EVALUATION OF ANTICANCER POTENTIAL OF SORGHUMS WITH DIFFERENT GENETIC CHARACTERISTICS AND LEVELS OF PHENOLIC COMPOUNDS

A Dissertation

by

SARA GUAJARDO FLORES

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

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Food Science and Technology

EVALUATION OF ANTICANCER POTENTIAL OF SORGHUMS WITH DIFFERENT GENETIC CHARACTERISTICS AND LEVELS OF PHENOLIC COMPOUNDS

A Dissertation

by

SARA GUAJARDO FLORES

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

DOCTOR OF PHILOSOPHY

Approved by:

Co-Chairs of Committee,	Lloyd W. Rooney
	Sergio Serna-Saldívar
Committee Members,	Alejandro Castillo
	Luis Cisneros-Zevallos
Chair of Food Science and Technology Faculty,	Jimmy T. Keeton

May 2008

Major Subject: Food Science and Technology

ABSTRACT

Evaluation of Anticancer Potential of Sorghums with Different Genetic Characteristics and Levels of Phenolic Compounds. (May 2008)

Sara Guajardo Flores, B.A., Instituto Tecnológico y de Estudios Superiores de Monterrey; M.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Lloyd W. Rooney
Dr. Sergio Serna-Saldívar

To evaluate the anticancer potential of sorghum phenolic compounds, different experiments including in vitro and in vivo tests were performed. A set of 25 sorghum samples was evaluated for phenolic (total phenols, flavonoids, anthocyanins and tannins) content, hydrophilic and lipophilic antioxidant capacity using de Oxygen Radical Absorbance Capacity assay (ORAC), and screened for citotoxic properties in mammary, colon and hepatic mammalian cancer cell lines in vitro. Results indicated that there was a wide variability in the phytochemical profile among the different sorghums. Among the 25 samples, sumac sorghum bran had the highest amount of phenolic compounds, flavonoids, tannins and the highest ORAC values. It exerted the highest percent inhibition (near 100%) in mammary, colon and liver cancer cell lines. Sumac sorghum bran was selected for further investigation. Methanolic extracts from sumac whole grain, bran and tannin removed bran were tested in vitro at different concentrations in hormone dependent MCF-7 mammary cancer cells and non hormone dependent Caco2 and HepG2 colon and liver cancer cells. Results indicated that the methanolic extract from sumac bran inhibited 100% of MCF-7 cancer cells at a concentration of 0.5 mg/ml and that the citotoxic effect could be partially due to the tannin content of the extract. Concentrations of 0.5 and 1.5 mg/ml were selected for an in vivo preventive cancer study with 7,12-dymethylbenz(*a*)-anthracene (DMBA) induced female rats. Bran at low and high concentrations and the correspondent amount of methanol extracts were included in the diet. It was observed that sumac methanol extract at low concentration promoted tumor appearance and development, whereas sumac bran had a preventive effect, however, there were no significant differences in rats treated and un-treated with sumac. Differences between in vitro and in vivo results

could be due to the degree of absorption of tannins during the in vivo experiment. To obtain additional data about the effect of sumac extracts on cancer development, a quinone reductase enzyme bioassay was performed. Methanol and hexane extracts from sumac bran induced phase II enzymes in vitro. Phytochemicals of sumac bran sorghum including phenolic compounds and lipid like compounds appeared to have potential for cancer prevention.

DEDICATION

To my husband Rodrigo

To my sons Rodrigo and Ignacio

ACKNOWLEDGMENTS

First of all, I want to thank my mom for taking care of my kids, every day, for more than a year. Thanks for all your help and for being an unconditional support to my family. I would like to thank my committee chairs, Dr. Lloyd Rooney and Dr. Sergio Serna-Saldívar. Dr. Rooney, thanks for giving me the opportunity, your unconditional support and your always wise guidance. Dr. Serna, thanks because you were the one who believed in and supported me from the beginning. Thank you for all the time and resources you dedicated to me. Thanks to Dr. Castillo who started this process, I still remember when you asked why not? and to Dr. Cisneros who agreed to be part of my committee. Thanks Daniel, for helping me during the experiments. Thanks to Janet, Ana and Santiago for ALL your help. Thanks to Ana Paola, because you were my eyes and voice at A&M. Thanks to my family: mom and dad, thank you for being there for me. Thank you to my brothers and sisters who are always behind me. Thanks to my father-and mother-in-law for your support. Thanks to my kids who are the light of my life, for giving me so much love and happiness. Thanks to my husband. You are an extraordinary man; thank you for all your support and help. Thanks because you believed in me even when I didn´t. Thank you for always being there for me, for your words, your company, your love. In all this time, there were many people who helped me in one way or another, thanks to all of them. Thanks to God for giving me so much.

TABLE OF CONTENTS

		Page
ABSTRACT		iii
DEDICATION		v
ACKNOWLEDGMENTS		vi
TABLE OF CONTENTS		vii
LIST OF FIGURES		ix
LIST OF TABLES		x
CHAPTER		
I	INTRODUCTION	1
II	SCREENING AND CHARACTERIZATION OF DIFFERENT SORGHUMS FOR ANTICANCER POTENTIAL	3
	Literature Review	3
	Materials and Methods	6
	Results and Discussion	12
III	IN VITRO EVALUATION OF ANTICANCER PROPERTIES OF SUMAC SORGHUM	27
	Literature Review	27
	Materials and Methods	32
	Results and Discussion	33
IV	IN VIVO EVALUATION OF SUMAC SORGHUM ON DMBA INDUCED FEMALE RATS	45
	Literature Review	45
	Materials and Methods	48
	Results and Discussion	50
V	EVALUATION OF SUMAC SORGHUM EXTRACTS WITH A PHASE II ENZYME-INDUCING BIOASSAY	61
	Literature Review	61
	Materials and Methods	63
	Results and Discussion	66
VI	CONCLUSIONS	71
REFERENCES		73
APPENDIX		82

Page
VITA.. 84

LIST OF FIGURES

FIGURE		Page
1	Total Phenol Content of Sorghum Samples…………………………..	13
2	Flavonoid Content of Sorghum Samples……………………………..	15
3	Anthocyanin Content of Sorghum Samples…………………………..	16
4	Tannin Content of Sorghum Samples…………………………………	18
5	In vitro Citotoxic Effect of Sorghum Extracts on Different Cancer Cell Lines……………………………………………………………….	24
6	Growth Control in Normal and Cancer Cell Division…………………	28
7	Citotoxicity Curves of Different Sumac Sorghum Extracts on MCF-7 Cancer Cells……………………………………………………	34
8	Citotoxicity Curves of Different Sumac Sorghum Extracts on Liver HepG2 Cancer Cells………………………………………………….	36
9	Citotoxicity Curves of Different Sumac Sorghum Extracts on Caco2 Cancer Cells……………………………………………………….	37
10	Evaluation of Sumac Bran Extract on MCF-7 Mammary Cancer Cells………………………………………………………………..	39
11	Evaluation of Anticancer Effect of Sumac Bran Extract on Caco2 Colon Cancer Cells……………………………………………………	41
12	Evaluation of Anticancer Potential of Sumac Bran Extract on HepG 2, Liver Cancer Cells………………………………………………….	42
13	Average Weight of Rats by Block During the 20 Week Study………	52
14	Average Weight of Rats by Treatment During the 20 Week Study…	53
15	Cumulative Tumor Incidence by Treatments………………………….	54
16	Principle of the NQ01 Assay…………………………………………	61
17	Quinone Reductase Activity Induced by Methanol Extract of Sumac Bran …………………………………………………………………	67
18	Quinone Reductase Activity Induced by Sumac Bran Extract………	68
19	Quinone Reductase Activity Induced by the Hexane Extract of Sumac Bran…………………………………………………………	69

LIST OF TABLES

TABLE		Page
1	Set of Sorghum Samples Selected for Characterization and in vitro Screening…………………………………………………………..	7
2	ORAC Values for the 25 Sorghum Sample Set, Sorghum Type Is Included………………………………………………………………..	20
3	Correlation of Tannins, Total Phenolics, Anthocyanins, Flavonoids and ORAC Values of the 25 Sorghum Sample Set with Inhibition of Three Different Cancer Cell Lines………………………………………	22
4	Treatments Selected to Test the Preventive Effect of Sumac Bran and 80% Methanol Extracts Using DMBA Induced Female Rats…………………………………………………………………....	48
5	Distribution of Rats per Treatment for the in vivo Experiment………	49
6	Diet Formulation per Treatment for the in vivo Study Conducted with DMBA Induced Rats……………………………………………….	50
7	Rats Distribution for in vivo Study………………………………………	51
8	Tumor Incidence, Latency Period, Tumor/Tumor-Bearing Rat and Tumor Weight in Rats Treated with DMBA……………………………	57
9	Amount of Extracts and Dilutions Used for the Phase II Enzyme Bioassay………………………………………………………………..	65

CHAPTER I
INTRODUCTION

Cancer is one of the main causes of death in the USA and the world. In 2004, cancer was the second cause of death in the USA, just below cardiovascular diseases, with 553, 888 deaths. In women, breast cancer is the second most important cause of cancer related deaths. It was estimated that in 2007, about 1.4 million new cases of cancer were diagnosed, cancers of the prostate and breast being the most frequently diagnosed in men and women, respectively, followed by lung and colorectal cancers both in men and in women (American Cancer Society 2007).

Cancer chemoprevention is an approach that has been studied for the last years in order to address the disease. Many natural compounds have been studied; among them phenolic compounds from fruits and vegetables have been extensively studied for their antioxidant properties related to oxidative stress and cancer prevention. Cancer preventive phytochemicals have been shown to suppress or block carcinogenesis by a variety of mechanisms including acting as antioxidants or antiproliferative agents (Singletary and others 2003). Polyphenols are reducing agents, and together with other dietary reducing agents, referred to as antioxidants, protect the body´s tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation (Tapiero and others 2002).

Sorghum is a cereal grown in different parts of the world, it has economically advantages over other grains such as drought resistance, water efficiency, with significant low levels of fumonisins and aflatoxins. Some sorghum varieties have been screened for its phenolic composition. These compounds are mainly localized in the bran in amounts sometimes equal or greater than the most studied vegetal products. The phenolic compounds of sorghum can be divided into three basic groups: simple phenolics, flavonoids, and tannins. All sorghums contain phenolic acids and most contain flavonoids, but only certain varieties contain tannins (Hahn and others 1984).

This dissertation follows the style of Journal of Food Science.

There is plenty of literature relating polyphenol-rich foods properties with the prevention of various types of cancers and oxidative stress, however, the information related to sorghum is too limited to draw reasonable conclusions. In vitro data as well as controlled animal studies are necessary to understand how the levels and composition of polyphenols in sorghum prevent cancer, and which specific components are responsible (Awika and Rooney 2004). The objective of the present study is to evaluate the potential of sorghum phenolic compounds as inhibitors of cancer cell growth and phase II enzymes induction in vitro and their chemopreventive properties on tumor development after 7,12-dimethylbenz[a]anthracene (DMBA)-initiation of female rats.

CHAPTER II
SCREENING AND CHARACTERIZATION OF DIFFERENT SORGHUMS FOR ANTICANCER POTENTIAL

Literature Review

Sorghum

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cereals in the world, more than 35% is grown directly for human consumption (in Asia and Africa is used primarily as a food crop), the rest is used for animal feed, alcohol and industrial products; generally is more economical to produce, resist drought and other environmental stresses (Awika and Rooney 2004).

Sorghum kernel, as the rest of the cereals, consists of three anatomical components: pericarp (outer layer), endosperm (storage tissue) and germ (embryo). The proximate composition of sorghums varies due to genetics and environment, but it is well known that the pericarp is rich in fiber, whereas the germ is high in protein, fat and ash. The endosperm contains mostly starch, some protein and small amounts of fat and fiber. Below the pericarp of sorghum is the seed coat or testa, in certain types of sorghum, phenols and tannins are concentrated in this part. The endosperm is composed of the aleurone layer, peripheral, and corneous and floury areas (depending on the arrangement of starch and protein). Germ consists of the embryonic axis and scutellum. (Rooney and Waniska 2000).

Based upon their genetics and chemical analysis sorghum varieties can be divided into three different groups: Types I, II and III. Type I sorghums do not have a pigmented testa, therefore contain low levels of phenols and are practically devoid of tannins. Types II and III have a pigmented testa and contain considerable levels of condensed tannins. Tannins from Type II sorghums are extracted with acidified methanol (1%HCL methanol) while tannins in Type III are extracted with either methanol or acidified methanol (Dykes and Rooney 2006).

Sorghum phytochemicals

In recent years sorghum has attracted attention and has been studied because it is a potential source of different phytochemicals. Phytochemicals are defined as bioactive nonnutrient plant compounds associated to fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases (Liu 2004). Sorghum contains phenolic compounds, waxes (lipid compounds), phytosterols and proteins that have been related to health benefits.

<u>Phenolic compounds</u>

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are the products of secondary metabolism in plants, in many cases are formed as defense mechanisms. These compounds exert their beneficial effects as free radical scavengers and chelators of pro-oxidant metals, these features have relate the consumption of foods rich in phenolic compounds with reduced health problems.

Some sorghum varieties contain phenolic compounds in amounts that equals or exceed the ones reported for fruits and vegetables. The amount and type of phenols varies between cultivars and is influenced by genotype and environment in which it is grown (Dykes and others 2005). Phenols in sorghum fall under two categories: phenolic acids and flavonoids including tannins (Awika and Rooney 2004).

Phenolic acids consist of two classes: hydroxybenzoic and hydroxycinnamic. Hydroxybenzoic acids are derived from benzoic acid and include gallic, protocatehuic, *p*-hydroxybenzoic, gentisic, salicylic, vanillic and syringic acids. Hydrozycinnamic acids include ferulic, caffeic, *p*-coumaric, cinnamic and sinapic acids. Phenolic acids are located in the pericarp, testa, aleurone layer (free phenolics) and endosperm of the kernel (bound phenolics) (Dykes and Rooney 2006).

The major flavonoids studied in sorghum include tannins and anthocyanins. Anthocyanins of sorghum are called 3-deoxyanthocyanins, because they lack a hydroxyl group in the 3-position of the C-ring (Dykes and Rooney 2006), the two common 3-deoxyanthocyanins are the apigeninindin (yellow) and the luteolinidin (orange) (Awika and others 2004). Anthocyanins are concentrated in the bran; sorghums with a black pericarp have the highest amount of 3-deoxyanthocyanins reported.

Tannins (proanthocyanidins) occur only in the outer layers of the kernel (pericarp, pigmented testa and glumes) of some Type II or III sorghums (Rooney and Waniska 2000). These tannins are of the condensed type, hydrolysable tannins have never been found in sorghum. Levels of tannins vary among genotypes, in general, Type II and III sorghums have tannin levels of 0.02-0.19mg/100mg and 0.4-3.5mg/100mg catechin equivalents, respectively.

Waxes

Waxes are defined as esters formed between long chain fatty acids and long chain alcohols; plant waxes contain besides wax esters, non esterified very long chain hydrocarbons, alcohols, aldehydes and acids (Hargrove and others 2004). Traditionally food waxes includes that from cereal grains, nuts, unrefined oil, sugar cane and honey. Wax compounds as octacosanol has been related to cholesterol lowering properties. In sorghum, wax comprises about 0.2-0.3% of the kernel, is mainly formed of long chain aldehydes. The major components of the long-chain lipids are policosanols (≈40%), aldhehydes (≈50%) and acids (≈4%). Octacosanol (28:0) and triacontanol (30:0) comprises over 80% of the policosanols in sorghum grain, other components are hexacosanol (26:0), dotriacontanol (36:0), lignoceryl alcohol (24:0) and nonacosanol (29:0) (Hwang and others 2004). Christiansen and others (2007) reported that sorghum grain contains besides policosanols, other health-promoting lipids as phytosterols, and omega 3-fatty acids. Phytosterols are cholesterol like compounds that are structural components of plant cell membranes. In sorghum the free phytosterols identified include sitosterol, campesterol and stigmasterol (Awika and Rooney 2004).

Proteins

Kafirin is the main storage protein in sorghum, three classes of kafirin: the *α*, *β* and *γ* forms have been identified at the protein level, they are the most hydrophobic of the prolamins and exhibit extensive cross-linking by disulphide bonds. Certain kafirin (*α*) has been studied as a compound that prevents blood vessels narrowing (Kamath and others 2005)

Materials and Methods

Different sorghum samples (Table 1) were characterized for phenolic, flavonoids, anthocyanins and tannin content (Robledo and others 2007). Samples included whole grain and bran from different sorghums (Type I, Type II and Type III) (Robledo and others 2007). To measure the antioxidant capacity the ORAC assay was performed. Samples were then screened for anticancer potential using in vitro colon (Caco 2), liver (HepG2) and hormone-dependent mammary cancer (MCF-7) cell lines.

Sample preparation

Whole grain samples or bran samples were ground through a cyclotec mill (UDY Corp. Fort Collins, CO) equipped with a 1 mm mesh. To obtain bran, sorghum (sumac and black) was decorticated and milled using procedures described by Awika and others (2005). Cleaned representative sorghum samples were decorticated using the tangential abrasive dehulling device (TADD) (model 4E-230, Venable Machine Works, Saskatoon, Canada) for a total of 6 min to obtain bran. Percent removal was 12% and was calculated as [initial sample weight – decorticated grit weight/ initial sample weight].

Table 1 – Set of sorghum samples selected for characterization and in vitro screening

Sample ID	Sample Name	Sorghum Type	Location	Year
CQL 01	ATX635 x RTX 436	1	College Station	2001
CQL 02	SC748	1	Lubbock	2005
CQL 03	Hegari	2	College Station	2003
CQL 04	TX2911	1	College Station	2005
CQL 05	TX430 Black	1	College Station	2001
CQL 06	PI Black Tall	3	College Station	2005
CQL 07	Sumac	3	College Station	2005
CQL 08	Sumac Bran	3	College Station	2005
CQL 09	TX430 Black Bran	1	College Station	2001
CQL 10	SC719-11E	3	College Station	2005
CQL 11	SC650	1	College Station	2005
CQL 12	BRON 176	1	Lubbock	2005
CQL 13	SC109-14E	3	College Station	2005
CQL 14	Shawaya (Mostly Black)	1	College Station	2005
CQL 15	Shawaya (Brownie Tan)	3	College Station	2005
CQL 16	NK 121 A	3	UMES	2004
CQL 17	NK 180	3	UMES	2004
CQL 18	NK 8830	3	UMES	2004
CQL 19	XM 217	3	UMES	2004
CQL 20	EBA 3	1	Lubbock	2005
CQL 21	SC575	2	College Station	2005
CQL 22	SC103	3	College Station	2005
CQL 23	SC630ii	1	College Station	2005
CQL 24	SC1038	1	College Station	2005
CQL 25	SC630 II	1	College Station	2005

Sample extraction for characterization of phenolic content, flavonoids, anthocyanins and hydrophilic ORAC determination

Sample (1 g) was extracted with 80% aqueous methanol (10ml) for 4 hr at room temperature under continuous agitation at 200 rpm. The resulting sample-solvent mix was filtered through Whatman No. 1 filter paper in a Buchner funnel under vacuum and the solvent recovered. The spent grain was then washed four consecutive times with 80% methanol to assure the complete removal of the soluble compounds. Resulting extracts were used for phenolic content, flavonoids, anthocyanins, tannins and hydrophilic ORAC determination.

Total phenolic content determination

Total phenolic content of sorghum samples was measured using a procedure similar to the one reported by Wolfe and others (2003). The method is a modified colorimetric Folin-Ciocalteu. A volume of 20 µl of methanol extract was added to a test tube. Folin-Ciocalteu reagent (200 µl) was added to the solution and allowed to react for 5 min. Then, 1.27 ml of 7% sodium carbonate solution was placed into test tubes, and the mixture was diluted to 3 ml with deionized water. The color developed for 20 min, and the absorbance was read at 750 nm using a spectrophotometer (Beckman Coulter DU 800 Beckman Coulter Inc, Fullerton California U.S.A.). The measurement was compared to a curve prepared with standard gallic acid solutions and expressed as milligrams of gallic acid equivalents per gram.

Flavonoid content determination

Flavonoid content of sorghum samples was measured using a modified colorimetric method reported by Wolfe and others (2003). A diluted methanol extract (20 µl), prepared as described previously, was added to a test tube containing 100 µl of distilled water. A 5% sodium nitrite solution (6 µl) was added to the mixture and allowed to stand for 5 min. Then, 12 µl of 5% aluminum chloride was added. After 6 min, 40 µl of 1 M sodium hydroxide was added, and the mixture was diluted with another 22 µl of distilled

water. Absorbance of the mixture was immediately measured at 510 nm using a spectrophotometer Beckman Coulter DU 800 (Beckman Coulter Inc, Fullerton California U.S.A.) and compared to a curve prepared with standard catechin solutions. The flavonoid content was expressed as milligrams of catechin equivalents per gram.

Anthocyanin content

Anthocyanin content of sorghum samples was measured using a pH differential protocol reported by Wolfe and others (2003). Sorghum methanol extracts were mixed thoroughly with 0.025 M potassium chloride pH 1 buffer in 1:3 or 1:8 ratio of extract to buffer. Then the absorbance was measured at 515 and 700 nm against a distilled water blank. Sorghum extracts were then combined similarly with sodium acetate buffer pH 4.5, the absorbance of these solutions was measured at the same wavelengths. Anthocyanin content was calculated as follows: Total monomeric anthocyanin (mg /100 g sample) = AxMWx1000/(εxC) where A is absorbance = $(A_{515} - A_{700})_{pH1} - (A_{515} - A_{700})_{pH\ 4.5}$; MW is molecular weight of cyaniding 3-glucoside = 449.2. ε is the molar absorptivity of cyanidin 3-glucoside = 26900; and C is the concentration of the buffer in milligrams per milliliter. Anthocyanin content was expressed as milligrams of cyanidin 3-glucoside equivalents per g of sample.

Tannin content determination

Tannins were determined on sorghum samples following the method suggested by Price and others (1978) with some modifications. Extracts of sorghum samples were prepared by continuously rotating 200 mg of ground grain with 10 ml of 80% aqueous methanol in test tubes at room temperature for 20 min. Methanol extracts were dissolved in water and an aliquot of vanillin reagent was added. Absorbance was measured at 500 nm using a spectrophotometer Beckman Coulter DU 800 (Beckman Coulter Inc, Fullerton California U.S.A.). Tannin content was expressed as milligrams of catechin equivalents per g of sample.

Antioxidant activity quantification by the Oxygen Radical Absorbance Capacity (ORAC) assay

Lipophilic and hydrophilic ORAC assays were performed essentially following the procedure by Prior and others (2003). Ground samples (1 g) were extracted with 10ml of hexane for 4 hr at room temperature. A sonicator (SC-15OT, Sonicor Instrument Corporation, NY, USA) was used to enhance extraction. The resulting sample-solvent mix was filtered and the solvent recovered. Spent grain was washed four consecutive times. Solvent was then evaporated by a nitrogen flush and the resulting solids resuspended in 50% aqueous acetone. Hydrophilic assay was performed in 80% aqueous methanol extracts. For the lipophilic assay, dried hexane extract was dissolved in acetone followed by RMCD solution. For the hydrophilic assay dilutions were made with a phosphate buffer. A Synergy^{TM} HT Multi-Detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) was used with fluorescence filters set to an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by KC4 3.0 software. Antioxidant activity was expressed as µmoles of Trolox per gram of sample.

Sample extraction for in vitro *analysis*

Samples were extracted with aqueous methanol (80%). Solvent (50 ml) was added to 5 g of sample in a 250 ml flask and placed in the shaker for 3 h at 150 rpm at 25°C for extraction. Samples were filtered through Whatman 1 filter paper in a Buchner funnel under vacuum, filtrate was concentrated using a rotary evaporator Yamato RE200 (Yamato Scientific America Inc., Orangeburg, N.Y. U.S.A.) set at 40°C to near dryness and then lyophilized with a Freezemobile FM25EL-85 (SP Industries, Germany) remove water. Dry samples were labeled as crude extracts and stored at -80°C until analysis.

In vitro cancer assay

MCF-7 breast, HepG2 liver and Caco-2 colon cancer cells were cultured as described by Romo-Lopez, (2005). Cell viability was estimated by trypan blue exclusion test. A 100 µl Cells (1 x 10^5 cell/ml) were injected in a 96-well plate, maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U/ml penicillin, and 100 µg/ml streptomycin. After 24 h incubation at 37ºC under a humidified 5% carbon dioxide atmosphere to allow cell attachment, cells were treated with sorghum crude extracts and incubated for 72 h under same conditions. (Romo-Lopez 2005)

Methanolic extracts were used to measure the ability of sorghum samples to inhibit cancer cell proliferation. Cell cultures were exposed to 0.5 mg /ml concentration of the extracts. Cell proliferation was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (Promega Corporation, Madison, WI) colorimetric based assay. Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS) to formazan. The absorbance at 490nm was measured using a Synergy™ HT Multi-Detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Statistical analysis

For the characterization and screening part results are means of 3 determinations ± standard error. Data were analyzed using a Microsoft Excel (Microsoft, 2007) spreadsheet.

Results and Discussion

The phenolic content of 25 different samples was evaluated. Among samples, sample #8 corresponding to the sumac bran, had the highest amount of phenolic compounds followed by sample #7 sumac grain and sample #9 black bran (Figure 1). Sumac is a type III, brown sorghum with pigmented testa with high amounts of phenolic compounds (Awika and others 2005). Compared to sumac sorghum which had almost 12 mg of gallic acid equivalents (GAE), sumac bran had approximately 17 mg. This was expected, since phenolic compounds are concentrated in the outer layers of the kernel (Awika and others 2005), and therefore the abraded bran would have the compounds concentrated. Sample 9, which is also a bran but from black sorghum, had values of approximately 8 mg.

Samples of whole grains were classified by the presence and absence of the pigmented testa and the pericarp color (Figure 1). Sorghum samples with pigmented testa had in general higher values of mg GAE. Sorghum samples without pigmented testa and black pericarp had an average value of 2.8 mg of GAE, samples with red or yellow pericarp and no testa had average values of 2.3 mg of GAE. A white sorghum (sample 1) had the lowest content of phenolics. Values of GAE were in general lower than values previously reported for the same samples, this was especially true for sorghums samples with pigmented testa that had an average GAE 3.45 vs 10.72 (Unpublished data, CQL). Differences in phenol content determination can be attributed to the method of extraction. Extraction of samples for Folin-Ciocalteu are commonly performed in 80% acetone, in this case extraction was made with 80% methanol. For this study samples for total phenols, flavonoids, anthocyanins, tannins and hydrophilic ORAC were extracted in 80% methanol in order to have the same extract composition analyzed and tested in the cancer cells.

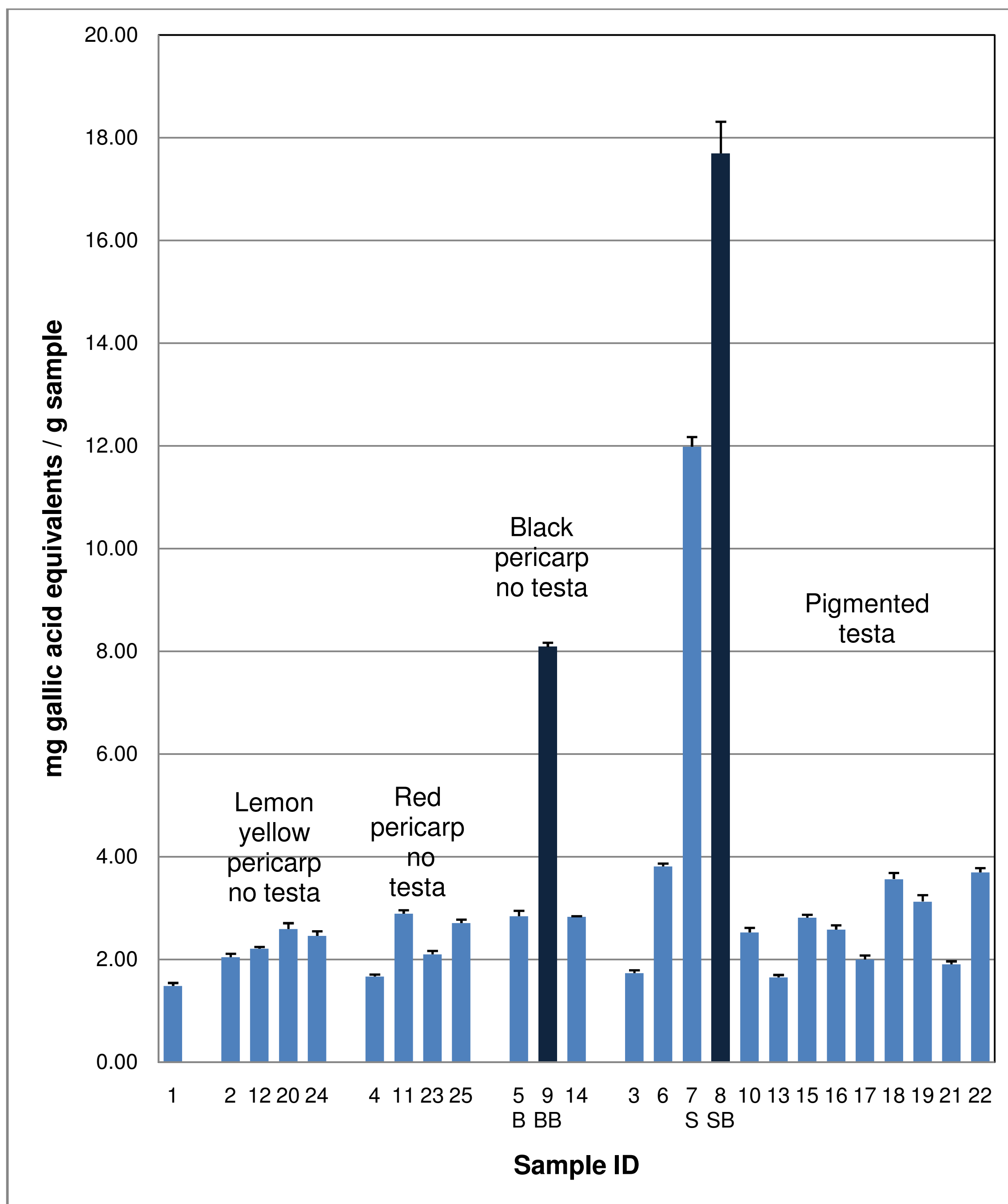

Figure 1 - Total phenol content of sorghum samples. Samples are grouped by pericarp color and the presence/absence of pigmented testa. Sample 1 is a white sorghum without testa. The characteristic of each sorghum entry tested is in Table 1. Error bars denote standard error.

Flavonoid, anthocyanin, and tannin contents were determined to characterize the type of phenolic compounds present in each sample. Flavonoids measured as mg of catechin equivalents (CE), were high for the bran samples 8 and 9 (Figure 2), sumac and black brans had 20 mg and 7 mg of CE respectively.

The average value of flavonoids for type II and type III sorghums (pigmented testa) was 1.68, this value was higher compared to red and yellow type I sorghums which had average values of 0.9 and 0.67 respectively. Black sorghums without pigmented testa had an average value of 1.44. Flavonoids reported in sorghum grains include anthocyanins (apigeninidin, luteolinidin), flavan-4-ols (luteoforol, apiforol), flavones (apigenin, luteolin), flavanones (eriodictyol, naringenin), flavonols (kaempferol), dihydroflavonols (taxifolin) proanthocyanidin monomer/dimmers (catechin, procyanidin B-1), and proanthocyanidin polymers (epicatechin) (Dykes and Rooney 2006). Type II and III sorghums had been recognized for their proanthocyanidin content, while black sorghums for the high amount of 3-deoxyanthocyanins (Dykes and Rooney 2006).

Anthocyanin content of samples was determined (Figure 3). Sample 9, black bran sorghum had the highest amount of anthocyanin with approximately 67 mg of cyanidin equivalents per gram (CAE). Sample 6, another black sorghum but with pigmented testa, had approximately 16 mg CAE. Black sorghums contained significantly more anthocyanin pigments than other types of sorghums (Awika and others 2004). Apigeninidin and luteolinidin are the major 3-deoxyanthocyanins found in this type of sorghums (Dykes and Rooney 2006). Sumac bran (sample 8) had almost 18 mg of CAE, the amount of anthocyanins in this sample can be attributed to a concentration effect. Sorghum with pigmented testa had an average of 5.31 mg of CAE. Luteolinidin and apigeninindin have been reported in red and brown sorghums (Dykes and Rooney 2006).

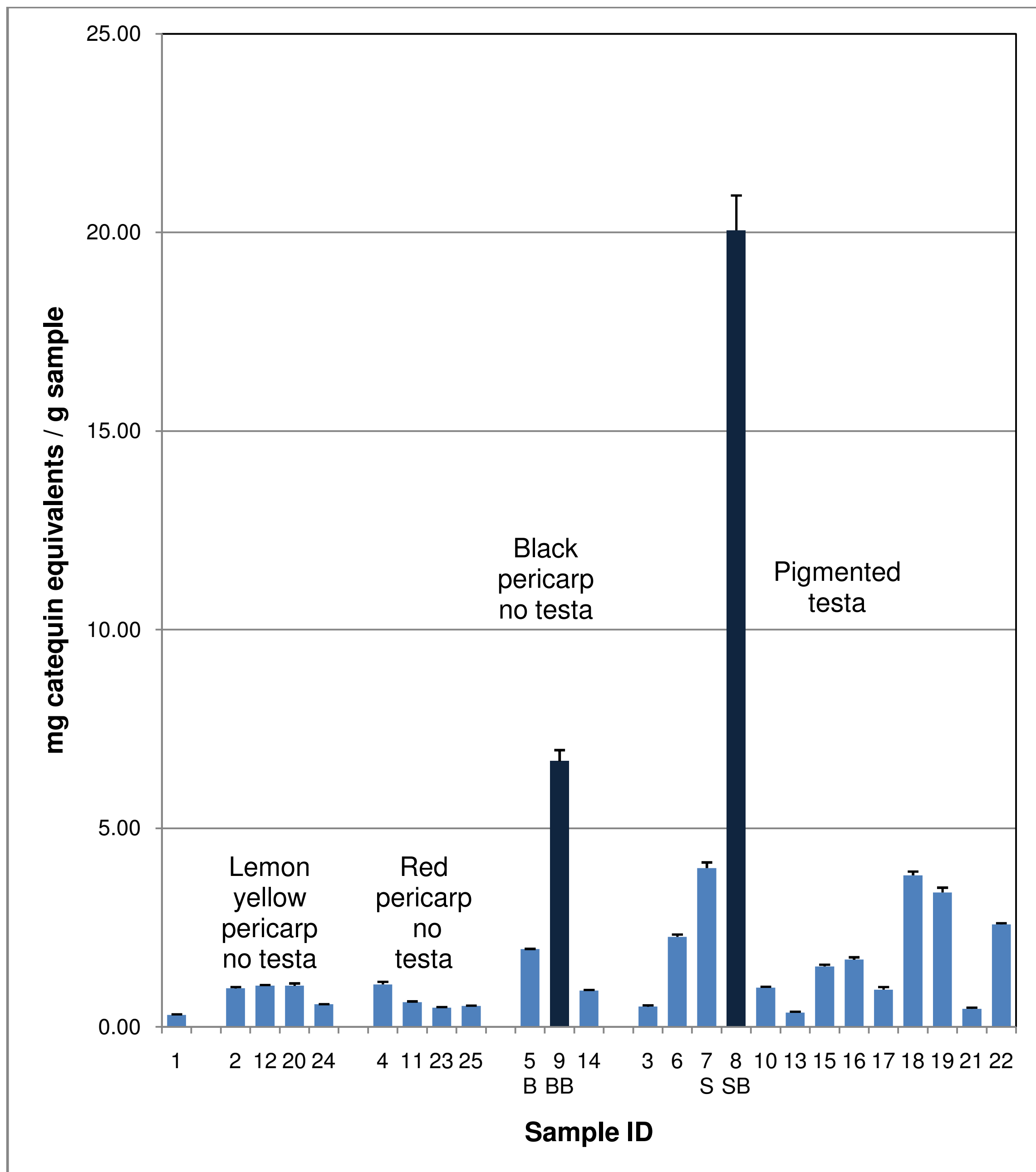

Figure 2 – Flavonoid content of sorghum samples. Samples are grouped by pericarp color and the presence/absence of pigmented testa. Sample 1 is a white sorghum without testa. The characteristic of each sorghum entry tested is in Table 1. Error bars denote standard error.

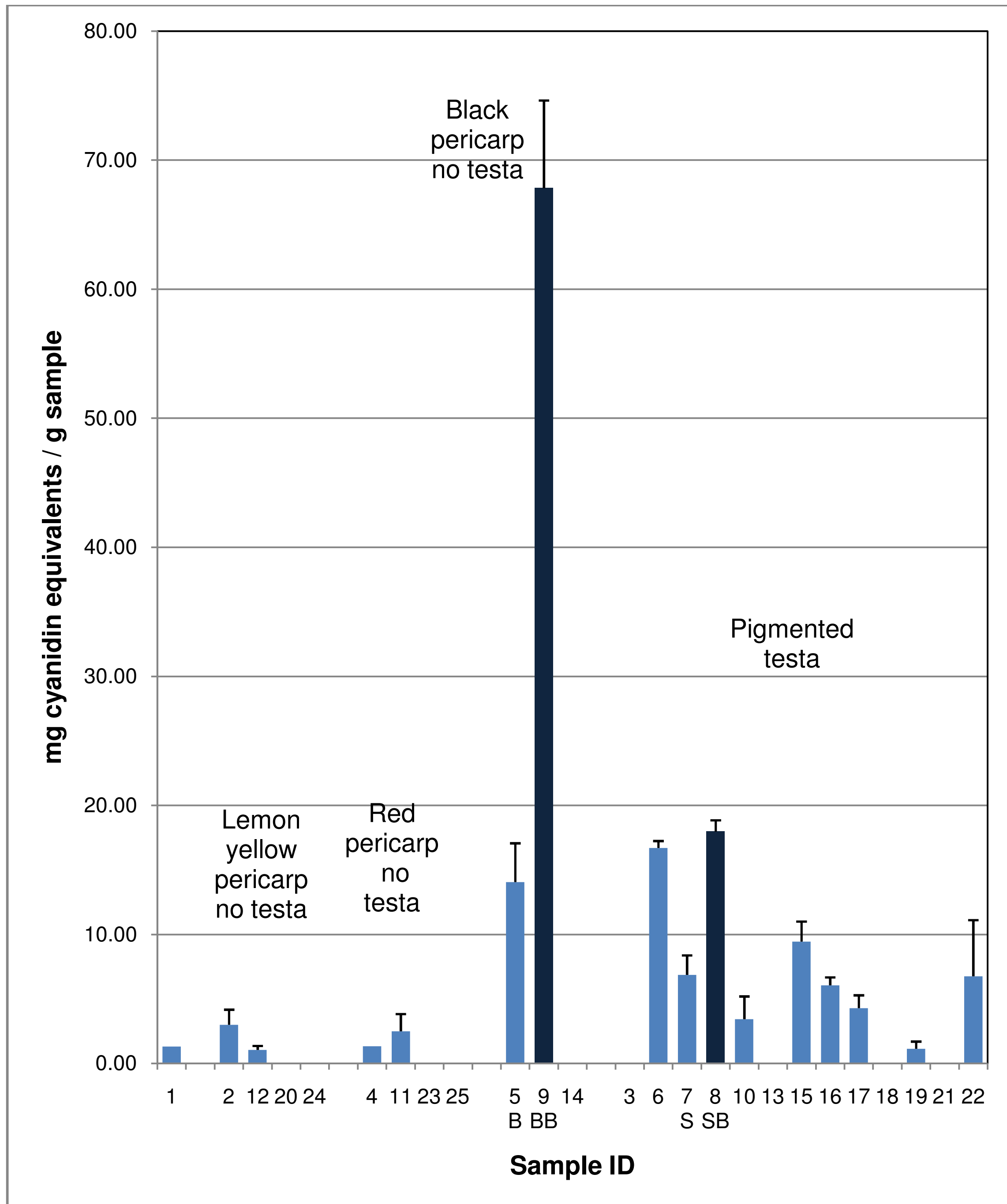

Figure 3 – Anthocyanin content of sorghum samples. Samples are grouped by pericarp color and the presence/absence of pigmented testa. Sample 1 is a white sorghum without testa. The characteristic of each sorghum entry tested is in Table 1. Error bars denote standard error.

Tannins from sorghum are only of the condensed type, hydrolysable tannins have not been reported in sorghum, although some early authors reported data on tannic acid equivalents (Dykes and Rooney 2006). Sumac bran (sample 8) had the highest amount of tannins (247 mg CAE/ g sample), this was expected since brown type III sorghums have the highest amount of tannins, which are concentrated in the bran (Figure 4). Samples 7, 15, 16, 18, 19 and 22 type III sorghums had a tannin content of 17 to 86 mg of CE. In type I sorghums tannins were not detected. These results were previously reported. Sorghums with the $B_1_B_2_$ gene (type II and type III) contains tannins while type I contain no tannins (Dykes and Rooney 2006). Samples 3, 6, 10, 13, 17 and 21 are also type III sorghums but tannins were not detected as it was expected. The tannin content of some of the samples was underestimated by the method of extraction used. For tannin extraction, aqueous methanol was used instead of acidic methanol. This decision was taken because at the beginning of the experiments we were expecting to correlate the flavonoid content of the samples with their antiproliferative effect on cancer cells. Another reason was that previously HCL showed a toxic effect on the cancer cell lines and HCL was difficult to remove from extracts without compromising the total phenolic content of the sample. It is well known that acidic methanol is used to extract tannins from sorghums. Methanol is not as efficient in extracting tannins as the acidic methanol especially in type II sorghums where the pigmented material is deposited in vesicles instead of being deposited along the cell walls like in type III sorghums. This difference makes tannins more difficult to extract from type II sorghums (Earp and others 2004). Additionally it has been reported that water interferes with vanillin in the tannin determination method giving lower values of tannin content (Terrill and others 1990).

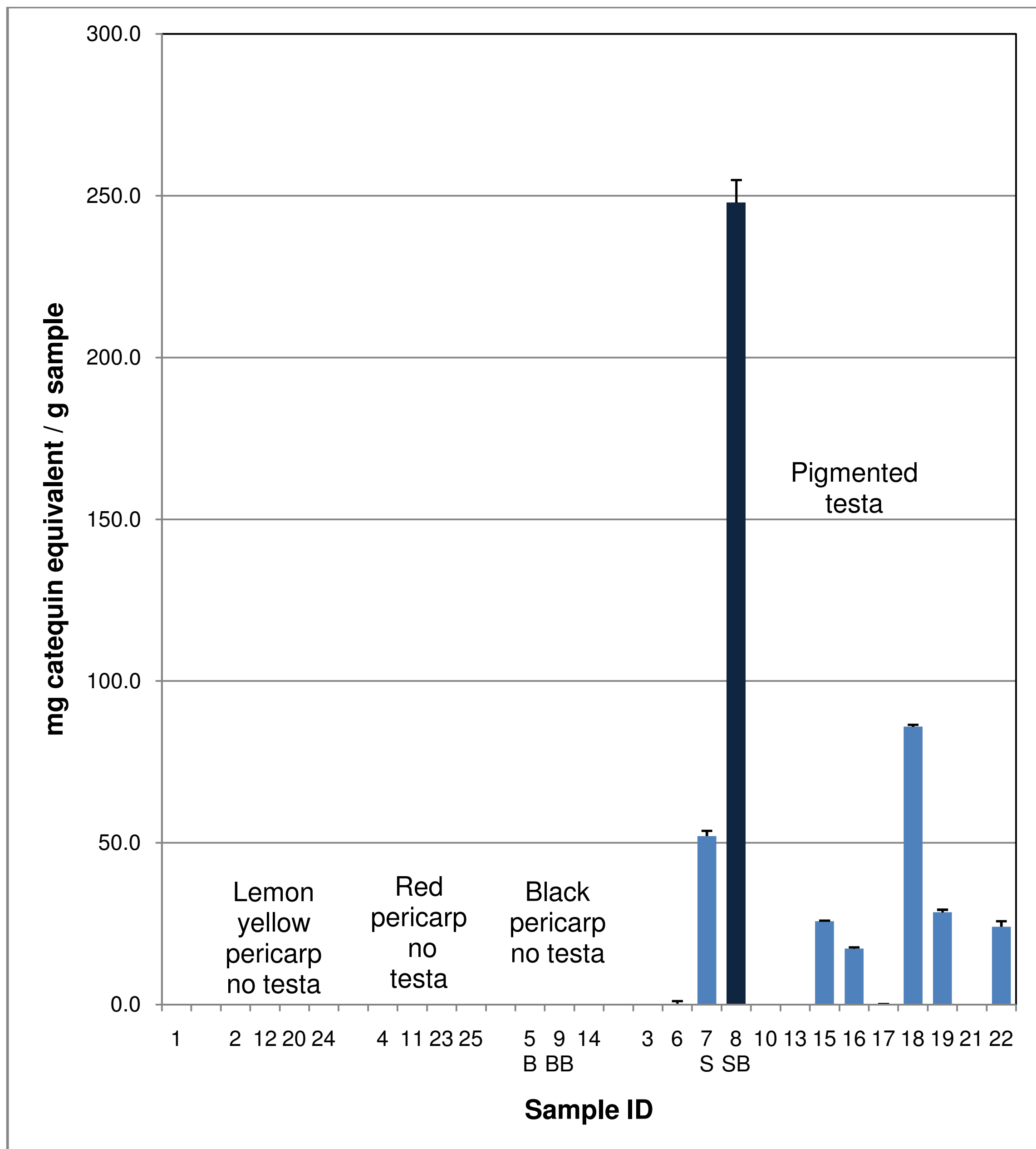

Figure 4 - Tannin content of sorghum samples. Samples are grouped by pericarp color and the presence/absence of pigmented testa. Sample 1 is a white sorghum without testa. The characteristic of each sorghum entry tested is in Table 1. Error bars denote standard error.

Phenolic and polyphenolic compounds had been recognized as potent natural antioxidants. The antioxidant capacity of these compounds have been related to health benefits such as counteracting the risk of cardiovascular diseases, cancer and cataract as well as a number of other degenerative diseases (Sahidi 2003).

Extracts from different sorghums samples had high antioxidant activities (Table 2), ranging from 91.1 to 1088 trolox equivalents/g. Differences in antioxidant activity among the 25 samples could be related to the type and amount of phenolics present on each sample. Phenolic compounds includes phenolic acids, flavonoids (flavonols, flavones, catechins, anthocyanins) and tannins, and each type exert different antioxidant activity. The antioxidant capacity of an extract is dependent on the profile (type and amount) of phenolic compounds (Reyes and Cisneros 2007).

Sumac sorghum had an ORAC value of 829, which is similar to values reported by Awika and others (2003), where sumac sorghum also had higher antioxidant activities than other samples. ORAC values for the sumac and black sorghum brans were 1133 and 930 respectively, these values are higher since phenolic compounds are concentrated in the bran.

For sumac bran (sample 8), it appears that tannin content is a considerable part of the phenolics reported in Figure 1. The high antioxidant activity of this sample could be related to the tannin content. Hagerman and others (2004) suggested that tannins are more potent antioxidants than simple monomeric phenolics. Tannins are likely responsible for the high activity in the brown sorghums (Awika and others 2003). It was expected that type II and III sorghum samples had higher average ORAC values than the type I sorghums since these samples had relatively no tannins. Results were not as expected probably because the use of aqueous methanol was not efficient for tannin extraction in all the samples and therefore the antioxidant capacity exerted by these compounds was affected, and not measured by the ORAC. For the samples were tannins were determined there was a positive correlation with the antioxidant capacity (Table 3), it is possible that this correlation were stronger if an efficient extraction of the tannins were achieved in all extracts.

Table 2 - ORAC values for the 25 sorghum sample set, sorghum type is included

Sample	Description	ORAC[1]			Sorghum Type
		Hidrophilic	Lipophilic	H+L	
1	ATX635 x RTX 436	228.79±19.57	20.40±0.53	249.19±20.11	1
2	SC748	285.99±6.89	19.91±0.12	305.9±7.01	1
3	Hegari	80.53±10.11	11.39±0.26	91.92±10.37	2
4	TX2911	552.65±6.68	4.91±0.14	557.56±6.82	1
5	TX430 Black	310.65±7.91	21.30±0.42	331.95±8.33	1
6	PI Black Tall	243.32±2.82	27.44±1.38	270.76±4.20	3
7	Sumac	811.74±6.80	17.21±0.43	828.95±7.23	3
8	Sumac Bran	1139.09±31.5	25.78±2.31	1133.08±33.81	3
9	TX430 Black Bran	920.04±31.41	24.90±1.56	920.04±32.97	1
10	SC719-11E	600.34±50.77	20.87±0.47	621.21±51.24	3
11	SC650	194.96±4.80	22.22±0.77	217.18±5.57	1
12	BRON 176	364.71±5.61	25.37±0.45	390.08±6.06	1
13	SC109-14E	95.34±2.91	20.43±0.90	115.77±3.81	3
14	Shawaya (Mostly Black)	244.81±5.39	22.70±0.99	267.51±6.38	1
15	Shawaya (Brownie Tan)	162.27±1.16	30.85±0.97	193.12±2.13	3
16	NK 121 A	138.72±2.02	22.28±0.33	161±2.35	3
17	NK 180	294.77±11.55	23.62±0.85	318.39±12.40	3
18	NK 8830	334.67±3.88	16.68±0.49	351.35±4.37	3
19	XM 217	302.86±10.26	19.32±0.40	322.18±10.66	3
20	EBA 3	953.94±3.57	24.98±0.38	978.92±3.95	1
21	SC575	951.38±33.59	12.36±0.39	963.74±33.98	2
22	SC103	1035.43±34.12	18.51±0.99	1053.94±35.11	3
23	SC630ii	442.23±9.28	15.80±0.64	458.03±9.92	1
24	SC1038	1065.75±7.90	21.97±0.49	1087.72±8.39	1
25	SC630 II	431.10±26.12	32.04±0.14	463.14±26.26	1

[1]mmol TE (trolox equivalents)/g, values are the sum of hydrophilic and lipophilic

Samples 20 and 24, type I sorghums, had surprisingly high ORAC value of 987 and 1087 respectively although they did not contain tannins or anthocyanins, and the amount of flavonoids and total phenols were also lower compared with other samples. These are lemon yellow pericarp sorghums, and it is possible that these specific samples had other high antioxidant compounds different from phenolic compounds exerting the antioxidant activity. Other high antioxidant compounds that have been detected in sorghum include carotenoids and tocopherols. Carotenoids are an important group of phytochemicals which has been recognized as natural antioxidants together with vitamin E (tocopherol), polyphenolics and flavonoids (Liu 2004). Carotenoid content of some sorghum cultivars has been reported to be 0.010 to 0.315 mg/kg. Among carotenoids, zeaxanthin has been recognized as the most abundant followed by lutein (Kean and others 2007); α tocopherol has also been detected in whole sorghum lipid extracts in concentrations of 0.219 to 0.577 µg/mg (Christiansen and others 2007). Non-polar compounds such as carotenes, xantophylls and tocopherols could be present in the methanol extracts even in small amounts. Choi and others (2006) studied the antioxidant contents and antioxidant activities of methanolic extracts from different grains including red sorghum. Extracts were prepared by placing 10g of ground grain with 200ml of methanol in a shaker for 24 h with subsequent centrifugation, evaporation and sample freeze storage. The polyphenol content for the red sorghum sample was 733mg/100g gallic acid equivalents, per 100g. Carotenoids and tocopherols were also present in the extract in amounts of 22 µg and 1.79 mg/100g of sample respectively. It is possible then, that methanol extracts contained other compounds besides phenolics.

Phenol contents of sorghums correlate strongly with their antioxidant activity measured either by the ABTS, DPPH or ORAC assays, Awika and others (2003) indicated that phenol contents of sorghum can be good predictors of their antioxidant activities. The ORAC method is reported to mimic antioxidant activity of phenols in biological systems better than other methods since it uses biologically relevant free radicals and integrates both time and degree of activity (Awika and others 2003). In this study, a good correlation between ORAC values (lipophilic+hydrophilic) (Table 2) and phenolic content was not found (Table 3), there was however a tendency, the higher the

phenolics (total phenols, flavonoids, anthocyanins and tannins) the higher the antioxidant capacity. Differences in these values with previous reports on sorghum could be due to the difference in the extraction procedures resulting in different amount/presence of phenolic compounds. Another reason that has been addressed previously (Kamath and others 2004; Wu and others 2004), is that there are other compounds besides phenolics measured by the Folin-Ciocalteu method that are responsible for the antioxidant activity. Wu and others (2004) reported that there are some foods were the correlation between total phenolics and ORAC values is not high, and this is the result from the presence of other compounds with antioxidant activity that are not phenolic, or because some phenolics are more effective than others.

Table 3 - Correlation of tannins, total phenolics, anthocyanins, flavonoids and ORAC values of the 25 sorghum sample set with inhibition of three different cancer cell lines

	ORAC h+l	**MCF-7 (breast)**	**Caco2 (colon)**	**HepG2 (liver)**
Tannins	0.61694	0.96596	0.96352	0.90840
Total phenolics	0.52824	0.32321	0.67531	0.78371
Anthocyanins	0.45071	-0.03231	0.96721	0.93595
Flavonoids	0.46878	0.34875	0.62764	0.97806
ORAC h+l	.	-0.28386	0.23715	0.87109

A screening of the antiproliferative effect of methanol sorghum extracts at 0.5 mg/ml concentration on different cancer cell lines was performed. Antiproliferative in vitro screening models provides preliminary data to select extracts with potential anticancer properties for further studies (Russo and others 2005)

The methanol extract of Sumac bran (Figure 5) had the highest percent of inhibition in mammary, colon and hepatic cancer cell lines (near 100%), compared to other samples. Sumac bran extract was the only one that inhibited all cancer cell lines. This

effect could be attributed to the phenolic content of the sample, especially the tannin content, since the methanol extract contained significant amounts of phenolics and tannins (Figures 1 and 4). Oxidative stress has been implicated in apoptosis and pathogenesis of cancers and antioxidants including proanthocyanidins can act as cancer chemopreventive and/or anticarcinogenic agents (Vlietnick and others 2004). The citotoxic effect of tannins in different cancer cell lines has been previously reported. Cranberries proanthocyanidins rich extracts have shown antiproliferative activity against the MCF-7 cancer cells (Yan 2007). Bawadi and others (2005) concluded that water-soluble condensed black bean tannins inhibited the proliferation of Caco-2 (colon), MCF-7 and Hs578T (breast), and DU 145 (prostatic) cancer cells. A proanthocyanidin rich fraction isolated from grape seeds was used in MDA-MB468 breast carcinoma cells resulting in highly significant inhibition (Awargal and others 2000). Sorghums methanol extracts with tannin content were highly correlated with the percent inhibition in MCF-7 cells (Table 3).

In general, whole sorghum extracts had a greater inhibitory effect on mammary than on colon and liver cancer cell lines. Samples 1, 2, 3, 5 and 13 caused approximately 60% or higher inhibition on MCF-7 cancer cells (Figure 5). A similarity between the samples besides the antiproliferative effect in the mammary cancer cells was not found. These samples had no tannins (Figure 4), the anthocyanin content for samples 1, 2 and 5 was not significantly different from the rest of the set, samples 3 and 13 had no anthocyanins (Figure 3); the phenolic and flavonoid content of the samples was similar to other extracts (Figures 1 and 2). These are type I sorghum samples (Table 1) except for sample 3 and 13 which are type II and III respectively. It is possible then, that these samples had a very potent type of phenolic (possibly flavonoid) exerting the inhibitory effect on the mammary cancer cells.

The antioxidant activity of sample 3 was the lowest of the 25 sample set. This data suggests that antioxidant activity of the samples is not necessarily highly related to the in vitro antiproliferative activity. A strong correlation between ORAC values and in vitro citotoxicity for the mammary, colon and hepatic cancer cell lines was not found (Table 3). Thus is possible that just a small amount of an undetermined compound has the

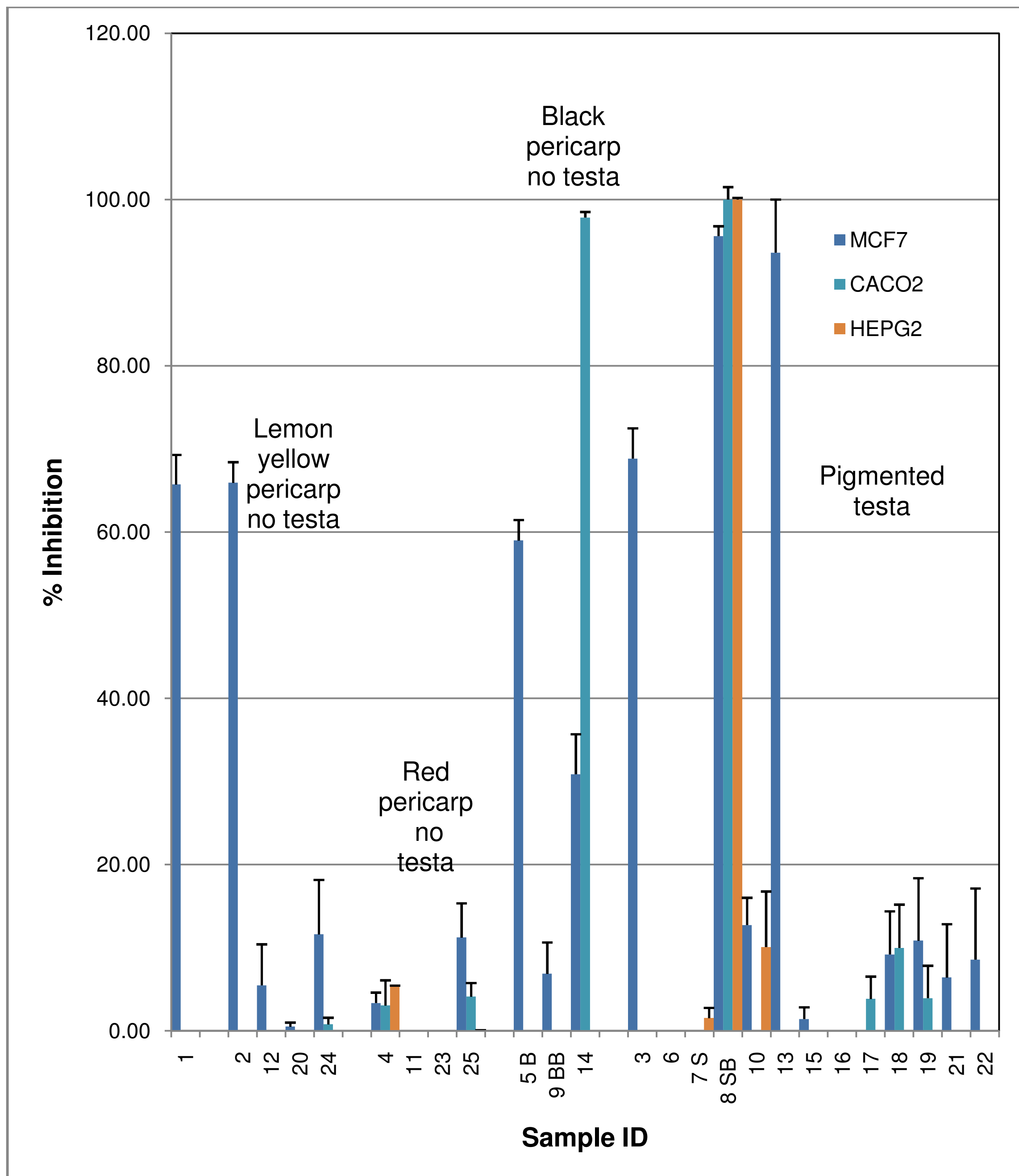

Figure 5 – In vitro citotoxic effect of sorghum extracts on different cancer cell lines. The characteristics of each sorghum entry tested are in Table 1. Error bars denote standard error.

potential to inhibit cancer cells by other mechanisms. The anticancer potential of phenolic compounds had been attributed to other mechanisms besides antioxidant activity, including modulation of phase 1 and phase 2 enzyme activities, anti-inflammatory activity, induction of apoptosis, enhancement or prevention of DNA damage, and estrogenic and antiestrogenic activity (Stoner and Casto 2004).

MCF-7 cancer cells are hormone dependent compared to Caco2 and HepG2 which are hormone independent cancer cells. MCF-7 cancer cells are very sensitive to estrogens and anti-estrogens compounds since they contain high levels of estrogen receptors (ER) (Coezy and others 1982). Estrogen binds to these ER forming an estrogen-receptor unit that begins a series of events that can instruct the cell to divide increasing the chance of a DNA mutation that could lead to cancer. It is possible that certain sorghums phytochemicals have anti-estrogen activity preventing estrogen to bind to ER. These compounds could be phenolics that are present in the extracts even in small amounts. Phenolic compounds that are known to be present in sorghum have been studied for their effects on hormone dependent cancers. Kaempferol significantly reduced the number of viable estrogen receptor-positive MCF-7 breast cancer cells (Nichenametla and others 2006). Green tea extracts (which are rich in catechin, epicatechins among others) inhibited growth of MCF-7 cells by inhibiting the interaction of estrogen with its receptors (Komori and others 1993).

Colon cancer cells inhibition was correlated to the tannin and anthocyanin content of the sorghum methanol extracts (0.9635 and 0.9672); by looking at the scatter charts there is a tendency of an increase in cell inhibition by the increase in the content of these compounds, however, not all the samples with tannins and/or anthocyanins promote Caco2 cells citotoxicity.

From the 25 sample set, just three sorghum grain extracts and the sumac bran inhibited liver cancer cells. Apparently flavonoid and tannin content were also correlated with the inhibition of this type of cells (0.9780 and 0.9084) but there were not a significant number of observations to draw conclusions. A study by Liu and others (2002) reported

that raspberry extract rich in phenolic compounds significantly inhibited in a dose-dependent manner the proliferation of HepG2 human liver cancer cells.

There is a possibility that other non phenolic compounds present in the methanol extract exerted the inhibitory activity. Besides phenolics, carotenes and tocopherols, there have been reports of other sorghum phytochemicals like the lipid-like compounds present in the wax that covers the pericarp of the kernel. Studies have shown that this wax is high in sterols and policosanols and that these compounds have been used for health purposes (Hwang and others 2004; Christiansen and others 2007). Gutierrez-Uribe and others (2008) showed that the highest anticancer activity from a type III sorghum extract was from a lipid-like compound. Even in very small quantities, the presence of these sorts of compounds on the methanol extract is possible. Sorghum waxes can be extracted using relatively non polar solvents (hexane, benzene, chloroform, acetone) because surface waxes are largely in a hydrophobically associated form, however small amounts of wax have been removed by just dipping plant leaves or whole plants in a solvent for less than 1 min,. In a study by Hwang and others (2002) ethanol was used for wax extraction obtaining yields similar for hexane.

In summary, extract from sumac bran had the highest amount of phenolic compounds, flavonoids and tannins. It also exerted the highest percent of inhibition (near 100%) in mammary, colon and liver cancer cell lines; these results could be related to the phenolic content of this specific sample, specially tannin content, although other samples with high levels of phenols did not show the same inhibitory effect. The phenolic content and the ORAC values of the sorghum set were not highly correlated, as in previous studies. Antioxidant activity of the samples was not correlated with the inhibition of cancer cells. Sorghum extracts inhibited more effectively the hormone dependent mammary cancer cells, this effect can be related to a specific anti-estrogen compound. The inhibitory effect could be due to specific compounds with known anticancer properties such as proanthocyanidins, flavonoids (kaempferol) and phenolic acids (ferulic acid) or to the presence of other compounds different from the phenolics that could exert the anticancer effect alone or in combination with other phytochemicals by mechanisms different from antioxidant capacity.

CHAPTER III
IN VITRO EVALUATION OF ANTICANCER PROPERTIES OF SUMAC SORGHUM

Literature Review

Cancer

Cancer is the largest single cause of death in both men and women (Russo and others 2005), among types of cancer, breast is a major malignancy, it affects one in every eight women in the USA, Europe, Australia, and Latin America; the disease kills about one fourth of the affected women (Bawadi and others 2005).

Investigators are increasingly describing cancer as a disease that involves both excessive cell proliferation and inhibition of the cell´s ability to die. Cancer cells lost cell´s ability to undergo cell suicide by a process called apoptosis. Apoptosis is the mechanism by which old or damaged cells normally self-destruct (Figure 6).

Cancer is ultimately the end stage of a chronic disease process characterized by abnormal cell and tissue differentiation: starts as an increase in the number of abnormal cells derived from a given normal tissue, then invasion of adjacent tissues by these abnormal cells, and lymphatic or blood-borne spread of malignant cells to regional lymph nodes and to distant sites occurs. This process which eventually leads to the final outcome of invasive and metastatic cancer is carcinogenesis (Sporn and Suh 2000).

Histological, epidemiological and experimental data suggest that breast carcinogenesis starts with hyperplasia, progressing through atypical hyperplasia to *in situ* and invasive carcinoma. The time course of these changes is difficult to estimate because, during this multistep process, unknown factors may stop progression and the hyperplastic lesions may regress and never undergo a malignant transformation (Bai and others 2001).

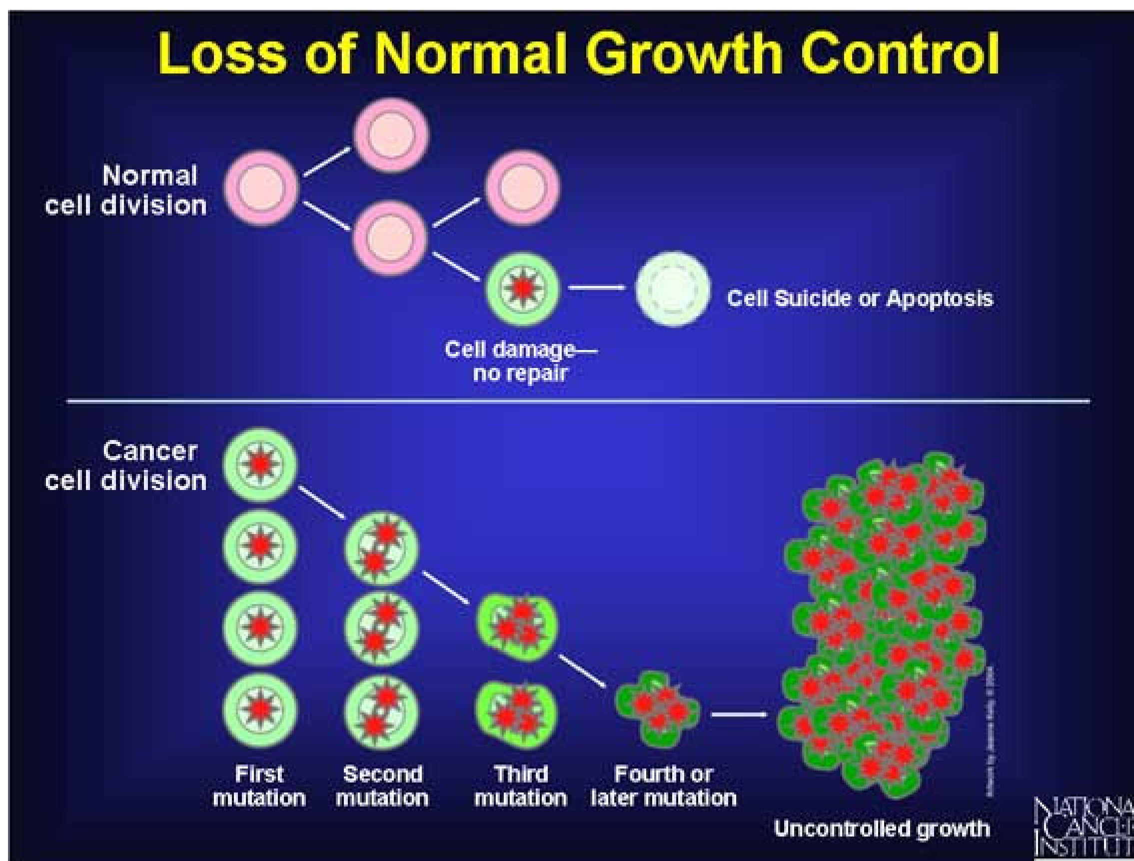

Figure 6 – Growth control in normal and cancer cell division. Normal cells performed apoptosis while cancer cells have an uncontrolled growth (American Cancer Society**, 2007).**

Causes of cancer include environmental (pollution, industrial), lifestyle (tobacco, diet), infection and genetic among others; the mechanisms are unclear: activation of oncogenes, loss of function of tumor suppressor genes, alteration in cell signaling pathways are some of the mechanisms studied. The oxidative effect of many compounds in cells has been studied. Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents may be present in air, food, and water, or they may be produced by metabolic activity within cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions. Overproduction of oxidants can cause an imbalance, leading to oxidative stress, especially in chronic bacterial,

viral, and parasitic infections. Oxidative stress can cause oxidative damage to large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for cancer and cardiovascular disease (Liu 2004). This potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants (Liu 2004).

Chemoprevention

Chemoprevention is a pharmacological approach to intervention in order to arrest or reverse the process of carcinogenesis (Sporn and Suh 2000), is a preventive strategy used to reduce the incidence of human cancer by inhibiting the initiation and spread of carcinogenesis or by preventing exposure to high levels of carcinogens (Kim and others 1999).

Pharmacologic or natural agents are used to inhibit the development of invasive cancer, either by blocking the DNA damage that initiates carcinogenesis, or by reversing the progression of premalignant cells in which such damage has already occurred. The goal of chemoprevention can be accomplished at three different levels: (1) at the clinical level, development of cancer must be prevented or delayed; (2) at the tissue level, preinvasive or premalignant lesions must be suppressed or reversed; and (3) at the cellular level, atypical clones must be eradicated (Lotan and Hong 2001).

Chemoprevention as a serious and practical approach to the control of cancer has been greatly enhanced by publication of three major randomized clinical trials in the field of breast cancer. Three different agents, namely tamoxifen, raloxifene and fenretinide have shown effective agents for prevention of breast cancer in women of varying degrees of risk (Sporn and Suh 2000). Incidence of breast cancer has increased in the last years and chemoprevention constitutes a valuable approach for its control.

Many substances derived from dietary or medicinal plants are known to be effective and versatile chemopreventive and antitumoral agents in a number of experimental models of carcinogenesis. Among the many classes of plant chemicals studied, phenolic compounds have been identified as anticancer agents and are consumed by humans in

a variety of plant foods and beverages (Singletary and others 2003). Hirose and others (1994) found that green tea catechins (GTC) in particular, inhibit rat mammary gland carcinogenesis. Purdue Research Foundation (2002) presented a US patent related to tea catechins as cancer specific proliferation inhibitors. Singletary and others (2003) studied the effects of concord grape juice, concluding that can inhibit the promotion stage of (DMBA)-induced rat mammary tumorigenesis in part by suppresing cell proliferation. The potential health enhancing properties of condensed tannins from black beans as inhibitors of angiogenesis was discussed by Bawadi and others (2005). Seeram and others (2005), studied polyphenols found in pomegranate juice for in vitro antiproliferative, apoptotic and antioxidant activities. Ademawobo and others (2005) hypothesized that flavonols reduce the risk of breast cancer through their effects on several biochemical pathways involved in carcinogenesis.

There have been numerous mechanisms by which plant phenolics, are hypothesized to be acting, including suppression of cellular oxidation, modulation of mitogenic signaling, suppression of aromatase activity, inhibition of inflammatory processes and angiogenesis, and induction of apoptosis (Singletary and others 2003).

In vitro studies

For the screening of potential chemopreventive agents, in vitro short-term tests can be applied because they are less time consuming, and also inexpensive and simple. In vitro tests can serve as biomarkers or endpoints of cancer to provide valuable insights into the mechanisms underlying carcinogenic processes and help screen for new chemopreventive agents (Kim and others 1999).

Cell lines are widely used in many aspects of laboratory research and particularly as in vitro models in cancer research (Burdall and others 2003). Techniques required to allow cells to grow and be maintained outside the body have been developed throughout the 20th century. Although some caution is required in interpreting data obtained by studying cells in vitro, it has allowed investigation of a complex disease such as cancer to be simplified to its component parts.

Citotoxic assays

Much effort has been made in the search for cancer chemopreventive agents and in vitro short-term tests are a valuable tool in the process. By using bioassays, fractions of active compounds can be tested in a specific cell line, to evaluate whether or not they possess a citotoxic effect.

The majority of breast cancer research uses established mammary cancer cell lines as in vitro models. MCF-7 is a breast cancer cell line derived in the Michigan Cancer Foundation in 1973 from a pleural effusion and is the most commonly used cancer cell line worldwide (Burdall and others 2003).

The human intestinal Caco-2 cell line has been also extensively used over the last twenty years as a model of the intestinal barrier. Caco-2 cells were derived from a human colon adenocarcinoma, and they differentiate spontaneously in vitro under standard culture conditions thereby exhibiting, enterocyte-like structural and functional characteristics. In differentiated state, they mimic typical characteristics of the human small intestinal epithelium, like a well-developed brush border with associated enzymes such as alkaline phosphatase and sucrase isomaltase (Lenaerts and others 2007).

The cell line HepG2 is derived from a well differentiated human hepatoblastoma, which retains many of the morphological characteristics of liver parenchymal cells. The ability to measure mutagenicity and genotoxicity directly in a single cell line of human origin and epithelial cell type, combined with the capacity for metabolic activation and proliferation makes the HepG2 cell line relevant as a model system for short-term testing of human mutagens and carcinogens (Eddy and others 1987)

Materials and Methods

Sample extraction for in vitro analysis

Sumac bran and whole sumac sorghum were extracted with aqueous methanol (80%). Solvent (100 ml) was added to 10 g of finely ground sample in 250 ml Erlenmeyer flask and placed in the shaker for 3 h at 150 rpm at 25℃ for extraction. Sample was then filtered through Whatman 1 filter paper in a Buchner funnel under vacuum. Solids were resuspended twice in fresh solvent and extraction was repeated. Filtrate (100 ml from each extraction) was concentrated using a rotary evaporator set at 40℃ to near dryness. Sample was resuspended with distilled water, sonicated and then lyophilized with a Freezemobile FM25EL-85 (SP Industries, Germany). Dry samples were weighed and labeled as crude extracts.

A sample of tannin extracted bran was prepared by diluting 2 g of crude extract from sumac bran in 10 ml of water, the solution was then homogenized and passed through a C_{18} Sep-pack column, then methanol was passed through the column and the eluent passed through a NH_2 column, tannins were retained and the eluent lyophilized and labeled, and saved for further analysis.

In vitro analysis

Cells were cultured as described by Romo-Lopez (2005). Cell viability was estimated by trypan blue exclusion test. A 100 µl Cells (1 x 10^5 cell/ml) were injected in a 96-well plate, maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U/ml penicillin, and 100µg/ml streptomycin. After 24 h incubation at 37ºC under a humidified 5% carbon dioxide atmosphere to allow cell attachment, cells were treated with sumac extract and incubated for 72 h under the same conditions.

Extract was used to measure the ability of sumac bran to inhibit cancer cell proliferation. Cell cultures were exposed to 0.06, 0.125, 0.25 and 0.5 mg/ml concentration of the

extract. Cell proliferation was measured using the CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation (Promega Corporation, Madison, WI) colorimetric based assay. Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS) to formazan. The absorbance at 490nm was measured using a Synergy™ HT Multi-Detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The concentration inhibiting 50% growth (IC50) was determined and expressed as milligrams of extract per milliliter.

Statistical analysis

Results are means of 2 replications, 3 determinations per replicate ± standard error. Data were analyzed using a Microsoft Excel (Microsoft, Redmon WA) spreadsheet.

Results and Discussion

Screening of different types of sumac sorghum extracts was performed to identify the one with the highest citotoxicity against cancer cell lines. Extracts of sumac whole grain, sumac bran with and without tannins were compared.

When tested on MCF-7 cells, methanol extract from sumac bran had higher percent inhibition compared to extracts obtained from whole grain, and the tannin free bran extract (Figure 7). As concentration of the extract increased, percent inhibition increased; at 0.50 mg/ml 100% inhibition was obtained. The whole sorghum extract did not show good inhibition, maximum inhibition obtained was near 5%, this may be due to a dilution effect of the active compounds present in the bran or to the presence of other compounds that inhibited or reduced the citotoxic effect.

When the tannins were removed the inhibition was significantly reduced, with maximum inhibition at 40%. Thus tannins play an important part of the citotoxic effect of the sumac methanol extract, either by themselves or acting synergistically with other compounds present in the extract.

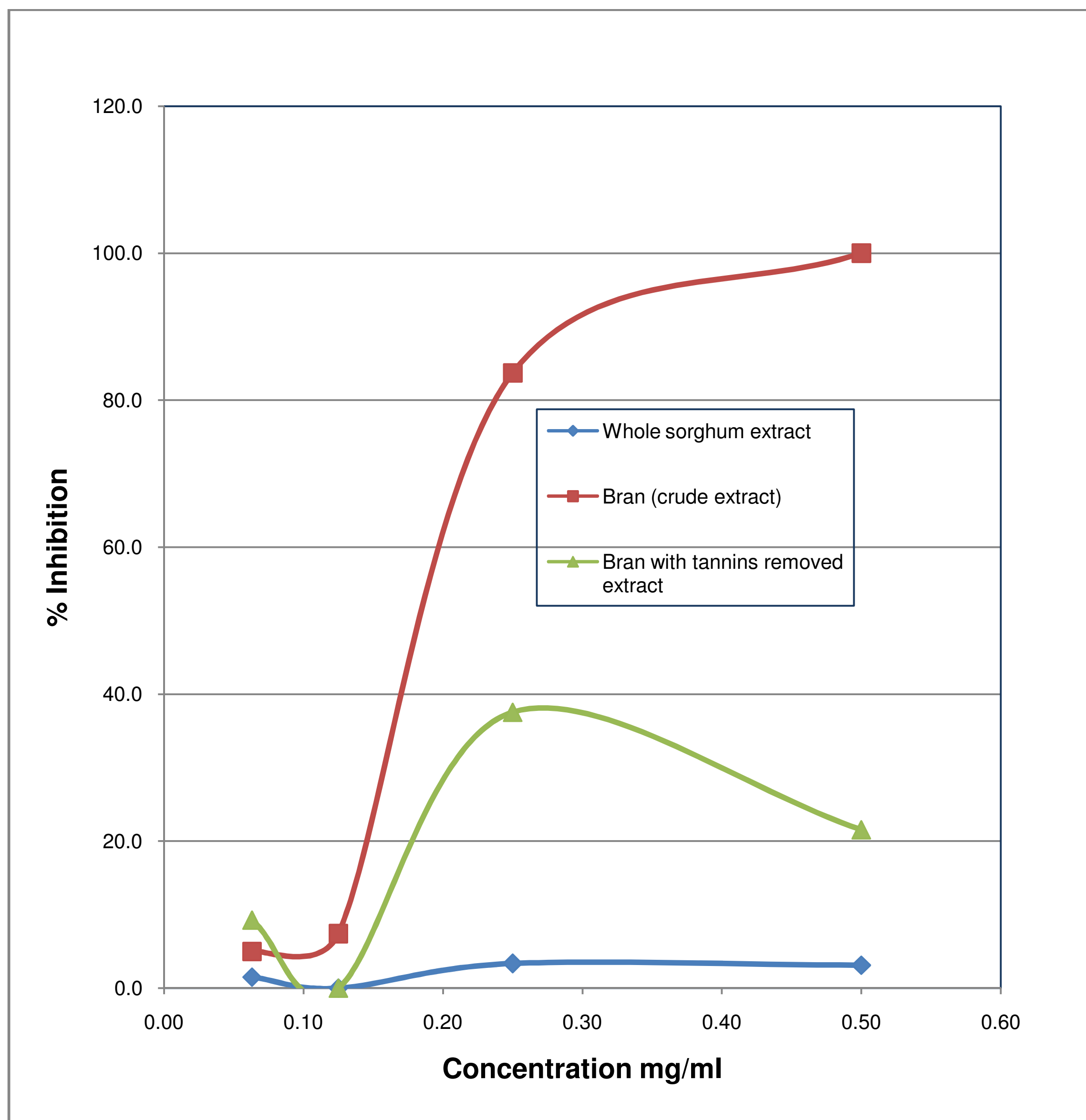

Figure 7 – Citotoxicity curves of different sumac sorghum extracts on MCF-7 cancer cells. Each point value is the average of two determinations.

When extracts were tested on liver cancer cells, the sumac bran extract also showed the highest percent of inhibition (100%) while the tannin free bran and whole sorghum extracts had 70 and 60% inhibition respectively (Figure 8). In this case whole sorghum and detannified extracts were almost as effective against HepG2 cells. In all cases as concentration of extracts increased the percent inhibition increased. Even though this is a different type of cancer cell line (non-hormone dependent) the phytochemicals associated to the sorghum bran extract exerted a similar inhibitory trend compared with the MCF-7 cells in vitro assay, the crude bran extract inhibited cancer cell growth more efficiently than the tannin free bran extract and the whole sorghum extract respectively. As concentration of extracts increased, the percent inhibition of colon cancer cells increased (Figure 9). The whole sumac grain extract exhibited the highest percent of inhibition (almost 100%) at a concentration of 0.5 mg/ml in Caco2 cancer cells. Bran and tannin free bran extracts also had good inhibition at high concentrations (above 70%). It appears that in colon cancer cells the component that promotes cells death is present regardless of the material used for extraction and in an amount sufficient to inhibit cancer cells; these compounds could be condensed tannins.

From these results (Figures 7 and 8) it was evident that tannins had an important role in the citotoxic effect of the extracts against MCF-7 breast and HepG2 liver cancer cells. In the last years studies have been performed in where polyphenols (proanthocyanidins – tannins) from food sources different from sorghum such as wine and grape seeds have been tested for anticancer properties. The degree of polymerization of the tannins and how they absorbed in the body would affect the anticancer properties of these compounds in in vivo studies.

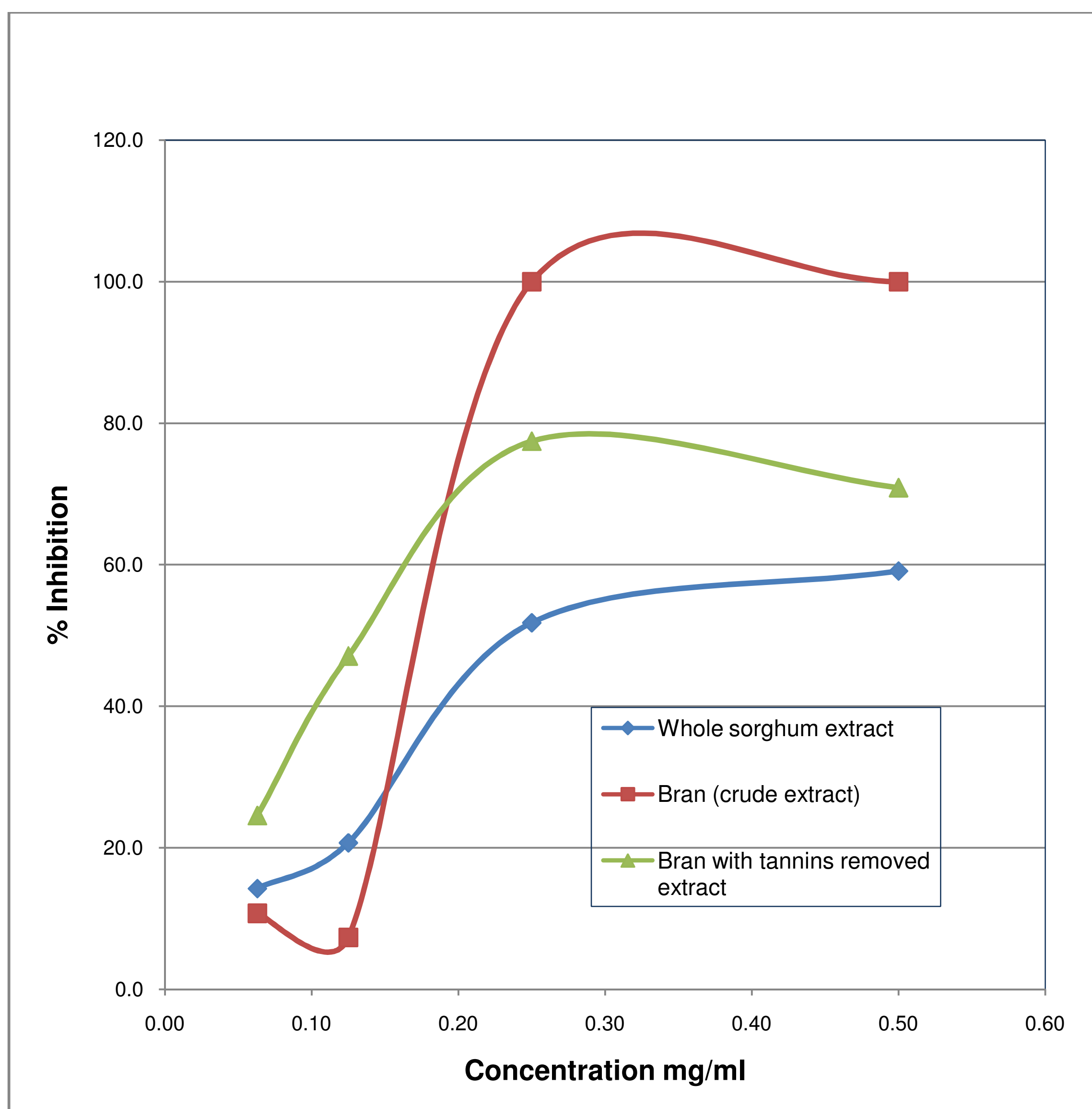

Figure 8 – Citotoxicity curves of different sumac sorghum extracts on liver HepG2 cancer cells. Each point value is the average of two determinations.

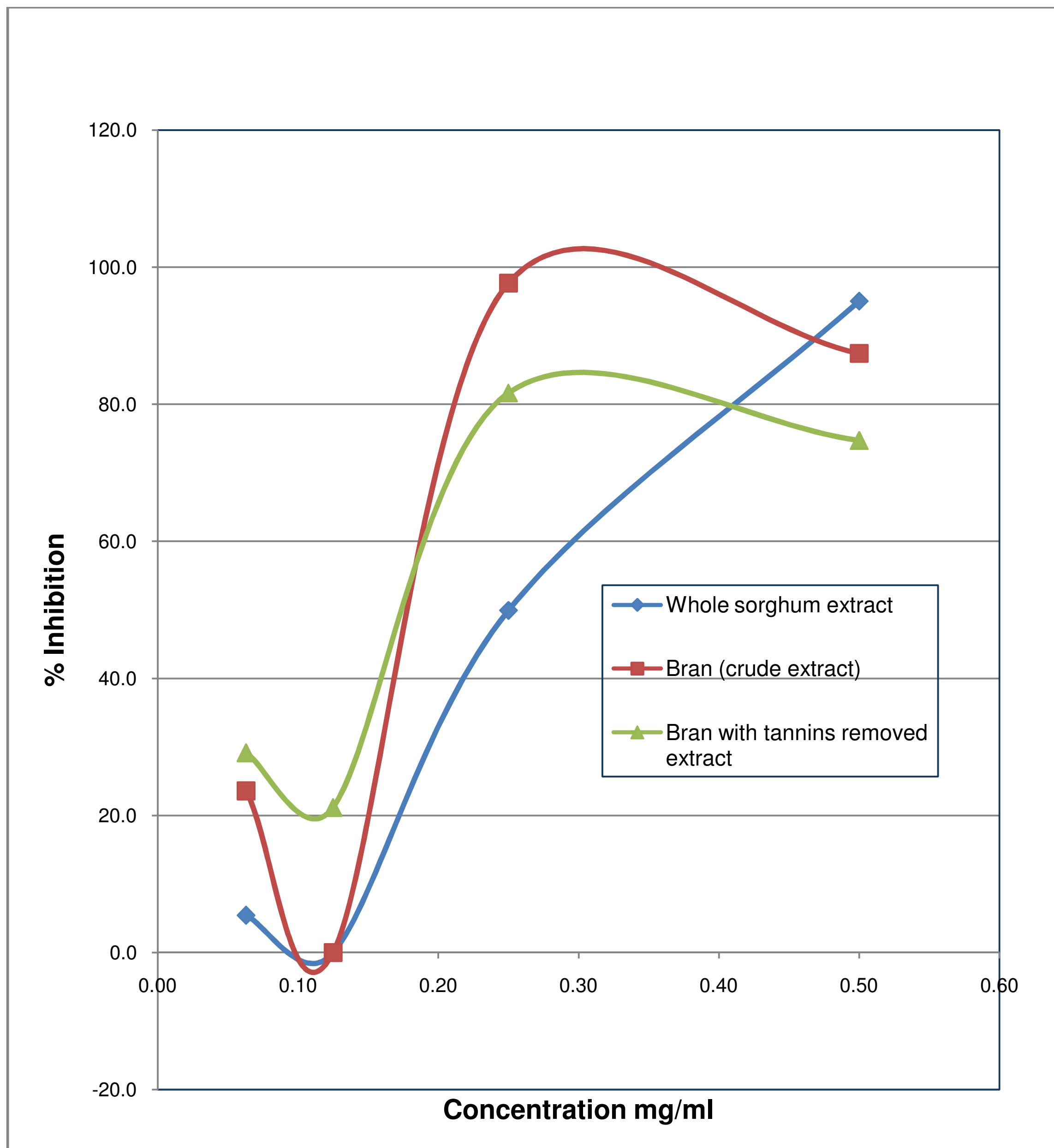

Figure 9 – Citotoxicity curves of different sumac sorghum extracts on Caco2 cancer cells. Each point value is the average of two determinations.

Tannins (proanthocyanidins) in sorghum are mainly B-type homopolymers of catechin/epicatechin. A-type proanthocyanidinds where the flavan-3-ol units are linked by C4-C8 interflavan bonds and by additional ether bond between C2-C7 have also been identified. A- and B- type heteropolymers with both catechin/epicatechin and gallocatechin/epigallocatechin hydroxylation patterns and glucosylated heteropolyflavans with proluteolinidin or proapigeninidin as extension units and the flavonones eriodictyyol or eriodictyol 5-O-β glucoside as terminal units have been reported (Dykes and Rooney 2006 ; Krueger and others 2003).

In a recent study, extracts from red wine containing mainly B-type proanthocyanidin dimmers were tested in vitro and in vivo, it was demonstrated they could suppress estrogen biosynthesis, therefore could be used as chemopreventive agents against breast cancer (Eng and others 2003). Grape seed extracts containing dimmers, trimers and other oligomers of flavan-3-ols were tested against MDA-MB468 breast cancer cells resulting in a significant growth inhibition (Agarwal and others 2000).

Tannins have shown to act as anti-initiators or antipromotores of cancer. A grape seed polyphenol extract (5, 10 and 20 mg) resulted in 30, 40 and 60% inhibition of skin tumor incidence in DMBA-initiated mouse skin. In addition, the number of tumors per mouse decreased by 63%, 51%, and 94% (Stoner and Casto 2004). The basic components of tannins (catechins, epicatechins, gallocatechins, epigallocatechins) have been related to cancer preventive properties. Several in vitro studies have shown that catechins and other structurally related compounds exhibited antiproliferative and growth controlling properties in cancerous and normal cell lines. Epigallocatechin and gallocatechin have been shown to exhibit antiproliferative action against MCF-7 breast cancer cells (Niechenametla and others 2006).

In summary, the methanol extract from sumac bran had the highest percent inhibition in all three types of cancer cell lines, especially against the hormone dependent mammary cancer cells (MCF-7). The major effect of the extract could be attributed to the tannin content. This extract was selected, and citotoxic bioassays were performed at different concentrations to determine doses to be used in an in vivo rat study.

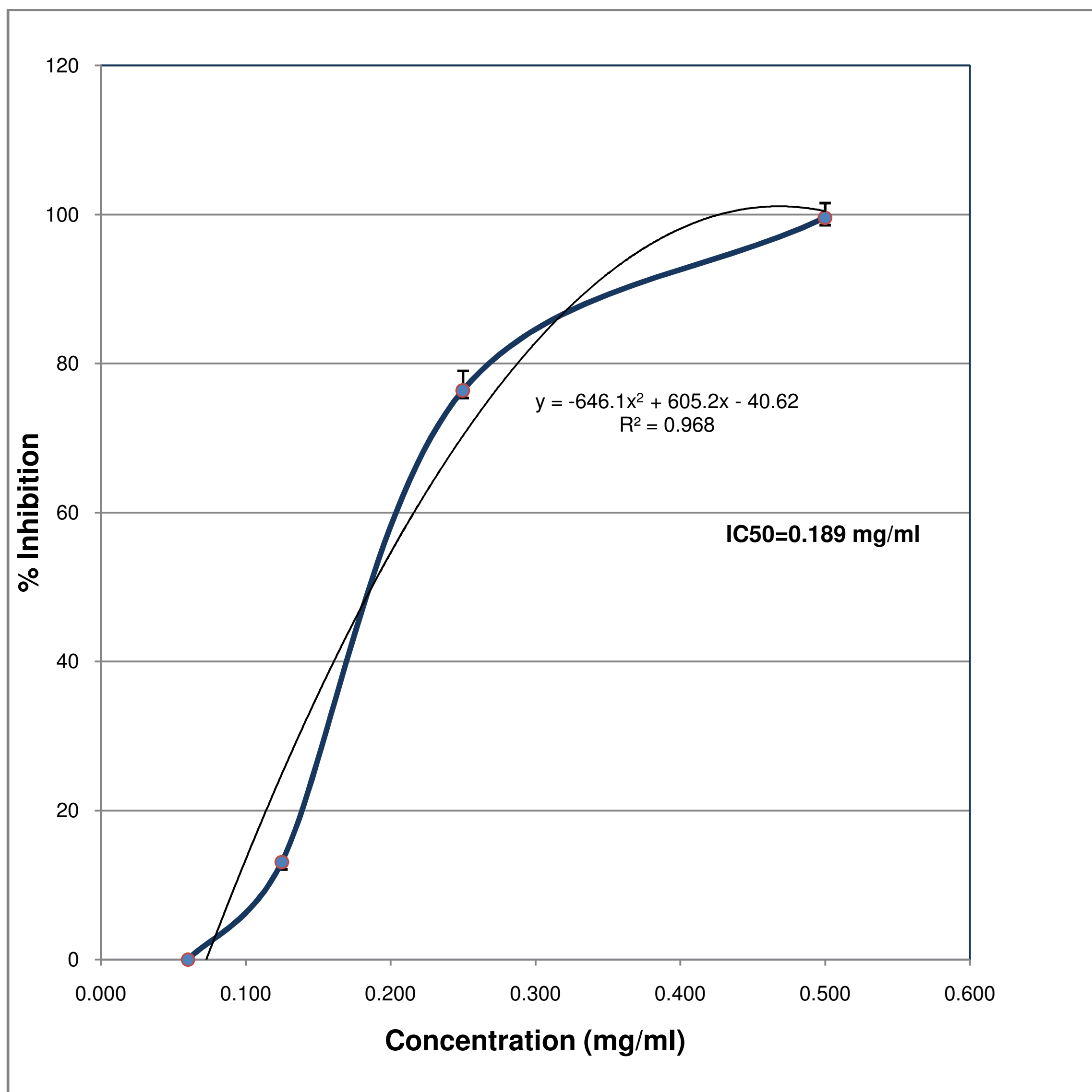

Figure 10 – Evaluation of sumac bran extract on MCF-7 mammary cancer cells. Results are means of three replicates (four determinations per replicate). Error bars denote standard error.

The anticancer effect of sumac bran extract was evaluated in MCF-7 cancer cells at different concentrations. As the concentration of the extract increased, antiproliferative effects increased (Figure 10). Almost 100% inhibition was obtained when using a concentration of 0.5 mg/ml. An IC_{50} value of 0.189 mg/ml was calculated from the curve; the curve shape was similar to the one observed in the screening part.

Growth inhibitory potency of different compounds in breast cancer cells have been reported in the literature. Genistein which is an isoflavone with chemopreventive properties has IC_{50} reported values of 0.0065 to 0.0105 mg/ml for (Peterson 1995). The effective concentration of catechins to exert in vitro antiproliferative activity seems to be 0.005mg/ml to 0.03mg/ml based on the type of cells (Nichenametla 2006). The IC_{50} for the inhibition of proliferation of MCF-7 by α-tocopherol was reported as 0.125mg/ml (Guthrie and others 1997). Compared to these data, the IC_{50} value of 0.189 mg/ml calculated for the methanol extract of sumac bran can be considered low, because this is a crude extract and not an isolated fraction or compound. The IC_{50} for MCF-7 cells was smaller than values for liver and colon cell lines. Therefore, the methanol sumac bran extract had a more potent effect on the hormone dependent cancer cells. The inhibitory effect of this extract could be due to a specific phenolic or a combination of phenolic components. From previous results it appeared that tannins had an important role in the observed citotoxic effect.

Other phenolic components present in sorghum like hydroxicinnamic acids (ferulic, caffeic, *p*-coumaric) and hydroxybenzoic acids (gallic, protocatechuic, vanillic) (Dykes and Rooney 2006), have been obtained from different fruits and have been reported to possess anticancer properties. Research was conducted with blackberries, raspberries, lemons and oranges, among other fruits. An extract containing *p*-coumaric, caffeic, ferulic, sinapic, and methoxycinnaic acids in combination with *p*-coumaric and vanillic acids inhibited viability and the colony-forming capacity of human breast and colon cancer cells in vitro. Ferulic acid added to diet on male rats significantly reduced the frequency of tongue carcinomas, and was proposed as an effective agent for oral cancer chemoprevention (Stoner and Casto 2004).

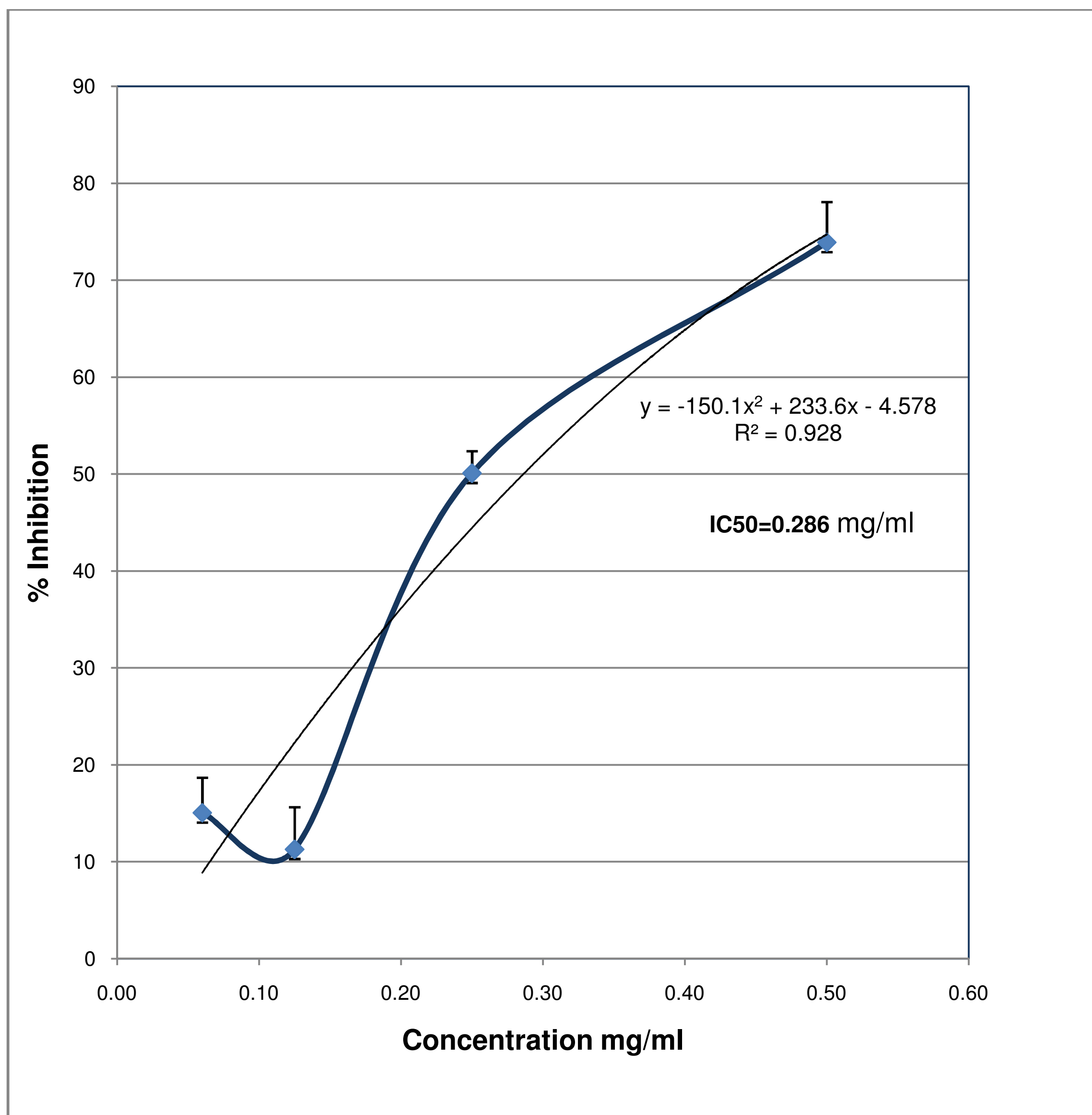

Figure 11 – Evaluation of anticancer effect of sumac bran extract on Caco2 colon cancer cells. Results are average of three replicates (four determinations per replicate). Error bars denote standard error.

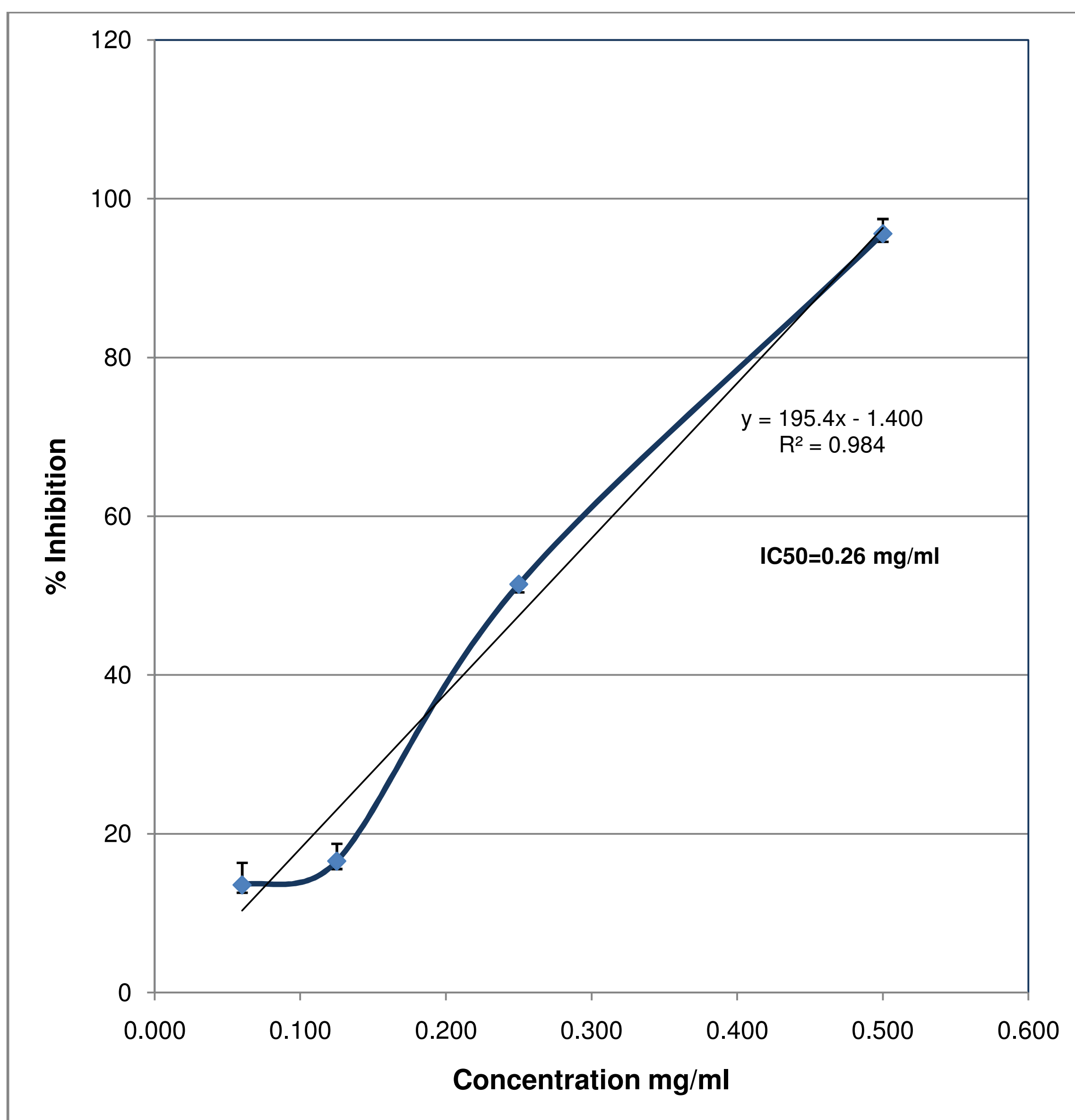

Figure 12 – Evaluation of anticancer potential of sumac bran extract on HepG 2, liver cancer cells. Error bars denote standard error.

When tested against colon cancer cells, as the concentration of the methanol extract from sumac bran increased, percent inhibition of cells also increased. In this case, a 100% inhibition could not be reached; almost 75% inhibition was obtained at 0.5mg/ml concentration (Figure 11). These results were consistent with the ones observed in the screening part, where maximum inhibition was around 85%. Differences in percent inhibition among cell lines are due to the nature of the cancer cells, MCF-7 cells are hormone dependant, Caco2 cells are not. IC_{50} value obtained from the curve was of 0.286 mg/ml. This is a low value taking into account that we tested and extract and not a fraction of a specific compound. These results are consistent with different in vitro studies. A blueberry extract rich in tannins and flavonoids tested in Caco2 cells had an IC_{50} value of 0.1 mg/ml. A grape seed extract rich in proanthocyanidins was tested on human colorectal HT29 and LoVo cancer cells; concentrations of 0.1mg/ml inhibited near 40 and 60% of the HT29 and LoVo cells respectively (Kaur and others 2006). In the case of isolated compounds, like the flavones apigenin and luteolin reported to be present in sorghum, IC_{50} values of 0.025 and 0.031 mg/ml respectively when used in Caco2 colon cancer cells, were reported by Kuntz and others (1999), the effect was associated with induction of the apoptotic pathway of cells. Results of the inhibitory effect of the methanol sumac bran extract also are consistent with an in vivo colon cancer study were brown and black sorghum brans administered in the diet reduced colon carcinogenesis in rats (Turner and others 2006).

For HepG 2 cells, a maximum percent inhibition of 95 was reached at a concentration of 0.5 mg/ml. IC_{50} value for HepG 2 cells was 0.26 mg/ml (Figure 12). When extracts from different raspberries varieties were tested in HepG2 cancer cells, IC_{50} antiproliferative values of ≈ 15 to 30 mg/ml were reported (Liu and others 2002). Compared to these values sumac bran methanol extract is a more potent antiproliferative agent compared to raspberries.

In summary, methanol extract from sumac bran had good inhibition potency against the three cancer cell lines, especially against the hormone dependent breast cancer cells MCF-7. Extract at a concentration of 0.5mg/ml inhibited 100% mammary cancer cells. The effect of the extract could be attributed at least in part to the tannin content. This concentration was selected in order to determine bran and extract concentrations for the DMBA induced rat in vivo study.

CHAPTER IV
IN VIVO EVALUATION OF SUMAC SORGHUM USING DMBA INDUCED FEMALE RATS

Literature Review

Sorghum and health

Several health and pharmaceutical benefits of sorghum have been reported recently (Dykes and others 2005). Compounds studied include protein fractions, sterols and policosanols and different sorts of phenolic compounds; these compounds have been related to health benefits such as cholesterol lowering properties and positive effects on cancer among others.

In a study of Kamath and others (2005) the α kafirin of sorghum was treated with chymotrypsin and separated yielding four different fractions that showed to possess angiotensin I converting enzyme inhibitory activity. Angiotensin I by itself is inactive, but when converted by the angiotensin-converting enzyme (ACE) to angiotensin II (active form), it causes narrowing of small blood vessels resulting in an increase in blood pressure.

Sorghum sterols and policosanols recently have been studied for its anti-cholesterolemic effects. A lipid extract from sorghum kernel tested on male hamsters significantly reduced plasma non HDL-cholesterol concentrations; sterols and policosanols were detected in the extract in concentrations of 0.35 and 8.0 g/100g of extract, respectively. Authors suggested that sterols reduced cholesterol absorption while policosanols inhibited endogenous cholesterol synthesis (Carr and others 2005). In other study by Burdette and others (2007), a sorghum fraction was reported to have a preventive effect on plasma lipids in the hyperlipidemic Syrian hamster model.

The anti-inflammatory effects of sorghum phenolic extracts in a topical inflammatory animal model were studied by Burdette and others (2007), extracts of sumac and black

sorghum brans had anti-inflammatory effects statistically similar to that produced by indomethacin (a non steroidal anti-inflammatory drug).

The chemopreventive properties of procyanidins from different fruits and vegetables have been widely studied with positive results (Stoner and Casto 2004). Bran from certain sorghums is a rich source of this type of phenolic compounds, commonly referred as tannins. Turner and others (2006) showed that induced rats fed with black and tanning sorghum bran had fewer aberrant colon crypts than those fed diets containing cellulose or white sorghum bran. Since tannins are very large molecules they are not absorbed intact. Recent studies have demonstrated that monomers and 3-mers are the major forms of procyanidins absorbed (Prior and others 2007). Awika and others (2004) discussed the positive effects of sorghum consumption on cancer, they suggested sorghum contains anticarcinogenic compounds, and that additional in vitro and in vivo studies are necessary to understand how polyphenols in sorghum affect cancer.

In vivo studies

Animal models of breast cancer have been widely used to study various aspects of breast cancer biology. The two most widely used experimental systems for the study of rat mammary tumorgenesis are the models in which tumors are induced in Sprague-Dawley (S-D) rat by 7,12-dymethylbenz(*a*)-anthracene (DMBA) or in the S-D or Fischer 344 rat by N-methylnitrosourea (NMU) (Russo and Russo 1996). DMBA is a synthetic polycyclic aromatic hydrocarbon that has been used extensively as a prototype agent in mutation and cancer research (Izzotti and others 1999)

Chemically induced mammary tumors develop by a multistep process. The initial step is a biochemical lesion caused by the interaction of the carcinogen with cellular DNA. In this interaction the DNA is damaged, and if the damage is not repaired efficiently, the result is a mutation, chromosomal translocation, inactivation of regulatory genes or more subtle changes not well identified as yet. Neoplastic development requires that the lesion becomes fixed, aided by cell proliferation, progressing to a third stage of

autonomous growth, resulting in cancer, when the lesion acquires the capacity to invade and metastasize (Russo and Russo 2004)

DMBA given by gavage in a single dose of 2.5 to 20 mg, induces tumors with latencies that generally range between 8 and 21 weeks with final tumor incidences close to 100% if sufficient time elapses before necropsy (Russo and Russo 1996). The susceptibility of the mammary gland to DMBA or NMU induced carcinogenesis is strongly age-dependent, and is a maximal when the carcinogens are administered to animals between the ages of approximately 45 and 60 days, that is the age of sexual maturity. (Russo and Russo 1996) Mammary tumors induced are hormone-dependent adenocarcinomas arising from terminal end buds (TEBs) on incompletely differentiated glands. These tumors bear a close resemblance to human breast cancer in their histologic and hormone-response patterns (Dias and others 2000).

The DMBA rat mammary model has found a broad application as a tool for assessing the efficacy of chemopreventive agents in inhibiting the formation of mammary tumors in preclinical studies (Izzotti and others 1999) The susceptibility of the mammary gland to carcinogenesis is modulated by the following parameters: 1) the presence of terminal end buds; 2) the size of the proliferative compartment; 3) the amount of binding of the carcinogen to the DNA; and 4) the ability of the cells to repair the DNA damaged by the carcinogen (Russo and Russo 1996)

The model has been widely used to assess the preventive cancer properties in different studies. Many popular dietary supplements enriched in polyphenols such as soy isoflavones, tea catechins, and resveratrol, have shown chemopreventive activity in cellular models of cancer. The health effects of green tea catechins in in vivo animal models were reviewed by Crespy and Williamson (2004), they concluded that studies using animal models showed that green tea catechins provided some protection against degenerative diseases, could act as antitumorigenic agents and could act as preventive agents against mammary cancer postinitiation.

Materials and Methods

A preventive mammary cancer study was conducted with laboratory rats. Four different treatments and a control were included (Table 4). Treatments were selected from the first two sections of the research by evaluating the anticancer potential of the samples in vitro. Treatments included sumac bran extract at 0.5 and 1.5 mg/ml concentration and the correspondent amount of bran. Extract concentrations were the ones that had 100% in vitro MCF-7 inhibition and 3X that amount, respectively.

Table 4 - Treatments selected to test the preventive effect of sumac bran and 80% methanol extracts using DMBA induced female rats

Identification	Treatment*
Control	No extract or bran added. Negative control.
Low extract	0.5 mg/ml of sumac bran extract, equivalent to 0.05% of diet
High extract	1.5 mg/ml of sumac bran extract, equivalent to 0.15% of diet
Low bran	40 mg of sumac bran, amount necessary to obtain 0.5mg of extract, equivalent to 0.4% of diet
High bran	120mg of sumac bran, amount necessary to obtain 0.5mg of extract, equivalent to 1.2% of diet.

*The bran concentration was determined considering a 12.5% yield of bran to extract. The extract concentration was determined considering 10ml of serum/rat, 10gr of diet/day/rat. DMBA 10mg/kg of body weight.

Extracts for in vivo study were obtained as described in the section of in vitro evaluation of anticancer properties of sumac sorghum, (sample extraction for in vitro analysis). In this case extractions were performed using 80 g of finely ground bran placed in a 1000 ml Erlenmeyer flask with 800 ml of aqueous methanol.

A total of 105 Wistar female rats (20-23 rats per treatment), 36-d-old weighing 75-100 g, were acclimated to the facility. Rats were blocked according to initial body weight. Then animals were randomly assigned to treatments and divided into groups of 4-6 in stainless steel cages and placed in a constant temperature and lighting environment (Table 5).

Table 5 - Distribution of rats per treatment for the in vivo experiment*

Treatment	High	Medium	Low	Super low	Rats/treatment
Control	5	5	5	8	23
Low extract	5	5	5	6	21
High extract	5	5	5	6	21
Low bran	5	5	5	5	20
High bran	5	5	5	5	20
Total				n=	105

*Initial number of rats per treatment, some rats died during experiment.

Rats had free access to diet and water. Beginning at 36-d-old (a week before DMBA induction) until the end of the experiment sorghum treatments were administered in the diet. Diets were formulated isocaloric, isonitrogenous, isolipidic and isofibrous (Table 6). Bran was analyzed to make proper adjustments (proteins 12.46%, lipids 7.61% and crude fiber 6.31%). Pellets were made to deliver diet-treatment to rats. A pelletizer (cellulose base) was included in the formula to enhance pellet formation (0.15%). Dry ingredients were mixed in a Hobart mixer at 1 speed for 2.5 min. Then, water was added (40%) and mixing continued for 1 min approximately. The dough like material was immediately formed into a “cylinder” and cut in approximately 1.5 cm width pellets. Pieces were placed in baking sheets and put into a convection oven set at 50-55°C for 16 – 20 h. Dry pellets were placed in plastic bags and frozen until used.

At 50-d-old a single intra-gastric dose of 10 mg/kg body weight of DMBA suspended in corn oil was administered to rats. Mammary tumor development was evaluated weekly, beginning at 5 weeks after DMBA administration. Time of appearance, location, number and size of tumors were documented during the experiment. At 20 weeks after DMBA administration rats were weighed, anesthetized, and tumors were excised according to the procedures of Appelt and Reicks (1999) and Zavala (2005).

Table 6 - Diet formulation per treatment for the in vivo study conducted with DMBA induced rats*

INGREDIENTS	Control %	Low Extract %	High Extract %	Low Bran %	High Bran %
Starch	50	49.95	49.85	49.72	49.15
Casein	20	20	20	19.94	19.82
Sugar	15.2	15.2	15.2	15.2	15.2
Vegetable oil	5	5	5	4.97	4.91
Cellulose	5	5	5	4.97	4.92
Mineral mix	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1	1
DL-Methionine	0.3	0.3	0.3	0.3	0.3
Bran	-	-	-	0.4	1.2
Crude extract	-	0.05	0.15	-	-
TOTAL	100	100	100	100	100

*Starch (corn starch), casein (casein purified high nitrogen MP Biomedicals), sugar (brown sugar economax), vegetable oil (soy + canola oil, economax), cellulose (alphacel non-nutritive bulk MP Biomedicals), mineral mix (ain-76 mineral mixture MP Biomedicals), vitamin mix (ain vitamin mixture 76, MP Biomedicals), DL-methionine.

Results and Discussion

An in vivo study with female rats was conducted. Bran and methanol extract from sumac bran were tested for anticancer potential; these treatments were selected from the experiments performed in the “screening and characterization of different sorghum for anticancer potential” and the “in vitro evaluation of anticancer properties of sumac sorghum” sections. Sumac bran was selected from the 25 sample set and the methanol extract after analyzing data of the MCF-7 in vitro assays.

Table 7 – Rats distribution for in vivo study*

Treatment	High Weight	Medium Weight	Medium-Low Weight	Low Weight	Rats/treatment
Control	4	2	5	7	18
Low extract	5	2	5	6	18
High extract	2	3	5	6	16
Low bran	5	3	5	5	18
High bran	3	2	4	5	14
Total				n=	105

* Rats per treatment at the end of the experiment.

At the beginning of the experiment 105 rats were assigned to four different treatments or a control group and at least 20 rats were on each of the treatments. During the experiment rats from the first two blocks (high and medium weight) got sick and some died, 25 and 50% respectively (Table 7). Rats from the high and medium blocks did not gain weight as compared to medium-low and low blocks (Figure 13). While rats from the medium-low and low blocks at the end of the experiment weighed over 350 g; some of high and medium block rats did not reach 200g. For these reasons the first two blocks were eliminated from the in vivo results.

Figure 13 shows the differences in weekly weight gains of the rats assigned to the different blocks. At the beginning of the experiment animals weighed an average of 100g, differences between blocks was significant at week 10 until the end of the study. Rats belonging to the high-weight block were thin and did not gain enough weight during the experiment. Rats belonging to the medium-weight block were also underweight, with reduced weight gain compared to the other two blocks.

Differences in weekly-weight gains among treatments (diets) were not evident throughout the experiment (Figure 14) indicating that blocking by initial body weight reduced the experimental error. Average weight of rats belonging to control, low extract, high extract, low bran and high bran groups were very similar during the 20 weeks of the experiment. Figure 13 shows large body weight differences according to block and Figure 14 clearly demonstrates the different dietary treatments did not affect body weights throughout the 20 weeks of the experiment.

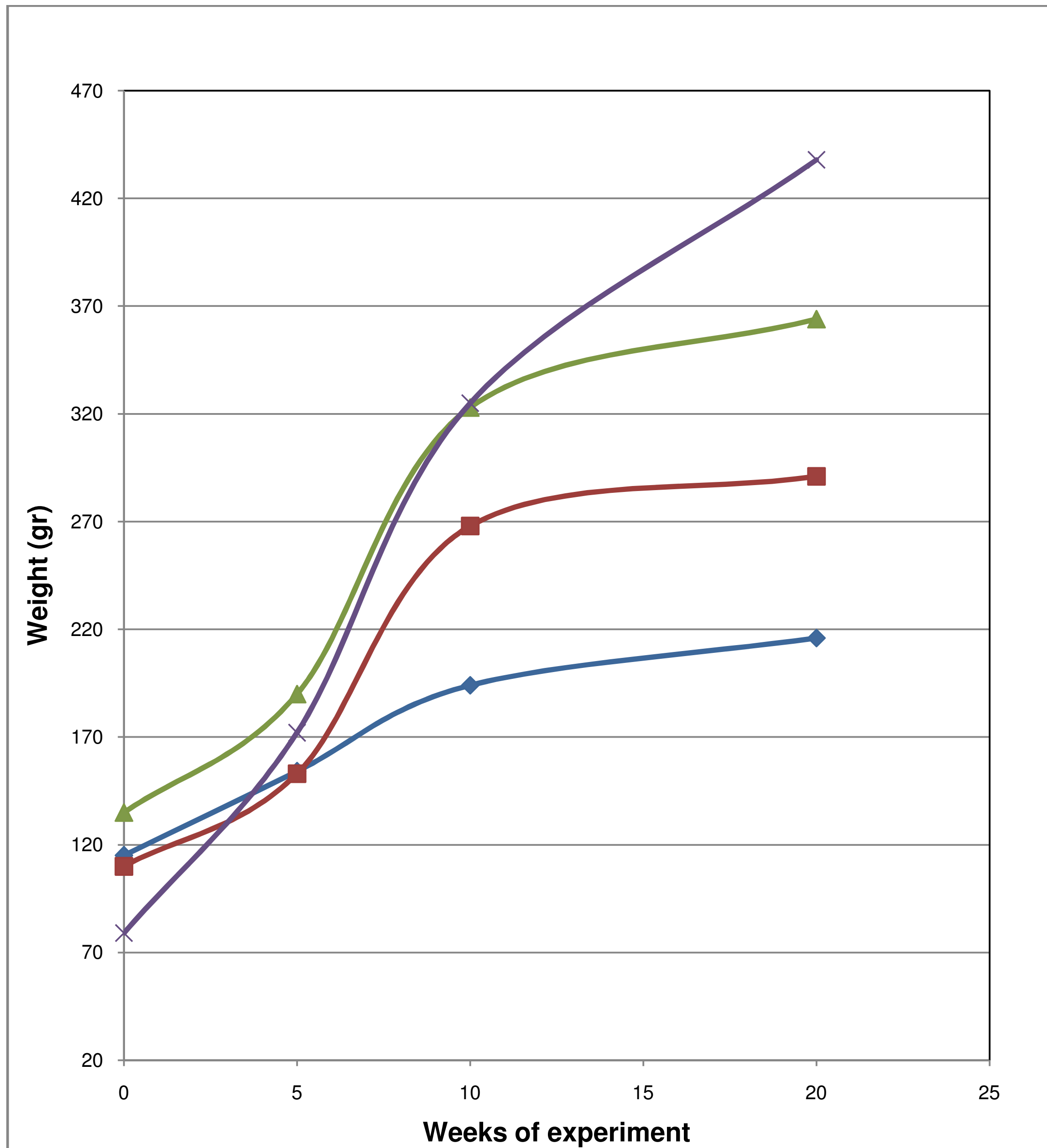

Figure 13 – Average weight of rats by block during the 20 week study.

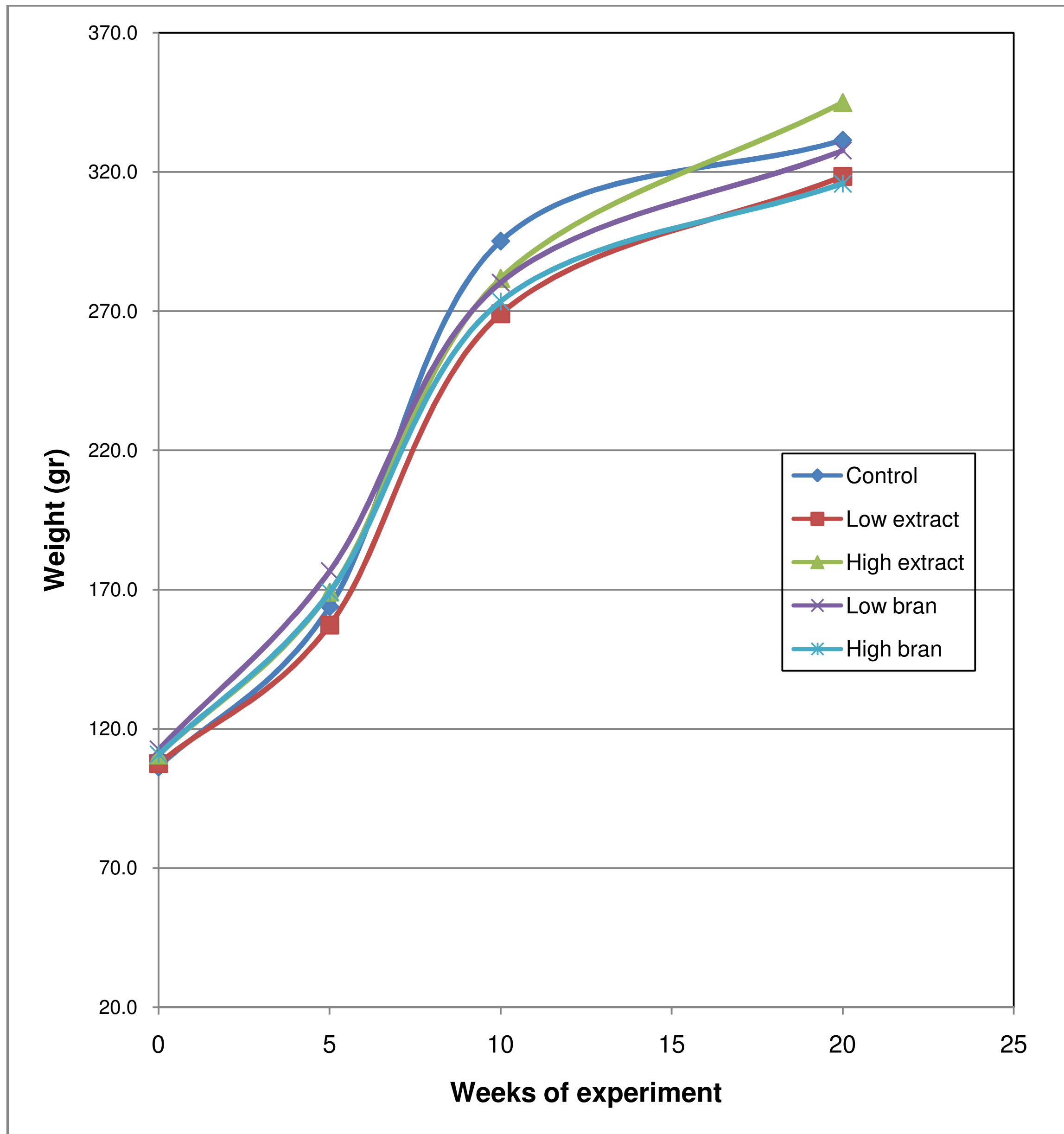

Figure 14 – Average weight of rats by treatment during the 20 week study.

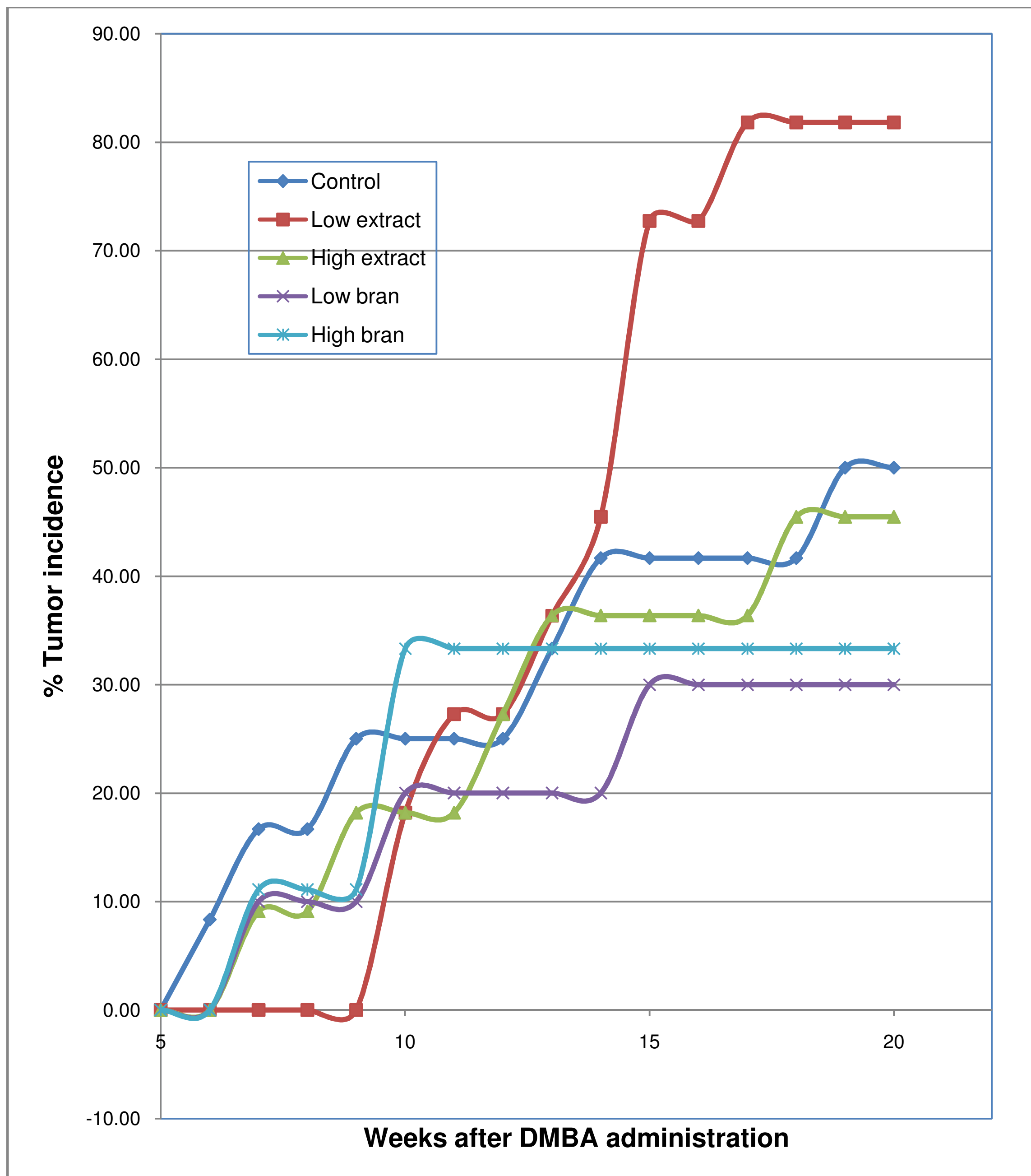

Figure 15 – Cumulative tumor incidence by treatments. Rats from blocks with medium–low and low initial body weight.

The percent tumor incidence during and at the end of the experiment was recorded for the four treatments and the control group (Figure 15). Surprisingly rats fed with the low extract diet had the highest percent of tumor incidence (80%). Fifty percent and 45% of the animals fed the control and high extract developed tumors while counterparts fed the high and low bran had 33 and 30% tumor incidence respectively.

Differences among treatments were not statistically different at any point. However a $P<0.10$ indicate that differences in tumor incidence between rats fed with extract and rats fed with bran were evident. Figure 15 clearly shows that animals fed the sorghum bran treatments had the lowest tumor incidence. Unexpectedly animals on the low methanol extract diets had more tumors than the rest of the treatments including the control. Probably during the process of extraction an estrogenic compound was concentrated, which promoted tumors at the concentration used in the diet (0.05%). The tumor incidence of animals fed the high extract diet was similar to the control group and lower compared to rats fed the low extract diet. The difference can be attributed to estrogenic compounds which according to concentration can exert or inhibit the development of hormone dependent tumors. Estrogen influences the growth, differentiation and function of many target tissues including the mammary gland. Estrogen is retained in certain target cells (such as the cells of the breast tissue) by the estrogen receptor. Estrogen receptors exist as subtypes α and β. Phytoestrogens (weakly estrogenic compounds) are believed to exert their chemopreventive action by interacting with estrogen receptors (ER) (Kuiper and others 1998), they prevent estrogen to form an estrogen-receptor unit that can instruct the cell to divide increasing the chance of a DNA mutation that could lead to cancer. Phytoestrogens however, depending on the concentration (may be present at high concentrations compared to that of endogenous estrogen) and the affinity to α or β receptor, can exert the prevention or promotion of cell division. It has been demonstrated that extracts with phytoestrogens had the ability to bind preferentially to ERβ stimulating the transcription of genes regulated by ER resulting in the induction of estrogen-dependent cancer cell growth (Boué and others 2003). Kaempferol for example, can act as an estrogen

receptor agonist (low concentrations) or growth inhibitor depending on concentrations used (Nichenametla and others 2006)

Both sorghum bran treatments had an inhibitory effect at high and low concentrations, compared to their methanol extracts and control group. Results of the inhibitory effect of the methanol sumac bran extract are consistent with an in vivo colon cancer study were brown and black sorghum brans administered in the diet reduced colon carcinogenesis in rats (Turner and others 2006).

The preventive effect could be attributed to the phenolic or to a different type of compounds. When preparing the extract, certain compounds in the bran were diluted in the methanol; other compounds were not extracted and remained in the insoluble mass that was discarded after extraction. From the in vivo data it appeared that bran contains compounds not obtained in the extract that had an inhibitory effect on tumors, these could be non-polar compounds such as the ones present in the sorghum wax (phytoesterols, policosanols) that could be acting alone or more probably together with other compounds as cancer preventive substances. Epidemiologic and experimental studies have suggested that dietary phytosterols may offer protection from common cancers such as colon, breast and prostate (Awad and Fink, 2000). Gutierrez-Uribe and others (2008) reported that a compound different from flavonoids with a molecular formula of $C_{17}H_{30}O_3$ or $C_{18}H_{34}O_2$ isolated from a type III sorghum had anticancer properties.

It appeared that bran treatments worked at the beginning of the carcinogenesis process in some of the animals. DMBA induced tumors develops by a multistep process. The initial step is a lesion caused by the interaction of the carcinogen with cellular DNA resulting in a promutagenic lesion (DNA adducts). Unless efficiently removed DNA adducts turns into tumors (Izzotti and others 1999). One of the possible mechanisms of cancer prevention in the bran treatment could be the prevention of adduct formation by the phenolic content of the sample. Phenolic compounds can enhance detoxification of carcinogens and decrease DNA damage (Jung and others 2006). Phenolics in purple grape juice (including proanthocyanidins) blocked the initiation stage of DMBA induced

rat tumors by suppressing mammary DMBA-DNA adduct formation, in part by an increase in the phase II enzyme metabolism (Jung and others 2006). Other phenolics like ferulic acid have been reported to deactivate carcinogens (Nichenametla and others 2006)

During evaluation of tumor(s) formation by palpation it was noticed that four rats belonging to the high and low bran concentrations diets had palpable follicle tumors that disappeared approximately 2 weeks afterwards. If tumors do not have enough blood to keep growing (vascularization) they could shrink and eventually disappear. This critical process in cancer biology is commonly known as angiogenesis. Some of the bran compounds may have inhibited tumor growth in these animals. Bran tannins could be the compounds acting as suppressing cancer agents, inhibiting blood vessel formation or angiogenesis. VEGF (vascular endothelial growth factor) has been shown to have an important role in the regulation of angiogenesis and water soluble condensed tannins from black bean inhibited the proliferation of cancer cells through inhibition of angiogenic factors such as VEGF (Bawadi and others 2005).

Table 8 - Tumor incidence, latency period, tumor/tumor-bearing rat and tumor weight in rats treated with DMBA[1]

Diet	n	Tumor incidence	Tumor latency period	Tumors/Tumor bearing rat	Tumor weight
		%	wk		g
Control	12	50.00	11.3±1.4	1.5±0.16	6.92±2.1
Low extract	11	81.82	13.3±0.8	1.6±0.22	6.89±1.4
High extract	11	45.45	11.8±1.3	1.2±0.13	3.20±0.5
Low bran	10	30.00	10.7±1.3	1.7±0.18	5.33±1.3
High bran	9	33.33	10.7±1.7	1.7±0.19	5.07±1.6

1 Values for tumor latent period, number of tumors/tumor-bearing rat, and tumor weight are means ±SEM. Tumor incidence was measured at the termination of the experiment (20 wk). Values are for palpable and non palpable tumors discovered upon termination of the experiment. None of the values are significantly different.

Tumor per rat, tumor weight, and tumor latency period were not statistically different among treatments (Table 8). The tumor latency period post DMBA administration was around week 10 for animals fed bran treatments and week 11 for control and high extract diets. Tumor latency of the low extract treatment was around week 13; even though it took longer for the tumors to appear, this treatment had the higher incidence of tumors.

Tumors per rat were between 1 and 2 in all cases. Bran treatments had the higher number of tumors per rats, although treatments with bran had the lowest incidence. Tumor bearing rats had in most cases 2 neoplasias. Control and low extract groups had higher tumor weight than the rest of the groups; tumors of rats were in some cases 10% of the live weight of the animal. Low extract compounds promoted growth of tumors. Tumors of rats on high extract diet were smaller than the other groups.

Results from the in vivo study were not as expected, because the in vitro data clearly suggested that the methanol extracts at different dosages had an inhibitory effect on hormone dependent and regular cancer cells. The in vivo study did not support results because rats fed the methanol extracts, especially the low concentration extract diet, had more tumors compared to the other treatments. One of the reasons for this is that for the in vitro studies, compounds (extracts) were directly put in contact with the cancer cells; in the animal study, compounds were delivered through the diet and were probably not completely absorbed and even when absorbed, they could be metabolized or modified in different ways. According to Manach and others (2004), polyphenols are not necessarily very active within the body either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition metabolites found in blood and target organs from digestive or hepatic activity may differ from the native substances in terms of biological activity.

In the previous experiments tannins appeared to be responsible (at least in some part) for the inhibitory effect of the methanol extract on the cancer cell lines. Tannins are large molecules containing multiple subunits from 1 to over 100 (Prior and others 2007).

Different studies have demonstrated that tannin bioavailability is low, and that it need to be depolymerized to be absorbed. Gu and others (2004) reported that in pigs proanthocyanidins were stable in the stomach and small intestine but depolymerized and degraded rapidly in the cecum and large intestine by bacteria. Prior and others (2007) conclude that the major form of tannin absorption was in molecules possessing 1 to 3 subunits. In a recent study by Gu and others (2007) sorghum bran was fed to piglets via gastric gavage. Sorghum bran had 44 mg/g of procyanidins. More than 80% were polymers with more than 10 catechin or epichatechin units. Results showed that procyanidins were absorbed as catechins in the weaning pigs, 47% of them stayed in the digesta after 4 hours of feeding, when bran was previously extruded, procyanidin content in the sample decreased and bioavailability was improved. The degree of polymerization for sumac bran has been previously reported, from a total of 58.44 mg/g of procyanidins just 3.27 mg/g are oligomers of 1 to 3 units (Dykes and Rooney 2006). It is possible then that the availability of these compounds during the in vivo study affected the results. In bran treatments, the bulking effect of the fiber could gave more time in the intestine for the absorption of the compounds and therefore had better results.

Another reason for the difference between in vitro and in vivo results could be diet preparation. Care was taken during formulation and preparation especially drying temperature control (50 and 55°C). All basic ingredients were kept constant and the mixing protocol standardized. However, the availability of the compounds may have been reduced in the process. Direct interactions between polyphenols and some components of food, such as binding to proteins and polysaccharides, can occur, and these interactions may affect absorption. Furthermore, more indirect effects of the diet on various parameters of gut physiology (pH, intestinal fermentations, biliary excretion, transit time) may have consequences on the absorption of polyphenols (Manach and others 2004). Kim and others (2004) suggested that weather or not a compound was chemopreventive depended on the diet in which the agent was administered.

In vitro studies can be well controlled and standardized, in vivo studies can only be controlled to a certain extent, because animals are subject to external factors. However,

the mammalian in vivo model is by far more useful and reliable in preparation for clinical trials with humans. Unfortunately, the in vivo results are inconclusive but can be useful for further planning.

For further in vivo testing I would recommend to fractionate the methanol extract and to investigate a lipid fraction for potent anticancer activity. I suggest to administer the selected compounds to rats by gavage. Since is possible that the anticancer properties are from just not one but the synergistic effect of different compounds, and because of the "good" results from the bran treatments, I would also suggest to repeat the experiment with bran, but in this case with processed (extruded) bran and to increase the percent in the diet to ensure the availability of the active compounds. Extrusion process increases bioavailability (Gu and others 2007), and sorghum bran at 2-5-5% in the diet could be an appropriate dose for studying health effects of sorghum procyanidins (Gu and others 2006).

In summary, the sumac bran methanol extract did not exert the expected anticancer effect on the DMBA induced rats, probably because of the poor absorption or availability of the polyphenols. Animals fed the experimental bran diets had fewer incidence of tumors compared to counterparts fed the control and methanol extract treatments. It appeared that the bran contains certain compounds that did not solubilize in the 80% methanol extract that could have a positive effect in the prevention of mammary cancer.

CHAPTER V
EVALUATION OF SUMAC SORGHUM EXTRACTS WITH A PHASE II ENZYME-INDUCING BIOASSAY

Literature Review

Phase II enzymes

Organisms have multiple defense systems, that ensure protection against the toxic effects of intrinsic and extrinsic oxidants and electrophiles to which they are exposed. These systems may be divided into four categories: phase 1 enzymes, phase 2 enzymes, phase 3 efflux transporters and thiol-containing molecules (Dinkova-Kostova and others 2005).

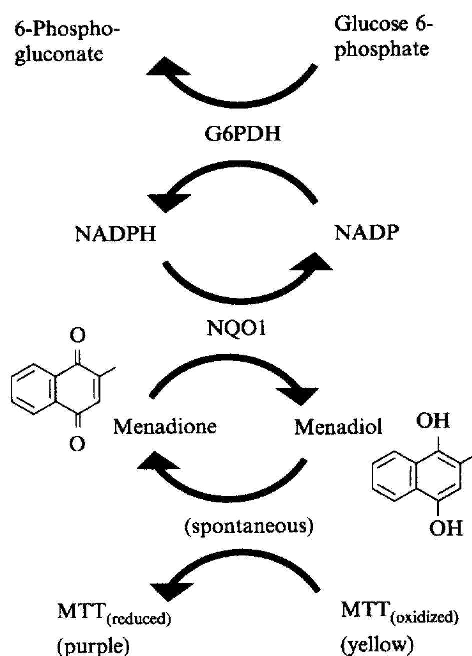

Figure 16 - Principle of the NQ01 assay. Glucose 6-phospate and glucose-6-phosphate dehydrogenase generate NADPH continually. This NADPH is used by the quinone reductase to transfer electrons to menadione. The menadiol formed reduced MTT spontaneously, to the purple formazan which can be measured spectrophotometrically (Fahey and others, 2004).

One specific mechanism of cancer chemoprevention is the ability to induce detoxifying enzymes (Phase I and/or Phase II enzymes) (Wettasinghe and others 2002).

Phase I enzymes, introduce functional groups onto largely hydrophobic organic molecules, and are usually cytochrome P450 enzymes - in some instances, these enzymes produce highly reactive products that are toxic to the cell. Phase II enzymes, such as glutathione S-transferases (GSTs) and UDP- glucuronosyl transferases, conjugates the products of phase I enzymes with hydrophilic groups in order to facilitate their excretion, and also enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, which inactivate reactive oxygen species (Dinkova-Kostova and others 2005). These enzymes are key in the prevention of cancer because they act as blocking agents and inhibitors of tumor initiation.

A simple cell culture system, developed to detect and quantitate the potency of phase II enzyme inducers, measures the elevation of NAD(P)H: quinone reductase (QR: a typical phase 2 enzyme) in murine hepatoma cells grown in 96-well microliter plates. This assay has been used for the isolation of the isothiocyanate sulforaphane as the principal and exceedingly potent monofunctional enzyme inducer in broccoli (Fahey and others 1997).

Figure 16 represents the principle of the NQ01 bioassay. For the test Hepa 1c1c7 line is used because cells have many characteristics of normal tissues, in particular the capacity for carcinogen activation and xenobiotic metabolism. The use of this assay has led to the discovery of a number of potent chemoprotective agents from plants (Fahey and others 2004).

Materials and Methods

Preparation of sumac bran extracts

Different extracts (3) were obtained from sumac bran for testing, including aqueous methanol, acidic methanol and hexane. The aqueous methanol extract was obtained as described previously. Procedures to obtain extracts using acidic methanol were the same as aqueous methanol but instead of using 80% methanol, the solvent was 1% HCL methanol. In these two-cases filtrates were concentrated to half the volume using a rotary evaporator Yamato RE200 (Yamato Scientific America Inc., Orangeburg, N.Y. U.S.A.) set at 40°C. The concentrated filtrate was used for the Phase II enzyme bioassays.

Hexane extract was obtained with some modifications of the procedure reported by Hwang and others (2004). Sorghum bran (50 g) was refluxed with hexane (335 ml) for 5 h. The mixture was filtered through a coffee filter paper lying on top of a Whatman No. 2 filter paper. The filtrate was stored at -18°C for 14 h. The cold miscella was filtered again through a Whatman No. 42 filter paper, and then desolventized using a rotary evaporator. The precipitate of long-chain lipids was collected and used for bioassay.

Bioassay

Induction of quinone-reductase was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates following the “Prochaska” microtiter plate bioassay for inducers of NQ01 (Fahey and others 2004). Stock solutions included, cultured medium, bioassay medium, trypsin, digitonin, dicumarol, BCA (bicinchoninic acid) and MTT (3-[4,5-dimethylthizo-2-yl]-2,5-diphenyltetrazolium bromide; a tetrazolium dye that is reduced non-enzymatically by menadiol) buffers. Methanol and hexane extracts were concentrated at 25 and 50 mg/ml respectively, these concentrations changed because of the dilution with solvent and medium. For methanol extract, concentrations were 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.35 and 16.7 µg/well. For hexane extract, concentrations evaluated were 0.15, 0.31, 0.6, 1.25. 2.5, 5, 10, and 20 µg/well.

Procedure

Day 1. Cell culture in 96-well microtiter plates

Culture medium was removed from cells (stock cells placed in petri dish at a concentration of 1 x 10^7 cells/ml); then 8 ml of phosphate buffer (PBS) was placed in the dish twice and discarded. A 1 ml solution of 0.05% of trypsin was added and the dish was placed in an incubator at 37℃ and 5% CO_2 for 5 min (standard conditions). After incubation 5 ml of maintaining medium was added to the petri dish to remove adhered cells. The suspension was placed in a 15 ml Falcon tube. Suspension was homogenized, and 1 ml aliquot was taken to perform cell quantification using the hematocitometer protocol. Dilutions were made to obtain 50,000 cells/ml. Medium containing 50,000 cells/ml was placed in a sterile reservoir and with a micropipette 200 µl were placed in each well (96-well plaque) from column 2 to 12 leaving column 1 empty. The plate was then placed in an incubator for 24 h at 37℃ and 5% CO_2 atmosphere.

Day 2. Extract addition

After 24 h, to allow cells to adhere, the plate was removed from the incubator and medium was discarded. Culture medium was then replaced with fresh medium (20ml per plate) containing antibiotics to permit the introduction of non-sterile extracts. Depending on the solvent used to prepare extracts, methanol or DMSO (for hexane extraction) was added to medium in a minimum concentration to avoid cell damage (Table 9). Medium (150 µl) was added to all plaque columns, except column 3. Extracts were then placed in prepared medium in volumes according to the solvent used (Table 9) and then added to the plate, column 3 with maximum concentration, and from column 4 to 10 with a dilution of 1:2 from the previous column. Plates were then incubated for 48 h under same conditions.

Table 9 – Amount of extracts and dilutions used for the Phase II Enzyme Bioassay

Solvent*	Minimum dilution	ml of medium used to add test compound	Amount of extract
ACN	1:200	4 ml	20 µl
Methanol	1:300	6 ml	20 µl
THF, DMSO	1:500	4 ml	8 µl

*ACN (acetonitrile), THF (tetrahydrofuran), DMSO (dimethyl sulfoxide).

Day 3. BCA and MTT – Results

After 48 h the plate was removed from the incubator and the medium discarded, then the plate was "washed" twice by placing in DPBS (Dubelcco´s Phosphate Buffered Saline) containing plates. DPBS was discarded and 75 µl of digitonin added to plate (approximately 10 ml per plate). The plate was incubated for 10 min and then agitated at 250 rpm for 10 min, then 20 µl from the bottom of each well were transferred to the correspondent column of another plate previously labeled. This new plate was filled with 300 µl/well of BCA buffer (to measure cell density and citotoxicity) and placed in incubator for 30 min under standard conditions. After incubation, plate was equilibrated at room temperature for 15 min and then placed in the microplate, reading was made at 550 nm.

Simultaneously, MTT buffer was prepared and added (200 µl /well) to the first plate, after 5 min of incubation at room temperature 50 µl/well of dicumarol was added and reading was performed at 490 nm. Results are means of 4 measurements. The activity of Phase II enzymes was calculated dividing the protein concentration (absorbance from MTT) by the cell viability (absorbance from BCA) at different concentrations of the extract. One unit of inducer activity was defined as the concentration that doubles the specific activity of quinone reductase in a microtiter well. This concentration has been designated the "CD" value.

Results and Discussion

Results from the in vivo study were inconclusive; therefore, the effect of sumac bran was evaluated using a different bioassay that measures the potency of phase II enzyme inducers, which is one specific mechanism of cancer chemoprevention.

A hexane extract likely containing policosanols and lipophilic material was included in this part of the research to evaluate the effect of compounds not present in the methanolic extract but that were originally present in the bran. The choice of solvent and extraction protocol to isolate bioactive agents is not a trivial one, and a single solvent may not extract all bioactive agents from a tissue matrix (Wettansigue and others 2002).

Figure 17 shows the behavior of the methanol sumac bran extract at three different days of experiments. Extract was made once, and tested three different times. The first time, activity results were high, however the activity changed in the second and third time the extract was tested. Storage of the extract was not properly done because as seen in the figure, some important compounds that promoted the quinone reductase activity were lost. Thus, only the first replicate was used for the results (Figure 18).

The Sumac bran methanol extract was tested in vitro for phase II enzyme activity. Measured as quinone reductase, the extract induced enzymes at concentrations ranging from 0.13 to 16.7 µg/well (Figure 18). As concentration increased activity increased. A CD value (concentration of a compound required to double the QR specific activity in Hepa 1c1c7 murine hepatoma cells) of 0.04mg/ml was determined from the chart. Faulkner and others (1998) reported a CD value of 0.01mg/ml for a broccoli plant extract; considering broccoli has been recognized for its content of isothiocyanates, a very potent phase II enzyme inducers, comparatively this methanol extract induced phase II enzymes very effectively. It is very likely that the phenolics present in the extract were responsible for the induction of the quinone reductase, since extraction with methanol concentrates these compounds. Some antioxidants have been

previously recognized as phase II enzyme inducing agents (Wettasinghe and others 2002).

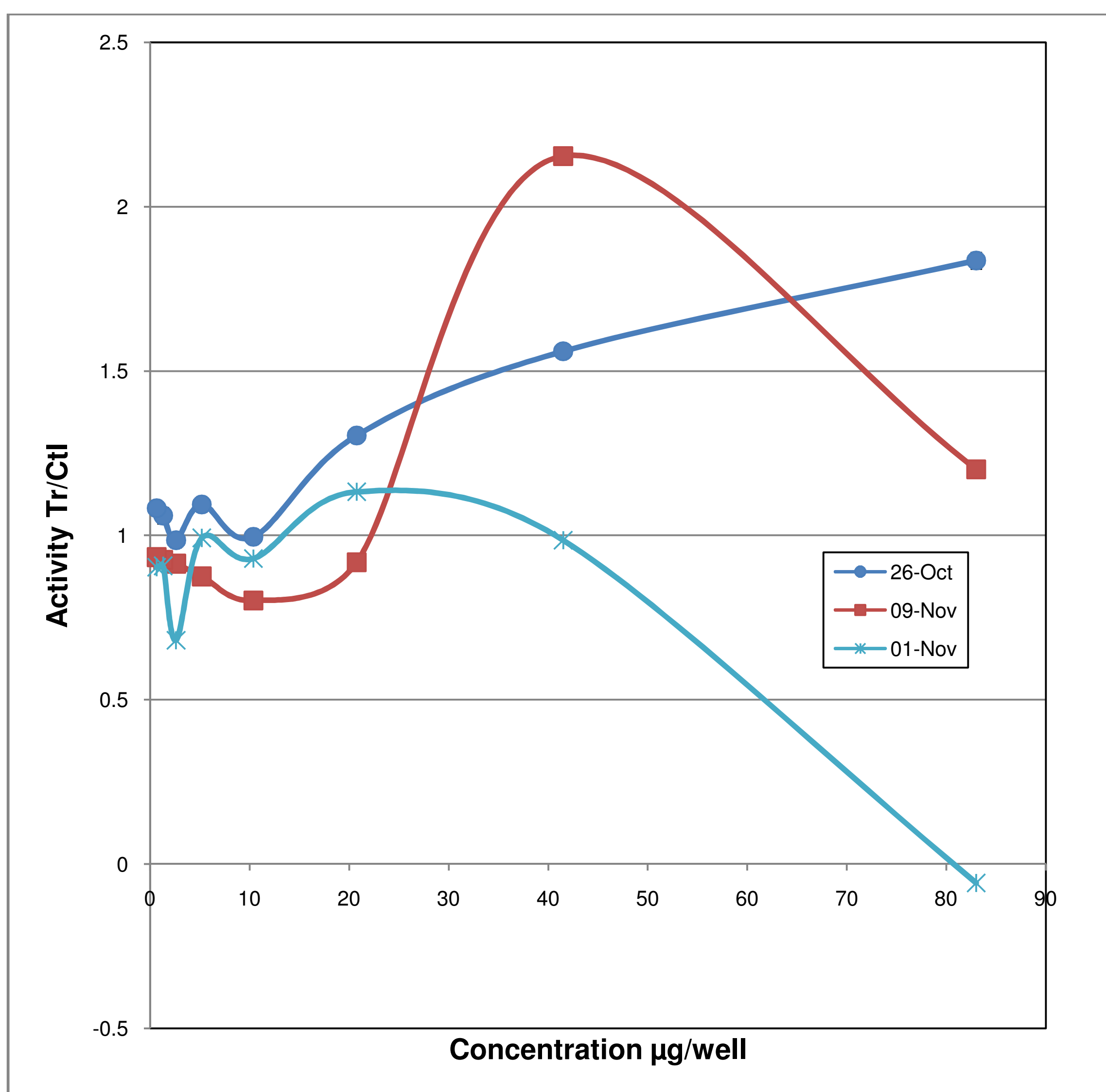

Figure 17 - Quinone reductase activity induced by a methanol extract of sumac bran. Each curve represents a replicate (four determinations per replicate).

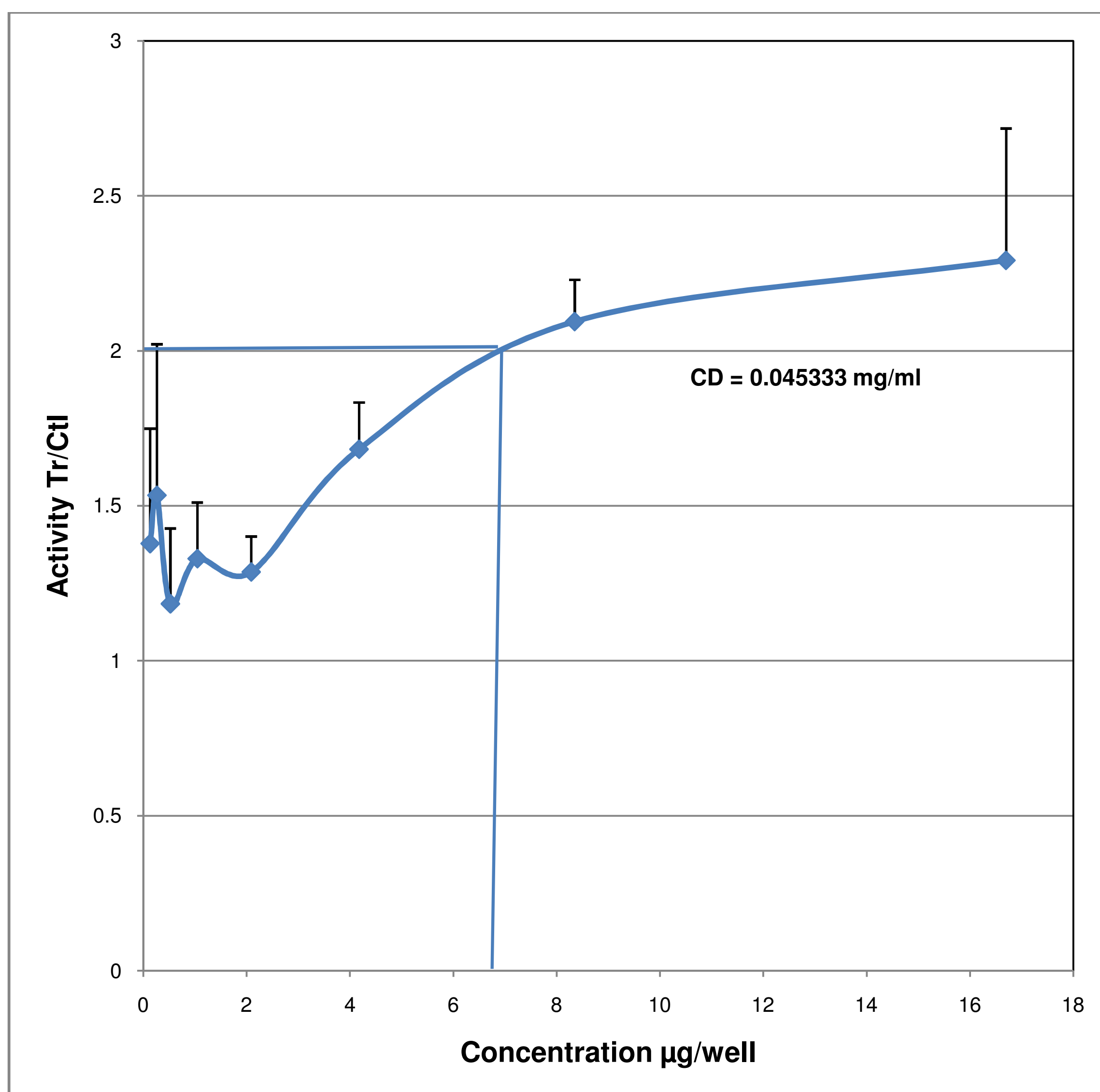

Figure 18 – Quinone reductase activity induced by sumac bran extract. Error bars denote standard error.

To ensure the presence of tannins, an acidic methanol extract was also tested for phase II enzyme activity, three different replicates were performed (data not shown). In two of the replicates extracts did not induce quinone reductase activity; in another replicate cell viability was too low to have reliable results. Thus, results were not conclusive and they were not included.

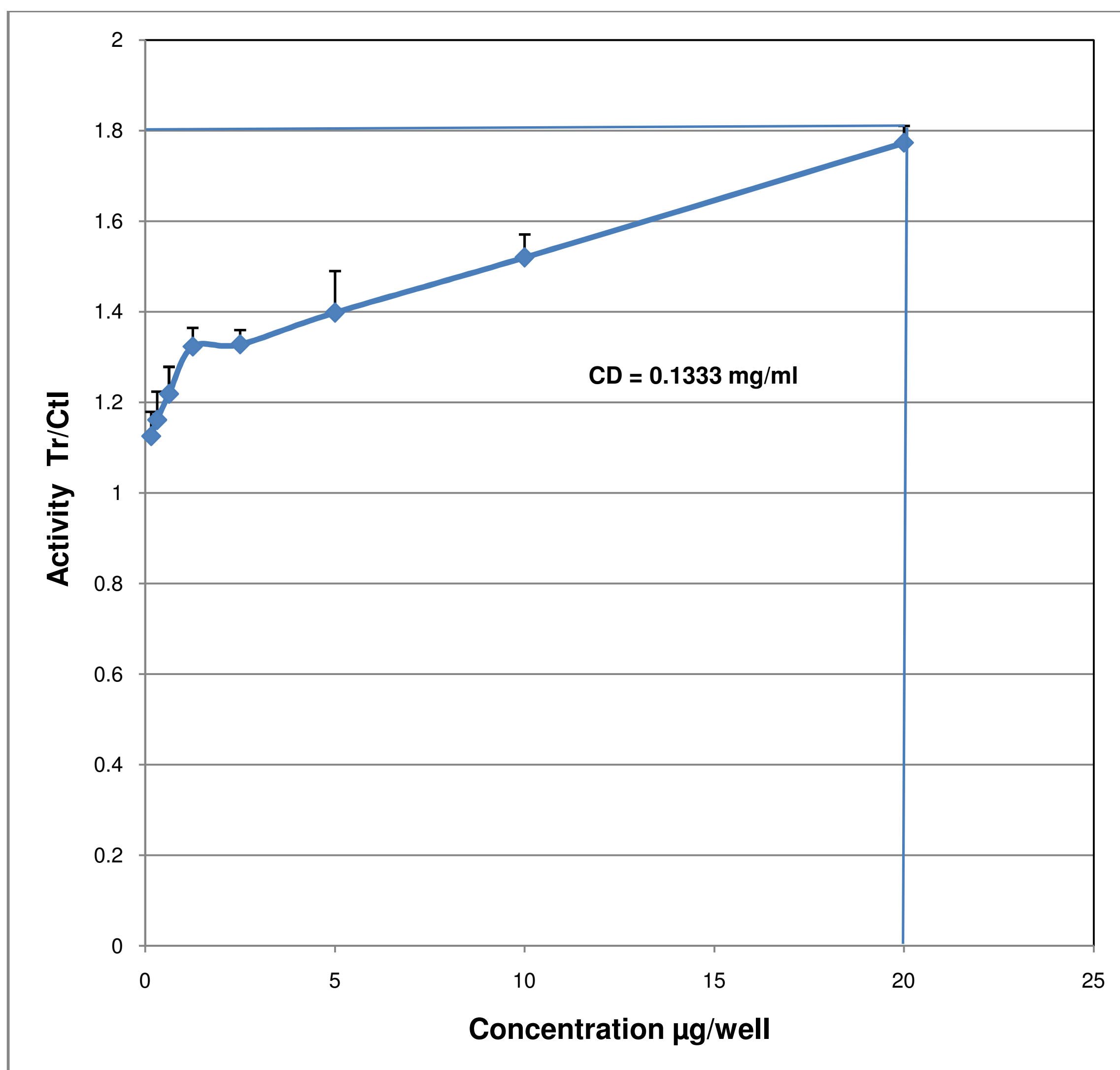

Figure 19 – Quinone reductase activity induced by the hexane extract of Sumac bran. Curve is an average of two replicates (four determinations per replicate). Error bars denote standard error.

The hexane Sumac extract promoted activity on a range of 0.15 to 20 µg/well (Figure 19). The extract tested was made at the beginning of the experiments and used in different replicates, curves obtained were similar since the components did not degrade under the same conditions as the hydrophilic extracts. As concentration increased activity also augmented. In this case, with the concentrations tested, activity of the enzymes could not be doubled. At maximum concentration a CD value of 0.1333 mg/ml was determined. The observed positive activity can be attributed to the lipophilic compounds of the extract. Since the extraction procedure was reported to be for policosanols, the policosanol content in the extract should be high; other lipophilic compounds probably present include phytosterols and aldehydes (Hwang and others 2004). Policosanols are a mixture of high molecular weight aliphatic alcohols that are part of the wax components of the plants. Policosanols represent 19-46% of the sorghum wax, with octacosanol and triacontanol as the most abundant (Awika and Rooney 2004). Policosanols from sugarcane wax have been studied for its health benefits. These compounds have cholesterol-lowering properties and other positive benefits on lipid peroxidation.

Results could be related with the observations in the in vivo experiment where bran diets exhibited a reduction in tumor incidence compared to methanol extract treatments and control. The methanol and lipid fraction present in the bran appeared to have a positive effect in cancer prevention by inducing phase II enzyme activity; this demonstrates that either alone or synergistically, bran compounds can prevent cancer.

In summary, methanol and hexane extracts of sumac bran induced quinone reductase activity. Methanol extract was a more potent inducer of phase II enzymes than the hexane extract. Bran from sumac sorghum contains phytochemicals that induced detoxifying phase II enzymes. Phytochemicals included phenol like compounds, lipophilic compounds (policosanols) and other unknowns.

CHAPTER VI
CONCLUSIONS

For the screening part, the Sumac bran methanol extract had the highest amounts of phenolic compounds and tannins whereas the black sorghum methanol extract had the highest amount of anthocyanins. When tested in vitro against 3 different cancer cell lines, the methanol extracts from sumac sorghum bran inhibited 100% of mammary, colon and hepatic cancer cells. In general sorghum extracts inhibited more easily the hormone dependent mammary cancer cells, this effect could be related to a specific anti-estrogen compound present in the samples.

The methanol extracts of sumac bran had higher percent inhibition in mammary and hepatic cancer cell lines than the extracts from the tannin free bran and the whole grain; this suggests that tannins have a citotoxic effect on MCF-7 and HepG2 cancer cells. It appeared that for sumac bran, tannins are partially responsible of the inhibitory effect, probably for its high antioxidant capacity. The Sumac bran, tannin free bran and the whole grain extracts had around 80% inhibition at a concentration of 0.5mg/ml on colon cancer cells; apparently for Caco2 cells a different compound is exerting the inhibitory effect.

The Sumac bran methanol extracts did not inhibit the tumor incidence in female rats treated with DMBA. Diets containing ground bran had lower tumor incidence compared to the corresponding methanol extracts treatments and control group. Differences between in vitro and in vivo experiments could be due to phenolic compounds absorption, availability and changes during metabolism.

Animals fed the bran diets had the lowest tumor incidence. It appeared that bran contains other compounds with anticancer properties different from the easily extractable phenolic compounds acting alone or combined.

The Sumac bran methanol and hexane extracts induced phase II enzyme activities determined by the quinone reductase assay. Phenolic compounds and policosanols or

unknown compounds present in methanolic and hexane extracts respectively have the potential to protect against carcinogenesis by promoting detoxifying enzymes.

Data from these experiments suggested that several mechanisms can be involved in the inhibition of carcinogenesis by sorghum phytochemicals. Phenolic compounds of sumac bran sorghum had a citotoxic effect on cancer cells, probably through their antioxidant activity; and a preventive effect as phase II enzyme inducers. Incidence of tumors in the in vivo experiment was reduced by the phytochemical content of the bran. It appeared that methanol and hexane extracts of sumac bran can prevent carcinogenesis by inducing phase II enzymes.

REFERENCES

Adebamowo AC, Cho E, Sampson L, Katin MB, Spiegelman D, Willet WC, Holems MD. 2005. Dietary flavonols and flavonol rich foods intake and the risk of breast cancer. Int J Cancer 114:628-33.

Agarwal C, Sharma Y, Zhao J, Agarwal R. 2000. A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitoge-activated protein kinases activation and inducing G1 arrest and differentiation. Clinical Cancer Research 6: 2921-2930.

American Cancer Society (ACS). 2007. Cancer statistics 2007. Retrieved July 8, 2007. http://www.cancer.org/docroot/stt/stt_0.asp.

Appelt LC, Reicks MM. 1999. Soy induces phase II enzyme but does not inhibit dimethylbenz[a]anthracene-induced carcinogenesis in female rats. J Nutr 129:1820-26.

Awad A, Fink C. 2000. Phytosterols as anticancer dietary components: evidence and mechanism of action. J Nutr 130: 2127-2130.

Awika JM, Rooney LW, Wu X, Prior RL, Cisneros-Zevallos, L. 2003. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. J Agric Food Chem 51:6657-6662.

Awika JM, Rooney LW. 2004. Sorghum phytochemicals and their potencial impact on human health. Phytochemistry 65:1199-1221.

Awika JM, Rooney LW, Waniska RD. 2004. Anthocyanins from black sorghum and their antioxidant properties. Food Chemistry 90:293-301.

Awika JM, McDonough CM, Rooney LW, 2005. Decorticating sorghum to concentrate healthy phytochemicals. J Agric Food Chem 53:6230-34.

Bai M, Agnantis NJ, Kamina S, Demos A, Zagorianakou P, Katsaraki A, Kanavaros P. 2001. In vivo cell kinetics in breast carcinogenesis. Breast Cancer Res 3:276-283.

Bawadi HA, Bansode RR, Trappey IIA, Truax RE, Losso JN. 2005. Inhibition of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. Cancer Letters 218:153-162.

Bouea SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Wintjes CH, Shih BY, McLachlan JA, Cleveland TE. 2003. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. J Agric Food Chem 51:2193-2199.

Burdall SE, Hanby AM, Lansdown MRJ, Speirs V. 2003. Breast cancer cell lines: friend or foe? Breast Cancer Res 5: 89-95.

Burdette AM, Greenspan P, Hargrove JL, Hartle DK. 2006. Hypolipidemic effect of a sorghum fraction in combination with other natural nutraceutical ingredients in the hyperlipidemic Syrian hamster. FASEB J 20:A1027.

Carr TP, Curtis LW, Schlegel VL, Cuppett SL, Guderian DM, Johnson KR. 2005. Grain sorghum lipid extract reduces cholesterol absorption and plasma non-HDL cholesterol concentration in hamsters. J Nutr 135:2236-2240.

Chen F, Cole P, Mi ZB, Xing LY. 1993. Corn and wheat-flour consumption and mortality from esophageal cancer in Shanxi, China. International Journal of Cancer 53:902-6.

Choi Y, Jeon HS, Lee J. 2007. Antioxidant activity of methanolic extracts from some grains consumed in Korea. Food Chemistry 103: 130-138.

Christiansen KL, Weller CL, Schlegel VL, Cuppett SL, Carr TP. 2007. Extraction and characterization of lipids from the kernels, leaves, and stalks of nine grain sorghum parents lines. Cereal Chemistry 84(5): 463-470.

Coezy E, Borgna JL, Rochefort H. 1982. Tamoxifen and metabolites in MCF7 cells: correlation between binding to estrogen receptor and inhibition of cell growth. Cancer Research 42: 317-323.

Crespy V, Williamson GJ. 2004. A review of the health effects of green tea catechins in in vivo animal models. J. Nutr. 134: 3431S-3440S.

Dias MF, Sousa E, Cabrita S, Patrício J, Oliveira CF. 2000. Chemoprevention of DMBA-induced mammary tumors in rats by a combined regimen of alpha-tocopherol, selenium, and ascorbid acid. The Breast Journal 6:14-19.

Dinkova-Kostova AT, Hotlzclaw WD, Kensler TW. 2005. The role of Keap1 in cellular protective responses. Chem Res Toxicol 18(12): 1779-91.

Dykes L, Rooney LW, Waniska RD, Rooney WL. 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. J Agric Food Chem 53: 6813-6818.

Dykes L. Rooney LW. 2006. Sorghum and millet phenols and antioxidants. Journal of Cereal Science 44: 236-251.

Earp CF, McDonough CM, Awika J, Rooney LW. 2004. Testa development in the caryopsis of *Sorghum bicolor* (L.) Moench. Journal of Cereal Science 39: 303-311.

Eddy EP, Howard PC, McCoy GD, Rosenkranz HS. 1987. Mutagenicity, Unscheduled DNA synthesis, and metabolism of 1-nitropyrene in the human hepatoma cell line HepG2. Cancer Research 47: 3163-3168.

Eng ET, Ye JJ, Williams D, Phung S, Moore RE, Young MK, Gruntmanis U, Braunstein G, Chen S. 2003. Supression of estrogen biosynthesis by procyanidin dimmers in red wine and grape seeds. Cancer Research 63: 8516-8522.

Fahey JW, Zhang Y, Talalay P. 1997. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci USA 94:10367-72.

Fahey JW, Dinkova-Kostova AT, Stephenson KK, Talalay P. 2004. The “Prochaska” microtiter plate bioassay for inducers of NQ01. In: Packer L, editor. Methods in Enzymology, Vol. 382, Quinones and quinone enzymes, Part B, New York: Academic Press pp 243-258.

Faulkner K, Mithenl R, Williamson G. 1998. Selective increase of the potential anticarcinogen 4-methylsuphinylbutyl glucosinolate in broccoli. Carcinogenesis 19:605-609.

Gu L, House SE, Prior RL. 2006. Effects of level of dietary sorghum procyanidins on early growth and development of rats. FASEB J 20(4): A154.

Guthrie N, Gapor A, Chambers AF, Carroll KK. 1997. Inhibition of proliferation of estrogen receptor negative MDA-MB-435 and positive MCF-7 human breast cancer cells by palm oil tocotrienols and tamoxifen, alone and in combination. J Nutr 127: 544S-8S.

Gutierrez-Uribe JA, Robledo AL, Serna-Saldívar S, Rooney LW. 2008. Fractionation of anti-carcinogenic phytochemicals from type III sorghum bran using fast centrifugal partition chromatography. Poster presentation CONACYT-TAMU meeting.

Hagerman AE, Riedl KM, Jones A, Sovik KN, Ritchard NT. 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J Agric Food Chem 46: 1887-1892.

Hahn DH, Rooney LW, Earp CF. 1984. Tannins and phenols of sorghum. Cereal Foods World 29:776- 7779.

Hargrove JL, Greenspan P, Hartle DK. 2004. Nutritional significance and metabolism of very long chain fatty alcohols and acids from dietary waxes. Experimental Biology and Medicine 229:215-226.

Hirose M, Hoshiya T, Akagi K, Futakuchi M, Ito N. 1994. Inhibition of mammary gland carcinogenesis by green tea catechins and other naturally occurring antioxidants in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz[a]anthracene. Cancer Letters 83:149-156.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid

handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 50:4437-4444.

Hwang KT, Cuppett SL, Weller CL, Hanna M. 2002. Properties, composition and analysis of grain sorghum wax. JAOCS 79: 521-527.

Hwang KT, Weller CL, Cuppett SL, Hanna MA. 2004. Policosanol contents and composition of grain sorghum kernels and dried distillers grains. Cereal Chem 81(3):345-9.

Izzoti A, Camoirano A, Cargiglia C, Grubbs CJ, Lubet RA, Kelloff GJ, Flora SD. 1999. Patterns of DNA adduct formation in liver and mammary epithelial cells of rats treated with 7,12-Dimethylbenz(a)anthracene, and selective effects of chemopreventive agents. Cancer Research 59:4285-4290.

Jung KJ, Wallig MA, Singletary KW. 2006. Purple grape juice inhibits 7, 12-dimethylbenz[a]anthracene (DMBA)- induced rat mammary tumorigenesis and in vivo DMBA-DNA adduct formation. Cancer Letters 233: 279-288.

Kamath V, Niketh S, Chandrashekar A, Rajini PS. 2007. Chymoryptic hydrolysates of α-kafirin, the storage protein of sorghum (*Sorghum bicolor*) exhibited angiotensin converting enzyme inhibitory activity. Food Chemistry 100 (1): 306.311.

Kamath VG, Chandrashekar A, Rajini PS. 2004. Antiradical properties of sorghum (*Sorghum bicolor* L. Moench). Journal of Cereal Science 40: 283-288.

Kean EG, Ejeta G, Hamaker B, Ferrruzzi MG. 2007. Characterization of carotenoid pigments in mature and developing kernels of selected yellow-endosperm sorghum varieties. J Agric Food Chem 55: 2629-2626.

Kim HS, Kacew S, Lee BY. 1999. In vitro chemopreventive effects of plant polysaccharides *(Aloe barbadensis* Miller*, Lentinus edodes, Ganoderma lucidum* and *Coriolus versicolor).* Carcinogenesis 20:1637-1640.

Komori A, Yatsunami J, Okabe S, Abe S, Hara K, Suganuma M, Kim SJ, Fujiki H. 1993. Anticarcinogenic activity of green tea polyphenols. Japanese Journal of Clinical Oncology 23: 186-190.

Krueger CG, Vestling MM, Reed JD. 2003. Matrix-assited laser desorption/ionization time of flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [*Sorghum bicolor* (L.) Moench]. J Agric Food Chem 51: 538-543.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, Saag PT. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139: 4252-4263.

Lenaerts K, Bouwman FG, Lamers WH, Renes J, Mariman EC. Comparative proteomic analysis of cell lines and scrapings of the human intestinal epithelium. 2007. BMC Genomics 8:91.

Liu M, Xin QL, Weber C, Lee CY, Brown J, Liu R. 2002. Antioxidant and antiproliferative activities of raspberries. J Agric Food Chem 50: 2926-2930.

Liu R. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 134: 3479-3485.

Lotan R, Hong WK. 2001. Molecular mechanisms of chemoprevention and differentiation therapy. In: Howley M, Liotta I. (Eds). The molecular basis of cancer 2nd. Edition. W.B. Saunders Company, Philadelphia, pp 621.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. 2004. Polyphenols: food sources and bioavailability. Am J Clin Nutr 79: 727-747.

Nichenametla SN, Taruscio TG, Barney DL, Exon JH. 2006. A review of the effects and mechanisms of polyphenolics in cancer. Critical Reviews in Food Sci and Nutr 46:161-183.

Peterson G. 1995. Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 125: 784S-789S.

Price ML, Scoyoc SV, Butler LG. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J Agric Food Chem 26:5.

Prior RL, Hoang G, Gu L, Wu X, Bacchiocca M, Howard L, Hampsch-Woodill M, Huang D, Ou B, Jacob R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. J Agric Food Chem 51:3237-3279.

Prior RL, Howard L, Gu L, Rooney LW, Hage A. 2007. Procyanidins: effects of source and extrusion conditions on structure, degradation and absorption/metamolism. American Chemical Society 234th National Meeting and Exposition. Abstract 209.

Reyes LF, Cisneros-Zevallos L. 2007. Electon-beam ionizing radiation stress effects on mango fruit (*Mangifera indica L.*) antioxidant constituents before and during postharvest storage. J Agric Food Chem 55:6132-6139.

Robledo AL, Zapata-Mairena MG, Gutierez-Uribe JA, Serna-Saldivar SO. 2007. Phenolic profile, antioxidant activity and anticancer properties of different types of sorghums and brans. Cereal Foods World 52:A61.

Romo-Lopez I. 2005. Efecto inhibitorio en el crecimiento de células de cancer de mama (MCF-7) del extracto crudo acetónico de *Phiecellobium flexicaule* y *Vigna unguiculata.* M.S. Tesis, Instituto Tecnológico y de Estudios Superiores de Monterrey, México.

Rooney LW, Waniska RD. 2000. Structure and chemistry of the sorghum caryopsis. In: Smith CW, Frederiksen RA. (Eds.), Sorghum: Origin, History, Technology, and Production. Wiley, New York, pp. 649-688.

Russo A, Cardile V, Lombardo L, Vanella L, Vanella A, Garbarino JA. 2005. Antioxidant activity and antiproliferative action of methanolic extract of *Geum quellyon* Sweet roots in human tumor cell lines. Journal of Ethnopharmacology 100:323-332.

Russo J, Russo IH. 1996. Experimentally induced mammary tumors in rats. Breast Cancer Research and Treatment 39:7-20.

Russo J, Russo IH. 2004. Animal models for human breast cancer. In: Molecular Basis of Breast Cancer Prevention and Treatment. Springer, New York. pp. 183-184.

Sahidi F, Naczk M. 2003. Phenolics in food and nutraceuticals. Boca Raton FL: CRC Press. p. 403.

Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D. 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. Journal of Nutritional Biochemistry 16: 360-367.

Singletary KW, Stansbury MJ, Giusti M, Van Breemen RB, Wallig M, Rimando A. 2003. Inhibition of rat mammary tumnorigenesis by concord grape juice constituents. J Agric Food Chem 51:7780-7286.

Sporn MB, Suh N. 2000. Chemoprevention of cancer. Carcinogenesis 21:525-530.

Stoner GD, Casto BC. 2004. Chemoprevention by fruit phenolic compounds. In: Keloff GJ, Hawk ET, Sigman CC (Eds). Cancer Chemoprevention. Vol 1. Humana Press, New Jersey, pp. 419-431.

Tapiero H, Tew K., Nguyen BaG, Mathé G. 2002. Polyphenols: do they play a role in the prevention of human pathologies? Biomed Pharmacoter 56: 200-207.

Terrill TH, Windham WR, Evans JJ, Hoveland CS. 1990. Condensed tannin concentration in sericea lespedeza as influenced by preservation method. Crop Sci 30:219-224.

Turner ND, Diaz A, Taddeo SS, Vanamala J, McDonough CM, Dykes L, Murphy ME, Carroll RJ, Rooney LW. 2006. Bran from black or brown sorghum suppresses colon carcinogenesis. FASEB Journal 20, A599.

Vlietnick AJ, Cos P, Bruyne TD, Hermans N, Apers S, Vanden-Berghe D. 2003. Proanthocyanidins in health care: current and new trends. Current Medicinal Chemistry 10: 1345-1359.

Wettasinghe M, Boling B, Plhak L, Parkin K. 2002. Screening for phase II enzyme-inducing and antioxidant activities of common vegetables. Journal of Food Science 67(7):2583-8.

Wolfe K, Wu X, Liu RH. 2003. Antioxidant activity of apple peels. J Agric Food Chem 51:609-614.

Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the united states. J Agric Food Chem 52, 4026-4037.

Yan X, Murphy BT, Hammond GB, Vinson JA, Neto C. 2002. Antioxidant activities and antitumor screening of extracts from cranberry fruit (*Vaccinium macrocarpon*). J Agric Food Chem 50:5844-5849.

Zavala-Arcos J. 2005. Efecto preventivo de los flavonoides del frijol negro (*Phaseolus vulgaris*) germinado sobre el cancer de mama inducido en un modelo animal. M.S. Tesis, Instituto Tecnológico y de Estudios Superiores de Monterrey, México.

APPENDIX

Table 9 – Total phenolics, flavonoids, anthocyanin, tannins and ORAC data for the screening and characterization of different sorghums for anticancer potential chapter

Sample	Description	Total phenolics[1]	Flavonoids[2]	Anthocyanin[3]	Tannins[2]	ORAC[4]
1	ATX635 x RTX 436	1.49±0.06	0.3±0.02	1.32±0.00	ND	249.19
2	SC748	2.05±0.06	0.97±0.04	3.00±1.16	ND	305.90
3	Hegari	1.74±0.05	0.51±0.03	ND	ND	91.92
4	TX2911	1.67±0.04	1.07±0.07	ND	ND	557.56
5	TX430 Black	2.84±0.10	1.96±0.00	14.06±3.00	ND	331.95
6	PI Black Tall	3.81±0.06	2.27±0.06	16.69±0.54	ND	270.76
7	Sumac	11.98±0.18	4.00±0.14	6.87±1.50	52.09±1.62	828.95
8	Sumac Bran	17.69±0.61	20.05±0.88	17.98±0.86	247.8±6.99	1133.08
9	TX430 Black Bran	8.08±0.08	6.69±0.28	67.83±6.79	ND	920.04
10	SC719-11E	2.53±0.08	0.99±0.02	5.15±0.66	ND	621.21
11	SC650	2.89±0.06	0.62±0.02	2.49±1.33	ND	217.18
12	BRON 176	2.21±0.03	1.04±0.02	1.05±0.17	ND	390.08
13	SC109-14E	1.65±0.04	0.36±0.02	ND	ND	115.77
14	Shawaya (Mostly Black)	2.83±0.01	0.91±0.02	ND	ND	267.51
15	Shawaya (Brownie Tan)	2.81±0.06	1.52±0.05	9.44±1.55	25.73±0.22	193.12
16	NK 121 A	2.58±0.08	1.69±0.06	6.05±0.61	17.30±0.40	161.00
17	NK 180	2.00±0.08	0.94±0.07	4.28±1.00	ND	318.39
18	NK 8830	3.56±0.12	3.81±0.10	ND	85.9±0.60	351.35
19	XM 217	3.13±0.12	3.38±0.12	1.69±0.00	28.52±0.81	322.18
20	EBA 3	2.59±0.12	1.04±0.06	ND	ND	978.92
21	SC575	1.91±0.06	0.46±0.03	ND	ND	963.74
22	SC103	3.70±0.08	2.58±0.03	6.76±4.34	24.07±1.70	1053.94
23	SC630ii	2.10±0.06	0.48±0.02	ND	ND	458.03
24	SC1038	2.46±0.09	0.57±0.00	ND	ND	1087.72
25	SC630 II	2.71±0.07	0.53±0.01	ND	ND	463.14

Table 10 – In vitro screening of different sorghum samples data

	% Inhibition		
Sample No.	**MCF7**	**CACO2**	**HEPG2**
1	65.73	0.00	0.00
2	65.93	0.00	0.00
3	68.83	0.00	0.00
4	3.34	3.04	5.36
5	59.00	0.00	0.00
6	0.00	0.00	0.00
7	0.00	0.00	1.53
8	95.61	102.64	101.02
9	6.87	0.00	0.00
10	8.05	0.00	10.07
11	0.00	0.00	0.00
12	5.47	0.00	0.00
13	62.41	0.00	0.00
14	43.30	65.24	0.00
15	1.42	0.00	0.00
16	0.00	0.00	0.00
17	0.00	3.84	0.00
18	9.18	9.97	0.00
19	10.85	3.92	0.00
20	0.50	0.00	0.00
21	6.42	0.00	0.00
22	8.56	0.00	0.00
23	0.00	0.00	0.00
24	11.62	0.79	0.00
25	11.23	4.11	0.05

VITA

Name:	Sara Guajardo Flores
Address:	Einstein 2772 Col. Contry Silla, Guadalupe N.L. México. CP 67170
Email Address:	sgf73@hotmail.com
Education:	B.A., Food Engineering, Instituto Tecnológico y de Estudios Superiores de Monterrey, 1994 M.S. Food Science and Technology, Texas A&M University, 1998