A PROPOSAL SUBMITTED TO NATIONAL CANOLA RESEARCH PROGRAM
FY 2007-08

Project Title: **Evaluation of Anti-Oxidant Activity, Food Efficacy and Health Aspects of Canola**

Principle Investigators: **Padu Krishnan, Ph.D.**
Chandradhar Dwivedi, Ph.D.

Amount Requested: **Year 2 - $35,984 (this is a year 2 continuation request)**

Mailing Address: **Padu Krishnan, Ph.D.**
Department of Nutrition, Food Science and Hospitality
PO Box 2275A
South Dakota State University
Brookings, SD 57007
Padmanaban_Krishnan@sdstate.edu
Tel . (605)688-4040
Fax. (605) 688-5603

Introduction, Statement of the Problem

The value of traditional cash crops is significantly enhanced by demonstrating new commercial uses or disease-preventative attributes that are unique to the crop (e.g. isoflavones in soybean, beta-glucan in oats, fiber in wheat, antioxidants in canola). Fruits, vegetables, spices, nuts, seeds, leaves, roots and barks have been exploited as potential sources of natural antioxidants (Pratt and Hudson, 1990). *We propose to explore the food, nutraceutical and disease-preventative aspects of canola and canola fractions. This study will be a natural extension of a previous Canola project aimed at identifying promising key areas for exploration. (see Progress Report sent as a separate document).*

Canola is unique in having significantly desirable fatty acid profile and significant physiological levels of phenolic compounds relative to other crops (Krishnan et at , 2003 Canola Progress Report). *Indeed, values for anti oxidant activity range as high as 50% (of Quercetin) have been noted in our work as compared to 4.7% (soybean) and 17.2% (oats)* These compounds ensure the integrity of cell membranes, a possible mechanism for the prevention of cancer and heart disease. They also increase the shelf stability of vegetable oils. Phenolic compounds occur at relatively low levels but have high antioxidative activity and physiological effect. Different fractions may harbor different compounds that can be optimally enriched.

Advances in dehulling of canola/rapeseed may soon bring about the introduction of dehulling to the canola/rapeseed industry. The subsequent use of hulls as component of feedstuffs may be one way of their utilization. Canola hulls have been reported to contain 6% of phenolic compounds such as phenolic acids, flavonoids, and soluble and insoluble condensed tannins (Naczk and Shahidi, 1998). The antioxidant activity of crude ethanolic extract of canola meal on canola oil was equivalent to that of TBHQ and stronger than that of BHA, BHT and BHA/BHT/monoiacylgylyceride citrate (MBC) (Wanasundara and Shahidi (1994). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are commonly used as synthetic antioxidants in lipid-containing foods

(Sherwin, 1990). In the last 2 decades, there has been an increasing interest in replacing these synthetic antioxidants with natural alternative (Howell, 1986), because of their possible role as promoters for carcinogenesis (Ito et al., 1986). The most active component of these extracts is identified as 1-O-B-glucopyranosylsinapate (Wanasundara et al, 1994). Addition of 0.5-5% canola flour to meat resulted in 73-97% inhibition of fat oxidation as determined by 2-thiobarbituric acid (TBA) assay (Shahidi et al, 1995). It has also been reported that low tannin hull extracts exhibit greater anti-oxidative activity than high-tannin hull extracts (Amarowicz et al, 2000). The proportion of anti oxidants separating with the oils and the destruction of antioxidant activity from oil processing needs further investigation as well.

Justification:

Evaluation of the anti-oxidative activity of canola is an important factor in increasing the value of canola crop. Comparison of canola crops from different regions in North Dakota, South Dakota and Minnesota may high-light an additional value-added factor that might be exploited commercially.

Health benefits attributable to canola meal and oil constituents can be established using experimentally sound small animal studies. Cause-effect relationships can be between feed and disease rates as evidenced by clinical indicators (tumors, chemical markers, etc).

The proposed study will employ two newly acquired state-of- the-art Mass Spectrometers to fingerprint, identify, and reliably measure oil and canola meal constituents. These tools will then be used to monitor success in developing valuable fractions from a variety of milling techniques.

Research Objectives:

a. To determine the content & composition of phenolic and other bioactive compounds that will increase the food and economic value of canola.

b. To develop functionally enriched canola fractions and to demonstrate their effectiveness in food systems and for use in nutraceutical products.

c. To assess the potential cancer preventive effects of canola meal flour and oil in experimental animals.

d. To investigate the mechanism of action of chemoptreventive effects of canola oil on colon cancer development.

Procedures

Sampling

A representative sampling of canola varieties will be acquired. Commercial and experimental lines will be included to ensure a diverse sampling. Sampling from 3 North Central growing regions will be attempted. These samples will have additional data on agronomic and seed characteristics. Samples will be cleaned using accepted methods and then stored in a walk-in cooler to ensure sample stability. The lead investigator will negotiate the acquisition and shipment of samples. A statistician will be consulted at all stages of the

investigation to ensure appropriate experimental design.

The overall goal is to develop canola meal or oil fractions that are highly enriched in naturally occurring functional ingredients. These compounds will have useful health benefits or may serve a protective role in the stability of foods. *Recent findings show that the total phenolic content in canola meal are highly correlated to antioxidant activity . High correlation coefficients (R^2 =0.76) have been found in samples from the 2003 harvest. We have developed extraction strategies that preserve the bioactivity of canola phenolics. The goal now is to optimize increases in canola functionality while preserving taste and other kinesthetic features desirable in common food stuffs.* A variety of milling techniques (both dry and wet) will be used to separate fractions enriched with selective constituents. These fractions will be achieved using a number of physical conditions. Steam treatments will be compared to facilitate extraction. Antioxidants will be extracted and purified from a variety of fractions. Hexane extractions of oils will be optimized to recover the maximum amount of target ingredients (low polarity compounds). Super critical Fluid Extraction employing food grade carbon dioxide will be used to isolate useful compounds. Selectively isolated compounds will be characterized by mass spectrometry. Mass spectra of these compounds will be stored in the spectral library of the mass spectrometer for use in their accurate measurement in the various enriched fractions. Ethanol extraction of pigmented compounds will be dried down and concentrated before purification using chromatography on select columns.

Antioxidant activity will be evaluated by free radical scavenging activity as follows: Five grams of canola hulls will be extracted first by homogenization in a high-speed homogenizer with 70% ethanol for 5 minutes. Filtrate will be evaporated under nitrogen and finally the extracted will be freeze-dried. Freeze dried extract will be used to evaluate the antioxidant activity. Free radical scavenging activity will be measured using Mellors and Tappel method (Mellors & tappel, 1966). DPPH (2,2-dihpenyl-1-picrylhydrazyl), a stable free radical will be dissolved in ethanol to give a 100 uM solution. To 3.0 ml of ethanolic solution of DPPH, add 0.5 ml of the test solution in ethanol. The decrease in DPPH absorption at 517 nm will be measured after 10 minutes. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The assay will be conducted in triplicate for each sample. Total antioxidative activity will be compared against synthetic antioxidant such as BHA, BHT and TBHQ.

Food Efficacy:

Purified enriched extracts will be applied to food products at the rate of 1% of food lipids. Sensory testing will be carried out to determine the onset of rancid odor. A variety of traditional antioxidants (such as BHT, BHA,TBHQ,etc.) will be similarly applied to compare the effectiveness of antioxidants in increasing shelf stability.

Facilities:

Food Product Development facilities will be used in conjunction with the Test Kitchen, Taste Panel and Analytical Laboratories. Food product development is both a creative and scientific process. Many a product has failed in the market place owing to inadequate product testing. In food companies, only one in six concepts survives the rigorous product testing and pre-commercialization filters. The public is likely to show slow acceptance of new and unfamiliar food products. Acceptance of such products will therefore be based on product familiarity, ease of preparation, convenience, taste, and nutrition. A more recent driving force in food product acceptance is the health promoting effects of certain ingredients or their disease prevention

attributes.

An additional objective of this grant is to extend SDSU's experience in product development to canola-based foods. The Department of Nutrition, Food Science and Hospitality has a fully developed facility for this purpose. The Lead PI is proficient in food product development. Past experiences include:

1. the development of a fiber source from sugar beet (DuoFiber) in collaboration with the American Crystal Sugar Co. Moorhead MN)
2. the production of a food grade dye from sunflower hulls
3. the development of a food grade ingredient from Distiller's Spent Grain.
4. Successful development of soy-based ingredients in traditional Mid-western food products.

The strategy employed in this study will be to introduce modest increments of canola ingredients in a wide range of traditional foods as opposed to extremely high fortification levels in a limited choice of foods. When substitutions are made in conventional food formulations, careful testing will be done to determine the robustness of recipes. An in-house test kitchen and taste testing will establish the acceptability of recipes and ingredients. Characteristics tested in sensory analysis will include: overall appearance, texture or mouth feel, taste, off flavor, and aroma. Information gleaned from this evaluation will be used to further tweak the recipes. Empirical tests will be done to determine product stability and shelf life as this will have a bearing on acceptability. The Water Activity of fresh products will be analyzed to establish potential for microbial degradation. Products tested will include baked goods, ingredients for assembly in home kitchens, and selected formulations which attempt meat replacement with vegetable analogues. Recipes will also be tested to determine the upper limits of canola meal substitution. Optimal substitution levels will be determined which do not compromise food quality or taste. Nutritional analysis will be done to determine the content of lipids, protein, carbohydrates (by difference), sugars, dietary fiber, and trace minerals in order to assess contribution of soy enhanced foods to the diet.

Stability Tests:

Accelerated storage tests of the various enriched fractions will be done. Samples will be incubated at 63 ^{0}C in a forced-air convection oven. Samples will be removed at pre-determined intervals for a period of 35 days. Stability of oils and oil-bearing fractions will be evaluated using the traditional chemical tests. Changes in free fatty acids and by-products of lipid breakdown will be monitored using the gas chromatograph.

Mass Spectroscopy:

This is an instrumental technique that permits the researcher to measure minute quantities of organic compounds that occur naturally. Such analysis is done with a high degree of compound certainty as the molecular fingerprint of the target compound is known or can be determined. Molecules of samples are subjected to high voltage (3000 volts) and heat (500 degrees C) to reveal their mass spectral signatures. The mass information is then compared to mass spectra of other compounds stored in an electronic library. A match is then found to possitively identify the target compound and to measure its accurate content or concentration. South Dakota State University currently has two such mass spectrometers which are capable of determining a wide range compounds in their natural levels and states.

Colon Cancer Effect Studies: Methodology:

The results obtained from first year study suggest that dietary (15%) canola meal (specially unroasted) and canola oil (15%) provide significant chemopreventive effects on colon tumor development in experimental rats. Evidence in the literature suggests that omega-6 polyunsaturated fatty acid (linoleic acid) enhance and omega-3 polyunsaturated fatty acids (α-linolenic acid) reduce the colon tumor development (10, 11, 12,). Since both omega-6 and omega-3 polyunsaturated fatty acids are essential component of diet, an optimal ratio of omega-6:omega-3 polyunsaturated fatty acids have to be maintained. As such, α-linolenic acid present in canola meal and oil may be contributing to the chemopreventive effects of canola products.

Furthermore, omega-6 polyunsaturated fatty acids increase the synthesis of prostglandins series 2 by cyclooxyagenase (COX) enzyme. The prostaglandin of series 2 promotes the colon tumor development. Omega-3 polyunsaturated fatty acids decrease the production of prostglandins series 2 and facilitates the synthesis of series 3 prostglandins that inhibits COX. Thus, omega-3 polyunsaturated fatty acids may be producing their chemopreventive effects by increasing the synthesis of series 3 prostglandins and decrease COX activity. Furthermore, during roasting process, omega-3 polyunsaturated fatty acids may be either get oxidized or decomposed, leading to a lower chemopreventive effect than unroasted canola seeds. Following experiments are planned to test the proposed hypothesis to elucidate the mechanism of action of dietary canola products.

Microsomes Preparation

The colon samples collected from adjacent to tumors from all rats will be washed with 1.15% ice-cold potassium chloride (KCI) and blotted. The samples will be minced and homogenized using an Omni GLH homogenizer (Omni International, Inc., Warrenton, VA). The homogenates will then be centrifuged at 10,000 *g* for 15 min in a J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, CA). The supernatant obtained will be recentrifuged in on Optima LE-80K preparative ultracentrifuge (Beckman Instruments, Inc.) at 105,000 *g* for 60 min under refrigeration. The pellet will be washed two to three times with ice-cold KCI and suspended in 1.15% KCI, which will then be used for extraction of fatty acids (10, 11, 12).

Fatty Acid Extraction

All meals (100 mg) or samples of serum or colon microsomes (160 µ1) from all rats combined with distilled water (1.6 ml) will be extracted as reported by Dwivedi et al. (10, 11,

12).

Determination of Fatty Acids

Fatty acid analysis of the meals, serum, and colon samples will be based on the procedures reported by Dwivedi et al. (10, 11, 12). Results will be reported as the percentage of mean values obtained from at least five individual samples.

Determination of COX-1 and COX-2 Expression

Colon microsomes will be resuspended in Tri reagent, and the protein pool will be isolated based on the manufacture's instructions (Sigma Chemical Company). The protein will be precipitated with isopropanol, washed, and then dissolved in sodium dodecyl sulfate (SDS). The protein in the samples will be quantitated by protein assay kit (BioRad, Richmond, CA). Sixty micrograms of protein will be resuspended in sample buffer, loaded on 7.5% gels, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The proteins will be then transferred to nitrocellulose membranes and probed with primary antibodies against COX-1 and COX-2 (Cayman Chemical Company, Ann Arbor, MI). The secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA) will be used for development with the enhanced chemiluminescence detection kit. The Western blots will be quantitated using a UVP Biochem Gel Documentation system (UVP, Inc, Upland, CA). (11, 12). Our laboratories are equipped to perform these experiments.

Project Timetable:	2006/07	**2007/08**
Activity	Year 1	**Year 2**
Milling and fractionation	X	**X**
Solvent Extraction	X	
Evaluation of hexane & ethanol extracts	X	
Phenolics Assay	X	**X**
Antioxidant Activity	X	**X**
Stability Tests		**X**
Small Animal Cancer Studies	X	**X**
Mass spectral analysis		**X**
Scale-up fractionation		**X**
Food product evaluation	X	**X**
Data reporting	X	**X**
Publication		**X**

This proposal is for continued 07/08 funding

Investigators:
Dr. Padu Krishnan is a Cereal Chemist with a range of experience in grain milling, food product

development & testing, food analysis, and sensory evaluation.

Dr. Dwivedi is a Professor of Pharmaceutical Sciences with considerable experience in implementing cancer studies involving animal models His most recent work involves tumor prevention activity of mustard oil and flaxseed meal and oil (10, 11, 12).

Technology Transfer:

Every effort will be made to disseminate information learned from this research. Findings will be reported in technical reports, brochures and news briefs. Producers and entrepreneurs will be invited to provide input in all stages of the development of the research.

Table 1. Health Food Market Indicators

Type	Growth % Per Year	1999 market Billion $	Projected Market Billion $
Natural	15	25.4	29.4
Organic	24	4.2	6.6
Vitamins/minerals	6	13.2	16.7
Herbs	16	3.6	6.6
Functional Foods	11	14.2	17.6

Source:
The Nutraceutical Market, Data Monitor, New York, 1998.
Dietary Supplement Projections. The Hartman Group, Bellevue, WA 1999.
General Supermarket Sales Statistics. A.C. Nielsen, Chicago, 1999.

Budget:

Item	**2007/08**
Salaries	19,585
Fringe Benefits 5%	979
Materials & Supplies	12,420
Travel	3,000
Total	**35,984**

Budget Justification:
Salaries will be used to hire a research assistant to accomplish laboratory tasks associated with the project. Work will involve small animal handling for expanded cancer studies and chemical analysis on feed and animal tissue. Analysis on food product is also included.

Material & Supplies costs will include special feeds for animal studies, organic food-grade solvents, distillation and column fractionation, pure standards, etc.

Travel costs will involve travel out of state for presentation of data at national and regional conferences.

Selected References:

1. Sherwin, E.R., Antioxidants. In: Food Additives: Branen, R., Ed.; Marcel Dekker: New York, 1990; pp 139-193.

2. Howell, J.C., Food Antioxidant: International perspectives welcome and industry remarks. Food Chem. Toxicol. 1986, 24, 997.

3. Ito, N.; Hirose, M.; Fukishima, S.; Tsuda, H.; Shirai, T.; and Tatematsu, M. Studies on antioxidants: their anticarcinogenic and modifying effects on chemical carcinogenesis. Food Chem. Toxicol. 1986, 24, 1099-1102.

4. Pratt, D.E.; Hudson, B. J. F. Natural antioxidant not exploited commercially. In Food Antioxidant: Hudson, B. J. F. Ed; Elsevier, Amsterdam, 1990, pp 141-192.

5. Naczk, M.; Shahidi, F. Insoluble condensed tannins of canola. Book of Abstracts of the 58th Annual Meeting, Atlanta, GA, June-25-29, 1998.

6. Wanasundara, U. N.; Shahidi, F. Canola extracts alternative natural antioxidant for canola oil. J. Am. Chem. Soc. 1994, 71, 817-822.

7. Shahidi,F.; Wanasundara, U. N.; Amarowicz, R.; Naczk, M. Natural antioxidant from canola meal. Proceedings of the Ninth International Rapseed Congress, Cambridge, U.K. 1995, Vol.3, pp. 873-878.

8. Amarowicz, R.; Naczk, M.; Shahidi, F. Antioxidant activity of various fractions of no-tannin phenolic of canola hulls.J. Agric. Food Chem., 2000, 48, 2755-2759.

9. Mellors, A.; Tappel, A. L.; The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. J. Biol. Chem., 1996, 241, 4353-4356.

10. Dwivedi, C, Muller, L.A., Goetz-Parten, D.E., Kasperson,K and Mistry , V.V.; Chemopreventive effects of dietary musterd oil on colon tumor development. Cancer Letters, 2003,196.29-34

11. Dwivedi, C., Natarajan, K., and Matthees, D.P. Chemopreventive effects of dietary flaxseed oil on colon tumor development. *Nutrition and Cancer*, 2005,51,52-58.

12. Bommareddy, A., Arasada, B.L., Matthees, D.P., and Dwivedi, C. Chemopreventive effects of dietary flaxseed on colon tumor development. *Nutrition and Cancer*, 2006,54, 216-222 .

Progress Report Krishnan Dwivedi 2007 - Value Added Research in Canola – Evaluation of Anti-Oxidant Activity, Food Efficacy and Food Functionality of Canola Products.

Considerable progress was made in this project. Funding was received in October 2006. A 7-month rat feeding trial was already underway in May 2006 and concluded in December 2006. Total Phenol Content (TPC) and antioxidant activity (AA) determinations were made on canola samples that were subjected to heat treatment. It was previously reported in the literature that heating at 165C for five minutes increased canolol content, an antioxidant (Wakamatsu et al., 2005) . The objective of the current investigation was to determine the net increase in total phenol content and antioxidant activity.as a result of heat treatment in processing the meal . The possible cancer preventative role of such treatment was also investigated. A variety of samples were explored in TPC and AA analyses to provide comparisons with canola (oat, oat oil, dark chocolate, wheat, flax, Corn Distillers Grains, etc). Heat-treated canola was used in rat feeding experiments to determine the effect of a 15% diet supplemented with canola meal (and canola oil) in prevention of colon tumors. Canola Oil (commercial) was also used in feeding trials to determine if the type of oil contributed to prevention of tumors.

Roasting brought about increases in total phenol content in canola meal, oat groat, distillers grains, high-selenium wheat, and ground whole soybean in contrast the corresponding unroasted samples. (table 1). Studies are in progress to determine if this phenomenon is due to actual increases to phenolics or due to some artifact of heating and moisture-loss. Dry basis phenolic content comparison will better address the effects of heat on canola meal composition. Antioxidant activity of the same samples are reported in table 2. Anti oxidant activity is expressed as a percentage of free radical scavenging capacity relative to a pure antioxidant standard. Quercetin demonstrated an 89% antioxidant activity. Roasting appeared to reduce antioxidant activity in canola meal. Heat is known to be a catalyst in lipid oxidation and destruction of some antioxidants such as vitamin e isomers and ascorbic acid.

Cancer Prevention Study

A seven month rat study was conducted involving several diets (AIN diet, corn meal diet, corn oil diet, canola oil diet, roasted canola meal diet, and unroasted canola meal diet). The effects of these diets in preventing chemically induced tumor were measured by several indicators such as tumor incidence (% of rats having at least one tumor of any size), tumor size (in mm diameter), and tumor multiplicity (average number of tumor per rat). Roasted canola meal diet had a slight but statistically insignificant effect on colon tumor incidence. Of the treatment groups, both roasted and unroasted canola meals significantly reduced the tumor size ($P<0.05$). Unroasted canola meal provided a significant reduction in the number of tumors per rat compared to all other groups. The canola oil group had a significant effect on the decrease of the number of tumors. Results indicate that both canola meal (15% of diet) and canola oil (15% of diet) provided significant chemopreventative effects on colon tumor development in rats. Roasted canola meal did not have as dramatic an effect of lowering tumor in rats as much as the unroasted meal. This indicates the mechanism of tumor reduction may have more to do with fatty acids profile than the occurrence of specific antioxidants. More investigation is warranted to explore the efficacy of the 15% diet replacement with altered fatty acids profile.

Total Phenolics:

Blank	Conc.	Abs 1	Abs 2	Abs 3	Mean	STD. DEV	CV%
5	0.0625	0.052	0.052	0.054	0.053	0.001	0.022
4	0.125	0.117	0.124	0.122	0.121	0.004	0.030
3	0.25	0.239	0.234	0.241	0.238	0.004	0.015
2	0.5	0.48	0.487	0.486	0.484	0.004	0.008
1	1	0.996	1.01	1.007	1.004	0.007	0.007

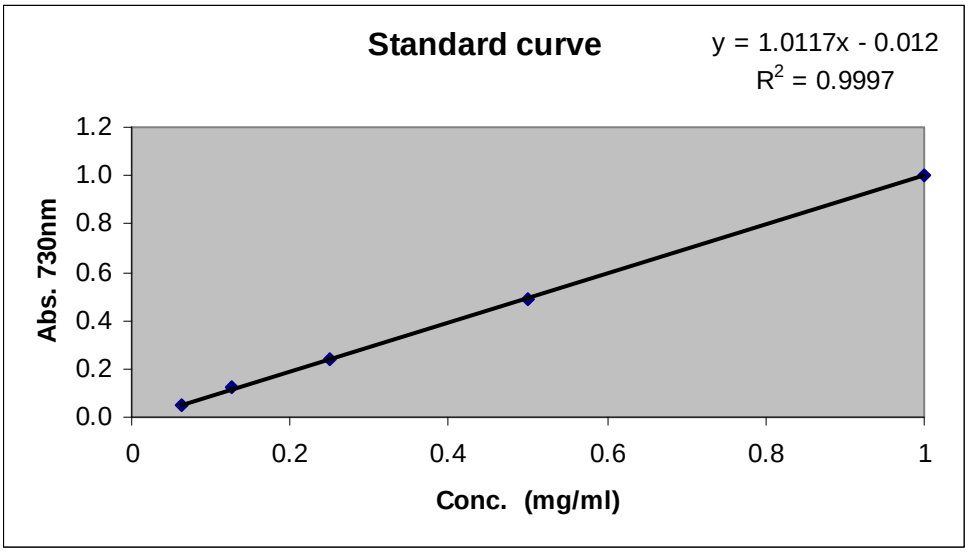


Table 1. Effect of roasting at 165 C for 5 minutes

Sample	Mean Conc. (mg of TAE/100g of Sample)	CV %
Roasted Canola	525.41	0.010
Un roasted Canola	427.48	0.021
Dark Chocolate	253.39	0.041
Roasted DDGS	218.31	0.013
Un roasted DDGS	196.89	0.003
Food Grade DDGS	185.04	0.010
Roasted Soybean	154.78	0.007
Milled Flax Seed	132.30	0.021
Un roasted Soybean	123.45	0.002
Roasted Oat Groats	116.28	0.002
Roasted High-Se Wheat	114.76	0.005
Un roasted Oat groats	112.94	0.009
Un roasted High Se Wheat	111.82	0.002
High-Se Scarified first fraction Wheat	108.28	0.001
Oat Oil	107.87	0.005
High-Se Scarified remaining wheat flour	98.14	0.005
Roasted dhulled Oat groat	42.16	0.010
Un roasted dehulled Oat groat	37.29	0.007

Antioxidant Activity:

	Abs 1	Abs 2	Abs 3	Mean	STDEV	CV%
Control	1.051	1.056	1.081	1.063	0.016	1.512
Quercetin	0.110	0.129	0.149	0.129	0.020	15.079
Quercetin %	89.534	87.784	86.216	**87.829**		

Table 2. Effect of roasting at 165C for 5 minutes

Sample	%Antioxidant value	CV%
Dark Chocolate	39.915	3.655
Un roasted Canola	38.221	1.831
Roasted Canola	35.304	2.983
Roasted DDGS	12.484	0.000
Un roasted DDGS	9.991	3.031
Food Grade DDGS	9.802	1.254
Roasted dehulled oat groat	6.791	4.926
High-Se Wheat Scarified first fraction	4.909	0.490
Milled Flax Seed	4.297	1.530
Roasted Oat Groats	4.297	0.000
Un Roasted Oat groats	4.015	0.832
Un roasted dehulled Oat groat	3.121	2.404
Roasted High-Se Wheat	2.886	0.274
Un roasted High Se Wheat	2.886	0.685
Oat Oil	2.415	2.591
Roasted Soybean	2.227	2.994
Un roasted soybean	1.521	2.365
High-Se Wheat Scarified remaining wheat flour	0.721	1.340

Rat Feeding Trials
Materials

Corn oil (Mazola brand) and canola oil (Crisco brand) were obtained from local super market. Corn meal was prepared by grinding yellow dent corn. Roasted and unroasted canola meals were used. Canola seeds were roasted in oven at 165°C for five minutes. Canola meals were prepared by grinding the roasted and unroasted canola seeds. AIN-93M was purchased from Dyets, Inc. (Bethlehem, PA). Azoxymethane was purchased from Sigma Chemical Company (Saint Louis, MO).

Animals

Male Fischer rats (10 wk old; Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD) were used in this study. Rats were housed in the College of Pharmacy animal room facility (temperature 22 ±1°C, humidity 40-60%, and light from 6:00 AM to 6:00PM) and provided food and water ad libitum.

Colon Carcinogenesis Protocol

Male Fischer rats (10 wk old) were divided into six groups of 24 each and placed on AIN-93M meal supplemented with either corn meal/oil or canola meal/oil. Group assignments were as follows:

Group 1. Basal Diet (AIN-93M meal)
Group 2. Corn meal (AIN-93M +15% corn meal)
Group 3. Corn oil (AIN-93M +15% corn oil)
Group 4 Canola oil (AIN-93M + 15% canola oil)
Group 5. Roasted canola meal (AIN-93M +15% roasted canola meal)
Group 6. Unroasted canola meal (AIN-93M +15% unroasted canola meal)

Supplemented diets were prepared by combining AIN-93M meal with appropriate amounts of corn or canola oil or meal in a mechanical mixer and then storing in airtight containers at 4°C in a refrigerator. Diets were prepared twice a week. Peroxide content of the various diets did not change during the storage period. Carcinogenesis protocol described by Dwivedi et al. (1, 2) was used.

Rats were fed with respective diets for 1 wk to acclimatize them with the texture and taste of the diet. Carcinogenesis was initiated with subcutaneous injections of azoxymethane (15 mg/kg) once a week for 2 consecutive wk. The rats were fed their respective diets *ad libitum* throughout the duration of the experiment. Bowls filled with the respective diets were placed in the corresponding cages in the afternoon and replaced the following day. After 30 wk of initiation, rats were anesthetized with ether. Blood was collected by cardiac puncture. Gastrointestinal tract was removed and flushed thoroughly with ice-cold normal saline. The site, size, and number of tumors were recorded. Serum was prepared from blood by centrifugation. The serum samples will be analyzed for fatty acid composition. Colon and tumor samples will be used for fatty acid analysis and COX expression.

Statistical Analysis

The software INSTAT 3.0 (Graph Pad, San Diego, CA) was used to analyze the data. Analyses of variance (ANOVA) follow by Tukey post test was used to compare the data on tumor multiplicity, tumor size, and weight gain. χ^2 was used to compare tumor incidence. Significance in all cases were considered at $P<0.05$.

Results

Effects of various meals on azoxymethane induced colon tumor development in rats are presented in Figure 1. Tumor incidence (percentage of rats having at least one tumor of any size) in various meals group is shown in Figure 1a. Tumor incidence in AIN basal diet, corn meal, roasted canola meal, and unroasted canola meal was found to be 83%, 83%, 74%, and 58%, respectively. Unroasted canola meal group has significantly ($P<0.05$) lower tumor incidence when compared with other groups. Roasted canola meal diet has slight but insignificant effect on colon tumor incidence.

Figure 2b shows the effects of various diets on tumor multiplicity (average number of tumor per rat). Tumor multiplicity in AIN basal diet, corn meal, roasted canola meal, and unroasted canola meal was 1.8, 1.3., 1.7, and 0.8, respectively. Analysis of variance (ANOVA) followed by Tukey post test showed that unroasted canola meal provided a very significant reduction in number of tumors per rat in comparison with other experimental diets.

Effects of various dietary meals on tumor size are presented in Figure 1c. Tumor size in AIN-93M basal diet, corn meal, roasted canola meal, and unroasted canola meal was 21 mm^2, 44 mm^2, 11 mm^2, and 11 mm^2, respectively. Corn meal group has significantly ($P<0.05$) larger tumor size when compared with other dietary meals. Both roasted and unroasted canola meals significantly ($P<0.05$) reduced the tumor size.

Effects of various dietary oils on azoxymethane-induced colon tumor development in rats are presented in Figure 2. Tumor incidence in AIN-93M basal diet, corn oil, and canola oil group was 83%, 100%, and 80%, respectively. Corn oil group had significantly ($P<0.05$) higher incidence than AIN-93M basal diet or canola oil group (Figure 2a). Tumor multiplicity in AIN-93M basal diet, corn oil, and canola oil was 1.8, 3.1, and 1.3, respectively. Corn oil group again had significantly ($P<0.05$) higher number of tumors in comparison to other dietary groups. Canola oil group had significantly ($P<0.05$) decreased number of tumor and lowest of all treatment groups (Figure 2b).

Tumor size in AIN-93M basal diet, corn oil, and canola oil group was found to be 21 mm^2, 49 mm^2, and 16 mm^2, respectively. Corn oil group again had significantly ($P<0.05$) larger tumor size as compared to other diets. Canola oil group again had significant ($P<0.05$) decrease on tumor size (Figure 2c). There were no significant differences in the weight gain of rats on various diets (data not shown).

These results suggest that dietary (15%) canola meal (specially unroasted) and

canola oil (15%) provide significant chemopreventive effects on colon tumor development in experimental rats. Evidence in the literature suggests that omega-6 polyunsaturated fatty acid (linoleic acid) enhance and omega-3 polyunsaturated fatty acids (α-linolenic acid) reduce the colon tumor development (1, 2). Since both omega-6 and omega-3 polyunsaturated fatty acids are essential component of diet, an optimal ratio of omega-6:omega-3 polyunsaturated fatty acids have to be maintained. As such, α-linolenic acid present in canola meal and oil may be contributing to the chemopreventive effects of canola products.

Furthermore, omega-6 polyunsaturated fatty acids increase the synthesis of prostglandins series 2 by cyclooxyagenase (COX) enzyme. The prostaglandin of series 2 promotes the colon tumor development. Omega-3 polyunsaturated fatty acids decrease the production of prostglandins series 2 and facilitates the synthesis of series 3 prostglandins that inhibits COX. Thus, omega-3 polyunsaturated fatty acids may be producing their chemopreventive effects by increasing the synthesis of series 3 prostglandins and decrease COX activity. Furthermore, during roasting process, omega-3 polyunsaturated fatty acids may be either get oxidized or decomposed, leading to a lower chemopreventive effect than unroasted canola seeds. Following experiments are planned to test the proposed hypothesis to elucidate the mechanism of action of dietary canola products.

Microsomes Preparation

The colon samples collected from adjacent to tumors from all rats will be washed with 1.15% ice-cold potassium chloride (KCI) and blotted. The samples will be minced and homogenized using an Omni GLH homogenizer (Omni International, Inc., Warrenton, VA). The homogenates will then be centrifuged at 10,000 *g* for 15 min in a J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, CA). The supernatant obtained will be recentrifuged in on Optima LE-80K preparative ultracentrifuge (Beckman Instruments, Inc.) at 105,000 *g* for 60 min under refrigeration. The pellet will be washed two to three times with ice-cold KCI and suspended in 1.15% KCI, which will then be used for extraction of fatty acids.

Fatty Acid Extraction

All meals (100 mg) or samples of serum or colon microsomes (160 µ1) from all rats combined with distilled water (1.6 ml) will be extracted as reported by Dwivedi et al. (1, 2).

Determination of Fatty Acids

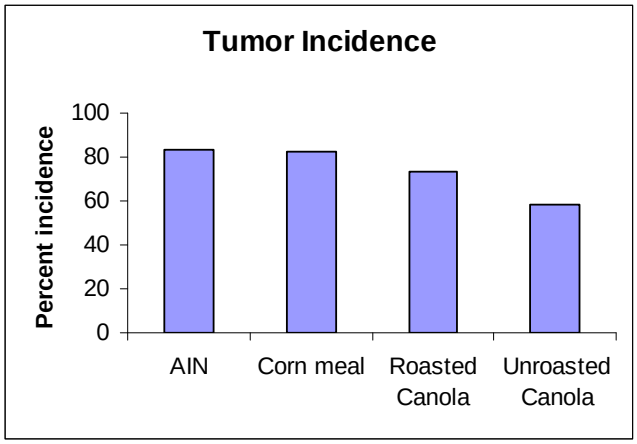
Fatty acid analysis of the meals, serum, and colon samples will be based on the procedures reported by Dwivedi et al. (1, 2). Results will be reported as the percentage of mean values obtained from at least five individual samples.

Determination of COX-1 and COX-2 Expression

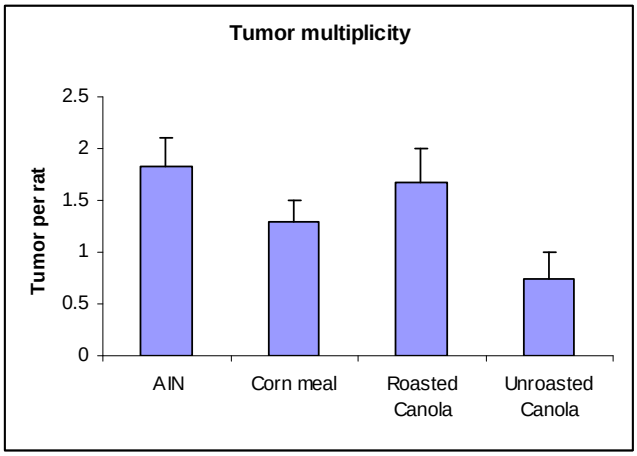
Colon microsomes will be resuspended in Tri reagent, and the protein pool will be isolated based on the manufacture's instructions (Sigma Chemical Company). The protein will be precipitated with isopropanol, washed, and then dissolved in sodium dodecyl sulfate (SDS). The protein in the samples will be quantitated by protein assay kit (BioRad, Richmond, CA). Sixty micrograms of protein will be resuspended in sample buffer, loaded on 7.5% gels, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The proteins will be then transferred to nitrocellulose membranes and probed with primary antibodies against COX-1 and COX-2 (Cayman Chemical Company, Ann Arbor, MI). The secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA) will be used for development with the enhanced chemiluminescence detection kit. The Western blots will be quantitated using a UVP Biochem Gel Documentation system (UVP, Inc, Upland, CA). (1, 2). Our laboratories

Figure1
Effects of various meals on colon tumor development in male Fischer rats

(a)



(b)



(c)

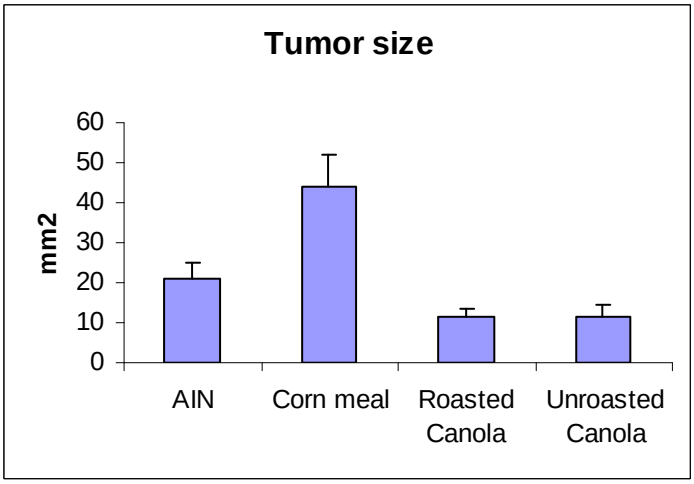
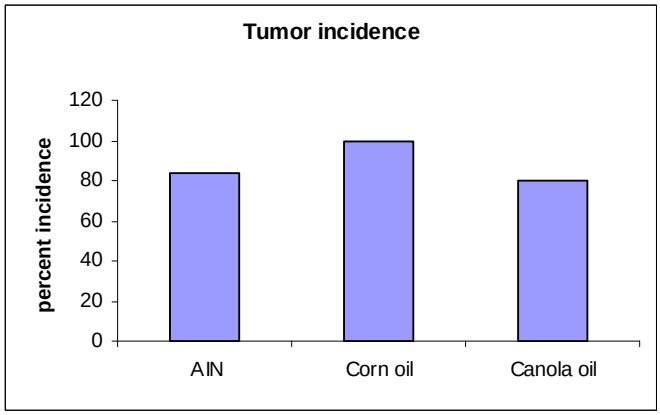


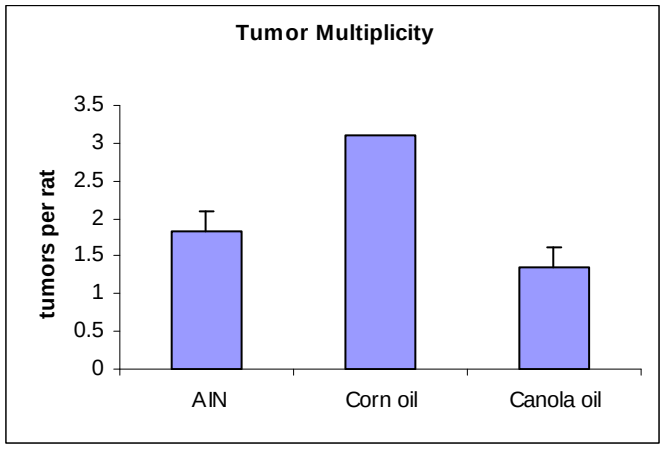
Figure 2

Effects of various oils on colon tumor development in male Fischer rats

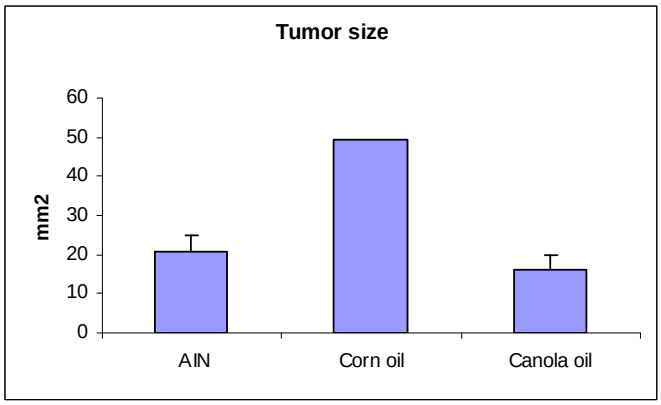
(a)



(b)



(c)



References

1. Dwivedi, C., Natarajan, K., and Matthees, D.P. Chemopreventive effects of dietary flaxseed oil on colon tumor development. *Nutrition and Cancer*, 51:52-58 (2005).

2. Bommareddy, A., Arasada, B.L., Matthees, D.P., and Dwivedi, C. Chemopreventive effects of dietary flaxseed on colon tumor development. *Nutrition and Cancer*, 54:216-222

3. D. Wakamatsu, S. Morimura, T. Sawa, K. Kida, C. Nakai, and H. Maeda. Isolation, Identification, and Structure of a Potent Alkyl-Peroxyl Radical Scavenger in Crude Canola Oil, Canolol. Bioscience, Biotechnology and Biochemistry. Vol. 69 (2005), No. 8 pp.1568-1574.