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Wheat Bran Oil and Its Fractions Inhibit Human Colon Cancer Cell Growth and Intestinal Tumorigenesis in *Apc*$^{min/+}$ Mice

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This study was designed to investigate the cancer preventive activities of wheat bran (WB) oil. We studied the colon cancer preventive effects of WB oil and its subfractions in the *Apc*$^{min/+}$ mouse model, a recognized mouse model for human colorectal cancer, and used human colon cancer cell lines (HCT-116 and HT-29) to identify possible active fractions in WB oil. Our results showed that the oil fraction of WB was more active than the water fraction against the growth of human colon cancer cell lines and that 2% WB oil significantly inhibited the overall tumorigenesis by 35.7% ($p < 0.0001$) in the *Apc*$^{min/+}$ mouse model. The WB oil was further fractioned into nonpolar lipids and phytochemicals and the phytochemical fraction was fractionated into phytosterols and phytosterol ferulates, 5-alk-(en)ylresorcinols, and unidentified constituents by normal phase silica gel column chromatography. Results on cell culture showed that the phytochemical fraction had a higher inhibitory effect on HCT-116 human colon cancer cells than that of WB oil, whereas the nonpolar lipid fraction had less growth inhibitory effectiveness. However, neither fractions showed a stronger inhibition than WB oil in the *Apc*$^{min/+}$ mouse model. The current results demonstrate, for the first time, the intestinal cancer preventive activity of WB oil. The active ingredients, however, remain to be identified.

KEYWORDS: Wheat bran oil; colon cancer; *Apc*$^{min/+}$ mice; phytochemicals; nonpolar lipids

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer death in the United States (*1*). Epidemiological studies have suggested that dietary fibers, especially fiber from cereal sources, may reduce the risk of colon cancer (*2*−*6*). The European Prospective Investigation into Cancer and Nutrition study showed that dietary fiber in foods was inversely related to incidence of colon cancer (*7*). The American Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial also indicated that a high intake of dietary fiber was associated with a lower risk of colorectal adenoma, after adjustment for potential dietary and non-dietary risk factors, and the inverse association was strongest for fiber from grains and cereals (*8*).

Human and animal studies indicated that not all cereal fibers are equally efficacious as cancer preventive agents. Intervention studies in humans consuming a high-fat/low-fiber diet demonstrated that adding WB to the diet favorably altered a number of biomarkers related to colon cancer risk, including fecal mutagenicity and secondary bile acids (*9*, *10*). In such studies, WB was more effective than corn bran or oat bran (*9*, *10*). Laboratory animal model studies have corroborated this concept (*11*). WB is the only cereal bran that shows consistent protection against colon cancer in laboratory animal models (*11*). It was reported that a diet containing 20% WB decreased the incidence of 1,2-dimethylhydrazine (DMH)-induced colon tumors in rats (*12*). Male F344 rats fed WB and given azoxymethane (AOM) or 3,2-dimethyl-4-aminobiphenyl (DMAB) had a lower incidence and multiplicity of small intestine and colon tumors than those fed the control diet and treated with carcinogens (*13*, *14*). Dietary supplementation with WB also protected against the formation of colonic aberrant crypt foci (ACF) induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline in the rat (*15*). In the *Apc*$^{min/+}$ (min: multiple intestinal neoplasia) mouse model, a diet containing 45% WB significantly decreased the tumor number in small intestine, and WB diets were found to work better than whole wheat diets (*16*). In contrast, oat and corn brans have generally been found to enhance carcinogenesis (*11*).

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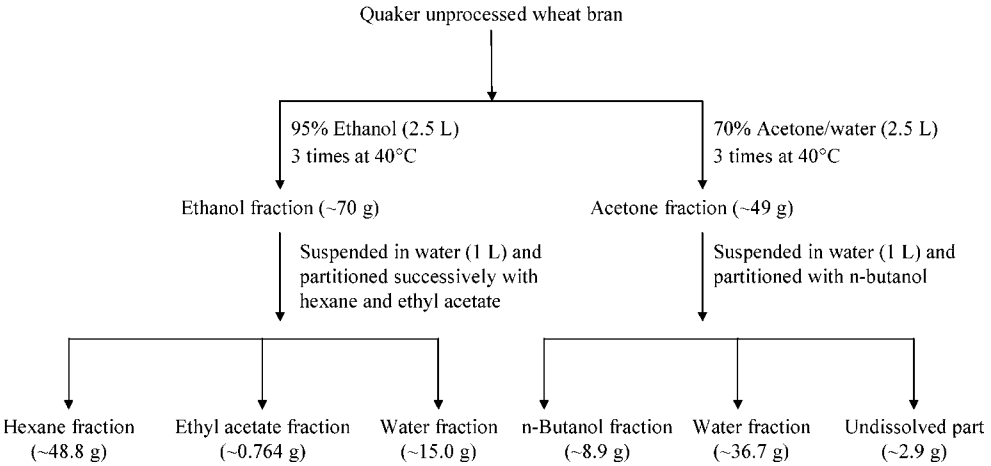

Figure 1. Extraction and fractionation procedure of wheat bran.

Although most human and animal studies show a correlation between WB consumption and reduction of the risk of colon cancer, the mechanism by which dietary WB protects against colon tumor development has not been fully explored. Most of the proposed mechanisms are based on the physiological function of fiber. A proposed mechanism for the action of WB is its fecal dilution effect (*17*). A second proposed mechanism for WB is its ability to accelerate the transit of fecal material through the colon, thus reducing access of luminal contents to the colonic epithelial cells (*17*). In addition, butyric acid and orthophenolic acids, which are the fermentation products of WB fiber in the colon, have been proposed to be protective agents (*16*, *17*). These proposed mechanisms are a subject of debate (*17*, *18*). Recently, Reddy and co-workers have studied the effect of WB oil on colon carcinogenesis in the AOM-induced rat tumor model (*19*). The results demonstrated that the removal of lipid and lipid soluble components from WB increased colon tumorigenesis as compared to the WB control group (10% WB), and fortification of a dephytinized and defatted WB (WB−PF) diet with WB oil significantly increased the inhibitory activity. These observations suggest that WB oil is a major active constituent of WB for the inhibition of colon tumorigenesis. However, these studies only provided indirect evidence for the preventive effect of WB oil itself against intestinal tumorigenesis.

The *Apc*$^{min/+}$ mouse model is recognized as a genetically relevant animal model mimicking human colon carcinogenesis, in which the most frequent molecular changes occur on *APC* and β-catenin (*20*−*24*). Even though the tumors develop mainly in the small intestine in *Apc*$^{min/+}$ mice, this model has been a useful system for testing the activities of many chemopreventive agents. Many chemopreventive agents, such as nonsteroidal anti-inflammatory drugs (NSAIDs), identified in the *Apc*$^{min/+}$ mouse model are also effective in humans (*25*−*27*). To test the efficacy of WB oil itself and identify its major active constituents in WB oil, the present study investigated the cancer preventive activities of WB oil and its subfractions in the *Apc*$^{min/+}$ mouse tumorigenesis model and in HCT-116 human colon cancer cell lines.

MATERIALS AND METHODS

Preparation of WB Extractions and Subfractions for Cell Line Studies. Quaker unprocessed wheat bran (904 g, purchased from a local supermarket) was extracted with 95% ethanol and 70% acetone/water at 40 °C for 24 h (3 times, each time 2.5 L), respectively (**Figure 1**). After evaporation, the ethanol residue (70 g) was suspended in water (1 L) and partitioned first with hexane (3 times, each time 1 L) and then ethyl acetate (3 times, each time 1 L) to obtain 38.8 g of a hexane fraction and 0.764 g of an ethyl acetate fraction. The acetone/water residue (49 g) was suspended in water (1 L), the undissolved material was filtered (2.9 g), and the water solution was partitioned with *n*-butanol (3 times, each time 1 L) to obtain 8.9 g of the butanol fraction.

Cell Culture Studies. Cell growth inhibition was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (*28*). The cells were plated in 96-well microtitre plates and allowed to attach for 24 h at 37 °C. The test samples (in DMSO) were added to McCoy's 5A medium (containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine) to desired final concentrations. After culturing for 24 h, the medium was aspirated, and the cells were treated with 100 μL of fresh medium containing 2.41 mmol/L MTT. Following incubation for 1−3 h at 37 °C, the MTT-containing medium was aspirated, 100 μL of DMSO was added to solubilize the formazan precipitate, and the plate was read at 595 nm on a microtitre plate reader. The reading reflected the number of viable cells and was expressed as % cell growth.

Large-Scale Preparation and Fractionation of WB Oil. WB (423 kg, provided by ConAgra Food Ingredients Company, NJ) was extracted twice by 95% ethanol at 40 °C to obtain 42.4 kg of ethanol extract (**Figure 3**). Part of the ethanol extract (1 kg) was then suspended in water and partitioned with ethyl acetate to obtain the WB oil (800 g). The WB oil fraction (300 g) was chromatographed on a normal phase silica gel column eluted with hexane/ethyl acetate (20:1) to give the nonpolar lipid fraction (170 g) and then methanol to give the phytochemical fraction (110 g). The phytochemical fraction was further fractionated into three subfractions using a normal phase silica gel column eluted with hexane/ethyl acetate (10:1 and 5:1) (fractions 1 and 2) and then methanol (fraction 3).

Animal Treatment and Tumor Harvesting. Experiments with mice were carried out according to a protocol approved by the Institutional Review Board for the Animal Care and Facilities Committee (IRB-ACFC no. 91-024) at Rutgers University. Male *Apc*$^{min/+}$ were obtained from our own breeding colony (Department of Chemical Biology) (*29*). The mice were housed 10 per cage and maintained in air-conditioned quarters with a room temperature of 20 ± 2 °C, relative humidity of 50 ± 10%, and an alternating 12 h light/dark cycle. At 6 weeks of age, male *Apc*$^{min/+}$ (C57BL/6J/min/+) mice were randomly placed in the control (AIN-93G diet, Research Diets, New Brunswick, NJ) and 2% WB samples (WB oil or nonpolar lipid and phytochemical fractions of WB oil) groups. The compositions of all the diets used in this study are listed in **Table 1**. Food and fluid intakes as well as body weight of mice were monitored on a weekly basis during the experimental periods. Mice were routinely checked for any abnormalities. At 14 weeks of age, all mice were sacrificed by CO_2 asphyxiation. The small intestine and colon were opened longitudinally, flattened on filter paper, and placed on dry ice briefly to freeze. The tumors displayed a distinct white color, were denser than normal tissue, and were scored by size and location (proximal, middle, and distal small intestine and colon) under a lighted magnifier.

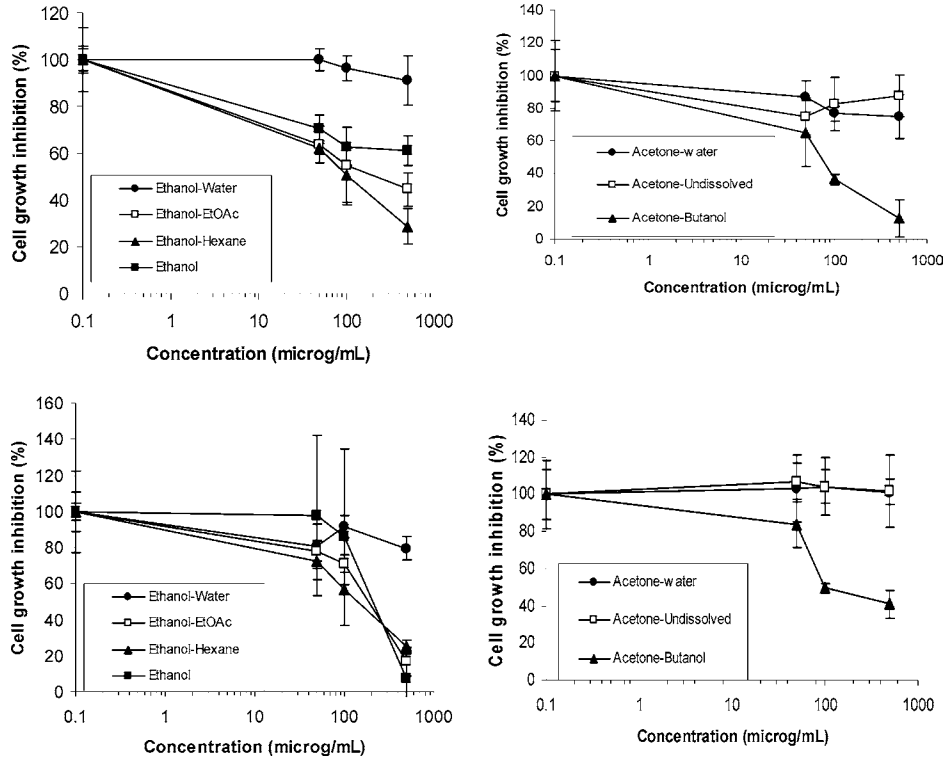

Figure 2. Cell growth inhibition in HCT-116 (upper panels) and HT-29 (lower panels) human colon cancer cell lines by different fractions of the extractions of WB. Cells were treated with 50, 100, and 500 μg/mL WB samples (the ethanol extraction, hexane, ethyl acetate, and water fractions of the ethanol extraction; butanol and water fractions and the undissolved part of the acetone extraction) for 24 h in the presence of 10% FBS at 37 °C. Cell growth inhibition was determined by MTT assay, $n = 6 \pm$ SD).

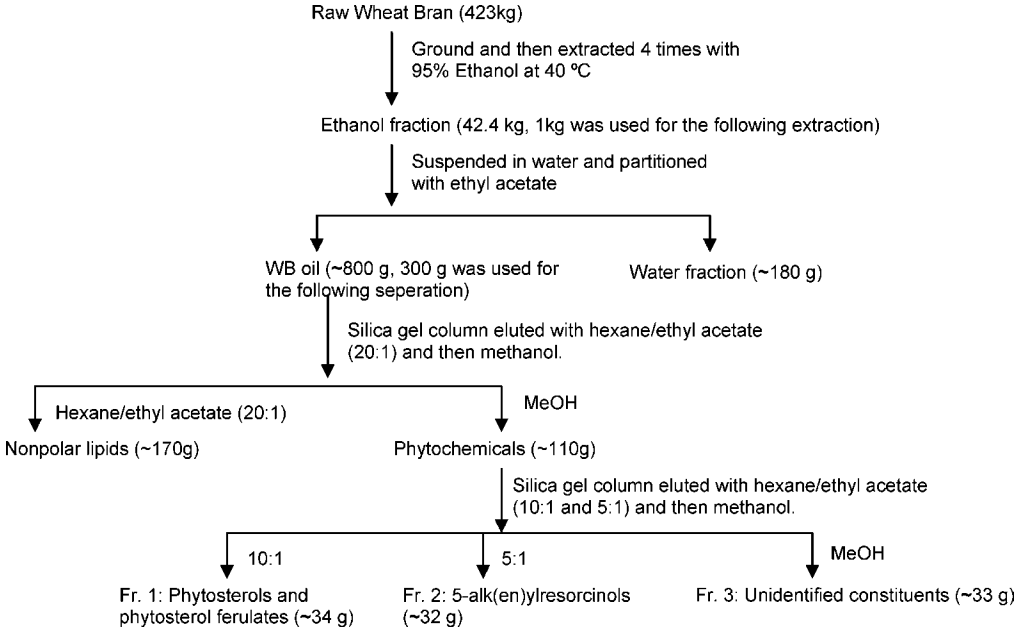

Figure 3. Large-scale preparation of WB oil and its subfractions.

Statistical Analysis. For simple comparisons between two groups, a two-tailed Student's *t*-test was used. One-way ANOVA with Tukey multiplicity adjustment was used for comparisons among multiple groups. The inhibition of tumorigenesis by WB oil was determined based on the Poisson linear model. A *p*-value of less than 0.05 was considered statistically significant in all the tests.

RESULTS

Cell Growth Inhibition by Different Fractions of the WB Extractions. On the basis of the MTT assay, the hexane fraction had the highest growth inhibitory activity, with IC_{50}-values of $\sim$100 μg/mL for both cell lines, HCT-116 and HT-29, after treatment for 24 h (**Figure 2**). The ethyl acetate fraction also showed inhibitory activity, and some of the activities are probably due to compounds that are also present in the hexane fraction. Since both hexane fraction and ethyl acetate fraction showed significant inhibitory effects and these two fractions contained all the lipids and lipid soluble phytochemicals, we used ethyl acetate to extract all the constituents in these two fractions and referred to it as WB oil (**Figure 3**) for further

Table 1. Composition of Diets Used in This Study

	AIN-93G		2% WBO		2% phytochemicals		2% nonpolar lipids	
diet	gm %	kcal %	gm %	kcal %	gm %	kcal %	gm %	kcal %
protein	20.3	20.3	20.3	20.3	20.1	20.3	20.3	20.3
carbohydrate	63.9	63.9	63.9	63.9	63.2	63.9	63.9	63.9
fat	7	15.8	7	15.8	8.1	15.8	7	15.8
total		100		100		100		100
kcal/gm	4		4		3.95		4	

ingredient	gm	kcal	gm	kcal	gm	kcal	gm	kcal
casein	200	800	200	800	200	800	200	800
L-cysteine	3	12	3	12	3	12	3	12
cornstarch	397.486	1589.9	397.486	1589.9	397.486	1589.9	397.486	1589.9
maltodextrin 10	132	528	132	528	132	528	132	528
sucrose	100	400	100	400	100	400	100	400
cellulose, BW 200	50	0	50	0	50	0	50	0
soybean oil	70	630	50	450	62	558	50	450
WBO	0	0	20	180	0	0	0	0
phyto-chemicals	0	0	0	0	20	72	0	0
nonpolar lipids	0	0	0	0	0	0	20	180
t-butylhydro-quinone	0.014	0	0.014	0	0.014	0	0.014	0
mineral mix S10022G	35	0	35	0	35	0	35	0
vitamin mix V10037	10	40	10	40	10	40	10	40
choline bitartrate	2.5	0	2.5	0	2.5	0	2.5	0
total	1000	4000	1000	4000	1012	4000	1000	4000

studies. The water fractions showed little or no inhibition on both cell lines. Interestingly, the butanol fraction from the more polar acetone/water extraction also showed potent inhibition on both cell lines.

Effect of WB Oil on Intestinal Tumorigenesis in *Apc*$^{min/+}$ Mice. On the basis of results from the previous cell culture studies, we prepared WB oil samples in a large scale for further animal study (**Figure 3**). In the min mice studies, there were no statistical significant differences of mouse body weight and food intake between control groups and WB oil treated groups. Our first set of experiments on the inhibition of tumorigenesis by WB oil in the *Apc*$^{min/+}$ mouse model indicated that 2% WB oil inhibited the formation of large tumors (>2 mm) in the small intestine (50.4% inhibition, $p < 0.05$) but that the inhibition of overall tumor formation was marginally significant (33.0%, $p = 0.09$), possibly because only 12 mice was used in each group. To further investigate the inhibitory effect of tumorigenesis by WB oil in this model, we conducted another set of studies using a larger number of mice ($n = 20$). The results (**Table 2**) showed that 2% WB oil significantly inhibited the overall tumorigenesis by 38.2% ($p < 0.01$). Since these two experiments were conducted under identical conditions, we conducted statistical analysis on the combined results of these two experiments. The response was the tumor number; the covariates included experiment, treatment, and interaction. The results showed that the mice in the 2% WB oil treated group had significantly fewer total tumors in the small intestine than those in the control group ($p < 0.0001$) with 35.6% inhibition after adjustment of the experimental effect. A greater inhibition was found in the distal small intestine (40.6% inhibition after adjustment of the experimental effect, $p < 0.0001$) than in the middle or in the proximal small intestine. Two percent WB oil also significantly ($p < 0.0001$) inhibited the formation of tumors when analyzed by size, with the greatest inhibition on large tumors (>2 mm, 46.7% inhibition after adjustment of the experimental effect).

Effect of WB Oil Subfractions on Intestinal Tumorigenesis in *Apc*$^{min/+}$ Mice. WB oil was chromatographed on a normal phase silica gel column eluted with hexane/ethyl acetate (20:1) to give fraction 1 and then with methanol to give fraction 2. On the basis of the analysis with thin layer chromatography (TLC) and information from the literature (*30*), fraction 1 contained most of the nonpolar lipids, such as fatty acids, triglycerides, and tocols, and fraction 2 contained all the phytochemicals in WB oil, such as phytosterol ferulates, phytosterols, 5-alk(en)ylresorcinols, and unidentified constituents. The activities of the phytochemical and lipid fractions were compared in the *Apc*$^{min/+}$ mice (**Table 3**). There were no statistically significant differences of mouse body weight and food intake between control groups and WB subfraction treated groups. Both the mice in the nonpolar lipid fraction and the phytochemical fraction treatment groups had fewer small intestinal tumors than those in the control group with 12.2 and 22.8% inhibition, respectively. The effects, however, were not statistically significant. Analysis of subsets of tumors revealed that the 2% phytochemical fraction significantly inhibited the formation of large tumors (>2 mm, 52.6% inhibition, $p < 0.05$) and tumors in the proximal small intestine (42.2% inhibition, $p < 0.05$).

Cell Growth Inhibition by Different Subfractions of the WB Oil. Since the phytochemical fraction showed a better inhibitory effect than the nonpolar lipid fraction in the *Apc*$^{min/+}$ mouse model, we further fractionated the phytochemical fractions on a normal phase silica gel column eluted with hexane/ethyl acetate (10:1 and 5:1) to give subfractions 1 (phytosterol ferulates and phytosterols) and 2 (5-alk(en)ylresorcinols) and then methanol to give subfraction 3 (unidentified constituents). On the basis of the MTT assay, the phytochemical fraction also showed a higher growth inhibitory effect than the nonpolar lipid fraction on HCT-116 cells (**Figure 4**). Among the three subfractions, the unidentified constituent fraction (fraction 3) had the highest inhibitory activity, and the phytosterol and phytosterol ferulate fraction (fraction 1) was less effective, but the 5-alk(en)ylresorcinol fraction (fraction 2) had activity only at much higher concentrations (**Figure 4**).

Table 2. Effects of 2% WB Oil on Tumorigenesis in the *Apc*$^{min/+}$ Mouse Model[a]

		small intestine (tumor numbers)								
		subtotal	region			size (mm)				
			proximal	middle	distal	≤1	1–2	≥2	colon (tumor numbers)	overall (tumor numbers)
experiment 1	control ($n = 12$)	40.2 ± 21.5	4.8 ± 2.1	12.5 ± 8.4	22.9 ± 15.8	13.4 ± 8.3	15.1 ± 11.5	11.7 ± 8.6	1.3 ± 1.3	41.5 ± 22.4
	2%WB ($n = 12$)	27.1 ± 13.8	4.8 ± 1.5	9.8 ± 6.0	12.5 ± 9.1	11.0 ± 4.5	10.3 ± 8.0	5.8 ± 4.1	0.8 ± 1.0	27.8 ± 14.5
experiment 2	control ($n = 20$)	28.4 ± 14.1	4.5 ± 2.8	9.1 ± 5.6	14.9 ± 10.7	11.7 ± 6.7	13.2 ± 10.8	3.5 ± 3.0	0.4 ± 0.75	28.8 ± 14.0
	2%WB ($n = 20$)	17.6 ± 9.8[b]	3.3 ± 2.4	4.8 ± 4.1	9.5 ± 6.1	8.0 ± 5.0	7.4 ± 5.4	2.1 ± 1.8	0.25 ± 0.55	17.8 ± 9.8[b]

[a] Two independent experiments (male *Apc*$^{min/+}$ mice, 6–14 weeks of age) were performed with different numbers of mice. Each value represents mean ± SD. [b] p-value < 0.01.

Table 3. Inhibitory Effect of 2% WB Oil Nonpolar Lipid and Phytochemical Fractions in *Apc*$^{min/+}$ Mouse Model

	small intestine (tumor numbers)								
	subtotal	region			size (mm)			colon (tumor numbers)	overall (tumor numbers)
		proximal	middle	distal	<1	1–2	>2		
control ($n = 20$)	36.9 ± 19.5	5.0 ± 2.9	12.0 ± 7.46	19.9 ± 11.3	13.4 ± 8.96	17.4 ± 10.2	6.10 ± 3.67	0.90 ± 1.07	37.8 ± 2.0
2% nonpolar lipids	32.6 ± 23.1	4.79 ± 3.52	11.1 ± 8.52	16.7 ± 12.6	12.4 ± 11.0	16.0 ± 12.0	4.68 ± 4.91	0.58 ± 0.90	33.2 ± 23.6
($n = 19$) (% inhibition)	(11.6%)	(4.2%)	(7.5%)	(16.1%)	(7.5%)	(8.0%)	(23.3%)	(35.6%)	(12.2%)
2% phytochemicals	28.4 ± 20.0	2.89 ± 2.25	9.6 ± 8.06	15.8 ± 12.2	9.67 ± 8.80	15.8 ± 11.5	2.89 ± 3.61	0.89 ± 0.96	29.2 ± 20.5
($n = 18$) (% inhibition)	(23.0%)	(42.2%)[a]	(20.0%)	(20.6%)	(27.8%)	(9.2%)	(52.6%)[a]	(1.1%)	(22.8%)

[a] p-value < 0.05.

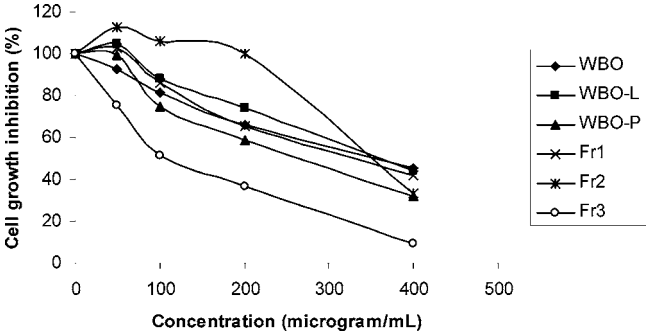

Figure 4. Cell growth inhibition in HCT-116 human colon cancer cell lines by wheat bran oil and its subfractions. Cells were treated with 50, 100, 200, and 400 μg/mL WB oils (WBO), its subfractions (the nonpolar lipid fraction (WBO-L), phytochemical fraction (WBO-P), and the subfractions of phytochemical fraction: fractions 1 (phytosterols and phytosterol ferulates), 2 (5-alk(en)ylresorcinols), and 3 (unidentified constituents) for 24 h in the presence of 10% FBS at 37 °C. Cell growth inhibition was determined by MTT assay, $n = 8 \pm$ SD).

DISCUSSION

In the present study, we demonstrated for the first time that 2% WB oil significantly inhibited intestinal tumorigenesis in the *Apc*$^{min/+}$ mouse model, especially for large tumors and tumors in the distal small intestine. To identify the major active constituents for colon caner preventive of WB oil, we further fractionated WB oil, using normal phase silica gel column chromatography, into two major fractions: nonpolar lipids and phytochemicals. We compared the inhibitory effects of tumorigenesis of the nonpolar lipid fraction and the phytochemical fraction in the *Apc*$^{min/+}$ mouse model. Our results indicated that the phytochemical fraction had a better inhibitory effect than the nonpolar lipid fraction (**Table 3**). However, the extent of inhibition by these fractions was not higher than that of WB oil as shown in **Table 2**. Because of the variability between experiments of the *Apc*$^{min/+}$ mouse model, in the absence of a WB oil group in **Table 3**, we cannot conclude that the subfractions are less active than WB oil. The subfractions, however, probably are not much more active than the WB oil preparation. This type of phenomenon has been observed in the fractionation of other natural products. For example, Luo et al. reported that the γ-oryzanol components in rice bran could be separated into two fractions, one fraction had much stronger cell growth inhibition against MCF-7 human breast carcinoma cells than the other fraction (IC_{50}: 82 vs 550 μg/mL) (*31*). However, the major components purified from the active fraction had similar cell growth inhibitory effects as the parent fraction. This suggests that the components in the active fraction had synergistic or additive effects. It is possible that some constituents in the nonpolar lipid and phytochemical fractions may act synergistically or that some minor components that have strong biological effects are lost during the fractionation.

Our cell culture studies indicated that the phytochemical fraction was more effective than WB oil at inhibiting the growth of HCT-116 human colon cancer cells at concentrations greater than 100 μg/mL, whereas the nonpolar lipid fraction showed a less inhibitory effect. Further studies on the growth inhibitory effects of the subfractions of the phytochemical fraction indicated that the unidentified constituent fraction showed strong inhibitory effects and that the phytosterols and phytosterol ferulates had moderate effects. The 5-alk(en)ylresorcinols had no significant effects at concentrations less than 200 μg/mL.

The present results in the *Apc*$^{min/+}$ mouse model are consistent with the previous results by Reddy et al. that WB oil is a major active constituent of WB for the inhibition of colon tumorigenesis (*19*). WB oil is effective on both the mouse and the rat models, which suggests that it may also be used to reduce the risk of colorectal cancer on humans. In human cancer prevention trials, WB oil is rather inexpensive, safe, and easy to use. It is also important to identify the major active constituents in WB oil for additional mechanistic studies and for better standardization of WB oil for future human use.

ABBREVIATIONS USED

AOM, azoxymethane; *Apc*, adenomatous polyposis coli; CRC, colorectal cancer; min, multiple intestinal neoplasia; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NSAIDs, nonsteroidal anti-inflammatory drugs; WB, wheat bran; WBO, wheat bran oil.

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