

Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin)

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It is estimated that one-third of Americans use dietary herbal supplements on a regular basis. Diets rich in bioactive phytochemicals are associated with reduced risk of certain cancers, notably, colon cancer. Herbal supplements have not been directly tested as sources of bioactive cancer preventives. Hence, this study compares the ability of four herbal flavonoids (quercetin, curcumin, rutin and silymarin) and one whole herb mixture (ginseng powder) to suppress aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced rat colon cancer model. Second, this study examines the effect of these herbal compounds on apoptosis and the mechanisms by which these compounds evoke apoptosis. The results of this study show that diets containing quercetin, curcumin, silymarin, ginseng and rutin decreased the number of ACFs by 4-, 2-, 1.8-, 1.5- and 1.2-fold, respectively compared with control. Histological analysis of the colon mucosa revealed that all the herbal supplements, except silymarin, induced apoptosis, with quercetin being the most potent (3× increase compared with control). Furthermore, ginseng and curcumin were region-specific in inducing apoptosis. The ability of quercetin and curcumin to modulate ACFs correlates well with their ability to induce apoptosis. Western blot analysis of caspase 9, Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins from the colon scraping suggests that quercetin and curcumin induce apoptosis via the mitochondrial pathway. Taken together, the results of this study suggest that these herbal supplements may exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions and inducing apoptosis in the large intestine.

Introduction

Colon cancer is the second leading cause of cancer-related deaths in USA. It has been estimated that this cancer will develop in >147 000 people and 56 000 will die from this disease (1). Colon cancer is a well-studied cancer but the progress in the field of preventing or curing this disease has not been significant. While there are chemotherapeutic drugs available for the treatment of this disease the majority of the patients do not respond to these drugs and side effects remain

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCAC, β -catenin accumulated crypts; IDV, integrated density value; MDF, mucin depleted foci; MTD, maximum tolerated dose.

problematic. Therefore, emphasis has been focused on a variety of clinical and basic studies of chemoprevention using naturally occurring dietary substances, since they might provide useful strategies to inhibit colon cancer with minimal toxicity (2).

The use of herbs as medicines dates back to the origin of civilized man. The earliest known records of herbal medicines were written by Sumerians on the medicinal use of opium poppy, thyme, licorice and mustard plant. Moreover, the Asian cultures have a long recorded history of herbal use dating back >3000 years. USA has a much shorter history of medicinal herbal use. However, it is interesting to note that the use of herbs and alternative medicine is more popular now than ever before. Recent estimates from National Institute of Health–Office of Dietary Supplements (NIH–ODS) suggest that 40–55% of Americans (>100 million people) use supplements on a regular basis, and 24% (>24 million people) of these people use herbal supplements. Herbal based dietary supplements contain a large array of phytochemicals with polyphenolic constituents (flavonoids) which might mediate physiological functions related to cancer suppression. Although evidence from epidemiological and animal studies (3) suggest that increased consumption of plant-based diet can reduce the risk of colon cancer (4,5), bioactive cancer preventives remain to be identified. Therefore, this study compares the ability of four herbal flavonoids (quercetin, curcumin, rutin and silymarin) and one whole herb mixture (American white ginseng powder) with indomethacin, an NSAID known to be chemopreventative for colon cancer, for their ability to suppress aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced rat colon cancer model. In addition, this study also examined the effects of these herbal compounds on apoptosis and suggests possible mechanisms by which these compounds induce apoptosis.

Materials and methods

Animals

Two hundred and thirty-five male F344 rats were purchased at 6 weeks of age from Charles River Laboratories (Frederick, MD). They were housed in the AAALAC-accredited laboratory animal facility at the University of South Carolina, School of Medicine (USC–SOM) and in accordance with the US Department of Health and Human Services (DHHS) Guide for the ‘Care and Use of Laboratory Animals’. The University of South Carolina, Institutional Animal Care and Use Committee (IACUC) approved the protocol for the study (AUP no. 1163). The rats were housed in standard cages with Bed-o-Cob bedding (Andersons, Toledo, OH). Animals were maintained on a 12 h dark and light cycle. The animal rooms were maintained at 20–24°C and 50–60% relative humidity. Drinking water and diet (AIN-76A diet alone or AIN-76A supplemented with herbal supplements) were supplied to the animals *ad libitum*.

Effect of dietary herbal supplements on ACFs

After the rats were acclimated for a week, at 7 weeks of age, animals were randomly divided into a carcinogen-treated group (130 rats) or a saline-treated group (35 rats). Both these groups were further divided into 13 and 7 subgroups with 10 and 5 rats per subgroup, respectively. The subgroups in

the carcinogen-treated group were: (1) positive control (no test reagent, with carcinogen), (2–7) treatment group [test agent at 80% maximum tolerated dose (MTD) and carcinogen] and (8–13) treatment group (test agent at 40% MTD and carcinogen); whereas, the subgroups in the saline-treated group were: (1) negative control (no test agent, no carcinogen) and subgroups 2–7 were treatment group (test agents at 80% MTD and no carcinogen). The positive and the negative controls were fed the standard AIN-76A diet (Dyets, Bethlehem, PA) and the treatment groups had either 40 or 80% of the MTD of each agent included in their diets; these doses were chosen based on published data. The test agents used in the study were purchased from Sigma Chemical (St Louis, MO) at the highest available purity and these compounds were incorporated in diet by Dyets. Upon arrival, the pellets were stored at -20°C prior to placement in the animal cages feeder bins. The diets were fed to the rats beginning 1 week prior to injection with AOM or saline, then continuously for the next 4 weeks. The positive control group and the two treatment groups were injected intraperitoneally with the carcinogen AOM purchased from Ash Stevens (Detroit, MI) once a week (weeks 2 and 3 of each experiment) at a dose of 15 mg/kg body wt. The negative control group was injected with saline. At the end of week 5, the rats were killed by decapitation and colons removed for evaluation of aberrant crypts.

Scoring of aberrant crypts

The colons were removed, flushed with cold phosphate-buffered saline (PBS), slit open along the longitudinal median and fixed flat between wet (PBS) Whatman no. 1 filter paper for 24 h in 10% buffered formalin prior to 30 s staining with 0.1% methylene blue (Sigma) dissolved in the PBS. Criteria used to identify aberrant crypt were increased size, elevated appearance from the surrounding mucosa and shape of the luminal opening. The number of ACF/colon and the number of aberrant crypts in each focus were determined by microscopic examination at $40\times$ magnification using a Nikon dissecting microscope with fiber optic light source to transilluminate the colon. Crypt multiplicity was defined as the number of aberrant crypts in each focus, categorized as either singlets, doublets, triplets or 4 or more (4+) aberrant crypts/focus. The scores were determined by an observer that was blinded to treatment groups during scoring of crypts.

Effect of dietary herbal supplements on apoptosis

For the second set of animal experiments, a total of 70 male F344 rats were randomly divided into 7 groups (10 rats per group). The first group was the negative control (no test agent, no carcinogen), the second group was the positive control (no test reagent, with carcinogen) and the remaining five experimental groups were treated with AOM plus herbal supplements. The concentration of herbal supplement that was most effective in inhibiting ACF formation in the first experiment was used. In addition, the colons harvested from these animals were used for both protein and histochemical analysis (5 rats per assay). For protein analysis, the colons were opened longitudinally and the mucosal layers were spread out over a Petri dish and scraped with a razor blade into a microcentrifuge tube containing 1 ml of RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 0.1 mg/ml PMSF), with protease inhibitors (aprotinin, leupeptin and pepstatin at $1\ \mu\text{g}/\text{ml}$ each) and phosphatase inhibitors ($0.1\ \mu\text{M}$ sodium orthovanadate and $1\ \mu\text{M}$ NaF); and the samples were rapidly frozen in liquid nitrogen and stored at -80°C until use. For histochemistry, colons were slit open along the longitudinal axis and rinsed in PBS. After rinsing, they were divided into three segments of 5–7 cm each, referred to as proximal colon (adjacent to the cecum), middle colon and distal colon (adjacent to the rectum). Each segment was fixed flat on wet (PBS) Whatman no. 1 filter paper for 24 h in 10% neutral buffered formalin and was routinely processed for embedding in paraffin. Three different segments (distal, middle and proximal) from 5 rats were randomly selected from each group for assessment of apoptosis by Feulgen's fast green analysis.

Detection of apoptosis

Histochemistry (Feulgen-fast green stain). Paraffin embedded tissues were cut into $5\ \mu\text{m}$ sections, deparaffinized and hydrated (Leica Autostainer), followed by acid hydrolysis in 5 N hydrochloric acid at room temperature for 1 h. After washing in distilled water, samples were treated with Schiff's reagent for 1 h at room temperature, rinsed in distilled water, treated for 10 min in fresh sulfonated water and dehydrated with ethanol by ascending (50–95%) ethanol series. The slides were then counterstained with fast green for 25 s, rinsed in 100% ethanol, treated with xylene and mounted. Apoptotic cells were identified in the longitudinal sections of the rat colonic mucosa under light microscopy by morphological features. These include: (i) nuclear and cytoplasmic condensation (micronuclei) and (ii) nuclear fragments (karyorrhexis). Apoptotic cells are counted under light microscopy by a scorer that was blinded to the treatment groups. The apoptotic index was determined by counting the number of apoptosis positive cells to total number of crypts evaluated for each

tissue section after counting at least 150 crypts per treatment group at 5 areas randomly selected in the distal, middle and proximal portion of the colon.

Western analysis. Mucosal scrapings from 35 rats (5 per treatment group) were thawed on ice, homogenized (10 s) and sonicated (5 s). The homogenate was centrifuged at $12\ 000\ g$ for 20 min at 4°C . Protein concentration in the supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). The supernatant ($50\ \mu\text{g}$ of protein) was separated by 15% SDS-PAGE, transferred and immobilized on a nitrocellulose membrane at 4°C . The membrane was blocked by incubation with 5% non-fat dry milk in PBS at room temperature for 3 h. The membrane was then washed three times (5 min/wash) in PBS containing 0.1% Tween-20 (PBS-T) and hybridized with rabbit antibody raised against rat Bax and Bcl-2 diluted 1:1000 or against rat caspase 9 (MBL International) diluted 1:500. Incubation with antibodies and detection of the antigen-antibody complex were performed using the ECL kit (Amersham) according to the manufacturer's instructions.

Statistical analysis

All data were analyzed using Sigmasat software (SPSS, Chicago, IL). All treatments were compared with the AOM-only group or indomethacin (50 p.p.m.) group using one-way ANOVA. If the variance was equal and a significant difference ($P < 0.05$) was observed, the Holm-Sidak method as a multiple comparison versus control group was used (6). For data with unequal variance, the Kruskal-Wallis one-way ANOVA on ranks was used. If a significant difference ($P < 0.05$) was observed, multiple comparisons versus the control group were performed using the Dunn's method. The χ^2 test was used to compare proportions among groups.

For quantitation of western analysis, the band intensity was measured using the Alpha Imager™ (Alpha Innotech, San Leandro, CA) with a constant measurement area around the protein bands. The digital numbers obtained were the integrated density value (IDV) of the intensity of the size of each band. The density of each band of interest (active caspase 9, Bax and Bcl-2) was normalized to the amount of β -actin band in the same gel lane. The values are expressed as mean \pm SD ($n = 3$ per treatment group). Data were analyzed using one-way ANOVA with Holm-Sidak comparison between means. Differences between means were considered significant when $P < 0.05$. For the Bax and Bcl-2 proteins, comparison of the mean \pm SD showed no statistical significance; hence, the changes in protein expression were expressed in percent relative to the basal levels of this protein in untreated sample.

Results

Effect of dietary herbal supplements on ACF incidence and multiplicity

Injecting rats twice with saline (negative control) or feeding rats a diet containing herbal supplements alone (subgroups 2–7 in the saline treated group) for a 5-week period did not affect the body weight of the animals, nor did it cause ACF formation in the colon (data not shown). Table I summarizes data for the carcinogen (AOM)-induced ACF in the colons of rats fed the control and the treated groups (subgroups 1–13). Except for subgroup 6 (rutin at 30 000 p.p.m.), administration of herbal supplements in the diet (subgroups 2–5 and 7–13), significantly suppressed the total number of ACF per colon compared with the positive control group (subgroup 1). The most effective concentrations for silymarin, quercetin, ginseng, curcumin, rutin and indomethacin were 5000, 30 000, 50, 8000, 15 000 and 50 p.p.m., respectively, and at these concentrations, the percent inhibition was 46, 75, 35, 52, 20 and 33%, respectively. In order to compare the effects of these dietary supplements with a pharmaceutical agent known to reduce ACF, we used indomethacin, that inhibits ACF by 42% (7). Although indomethacin (subgroup 7) significantly reduced the incidence of ACF at both of the concentrations tested (82% at 25 p.p.m. and 67% at 50 p.p.m.), quercetin (subgroups 3 and 9) and curcumin (subgroup 11) were more robust than indomethacin (subgroup 7) alone.

A summary of the number and multiplicity of ACF for the most effective concentrations of dietary herbal supplements are shown in Table II. It has been reported that large ACF

Table I. Chemopreventative effects of herbal supplements on AOM-induced ACF in rat colon

Treatment	Dose (p.p.m.)	n	Aberrant crypts/colon			
			Mean ± SEM	% of control	Compared with AOM group	Compared with indomethacin (50 p.p.m.)
Indomethacin	50	10	123 ± 9	67	$P < 0.001$	
	25	10	150 ± 14	82	$0.01 < P < 0.05$	NS
Quercetin	30 000	10	46 ± 8	25	$P < 0.001$	$P < 0.001$
	15 000	10	70 ± 7	38	$P < 0.001$	$P < 0.001$
Curcumin	16 000	10	148 ± 17	80	$0.01 < P < 0.05$	NS
	8000	10	89 ± 11	48	$P < 0.001$	$0.01 < P < 0.05$
Silymarin	10 000	10	126 ± 9	68	$P < 0.001$	NS
	5000	10	99 ± 9	54	$P < 0.001$	NS
Ginseng	50	10	119 ± 11	65	$P < 0.001$	NS
	25	10	128 ± 15	70	$P < 0.001$	NS
Rutin	30 000	10	150 ± 14	89	NS	$P < 0.001$
	15 000	10	148 ± 14	80	$0.01 < P < 0.05$	NS
AOM	0	10	184 ± 15	100		$P < 0.001$

NS, inhibition is not statistically significant, $P > 0.05$.

Table II. Incidence and multiplicity of ACF at the most effective concentrations of dietary herbal supplements

Treatment	Dose (p.p.m.)	Total no. of ACF	No. of foci containing							
			Singlets		Doublet		Triplets		4+	
			Number	%	Number	%	Number	%	Number	%
Ind	50	123 ± 9 ^a	46 ± 5 ^a	38	54 ± 3	44	17 ± 2	14	6 ± 1	5
Que	30 000	46 ± 8 ^a	20 ± 4 ^{a,c}	43	16 ± 3 ^{b,d}	36	6 ± 1 ^{b,d}	13	4 ± 1 ^{a,c}	8
Cur	8000	89 ± 11 ^a	37 ± 5 ^a	43	36 ± 5 ^b	40	12 ± 2 ^b	13	4 ± 1 ^{a,c}	5
Sil	5000	98 ± 10 ^a	51 ± 7 ^a	52	37 ± 3 ^b	37	10 ± 1 ^b	10	2 ± 0 ^{a,c}	2
Gin	50	120 ± 11 ^a	50 ± 5 ^a	42	49 ± 4	41	16 ± 2	14	4 ± 1 ^{a,c}	3
Rut	15 000	148 ± 14 ^a	62 ± 5 ^{a,c}	42	60 ± 6	41	19 ± 3	13	5 ± 1	4
AOM		184 ± 15	84 ± 7 ^c	45	72 ± 9	39	22 ± 2	12	6 ± 1	3

^aSignificantly different from subgroup 1 by Holm–Sidak method ($P < 0.05$).

^bSignificantly different from subgroup 1 by Dunn's Method ($P < 0.05$).

^cSignificantly different from subgroup 7 by Holm–Sidak method ($P < 0.05$).

^dSignificantly different from subgroup 7 by Dunn's Method ($P < 0.05$).

containing at least four crypts per focus (4+) are more likely to progress into tumors (8). Compared with the positive control group (subgroup 1), quercetin (subgroup 3), ginseng (subgroup 4), silymarin (subgroup 8) and curcumin (subgroup 11) caused a significant reduction in the number of ACF containing 4+ crypts per focus. However, only silymarin (subgroup 8) was effective at significantly reducing (98%; $P = 0.004$) the proportions of aberrant crypts that progress to 4+ crypts per focus. The majority of the ACF observed in this treatment group were singlets. The ability of silymarin to inhibit progression was significantly stronger than those of indomethacin (subgroup 7) and quercetin. The number of foci containing three crypts (triplets) was also significantly decreased by feeding quercetin (subgroup 3), silymarin (subgroup 8) and curcumin (subgroup 11) after administration of AOM (subgroup 1). Again, only silymarin was more potent than indomethacin (subgroup 7) at inhibiting the number of aberrant crypts that convert to triplets.

Effects of dietary herbal supplements on apoptosis

To assess the frequency and distribution of apoptotic cells in the colon, paraffin embedded longitudinal sections of rat colon (>150 crypts per treatment group) were analyzed with

Table III. Apoptotic indices of colonic crypt in normal and AOM-treated rats fed dietary herbal supplements

Treatment	Dose (p.p.m.)	Feulgen's fast green staining index (%)			
		Distal	Middle	Proximal	Average
AOM+76-A	0	24.2 ± 2.7	24.2 ± 3.8	27.1 ± 5.9	25.2 ± 2.2
76-A only	0	43.1 ± 4.5	33.2 ± 3.0	40.3 ± 2.2	38.9 ± 2.2 ^a
Indomethacin	50	28.7 ± 7.8	47.4 ± 1.0	53.1 ± 2.0	44.8 ± 4.0 ^a
Quercetin	30 000	83.3 ± 19.2	101.8 ± 2.6	95.3 ± 4.5	93.5 ± 6.4 ^b
Curcumin	8000	93.4 ± 0.8 ^c	39.2 ± 7.9	55.1 ± 8.1	61.8 ± 8.0 ^b
Silymarin	5000	41.8 ± 3.2	37.2 ± 1.8	33.8 ± 1.6	38.1 ± 1.7
Ginseng	50	50.5 ± 3.3	64.1 ± 2.3 ^c	51.1 ± 2.1	55.2 ± 2.6 ^b

Apoptotic cells were identified in the longitudinal sections of the rat colonic mucosa under light microscopy by morphological features noted in the Materials and methods section ($n = 5$ per treatment group). The apoptotic index was determined by counting the number of apoptosis positive cells to total number of crypts evaluated for each tissue section after counting at least 150 crypts per treatment group in five areas randomly selected in the distal, middle and proximal portions of the colon. Results are expressed as percentage of apoptotic cells per crypt.

^aSignificantly different from AOM+76-A by Holm–Sidak method ($P < 0.01$).

^bSignificantly different from AOM+76-A by Dunn's Method ($P < 0.05$).

^cSignificantly different from other regions by Holm–Sidak method ($P < 0.01$).

Feulgen nuclear stain and counterstained with fast green. Table III shows the frequency and distribution of apoptotic cells in colonic mucosa of rats kept on various dietary regimens. The number of apoptotic cells/crypt was markedly decreased ($P < 0.01$) in the AOM-treated groups compared with untreated animals. Similarly, apoptotic cells along the colonic crypt was significantly higher in all treated groups compared with that observed in rat fed the 76-A diet alone.

ACF are thought to be predominantly expressed in the middle and distal colon (9,10); therefore, we sought to investigate whether herbal supplement-induced apoptosis was specific to a certain region of the large intestine. Analysis of the distribution of apoptotic cells in the rat colon revealed that of the four dietary herbals tested (quercetin, ginseng, silymarin and curcumin), only curcumin and ginseng were region specific at inducing apoptosis. As shown in Table III, in rats fed a diet containing curcumin, the majority of apoptotic cells were observed in the distal colon ($P < 0.05$) as compared with the middle and proximal region; whereas, with ginseng, most of the apoptotic cells were observed mainly in the middle portion of the colon ($P < 0.05$) as compared with distal and proximal.

In order to investigate the mechanisms by which these herbal supplements evoke apoptosis, proteins isolated from colon mucosa of the animals fed various herbals supplements were subjected to western blot analysis of caspase 9, an initiator caspase known to play a major role in the mitochondria-induced apoptotic pathway. As shown in Figure 1A, a 35-kDa band representing active caspase 9 was detected in all (control and treatment) groups. However, densitometric analysis of

the bands revealed that the protein levels of active caspase 9 was significantly higher ($P < 0.001$) in rats fed quercetin (at 30 000 p.p.m.) and curcumin (at 8000 p.p.m.) compared with the unsupplemented diet (76-A alone). In order to determine whether these differences correlate with Bax (a 24-kDa proapoptotic protein) and Bcl-2 (a 27-kDa prosurvival protein) expression, 50 μ g of protein was separated on an SDS-PAGE, transferred to a PVDF membrane and probed with polyclonal antibodies that recognize anti-rat Bax and Bcl-2 proteins. Analysis of Bax and Bcl-2 band intensity showed no statistical significance in expression levels of either of these proteins by any of the groups (data not shown). However, the ratio of Bax: Bcl-2 protein levels were 7% higher in quercetin fed group and 13% higher in ginseng fed group compared with the animals fed 76-A diet alone.

Discussion

The concern over the risk of surgical procedures and toxicity of chemotherapeutic drugs has led to a focus on avenues for prevention of colon cancer. Chemoprevention seems to be a promising strategy because other therapies have not been effective in controlling either the high incidence or low survival rate of colon cancers. Recently, emphasis has been on a variety of clinical and basic studies of chemoprevention using naturally occurring substances that are found in normal diets, since they might provide useful strategies to inhibit colon cancer with minimal toxicity. Moreover, modifications in dietary habits appear to be particularly influential in the prevention

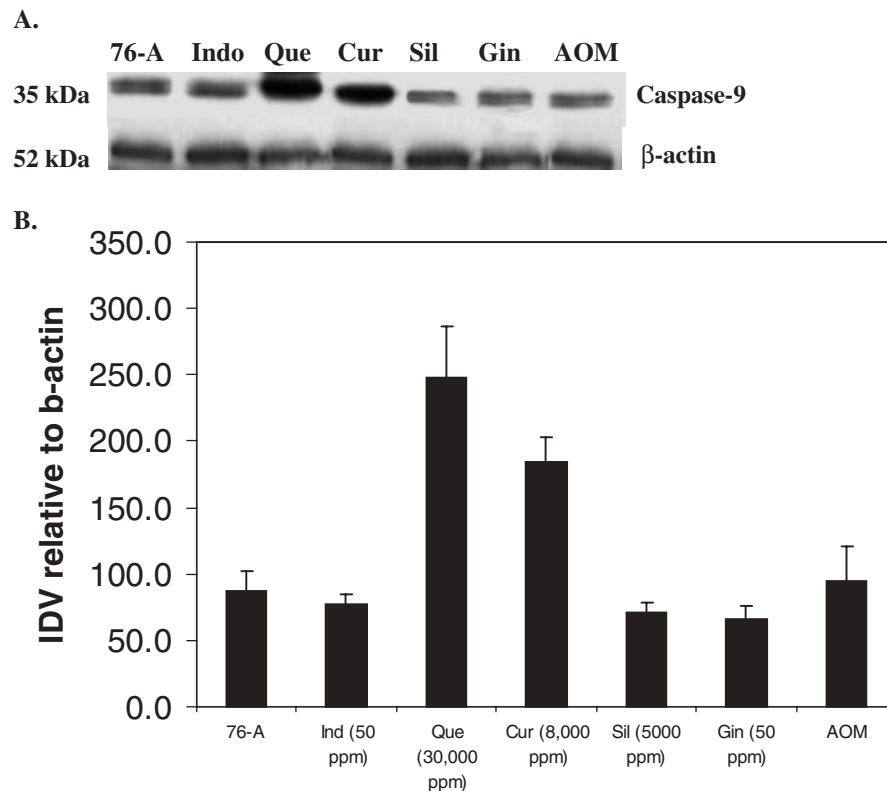


Fig. 1. (A) Western blot analysis of active caspase 9 proteins in rats injected with AOM and fed various dietary herbal supplements (indomethacin at 50 p.p.m., quercetin at 30 000 p.p.m., curcumin at 8000 p.p.m., silymarin at 5000 p.p.m., ginseng at 50 p.p.m. and AOM at 15 mg/kg body weight). All samples in gels are equally loaded at 50 μ g of total protein and β -actin as an internal control for equal protein loading. A 35-kDa active caspase 9 was detected in all samples. (B) Densitometric analysis was performed and the results expressed in terms of integrated density value (IDV) normalized to β -actin. Three representative samples from each group are shown and the values are expressed as mean \pm SD.

of colon cancer. Therefore, the present study was undertaken to evaluate the chemopreventive efficacy of five commonly used dietary supplements, four herbal supplements (quercetin, curcumin, rutin and silymarin) and one whole herb mixture (white ginseng powder). These herbal supplements were chosen for study because of widespread interest in their use as dietary supplements and as possible chemopreventive agents. The concentrations of the herbal supplements used in this study are 40 and 80% of the MTD for these compounds as specified by the National Cancer Institute's Chemoprevention Agent Development Program and fall well within the ranges consumed by humans. For instance, quercetin and rutin are sold as dietary supplements with a 'recommended' dose of 1200–1500 mg/day (400–500 mg, 3 times a day) and in our study we used 30 000 p.p.m. (30 g/kg diet). Considering that an average male F344 rat consumes ~20 g of chow daily, the rats in our treatment group would have consumed ~600 mg of quercetin and rutin per day. However, there may be differences on a per kg body weight basis, and differences due to genetics of metabolism. None of these compounds used in this study has ever been reported to be toxic, even at supra-physiological concentrations. Thus, the purpose of our study was to confirm the chemopreventive activity of these herbal supplements at doses previously used by our laboratory and by others (11–15). The data presented in this study are used to set the upper limits of efficacy for future human studies, but are not to be construed to replicate human intake levels, or faithfully recapitulate human metabolism. This is the limitation of all animal models of cancer chemoprevention, but the dose levels chosen for our study allow comparisons with previously peer-reviewed published research. Therefore, the results of our study suggest that administration of these herbal supplements prior to exposure of colon specific chemical carcinogen (AOM), can drastically reduce the incidence of ACF (putative preneoplastic lesion).

ACF were first reported in rodents injected with AOM by Bird in 1987 and similar lesions were characterized in humans in 1991 and 1994 by Pretlow; since then, the AOM-induced ACF model has been the most widely used animal model system for evaluating naturally occurring compounds (flavonoids, carotenoids, green tea, etc.) as well as synthetic chemicals [cyclooxygenase (COX) 2 inhibitors, nitric oxide synthase (NOS) inhibitors and peroxisome proliferators-activator receptor (PPAR) γ] for their colon cancer chemopreventive efficacy. The growth dynamics, morphological and molecular features of ACF support the contention that ACF are putative preneoplastic lesions. For instance, ACF have a hyperproliferative epithelium and their size increases with time (16–18). The nuclear atypia observed in some ACF are similar to those seen in the crypts of adenocarcinomas in colons (18). Furthermore, identification of dysplasia and monoclonality strongly links this lesion to neoplastic progression (19). Recently, two new types of lesion have been described in the AOM-induced ACF model. Mori *et al.* (20) have identified β -catenin accumulated crypts (BCAC) by using immunohistochemical methods and Caderni *et al.* (21) have identified mucin depleted foci (MDF) in unsectioned colon stained with high-iron diamine alcian blue (HID-AB). These newly described lesions are not yet well characterized and we do not know if BCAC and MDF are related lesions. It is interesting to note that BCAC, like MDF, have a low production of mucins and are thought to be premalignant lesions rather than preneoplastic lesions. Since, ACF are widely accepted as a reliable end point in

experimental colon carcinogenesis, this study reports the effects of herbal supplements on the 'classical' ACF.

All the herbal supplements tested reduced significantly the incidence of ACF in at least one of the two doses tested; three of the five (silymarin, quercetin and curcumin) were effective at both doses tested. In fact, these three compounds were more effective at reducing the incidence of ACF than indomethacin, known to reduce the formation of ACFs. Comparison of the most effective concentrations shows that quercetin was the most potent supplement tested and rutin was the least effective. These results are consistent with our previous report using the same model system, where we observed that quercetin was one of the most effective compounds screened (22) and rutin was not as effective as the other compounds (23). In addition, a recent study conducted by Yang *et al.* (24), also found that dietary quercetin reduced the number of focal areas of dysplasia (ACF) by 44%; whereas, dietary rutin was only 33% effective. It has been speculated that the rutinosyl sugar moiety on the C ring markedly influences the pharmacokinetic profile of rutin, i.e. the absorption of rutin by the colonic epithelial cells is delayed in comparison with quercetin because the sugar moiety must be hydrolyzed by the microflora in the large intestine prior to absorption (25).

Colon tumor incidence in rats correlates best with multicrypt ACF (≥ 4 crypts/focus), which are more likely to persist, increase in size through multiplication and develop into tumors. In the present study, although all the herbal supplements were effective at reducing the number of 4+ crypts, only silymarin was effective at reducing the percent of ACF that progress to four or more, suggesting that this compound interferes with the initiation and the progression stages of carcinogenesis. Indeed, Kohno *et al.* (14) reported that in both a short- and a long-term experiment, dietary feeding of silymarin during the initiation or post-initiation phase of AOM-induced colon carcinogenesis reduced the incidence and multiplicity of colonic adenocarcinoma (14).

Modulation of apoptosis provides a protective mechanism against intestinal neoplasia. Our data show that the herbal supplements we tested significantly reduced the incidence of ACF. This regression of precancerous lesions induced by treatment with herbal supplements provides indirect evidence that these polyphenols can arrest cell growth or stimulate apoptosis. Therefore, morphological assessment of apoptosis was performed. Longitudinal sections of rat colon were stained with Feulgen nuclear stain which revealed fragmentation in apoptotic nuclei. Quercetin, curcumin and ginseng were the most effective at inducing apoptosis and the degree of apoptosis induced by these compounds strongly correlated with the decrease in ACF. Interestingly, silymarin was able to suppress the incidence and multiplicity of ACF but had no effect on apoptosis indicating that this compound inhibits preneoplastic lesions (ACF) by other anticancer mechanisms. Consistent with this idea, a study conducted on HT-29 cells showed that silybinin, the pure active agent in silymarin, was only 15% effective at inducing apoptosis and was more efficient at inhibiting cell-cycle progression (26). Furthermore, Kohno *et al.* (14) demonstrated that silymarin elevated rat liver glutathione *S*-transferase (GST) and quinone reductase (QR) activity in a dose-dependent manner.

The structural and functional properties of the colon are not the same throughout its entire length. For example, the proliferating stem cells in the proximal rat colon are located in

the mid-crypt base and migrate up toward the luminal surface; whereas in the distal colon the stem cells are located in the crypt base and these cells migrate up toward the luminal surface (27). Also, in response to carcinogens, ACF are thought to be mainly expressed in the middle and distal colon. In this study, only curcumin and ginseng appeared to be region specific at inducing apoptosis; curcumin was more effective at inducing apoptosis at the distal end of the colon, whereas ginseng was more effective at the middle portion of the colon. The specificity of these compounds could possibly be explained by the autoxidation of these flavonoids. It has been shown that the rate of autoxidation of flavonoids decreases with increasing number of hydroxyl group at a neutral pH (ability to chelate oxygen radicals) (28). This mechanism needs to be examined further.

In order to elucidate the mechanism by which these herbal supplements induce apoptosis, proteins isolated from the colon scrapings were analyzed by western blotting. The results from this experiment revealed that quercetin was able to increase active caspase 9 and Bax expression along with a concomitant decrease in Bcl-2 proteins levels, strongly suggesting that this compound induces apoptosis via the mitochondrial pathway. Similarly, curcumin was able to increase the protein levels of active caspase 9 indicating the involvement of the mitochondrial pathway; however, the low Bax:Bcl-2 ratio indicates that other Bcl-2 family members might be responsible for the observed increase in activity caspase 9 protein levels. An *in vitro* study conducted by Ruby *et al.* (29) showed that curcumin can activate both mitochondrial and death receptor pathways by inducing BID (a member of the Bcl-2 family) cleavage and negatively regulating Bcl-XL.

In conclusion, the evidence presented in this study suggests that polyphenolic plant constituents may exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions and inducing apoptosis in the large bowel. The ability of quercetin to decrease the incidence of ACF by 75%, along with the ability of silymarin to reduce crypt multiplicity combined with curcumin and ginseng to induce apoptosis in the region of the colon where most of these preneoplastic lesions accumulate, suggests that combination of these herbal supplements rather than any one alone might be the ultimate chemopreventive agent. Furthermore, this study also showed that the two most effective herbal supplements in our study, quercetin and curcumin, reduce the number of preneoplastic by inducing apoptosis via the mitochondrial pathway.

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References

- Jemal,A., Tiwari,R.C., Murray,T., Ghafoor,A., Samuels,A., Ward,E., Feuer,E.J. and Thun,M.J. (2004) Cancer statistics, 2004. *CA Cancer J. Clin.*, **54**, 8–29.
- Kelloff,G.J., Crowell,J.A., Steele,V.E. *et al.* (2000) Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J. Nutr.*, **130**, 467S–471S.
- Mahmoud,N.N., Carothers,A.M., Grünberger,D., Bilinski,R.T., Churchill,M.R., Martucci,C., Newmark,H.L. and Bertagnolli,M.M. (2000) Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis*, **21**, 921–927.
- Mutoh,M., Takahashi,M., Fukuda,K., Komatsu,H., Enya,T., Matsushima-Hibiya,Y., Mutoh,H., Sugimura,T. and Wakabayashi,K. (2000) Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: structure-activity relationship. *Jpn. J. Cancer Res.*, **91**, 686–691.
- Wenzel,U., Kuntz,S., Brendel,M.D. and Daniel,H. (2000) Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res.*, **60**, 3823–3831.
- Ludbrook,J. (1998) Multiple comparison procedures updated. *Clin. Exp. Pharmacol. Physiol.*, **25**, 1032–1037.
- Brown,W.A., Skinner,S.A., Malcontenti-Wilson,C., Misajon,A., DeJong,T., Vogliagis,D. and O'Brien,P.E. (2000) Non-steroidal anti-inflammatory drugs with different cyclooxygenase inhibitory profiles that prevent aberrant crypt foci formation but vary in acute gastrotoxicity in a rat model. *J. Gastroenterol. Hepatol.*, **15**, 1386–1392.
- Pretlow,T.P., O'Riordan,M.A., Somich,G.A., Amini,S.B. and Pretlow,T.G. (1992) Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis*, **13**, 1509–1512.
- Furukawa,F., Nishikawa,A., Kitahori,Y., Tanakamaru,Z. and Hirose,M. (2002) Spontaneous development of aberrant crypt foci in F344 rats. *J. Exp. Clin. Cancer Res.*, **21**, 197–201.
- Rodrigues,M.A., Silva,L.A., Salvadori,D.M., De Camargo,J.L. and Montenegro,M.R. (2002) Aberrant crypt foci and colon cancer: comparison between a short- and medium-term bioassay for colon carcinogenesis using dimethylhydrazine in Wistar rats. *Braz. J. Med. Biol. Res.*, **35**, 351–355.
- Fukushima,S., Wanibuchi,H. and Li,W. (2001) Inhibition by ginseng of colon carcinogenesis in rats. *J. Korean Med. Sci.*, **16** (Suppl), S75–S80.
- Li,W., Wanibuchi,H., Salim,E.I., Wei,M., Yamamoto,S., Nishino,H. and Fukushima,S. (2000) Inhibition by ginseng of 1,2-dimethylhydrazine induction of aberrant crypt foci in the rat colon. *Nutr. Cancer*, **36**, 66–73.
- Kawamori,T., Lubet,R., Steele,V.E., Kelloff,G.J., Kaskey,R.B., Rao,C.V. and Reddy,B.S. (1999) Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res.*, **59**, 597–601.
- Kohno,H., Tanaka,T., Kawabata,K., Hirose,Y., Sugie,S., Tsuda,H. and Mori,H. (2002) Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int. J. Cancer*, **101**, 461–468.
- Tao,L., Kramer,P.M., Wang,W., Yang,S., Lubet,R.A., Steele,V.E. and Pereira,M.A. (2002) Altered expression of c-myc, p16 and p27 in rat colon tumors and its reversal by short-term treatment with chemopreventive agents. *Carcinogenesis*, **23**, 1447–1454.
- Roncucci,L., Pedroni,M., Fante,R., Di Gregorio,C. and Ponz de Leon,M. (1993) Cell kinetic evaluation of human colonic aberrant crypts. (Colorectal Cancer Study Group of the University of Modena and the Health Care District 16, Modena, Italy). *Cancer Res.*, **53**, 3726–3729.
- Dashwood,R.H., Xu,M., Orner,G.A. and Horio,D.T. (2001) Colonic cell proliferation, apoptosis and aberrant crypt foci development in rats given 2-amino-3-methylimidaz. *Eur. J. Cancer Prev.*, **10**, 139–145.
- McLellan,E.A., Medline,A. and Bird,R.P. (1991) Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis*, **12**, 2093–2098.
- Siu,I.M., Robinson,D.R., Schwartz,S., Kung,H.J., Pretlow,T.G., Petersen,R.B. and Pretlow,T.P. (1999) The identification of monoclonality in human aberrant crypt foci. *Cancer Res.*, **59**, 63–66.
- Mori,H., Yamada,Y., Hirose,Y., Kuno,T., Katayama,M., Sakata,K., Yoshida,K., Sugie,S., Hara,A. and Yoshimi,N. (2002) Chemoprevention of large bowel carcinogenesis; the role of control of cell proliferation and significance of beta-catenin-accumulated crypts as a new biomarker. *Eur. J. Cancer Prev.*, **11**(Suppl 2), S71–S75.
- Caderni,G., Femia,A.P., Giannini,A., Favuzza,A., Luceri,C., Salvadori,M. and Dolara,P. (2003) Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. *Cancer Res.*, **63**, 2388–2392.
- Wargovich,M.J., Harris,C., Chen,C.D., Palmer,C., Steele,V.E. and Kelloff,G.J. (1992) Growth kinetics and chemoprevention of aberrant crypts in the rat colon. *J. Cell Biochem. Suppl.*, **16**(G), 51–54.
- Wargovich,M.J., Chen,C.D., Jimenez,A., Steele,V.E., Velasco,M., Stephens,L.C., Price,R., Gray,K. and Kelloff,G.J. (1996) Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. *Cancer Epidemiol. Biomarkers Prev.*, **5**, 355–360.

24. Yang, K., Lamprecht, S.A., Liu, Y., Shinozaki, H., Fan, K., Leung, D., Newmark, H., Steele, V.E., Kelloff, G.J. and Lipkin, M. (2000) Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. *Carcinogenesis*, **21**, 1655–1660.
25. Hollman, P.C., de Vries, J.H., van Leeuwen, S.D., Mengelers, M.J. and Katan, M.B. (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.*, **62**, 1276–1282.
26. Agarwal, C., Singh, R.P., Dhanalakshmi, S., Tyagi, A.K., Tecklenburg, M., Sclafani, R.A. and Agarwal, R. (2003) Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene*, **22**, 8271–8282.
27. Sato, M. and Ahnen, D.J. (1992) Regional variability of colonocyte growth and differentiation in the rat. *Anat. Rec.*, **233**, 409–414.
28. Miura, Y.H., Tomita, I., Watanabe, T., Hirayama, T. and Fukui, S. (1998) Active oxygens generation by flavonoids. *Biol. Pharm. Bull.*, **21**, 93–96.
29. Ruby, A.J., Kuttan, G., Babu, K.D., Rajasekhavan, K.N., Kuttan, R. (1995) Anti-tumor and antioxidant activity of natural curcuminoids. *Cancer Lett.*, **94**, 79–83.

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