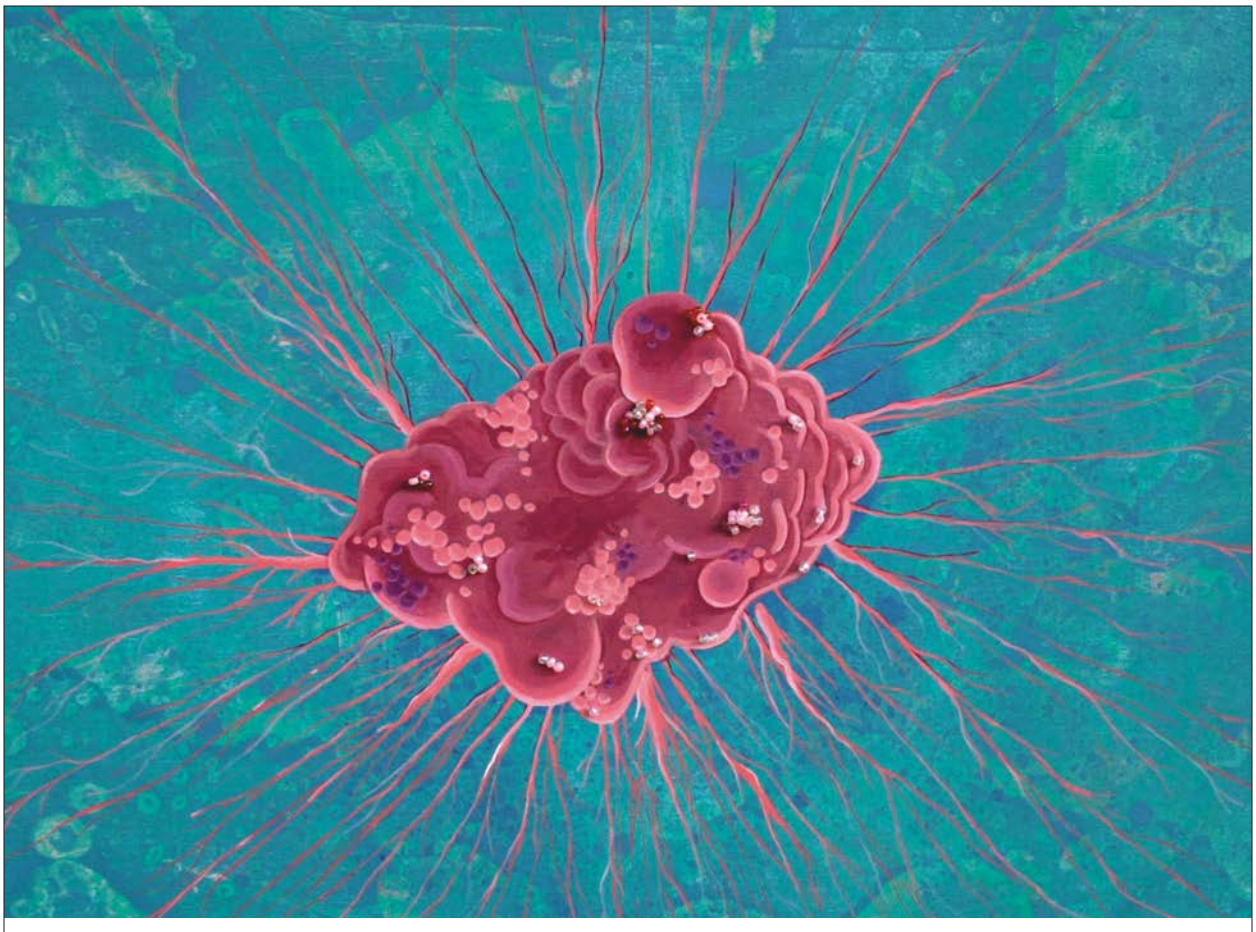

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Feeding Almonds and Pecans Reduced Development of Azoxymethane Induced Precancerous Lesions

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Abstract: The aim of the study was to test the chemopreventive potential of almonds and pecans on (AOM) induced aberrant crypt foci (ACF). Following a 1 week period of acclimatization, 30 Fisher 344 male rats were randomly divided into 5 groups. One group was fed AIN93G (growth) diet as Control(C) and the other groups were fed almonds (A) and pecans (P) at 5% (5 g/100 g level) and 10% (10 g/100 g level). At 7 and 8 weeks of age rats received subcutaneous injections of Azoxymethane (AOM) at 16 mg kg^{-1} body weight and were killed by CO_2 asphyxiation at 17 weeks of age. Selected enzyme activities such as, glutathione-s-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) were determined. ACF incidence in rats fed Pecans and Almonds at 5 and 10% dose levels were significantly ($p<0.05$) lower than rats fed control. ACF was reduced by 46-61% in the treatment groups compared to the control. GST and CAT activities ($\mu mol\ g^{-1}$) in rats fed Pecan and Almonds at 5 and 10% dose levels were significantly higher ($p<0.05$) compared to control fed rats and ranged from 22.03 to 26.44 and 2.38 to 2.98, respectively. We also noted a significant increase in SOD activity ($\mu mol\ g^{-1}$) in rats fed the treatment diets compared to those fed control. Present data indicate that feeding almonds and pecans significantly ($p<0.05$) reduced incidence of AOM induced ACF which are precancerous lesions.

Key words: Edible nuts, antioxidant enzymes, aberrant crypt foci (ACF), Azoxymethane (AOM)

INTRODUCTION

Colorectal cancer is a term used to refer to cancer that develops in the colon or the rectum. These cancers are sometimes referred to separately as colon cancer or rectal cancer, depending on their origin. The American Cancer Society estimates that about 108,070 new cases of colon cancer and 49,690 deaths will be attributed to this disease. Several modifiable factors have been reported to increase the risk of developing colorectal cancer, of this, diet has been implicated in the prevention or progression of colon cancer in comparison to other cancers of the intestinal tract (Verghese *et al*., 2005).

Observational studies suggest that nut consumption is inversely associated with several diseases associated with metabolic syndrome (Jiang *et al*., 2006; Chen and Blumberg, 2008; Kris-Etherton *et al*., 2008; Li *et al*., 2009; Kendall *et al*., 2010). Nuts are a rich source of protein, monounsaturated and polyunsaturated fatty acids, vitamin E, phenolic compounds,

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selenium, fiber, folic acid and phytoestrogens. Healthy fats (i.e., unsaturated fatty acids) in nuts contribute to the beneficial associations of frequent nut intake observed in epidemiologic studies, prevention of Coronary Heart Disease (CHD), diabetes and sudden death and other CVD risk factors. Due to their desirable nutritional profile, it is thus, likely that adding nuts to the diet could enhance the nutritional quality of the diet (Kendall *et al*., 2010).

Nuts are complex food matrices that also are sources of other bioactive compounds. It has been reported that they contain several compounds such as, phenolic acids, flavonoids and phytosterols to mention a few (King *et al*., 2008). These compounds are linked to several bioactivities including antioxidant, antiviral, antiproliferative, hypocholesterolemic and antiinflammatory actions; which are associated with the initiation and progression of several pathogenic processes (Jiang *et al*., 2006; King *et al*., 2008; Yang *et al*., 2009). In fact as pointed out by Yang (2009), several studies, including *in vitro*, *in vivo* as well as observational epidemiological studies suggest that antioxidants can prevent the development of cancer and cardiovascular diseases.

Since, oxidative stress is the causative factor in many chronic diseases, it is assumed that dietary antioxidants may explain this protective effect. Healthy fats (i.e., unsaturated fatty acids) in nuts contribute to the beneficial associations of frequent nut intake observed in epidemiologic studies prevention of Coronary Heart Disease (CHD), diabetes and sudden death and other CVD risk factors.

Almonds (*Prunus amygdalus*) and pecans (*Carya illinoinensis*) are one of the best food sources of α-tocopherol (vitamin E), which may protect against oxidative stress (Hu and Stampfer, 1999). When added to the diet, almonds were shown increase HDL cholesterol and reduce LDL cholesterol levels in humans (Hyson *et al*., 2002; Jahanban *et al*., 2009). Recently, Mandalari *et al*. (2010) showed that almond skins reduced the development of inflammation and tissue injury following spinal cord injury in animal model. In another study, extracts from different parts of the almond were shown to have potent radical-scavenging capacities (Amarowicz *et al*., 2005; Wijeratne *et al*., 2006; Moure *et al*., 2007; Jahanban *et al*., 2009) as well as Cytoprotection of hepatocyte cytotoxicity induced by hydroperoxide (Dong *et al*., 2010). In addition to lowering blood LDL cholesterol levels pecans were reported to possess antiproliferative properties in cancer cells (Yang *et al*., 2009). Pecans are a good source of ellagic acid. This polyphenol has been shown to inhibit chemically induced cancer in the liver, skin, lung and esophagus of rodents through a variety of mechanisms which include inhibition of P450 enzymes, stimulation of glutathione-*S*-transferase, scavenging the reactive metabolites of carcinogens and direct binding to DNA.

Foods such as almonds and pecans are rich in phytochemicals which may prevent severe chronic diseases such as cancer. It is important to utilize foods that contain bioactive components and study their potential role in colon cancer prevention. Nevertheless, the chemopreventive potential of almonds and pecans is sparsely known.

The objective of the study was to test the chemopreventive potential of almonds and pecans (5 and 10% levels) on (AOM) induced Aberrant Crypt Foci (ACF) and to determine the effect on selected detoxification and antioxidant enzymes. The levels of the dietary nuts were selected to correspond to dietary intake of 1- 2 servings of these nuts.

MATERIALS AND METHODS

Chemicals and Dietary Ingredients

All biochemicals were obtained from Sigma Chemical Company (St. Louis, MO). Dietary ingredients were obtained from MP Biomedicals (Costa Mesa, CA). Azoxymethane was

Table 1: Composition of diets[1]

Ingredients (g kg^{-1})	Control	Almonds 5%	Almonds 10%	Pecans 5%	Pecans 10%
Corn starch	397.0	390.1	383.9	393.8	390.1
Casein	200.0	189.9	178.8	195.5	191.0
Dextrose	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	98.0	96.0	98.0	96.0
Soybean oil	70.0	45.5	21.0	35.0	0.0
Fiber	50.0	44.0	37.8	45.2	40.4
Mineral mix	35.0	35.0	35.0	35.0	35.0
Vitamin mix	10.0	30.0	30.0	10.0	10.0
Cystine	3.0	9.0	9.0	3.0	3.0
Choline	2.5	2.5	2.5	2.5	2.5
Almonds/Pecans	0.0	50.0	100.0	50.0	100.0

[1]Formulations of diets based on AIN-93G (Reeves *et al*., 1993a, b)

obtained from NCI Chemical Repository, (Kansas City, MO). All protocols involving rats were approved by the Institutional Animal Care and Use committee of Alabama A and M University, 2008.

Animals and Housing

Fisher 344 male weanling rats were obtained from Harlan, IN. The animals were housed in stainless steel wire cages at 2 rats per cage in the Alabama A and M University Small Animal Laboratory Facility. The temperature and relative humidity were maintained at 21°C and 50%, respectively and light and dark cycles were maintained at 12 h each. The animals were given a 1 week acclimatization period to the conditions mentioned above. After this period, rats were randomly assigned to 5 groups (n = 6) and given access to their prescribed (Table 1) diets throughout the experimental period. Modifications of the treatment diets (5%-5 g/100 g and 10%-10 g/100 g pecan and almonds) were made to cornstarch, casein, sucrose, soybean oil and fiber in order to keep diets isocaloric. Treatment diets were added to AIN93G in the form of a meal. All diets were prepared fresh weekly and stored at 4°C. Biweekly weight gains and daily feed intakes were recorded.

Carcinogenic Injection

For induction of Aberrant Crypt Foci (ACF) all animals received 2 subcutaneous injections of azoxymethane (AOM) (NCI Chemical Repository, Kansas City, MO) in saline 16 mg kg^{-1} b.wt., 1 dose at 7 weeks and another at 8 weeks of age.

Sample Collection

Rats were killed by CO_2 asphyxiation at 17 weeks of age and colons were removed for ACF enumeration. Livers were excised and stored at -80°C for determination of enzyme activities. Cecum was removed and cecal contents and weights of cecal wall were determined.

Enumeration of Aberrant Crypt Foci (ACF)

Colons from each group were rinsed in PBS (0.1 M, pH 7.2), split longitudinally and fixed in 10% buffered formalin (Fisher Scientific, Suwanee, GA). Colons were sectioned and stained with methylene blue. Total number of ACF and the number of crypts per focus were enumerated as described by Bird (1987).

Glutathione S-Transferase (GST) Assay

Hepatic GST (a phase II detoxification enzyme) was assayed by following the procedure outlined by Habig *et al*. (1974). The assay mixture contained 1 mL of potassium phosphate

buffer, 1-chloro 2, 4-dinitrobenzene (1 mmol L^{-1}) and glutathione (1 mmol L^{-1}). The assay mixture was analyzed for enzyme activity at an absorbance of 340 nm.

SOD and CAT Assays

Liver samples were rinsed and homogenized in Phosphate Buffered Saline (PBS) solution, pH 7.4. One gram of tissue sample was homogenized in 10 mL ice-cold buffer (50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA) (1:10 v/w). The homogenate (1 mL) was placed into eppendorf microcentrifuge tubes and centrifuged at 10, 000 x g for 25 min at 4°C. The supernatant was removed and placed into a clean microcentrifuge tube and centrifuged a second time at 10,000-x g for 10 min at 4°C to remove any residual fatty deposits. The final supernatant was removed and used for SOD and CAT assays. SOD and CAT activities in liver and colonic mucosal tissues were estimated using a Superoxide Dismutase assay kit (Trevigen, Gaithersburg, MD) and Catalase assay kit (Cayman Chemicals, Ann Arbor, MI). The analyses were performed according to the manufacturer's protocol.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed on all analyses using SAS version 9.1.2 (SAS Institute, Inc., Cary, NC). Experimental data are the mean±SEM from three experiments and the results were statistically evaluated using Tukey's studentized range test. Differences between treatment groups were tested by student's t test and paired t-test. Unless otherwise indicated levels of significance were considered significant at $p<0.05$.

RESULTS

Effect of Pecans and Almonds on Body Weights Cecal Weight and Cecal pH

Table 2 shows weight gain, cecal weight and cecal pH in rats fed control and treatment diets. Present results showed no significant differences in weight gain between the treatment groups and the control, except for the group fed 5% pecan. We also found no statistical differences in cecal weight and cecal pH between the treatment groups and the control, even though the experimental group was fed a high fiber diet.

Effect of Pecans and Almonds on the Incidence of Aberrant Crypt Foci (ACF)

Data in Table 3 showed the incidence of Aberrant Crypt Foci (ACF) in rats fed experimental diets. Higher numbers of ACF were primarily observed in the distal colon of all the rats, regardless of the diets (Table 3). Research has shown that in humans or in rodents that have been experimentally induced with a chemical carcinogen, colon tumors appeared in the distal portions of the colon. The highest number of ACF was seen in the control group. The incidence of ACF in the proximal and distal colons was significantly higher ($p<0.05$) in the control compared to the treatment groups. Among the rats fed pecans and almonds, ACF

Table 2: Weight gain, cecal weight and cecal pH in Fisher 344 male rats

Groups	Weight gain (g/13 week)	Cecal weight (g)	Cecal pH
Control	202.45±3.80^{b}	0.90±0.10^{a}	8.03±0.70^{a}
5% Pecans	217.25±7.70^{a}	1.10±0.21^{a}	8.14± 0.13^{a}
10% Pecans	202.00±16.10^{b}	1.09±0.17^{a}	7.51±0.13^{a}
5% Almonds	184.25±3.68^{b}	1.00±0.182^{a}	7.63±0.10^{a}
10% Almonds	194.50±17.2^{b}	1.03±0.22^{a}	7.94±0.15^{a}

Values are means ± SEM, n = 6. Means in a column with the same superscript do not significantly differ ($p<0.05$) using Tukey's studentized test

Table 3: Effect of pecans and almonds on total number of AOM- induced Aberrant Crypt Foci (ACF) incidence in colon of fisher 344 rats

Location	Control	Pecans (5%)	Pecans (10%)	Almonds (5%)	Almonds (10%)
Proximal	32.26±6.42^{a}	23.14±3.10^{b}	22.26± 3.04^{b}	19.05±3.06^{c}	16.43±3.01^{c}
Distal	96.40±9.87^{a}	46.0±5.08^{b}	44.22±4.26^{b}	34.10±4.10^{c}	33.92±3.18^{c}
Total	128.52±12.11^{a}	69.15±8.99^{b}	66.48±7.02^{b}	53.15±6.11^{c}	50.35±6.98^{c}

Values are means±SEM, n = 6. Means in a row with the same superscript do not significantly differ ($p<0.05$) using Tukey's studentized test

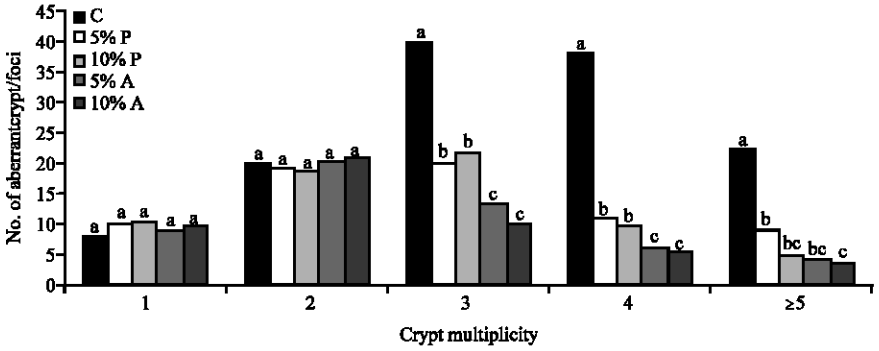

Fig. 1: Effect of diets on number of crypt multiplicity in colon of Fisher 344 male rats. Number of crypts = 3 is significantly higher in rats fed control diet. ACF consisting of = 4 crypts have been reported to progress into putative premalignant lesions. Values are means ± SEM, n = 6. ^{abc}Means on a bar with the same superscript do not significantly differ ($p<0.05$) using Tukey's studentized test

was significantly ($p<0.05$) lower in the rats fed almonds at 5 and 10% levels in both proximal and distal colon compared to the other groups (Table 3). Compared to the control, reductions in ACF ranged from 46.19% in the group fed pecans at 5% level to 61% in the group fed almonds at 10% dose level.

Effect of Pecans and Almonds on Crypt Multiplicity

Although, ACF with 1 and 2 crypts did not significantly differ between the experimental groups (Fig. 1), we noted that rats fed the control diet had significantly ($p<0.05$) higher number of ACF containing 3, 4, 5 crypts/foci compared to the treatment groups. The reduction in crypt multiplicity observed in the groups fed almonds and pecans is critical since those ACF are more likely to develop into tumors over time. These results indicate that both treatment diets, i.e., pecans and almonds, were effective in reducing precancerous lesions.

Effect of Pecans and Almonds on Hepatic Glutathione-s-Transferase, Catalase and Superoxide Dismutase Activities

Hepatic Glutathione-s-transferase (GST), Catalase (CAT) and Superoxide Dismutase (SOD) activities (μmol mg^{-1}) were significantly ($p<0.05$) lower in control fed rats compared to the treatment groups (Table 4). However, we observed no significant differences in enzyme activities (μmol mg^{-1}) among treatment groups. Hepatic GST, CAT and SOD activities in the treatment groups (pecan and almond fed rats) were over 2 fold higher compared to the control.

Table 4: Effect of pecans and almonds on hepatic glutathione S-Transferase (GST), Catalase (CAT) and superoxide dismutase (SOD) activities in fisher 344 male rats

Groups	GST activity (μmol mg^{-1})	CAT activity (μmol mg^{-1})	SOD activity (μmol mg^{-1})
Control	10.34±0.79^{b}	1.02±0.10^{b}	1.20±0.20^{b}
5% Almonds	26.44±0.97^{a}	2.38±0.11^{a}	3.00±0.97^{a}
10% Almonds	23.85±1.96^{a}	2.60±0.29^{a}	2.65±3.22^{a}
5% Pecans	24.05±3.22^{a}	2.80±0.34^{a}	2.95±0.21^{a}
10% Pecans	22.03±0.21^{a}	2.98 ±0.26^{a}	2.16±1.96^{a}

Results showed are specific enzyme activities in liver of rats. Values are means±SEM, n = 6. Means in a column with different letters significantly differ ($p<0.05$) using Tukey's test

DISCUSSION

There is a critical need for better understanding of the role of foods containing fats and fiber in colon cancer risk. The objective of the study was to determine the effects of feeding almonds and pecans on AOM induced aberrant crypt foci in Fisher 344 rats. Epidemiological evidence supports the hypothesis that 50-80% cases of colon cancer can be prevented by diet modifications (McCann *et al*., 2007). The current study found that rats fed almonds and pecans showed a significant decrease in colonic ACF. Although the exact mechanism by which these tree nuts inhibited preneoplastic lesions in the colon is not fully known we speculate that almonds and pecans contain several polyphenols and phytochemicals which studies have shown to have anticarcinogenic effects. These compounds may act as antioxidants, thereby preventing DNA damage caused by reactive oxidant species. In addition, these nuts are good sources of protein, monounsaturated fatty acids, Vitamin E, phenolic compounds, selenium, fiber, folic acid and phytoestrogens (Gonzalez and Salas-Salvado, 2006).

ACF are precursor lesions which indicate the risk of premalignant cancer. Aberrant crypt foci are good predictors of tumor outcome because they can be detected in the early stages of carcinogenesis (Ishizuka *et al*., 2003). However, crypt multiplicity takes into account the development of ACF into larger crypts ultimately resulting in tumor development over time. Feeding almonds and pecans reduced crypt multiplicity (>3 crypts/focus) compared to the control fed rats. It has been suggested that larger ACF are more predictive of colon cancer than the total number of ACF (Govers *et al*., 1993). As the number of crypts/foci increase, there is an increase in tumor incidence, with ACF containing = 3 crypts/foci correlating to a >50% tumor incidence and < 3 crypts/foci correlating to <30% tumor incidence (Alabaster *et al*., 1996). These results are in line with a previous study where we indicated that feeding peanuts significantly reduced AOM-induced ACF in Fisher 344 male rats (Guyton *et al*., 2008). As initially indicated, almonds and pecans are significant sources of vitamin E. Vitamin E has been shown to offer protection to cells from carcinogens perhaps through synergistic interactions with inhibitors of prostaglandin synthesis (Weitberg, 1987). Thus, vitamin E may have reduced the number of ACF and thus crypt multiplicity, by possibly neutralizing reactive oxygen species and other free radicals known to cause DNA damage (Jacobs *et al*., 2001) and also by probably inhibiting Cox-2 activity (O`Leary *et al*., 2004).

In a study conducted by the American Heart Association in individuals with high cholesterol levels, a pecan-enriched diet lowered total cholesterol by 11.3% and LDL cholesterol by 16.5%, without any associated weight gain (Lupu, 2006). According to King *et al*. (2008), individuals who consume nuts are inclined to have higher energy intakes, however, their body weight are no less higher than individuals who do not consume nuts. This could explain the lower weight gain observed in the groups fed 10% pecans thus,

suggesting mechanisms which may include satiation which may lead to spontaneous reduction in food intake and an increase in resting energy expenditure (King *et al*., 2008; Mattes *et al*., 2008).

The present research also demonstrated that consumption of almonds and pecans had some modulatory effects on the activity of selected hepatic enzymes; GST, CAT, SOD. One of the probable mechanisms of chemoprevention is the induction of GST. GST is a principal detoxifying enzyme involved in conjugating reactions of phase II metabolism and also used as a marker enzyme to monitor the severity of carcinogenesis (Jakobisiak *et al*., 2003). In our study GST ($\mu mol\ mg^{-1}$) activity in the groups fed dietary nuts was significantly higher compared to the control fed group (Table 4). There is an inverse relationship between GST activity and the number of ACF, linking GST activity as one of the possible mechanisms in reducing colonic ACF (Williams *et al*., 2006; Boateng *et al*., 2007; Sunkara *et al*., 2009).

Both CAT and SOD play an important role in the cellular defense system against free radical damage. Several studies have showed that tumor cells lack complex enzyme systems which exert protection by scavenging free radicals such as superoxide, hydrogen peroxides and lipid peroxides (Sengottuvelan and Nalini, 2006; Cengiz *et al*., 2007; Boateng *et al*., 2007). In this regard, the increase observed in CAT and SOD activities in rats fed dietary nuts could possibly be attributed to the antioxidative compounds such as, phenolic acids, flavonoids and phytosterols.

Overall, the results from the study indicate that the utilization of almonds and pecans may have implications in chemoprevention. A long-term study (tumor model) on the chemopreventive benefits of almonds and pecans on the modulation of colon cancer will provide further conclusive evidence. Regular consumption of nuts such as almonds and pecans may therefore have implications in the prevention of chronic diseases such as cancer.

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