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The Effect of Pine Needle Powder on AOM-induced Colon Aberrant Crypt Formation and Antioxidant System in Fisher 344 Male Rats*

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Pine needles are known as a traditional medicine and their ingestion has been shown to be beneficial to human beings. Following induction of the neoplastic process in rats by azoxymethane (AOM), we determined the effects of pine needle supplementation on colon carcinogenesis and on antioxidant systems in the blood and liver. Five week old male Fisher 344 rats were injected with AOM (15 mg/kg) once a week for two weeks. After the second injection, 18 rats were randomly assigned into two groups and were fed a casein-based high-fat diet (120 g fat and 1 g cholesterol/kg diet) with or without pine needle powder (10% w/w). After 6 weeks, rats receiving pine needle powder showed a 40% lower incidence of the number of colonic preneoplastic lesions (aberrant crypts) and a 52% lower incidence of aberrant crypt foci ($p<0.01$). A significantly elevated level of erythrocyte catalase activity was observed in the pine needle supplemented group (17.4 ± 1.1 vs. 24.5 ±1.5, $p<0.01$). Pine needle supplementation also increased liver glutathione peroxidase activity (7.5 ± 0.6 vs. 14.6 ± 0.6, $p<0.01$). Other antioxidant parameters such as erythrocyte glutathione peroxidase, liver catalase activity, and plasma total antioxidant potential (TRAP), showed no statistical differences between the two groups. Our data demonstrate that pine needle supplementation improves the antioxidant system and suppresses the formation of colonic preneoplastic lesions in AOM-treated rats. This result provides additional insights into the chemo-preventative properties of pine needles.

Key words : Pine needle, Colon carcinogenesis, Aberrant-crypt foci, Azoxymethane, Chemoprevention, Antioxidant Enzymes

INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm in the world,[1,2] and is the fourth most common cancer in Korea.[3,4] Although the incidence of and mortality from colorectal cancer are lower in Korea than in Western countries, its incidence in Koreans has increased recently, from 5.8% among all cancers in 1980 to 11.2% in 2002.[5] In 2002, 11,097 people in Korea were diagnosed with colon cancer, and 5,113 people died from this disease.[3,5]

Diet is the greatest contributor to human cancer, including colon cancer, and may be associated with 35-70% of the incidence of the disease.[6] In line with this it is now widely known that high intakes of red meat play an important role in the etiology of colon cancer.[7] On the other hand, there is an abundance of evidence suggesting that dietary fiber or phytochemicals may reduce the risk of colon cancer.[8,9] Therefore, much attention has been focused on reducing colon cancer risk through the medical properties of natural compounds that could act as anticarcinogens.

Pine needles (*Pinus densiflora Sieb. et Zucc.*) have been regarded as an important oriental medicine for more than one thousand years.[10] Analysis of the chemical composition of pine needles has identified compounds such as terpenoids, phenolic compounds (benzoic acid, caffeic acid, salicyclic acid, vanilic acid, and cinnamic acid), and tannin.[11-13] Recently, diverse benefits have been claimed for pine needles, such as antibacterial, cholesterol lowering, antimutagenic, antioxidant and antitumoral effects.[14-18]

During colon carcinogenesis, aberrant crypts are recognized as early neoplastic lesions in the colons of both rodents and humans.[19] A number of natural chemopreventive agents or medicinal plants that inhibit aberrant crypt foci (ACF) development have been proved to prevent colon cancer in rodents,[20,21] suggesting that the ACF assay in the rodent colon can be used as a good biomarker in colon carcinogenesis.

It is also known that free radicals or reactive oxygen molecules play a role in colon cancer.[22-24] Thus, we assumed that the antioxidant properties of pine needles might reduce the formation of mutagenic peroxidation products in the colon by countering free radicals. Therefore, the present study was designed to investigate the effects of pine needle supplementation on antioxidant systems and on colonic ACF formation in AOM-treated F344 male rats.

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MATERIALS AND METHODS

Animal and Diets

Five-week-old male F344 rats (n=18) were purchased from Samtako Inc. (Osan, Korea) and were housed individually in hanging wire cages in a room controlled for humidity (55%), temperature (25 ℃) and light (12/12h light-dark cycle). The rats were allowed free access to water and fed a commercially prepared pelleted diet for 1 week of adjustment. All animals received the carcinogen azoxymethane (AOM, Sigma) s.c. once a week for two weeks at a dose of 15mg/kg body weight (Fig. 1) and fed a high-fat and high-cholesterol control diet (12 g lard + 0.1 g cholesterol/100 g diet). After the second injection of AOM, the rats were randomly assigned into two groups (n=9) and fed a high-fat and high-cholesterol diet with or without 10% (w/w) pine needle powder for 6 weeks. The pine needle powder was purchased from Myongjin Farm (Inje, Gangwon-do, Korea) where pine needles had been gathered and freeze-dried. The compositions of the diets are shown in Table 1. Animals were monitored daily for general health, and body weights were recorded every week for the duration of the study. At the end of the experimental period, the rats were anesthetized with ethyl ether and blood was collected from the abdominal artery into heparinated sterile tubes. Plasma was obtained from the blood samples by centrifugation (1500 rpm for 30 minutes) and stored at -80 ℃ until further analysis. Erythrocytes were washed three times with isoosmotic phosphate buffered saline (PBS), pH 7.4, and resuspended to the original volume. The erythrocyte suspensions were frozen at -80 ℃ until final analysis. The liver was removed and washed with ice-cold saline and then stored at -80 ℃ before analysis. The entire colon was collected for aberrant crypt analysis.

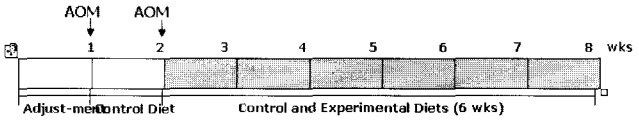

Fig. 1. Experimental design and protocol for evaluation of chemo-preventive effect of pine needle supplementation on colon carcinogenesis. Male F344 rats were fed control diet or 10% pine needle supplemented diet after being exposed to the second injection of AOM until termination of the study 6 weeks after AOM treatment.

Animals : Five-week-old male F344 rats (9/group)
Carcinogen : Azoxymethane (AOM), s.c. at a dose of 15 mg/kg body weight
Control diet : High fat-high cholesterol diet (12 g lard+ 0.1 g cholesterol/100 g diet)
Experimental diet : High fat-high cholesterol diet + 10% pine needle powder

Table 1. Diet composition (g/100 g)

	Control diet	Experimental diet
Corn starch	43.849	33.849
Casein	20	20
Sucrose	10	10
Corn oil	5	5
Lard	12	12
Cellulose	4	4
Mineral mix[1)]	3.5	3.5
Vitamin mix[2)]	1	1
L-Methionine	0.3	0.3
Choline bitartarate	0.25	0.25
Butylated hydroxy toluene	0.001	0.001
Cholesterol	0.1	0.1
Pine needle powder	-	10

1) AIN 76 mineral mixture contained (in g/kg of mixture): calcium phosphate, dibasic 500; sodium chloride 74; potassium citrate, monohydrate 220; potassium sulfate 52; magnesium oxide 24; manganouscarbonate (43-48% Mn) 3.5; ferric citrate (16-17% Fe) 6; zinc carbonate (70% ZnO) 1.6; cupric carbonate (53-55% Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulfate 0.55; sucrose, finely powdered 118.03

2) AIN 76 vitamin mixture contained (in g/kg of mixture): thiamine HCl 0.6; riboflavin 0.6; pyridoxine HCl 0.7; niacin 3; d-calcium pantothenate 1.6; folic acid 0.2; d-biotin 0.02; cyanocobalamin (vitamin B_{12}) 0.001; dry vitamin A palmitate (500,000 U/d) 0.8; dry vitamin E acetate (500U/d0 10; vitamin D3 trituration (400,000 U/g) 0.25; menadione sodium bisulfite complex 0.15; sucrose finely powdered 981.08

Erythrocytic and Hepatic Antioxidant Enzyme Activities

Tissue sample preparation

1 g of liver tissue was homogenized in 10 volumes of 50 mM phophate-0.25 M sucrose-0.5 M EDTA and was then centrifuged at 10,000 g for 20 min at 4 ℃. 3 mL of the supernatant (A) was centrifuged at 105,000 g for 50 min at 4 ℃ to obtain a cytosol supernatant for the GSH-Px assay. 5 mL of the supernatant (A) was disrupted by ultra-sonication and mixed with 800 μL of chloroform:ethanol (5:3) and centrifuged at 20,000 g for 20 min at 4 ℃ to obtain a supernatant for the SOD assay. For the catalase assay, 0.2 g of liver tissue was homogenized on ice in 4 mL of 25 mM KH_2PO_4 buffer solution (pH 7.0). The homogenate was diluted in 60 volumes of 25 mM KH_2PO_4 buffer solution and was then twice treated in an ultrasonic ice bath for 15 seconds. The protein contents of the tissue preparations were determined by the method of Bradford[25)] using a kit reagent (Bio Rad, USA) with bovine serum albumin as the standard. Erythrocytic hemolysates were prepared by diluting erythrocytes with distilled water as follows: 1:7 for the SOD assay; 1:20 for GSH-Px; and 1:500 for CAT. The hemoglobin content of erythrocytic hemolysate was assayed according to the method of Van Kampen and Zijlstra[26)] using a kit reagent (Roche, Switzerland).

Superoxide dismutase activitiy (SOD)

SOD was assayed in erythrocyte suspension by the procedure of Marklund and Marklund.[27)] 500 μL of the erythrocytic hemolysate or liver tissue preparation was combined with 3.5 mL of water, 1 mL of ethanol and 0.6 mL chloroform. After centrifugation at 3000 U/min for 2 minutes, various dilutions were prepared from the supernatant. 20 μL of 10 mM pyrogallol were added to each dilution after incubation at 37 ℃ for 10 minutes. The reaction was monitored spectrophotometrically at 320 nm for 2 minutes. The unit of the enzyme was defined as that amount which inhibits the

autoxidation of pyrogallol by 50%.

Glutathione peroxidase activitiy (GSH-Px)

GSH-Px was determined by the use of a method described by Beutler.[28] 10 μL of erythrocytic hemolysate or liver tissue preparation was added to 100 μL of 1 M Tris-HCl-5 mM EDTA buffer (pH 8.0), 20 μL of 0.1 M glutathione, 100 μL of 10 U/mL glutathione reductase, and 100 μL of 2 mM NADPH; water was then added to this mixture, to make it up to 1 mL. After incubation at 37 ℃ for 10 minutes, the reaction was initiated by the addition of 10 μL of t-Butyl hydroperoxide, and the absorbance was measured at 340 nm. The reaction was continued for 90 seconds, and the loss of NADPH was monitored by the change in A_{340nm}/minute.

Catalase activitiy (CAT)

Catalase activitiy was measured by the method of Aebi.[29] 100 μL of erythrocytic hemolysate or liver tissue preparation was dissolved in 50 mL of 50 mM phosphate buffer (pH 7), and 2 mL of the mixture was then added to a cuvette. The reaction was initiated by the addition of 1 mL 30nM H_2O_2 at 20 ℃. The decomposition rate of H_2O_2 was measured at 240 nm for 30 seconds using a spectrophotometer.

Plasma total radical trapping antioxidant potential (TRAP)

TRAP was measured by a modification of a photometric method according to Rice-Evans and Miller.[30] The method for measuring antioxidant activity is based on the inhibition by antioxidants of the absorbance of the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^+$). The $ABTS^+$ radical cation is formed by the interaction of $ABTS^+$ (150 μM) with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin (2.5 μM) with H_2O_2 (75 μM). 10 μL of sample/buffer/Trolox standard were added to the tubes that contained 400 μL of PBS buffer, 20 μL of metmyoglobin and 400 μL of ABTS; these ingredients were then mixed by vortexing. The reaction was initiated by the addition of 170 μL of H_2O_2. Absorbance was measured at 734 nm after 6 minutes of incubation using a spectrophotometer. Values were expressed as TEAC (Trolox equivalent antioxidant capacity), defined as the mM concentration of the Trolox antioxidant capacity of a calibration curve.

Baseline conjugated dienes in LDL

Baseline LDL conjugated dienes were determined according to Ahotupa et al., with little modification.[31] 100 μL of plasma was added to 700 μL heparin citrate buffer (0.064 M trisodium citrate, 50,000 IU/L heparin, pH 5.05), and the suspension was allowed to stand for 10 minutes at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1000 g for 10 minutes. The resulting pellet was resuspended in 100 μL of 0.1 M Na-phosphate buffer containing 0.9% NaCl (pH 7.4). Lipids were extracted from the 100 μL of LDL suspension by chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm. Oxidation during sample preparation was prevented by adding EDTA.

Aberrant crypt analysis

The determination of aberrant crypts was performed on a 5-cm-long segment corresponding to the distal part of the colon. The segment was washed with physiological saline (0.9% NaCl), cut open, and fixed in 10% buffered formalin solution for at least 24 hours. Later, the colon samples were stained with 0.2% methylene blue for five minutes; the mucosal side was then placed on a glass slide and examined microscopically using the × 10 objective for assessment of the number of aberrant crypts, following a procedure described by Bird (Fig. 2).[19]

Statistical analysis

Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as the mean ± standard error (S.E.). The mean values of the data from the control group and the pine needle supplement group were compared using the Student's *t*-test and the differences were considered significant at a level of $P < 0.05$.

RESULTS

1. Food intakes and body weight

During the experiment, no treatment-related signs of adverse effects in the clinical appearance of the animals were observed. The body weight was found to be similar between the two groups in the pre-initiation period. Although pine needle supplementation caused a statistically significant decrease in food consumption (12.09 ± 0.21 vs. 14.45 ± 0.28, p<0.001), no differences in final body weight and food efficacy ratio (FER) were seen between the groups (Table 2).

Table 2. Effects of Pine needle supplementation on body weight, food intakes, weight gain and food efficacy ratio in F344 male rats

	Control group	Pine needle group
Initial body weight (g)	209.7±3.1[1)]	209.9±3.8
Final body weight (g)	286.1±7.5	273.2±5.3
Body weight gain (g/d)	1.56±0.15	1.29±0.12
Food intake (g/d)	14.45±0.28	12.09±0.21**
Food efficacy ratio	0.11±0.02	0.11±0.01

1) Values represent mean ± SE.
Significantly different from the control group: ** p<0.001 (Student's t-test)

2. Colon aberrant crypt foci formation

As shown in Figure 2 (B), all rats injected with AOM developed abnormal and hyperplastic crypts in the colon. The frequency of ACF in the colon was 52% less (significant at p<0.01) in the group supplemented with pine needles (Fig. 3A). Each ACF contained one to four AC. The total number of AC in the colon was 40% lower (significant at p<0.05) in the

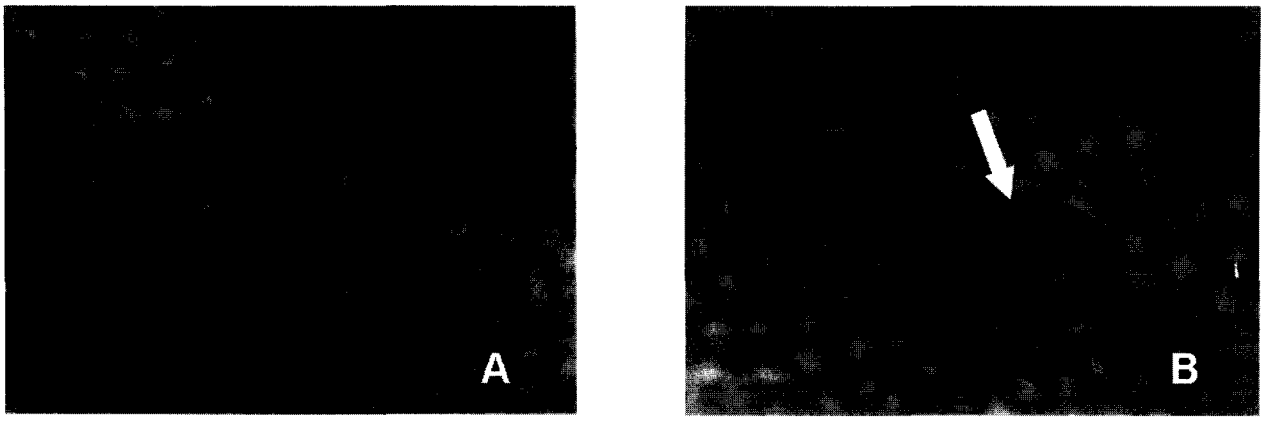

Fig. 2. Aberrant crypt foci (Preneoplastic lesion of colon) surrogate intermediate biomarker of colon cancer. A: Normal colon without any ACF, rats were treated with a vehicle (saline), Magnification × 10. B: Colon with two AC (arrow), rat were treated with AOM (carcinogen of colon), Magnification × 10.

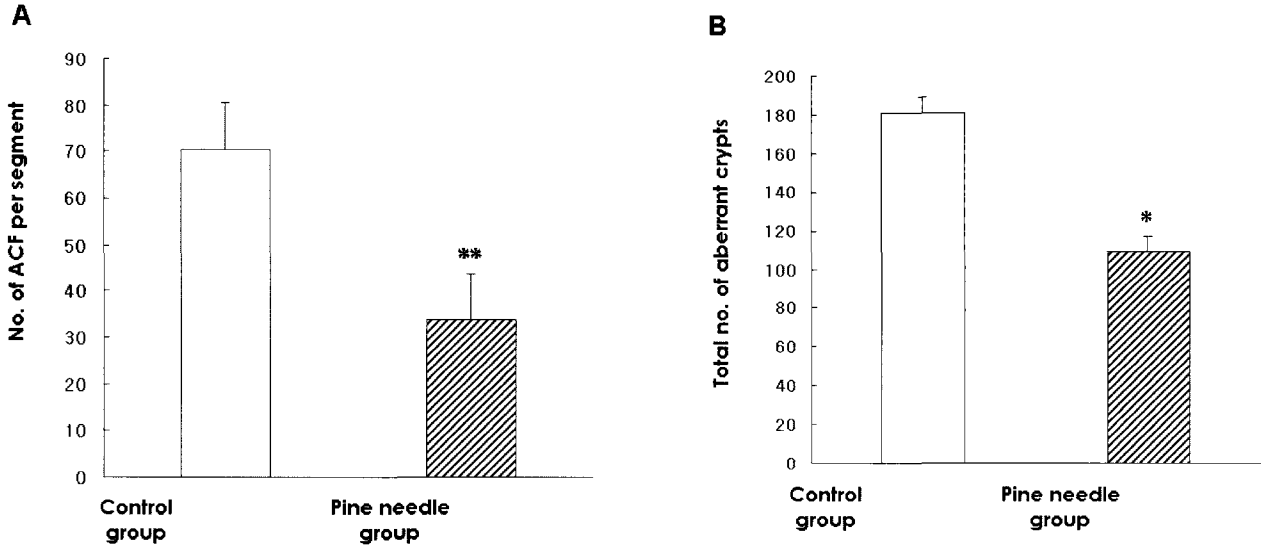

Fig. 3. Effect of pine needle supplementation on no. of aberrant crypt foci (ACF) (A) and total no. of aberrant crypts (B) in distal colon. Bars represent mean ± SE. Significantly different from the control group: * $p<0.05$, ** $p<0.01$ (Student's t-test)

pine needle supplemented group compared to the control group (Fig. 3B).

3. Antioxidant system

The antioxidant enzyme activities for the erythrocytes are presented in Table 3. Six weeks of pine needle supplementation resulted in increased catalase activity in erythrocytes, by 41% ($p<0.001$), compared to the control group. GSH-Px and SOD activities in erythrocytes were higher in the pine needle supplemented group, but these differences were not statistically significant. Plasma levels of TRAP and conjugated dienes did not differ between the two groups (Table 3). In the liver, GSH-Px activity was significantly higher, by 77%, for the pine needle supplementation group ($p<0.01$). However, catalase and SOD activities did not appear to be influenced by pine needle supplementation (Table 4).

Table 3. Effects of Pine needle supplementation on erythrocytic antioxidant enzymes and plasma antioxidant system in F344 male rats.

	Control group	Pine needle group
Erythrocyte		
Glutathione peroxidase(IU/g Hb)	1186.8±41.9[1)]	1219.6±59.1
Catalase(k/g Hb)	17.4±1.1	24.5±1.5**
Superoxide dismutase(U/g Hb)	2489.5±317.6	2900.3±344.2
Plasma		
TRAP(mM)	1.12±0.01	1.07±0.03
Conjugated dienes(μM)	10.9±1.9	8.2±0.3

1) Values represent mean ± SE.
Significantly different from the control group: ** $p<0.01$ (Student's t-test)

Table 4. Effects of Pine needle supplementation on hepatic antioxidant enzymes in F344 male rats.

	Control group	Pine needle group
Glutathione peroxidase (IU/mg pro)	8.47±1.20[1)]	14.96±1.34**
Catalase (k/mg pro)	2.47±0.56	1.99±0.43
Superoxide dismutase (U/mg pro)	12.03±3.91	9.90±4.71

1) Values represent mean ± SE.
Significantly different from the control group: ** $p<0.01$ (Student's t-test)

DISCUSSION

Previously, pine needle extracts have been shown to inhibit the mutagenic effect of cyclophosphamide on mouse bone marrow and human lymphocytes.[16] It has also been reported that pine needle extract inhibits the *in vitro* growth of a variety of animal and human tumor cells, including mice leukemic cancer cells (L1210), sarcoma 180 cells, human monocyte-like cancer cells (U937), human breast cancer cells (T47D, MDA-MB-231, MH7A and MCF-7), hepatoma cells (Hep3B, SNU 354 and SK-HEP-1) and lung cancer cells (3LL, A549).[32-34]

Several *in vivo* experiments have also demonstrated the anti-carcinogenic properties of pine needles. For example, Kil et al. have reported that 10% of pine needle supplementation for 4 weeks suppressed the growth of tumors in transplanted sarcoma-180 cells in mice.[18] However, the effect of pine needles on colon carcinogenesis has not been reported. In this study, we investigated the effect of pine needle supplementation on both the antioxidant system and on colon carcinogenesis in rats.

Our present results clearly indicate that feeding pine needle powder in the diet effectively suppresses the occurrence of colonic ACF induced by AOM, when administered after the carcinogen treatment. AOM is an intermediate product of the colonic carcinogen 1,2-Dimethylhydrazine (DMH) that produces a high incidence of precancerous lesions, referred to as ACF, in rats. ACF also has been identified in the human colon and is associated with carcinomas.[35,36] Because of the potential progression of early changes to malignancy, the study of premalignant hyperproliferative lesions and of aberrant crypts is crucial for the understanding of the pathogenesis of colon cancer.[37]

It was reported that DMH induces carcinogenesis in rats and mice by the increased production of reactive free radicals and a decreased activity of some antioxidant enzymes.[38] DMH is activated in a series of oxidative steps to indirect electrophilic carcinogens.[39] Much of the evidence has come from the facts that antioxidants that scavenge free radicals directly, or that interfere with the generation of free radical mediated events, inhibit the neoplastic process, and that the activities of antioxidant enzymes change during tumor formation.[40] When compared to their appropriate normal cell counterparts, tumor cells are almost always low in antioxidant enzyme activity.[22-24]

In this sense, the one possible mechanism for the reduction of ACF by pine needles could be ascribed to their antioxidant effects. In this study, pine needle supplementation increased catalase activity in erythrocytes and GSH-Px in the liver, compared to the control diet. The antioxidative activity of pine needles has been found by several researchers. For example, Choi et al. found that pine needle extract increased SOD, catalase and GSH-Px in the serum and liver of male SD rats.[41,42] Boo et al. isolated an antioxidant, 4-hydroxy-5-methyl-3[2H]-furanone, from pine needles.[43] In future studies, antioxidant activity should be measured in the colon, the target organ for AOM-induced carcinogenesis, to investigate the exact mechanism of the antioxidative effects of pine needles.

Another possible mechanism for the reduction of ACF by pine needles may be explained by the presence of fibrous matter in pine needles, which contain 13.3% of crude fibers.[44] It is known that fiber reduces exposure of the intestinal wall to potential carcinogens; assists in sequestration of bile acids and other carcinogens of endogenous or exogenous origin, and accelerates their excretion; increases the volume and dilution of the intestinal content; and accelerates transit through the colon and stimulation of defaecation.[45-47]

In conclusion, the results of the present study suggest that pine needles may have a protective effect on the early phase of colon carcinogenesis induced by AOM, possibly through increased antioxidant enzyme activities.

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