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

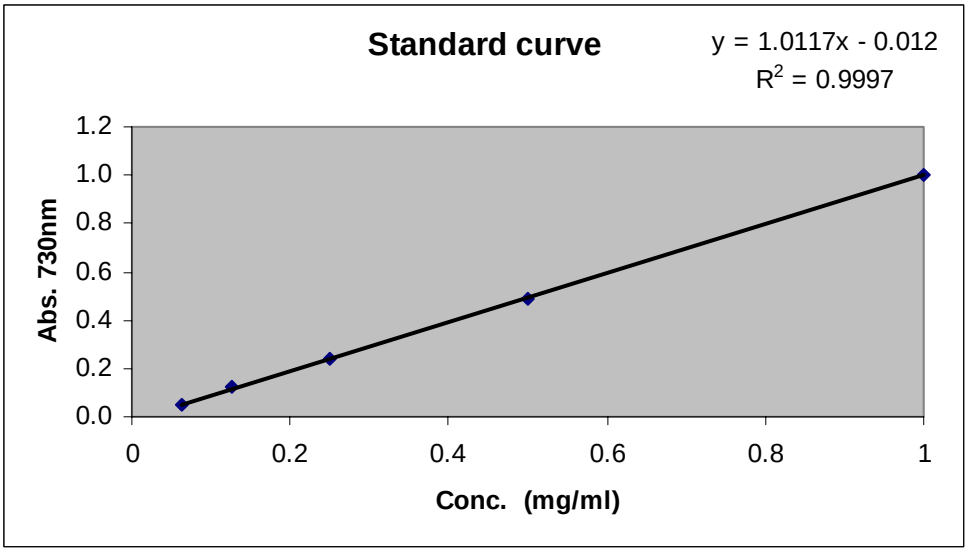


Table 1. Effect of roasting at 165 C for 5 minutes

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

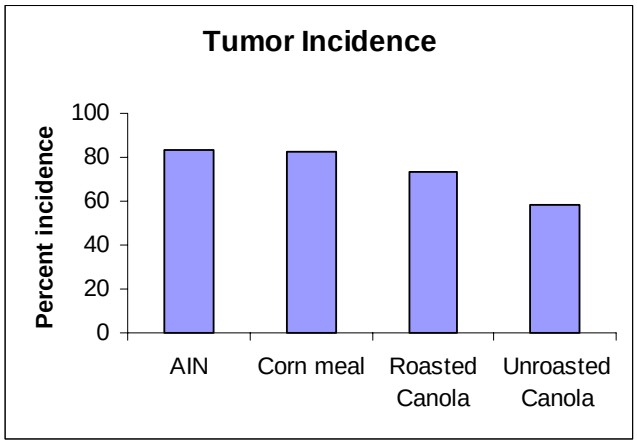
Fatty acid analysis of the meals, serum, and colon samples will be based on the procedures reported by Dwivedi et al. (1, 2). Results will be reported as the percentage of mean values obtained from at least five individual samples.

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)



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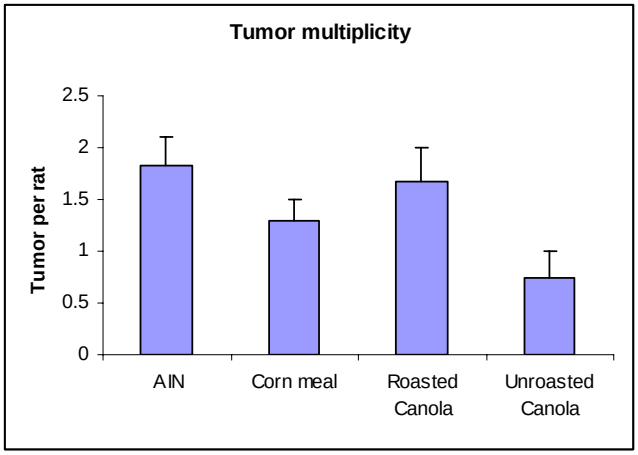

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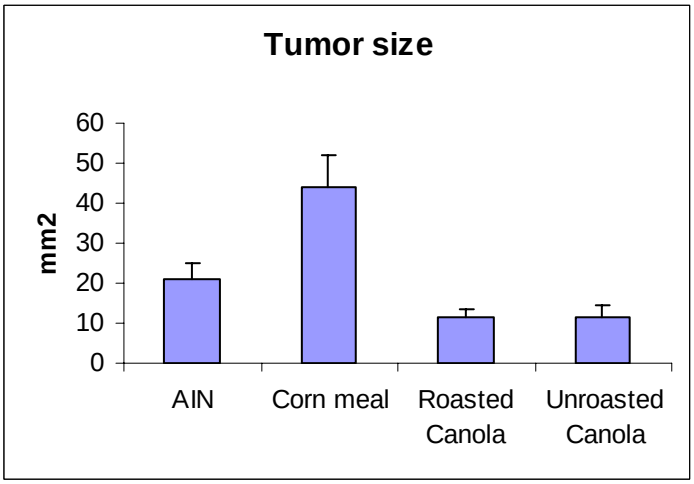
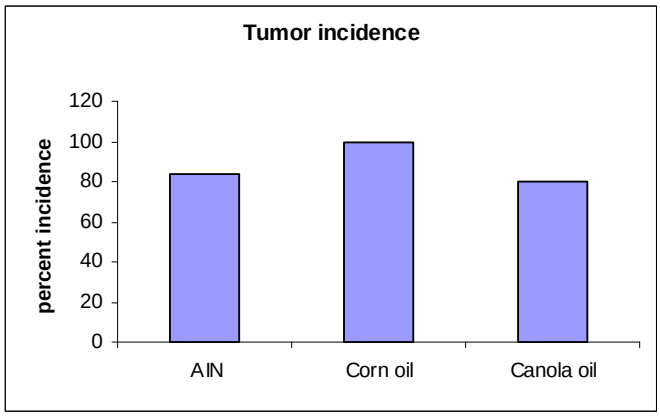


Figure 2

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

(a)



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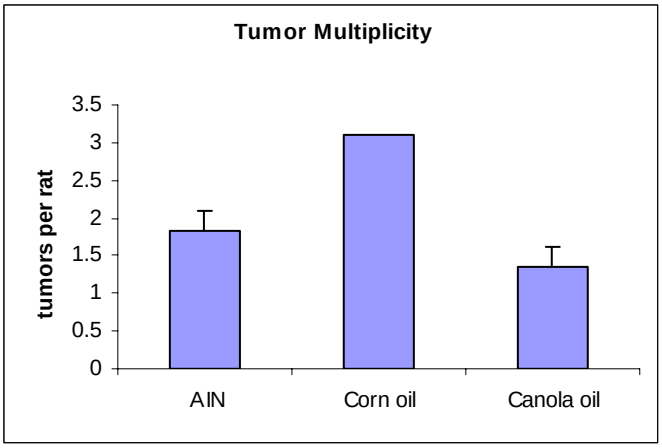

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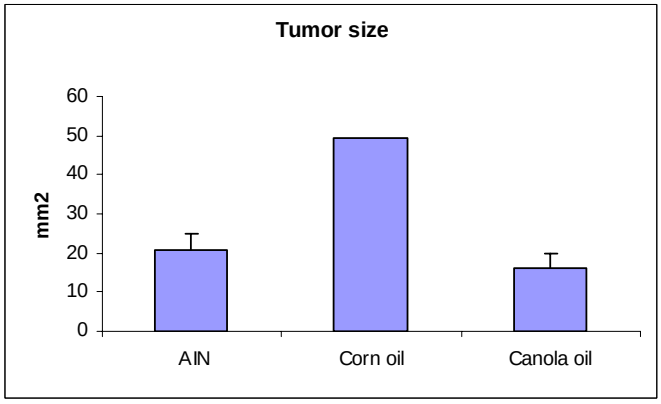

References

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