

**CHEMOPREVENTIVE POTENTIAL OF SORGHUM WITH DIFFERENT  
PHENOLIC PROFILES**

A Thesis

by

LIYI YANG

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Food Science and Technology

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## ABSTRACT

Chemopreventive Potential of Sorghum with Different Phenolic Profiles.

(December 2009)

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Epidemiological evidence has correlated consumption of sorghum with reduced incidences of gastrointestinal (GI) tract cancer, especially esophageal cancer. There is little evidence on how phenols of sorghum may affect chemoprevention. Seventeen sorghum varieties were screened for phenolic profiles and antioxidant capacity. The ability of crude sorghum extracts to induce NAD(P)H:quinone oxidoreductase (QR, a phase II protective enzyme), and inhibit proliferation of colon (HT-29) and esophageal (OE33) carcinoma cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and PicoGreen assays, were determined *in vitro*. 3-Deoxyanthocyanidins, apigeninidin, luteolinidin and their methoxylated derivatives were also investigated for antioxidant capacity, QR inducing and antiproliferative potential.

Tannin sorghum generally showed higher antioxidant capacity than non-tannin sorghum varieties. Sorghum varieties containing extractable condensed tannins did not show any significant QR inducing potential; on the other hand, non-tannin sorghums increased QR activity by 1.5-2.7 times; black sorghum (Tx430) was most potent (doubled QR activity at 25  $\mu\text{g}/\text{mL}$ , 2.7-fold increase at 100  $\mu\text{g}/\text{mL}$ ). All sorghum

extracts showed relatively strong antiproliferation activity with  $IC_{50}$ s (the concentration needed to inhibit cancer cell growth by 50%) of 49.7-883  $\mu\text{g/mL}$ . Tannin-containing sorghums had stronger cancer cell proliferation inhibitory potential ( $IC_{50}$ s 49.7-131  $\mu\text{g/mL}$ ) than non-tannin sorghums ( $IC_{50}$ s 141-883  $\mu\text{g/mL}$ ). Total phenol content and antioxidant capacity of crude sorghum extracts positively correlated with their antiproliferative potential ( $r^2$  0.71-0.97).

Among tested 3-deoxyanthocyanidins, methoxylation on A-ring improved QR inducing potency. 5,7-Dimethoxyluteolinidin had the greatest QR inducing potency (4.3-fold at 100  $\mu\text{M}$ ). Methoxylation also improved their antiproliferation potential; the  $IC_{50}$ s trend was di-methoxylated (8.3-105  $\mu\text{M}$ ) > mono-methoxylated (40.1-192  $\mu\text{M}$ ) > apigeninidin and luteolinidin (81.5-284  $\mu\text{M}$ ).

This study provides information for how phenolic compositions of sorghum and 3-deoxyanthocyanidin structure affect their capacity to induce QR activity and inhibit GI tract cancer cell proliferation. The information is useful to promote the utilization of sorghum in functional foods, beverages, dietary supplements, and other health-related industries. Further study will focus on, fractioned and isolated sorghum phenols, the effect of food processing on their chemopreventive potential, as well as cellular mechanisms involved.

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## CHAPTER I

### INTRODUCTION

Cancer is the second leading cause of death in the US and 1.4 million new cases of cancer were expected to be diagnosed in 2008 (1). Eighty-nine billion dollars was spent on direct cancer-related medical care in 2007 in the US (2). Most cancer incidence (90-95%) is related to diet, lifestyle and environment (3). Hence effective use of dietary components to reduce the risk of developing cancer is desirable.

Diets rich in fruits and vegetables reduce the risk of cancer in various sites (4-7). The plausible mechanism is not clear but is possibly related to intake of fiber, vitamins, minerals and dietary antioxidants (8). Consumption of whole grains is also associated with lower incidence of cancer, especially digestive system cancers. Complex carbohydrates, vitamins, minerals and phenolic compounds in whole grain are partly responsible (9-13).

Epidemiological studies have shown that a staple diet of sorghum correlates with low incidence of esophageal cancer in areas where this type of cancer is endemic (14-18). However, how sorghum contributes to this chemoprevention and which components of sorghum are responsible is unclear. Sorghum has a unique profile of polyphenols, such as condensed tannins, flavones and 3-deoxyanthocyanins, not commonly found

in wheat, corn, rice, and other cereals. These polyphenols may be responsible for the chemopreventive properties of sorghum. For example, 3-deoxyanthocyanins were recently reported as stronger cancer cell proliferation inhibitors than their anthocyanin analogs (19). The unique compounds in sorghum should be evaluated for their chemoprevention potential.

Carcinogenesis generally involves three main steps: initiation, promotion and progression (20). Chemopreventive agents could target any of these three steps, such as blocking activation of carcinogens, activating transcription factors that regulate the expression of protective enzymes, and suppressing cancer promotion and/or progression (21). Thus dietary compounds that have such properties may be important in chemoprevention. For example, dietary antioxidants could prevent or block the insults from carcinogens and oxidative stresses *in vitro*. Dietary compounds that induce phase II detoxifying enzymes could directly protect normal cells by detoxifying potential carcinogens. Additionally, the capacity of food components to directly inhibit cancer cell proliferation may indicate their ability to suppress preformed cancer promotion and progression.

This study aims to evaluate the primary chemopreventive effects of different types of sorghums and how their phenol profile affects this potential via *in vitro* cell culture studies. This will allow for selection of sorghum varieties with the best potential for chemoprevention for further studies of the molecular mechanisms involved, as well as effect of food processing conditions on activity and bioavailability of the active components of sorghum.

The objectives of this study were to:

1. Compare the capacity of different varieties of sorghum to induce phase II enzyme activity *in vitro*;
2. Compare the capacity of different varieties of sorghum to inhibit colon and esophageal cancer cell proliferation *in vitro*;
3. Determine the effect of sorghum 3-deoxyanthocyanidin structure on phase II enzyme activity and GIT cancer cell growth inhibition *in vitro*.

## CHAPTER II

### LITERATURE REVIEW

#### **The Prevalence of Cancer**

North America has the highest prevalence of cancer, with 1.5% of the population (over 3.2 million individuals) affected during the previous 5 years (22). Western Europe, Australia and New Zealand have similar percentages of affected population (1.2% and 1.1%, respectively), while Japan and Eastern Europe have 1.0% and 0.7%, respectively, followed by Latin America and the Caribbean (0.4%), and other regions (0.2%) (22). Developed countries generally have similar prevalence in men and women; while in developing countries, the prevalence in men is 25% greater than in women. This reflects a preponderance of cancer sites with poor survival in males, such as liver, esophagus and stomach in developing countries (22). Overall, in 2006, there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer in the world. The most common causes of cancer deaths worldwide are lung cancer (1.18 million deaths in 2006), stomach cancer (700,000), and liver cancer (598,000) (23).

In the US, cancer is the second leading cause of death (after cardiovascular disease) (1). It is a growing burden on public health: approximately one in two men and one in three women in the US will develop cancer during their lifetime. Nearly 294,120 men and 271,530 women will die of cancer in the US in 2008 (1) and 1.44 million new cases of cancer will be diagnosed in 2008 (1).

On a worldwide basis, gastrointestinal tract (GIT) cancers account for approximately 20% of all cancers (24) and are a major cause of death in cancer patients (25). The incidence of certain gastrointestinal cancers, such as esophageal adenocarcinoma, liver and intrahepatic bile duct cancer, increases yearly (24). In general, rates of incidence of colorectal cancer are increasing rapidly in countries where overall risk was formerly low (especially in Japan, but also elsewhere in Asia), while in high risk countries, trends are either gradually increasing, stabilizing (North and West Europe), or declining with time (North America) (26). Among US men and women colorectal cancer is the most prevalent gastrointestinal tract cancer, accounting for 8-9% of total cancer deaths. Pancreatic, liver and intrahepatic bile duct cancer, and esophageal cancer accounted for about 6%, 4%, and 4% of total cancer deaths, respectively (1). While the incidence of colorectal cancer has decreased since the last decade, the incidence of esophageal, and liver and intrahepatic bile duct cancer is still increasing (1). Moreover, while colorectal cancer and liver and intrahepatic bile duct cancer similarly affect males and females, esophageal cancer mainly affects males. African Americans are at higher risk of GIT cancer than any other ethnic groups (1).

The cost of cancer is one of the most important contributors of health care expenditures in the US. In 2007, \$219 billion was spent on overall annual costs of cancer, with \$89 billion direct medical care; \$18 and \$ 112 billion on costs of lost productivity due to illness and premature death, respectively (2). Since direct medical treatments, for example surgery, chemo- and radiation therapy or the combination of

single treatments, for cancer are hardly affordable for cancer patients, effective ways to prevent cancer or at least, to reduce the risk of cancer are needed.

### **Causes of Cancer**

Cancer is a group of diseases involving cells that have defects in regulatory circuits, which governs normal cell proliferation and homeostasis. More than 100 distinct types of cancer and subtype tumors are found in specific organs (27). Cancer cells have six common characteristics: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Due to the malignant growth, cancer cells form a microenvironment to suffice their own growth needs and nutrient supply, which impairs the normal metabolism of the specific tissue where the tumor locates (27).

Carcinogenesis is a multistep and complex process. In an oversimplified way, carcinogenesis is divided into initiation, promotion and progression. Initiation is the damage of genetic materials: DNA or RNA, which leads to unregulated cell growth. This step is rapid, reversible and affected by both intracellular (inherited genes, oxidative stress, inflammation, hormones) and extracellular (environmental including exposure to carcinogens, diet and lifestyle) factors. A single malignant cell reproduces itself to form more malignant cells in the process called promotion. This process is reversible and slow, sometimes taking up to 10 years (20). Finally, in the process of progression,



preneoplastic cells undergo neoplastic transformation, which forms a tumor with invasive and metastasis potential (20).

Leading causes of cancer in general include smoking, alcohol abuse, physical inactivity, malnutrition, chronic infection, over-exposure to sunlight, and exposure to occupational or environmental carcinogens (28). Causes of gastrointestinal tract cancer are tightly associated with dietary components, long-term/persistent inflammation in the gut and environmental risk factors, which possibly lead to oxidative damage of the epithelial cells in the gastrointestinal tract (25).

Smoking and alcohol abuse account for 75% of oral cancer occurrence in USA men. They are the two most important risk factors for oral cancer in developed countries (29). Alcohol consumption has been identified as a risk factor for esophagus, gastric cancer and colorectal cancer (30). Increased consumption of red and processed meats initiate oxidative damage to normal cells, activate carcinogenic compounds, or increase risk of cancer in the distal portion of the large intestine (31-33).

Increased incidence of cancer in the gastrointestinal tract has been associated with exposure to high fat diet induced bile acids (34). The primary bile acids are synthesized from cholesterol in the liver, and mainly contain cholic and chenodeoxycholic acid (35). Deoxycholate and other hydrophobic bile acids, which are induced by high fat diets, generate reactive oxygen/nitrogen species (ROS/RNS) (36). ROS/RNS increase the oxidative stress *in vivo*, which causes damage to DNA and eventually DNA mutation (37).

Major chronic inflammation of the GI tract is linked to *Helicobacter pylori* (*H. pylori*) infection, and/or inflammatory diseases such as celiac disease, inflammatory bowel disease, chronic pancreatitis, primary sclerosing cholangitis, ulcerative colitis, and Crohn's disease (25). Infection of viruses (mainly hepatitis B and C viruses, some Epstein–Barr virus and the oncogenic JC virus) also may cause chronic inflammation (38). Though it is still not clear how and why chronic inflammation triggers carcinogenesis, it has been proposed that ROS generated from inflamed epithelia causes oxidative damage to DNA (39). Normally, the gut epithelia are uniquely structured to manage interaction with a wide range of stimuli. However, repeated and/or over stimulation by diet and from the gut environment leads to malfunctioned responses of gut epithelia. Chronic inflammation begins with a trigger (dietary or environmental) acting on the epithelium to invoke an inflammatory response. This results in the activation of a number of different inflammatory cells and the generation of oxidative stress. Then the existing inflammation may lead to genetic and/or epigenetic alterations. Though repair and regeneration could help on a limited basis at early stages, over a longer term, the damage to DNA may result in cellular dysplasia, which finally leads to carcinogenesis (25).

In the “cancer belt” extending from Northeastern China through Central Asia into Northern Iran and Eastern Turkey (40) where upper gastrointestinal cancers are endemic, poor socioeconomical conditions, barren lands, heavy metals, and soil contaminated by industrial toxins are major environmental risk factors for these specific cancers (41).

High prevalence of gastrointestinal cancers has been observed in areas where heavy metals and radioactive elements are ubiquitous (42, 43).

### **Principles of Chemoprevention**

The concept of chemoprevention entails protective actions, including retarding, blocking or even reversing carcinogenesis (44). The mechanisms of cancer prevention target any of the six physiological changes required for development of a tumor: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (27). Wattenberg (21) proposed that cancer chemopreventive compounds could be divided into two basic categories, cancer initiation blockers and cancer promotion suppressors.

Blocking the insult that causes damage to DNA is a major approach to preventing cancer initiation. Cancer initiation blockers can prevent the formation of carcinogens from precursor substances (21). The blockers, which are mainly antioxidants, can scavenge reactive ROS/RNS species or environmental carcinogens, which in turn, prevent potential damage from free radicals to cells (45). The cancer initiation blockers can also stimulate molecular pathways, such as Nrf2 (nuclear factor-erythroid 2p45 (NF-E2)-related factor 2) to activate the production of protective phase II enzymes, such as acetyltransferases, glutathione-S-transferases (GSTs), UDP-glucuronyltransferases, NAD(P)H:quinone oxidoreductase and sulphotransferases (45). The phase II enzyme

induction system is responsible for the removal of a diverse array of electrophilic and oxidative toxicants.

Phase II enzymes, which catalyze conjugation reactions producing molecules that can be excreted in bile or urine, can detoxify xenobiotic chemicals, including carcinogens (20). Dietary antioxidants perform their protective effects not only by scavenging ROS, but also by inducing *de novo* expression of genes that encode detoxifying/defensive proteins, including phase II enzymes (44). These xenobiotics-activated stress response genes contain a common *cis*-element, known as antioxidant response elements (AREs). Basic leucine zipper (bZIP) transcription factors — including NRF (NF-E2 related factor)— bind to these ARE sequences and modulate expression of stress-response genes. NRF2-KEAP1 (Kelch-like ECH-associated protein 1, a negative regulator of NRF2) complex pathway is the one that most commonly responds to dietary phytochemical stimulation (46).

The cancer promotion suppressors can activate cellular pathways to over express signaling molecules to induce apoptosis in abnormal cells. They can repair the damaged genetic materials to terminate the malignant cell growth (45). Among the intracellular signaling pathways related to regulation of cell proliferation and differentiation, mitogen-activated protein kinases (MAPKs), which belongs to the family of proline-directed serine/threonine kinases, is one of the central cellular signaling systems maintaining *in vivo* homeostasis (47).

Dietary phytochemicals targeting different signaling compounds in MAPKs, can activate or silence part of the signaling system; hence they can prevent abnormal cell

proliferation. Dietary phytochemicals can also target other cell-signaling kinases such as protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) (48). These upstream kinases activate a distinct set of transcription factors, including nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein 1 (AP1) (48). Aberrant activation or overexpression of NF- $\kappa$ B has been linked to resistance to apoptosis, stimulation of proliferation and neoplastic transformation in cells. Similarly, functional activation of AP1 also results in malignant transformation and tumor promotion. A vast group of phytochemicals target these two cellular-signalling cascades to suppress the gene expression associated with carcinogenesis (49, 50). Epigallocatechin gallate (EGCG), genistein, resveratrol, curcumin, [6]-gingerol and capsaicin have been suggested as involved in MAPKs and its downstream pathways (28).

### **Evidence of Chemoprevention from Dietary Substances**

It is believed that 90-95% of cancer is related to our diet and environment, only 5-10% is genetic (3). More than adequate intake of red meat, processed meat (31), saturated fat, fried foods, or salt-preserved foods, inadequate intake of vegetables and fruits (28), and abuse of alcohol (29) are risk factors of cancer. On the other hand, consumption of green tea, colored vegetables and fruits, and whole grains reduces risk of some types of cancer (51, 52).

Increased intake of fruits and vegetables has been associated with lower occurrence of lung, stomach and colon cancer (4, 5). In a large nutrition cohort study performed in the US from 1992 to 1997, a 30% reduction in colon cancer risk was

observed in men with the highest vegetable intakes (>3.3 servings) per day (6). The study also suggested that very low intakes of plant foods may increase colon cancer risk. High intakes of glucosinolates, indoles,  $\beta$ -carotene, lutein, lycopene and vitamin C phytochemical subgroups may decrease colon cancer risk.

Mediterranean diets high in fruit, cereals, olive oil as the main source of fat, and relatively low in meat and dairy products (53, 54) have been linked with lower incidence of GIT cancer (55). De Lorgeril and others (56) found a 61% reduction of all-site cancer risks in French coronary heart disease patients consuming a Mediterranean diet. Gallus and others (57) identified consumption of fruits and vegetables, fish, wholegrain foods and olive oil in a typical Mediterranean diet as potential protective factors against cancer, especially digestive tract cancer. A case control study in Italy by la Vecchia and others (58) showed that consumption of olive oil and other unsaturated oils and fish were preferable over saturated oil, red meat and refined carbohydrates in terms of lowering the risk of epithelial cell cancers. Dietary antioxidants such as  $\beta$ -carotene, vitamin E and minerals, such as calcium, showed an inverse relationship with breast cancer risk. Bosetti et al. (59) investigated the diversity of the components of Mediterranean diet and upper gastrointestinal tract cancer risk in three epidemiological studies in Italy. They reported a lower risk of upper aerodigestive tract cancers in people whose diet contained at least six of the components in a typical Mediterranean diet related to those that contained less than three.

An epidemiological study revealed that drinking over 10 Japanese-size cups (about 80–120 ml) of green tea per day delayed the average age of cancer onset in Japan.

The recurrence of human breast cancer was also delayed with increased consumption of green tea (60-62). Experiments performed on tumorigenesis in APC mice showed that green tea polyphenol extracts, such as epigallocatechin gallate (EGCG), decreased proliferation and induced apoptosis in tumor cells (63).

### **Whole Grains and Chemoprevention**

Cereals are the edible seeds or grains of the grass family, *Gramineae*. The predominant cereals consumed around the world are wheat, rice, corn (maize), millets, oats, rye, sorghum and barley. Cereals provide mainly carbohydrate, protein, fiber as well as vitamins (mainly E and B family) and minerals (zinc, magnesium and selenium) in the diet (64). The anatomical structure of all cereals is similar: the grain is made up of three components, the endosperm, the germ and the bran. Though the endosperm constitutes almost 80% of the whole seed and provides protein and starch, the bran and the germ account for most vitamins, minerals and phytochemicals (65, 66). Whole grain products use all three parts in processing. The Food and Drug Administration requires products to be low-fat and contain at least 51% whole grains by weight, to make the “whole grain” claim on the label (67).

Epidemiological studies reveal a strong protective effect of whole grain food against GIT cancers (10). Jacobs and co-workers (10) concluded that intake of whole grain foods consistently reduced the risk of oral cavity and pharynx, oesophagus, stomach, colon, rectum and liver neoplasms by 30–70 %. A cohort study among 34,651 postmenopausal women showed that intake of a high amount of whole grains (7 servings

per day) and yellow/orange vegetables (0.5 serving per day) was inversely related to risk of overall upper aerodigestive cancers, such as oropharyngeal, laryngeal, nasopharyngeal/salivary, esophageal and gastric cancers (12). A case-control study in Switzerland found that consumption of whole grain cereals correlated with decreased risk of cancer of the oral cavity and pharynx, esophagus and larynx. In contrast, refined grains were associated with higher risk of these cancers (68). A population-based cohort study among Swiss women concluded that high consumption of whole grain (>4.5 servings per day) could reduce the risk of colon cancer (69).

Whole grains may contribute to cancer prevention by stimulating a healthy gut environment, with dietary fiber providing antioxidants (66), potentially binding of carcinogens, modulating glycemic response, and producing hormone like effects (from phytoestrogens) (65).

Complex carbohydrates in grains may lower the risk of large bowel cancer (70). Insoluble non-starch polysaccharides, resistant starch, and oligosaccharides are large bowel laxatives, microflora fermentation substrates and prebiotics that promote gut health (70). Dietary fiber can be fermented in the gut, producing short-chain fatty acids (SCFA), which lower colonic pH. They also serve as an energy source for colonocytes and may alter blood lipids. These improvements in the gut environment may provide immune protection beyond the gut (71). Dietary fiber is also protective by diluting or adsorbing carcinogens (72), reducing colonic transit time, and altering bile acid metabolism (73).



An inverse relation between intake of dietary fibers and incidence of large bowel cancer was observed (7). It suggested that colorectal cancer risk could be reduced by 40% by increasing fiber consumption from 15 g to 30 g per day (7). McIntyre and others (74) found that rats fed with wheat bran and oat bran had a lower incidence of large bowel cancer than rats fed with non-fiber diets. Butyrate produced by gut microflora through fermenting fiber in bran was proposed as the preventive mechanism.

Various phytochemicals and minerals present in whole grains provide antioxidant benefits to the gastrointestinal tract epithelia. Important groups of phytochemicals found in whole grains are phenolics, carotenoids, vitamin E compounds, lignans,  $\beta$ -glucan, and inulin (75). Major antioxidant minerals in cereals are Zinc and Selenium (64). A 16-year follow up study in 34,708 postmenopausal women in Iowa indicated that zinc intake was negatively related to upper digestive tract cancer (76). Whole grain phytochemicals, such as ferulic acid and diferulates, complement those in fruits and vegetables. The antioxidant compounds in grains are mainly substituted benzoic and cinnamic acid compounds, caffeic and ferulic acid esters of long chain mono and dialcohols, tocopherols, and flavonoids (75). Most of these phytochemicals are bound to cell wall materials (75). Due to their insolubility, whole grain phytochemicals survive digestion in the upper gastrointestinal tract and reach the colon. Thus colonic microflora digestion may release them and make them available (77). For example, ferulic acid and diferulic acids from cereal bran can be released by gastrointestinal esterase from intestinal mucosa and microflora (78). Dietary fiber, condensed tannins and phytic acids may bind excessive iron and heavy metals in diet thus provide antioxidant benefits (65).

Phytoestrogens present in legumes, whole grains, fruits, and vegetables could induce detoxifying phase II enzymes hence provide chemopreventive effects. Jacobs and others (79) concluded that consuming whole grains in the diet regularly could increase serum enterolactone concentrations, which has been associated with reduced cancer risk (80). The elevated enterolactone concentrations in serum was mainly due to breakdown of the lignans contained in the whole grains (81). Wang et al. (82) determined the capacity of enterolactone to induce NAD(P)H:quinone oxidoreductase (QR) in human colon cancer cells and found a 6-fold induction of QR activity at 10.0  $\mu\text{M}$  of enterolactone.

Whole grains reduce glycemic index and glycemic loads while refined carbohydrates elevate them. A persistent high glycemic load from the diet causes abnormal glucose metabolism and probably dyslipidemia, which are risk factors for many chronic diseases (83, 84). A case-control study in Italian women showed an inverse association between glycemic index, and glycemic loads of the diet and colorectal cancer risk (85).

### **Chemoprevention Potential of Sorghum**

Sorghum (*Sorghum bicolor*) is the fifth most important cereal crop in the world (86). It is a staple food and important cereal in many parts of Africa, Asia, and the Middle East (65). Sorghum in these areas is mainly consumed in the forms of couscous, porridge, baked goods (bread, flat bread, cookie, and steam bread), and fermented drinks. Sorghum is an important alternative grain to use in gluten-free diets (87).

Sorghum is fairly resistant to pests and diseases, which is partly linked to its unique profile of phytochemicals (88).

In Africa and parts of Asia, regular consumption of sorghum and millets was associated with lower esophageal cancer incidence compared to areas where wheat and corn were the major cereals consumed (15, 18). Chen and others (17) reported a correlation between decreased incidence of esophageal cancer and consumption of sorghum and millet as opposed to corn and wheat in the diet in Shanxi province, China. Isaacson (16) proposed that the increase in esophageal cancer among South African blacks was due to their substitution of sorghum with corn as a staple diet. These observations suggested that sorghum and/or sorghum components have preventive properties against esophageal cancer that other cereal grains do not possess. It is possible that this beneficial effect could extend to other GIT cancers. However, information on the possible mechanisms of sorghum chemoprevention and the components of sorghum that contribute to this effect are limited.

Phenolic acids (89, 90) and flavonoids (3-deoxyanthocyanins (91, 92), flavones (93), flavanones (93), and condensed tannins (94)) have been found in sorghum. The total phenol contents in colored pericarp sorghums ranged from 6.4-19.8 mg gallic acid equivalent (GAE)/g (95). Total phenol content of brans from those sorghums ranged from 19.9-66.3 mg GAE/g (95). Total phenols in sorghum are higher than other common cereal grains, such as wheat (0.5-0.6 mg GAE/g), barley (0.9 mg GAE/g), millet (1.4 mg GAE/g), and rye (1.0 mg GAE/g) (96, 97).

Two classes of phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids, are the major phenolic acids in sorghums (98). Free phenolic acids are found in the outer layers of the kernel while bound phenolic acids are attached to the cell walls of the grain matrix. The ratio of free to bound phenolic acids range from 1:2-1:5, depending on the varieties (89).

Sorghum flavonoids include 3-deoxyanthocyanins (apigeninidin, luteolinidin and their derivatives) (91, 92), flavones (apigenin, luteolin) (99), flavanones (eriodictyol (100), naringenin (101)), flavonols (kaempferol 3-rutinoside-7-glucuronide (91), dihydroflavonols taxifolin (93), taxifolin 7-glucoside (93)), and flavan-4-ol compounds (luteoforol, apiforol) (102).

Based on appearance and total extractable phenols, sorghums can be classified as white (with no detectable tannins or anthocyanins and very low levels of phenols); red (with no tannins but significant levels of phenols and a red pericarp); black (with very high levels of 3-deoxyanthocyanins and a black pericarp) and tannin sorghums (with significant levels of tannins and different degrees of pericarp pigments) (86). The high pigment content in black pericarp sorghums were correlated with high antioxidant capacity (86).

The pigments from black pericarp sorghums, 3-deoxyanthocyanins, are unique compared to anthocyanins from other plant sources. The 3-deoxyanthocyanins lack a substitution at the C-3 position of the C-ring, which makes them more stable as colorants (103, 104), and more cytotoxic than their anthocyanin analogs (19). 3-Deoxyanthocyanins identified in sorghum mainly include apigeninidin, luteolinidin (86,

92, 105); their glucosides, apigeninidin-5-glucoside and luteolinidin-5-glucoside (91, 92, 105); acylated forms (106, 107); and methoxylated derivatives, 5-methoxyluteolinidin, 7-methoxyapigeninidin, 5-methoxyluteolinidin-7-glucoside (105), 5-methoxyapigeninidin, 7-methoxyluteolinidin (99), and 7-methoxyapigeninidin-5-glucoside (105, 108). Black pericarp sorghum has the highest level of 3-deoxyanthocyanins ranging from 173-1054  $\mu\text{g/g}$ ; red pericarp sorghum contains 32-180  $\mu\text{g/g}$ ; lemon-yellow and white pericarp sorghum have very low level of 3-deoxyanthocyanins (109). Compared with other pigmented cereal grains, black sorghum has similar content of anthocyanins as purple corn (965  $\mu\text{g/g}$ ) and at least twice the level in pink (93  $\mu\text{g/g}$ ), red (558  $\mu\text{g/g}$ ), and blue corns (225  $\mu\text{g/g}$ ), blue barley (4  $\mu\text{g/g}$ ), and blue (106-156  $\mu\text{g/g}$ ) and purple wheat (13-139  $\mu\text{g/g}$ ) (96, 110).

Condensed tannins, also known as proanthocyanidins, are high-molecular weight polyphenols that consist of polymerized flavan-3-ol and/or flavan-3,4-diol units (98). Tannins in sorghum are of condensed type. Because of their ability to bind free radicals, sorghums containing tannins have higher antioxidant capacity than most non-tannin sorghums (86).

Different genotypes of sorghum with a wide range of phenol profiles have been recognized as strong free radical scavengers *in vitro* (109). The antioxidant capacity (evaluated by ABTS) of sorghums, especially black and high tannin sorghums (75-340  $\mu\text{mol Trolox equivalent /g}$ ) are the highest among common cereals such as red/purple/white wheat (20-30  $\mu\text{mol Trolox equivalent /g}$ ), barley (10-40  $\mu\text{mol Trolox equivalent /g}$ ), finger and pearl millets (40-50  $\mu\text{mol Trolox equivalent /g}$ ), black/red rice

(50-120  $\mu\text{mol}$  Trolox equivalent /g), and blue/red/yellow corn (20-30  $\mu\text{mol}$  Trolox equivalent /g) (96, 97).

Antioxidant capacity is insightful in evaluating chemopreventive potential because antioxidants can block initiation of carcinogenesis by scavenging or reduce the oxidative species and/or carcinogens. Different genotypes of sorghum with a wide phenol profile have been recognized as strong free radical scavengers. Brans from tannin sorghum, red, black and white pericarp sorghum varieties exhibited anti-inflammatory potential (111). Hence sorghum phenols may have a potential in chemoprevention. It is necessary to determine how composition affects their ability to prevent carcinogenesis and/or inhibit cancer growth.

## **Methods for Evaluating Chemopreventive Potential of Phytochemicals**

### *Antioxidant (Free Radical Scavenge) Capacity Assays*

Free radical (or oxidant) scavenging assays determine how effective an antioxidant is at preventing initiation and prolongation of radicals or oxidation (112). Free radical scavengers have strong potential to block the insults from carcinogens and oxidative stress (21). Antioxidant assays are divided into two major categories: single electron transfer reaction based assays (ET) and hydrogen atom transfer reaction based assays (HAT) (112).

The ET-based assays involve one redox reaction between the antioxidant and the oxidant (also as the probe for monitoring the reaction). The oxidant attracts an electron

from the antioxidant then changed color. The color change is proportion to the concentration of the antioxidant (112). The ET-based assays include DPPH (diphenyl-1-picrylhydrazyl) radical scavenging capacity (113), FRAP (ferric ion reducing antioxidant parameter) (114), and TEAC (Trolox equivalent antioxidant capacity) also known as ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (115) assay. Because of lack of a competitive reaction between the antioxidant and an oxygen radical in ET-based assays, it is assumed that antioxidant capacity measured by these assays is indeed the reducing capacity of the compound (116).

The HAT-based assays generally evaluate how competitive an antioxidant is to trap free radicals. Major assays in this category include TRAP (total radical trapping antioxidant parameter) assay (117), ORAC (Oxygen Radical Absorbance Capacity) assay (118), IOU (inhibited oxygen uptake) assay (119), and inhibition of linoleic acid oxidation assay (120). Those assays monitor the kinetic competitive reaction in a system composed of a synthetic free radical generator, an antioxidant and a probe (also an oxidant) to indicate the progress of the reaction; and quantitation is derived from the kinetic reaction curve (112).

Among these assays, ABTS and ORAC assay are common methods used to evaluate free radical scavenging capacity and were demonstrated as suitable methods to evaluate phenolic compounds (95, 121, 122). The ABTS assay is based on the capacity of a compound to donate an electron to react with the probe ABTS, which causes a color change in the probe (112). The ABTS assay is relatively easy, fast, and inexpensive to perform. The ORAC assay was developed to directly assess the capacity of the

antioxidants to inhibit the oxidation of the molecular probe (fluorescein) by monitoring the kinetics of the reaction. The ORAC assay evaluates the radical chain-breaking antioxidant capacity. In general, ORAC assay applies a thermal radical generator (2,2'-azobis(2-amidinopropane) dihydrochloride, AAPH) to give a steady flux of peroxy radicals in air-saturated solution. An antioxidant competes with probes (fluorescein) for the radicals and inhibits or retards the probe oxidation (112, 118). Though the ORAC assay takes a relatively longer time to gather results and expensive instrument to complete analysis, its procedure is standardized hence results over different laboratories can be compared.

#### *Phase II Enzyme Inducing Capacity Assays*

Potential to activate cellular actions to block cancer initiation is an important aspect of chemoprevention (45). Determining phase II enzyme inducing capacity of a compound is an established method to determine its capacity to activate the ARE protective pathways (123). Phase II enzymes include glutathione-*s*-transferase (GST), UDP-glucuronyltransferases (UGTs), NAD(P)H: quinone oxidoreductase (QR) and heme oxygenase (HO-1) (124). Methods to evaluate phase II enzyme inducing capacity can target different sites along the synthesis and expression of these enzymes, such as measuring gene expression, measuring the amount of enzyme protein, or determining enzyme activity (45, 124).

NAD(P)H: quinone oxidoreductase (QR), a model phase II enzyme, promotes the obligatory two electron reduction of quinones to hydroquinones, which are then



degraded by glucuronidation. Thus QR protects cells from the toxicity of quinones and their metabolic precursors (125). One method to evaluate QR enzyme inducing capacity *in vitro* has been developed using murine hepatoma cell lines (123).

#### *Cancer Cell Proliferation Inhibition Assays*

Chemopreventive compounds have the potential to suppress the promotion and progression of malignant cells. Evaluating the potential of a compound to directly inhibit cancer cell proliferation determines the efficacy of the compound to suppress preformed tumors. Many assays are available to determine cell proliferation, for example, radioactive assays based on the capacity of viable cells to incorporate isotopes (such as [<sup>3</sup>H]-thymidine, [<sup>3</sup>H]uridine) into nucleic acids (126), measurement of nucleic acid and adenosine triphosphate (ATP) contents in lysed cells (127), and colorimetric assays based on the capacity of living cells to reduce a chemical (such as Alamar blue (128) and tetrazolium dyes (129)) then measure the color change.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a commonly used non-radioactive method to determine proliferation of cultured cells. The MTT assay (129) is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals, thus accumulates within healthy cells. The crystals formed are largely impermeable to cell membranes. The number of surviving cells is directly proportional to the level of the formazan product created. Adding a detergent to the system solubilizes the cells and the crystals at the same time. Then the color is quantified using a multiwell

scanning spectrophotometer (130-132). The MTT assay is easy and not labor intensive to perform. However, several reports have indicated that for some agents and cell lines, an increase of MTT reduction was observed without increased cell viability; e.g. with drug efflux inhibitors (133), ursolic acid (134), resveratrol (135), and high Abl kinase activity tumor cell lines (136). Hence it is important to use complementary cell viability assays along with MTT assay.

Cell proliferation can also be determined by measuring double-stranded DNA (dsDNA) content by fluorescent dyes (137). PicoGreen is a fluorescent probe that specifically binds to dsDNA. PicoGreen assay has a high sensitivity for measuring dsDNA (25 pg/mL dsDNA) (138, 139). Double-stranded DNA content is considered as a stable parameter in cells and the estimated dsDNA content from PicoGreen assay varies depending on the cell cycle of a tested culture (140), hence it is a reliable indicator of cell proliferation.

## CHAPTER III

### MATERIALS AND METHODS

#### **Sorghum Samples**

Seventeen sorghum varieties were screened for phenolic profile and antioxidant capacity. Among the 17 samples, some were selected for further cell culture studies. The samples included six tannin sorghums (Sumac, Hi-tannin (College Station, TX 2002); Seredo, Cadam and CR 35 (KARI, Kenya 2006); Black PI Tall (College Station, TX 2005), and 11 non-tannin sorghums selected based on different pericarp pigmentation: a black variety Tx430 (College Station, TX 2002), a commercially grown red variety from Missouri (Columbia, MO 2006) (“Mizzou”), three red varieties Tx2911, 98BRON155, and 99LGWO50 from Texas (College Station, TX 2008), a light red variety I CSV III from Kenya (KARI, Kenya 2006), two lemon yellow varieties SC748 and EBA3 (College Station, TX 2008), and three white varieties: KARI-Mtama (KARI, Kenya 2006), a commercially grown variety from Missouri (Columbia, MO 2006) (“Mizzou white”), and ATX635 × RTX436 (College Station, TX 2001). White commercial dent corn was used as a control because of its low phenol and carotenoids content. Sorghum grains were kept at -35°C till used.

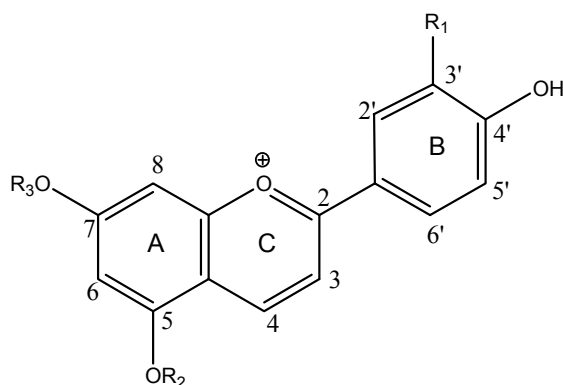
## Reagents and Experimental Materials

### *Chemicals and Reagents*

3-Deoxyanthocyanidin standards, apigeninidin, luteolinidin, 7-methoxyapigeninidin, 7-methoxyluteolinidin, 5-methoxyapigeninidin, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin were obtained from AlsaChim (Strasbourg, France). Figure 1 shows the structures of 3-deoxyanthocyanidin standards and the abbreviations used in this thesis. Genistein and sulforaphane were purchased from Sigma-Aldrich (St. Louis, MO).

Hexane, acetone, ethanol, methanol, hydrochloride acid (HCl), Folin-Ciocalteu reagent, ethanolamine, potassium phosphate, sodium chloride (NaCl), sodium dodecyl sulfate (SDS), crystal violet, Tris, and Tween-20 were of analytical grade and purchased from Fisher (Fisher Scientific, Fair Lawn, NJ). Gallic acid, catechin hydrate, vanillin, ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], sodium fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), flavin adenine dinucleotide (FAD),  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADH), and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) and digitonin were purchased from Acros Organics (Acros Organics, NJ). Menadione was purchased from Alexis Biochemicals (San Diego, CA). Dicoumarol was from EMD Biosciences (EMD

Biosciences, Inc., La Jolla, CA). Glucose-6-phosphate and yeast glucose-6-phosphate dehydrogenase were from MP Biomedicals (Solon, Ohio).



Compound	Abbreviation	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Apigeninidin	Api	H	H	H
5-methoxyapigeninidin	5-Api	H	CH <sub>3</sub>	H
7-methoxyapigeninidin	7-Api	H	H	CH <sub>3</sub>
5,7-dimethoxyapigeninidin	5,7-Api	H	CH <sub>3</sub>	CH <sub>3</sub>
Luteolinidin	Lut	OH	H	H
7-methoxyluteolinidin	7-Lut	OH	H	CH <sub>3</sub>
5,7-dimethoxyluteolinidin	5,7-Lut	OH	CH <sub>3</sub>	CH <sub>3</sub>

Figure 1. Skeleton structures of 3-deoxyanthocyanidins used in this study.

### *Cell Lines*

Two human gastrointestinal carcinoma cell lines were selected to represent the upper digestive tract (esophagus) and lower digestive tract (colon), which are common gastrointestinal tract cancer sites. The OE33 (a cell line established from the adenocarcinoma of the lower esophagus of a 73-year-old female patient) was purchased from Sigma (St. Louis, MO); HT-29 (a cell line established from colorectal adenocarcinoma of a 44-year-old female patient) was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

Murine hepatoma Hepa 1c1c7 (a murine hepatoma cell line derived from C57/L mouse) was purchased from ATCC (Manassas, VA). This cell line is useful for studying aryl hydrocarbon receptor and P450IA1 regulation. It has been used as a standard cell line in testing the inducing capacity of a compound to induce cellular NAD(P)H:quinone oxidoreductase activity.

### *Cell Culture Materials*

Media used included  $\alpha$ -MEM (minimum essential medium) (with 2mM L-glutamine without ribonucleosides or deoxyribonucleosides), McCoy's 5A medium (with 1.5mM L-glutamine) and RPMI 1640 medium (with 2mM L-glutamine). Media were purchased from Invitrogen (Carlsbad, California) and kept at 4 °C till use. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Dulbecco's phosphate-buffered saline (DPBS) and 0.05% trypsin were purchased from Invitrogen (Carlsbad, CA).

Three types of plates were used to culture cells: 96-well all clear tissue culture plates (FALCON, Franklin Lakes, NJ), 96-well black with clear bottom tissue culture plates (Costar) and regular all black 96-well plates (Costar, Corning Incorporated, NY).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay kit was purchased from ATCC (Manassas, VA). Quant-iT PicoGreen dsDNA assay kit was purchased from Invitrogen (Carlsbad, CA).

### **Preparation of Crude Sorghum Extracts**

Whole kernels of sorghum grain were ground in a cyclone mill (UDY, Boulder, CO) to pass through 0.1 mm screen before extraction. Ground samples were defatted using hexane at a ratio of 1:2 (w:v) by stirring for 2 h. The mixture was then centrifuged at  $3100 \times g$  and the residue was dried inside a fume hood over night at room temperature. Defatted samples were extracted with 70% (v/v) aqueous acetone with stirring for 30 min. Supernatant was collected by centrifuging ( $3100 \times g$ ) for 15 min at 4 °C. Acetone was immediately removed from the supernatant under vacuum at 40 °C. The aqueous phase was freeze-dried and used as a crude extract. Extracts were kept at -35°C till use.

### **Gross Phenolic Profile**

Ground grain samples were extracted with 1% HCl in methanol for 2 h to measure total polyphenol, tannin, and 3-deoxyanthocyanin contents. Freeze-dried crude sorghum extracts were dissolved in 1% HCl in methanol for these assays.

### *Phenol Content*

The Folin-Ciocalteu method described by Kaluza et. al. (141) was used. Aqueous samples (0.1 mL) was added into 1.1 mL distilled water then reacted with 0.4 mL Folin reagent and 0.9 mL 0.5 M ethanolamine for 20 min at room temperature. The absorbance was then read at 600 nm. Serial dilutions of gallic acid (0, 5, 10, 15, 20, 25 mg/mL in methanol) was used as standards. Results were calculated and expressed as mg gallic acid equivalent (GAE)/g sample.

### *Condensed Tannin Content*

The vanillin-HCl method described by Price et. al. (142) was used. Aqueous samples (1 mL) was added to 5 mL vanillin reagent (0.01 g/mL vanillin in methanol mixed with equal volume 8% HCl in methanol) and allowed to react for 20 min at 35 °C; a blank was prepared under the same reaction condition by reacting 1 mL the same aqueous sample with 5 mL 4% HCl in methanol. Absorbance was read at 500 nm and blank for each sample was subtracted. Serial concentrations of catechin (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL in methanol) were used as standards. Results were calculated and expressed as mg catechin equivalent (CE)/g sample.

### *3-Deoxyanthocyanin Content*

The method used was first described by Fuleki and Francis (143) and further detailed by Awika et al. (144). Absorbance of sorghum grain samples and extract samples were read at 480 nm. The concentrations of 3-deoxyanthocyanins were



calculated based on the absorbance of luteolinidin at 480 nm using the Lambert-Beer's Law:  $A = \epsilon CL$ , where  $A$  is the absorbance at 480 nm,  $\epsilon$  is the molar extinction coefficient ( $\epsilon$ ) of luteolinidin,  $C$  is sample concentration,  $L$  is the pathlength, which is 1 cm. The 3-deoxyanthocyanin content of a sample was calculated based on the formula  $C \text{ (mol/L)} = A / \epsilon$ . The mg luteolinidin equivalent of a sample =  $A / \epsilon \times 10^3 \times 270 \times$  dilution factor, where 270 is the molecular weight of luteolinidin and molar extinction coefficient ( $\epsilon$ ) of luteolinidin used was 29,157 (145).

### **Antioxidant (Free Radical Scavenging) Capacity Assays**

Antioxidant (Free radical scavenging) capacity was measured by ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and Oxygen Radical Absorbance Capacity (ORAC) assays. Ground grain samples were extracted in 70% aqueous acetone for 2 h and were used immediately in these assays. Freeze-dried sorghum crude extracts were dissolved in 70% acetone to be used in these assays. For both assays, the methods were described by Awika et al. (95).

#### *ABTS Assay*

The ABTS stock solution was prepared by reacting equal volume of 8 mM ABTS with 3 mM potassium persulfate in the dark for at least 12 h. The ABTS working solution must be prepared right before the assay by diluting 5 mL ABTS stock solution with 145 mL of pH 7.4 PBS buffer. The original absorbance of ABTS working solution at 734 nm should be around 1.5. Aqueous sample (0.1 mL) was added with 2.9 mL

ABTS working solution then allowed to react in the dark for 30 min at room temperature. The absorbance was read at 734 nm. A series dilution of Trolox (0, 100, 200, 300, 400, 500, 600, 700, 800, 1000  $\mu\text{M}$  in methanol) was used as standards. Antioxidant capacity was calculated and expressed as  $\mu\text{mol}$  Trolox equivalent /g sample.

#### *ORAC Assay*

The ORAC assay was conducted using a solid black 96-well plate and the reaction was monitored by a Biotek Synergy HT plate reader with automatic dispenser (Biotek, Winooski, VT). Sodium fluorescein working solution ( $4 \times 10^{-3}$   $\mu\text{M}$ ), AAPH (153 mM), Trolox standards (0, 6.25, 12.5, 25, 50, 100  $\mu\text{M}$ ) were prepared using PBS buffer (pH 7.4) right before performing the assay. Aqueous samples (25  $\mu\text{L}$ ) and Trolox standards (25  $\mu\text{L}$ ) were analyzed in triplicate on the same plate. Sodium fluorescein working solution (150  $\mu\text{L}$ ) was added to each well before the whole plate was incubated inside the plate reader at 37 °C for 30 min. One aliquot (25  $\mu\text{L}$ ) of 153 mM AAPH was automatically dispensed into each well as the free radicals generator to start the reaction after incubation. Fluorescence readings (excitation 485 nm, emission 528 nm) was taken every minute from the time AAPH was added for 90 min. Antioxidant capacity was calculated based on the area under the fluorescence curve (AUC),

$$\text{AUC} = 0.5 + (R_2/R_1) + (R_3/R_1) + (R_4/R_1) + \dots + (R_{90}/R_1),$$

where  $R_n$  is the fluorescent reading at the  $n^{\text{th}}$  minute.

A Trolox standard curve was obtained by plotting the concentrations of Trolox standards versus their AUC. Trolox equivalent of each sample was obtained by

interpolating the AUC of sample into the Trolox standard curve. The antioxidant capacity of ORAC assay was expressed as  $\mu\text{mol}$  Trolox equivalent / g. Each sample was measured on three different plates at three separate times.

## **Cell Culture**

### *General*

Murine hepatoma cells Hepa 1c1c7 were cultured in Gibco  $\alpha$ -MEM medium supplemented with 10% fetal bovine serum. Human colon carcinoma cells HT-29 were cultured in Gibco McCoy's 5A medium supplemented with 10% fetal bovine serum. Human esophageal carcinoma cells OE33 were cultured in Gibco RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell lines were subcultured every 3 or 4 d and incubated in a humidified incubator with 5%  $\text{CO}_2$  at 37 °C.

### *Preparation of Treatment Media*

Sorghum extract stocks were prepared by dissolving crude sorghum extracts in 500  $\mu\text{L}$  DMSO then diluted with DMSO into serial concentrations (5-800 mg/mL). Treatment medium was prepared by adding stocks at a ratio of 1:1000 (v/v) to corresponding growth medium. Final treatment medium contained 5-800  $\mu\text{g/mL}$  crude sorghum extracts. Sorghum 3-deoxyanthocyanidin standard stocks (0.2-200 mM) and treatment medium were prepared by the same method. Final treatment medium contained 0.2-200  $\mu\text{M}$  3-deoxyanthocyanidins.

## NAD(P)H:Quinone Oxidoreductase (QR) Inducing Capacity and Cytotoxicity

### Assay

This assay measured QR activity in both sample-treated and control cells. The QR activity was measured through the NAD(P)H-dependent QR oxidized menadiol-mediated reduction of MTT. Figure 2 shows the schematic principle of this assay.

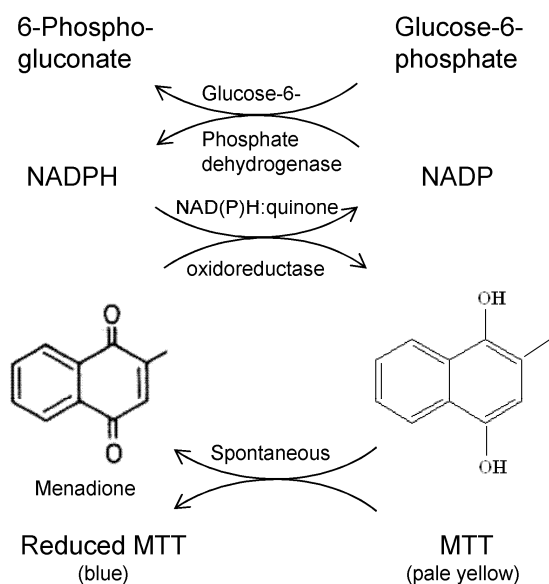


Figure 2. Principle of the NAD(P)H: quinone reductase (QR) activity assay (adapted from Prochaska and Santamaria (146)). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase continually generate NADPH, which is used by QR to transfer electrons to menadione. Menadione further transfers this electron to MTT then produces blue reduced MTT formazan dye, which can be measured at 610 nm. Both NADPH and menadione are regenerated, which obviates problems encountered with substrate depletion (146).

### *NAD(P)H:Quinone Oxidoreductase (QR) Inducing Capacity Assay*

The Hepa 1c1c7 cells were cultured in growth medium ( $\alpha$ -MEM medium supplemented with 10% fetal bovine serum) for 72 h before plating into a clear FALCON 96-well tissue culture plate at a density of 10,000 cells/well with 200  $\mu$ L growth medium. The cells were incubated at 37 °C with 5% CO<sub>2</sub> for 24 h before exposure to treatment media.

Six different treatments and one control were applied onto one 96-well plate. Each treatment was assigned to 12 wells while control was assigned to 16 wells, and the remaining 8 wells were blanks. Final treatment medium contained 5-400  $\mu$ g/mL sorghum crude phenol extracts or 0.2-200  $\mu$ M 3-deoxyanthocyanidin standards. Each well received 200  $\mu$ L treatment medium while control cells received 200  $\mu$ L growth medium containing 0.1% DMSO. The cells were grown for another 24 h (37°C, 5% CO<sub>2</sub>) before QR activity assay. After 24 h incubation, the medium in each well was aspirated and the cells were lysed with 50  $\mu$ L 0.8% digitonin in 2 mM pH 7.8 EDTA, incubated for 10 min at 37 °C then agitated on a shaker for another 10 min at room temperature. Lysed cells were used for QR activity assays.

Each treatment set was then divided into three different assays to evaluate different aspects of QR activity. The three assays were: 1) *standard enzyme activity assay*, 2) *prior stop enzyme activity assay*, and 3) *minus menadione assay*.

*Standard enzyme activity assay* evaluates the proportion of MTT reduction attributable to QR activity. MTT, a pale yellow tetrazolium dye, is reduced to its blue formazan form, which can be measured by the absorbance at 610 nm. *Prior stop enzyme*

*activity assay* evaluates the proportion of MTT reduction not attributable to QR activity. Since menadione is used in these two assays mentioned above as an electron transporter to MTT, *minus menadione assay* determines the proportion of MTT reduction attributable to QR activity but without menadione as an electron transporter. True cellular QR activity is determined using the QR activity obtained from *standard enzyme activity assay* subtracting the activity obtained from *prior stop enzyme activity assay* and *minus menadione assay*.

A *baseline reaction solution* was prepared for each set of QR activity assays. The composition of the *baseline reaction solution* was as follows: 5% (v/v) 0.5M Tris-HCl (pH 7.4), 6.67% (v/v) 1.5% Tween-20, 0.67% (v/v) 7.5mM FAD, 0.06% (v/v) 50mM NADP, 66.7% (g/L) bovine serum albumin, 30% (g/L) MTT, 6.67% (v/v) 150 mM glucose-6-phosphate (in 1% HCl acidified distilled water), 2 U/mL yeast glucose-6-phosphate dehydrogenase, and distilled water to make up to the final volume. A *standard reaction solution* used for *standard enzyme activity assay* was made by adding 1  $\mu$ L of 50 mM menadione (dissolved in acetonitrile) per mL of *baseline reaction solution* just before the *standard reaction solution* was dispensed into the wells. Dicoumarol (0.3mM in 0.5% DMSO and 5mM potassium phosphate (pH 7.4)) served as the *stop solution* to stop QR activity. The three assays to determine cellular QR activity were carried out on the same plate and at the same time. Figure 3 shows a schematic process of the three assays.

Once the cells were lysed, *stop solution* (50  $\mu$ L) was first added to *prior stop enzyme activity assay* wells. Then 200  $\mu$ L of *standard reaction solution* was added to

*standard enzyme activity assay* and *prior stop enzyme activity assay* wells. Finally 200  $\mu\text{L}$  of *baseline reaction solution* was added to *minus menadione assay* wells. The plate was then allowed to react in the dark for 5 min at room temperature. The reaction was arrested by dispensing 50  $\mu\text{L}$  of *stop solution* to both *standard enzyme activity assay* and *minus menadione assay* wells. The plate was then read in the BioTek Synergy HT Plate Reader at 610 nm. Absorbance of all the solutions from the *standard enzyme activity assay* was taken as blank reading.

#### *Cytotoxicity Assay (Crystal Violet Assay)*

Since samples screened for QR inducing activity could also suppress growth of the cells, thus affecting results, cell population was measured by the crystal violet assay (146). Plates were treated identically as for the QR inducing activity assay. After the cells were incubated with treatment medium for 24 h, the medium was decanted. Then 200  $\mu\text{L}$  of 0.2% crystal violet in 2% ethanol was dispensed into each well to stain the cells for 10 min at room temperature. The plate was rinsed 3 times with distilled water. Bound dye was solubilized by adding 200  $\mu\text{L}$  of 0.5% SDS in 50% ethanol in each well. The plate was then incubated at 37 °C for 1 hour, and absorbance was read at 610 nm. A higher absorbance reading in treated cells than the control cells indicated cell growth induced by treatment; while a lower reading indicated a reduction of cell population due to treatment. Hence cell population was used to correct QR activity obtained from treatments.

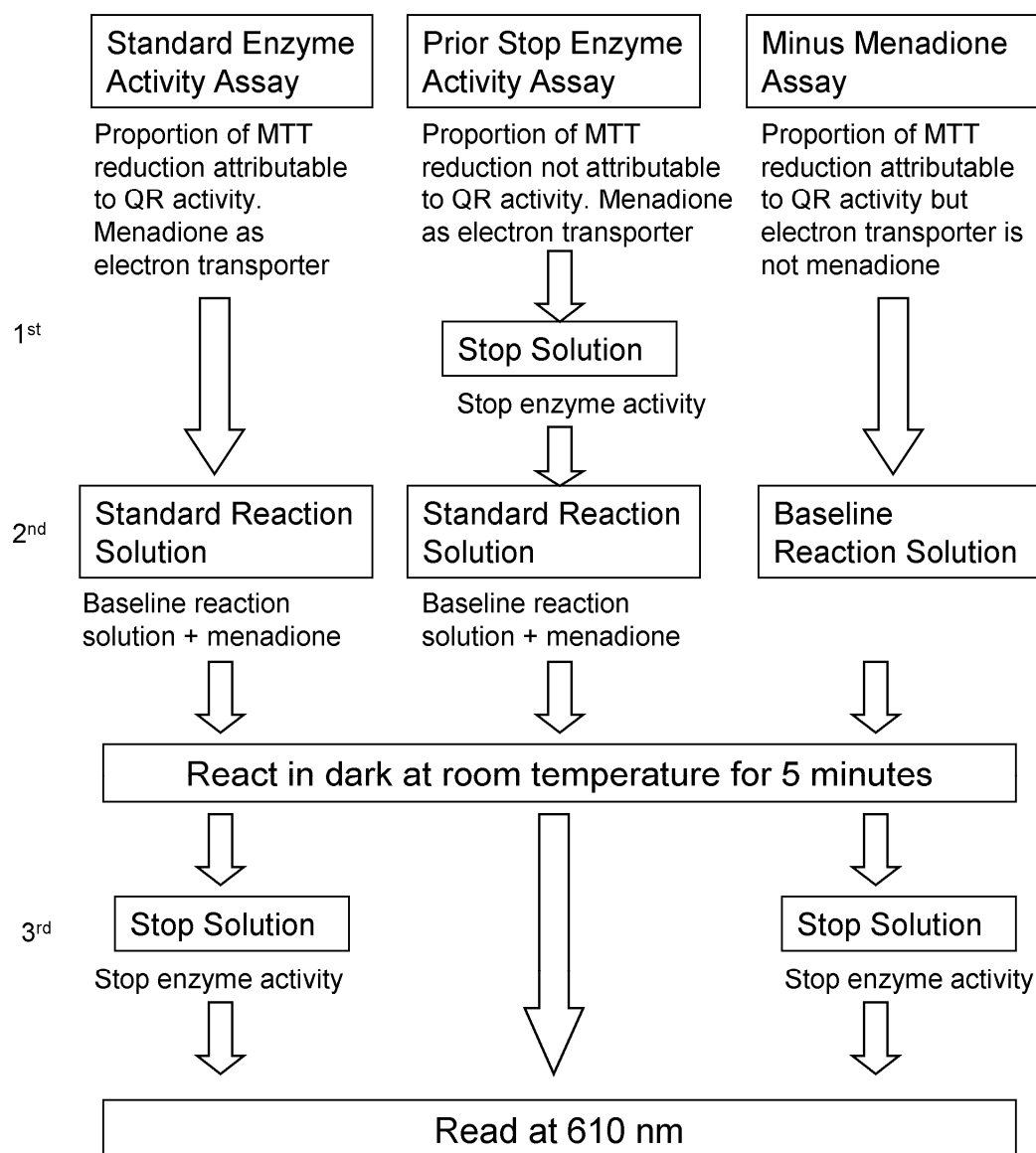


Figure 3. Schematic process of NAD(P)H:quinone oxidoreductase (QR) activity assay according to Prochaska and Santamaria's method (146).

### *Calculating QR Inducer Ratios*

The QR inducer ratio determines how well a treatment could raise the cellular activity of QR compared with non-treated cells (controls). The QR inducer ratio was



calculated as the ratio of cellular QR activity obtained from each treatment to the cellular QR activity from the control treatment conducted on the same plate. The formula below was used:

QR inducer ratio =  $(A_{610} \text{ of treatment} - A_{610} \text{ of control on the same plate}) / A_{610} \text{ of control on the same plate}$

QR specific inducer ratio = treatment QR inducer ratio  $\times$  treatment cell population / cell population of control for the treatment.

### **Cancer Cell Proliferation Assay**

Both MTT and PicoGreen assays were used because of their non-radioactive nature, convenience and accuracy.

#### *MTT Assay*

The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created. Adding a detergent into the system solubilized the cells and the crystals at the same time, which enabled the quantitative reading of the absorbance by a plate reader (130-132).

Both HT-29 and OE33 cells were grown in growth medium ( $\alpha$ -MEM supplemented with 10% FBS for HT-29 and McCoy's 5A supplemented with 10% FBS

for OE33) for 72 h before plating into a clear FALCON 96-well tissue culture plate at a population of 2,000 cells/well with 100  $\mu$ L growth medium. Final treatment medium contained 5-800  $\mu$ g/mL crude sorghum extracts or 0.2-200  $\mu$ M 3-deoxyanthocyanidin standards. The cells were incubated with treatment medium for 48 h. Each treatment used 4 wells and each well received 100  $\mu$ L of treatment medium while control cells received 100  $\mu$ L of growth medium containing 0.1% DMSO. After 48 h incubation, viable cells were determined using the ATCC MTT assay kit. The linearity of this MTT assay was 100-5000 cells per well (plated cell population, tested after 48 h incubation) for both cell lines. The MTT reagent (10  $\mu$ L) was added to each well and incubated for 3 h at 37  $^{\circ}$ C; the medium was then decanted and 50  $\mu$ L of DMSO was added to dissolve the purple crystals. Absorbance was read at 570 nm. Blank reading (10  $\mu$ L of MTT reagent plus 50  $\mu$ L of DMSO) was subtracted from each well. Sample blank reading was recorded as absorbance due to treatment medium only (without cells). Relative Growth (%) was calculated using the following formula:

$$\text{Relative Growth (\%)} = (\text{A}_{570} \text{ of test sample} - \text{blank} - \text{sample blank}) / \text{A}_{570} \text{ of control cells} \times 100 \%$$

#### *Double-Stranded (ds) DNA Content: PicoGreen Assay*

Since the dsDNA content in each cell is constant, by determining the contents of dsDNA using Quant-iT PicoGreen dsDNA assay indicates the living cell population. Both HT-29 and OE33 cells were treated using the same protocol described for MTT assay above, except cells were plated in 96-well black plates with a clear bottom. After

48 hour incubation, the medium was aspirated and cells were washed twice with DPBS buffer to wash out dead cells floating in the medium. Each well then received 50  $\mu\text{L}$  of 0.05% trypsin in 0.9% NaCl solution to detach cells at 37  $^{\circ}\text{C}$  for 1 hour. The plate was then frozen at -35  $^{\circ}\text{C}$  for 1 hour then thawed at room temperature to lyse cells. A standard curve of dsDNA (0, 1, 5, 10, 50, 100, 150 ng/well) was prepared in the same testing plate for each assay. Standard solution of dsDNA (100  $\mu\text{g}/\text{mL}$  in 10 mM Tris-HCl 1 mM EDTA, pH 7.5) were provided in the PicoGreen assay kit and diluted with 0.05% trypsin in 0.9% NaCl solution. PicoGreen fluorescent dye working solution was prepared in 10mM Tris-1mM EDTA buffer provided in the PicoGreen assay kit. After lysing the cells, PicoGreen working solution (50  $\mu\text{L}$ ) was added to each well including the standards then the whole plate was incubated at room temperature in the dark for at least 5 min. Fluorescence was read in the plate reader at excitation 480 nm and emission 520 nm. The content of dsDNA in each well was calculated using the standard curve obtained from each assay. Relative Growth (%) was calculated using the following formula:

$$\text{Relative Growth (\%)} = \frac{\text{dsDNA content of treatment cells}}{\text{dsDNA content of control cells}} \times 100 \%$$

### **Statistical Analysis**

Total phenol, condensed tannin, 3-deoxyanthocyanin pigment contents, ABTS and ORAC antioxidant capacity, and cancer cell growth inhibition assays were repeated three times. QR inducing capacity assay was repeated two times. Statistical analyses

were done using SPSS version 15.0 (SPSS Inc., Chicago, IL) with one-way Analysis of Variance (ANOVA). Post Hoc test (Dunnett's, Fisher's LSD, and Tukey's HSD) after ANOVA was used to compare treatments means.

## CHAPTER IV

### GROSS PHENOLIC PROFILES AND ANTIOXIDANT PROPERTIES OF SORGHUM GRAINS AND THEIR CRUDE EXTRACTS

#### **Gross Phenolic Profiles and Antioxidant Properties of Sorghum Grains**

##### *Gross Phenolic Profiles of Sorghum Grains*

The seventeen varieties of sorghum showed a wide range of phenolic compositions (Table 1). Based on the presence of condensed tannins, the seventeen sorghum grains can be divided into two groups: tannin and non-tannin sorghums. The tannin contents in tannin sorghums ranged from 7.0-23.3 mg catechin equivalent (CE)/g sample (db), which agreed with Dykes et al. (98) (6.4-50.2 mg CE/g sample). Only sorghums with a pigmented testa contain tannins (86, 147).

Tannin sorghums had varying contents of 3-deoxyanthocyanins (trace to 1.2 mg luteolinidin equivalents (LE)/g sample) (Table 1). Among non-tannin sorghums, 3-deoxyanthocyanin contents in red and black pericarp sorghums ranged from 0.07-4.5 mg LE /g sample (db) (Table 1), which was similar to what Awika et al. (103) reported in red and black sorghums (0.8-2.8 mg LE/g sample). White and lemon yellow pericarp sorghum grains did not contain significant amount of 3-deoxyanthocyanin pigments. Pericarp color of sorghum grains is significantly correlated with 3-deoxyanthocyanin contents: the darker and redder the grain pericarp color, the higher the 3-deoxyanthocyanin contents (148, 149). Pericarp color is regulated by the *R* and *Y* genes

and is not influenced by the presence of tannins (regulated by the dominant *B<sub>1</sub>B<sub>2</sub>* gene) (150), which explains why tannin content did not correlate with 3-deoxyanthocyanin content.

Total phenol content of tannin sorghums was 5.1 to 29.6 mg gallic acid equivalent (GAE)/g sample (db); while non-tannin sorghums ranged from 0.9-18.2 mg GAE/g sample (Table 1). Dykes (109) reported that total phenol contents of tannin sorghums was 8-20 mg GAE/g sample and that of non-tannin sorghums was 1.8-6.0 mg GAE/g sample. Tannin sorghums generally had higher total phenol content than non-tannin sorghums ( $p < 0.001$ ) (Figure 4), which agreed with previous reports (109, 151). This may be due to the chemistry behind the total phenol assay. The Folin-Ciocalteu assay involves redox reaction between phenolate and Folin-Ciocalteu reagent, and the assay actually measures a phenol's reducing capacity (112). Tannins presented in sorghum are of condensed type (94, 152, 153) and one condensed tannin molecule can have more phenolate groups than one molecule of other phenols, such as phenolic acids, flavones, 3-deoxyanthocyanins, and flavanones. This characteristic of condensed tannins in tannin sorghums can contribute to their higher total phenol contents than non-tannin sorghums. Since the Folin-Ciocalteu method for total phenol content has a similar chemical reaction with the single electron transfer reaction based antioxidant capacity assays (112), the results of total phenol content indicated that condensed tannins were stronger antioxidants than other sorghum phenols.

Table 1. Phenolic Content and Free Radical Scavenging Capacity of Sorghum Grains

	Condensed tannins (mg CE/g) <sup>a</sup>	3-DXA content (mg LE/g) <sup>b</sup>	Phenol content (mg GAE/g) <sup>c</sup>	ORAC (μmol TE/g) <sup>d</sup>	ABTS (μmol TE/g) <sup>d</sup>
White dent corn	nd	trace	0.6 ± 0.05	11.8 ± 1.2	7.29 ± 1.3
ATX635×RTX436 (white)	nd	trace	0.9 ± 0.09	24.3 ± 3.2	9.70 ± 1.0
KARI- Mtama (white)	nd	trace	8.1 ± 0.3	81.6 ± 1.8	63.9 ± 0.9
Mizzou White (white)	nd	trace	N. D.	N. D.	N. D.
Mizzou (red)	nd	1.0 ± 0.2	11.8 ± 0.6	95.3 ± 1.8	67.4 ± 1.8
Tx2911 (red)	nd	0.27 ± 0.01	3.1 ± 0.1	171 ± 16	44.6 ± 2.0
98BRON155 (red)	nd	0.07 ± 0.01	2.1 ± 0.1	113 ± 14	27.6 ± 1.8
99LGWO50 (red)	trace	0.20 ± 0.01	3.5 ± 0.2	178 ± 17	51.5 ± 1.6
I CSV III (red)	trace	0.14 ± 0.08	7.9 ± 0.1	102 ± 1.1	65.9 ± 0.5
Tx430 (black)	nd	4.5 ± 0.3	18.2 ± 1.3	126 ± 5.8	78.9 ± 4.3
SC748 (lemon yellow)	nd	0.05 ± 0.01	2.09 ± 0.14	132 ± 18	24.4 ± 2.8
EBA-3 (lemon yellow)	nd	0.03 ± 0.00	2.17 ± 0.11	122 ± 14	25.4 ± 2.5
Black PI Tall (tannin)	7.2 ± 1.8	1.2 ± 0.07	5.14 ± 0.18	203 ± 31	66.6 ± 4.4
Cadam (tannin)	18.3 ± 0.4	N. D.	N. D.	N. D.	N. D.
CR 35 (tannin)	7.0 ± 0.4	trace	16.5 ± 0.5	72.4 ± 6.3	61.6 ± 0.8
Seredo (tannin)	20.2 ± 0.4	0.7 ± 0.1	21.9 ± 0.4	171 ± 14	118 ± 1.2
Sumac (tannin)	23.3 ± 1.0	1.2 ± 0.09	29.6 ± 0.7	236 ± 21	125 ± 3.1
Hi-Tannin (tannin)	18.1 ± 0.6	0.5 ± 0.05	17.6 ± 0.2	182 ± 23	108 ± 4.6

<sup>a</sup>CE; catechin equivalents (Vanillin-HCl method). <sup>b</sup>3-Deoxyanthocyanin pigment content expressed as luteolinidin equivalents (LE). <sup>c</sup>GAE; gallic acid equivalents (Folin-Ciocalteu method). <sup>d</sup>TE; trolox equivalents. Values ± sd (db) reported for three separate experiments; nd – not detected; N.D. – not determined.

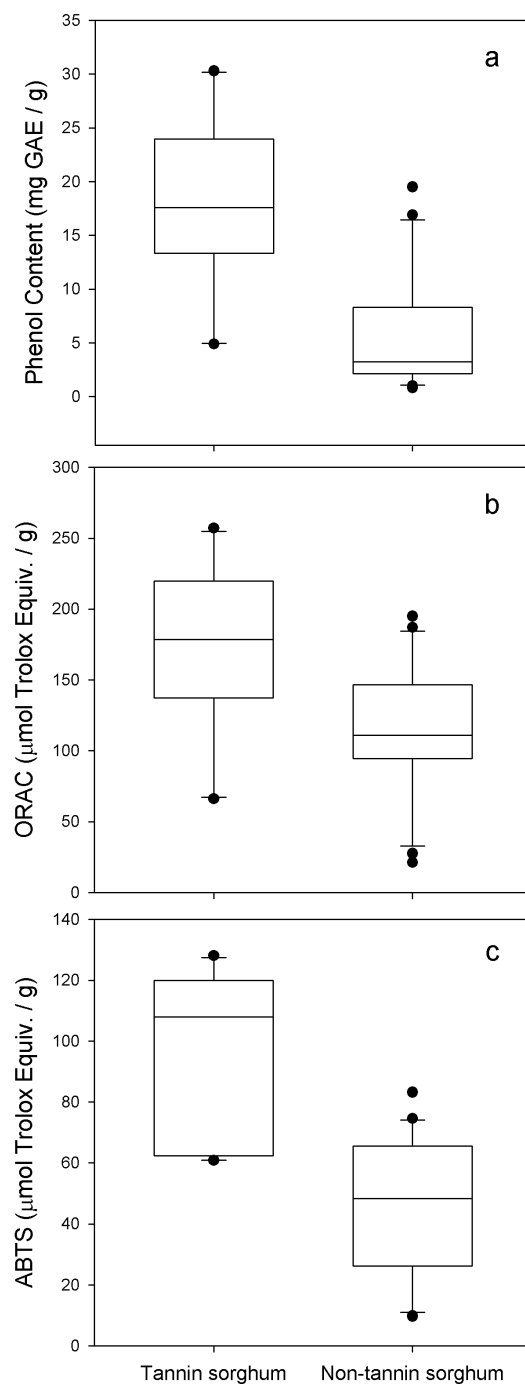


Figure 4. Box plot of total phenol contents (mg gallic acid equivalent/g sample) (a), ORAC (b) and ABTS (c) antioxidant capacity (μmol Trolox equivalent/g sample) between tannin and non-tannin sorghum grains. Tannin sorghum grains had higher total phenol content ( $p < 0.001$ ), ORAC value ( $p < 0.006$ ), and ABTS value ( $p < 0.001$ ) than non-tannin sorghum grains.



### *Antioxidant Capacity of Sorghum Grains*

The antioxidant capacity measured by ABTS assay of tannin sorghum grains ranged from 61.6-125  $\mu\text{mol}$  Trolox equivalent (TE)/g sample (Table 1). Dykes (109) reported tannin sorghum ABTS antioxidant value ranged from 125-340  $\mu\text{mol}$  TE/g sample using 1% HCl acidified methanol as the extraction solvent (ours was 70% aqueous acetone). The differences observed in this study could be first due to extraction solvent. Another reason can be the different varieties of grains and growth environments of the samples, such as year and location. The antioxidant capacity measured by ABTS assay of non-tannin sorghum grains ranged from 9.7-78.9  $\mu\text{mol}$  TE/g sample (Table 1), which was close to what Dykes (109) reported (8-75  $\mu\text{mol}$  TE/g sample). Tannin sorghums had higher antioxidant capacity measured by ABTS assay than non-tannin sorghum grains ( $p < 0.001$ ) (Figure 4). Plausible reason for this is that condensed tannins are stronger hydrogen donors because of the presence of more phenolate groups in one molecule than other phenols (94, 152, 153).

The antioxidant capacity measured by ORAC assay of tannin sorghum grains was 72.4 to 236  $\mu\text{mol}$  TE/g sample (Table 1). Awika et al. (95) reported ORAC antioxidant value for tannin sorghum grains as 440-853  $\mu\text{mol}$  TE/g sample using aqueous acetone as extraction vehicle. The differences can be due to different varieties of tannin sorghums or a different model of instrument used. The antioxidant capacity measured by ORAC assay of non-tannin sorghum grains ranged from 24.3 to 178  $\mu\text{mol}$  TE/g sample (Table 1), which was close to the ORAC values reported by Awika et al. (95) (21-205  $\mu\text{mol}$  TE/g sample). Similar to ABTS assay, ORAC antioxidant values of

tannin sorghum grains were higher than non-tannin sorghum grains ( $p < 0.006$ ) (Figure 4). Since ORAC assay measures a competitive reaction between an oxidant and an antioxidant, the structure of condensed tannins may make it more efficient in terminating the insults from free radicals than other phenols. Besides higher hydrogen donating capacity, condensed tannins have a greater metal chelating capacity because of the polymerized structures (154). However, some varieties of non-tannin sorghums, e.g. 99LGWO50 (red), Tx2911 (red), SC748 and EBA-3 (lemon yellow), had ORAC values almost as high as tannin sorghums. This suggested that other phenol groups present in these varieties, such as flavones and flavanones (109) may have strong free radical scavenging capacity as well.

Antioxidant capacity is an indicator of chemopreventive potential because dietary components which are antioxidants can reduce the oxidative stress and terminate the insults from free radicals or carcinogens. Hence the antioxidant capacity of these sorghum varieties may provide a direct insight into their chemopreventive potential. However, antioxidant capacity is not the only factor that influences this potential. It is important to determine how gross phenolic profile and antioxidant capacity affect chemoprevention by evaluating how the compounds interact with related gastrointestinal tract epithelial cells.

#### *Correlation between Gross Phenolic Profile and Antioxidant Capacity*

As previously stated, tannin sorghum grains had higher total phenol content ( $p < 0.001$ ), ABTS antioxidant capacity ( $p < 0.001$ ) and ORAC antioxidant capacity

( $p < 0.006$ ) than non-tannin sorghum grains (Figure 4). Total phenol content and ABTS antioxidant value correlated with the tannin contents among tested tannin sorghum grains (Table 2). Pearson's correlation determines strength of linear correlation while Spearman's rho (one of nonparametric correlations) evaluates how strong a correlation is regardless of its nature. The results suggested that ORAC values of tannin sorghum grains were not correlated with their tannin contents significantly. The reason could be ORAC assay based on a different chemical reaction than total phenol and ABTS assays.

Table 2. Correlation of Condensed Tannin Content with Total Phenol Content, ABTS and ORAC Antioxidant Capacity among Tannin Sorghum Grains

Correlation coefficient		Phenol	ABTS	ORAC
Pearson's correlation	Tannins	0.837**	0.995**	0.601
Spearman's rho	Tannins	0.964**	0.988**	0.600

\*\* correlation was significant ( $p < 0.01$ ) (2-tailed)

Among non-tannin sorghum grains, content of 3-deoxyanthocyanins (the degree of pigmentation) had a strong linear correlation with total phenol content and ABTS antioxidant capacity (Table 3). This correlation indicated that 3-deoxyanthocyanin content was an indicator of antioxidant capacity in non-tannin sorghum grains. However, pigment content in non-tannin sorghum grains only correlated with ORAC antioxidant capacity moderately by nonparametric correlation (Spearman's rho 0.474,  $p < 0.05$ ). This suggested that pigmentation itself was not good enough to explain the differences observed in ORAC assay. Since the chemical reaction of ORAC assay is quite different from total phenol and ABTS assays, it is assumed that other sorghum phenols which are

colorless are able to show some free radical scavenging capacity in the ORAC assay. The antioxidant capacity of the colorless phenols may be underestimated by ABTS assay because they are not strong reducing agents or their reaction with ABTS is slow. Hence it is important to consider pigmentation as an indicator of antioxidant capacity but 3-deoxyanthocyanins are not the only group of antioxidants in non-tannin sorghum grains.

Table 3. Correlation of 3-Deoxyanthocyanin Content with Total Phenol Content, ABTS and ORAC Antioxidant Capacity among Non-Tannin Sorghum Grains

Correlation coefficient		Phenol	ABTS	ORAC
Pearson's correlation	3-DXA pigment	0.853 <sup>**</sup>	0.587 <sup>**</sup>	0.109
Spearman's rho	3-DXA pigment	0.624 <sup>**</sup>	0.674 <sup>**</sup>	0.474 <sup>*</sup>

3-DXA: 3-deoxyanthocyanin content

\* correlation was significant ( $p < 0.05$ ) (2-tailed)

\*\* correlation was significant ( $p < 0.01$ ) (2-tailed)

On the whole, total phenol content of all tested sorghum grains correlated with their ABTS antioxidant capacity (Pearson's correlation 0.911,  $p < 0.001$ ). Since the chemical mechanisms of these two assays are similar, the results are expected. Total phenol content also correlated with ORAC antioxidant value (Pearson's correlation 0.401,  $p < 0.028$ ) but not very strongly, which indicated that reducing potential was not the only mechanism for the antioxidant capacity measured by ORAC assay. The ORAC assay also measures radical chain-breaking potential of antioxidants (112). The ABTS antioxidant capacity had similar moderate correlation with ORAC value (Pearson's correlation 0.599,  $p < 0.001$ ). This may be due to the different chemical principles behind these assays.

The correlations observed among gross phenolic profiles suggested that certain groups of phenols were strong antioxidants, such as condensed tannins and 3-deoxyanthocyanins. The correlation between phenolic profile and antioxidant capacity indicated that phenols in sorghum can serve as indicators as antioxidant capacity.

### **Gross Phenolic Profiles and Antioxidant Properties of Crude Sorghum Extracts**

Extracts from natural sources, for example, fruits, grains, leaves, roots, can have wider applications than the source itself. Because extracts are concentrated and dried, they are easier to store, transport, package and use as additives, ingredients, and dietary supplements than the bulk original source. Hence it is important to analyze the properties of extracts compared with the original material.

#### *Gross Phenolic Profiles of Crude Sorghum Extracts*

Crude sorghum extracts presented similar phenolic profiles compared with respective sorghum grains (Table 4). Generally speaking, the extracts were 5- to 30-fold higher in condensed tannins, 3-deoxyanthocyanins, and total phenols than the grains.

Table 4. Phenolic Content and Free Radical Scavenging Capacity of the Crude Freeze-Dried Sorghum Extracts

Extract (grain type)	Tannins (mg CE/g) <sup>a</sup>	3-DXA (mg LE/g) <sup>b</sup>	Phenol content (mg GAE/g) <sup>c</sup>	ORAC ( $\mu$ mol TE/g) <sup>d</sup>	ABTS ( $\mu$ mol TE/g) <sup>d</sup>
White dent corn	nd	0.1 $\pm$ 0.08	20.4 $\pm$ 2.4	776 $\pm$ 46	358 $\pm$ 31
ATX635 $\times$ RTX436 (white)	nd	0.3 $\pm$ 0.01	24.9 $\pm$ 4.3	1232 $\pm$ 162	403 $\pm$ 37
KARI-Mtama (white)	nd	0.2 $\pm$ 0.05	48.3 $\pm$ 0.3	1470 $\pm$ 97	506 $\pm$ 49
Mizzou White (white)	nd	0.4 $\pm$ 0.02	26.2 $\pm$ 2.1	933 $\pm$ 34	276 $\pm$ 31
Mizzou (red)	nd	9.7 $\pm$ 1.2	81.5 $\pm$ 3.2	2050 $\pm$ 200	1060 $\pm$ 98
Tx2911 (red)	nd	3.8 $\pm$ 0.5	88.4 $\pm$ 5.2	4758 $\pm$ 509	2043 $\pm$ 564
98BRON155 (red)	nd	1.0 $\pm$ 0.4	49.9 $\pm$ 15	4383 $\pm$ 690	1261 $\pm$ 160
99LGWO50 (red)	trace	4.3 $\pm$ 0.5	103 $\pm$ 8.7	6284 $\pm$ 298	2226 $\pm$ 302
I CSV III (red)	trace	4.8 $\pm$ 0.4	87.9 $\pm$ 8.1	2280 $\pm$ 210	1190 $\pm$ 140
Tx430 (black)	trace	25.8 $\pm$ 2.4	92.2 $\pm$ 4.3	2910 $\pm$ 210	2790 $\pm$ 170
SC748 (lemon yellow)	nd	0.9 $\pm$ 0.02	58.5 $\pm$ 3.3	3635 $\pm$ 365	1405 $\pm$ 215
EBA-3 (lemon yellow)	nd	0.8 $\pm$ 0.05	66.8 $\pm$ 3.5	2822 $\pm$ 219	1187 $\pm$ 64
Black PI Tall (tannin)	881 $\pm$ 36	18.5 $\pm$ 2.5	241 $\pm$ 7.5	2958 $\pm$ 227	2879 $\pm$ 262
Cadam (tannin)	nd	4.0 $\pm$ 0.8	45.0 $\pm$ 2.5	2944 $\pm$ 418	777 $\pm$ 46
CR 35 (tannin)	nd	0.9 $\pm$ 0.2	69.5 $\pm$ 1.6	1670 $\pm$ 52	648 $\pm$ 13
Seredo (tannin)	253 $\pm$ 10	7.1 $\pm$ 2.7	169 $\pm$ 7.0	2970 $\pm$ 6.0	3310 $\pm$ 185
Sumac (tannin)	394 $\pm$ 15	16.3 $\pm$ 8.1	308 $\pm$ 13	6333 $\pm$ 697	4420 $\pm$ 270
Hi-Tannin (tannin)	605 $\pm$ 23	5.3 $\pm$ 0.5	251 $\pm$ 14	2630 $\pm$ 190	4320 $\pm$ 420

<sup>a</sup>CE; catechin equivalents (Vanillin-HCl method). <sup>b</sup>3-Deoxyanthocyanin pigment content expressed as luteolinidin equivalents. <sup>c</sup>GAE; gallic acid equivalents (Folin-Ciocalteu method). <sup>d</sup>TE; trolox equivalents (antioxidant activity). Values  $\pm$  sd reported three separate experiments; nd – not detected.

Condensed tannin content in tannin sorghum extracts varied vastly: the crude extracts from Cadam and CR35 did not contain detectable condensed tannins; the rest of the crude tannin sorghum extracts had 253-881 mg CE/g sample. The difference should

be from the genetics of tannin sorghums. According to the presence of a spreader gene SS, tannin sorghums can be divided into two groups, type II sorghum (recessive spreader gene) and type III sorghum (with spreader gene) (155). The extractability of condensed tannins in these two groups is different: tannins in type II sorghum can only be extracted with acidified methanol because acid is needed to disturb the structure of the vesicles in the pigmented testa where tannins are located (156). The extraction solvent used to obtain the extracts was 70% aqueous acetone; hence condensed tannins in Cadam and CR35 were not extracted in corresponding freeze-dried extracts. So Cadam and CR35 crude extracts were grouped into non-tannin sorghum extracts within the discussion here.

3-Deoxyanthocyanin contents in crude sorghum extracts followed similar trends as the grains (Tables 1 & 4). Tannin sorghum extracts contained varying amounts of 3-deoxyanthocyanins (5.3-18.5 mg LE/g sample) because pigment and condensed tannin contents are controlled by different genes (150). Red and black pericarp sorghum extracts contained high levels of 3-deoxyanthocyanins, for example, Tx430 (black) (25.8 mg LE/g), Black PI Tall (tannin) (18.5 mg LE/g), and Mizzou (red) (9.7 mg LE/g). White and lemon yellow pericarp sorghum extracts contained low levels of 3-deoxyanthocyanins (less than 1 mg LE/g sample). Dark red pericarp color of sorghum grains is associated with higher 3-deoxyanthocyanin contents in the crude extracts.

Total phenol content in tannin containing sorghum extracts (169-308 mg GAE/ g sample) was higher than non-tannin containing extracts (24.9-103 mg GAE/ g sample) ( $p < 0.001$ ) (Figure 5). Based on the total phenol contents of crude sorghum extracts, the

antioxidant capacity of crude sorghum extracts were expected to have similar trends as the grains.

The phenolic profile in extracts from different natural sources varies depending on the source and extraction solvent. Yang et al. (157) reported that total phenolics of 80% aqueous acetone extracts from common edible nuts ranged from 1.5-15.8 mg GAE/g extract. Chen and Blumberg (158) used both acidified methanol and gastrointestinal juice mimics to extract the phenols from almond skin. They found the total phenol content of the extracts was 15.5 mg and 2.5 mg GAE/g for the two methods, respectively. Ajila and others (159) reported the total phenols from 80% acetone extracts of mango peel was 54.7-109.7 mg GAE/g extract. Dai and others (160) extracted Hull blackberry with acidified ethanol and reported total phenol content was 17.3 mg GAE/g dried extract. Compared with these studies, sorghum grains have higher total phenols, which indicate potential to use sorghum extracts in the functional food industry.



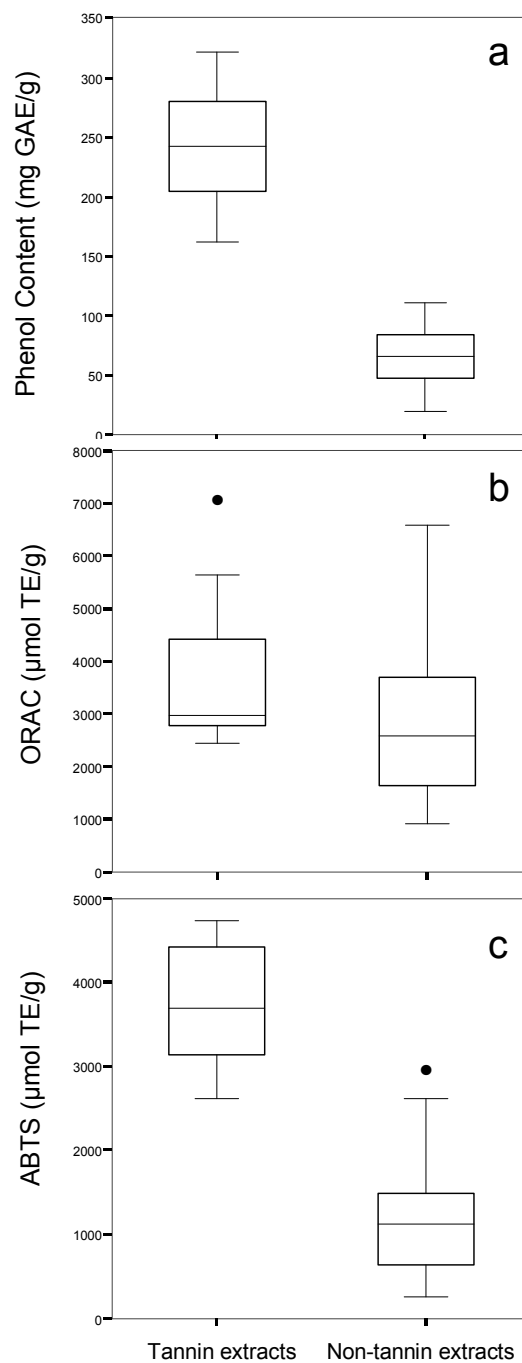


Figure 5. Box plot of total phenol contents (mg gallic acid equivalent/g sample) (a), ORAC (b) and ABTS (c) antioxidant capacity ( $\mu\text{mol Trolox equivalent/g sample}$ ) between crude tannin and non-tannin sorghum extracts. Crude sorghum extracts containing tannin had higher total phenol ( $p < 0.001$ ) and ABTS ( $p < 0.001$ ) antioxidant capacity than crude sorghum extracts which do not contain tannins, but the difference between ORAC values was not significant ( $p < 0.194$ ).

### *Antioxidant Capacity of Crude Sorghum Extracts*

The antioxidant capacity of crude sorghum extracts was 10 to 40 times higher than that of their grains. The antioxidant capacity measured by ABTS assay of tannin containing extracts ranged from 2879-4420  $\mu\text{mol TE/g}$  sample; while non-tannin containing extracts ranged from 276-2790  $\mu\text{mol TE/g}$  sample (Table 4). The trend was generally related to what was observed in sorghum grains.

The antioxidant capacity measured by ORAC assay of tannin containing extracts was from 2630 to 6333  $\mu\text{mol TE/ g}$  sample; while non-tannin containing extracts varied from 933- 6284  $\mu\text{mol TE/ g}$  sample (Table 4). The difference in ORAC values ( $p < 0.194$ ) between tannin containing and non-tannin containing crude sorghum extracts was not significant as total phenol content and ABTS antioxidant capacity ( $p < 0.001$ ). The possible reason was some other groups of phenol in non-tannin containing crude extracts were more efficient in the kinetic experimental settings using ORAC to evaluate antioxidant capacity. For example, the crude extract from 99LGWO50, a red thick pericarp sorghum with tan secondary plant color (109), showed ORAC value of 6284  $\mu\text{mol TE/g}$  sample, which is almost as high as Sumac (tannin) extracts (6333  $\mu\text{mol TE/g}$  sample). Also, crude extracts from Tx2911, 98BRON155, SC748 all showed relatively high ORAC values among non-tannin extracts, which was higher than the crude extract from Tx430 (black). This indicated that pigmentation alone can not explain the higher ORAC values in these crude sorghum extracts. Dykes (109) observed similar high antioxidant capacity trends among these sorghum grains. Further investigation into the phenolic profile found that flavanones, flavan-4-ols, and flavones content in

99LGWO50, Tx2911, 98BRON155, and SC748 sorghum grains were relatively high (109). Aqueous acetone extraction may concentrate these groups of phenols in the freeze-dried extracts.

Many studies report the antioxidant potential of extracts from different plant sources using varying methods; hence it is difficult to compare the antioxidant capacity values reported in literature. Yang et al. (157) reported that the antioxidant capacity of phenolic extracts from common nuts measured by Total Oxyradical Scavenging Capacity assay, which is similar to ORAC assay, ranged from 7.1-458.1  $\mu\text{mol}$  vitamin C equivalent/g. Chen and Blumberg (158) reported that ORAC value of almond skin extract was around 3.0  $\mu\text{mol}$  Trolox equivalent/  $\mu\text{mol}$  GAE. Dai and others (160) determined the antioxidant capacity of dried blackberry extract using ABTS was around 67.0  $\mu\text{mol}$  TE/g extract. By a rough comparison with similar studies, crude extracts from different sorghum grains exhibited relatively strong antioxidant potential. This characteristic makes crude sorghum extracts potent source of healthy food ingredients or dietary supplements.

#### *Correlation between Gross Phenolic Profile and Antioxidant Capacity*

Tannin content of crude sorghum extracts did not correlate with total phenol content, or ABTS or ORAC antioxidant capacity of crude tannin extracts (Table 5) though the correlation of tannin content was observed in tannin sorghum grains (Table 2). The results suggested that after 70% acetone extraction, removal of acetone, and freeze-drying, the phenolic profile of crude sorghum extracts was different than the

sorghum grains. Some phenols may be concentrated, and some may be lost during this process. Hence the impact of condensed tannins was not as great as it was in sorghum grains. Another possibility was small sample size. We only have 4 varieties of tannin sorghum included in this correlation. There may be a correlation if we could include more varieties.

Table 5. Correlation of Condensed Tannin Content with Phenol Content, ABTS, and ORAC Antioxidant Capacity among Crude Tannin Sorghum Extracts.

Correlation coefficient		Phenol	ABTS	ORAC
Pearson's correlation	Tannins	0.258	-0.302	-0.319
Spearman's rho	Tannins	0.310	-0.048	-0.238

Among crude non-tannin extracts, 3-deoxyanthocyanin content correlated with total phenol content, ABTS, and ORAC antioxidant capacity significantly (ORAC only significant for Spearman's rho correlation, Table 6). This correlation suggested that 3-deoxyanthocyanins were a major contributor to antioxidant capacity in the extracts. However, as mentioned above, some red pericarp sorghum (99LGWO50, Tx2911, and 98 BRON155) and lemon yellow pericarp sorghum contained other colorless phenols that also had strong antioxidant capacity.

Table 6. Correlation of 3-Deoxyanthocyanin Content with Phenol Content, ABTS, and ORAC Antioxidant Capacity among Crude Non-Tannin Sorghum Extracts.

Correlation coefficient		Phenol	ABTS	ORAC
Pearson's correlation	3-DXA pigments	0.519**	0.677**	0.098
Spearman's rho	3-DXA pigments	0.785**	0.703**	0.502**

\*\* correlation was significant ( $p < 0.01$ ) (2-tailed)

Overall, the total phenol content of all the crude sorghum extracts correlated significantly with ABTS and ORAC antioxidant capacity (Pearson's correlation 0.911,  $p < 0.001$  and 0.451,  $p < 0.007$ , respectively). The correlation suggested that phenols were the major components in the extracts that had antioxidant potential. Also, the two antioxidant assays correlated with each other (Pearson's correlation 0.570,  $p < 0.001$ ), which indicated that though the chemical basis is different in these two assays, the results reflected similar antioxidant potential of the crude extracts.

Based on the gross phenolic profile and antioxidant capacity of these seventeen sorghums, different genotypes presented varying phenolic profiles. The presence and amount of condensed tannins and 3-deoxyanthocyanins had major impact on antioxidant levels. Thus we studied how these two groups of compounds affect chemopreventive potential of sorghums. Capacity to scavenge free radicals such as ROS/RNS and carcinogens could minimize the oxidative damage to normal cells and reduce oxidative stress in the body (21). Hence sorghum varieties representing varying levels of 3-deoxyanthocyanins and condensed tannins were selected to further investigate how composition of sorghum affects chemopreventive potential on relevant cancer cells.

**CHAPTER V**

**PHASE II ENZYME INDUCING AND ANTIPROLIFERATIVE POTENTIAL OF  
CRUDE SORGHUM EXTRACTS**

**NAD(P)H:Quinone Oxidoreductase (QR) Inducing Capacity of Crude Sorghum  
Extracts**

Capacity of dietary phytochemicals to elevate the activity of phase II detoxifying enzymes in normal cells is an important cancer initiation blocking mechanism (45). The phase II enzyme inducing capacity of crude sorghum extracts should be a useful screening tool for determining chemopreventive potential of sorghum.

Nine sorghum varieties were used to measure the NAD(P)H:quinone oxidoreductase (QR) inducing potential. Four non-tannin sorghums were selected based on pericarp pigmentation: KARI-Mtama (white), Mizzou (red), I CSV III (red) and Tx430 (black). Five tannin sorghums included two type II tannin sorghum, Cadam and CR35; and three type III sorghum, Seredo, Sumac and Hi-Tannin. Because Hepa 1c1c7 cell growth was affected by the crude sorghum extracts, QR specific inducer ratio which was corrected for cell population was reported.

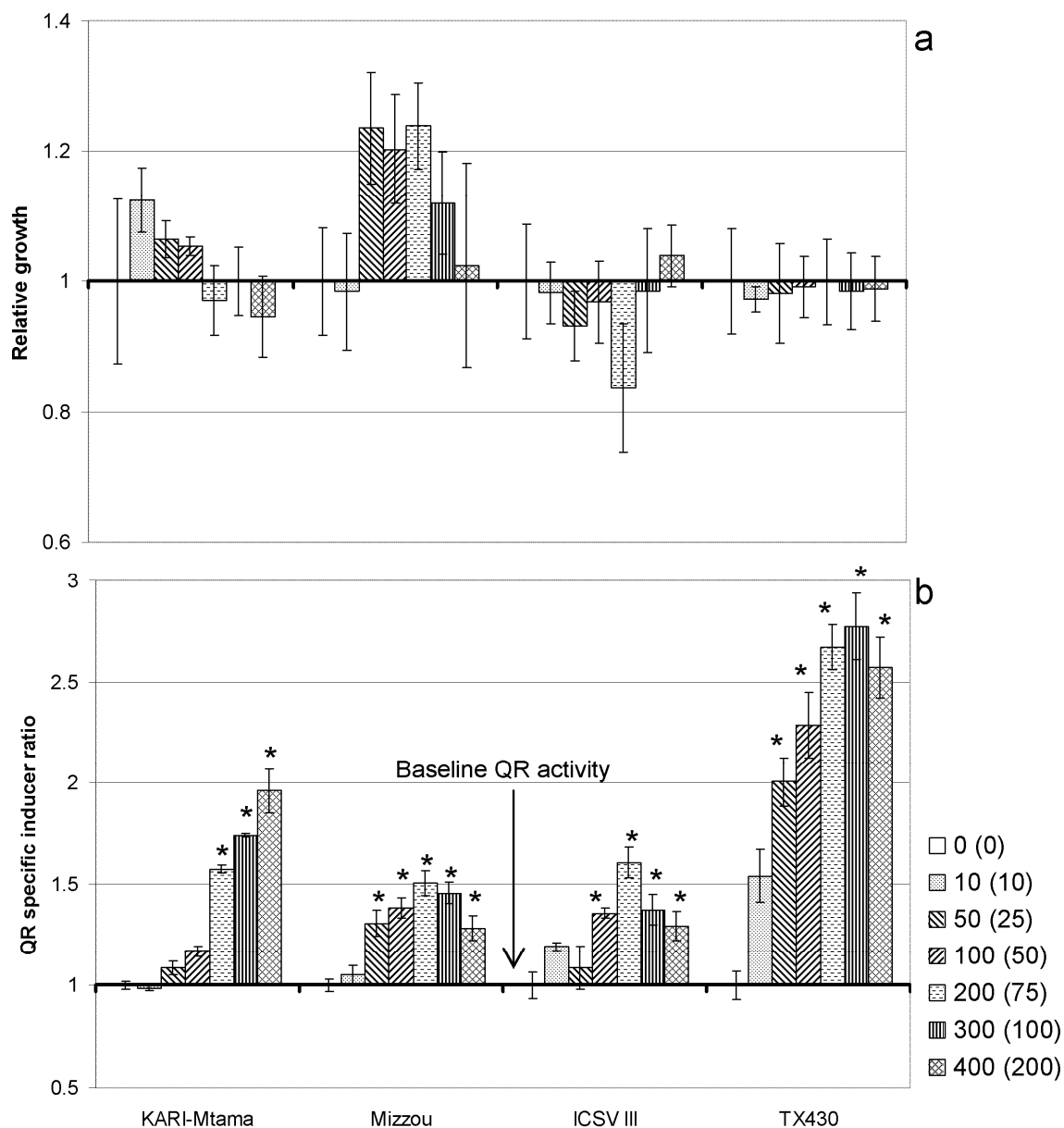


Figure 6. Effect of crude non-tannin sorghum extracts on (a) Hepa 1c1c7 murine hepatoma cells growth and (b) NAD(P)H:quinone oxidoreductase (QR) specific inducer ratio (after correction for cell population). Cells (10,000/well) were induced with extracts for 24 h before QR activity analysis. Legend represents concentrations ( $\mu\text{g/mL}$ ) of crude sorghum extracts used while concentrations of Tx430 (black) are shown in parentheses. Error bars represent  $\pm$  sd from two separate experiments. \* indicates significantly different QR inducer ratio from that in control cells ( $p < 0.05$ , Dunnett's multiple comparison, 2-sided).

All the tested crude non-tannin sorghum extracts showed a quadratic except KARI-Mtama (white) showed a dose-dependent QR inducing capacity (Figure 6). Tx430 (black) had the highest QR specific inducer ratio among the four varieties; it doubled QR activity at 25  $\mu\text{g}/\text{mL}$  and maximally increased QR activity 2.7-fold at 100  $\mu\text{g}/\text{mL}$ . Tx430 (black) did not show significant inhibition of Hepa 1c1c7 growth at the concentrations tested, which indicated that the QR activity induced by Tx430 (black) was independent of change in cell population. The Tx430 (black) extract contained the highest 3-deoxyanthocyanin contents (25.8 mg luteolinidin equivalent (LE)/ g of extract, Table 4) among all non-tannin sorghum extracts, which may partly explain its high QR inducing capacity.

Two red varieties (Mizzou and I CSV III) showed similar QR specific inducer ratio. Mizzou (red) maximized QR activity by 50% at 200  $\mu\text{g}/\text{mL}$ ; while I CSV III (red) increased QR activity by 60% at 200  $\mu\text{g}/\text{mL}$ . Mizzou (red) and I CSV III (red) extracts contained less 3-deoxyanthocyanin content than Tx430 (black) (9.6 and 4.8 mg LE/g, respectively). The difference in 3-deoxyanthocyanin contents could partly explain the different QR inducing potency. However composition of 3-deoxyanthocyanins and other flavonoids may also affect QR inducing potential in pigmented sorghum varieties.

Crude extract of white pericarp sorghum KARI-Mtama showed higher QR inducing potential than both red varieties; it increased QR activity by almost 60% at the concentration of 200  $\mu\text{g}/\text{mL}$  and almost doubled QR activity at 400  $\mu\text{g}/\text{mL}$ . This indicated that non-pigmented phenols in white sorghum likely induced QR activity in Hepa 1c1c7 cells.



The QR specific inducer ratio of five tannin sorghum is shown in Figure 7. The five tannin sorghum crude extracts did not show any QR activity inducing potential. Cadam increased QR activity by 20% maximally at 10  $\mu\text{g}/\text{mL}$  but this was not statistically significant; while CR35 increased QR activity by 40% maximally at 50  $\mu\text{g}/\text{mL}$ . Crude extracts from Cadam and CR35 did not contain detectable condensed tannins. Hence it is possible that other phenols which are also present in non-tannin sorghums were able to induce QR activity. Seredo and Sumac extracts did not increase any significant QR activity at concentrations tested; Hi-Tannin extracts exhibited a significant 20-30% QR activity increase at lowest concentration (1 and 5  $\mu\text{g}/\text{mL}$ ) but the QR activity decreased when concentration went up. An inhibitory effect of tannin containing sorghum extracts on Hepa 1c1c7 cells was observed when concentration was greater than 20  $\mu\text{g}/\text{mL}$ . This may partly explain why QR inducing potential was not seen in these three varieties. Another possibility is that condensed tannins can not induce QR activity or are inhibitors of QR activity.

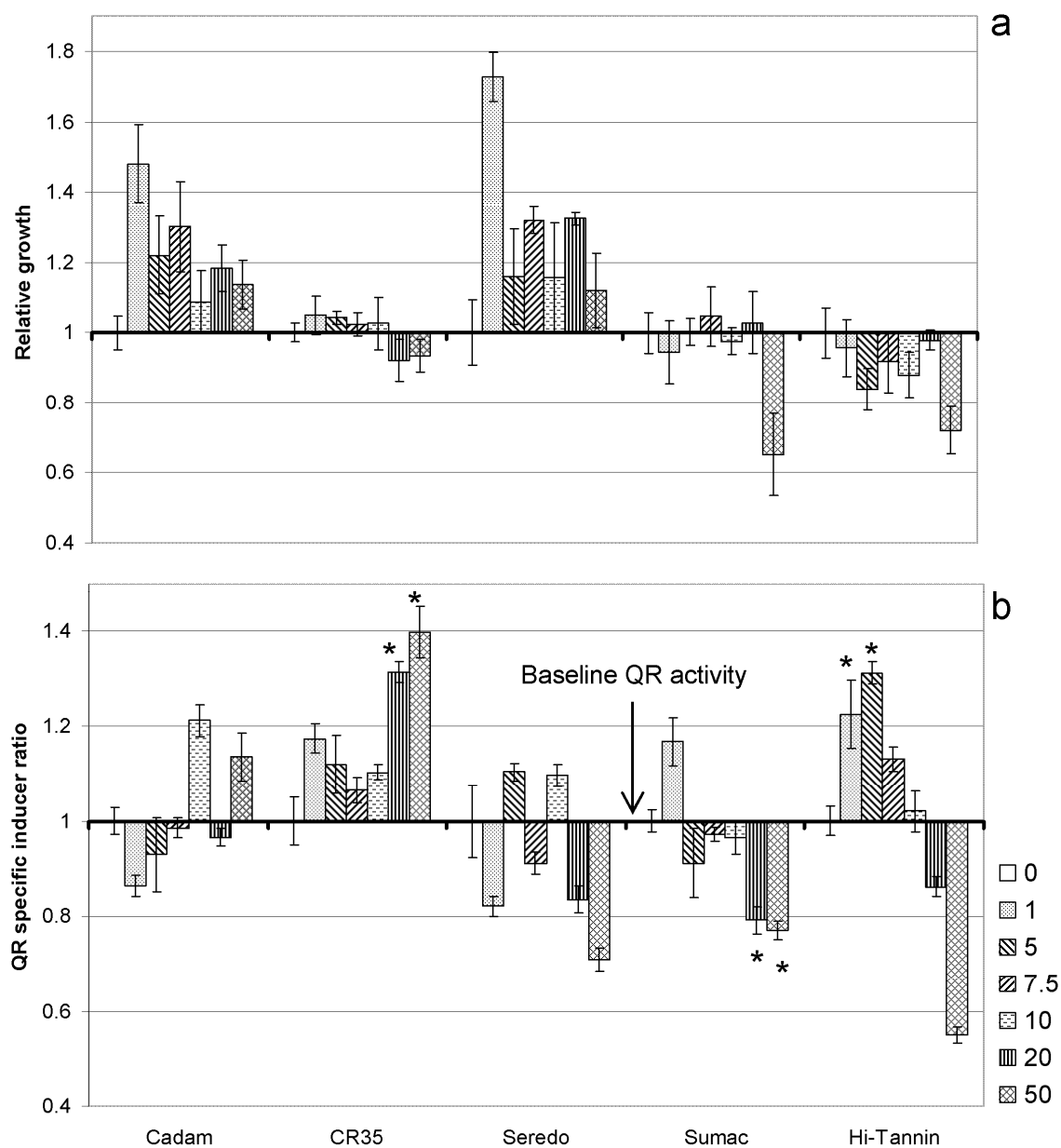


Figure 7. Effect of crude tannin sorghum extracts on (a) Hepa 1c1c7 murine hepatoma cells growth and (b) NAD(P)H:quinone oxidoreductase (QR) specific inducer ratio (after correction for cell population). Cells (10,000/well) were induced with extracts for 24 h before QR activity analysis. Legend represents concentrations ( $\mu\text{g/mL}$ ) of crude tannin sorghum extracts used. Error bars represent  $\pm$  sd from two separate experiments. \* indicates significantly different QR inducer ratio than that in control cells ( $p < 0.05$ , Dunnett's multiple comparison, 2-sided).

Among the nine sorghum varieties tested, QR specific inducer ratio varied according to their compositions: condensed tannins containing crude sorghum extracts were non-inducers. Among non-tannin sorghum crude extracts, Tx430 (black), a black pericarp sorghum containing the highest level of 3-deoxyanthocyanins, was the most potent QR inducer; red pericarp sorghum crude extracts were less efficient QR inducers; the QR inducing potential of white sorghum was better than the red ones but not better than the black variety. However, taking all the sorghum varieties into account, there was no correlation between QR inducing potential and total phenol content or antioxidant capacity of the crude sorghum extracts. Condensed tannin containing crude extracts had higher total phenol content and antioxidant capacity but no QR inducing potential; white sorghum extracts contained less total phenols with less antioxidant capacity but showed a moderate QR inducing capacity.

Yang and Liu (161) reported that acetone extracts from different varieties of fresh grapes were potent QR inducers with QR activity doubling concentration of 600-800  $\mu\text{g}/\text{mL}$  using Hepa 1c1c7 cells. Similarly, the authors suggested that there was no positive correlation found between QR induction and antioxidant capacity of these varieties of grapes. Additionally, some white varieties of grapes had higher QR induction potential than red varieties, which indicated that pigmentation of grapes was not correlated with QR inducing potential. Chen and Blumberg (158) used acidified methanol and gastrointestinal juice mimics extracted almond skin extracts to induce QR in Hepa 1c1c7 cells and found the two extracts behaved differently: acidified methanol extract was a QR inducer while gastrointestinal juice extract was QR inhibitor.

Srivastava et al. (162) did not find any phase II enzyme inducing potential from anthocyanin fractions of blueberry extracts. This indicated that activation of the cellular upregulation of phase II enzymes does not necessarily relate to higher antioxidant capacity but is more dependent on structure, steric bulk and partition coefficient (lipophilicity) (163) of a compound.

Sorghum is a rich source of different groups of phenolic compounds. It is apparent that different groups of phenols have different effects on phase II enzymes. Hence it is beneficial to determine the effect of different fractions of sorghum phenols on phase II enzyme inducing potential, such as 3-deoxyanthocyanins, flavones and flavanones, to obtain a complete picture for how phenolic profiles of various sorghum varieties may affect phase II enzyme inducing potential.

### **Inhibitory Effect of Sorghum Crude Extracts on Human Colon and Esophageal Carcinoma Cell Proliferation**

The capacity to inhibit cancer cell proliferation is an important aspect of chemoprevention (45). Seven crude sorghum extracts were selected: three condensed tannin containing crude extracts from tannin sorghums, Seredo, Sumac and Hi-Tannin; four crude extracts from non-tannin sorghums, KARI-Mtama (white), Mizzou White (white), Mizzou (red) and Tx430 (black). The two assays used in this study (MTT and PicoGreen assay) generally showed the same inhibitory trends for the extracts tested. However, we noticed that MTT assay consistently estimated higher viable cell population than the PicoGreen assay in preliminary tests. We suspected this was because

of the interference of the phenols present in the extracts. MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals. The number of surviving cells is directly proportional to the level of the formazan product created (129). However, phenols in sorghum may reduce MTT and form the dark blue color without the presence of viable cells. The color interference due to the extracts resulted in higher false reading. Blank sample using only the extracts were used to correct for the interference from phenols.

The inhibitory effect of the seven selected crude sorghum extracts generally followed a dose-response pattern but with different inhibitory endpoints (Figure 8 & 9). The inhibitory pattern of these extracts on both human colon cancer (HT-29) and esophageal cancer (OE33) cell lines was similar. In order to further compare the inhibitory effect of sorghum extracts,  $IC_{50}$ s (Table 7), a parameter estimates the concentration for a compound needed to inhibit 50% of cancer cell proliferation, were calculated. A lower  $IC_{50}$  indicated a stronger inhibitory capacity.

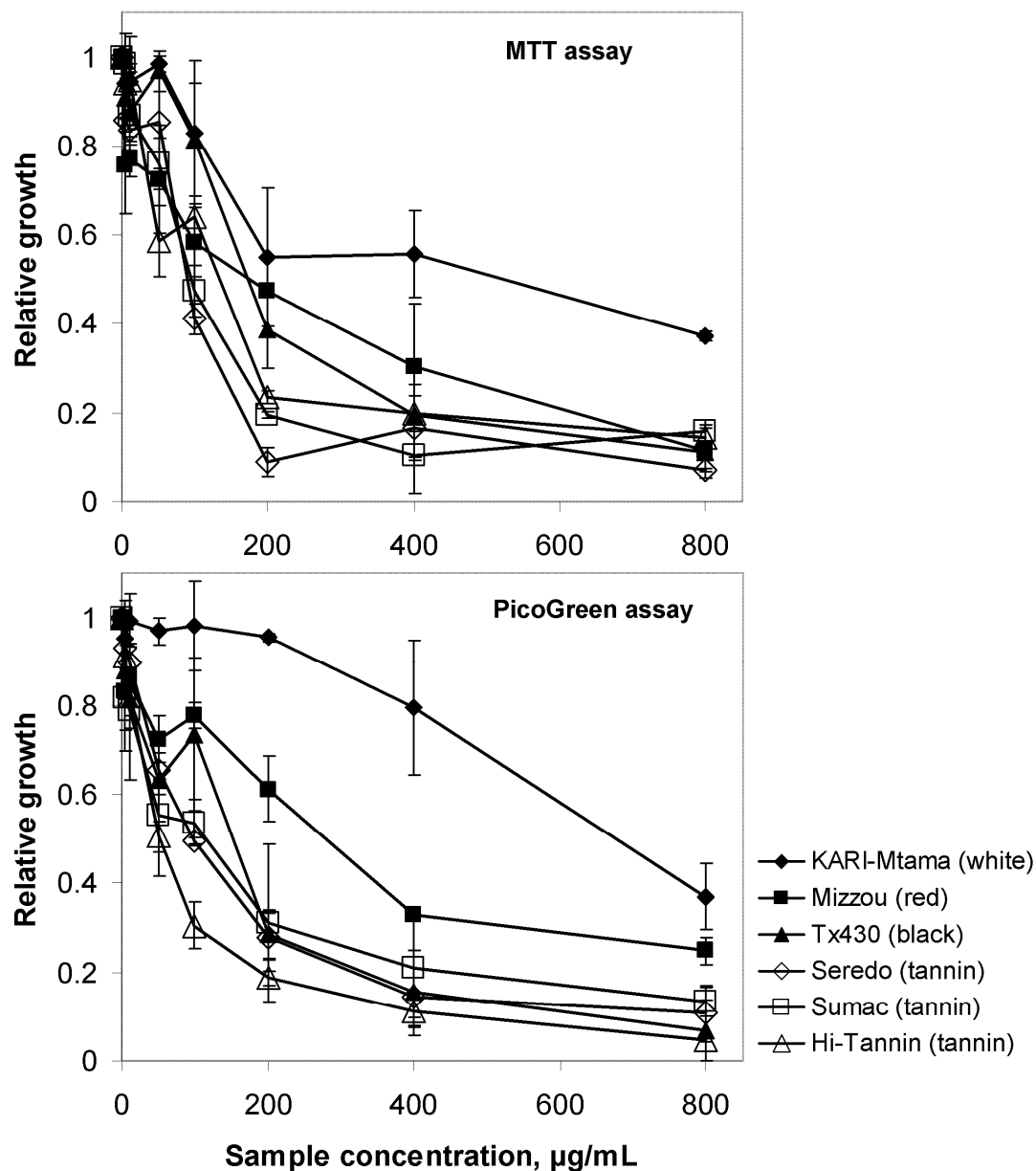


Figure 8. Inhibition of HT-29 human colon carcinoma cell proliferation by crude sorghum extracts (baseline to 0.1% DMSO) measured by MTT (top) and PicoGreen (bottom) assays. Cells (2000/well) were induced with extracts for 48h before proliferation assay. Error bars represent standard deviation from triplicates.

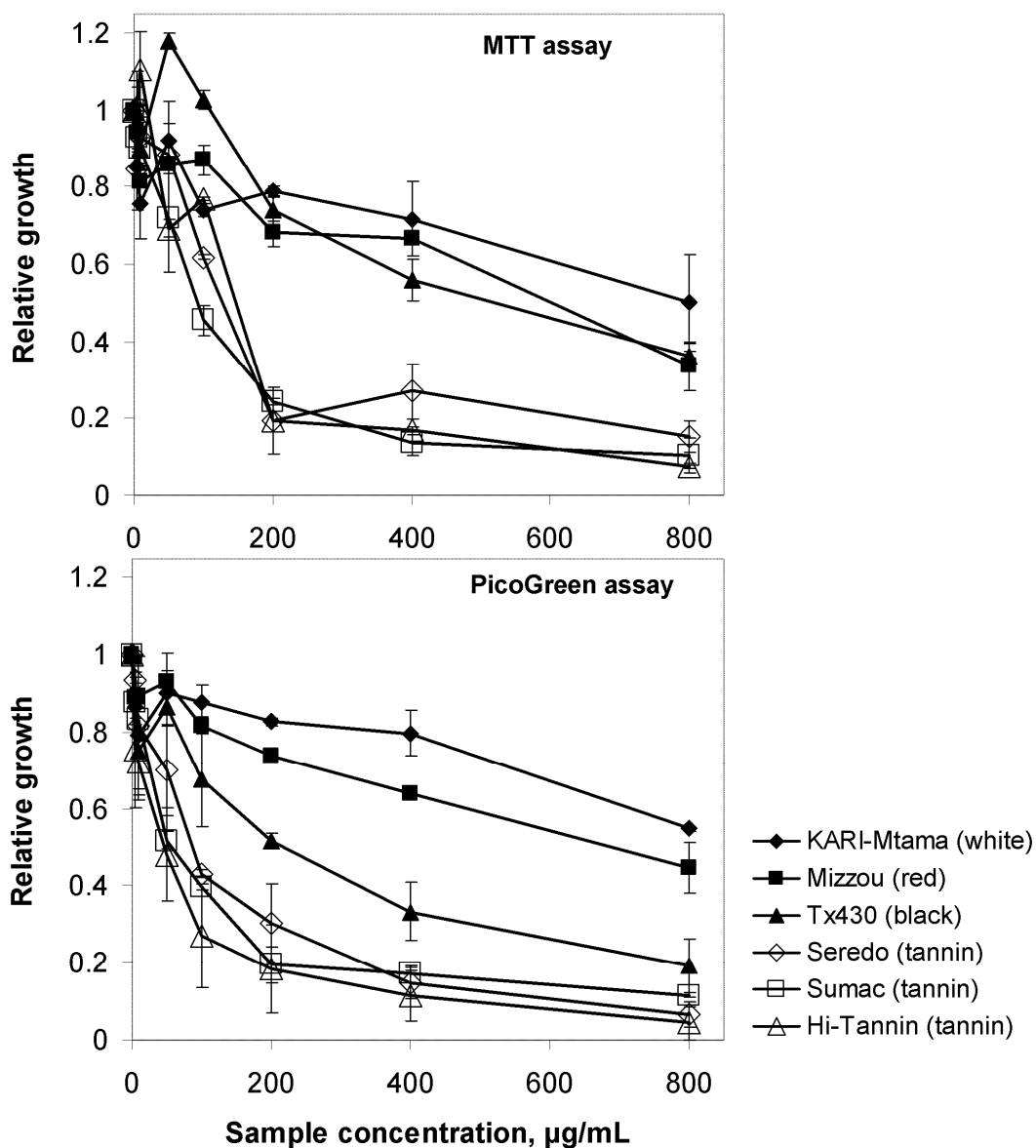


Figure 9. Inhibition of OE33 human lower esophageal adenocarcinoma cell proliferation by crude sorghum extracts (baseline to 0.1% DMSO) measured by MTT (top) and PicoGreen (bottom) assays. Cells (2000/well) were induced with extracts for 48h before proliferation assay. Error bars represent standard deviation from triplicates.

Table 7. Concentration ( $\mu\text{g/mL}$ ) of Crude Sorghum Extracts Needed to Inhibit 50% of HT-29 (colon) and OE33 (lower esophageal) Carcinoma Cell Proliferation ( $\text{IC}_{50}$ )

Sample (type)	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )			
	HT-29		OE33	
	MTT	PicoGreen	MTT	PicoGreen
KARI-Mtama (white)	$452 \pm 111^b$	$635 \pm 180^b$	$824 \pm 204^b$	$883 \pm 13.8^d$
Mizzou White (white)	$608 \pm 108^b$	$611 \pm 19.8^b$	$701 \pm 44.3^{a,b}$	$747 \pm 57.1^c$
Mizzou (red)	$190 \pm 0.3^a$	$262 \pm 50.1^a$	$604 \pm 57.8^{a,b}$	$553 \pm 18.4^b$
Tx430 (black)	$164 \pm 22.9^a$	$141 \pm 10.9^a$	$458 \pm 58.9^a$	$208 \pm 7.3^a$
<b>Overall mean (non-tannin)</b>	<b>354</b>	<b>412</b>	<b>647</b>	<b>598</b>
Seredo (tannin)	$85.0 \pm 4.7^a$	$99.0 \pm 1.4^b$	$131 \pm 1.9^b$	$88.0 \pm 1.6^b$
Sumac (tannin)	$95.6 \pm 10.3^a$	$59.1 \pm 18.6^{a,b}$	$91.9 \pm 6.6^a$	$51.9 \pm 2.4^{a,b}$
Hi-Tannin (tannin)	$130 \pm 3.0^b$	$50.6 \pm 3.3^a$	$125 \pm 13^b$	$49.7 \pm 11.4^a$
<b>Overall mean (tannin)</b>	<b>104</b>	<b>69.6</b>	<b>118</b>	<b>63.1</b>

Cells (2000/well) were induced with extracts for 48h before proliferation assay. Values  $\pm$  sd ( $\mu\text{g/mL}$ ) reported from three separate experiments. Tannin sorghum extracts had significantly lower  $\text{IC}_{50}$ s than non-tannin sorghum extracts ( $p < 0.05$ ). Within tannin and non-tannin sorghum extract groups, values with different letters within the same column were different (Fisher's LSD,  $p < 0.05$ ).

Tannin sorghum extracts inhibited almost 80% of both HT-29 and OE33 growth at around 200  $\mu\text{g/mL}$  and reached 90-95% inhibition at 800  $\mu\text{g/mL}$ . The  $\text{IC}_{50}$ s of tannin sorghum extracts for HT-29 ranged from 50.6-130  $\mu\text{g/mL}$  and 49.7-131  $\mu\text{g/mL}$  for OE33. Though the condensed tannin content in these three extracts differed from each other (Table 4), the inhibitory effect was generally similar. The black sorghum Tx430 showed the strongest inhibition potential among the three non-tannin sorghums, with



IC<sub>50</sub>s of 141-458 µg/mL (though statistical analysis was not significant on all assays). It inhibited HT-29 growth by more than 90% at 800 µg/mL, which was similar endpoint inhibition as the tannin sorghum extracts; it inhibited approximately 70% of OE33 growth at 800 µg/mL. The Mizzou (red) had IC<sub>50</sub>s from 190-604 µg/mL, and it achieved maximal inhibition of HT-29 (about 65% inhibition) and OE33 (about 55% inhibition) growth at 800 µg/mL. The two white varieties KARI-Mtama and Mizzou White had similar IC<sub>50</sub>s from 452 to 883 µg/mL. Both of them achieved 60% inhibition against HT-29 growth at 800 µg/mL and around 46% inhibition of OE33 proliferation at the same concentration.

Tannin sorghum extracts were stronger GIT cancer cell proliferation inhibitors than non-tannin sorghum extracts. For both MTT and PicoGreen assays, crude tannin sorghum extracts had significantly lower IC<sub>50</sub>s than crude non-tannin sorghum extracts ( $p < 0.05$ ) (Figure 10). Condensed tannins present in sorghum were probably responsible for the higher cancer cell proliferation inhibition. McDougall and others (164) determined the antiproliferative capacity of anthocyanin-rich and tannin-rich fractions of lingonberry extracts and concluded that tannin-rich fraction had a stronger inhibitory potential than anthocyanin-rich fraction.

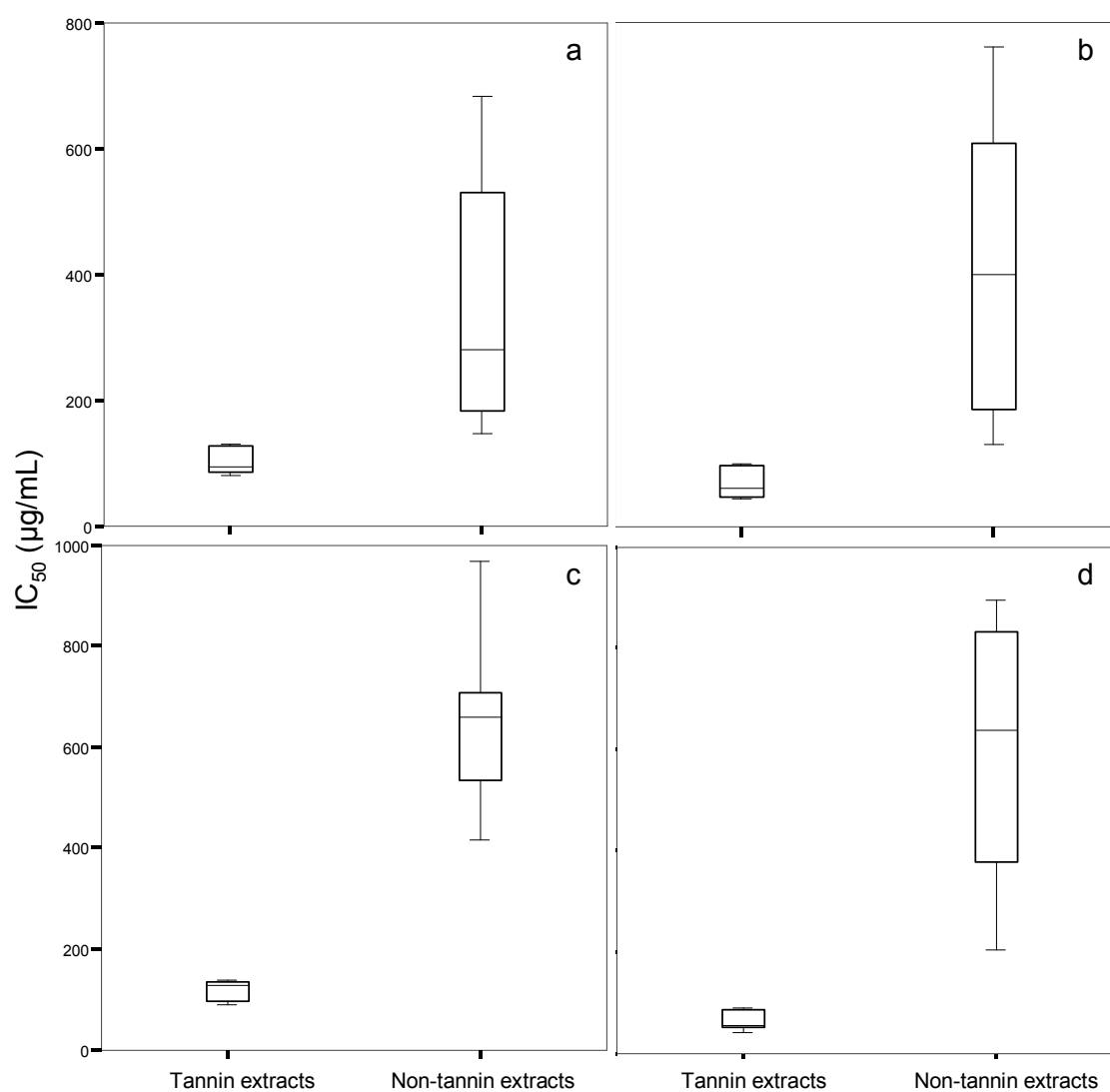


Figure 10. Box plot of IC<sub>50</sub>s (µg/mL) (concentration needed to inhibit 50% of cell growth) of crude tannin and non-tannin sorghum extracts, (a) IC<sub>50</sub>s on HT-29 proliferation measured by MTT assay; (b) IC<sub>50</sub>s on HT-29 proliferation measured by PicoGreen assay; (c) IC<sub>50</sub>s on OE33 proliferation measured by MTT assay; (d) IC<sub>50</sub>s on OE33 proliferation measured by PicoGreen assay. Crude tannin sorghum extracts had lower IC<sub>50</sub>s than crude non-tannin sorghum extracts (all p-values < 0.05).

Due to the stronger inhibition observed in Tx430 (black) and Mizzou (red) extracts among non-tannin sorghum extracts, 3-deoxyanthocyanins may have

contributed to the inhibitory potential among non-tannin pigmented sorghum varieties. Shih et al. (19) indicated aglycones of 3-deoxyanthocyanins, apigeninidin and luteolinidin, had stronger antiproliferative capacity than their anthocyanidin analogs. Additionally, non-pigmented sorghum flavonoids could have inhibitory effect against GIT cancer cell growth based on the inhibitory capacity observed in white sorghum extracts.

Overall, all sorghum extracts tested had relative strong inhibition capacity against HT-29 growth ( $IC_{50}$ s 50.6-635  $\mu\text{g/mL}$ ) than other natural phenol extracts reported in literature. Yi et al. (165) observed that crude extracts from various muscadine grapes had  $IC_{50}$ s of 1000-7000  $\mu\text{g/mL}$  (48 h incubation) against HT-29 growth measured by MTT assay. Netzel and others (166) reported that anthocyanin fraction from black carrots had an  $IC_{50}$  greater than 1000  $\mu\text{g/mL}$  (48 h incubation) against HT-29 growth using MTT assay. This suggests that compounds in sorghum extracts may have stronger cancer growth inhibitory potency than those found in other resources, which agrees with Shih et al. (19).

The antiproliferative effect of sorghum extracts was correlated with phenolic profiles and antioxidant properties. The  $IC_{50}$ s had a negative correlation with ABTS values (Pearson's correlation 0.778-0.941), total phenol content (Spearman's rho 0.844-0.944) and ORAC values of the crude extracts (Spearman's rho 0.713-0.867) ( $p$  all less than 0.05) (Table 8). Yang et al. (157) determined the antiproliferative capacity of crude extracts from nuts and concluded that inhibitory potency was correlated with phenolics and flavanoids contents as well as total antioxidant capacity of the extracts. By this

token, we can use phenol content and antioxidant capacity of a crude sorghum extract as an indicator of its antiproliferative potential. However, the correlation between antiproliferation and antioxidant potential of crude extract can be misleading because different groups of phenols may have different impact on antioxidant potential. For example, tannins are stronger hydrogen donors (94, 152, 153), but tannin content itself did not correlate with the antioxidant capacity of crude tannin extracts (Table 5). So phenolic composition should be considered when correlate phenol content and antioxidant potential, further when correlate antioxidant and antiproliferation potential. Thus we may only conclude that phenol composition affects GIT cancer growth by this correlation.

Table 8. Correlation between IC<sub>50</sub>s and Chemical Properties of Crude Sorghum Extracts

Correlation coefficient		IC <sub>50</sub> (μg/mL)			
		HT-29		OE33	
		MTT	PicoGreen	MTT	PicoGreen
Pearson's Correlation	ABTS	-0.778**	-0.839**	-0.904**	-0.941**
	ORAC	-0.666*	-0.652*	-0.759**	-0.709**
	Phenol content	-0.659*	-0.705*	-0.849**	-0.791**
Spearman's rho	ABTS	-0.820**	-0.944**	-0.860**	-0.965**
	ORAC	-0.841**	-0.713**	-0.867**	-0.727**
	Phenol content	-0.844**	-0.937**	-0.923**	-0.944**

\*correlation is significant at the 0.05 level (2-tailed).

\*\*correlation is significant at the 0.01 level (2-tailed).

For sorghum, separation of the crude extract into different phenol fractions would determine the role of each group of phenol in chemoprevention. Identification of the specific active components is also needed to determine how structure of these components affects the chemopreventive capacity. Based on this information, mechanisms involved in chemoprevention of sorghum phenols would be determined, i.e. mechanisms for inducing phase II enzymes; mechanisms for inhibit cancer cell proliferation, to see if apoptosis is induced or cell cycle is arrested. This will open up opportunities to utilize sorghum as a rich and economical source of ingredients for health-related industries.

## CHAPTER VI

### EFFECT OF 3-DEOXYANTHOCYANIDIN STRUCTURE ON PHASE II ENZYME INDUCING AND ANTIPROLIFERATIVE POTENTIAL

Uda et al. (167) reported that among natural flavonoids, structure had an impact on the capacity to induce phase II enzyme, NAD(P)H: quinone oxidoreductase (QR). They suggested a 2-3 double bond on the C-ring was essential to induce QR; i.e. catechin was not QR inducer but apigenin was. Shih et al. (19) reported that aglycones of 3-deoxyanthocyanins exhibited stronger antiproliferative capacity than their anthocyanidin analogs. Additionally, relatively strong QR inducer and antiproliferative potential of crude extract from Tx430 (black) sorghum made it essential to determine effect of structure on the chemopreventive potential of the unique 3-deoxyanthocyanins. Hence two groups of major sorghum 3-deoxyanthocyanin aglycones, apigeninidin and luteolinidin and their methoxylated derivatives, were used to determine the effect of 3-deoxyanthocyanidin structure on their QR inducing and human colon and esophageal cancer cell proliferation inhibition capacity.

#### **Effect of 3-Deoxyanthocyanidin Structure on Antioxidant Capacity**

The antioxidant capacity of 3-deoxyanthocyanidins is shown in Table 9. No meaningful correlation between structure and ABTS values was observed. The possible reason is antioxidant capacity of individual molecules is affected by various reaction factors, such as solvent system, reaction time, probes, protocols, etc. As to ORAC

values, methoxylation reduced ORAC antioxidant capacity for both apigeninidin and luteolinidin.

Table 9. Free Radical Scavenging (Antioxidant) Capacity of 3-Deoxyanthocyanidins. Results Were Expressed as mol Trolox Equivalent (TE)/ mol Sample.

	ORAC (mol TE/ mol sample)	ABTS (mol TE/ mol sample)
Apigeninidin	7.4 ± 0.7 <sup>a</sup>	4.0 ± 0.2 <sup>a,b,c</sup>
7-Methoxyapigeninidin	4.7 ± 0.9 <sup>a,b</sup>	3.7 ± 0.1 <sup>b,c</sup>
5-Methoxyapigeninidin	2.9 ± 0.4 <sup>b</sup>	2.6 ± 0.02 <sup>d</sup>
5,7-Dimethoxyapigeninidin	2.9 ± 1.0 <sup>b</sup>	3.6 ± 0.1 <sup>c</sup>
Luteolinidin	7.6 ± 1.0 <sup>a</sup>	4.3 ± 0.1 <sup>a,b</sup>
5,7-Dimethoxyluteolinidin	4.4 ± 0.6 <sup>a,b</sup>	4.6 ± 0.1 <sup>a</sup>

Mean ± Standard deviation was reported from three separate replicates. Within columns, values with different letters are different,  $p < 0.05$  (Turkey's HSD).

It has been reported that structure has an important impact on antioxidant capacity of flavonoids. Hydroxylation, especially *ortho*-(3',4'-) dihydroxyl group on the B-ring, enhances free radical scavenging capacity and metal chelating capacity (154, 168, 169). On the other hand, hydroxylation or methoxylation on the A-ring could decrease or increase antioxidant capacity (154, 168, 169). Our study found that methoxylated luteolinidin and apigeninidin derivatives generally had lower antioxidant capacity than non-methoxylated aglycones. We also observed that ORAC assay measured a bigger difference between apigeninidin/luteolinidin and their methoxylated derivatives than ABTS assay. The different chemical principle of the two assays may contribute to this difference observed. More 3-deoxyanthocyanins standards are needed

in order to determine the effect of different substitution pattern on their antioxidant capacity.

### **Effect of 3-Deoxyanthocyanidin Structure on NAD(P)H:Quinone Oxidoreductase (QR) Inducing Capacity**

The QR specific inducer ratios of apigeninidin and its derivatives are shown in Figure 11. Apigeninidin doubled QR activity at 20  $\mu\text{M}$ , which was also its maximal inducer ratio. 7-Methoxyapigeninidin showed higher maximal QR inducing potential, almost 2.7-fold increase at 50  $\mu\text{M}$ . 5-Methoxyapigeninidin maximally increased QR activity by 70% at 50  $\mu\text{M}$ . 5,7-Dimethoxyapigeninidin had the strongest inducing potency among apigeninidin derivatives: doubled the QR activity at 0.2  $\mu\text{M}$ ; and increased to 3-fold at 5  $\mu\text{M}$ .

The QR inducer ratio of luteolinidin and its derivatives are shown in Figure 12. Luteolinidin did not show significant increase of induced QR activity at tested concentrations. 5,7-Dimethoxyluteolinidin on the other hand increased QR activity by 2.4-fold at 50  $\mu\text{M}$  and maximally 4.2-fold at 100  $\mu\text{M}$ . We noticed some pure compounds used in this study, besides 5,7-dimethoxyluteolinidin, 7-methoxyapigeninidin, sulforaphane and genistein, inhibited Hepa 1c1c7 growth at most concentrations tested. It suggested that the upregulated QR activity may be due to the potency of those compounds or the stress from inhibition of proliferation. The QR activity can be induced in response of xenobiotics, antioxidants, oxidants, heavy metals, UV, light, stress conditions and ionizing radiation (170-172), and its activity is also upregulated in



malignant tissues (170). Hence the upregulated QR activity observed in this study may come from the effect of inhibited proliferation, not merely the direct inducing potency of the compound. Sulforaphane, the known QR inducer isolated from broccoli, doubled the QR activity between 0.2-0.5  $\mu\text{M}$  and had a maximal QR activity increase of 4.2-fold at 10  $\mu\text{M}$  (Figure 13), which confirmed to Prochaska's findings (123). Genistein, the isoflavone isolated from soybean, doubled QR activity at 10  $\mu\text{M}$  and had a maximal QR specific inducer ratio of 3.7 at 50  $\mu\text{M}$  (Figure 13), which agreed with what Yang and Liu reported (161).

The dimethoxylated forms of 3-deoxyanthocyanin aglycones had equivalent maximal QR specific inducer ratio as sulforaphane and genistein tested under the same experimental conditions. Gao and others (124) reported that nitric oxide-donating aspirin, the most extensively studied nitric oxide-donating non-steroidal anti-inflammatory drug, increased QR activity by 2.3-fold maximally at 100  $\mu\text{M}$ . Yang and Liu (161) reported only quercetin and resveratrol exhibited QR inducing capacity among common grape flavonoids, with maximal 3.5-fold increase at 30  $\mu\text{M}$  and 2.2-fold at 50  $\mu\text{M}$ , respectively. This suggested that dimethoxylated apigeninidin and luteolinidin have strong potential as chemopreventive agents.

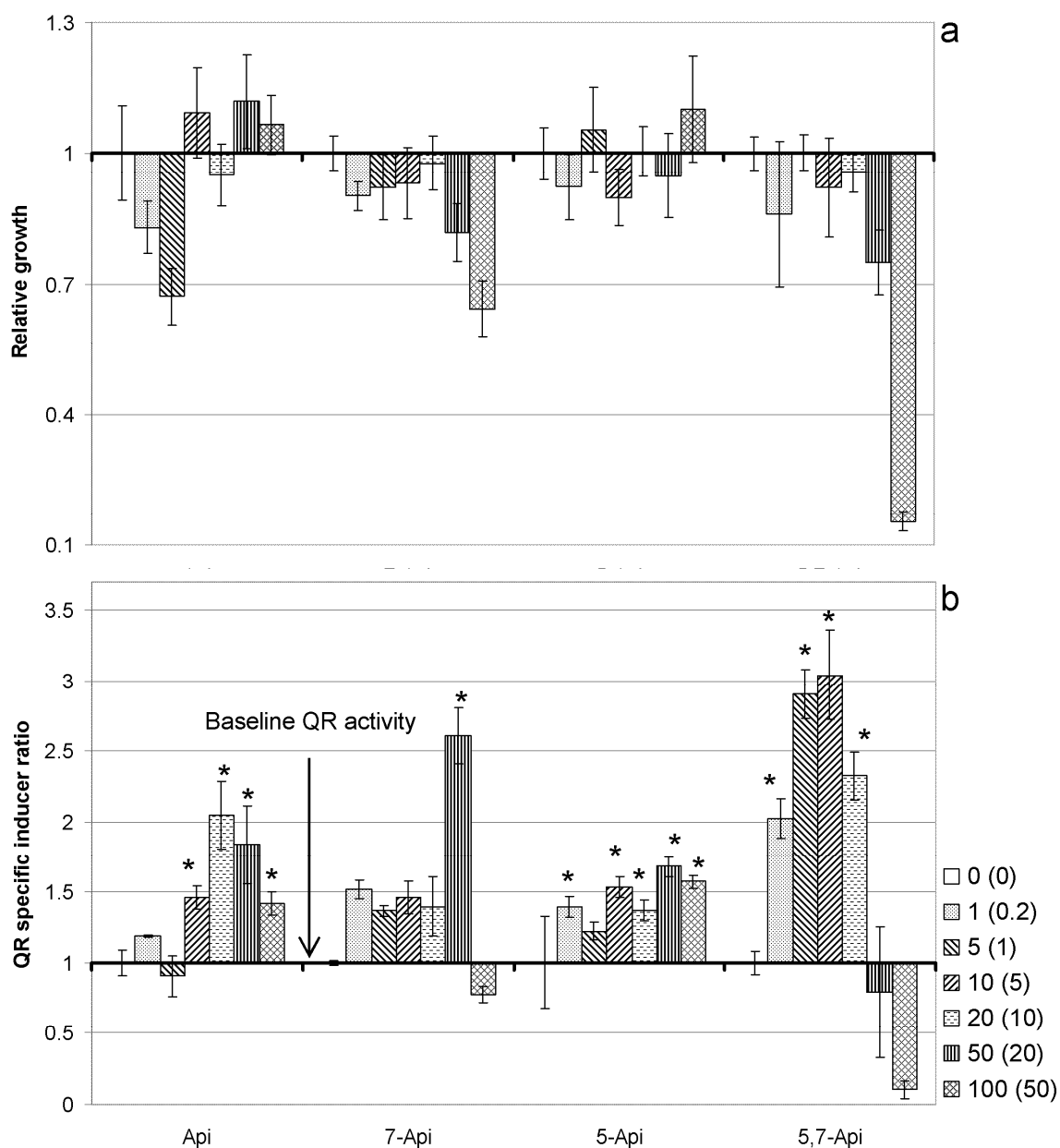


Figure 11. Effect of apigeninidin and its derivatives on (a) Hepa 1c1c7 murine hepatoma cells growth and (b) NAD(P)H:quinone oxidoreductase (QR) specific inducer ratio (after correction of cell population). Cells (10,000/well) were induced with extracts for 24 h before QR activity analysis. Legend represents concentrations ( $\mu\text{M}$ ) used while concentrations of 5,7-dimethoxyapigeninidin are shown in parentheses. Error bars represent  $\pm$  sd from two separate experiments. \* indicates significantly different QR inducer ratio than that in control cells ( $p < 0.05$ , Dunnett's multiple comparison, 2-sided).

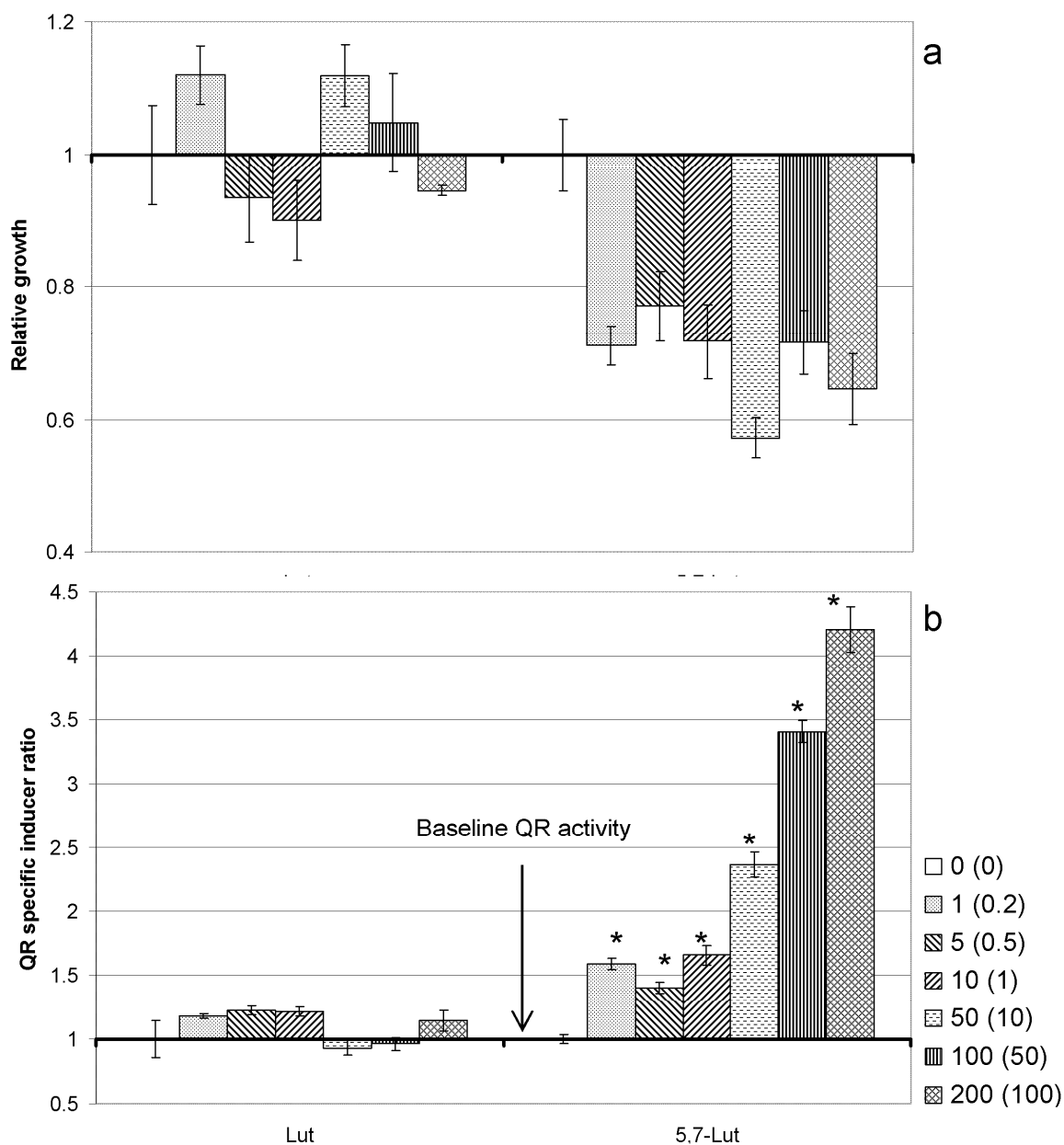


Figure 12. Effect of luteolinidin and 5,7-dimethoxyluteolinidin on (a) Hepa 1c1c7 murine hepatoma cells growth and (b) NAD(P)H:quinone oxidoreductase (QR) specific inducer ratio (after correction of cell population). Cells (10,000/well) were induced with extracts for 24 h before QR activity analysis. Legend represents concentrations ( $\mu\text{M}$ ) used while concentrations of 5,7-dimethoxyluteolinidin are shown in parentheses. Error bars represent  $\pm$  sd from two separate experiments. \* indicates significantly different QR inducer ratio than that in control cells ( $p < 0.05$ , Dunnett's multiple comparison, 2-sided).

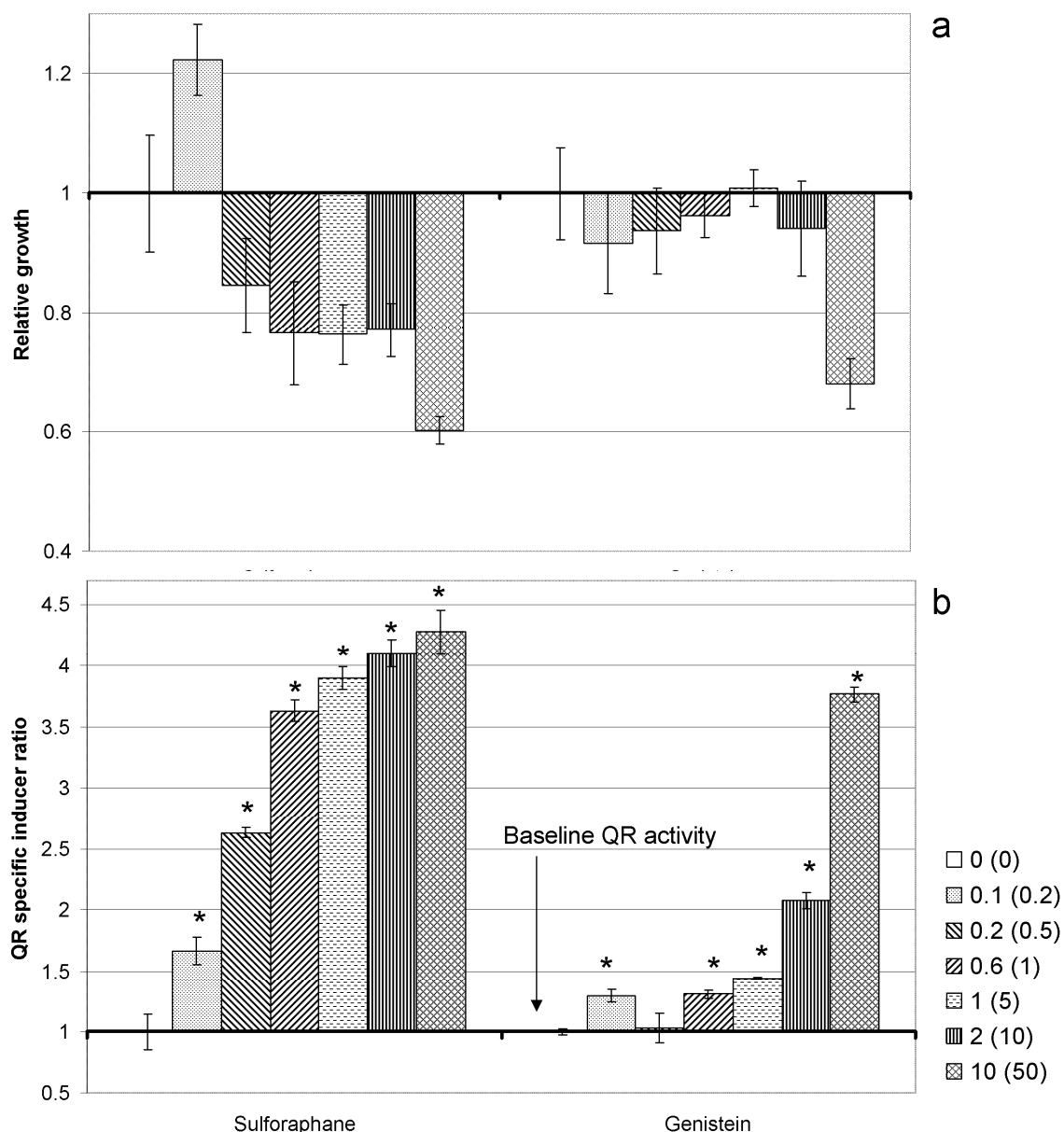


Figure 13. Effect of sulforaphane and genistein on (a) Hepa 1c1c7 murine hepatoma cells growth and (b) NAD(P)H:quinone oxidoreductase (QR) specific inducer ratio (after correction of cell population). Cells (10,000/well) were induced with extracts for 24 h before QR activity analysis. Legend represents concentrations ( $\mu\text{M}$ ) used while concentrations of genistein are shown in parentheses. Error bars represent  $\pm$  sd from two separate experiments. \* indicates significantly different QR inducer ratio than that in control cells ( $p < 0.05$ , Dunnett's multiple comparison, 2-sided).

The findings indicated a structure-activity relationship of QR inducing capacity of 3-deoxyanthocyanidins: methoxylation of the A-ring improved QR inducing capacity; position of methoxylation (like 7- or 5- on the A-ring) also influenced it. The results confirmed that the efficacy of a compound to induce phase II enzyme does not necessarily relate to its antioxidant capacity. The structure of the compound probably plays a more important role. Uda et al. (167) suggested that a double bond at the 2-3 position of the C-ring was essential for a flavonoid to induce QR activity and hydroxylation of the B-ring was not essential. Harris et al. (163) proposed that bioactivity of phenolics correlated with steric bulk (structural configuration) as well as lipophilicity (partition coefficient). At near neutral pH ranges, 3-deoxyanthocyanins exist primarily as quinoidal bases (173). Awika (174) reported that some of these sorghum 3-deoxyanthocyanin quinoidal bases contained a 2-3 double bond on their C-ring, which may partly explain the QR inducing capacity observed in this study. Methoxyl substitution of 3-deoxyanthocyanidins changes their steric configuration and enhances their lipophilicity, which may afford them better affinity to specific cell membrane binding sites or may favor their migration across the cell membrane. This can facilitate the expression of phase II enzymes.

The 3-deoxyanthocyanidins can be used as dietary supplements or chemopreventive agents. A systematic study of the structure-activity relationship of 3-deoxyanthocyanins is needed to understand how structure affects the activation of cellular mechanisms to enhance the expression of phase II enzymes. Other phenolic groups present in sorghum also require investigation of their structure-activity

relationship. Uda et al. (167) recognized flavonols as the most effective QR inducer among flavonoids. Lemon yellow sorghums are a rich source of flavonols (109), and if we demonstrate the effectiveness of sorghum flavonols to induce phase II enzymes, it will enhance opportunity to utilize sorghum.

### **Inhibitory Effect of 3-Deoxyanthocyanidin Structure on Human Colon and Esophageal Carcinoma Cell Proliferation**

According to Shih et al. (19), the aglycones of sorghum 3-deoxyanthocyanins, apigeninidin and luteolinidin, had greater inhibitory potency against human cancer cell growth than their 3-hydroxylated analogs, pelargonidin and cyanidin; moreover luteolinidin was a stronger inhibitor than apigeninidin. This evidence drove us to investigate how the structure of 3-deoxyanthocyanidins affected their antiproliferative potency. Genistein, the isoflavone in soybean was used as a positive control in the study.

To compare the effect of structure on antiproliferative potential of 3-deoxyanthocyanidins tested,  $IC_{50}$ s ( $\mu$ M) of each 3-deoxyanthocyanidin were calculated (Table 10). After 48 h incubation, apigeninidin inhibited approximately 60% and 50% of HT-29 and OE33 growth, respectively, at 200  $\mu$ M (Figures 14 and 15), with  $IC_{50}$ s from 81.5-284  $\mu$ M. 7-Methoxyapigeninidin had stronger inhibitory potential than apigeninidin: it inhibited almost 90% of HT-29 growth and 70% of OE33 growth at 200  $\mu$ M with  $IC_{50}$ s from 40.1-87.9  $\mu$ M. 5-Methoxyapigeninidin had similar inhibitory potency on both HT-29 and OE33 cell lines as its 7-methoxylated analog, with  $IC_{50}$ s from 48.4-57.7  $\mu$ M. 5,7-Dimethoxyapigeninidin had the strongest inhibitory potential

among apigeninidin derivatives: it almost inhibited 95% of HT-29 and OE33 growth at 50  $\mu\text{M}$ ,  $\text{IC}_{50}\text{s}$  were 8.3-23.4  $\mu\text{M}$  on both cell lines.

Among luteolinidin and its derivatives, luteolinidin reduced approximately 50% and 45% of HT-29 and OE33 growth at 200  $\mu\text{M}$ , respectively (Figures 14 and 15), with  $\text{IC}_{50}\text{s}$  from 172-219  $\mu\text{M}$ . 7-Methoxyluteolinidin was a stronger inhibitor than luteolinidin on both cell lines, with  $\text{IC}_{50}\text{s}$  91.4-192  $\mu\text{M}$ . 5,7-Dimethoxyluteolinidin had strongest inhibition capacity among luteolinidin derivatives: it inhibited 60-65% of HT-29 and OE33 growth at 200  $\mu\text{M}$  and had  $\text{IC}_{50}\text{s}$  from 47.1-105  $\mu\text{M}$ . Genistein, as a control, had  $\text{IC}_{50}\text{s}$  from 47.8-111  $\mu\text{M}$ .

The most obvious structure-activity relationship of 3-deoxyanthocyanidins was that methoxylation of the A-ring enhanced antiproliferative capacity. The  $\text{IC}_{50}$  trends were: di-methoxylated aglycones < mono-methoxylated < non-methoxylated (Figure 16). The position of methoxyl group on the A-ring did not affect this potential. Apigeninidin and its derivatives also generally had lower  $\text{IC}_{50}\text{s}$  than luteolinidin and its derivatives, though this was more apparent in the HT-29 cells (Figures 17).

Table 10. Concentration ( $\mu\text{M}$ ) of Sorghum 3-Deoxyanthocyanidins Needed to Inhibit 50% of HT-29 (colon) and OE33 (lower esophageal) Carcinoma Cell Proliferation ( $\text{IC}_{50}$ )

	$\text{IC}_{50}$ ( $\mu\text{M}$ )			
	HT-29		OE33	
	MTT	PicoGreen	MTT	PicoGreen
Apigeninidin	$81.5 \pm 11.6^c$	$106 \pm 11.8^c$	$284 \pm 11.4^d$	$276 \pm 11.8^c$
7-Methoxyapigeninidin	$50.3 \pm 7.4^b$	$40.1 \pm 1.9^b$	$87.9 \pm 10.6^c$	$51.9 \pm 0.8^b$
5-Methoxyapigeninidin	$53.6 \pm 12.6^b$	$48.4 \pm 6.3^b$	$57.7 \pm 0.6^b$	$55.0 \pm 10.6^b$
5,7-Dimethoxyapigeninidin	$13.7 \pm 0.1^a$	$8.3 \pm 0.7^a$	$23.4 \pm 3.4^a$	$22.7 \pm 0.9^a$
<b>Overall mean (apigeninidin and derivatives)</b>	<b>49.7</b>	<b>50.7</b>	<b>113</b>	<b>101</b>
Luteolinidin	$199 \pm 11.2^b$	$188 \pm 20.6^c$	$219 \pm 17.2^b$	$172 \pm 20.7^c$
7-Methoxyluteolinidin	$91.4 \pm 11.5^a$	$129 \pm 6.2^b$	$192 \pm 2.8^b$	$94.4 \pm 7.2^b$
5,7-Dimethoxyluteolinidin	$89.6 \pm 11.1^a$	$53.3 \pm 0.5^a$	$105 \pm 0.8^a$	$47.1 \pm 0.8^a$
<b>Overall mean (luteolinidin and derivatives)</b>	<b>127</b>	<b>123</b>	<b>172</b>	<b>104</b>
Genistein	$97.4 \pm 15.3$	$47.8 \pm 2.6$	$84.7 \pm 6.5$	$111 \pm 4.3$

Cells (2000/well) were induced with samples for 48h before proliferation assay. Genistein, an isoflavone isolated in soybean, was used as a positive control in this study. Values  $\pm$  sd ( $\mu\text{g}/\text{mL}$ ) reported from three separate experiments. Within apigeninidin and luteolinidin and their correspond derivatives, values with different letters within the same column were different, and genistein was not included in this Post Hoc test (Fisher's LSD,  $p < 0.05$ ).



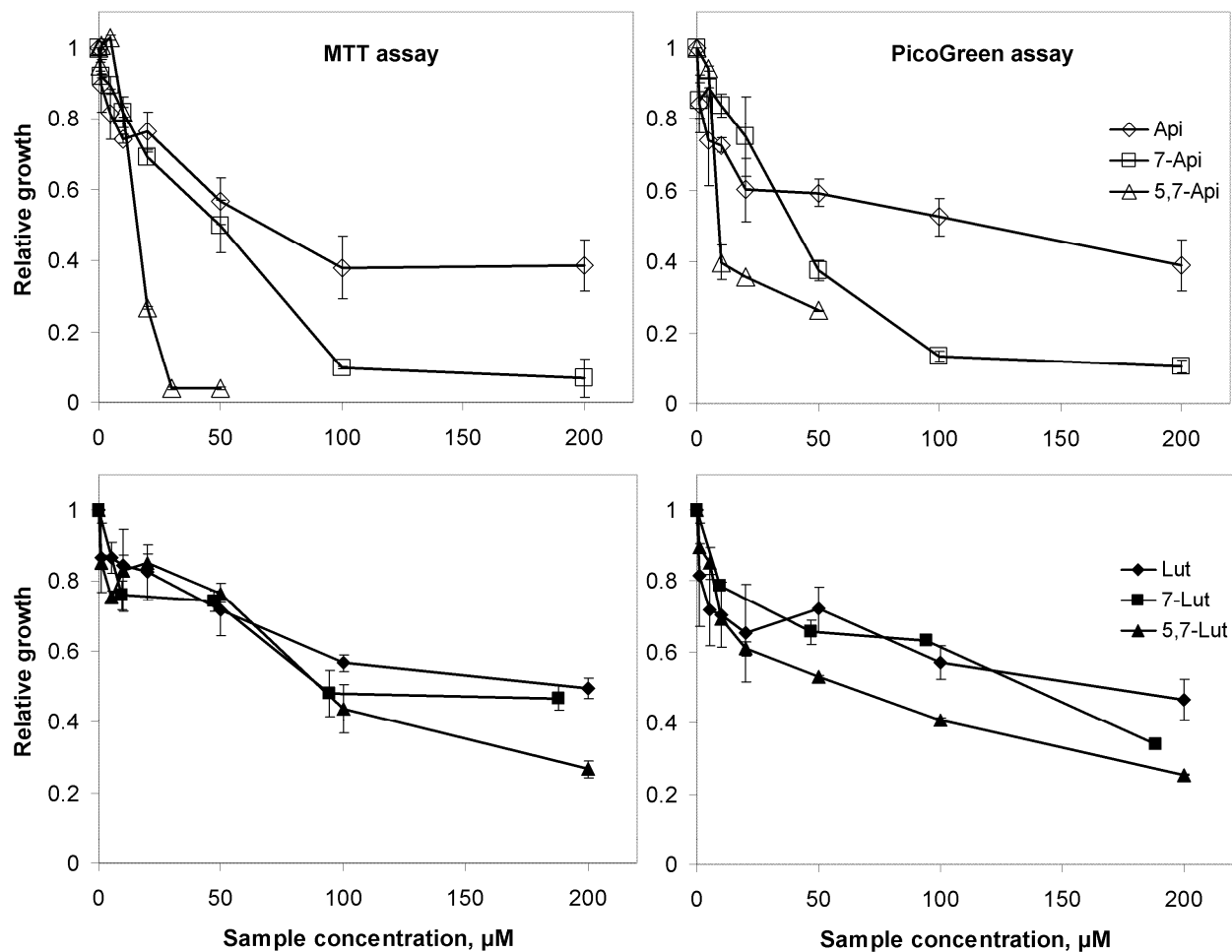


Figure 14. Inhibition of HT-29 human colon carcinoma cell proliferation by sorghum 3-deoxyanthocyanidins (baseline to 0.1% DMSO) measured by MTT (left) and PicoGreen (right) assays. Cells (2000/well) were induced with samples for 48h before proliferation assay. Error bars represent standard deviation from triplicates.

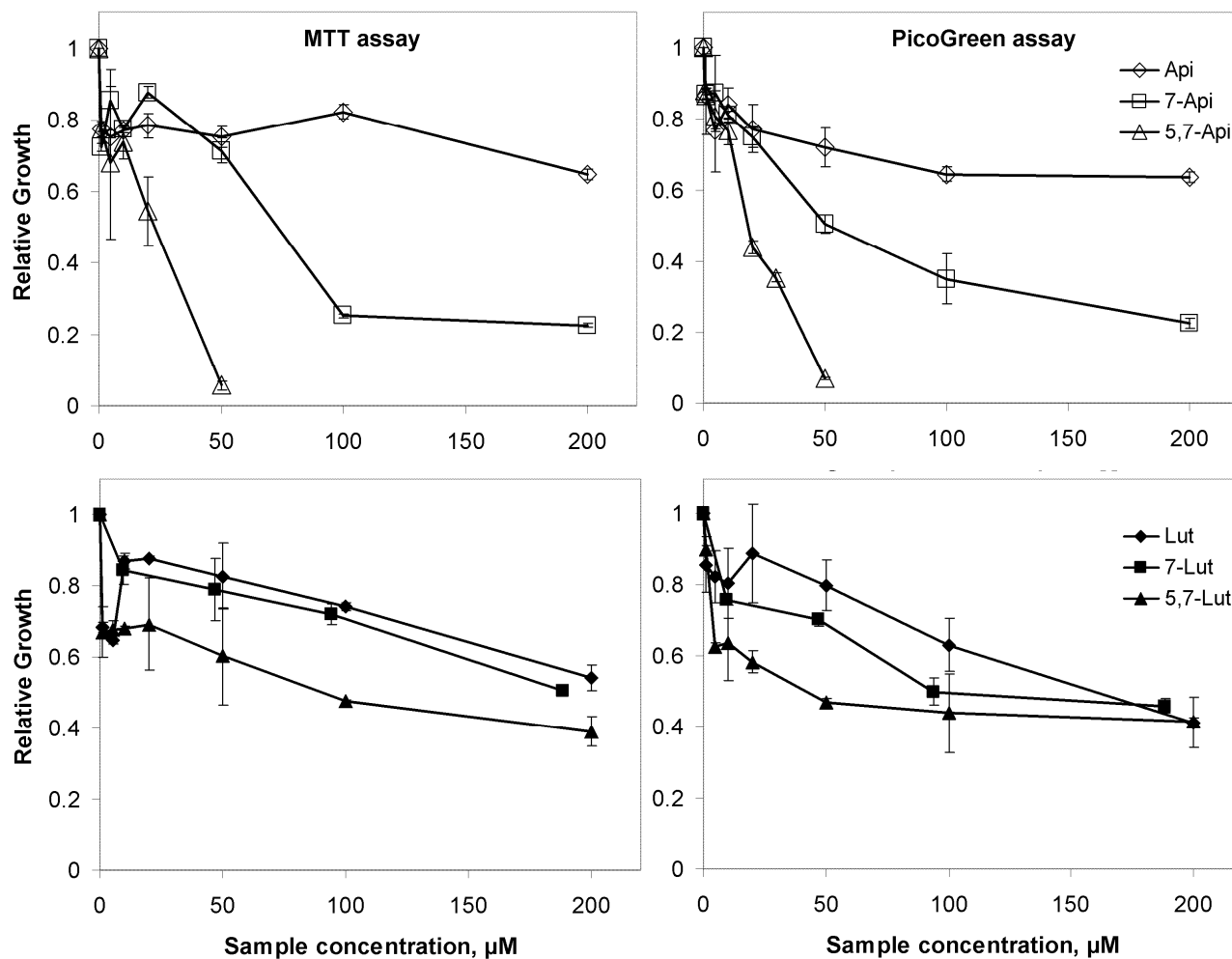


Figure 15. Inhibition of OE33 human lower esophageal adenocarcinoma cell proliferation by sorghum 3-deoxyanthocyanidins (baseline to 0.1% DMSO) measured by MTT (left) and PicoGreen (right) assays. Cells (2000/well) were induced with extracts for 48h before proliferation assay. Error bars represent standard deviation from triplicates.

To explain this structure-activity relationship of 3-deoxyanthocyanidins, we tried to correlate their inhibition capacity with antioxidant capacity (Table 11). The ABTS antioxidant capacity of 3-deoxyanthocyanidins had a positive non-linear correlation with  $IC_{50}$ s measured by MTT assay on both cell lines; while ORAC antioxidant capacity had a positive linear correlation with  $IC_{50}$ s measured by both MTT and PicoGreen assays and on both cell lines. The correlation suggested lower antioxidant capacity actually indicated stronger antiproliferative potential. The ORAC values seemed to be better predictors because methoxylation reduced ORAC values but enhanced antiproliferative potency. Since antioxidant capacity of a compound is highly related to its structure and the method used to measure it, antioxidant capacity may not have a direct relation with antiproliferative potential.

The antiproliferative potential of 3-deoxyanthocyanidins indicated a strong structure-activity relationship: methoxylation on the A-ring enhanced inhibitory potency. Hydroxylation on the B-ring could reduce inhibitory potency but this relationship may be cell line specific. There is little information on the structure-antiproliferation potential of 3-deoxyanthocyanidins. However, the inhibitory effect of anthocyanin aglycone

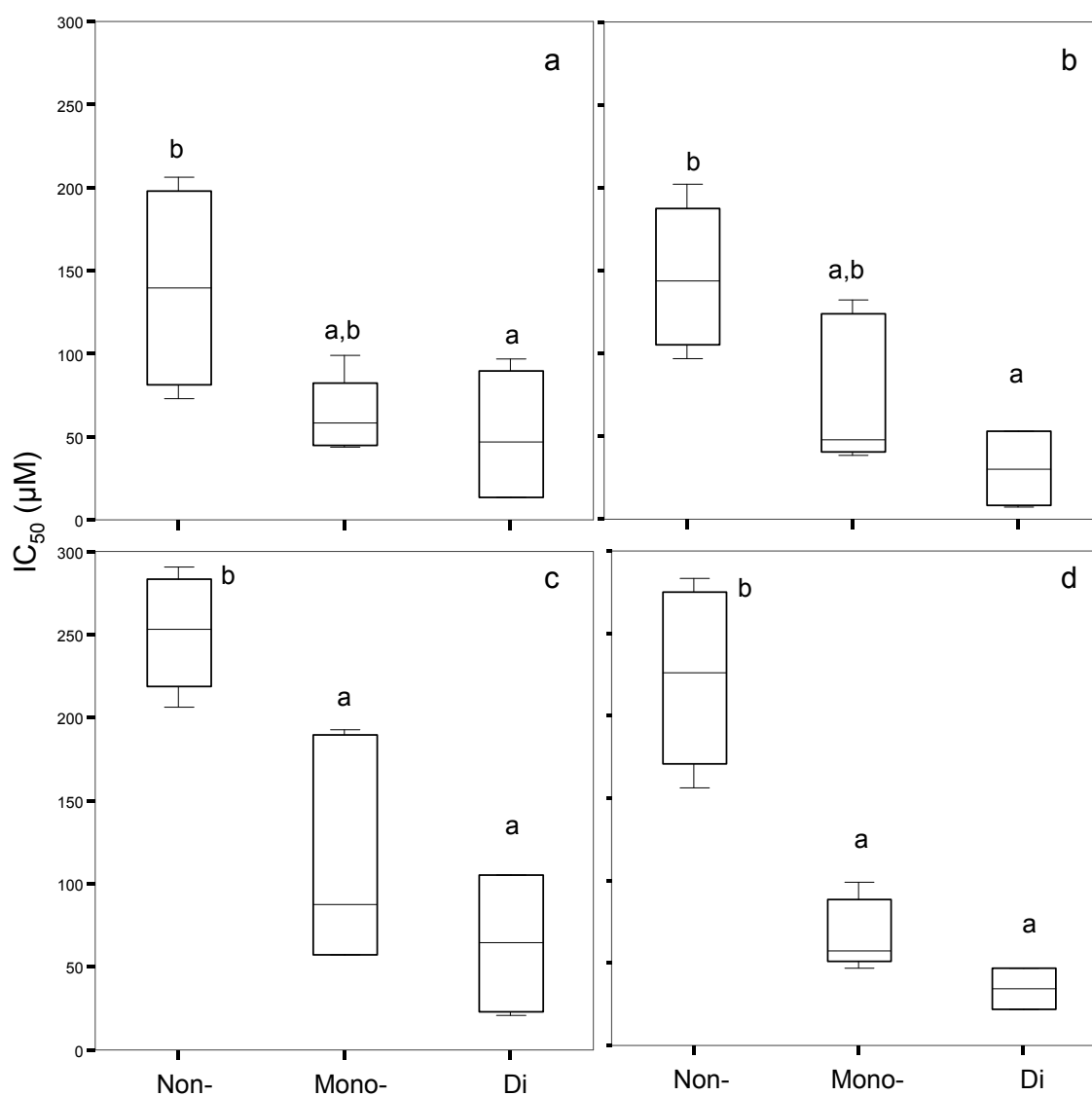


Figure 16. Box plot of IC<sub>50</sub>s (µg/mL) (concentration needed to inhibit 50% of cell growth) among non-, mono-, and di-methoxylated 3-deoxyanthocyanidins, (a) IC<sub>50</sub>s on HT-29 proliferation measured by MTT assay (p<0.072); (b) IC<sub>50</sub>s on HT-29 proliferation measured by PicoGreen assay (p<0.014); (c) IC<sub>50</sub>s on OE33 proliferation measured by MTT assay (p<0.002); (d) IC<sub>50</sub>s on OE33 proliferation measured by PicoGreen assay (p<0.001). Boxes with different letters within the same plot were different (p<0.05, Fisher's LSD). Non-, non-methoxylated 3-deoxyanthocyanidins, apigeninidin and luteolinidin; mono-, mono-methoxylated 3-deoxyanthocyanidins, 5-methoxyapigeninidin, 7-methoxyapigeninidin, and 7-methoxyluteolinidin; di-, di-methoxylated 3-deoxyanthocyanidins, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin.

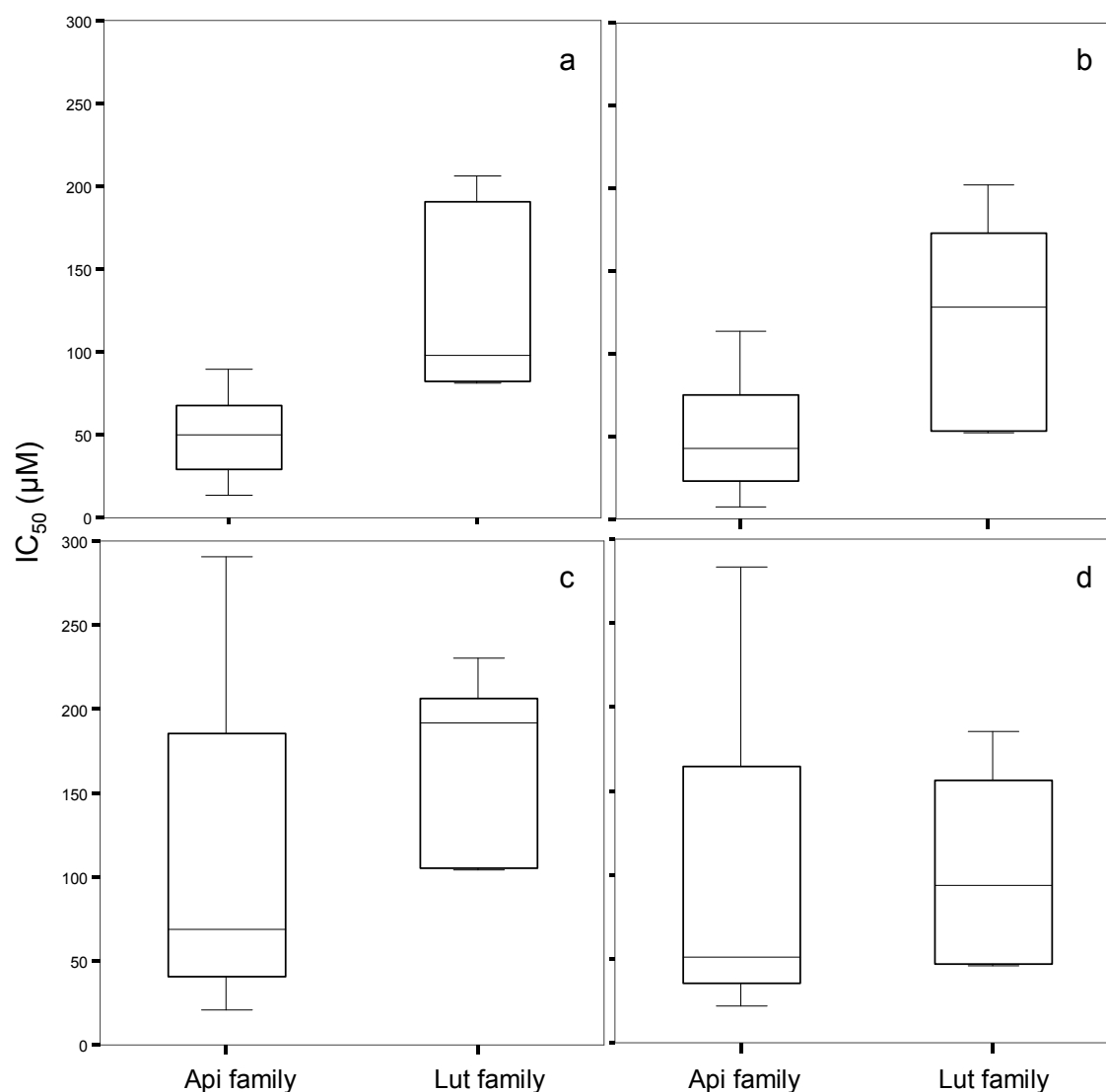


Figure 17. Box plot of  $IC_{50}$ s ( $\mu\text{g}/\text{mL}$ ) (concentration needed to inhibit 50% of cell growth) between apigeninidin derivatives and luteolinidin derivatives, (a)  $IC_{50}$ s on HT-29 proliferation measured by MTT assay ( $p < 0.014$ ); (b)  $IC_{50}$ s on HT-29 proliferation measured by PicoGreen assay ( $p < 0.042$ ); (c)  $IC_{50}$ s on OE33 proliferation measured by MTT assay ( $p < 0.473$ ); (d)  $IC_{50}$ s on OE33 proliferation measured by PicoGreen assay ( $p < 0.828$ ). Api family, apigeninidin, 5-methoxyapigeninidin, 7-methoxyapigeninidin, and 5,7-dimethoxyapigeninidin; lut family, luteolinidin, 7-methoxyluteolinidin, 5,7-dimethoxyluteolinidin.

Table 11. Correlation between IC<sub>50</sub>s and Antioxidant Properties of 3-Deoxyanthocyanidin Standards

Correlation coefficient		IC <sub>50</sub> (μM)			
		HT-29		OE33	
		MTT	PicoGreen	MTT	PicoGreen
Pearson's Correlation	ABTS	0.499	0.377	0.461	0.273
	ORAC	0.732**	0.772**	0.887**	0.804**
Spearman's rho	ABTS	0.747**	0.551	0.660*	0.239
	ORAC	0.687*	0.711**	0.799**	0.673*

\*correlation is significant at the 0.05 level (2-tailed).

\*\*correlation is significant at the 0.01 level (2-tailed).

structure on carcinogenesis is mostly affected by substitution on the B-ring: number of hydroxyl/methoxyl group on the B-ring of anthocyanin aglycone positively correlated with stronger cancer cell growth inhibitory action (175, 176). Hydroxylation also enhanced apoptosis inducing (177) and antiproliferative potential (178) of flavones. Our findings were in accordance with the literature on methoxyl substitution but did not match the literature on hydroxyl substitution. Other reports showed methoxyl substitution and substitution of other lipophilic groups enhanced antiproliferative potency of phenolic compounds (179, 180). Greater lipophilicity allows easier penetration into the cell (181). Hydroxylation of luteolinidin and its derivatives reduce their lipophilicity compared to apigeninidin and its derivatives hence luteolinidin and its derivatives may not easily get into cells.

For future study, a more complete structure-activity relationship of 3-deoxyanthocyanins is desired. Research on chemopreventive effect of hydroxylation and methoxylation at different positions on 3-deoxyanthocyanin aglycones is needed.

Because glycosylation of flavonoids reduced their inhibitory activity against human cancer cell lines (178), it is highly recommended to study the effect of glycosylation, the most common substitution pattern in sorghum 3-deoxyanthocyanins, on their chemopreventive potentials. Additionally, Marko and others (176) indicated that the effect of B-ring substitution of anthocyanidins might vary depending on different assays used. So the research on chemopreventive potential of 3-deoxyanthocyanins should involve different mechanisms (i.e. apoptosis induction, cell cycle arrest, and cell membrane sites binding) on various cell lines. Other phenolic compounds in sorghum, such as flavones and flavonols deserve further study on their chemopreventive potential. Daskiewicz and co-workers (182) reported that among 42 flavonoids with different substitution at various positions examined for human cancer cell antiproliferation capacity, flavones and flavonols possessed greater antiproliferative activity than chalcones and flavanones. The study on all major groups of sorghum phenols will be of great benefit to utilize sorghum in health related industries.

## CHAPTER VII

### SUMMARY

We observed a wide range of phenolic profiles among different varieties of sorghum. Tannin sorghums contained higher total phenols ( $p < 0.001$ ), ABTS ( $p < 0.001$ ) and ORAC ( $p < 0.006$ ) antioxidant capacity than non-tannin sorghums, similar to findings of Awika et al. (95) and Dykes (109). Among non-tannin sorghum grains, 3-deoxyanthocyanin content had a significant correlation with total phenol content ( $p < 0.01$ ), ABTS ( $p < 0.01$ ), and ORAC ( $p < 0.05$ ) antioxidant capacity. The crude 70% acetone extracts of sorghum grains had similar phenolic profiles as their grains. However, the ORAC antioxidant capacity of the extracts did not differ between tannin and non-tannin extracts ( $p < 0.194$ ) hence tannin content of the extracts did not correlate with their ORAC values. Some non-tannin sorghum extracts, such as 98BRON155, 99LGWO50, Tx2911, had high ORAC values as tannin sorghum extracts. The extraction process may concentrate some groups of phenols in these varieties (i.e. flavanones, flavones, flavan-4-ols (109)) that exhibited high ORAC values among those sorghums.

The NAD(P)H: quinone reductase (QR) inducing capacity of selected sorghum varieties was different between tannin and non-tannin sorghums. Tannin sorghums did not show QR inducing potential. Among non-tannin sorghums, black sorghum (Tx430) doubled QR activity at 25  $\mu\text{g/mL}$ , and increased the activity 2.7-fold at 100  $\mu\text{g/mL}$ . Two red varieties (Mizzou and I CSV III) increased QR activity by 50-60% at 200  $\mu\text{g/mL}$  while the white variety (KARI-Mtama) doubled QR activity at 400  $\mu\text{g/mL}$ . The QR



inducing capacity did not correlate with the phenol content or antioxidant capacity of selected varieties. This indicated that the QR inducing potential may be related to structure of active phenols present in sorghums. Further investigation into how phenol composition affects phase II enzyme inducing capacity is needed. Future study could focus on 1) the phase II enzyme inducing potential of fractionated sorghum extracts containing isolated phenols (i.e. 3-deoxyanthocyanins, flavones, flavanones), 2) the specific cellular mechanism of the inducing potential (i.e. measure expression of genes that upregulate phase II enzymes), and 3) other enzymes in phase II family.

All the crude sorghum extracts tested had a relatively strong antiproliferative potential against human gastrointestinal cancer cell proliferation, though inhibitory potential was different between tannin and non-tannin sorghum varieties. Tannin sorghums generally had a stronger inhibitory effect on cancer cell growth than non-tannin sorghums ( $IC_{50}$ s 49.7-131 vs. 141-883  $\mu\text{g/mL}$ ). Among non-tannin sorghums, black sorghum (Tx430) showed the strongest antiproliferative potential followed by red (Mizzou), then white (KARI-Mtama and Mizzou White) varieties. This inhibitory effect correlated with total phenol content and antioxidant capacity of the extracts. This indicated that the antioxidant capacity may be indicators of inhibitory potential. Some varieties of strong antioxidant non-tannin sorghum were not tested in our study, i.e. 98BRON155, 99LGWO50, and Tx2911, hence it will be beneficial if we can use them to confirm this theory. Future study could 1) determine the antiproliferative potential of different phenolic fractions of sorghum extracts, 2) target specific mechanisms of the antiproliferative effect, such as induction of apoptosis, arresting cell cycles, or

preventing DNA damage, 3) determine the antiproliferative potential of sorghum using additional gastrointestinal and other types of cancer cells, to have a better understanding of the chemopreventive properties of sorghum.

Among the 3-deoxyanthocyanidins tested, the activity trends for both QR inducing and antiproliferation were similar: methoxylation improved the potency of apigeninidin and luteolinidin. On the other hand, apigeninidin and its methoxylated derivatives generally have stronger antiproliferative potential than luteolinidin and derivatives. For future study, 1) more substitution pattern, i.e. glycosylation, hydroxylation, multi-methoxylation/hydroxylation, of 3-deoxyanthocyanidins need to be studied for a complete structure-activity relationship, 2) specific cellular chemopreventive mechanisms, such as affinity to bind specific cell membrane sites, permeability across the cell membrane, induction of apoptosis, cell cycle arrest, and repair of damaged DNA need to be studied, 3) other groups of phenols present in sorghum (i.e. flavones, flavanones, flavol-4-ols) need to be studied for their chemopreventive properties.

On the whole, we observed a relatively strong chemopreventive potential of sorghums with different phenolic compositions. Future studies should not only aim at the aforementioned chemopreventive aspects, but also focus on the effect of food processing, storage, and use of additives on the chemopreventive potentials of sorghum phenols. Also, determining the chemopreventive roles of sorghum on healthy population is desirable. This information can be used to promote the utilization of sorghum grains, crude extracts, and isolated compounds. Applications could target direct food use, use as

functional food ingredients, and dietary supplements. The information would also be useful to breeding programs to selectively breed sorghum varieties for specific health applications.

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