure−activity relationships have been studied for different structural conformations of anthocyanidins (*23*), flavones (*18*), flavans (*22*), and gallates (*25*), among others. Hou et al. (*23*) reported that increased hydroxylation on the B-ring enhanced activity of anthocyanins against various cellular mechanisms associated with cancer initiation and propagation. On the other hand, Uda et al. (*14*) demonstrated that the degree of hydroxylation on the B-ring of flavones inversely correlated with their capacity to induce NQO activity in Hepa1c1c7 cells. However, Harris et al. (*22*), in a detailed study of different classes of phenolics, demonstrated that the steric bulk of a molecule had an influence on how much lipophilicity and antioxidant capacity (related to number of hydroxyl groups) of the molecule affected its bioactivity. Thus, structure−activity relationships observed within a group of relatively similar molecules may not be universally applicable in predicting bioactivity of phenols. In the 3-DXA, the $-OCH_3$ substitution not only increases their partition coefficient (as evidenced by increased retention time on C-18 column) but also influences their steric bulk. Hence, more detailed studies will be required to establish the mechanisms behind the observed properties.

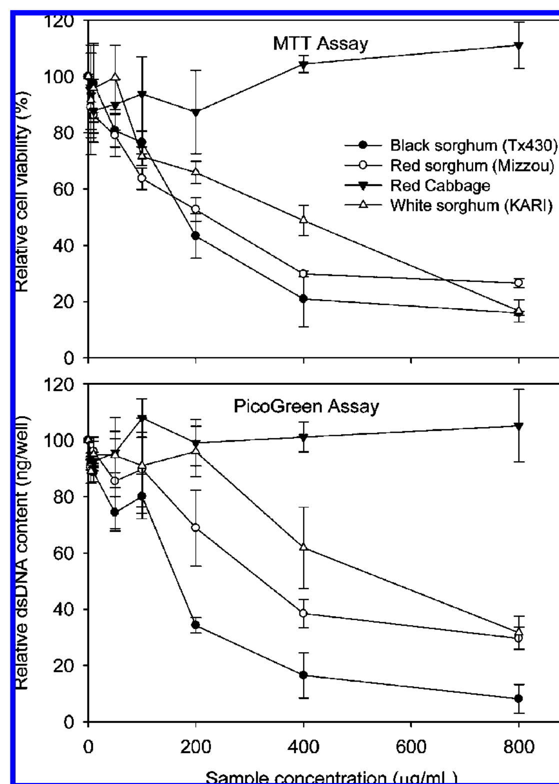

Figure 6. Inhibition of HT-29 colon carcinoma cell growth by various sorghum extracts compared to commercial red cabbage extract. MTT and PicoGreen assays were used to estimate viable cell population. Cells were incubated with samples for 48 h before assay. Error bars represent ±sd from three separate experiments.

Antiproliferative Activity of the 3-DXA against HT-29 Human Colon Cancer Cells. Elimination of tumors in early stages is considered to be an integral part of chemoprevention. We were thus also interested in how the sorghum extracts would inhibit proliferation of gastrointestinal epithelial cancer cells. Because we previously demonstrated that the black sorghum pigments are chemically very stable and have a good potential as natural food colorant (*2*), we compared the potency of the sorghum extracts against that of a commercially available natural anthocyanin colorant from red cabbage. This was important given that Shih et al. (*7*) recently demonstrated that the two primary 3-DXA from sorghum, apigeninidin and luteolinidin, were more potent against various human cancer cell lines compared to their more common anthocyanidin analogues, pelargonidin and cyanidin. All of the sorghum extracts showed relatively strong antiproliferative activity against the HT-29 colon cancer cells compared to the red cabbage pigment extract after 48 h of incubation (**Figure 6**). Black sorghum extract generally showed stronger antiproliferative activity ($P < 0.001$) than the red and white sorghum extracts. The IC_{50} for the black sorghum extract was 180 μg/mL (MTT assay) and 167 μg/mL (PicoGreen assay); the red sorghum extract had IC_{50} of 236 μg/

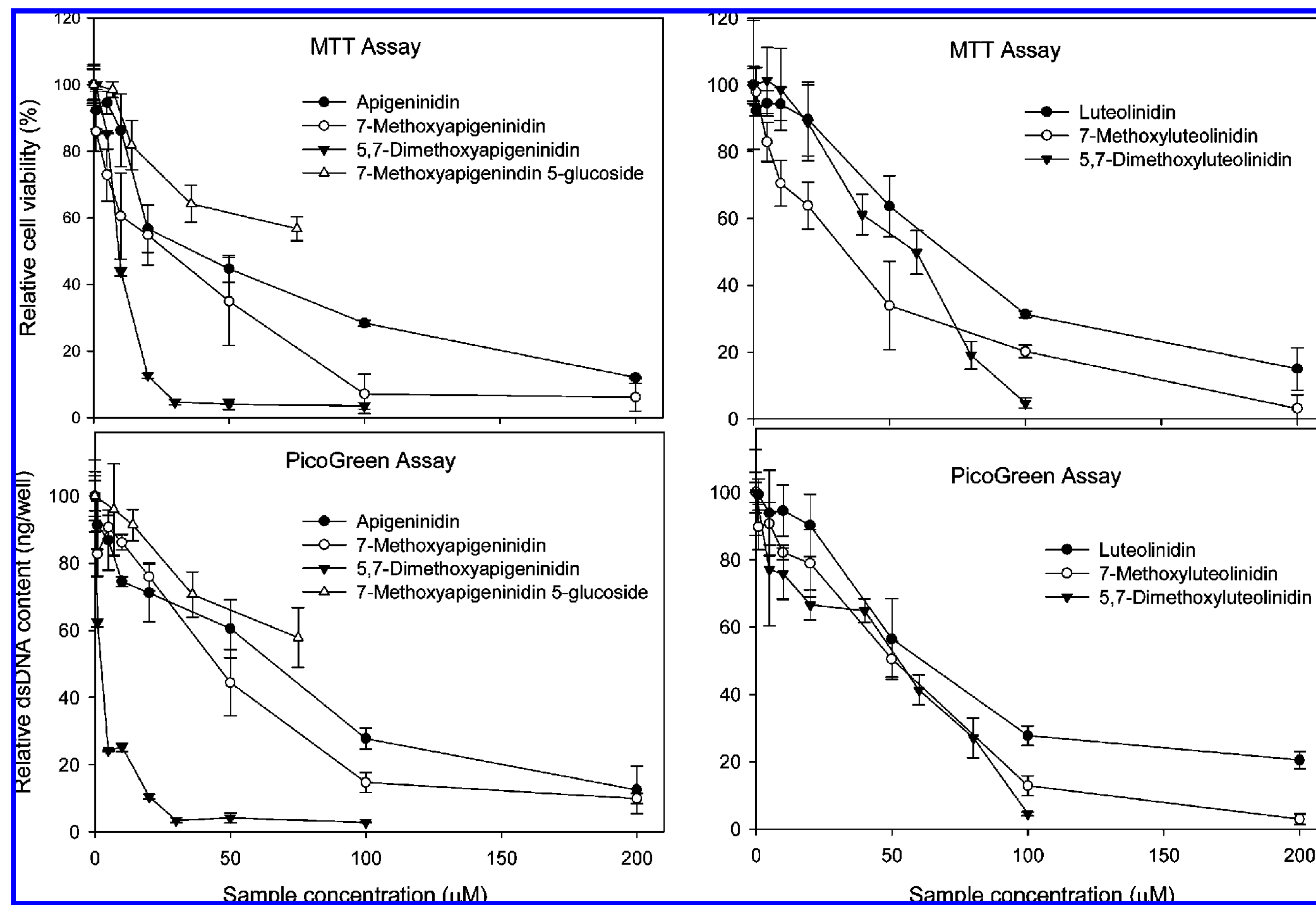

Figure 7. Inhibition of HT-29 colon carcinoma cell growth by various 3-deoxyanthocyanidin compounds. MTT and PicoGreen assays were used to estimate viable cell population. Cells were incubated with samples for 48 h before assay. Error bars represent ±sd from three separate experiments.

mL (MTT) and 326 μg/mL (PicoGreen), whereas the white sorghum extract had IC_{50} of 403 μg/mL (MTT) and 557 μg/mL (PicoGreen) at 48 h. The red cabbage extract did not have any antiproliferative activity at the concentrations tested.

Yi et al. (*26*) in a comparable study (48 h of incubation) observed that crude extracts from various grapes had IC_{50} of 1000−7000 μg/ mL against the HT-29 cell line by the MTT assay. Similarly, Netzel et al. (*27*) also reported that black carrot anthocyanins had IC_{50} > 1000 μg/mL against HT-29 cell proliferation using the MTT assay (48 h of incubation). Our findings partly confirm the data by Shih et al. (*7*) that the 3-DXA have stronger antiproliferative activity than their natural anthocyanin analogues commonly found in other food plants. Additionally, most of the nonpigmented sorghum flavonoids are 3-deoxy in nature (have no −OH substitution at position 3 on the A-ring) (see the review in ref *6*); this may help to explain why the white sorghum devoid of 3-DXA showed such relatively strong antiproliferative activity. Similar evidence for increased potency of 3-deoxyflavans compared to flavans against various cancer cell lines was recently reported by Li et al. (*24*).

With these interesting findings, we again set to establish how the structure of the 3-DXA affected their antiproliferative activity. All isolated natural 3-DXA compounds showed strong activity against HT-29 cell proliferation, with IC_{50} at 48 h of incubation of between 29.0 and 40.3 μM (MTT) and between 46.5 and 66.1 μM (PicoGreen) (**Figure 7**). For comparison, genistein used as positive control had IC_{50} values of 78.0 μM (MTT) and 61.6 μM (PicoGreen) under conditions used in this study (data not shown). Antiproliferation activity of apigeninidin and luteolinidin was apparently increased by methoxylation at position 7. We did not purify enough glycosides for complete antiproliferation tests, but on the basis of the individual glycoside tested (7-methoxyapigeninidin 5-glucoside), glycosylation appears to reduce the potency of the 3-DXA (**Figure 7**), which agrees with the findings of Meiers et al. (*28*), who reported that glycosides of anthocyanins were less potent against tumor growth than their aglycons. Dimethoxylation had a major effect on the potency of apigeninidin, with the synthetic 5,7-dimethoxyapigeninidin showing IC_{50}values of 9.3 μM (MTT) and 1.4 μM (PicoGreen), and nearly complete inhibition of cell growth at 30 μM. On the other hand, dimethoxylated luteolinidin had relatively high IC_{50} compared to the monomethoxylated analogue, but showed nearly complete inhibition of cell growth at 100 μM.

Various authors have attempted to define structure−activity relationships of anthocyanins against cancer initiation and progression (*23, 28, 29*). Substitution on the B-ring (hydroxylation pattern and/or methoxyl substitution), as mentioned earlier, was shown to significantly affect potency and mode of action of anthocyanins against cancer cell proliferation (*28, 29*). For example, Meiers et al. (*28*) demonstrated that the increased hydroxylation on the B-ring increased the antiproliferaton capacity of anthocyanidins against human tumor cells, with the most hydroxylated anthocyanidin, delphinidin, showing the strongest activity. Marko et al. (*29*) also reported that delphinidin (3-OH on the B-ring) was more potent against HT-29 cells than cyanidin (2-OH on the B-ring), which was in turn more potent than pelargonidin (1-OH on the B-ring).

On the basis of hydroxylation on the B-ring, our findings actually seem to indicate the opposite observation; that is, the

apigeninidin family of molecules (1-OH on B-ring) tended to have higher potency against HT-29 cell proliferation than their luteolinidin (2-OH on the B-ring) analogues; this observation was especially obvious in the dimethoxylated derivatives (**Figure 7**). Even though anthocyanins and 3-DXA belong to the same family of compounds, as previously discussed, due to their different hydration constants, their molecular orientation in near-neutral solutions is very different. This obviously produces different steric effects in the molecules that would be expected to influence their bioactivity patterns. For example, many authors have reported that increased hydroxylation is detrimental to the bioactivity of various molecules, whereas methoxylation is beneficial (*24, 25, 30*). Zheng et al. (*30*) reported that hydroxylating the triphenyl cancer drug, Tamoxifen, significantly reduced its potency against various cancer cell lines in vitro and that addition of methoxyl groups on the phenyl rings of the hydroxylated molecule reactivated their potency beyond that of the parent Tamoxifen. Li et al. (*24*) also observed that substitution of hydroxyl with methoxyl groups on the A-ring of 3-deoxyflavans considerably increased their potency against cancer cell growth.

Xiao et al. (*25*) studied structurally related gallic acid derivatives and observed that the compounds with the galloyl group fully substituted with methoxyl and benzoyl groups had increased potency against HepG2 cancer cells. They attributed the increased potency to the increased lipophilic nature of the substituted compounds, which made them permeate the cell membrane more easily. Indeed, when we consider each family of compounds (apigeninidin and luteolinidin) in our study, increased potency for both NQO enzyme activation and HT-29 cell growth inhibition tended to correspond to a decrease in hydrophilicity. On the other hand, Sergeev et al. (*31*) demonstrated that partial hydroxylation of the highly methoxylated and lipophilic polymethoxyflavones (PMF) found in orange peel oil significantly increased the rate at which they inhibited cancer cell growth (higher inhibition effect than fully methoxylated PMF after 3 days, but not after 6 days, of incubation). These different findings point to the fact that both steric bulk and partition coefficient interact to influence the bioactivity of molecules and that molecules with biphasic properties may offer the best promise as chemoprotective agents.

It is apparent that a combination of polarity and structural conformation plays a significant role in how the 3-DXA molecules interact with cells to effect chemoprotection. Methoxyl substitution on these molecules not only affords them significantly enhanced activity against HT-29 colon cancer cell proliferation but also transforms them to NQO inducer molecules. Thus, the natural black sorghum pigment extract that contained almost 50% methoxylated 3-DXA derivatives showed extraordinary capacity both to inhibit HT-29 cell growth and to enhance the chemoprotective NQO enzyme activity. The synthetic dimethoxylated 3-DXA molecules displayed even increased activity and are worth investigating further as potential therapeutic agents. Given that the 3-DXA are generally stable to food processing and handling conditions (*1, 4*), their chemoprotective potential may likely be retained when they are used in food products. These preliminary findings are very important in providing a basis for explaining the previous epidemiological evidence that linked sorghum consumption with reduced incidence of GIT cancer. We are currently proceeding with studies that will provide better understanding of how these compounds interact with each other and with other nonpigment molecules from sorghum to effect chemoprevention using a variety of cell lines derived from the human gastrointestinal tract. Specific cellular mechanisms involved as well as membrane permeability of these compounds are also being investigated.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; 3-DXA, 3-deoxyanthocyanins; NQO, NAD(P)H:quinone oxidoreductase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PMF, polymethoxyflavones.

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