

## Effect of dietary $\beta$ -sitosterol on fecal bacterial and colonic biotransformation enzymes in 1,2-dimethylhydrazine-induced colon carcinogenesis

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**Aim:** The present study was designed to investigate the modulatory effect of  $\beta$ -sitosterol administration on 1,2-dimethylhydrazine (DMH)-induced fecal bacterial and colonic biotransformation enzyme activities.

**Materials and methods:** The chemopreventive potential of  $\beta$ -sitosterol in colon carcinogenesis was assessed by injecting DMH (20 mg/kg body weight) subcutaneously into male Wistar rats and supplementing with  $\beta$ -sitosterol throughout the experimental period of 16 weeks at 3 different doses (5, 10, and 20 mg/kg body weight).

**Results:** Amplified activities of fecal bacterial ( $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, nitroreductase, hyaluronate lyase, and sulfatase) and colonic biotransformation ( $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, and nitroreductase) enzymes were considered hallmarks of colon carcinogenesis. DMH-induced animals showed increased activities of fecal bacterial and colonic biotransformation enzymes. Treatment with  $\beta$ -sitosterol markedly decreased the fecal bacterial and colonic biotransformation enzymes and reverted the colonic tissue to near normal.

**Conclusion:** The results of the present study revealed that  $\beta$ -sitosterol markedly inhibited DMH-induced colon carcinogenesis by its ability to ameliorate the fecal bacterial and colonic biotransformation enzymes. Hence,  $\beta$ -sitosterol can be a potential chemopreventive agent towards colon carcinogenesis.

**Key words:**  $\beta$ -Sitosterol,  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, nitroreductase, hyaluronate lyase, sulfatase

### Introduction

Lung, breast, and colon cancer are the 3 most common cancers worldwide, with an increasing annual incidence (1). Considerable interest has been focused on the metabolic activities of the intestinal microflora, especially in relation to the etiology of colon cancer. The intestinal bacteria play a crucial role in the development of carcinogenesis (2–5). Experimentally induced colon tumors involve activation of the procarcinogen to an active carcinogen mediated by bacterial enzymes. The carcinogens are excreted into the bile as glucuronides and are activated by glucuronidase in the intestinal

track by enzymatic hydrolysis. Inhibition of this glucuronidase reduced the incidence of colon cancer in rats (6–9).

1,2-Dimethylhydrazine (DMH)-induced colon carcinogenesis in male Wistar rats is a widely used experimental model among cancer chemoprevention studies. During the process of experimental colon carcinogenesis, dysplastic crypts appear in the early stage and eventually develop into tumors (10). The goal of cancer chemoprevention is to retard, block, or reverse the process of carcinogenesis through the use of natural or synthetic agents, including antioxidants. *Asclepias curassavica* powder is used

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to treat abdominal tumors in the traditional system of Indian medicine.  $\beta$ -Sitosterol and its glycosides, along with other compounds such as oleanolic acid, uzarigenin, calactin, calotropin, coroglaucigenin, calotropagenin, and uzarin have been isolated from *A. curassavica* (11).

$\beta$ -Sitosterol, isolated from various plants, promotes apoptosis by increasing Fas levels and caspase-8 activity (12), phosphorylation of extracellular-signal regulating kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) (13). Even at low concentrations with no cytotoxic effect on noncancerous cells (14),  $\beta$ -sitosterol works to inhibit cancer cell proliferation by modulating antioxidant enzyme levels in pathogenesis (15), arresting prostate cancer cells at the G2/M phase (16), and decreasing free radical generation in vitro (17).  $\beta$ -Sitosterol shows great cancer chemopreventive potential in DMH-induced animals (18). A large number of medicinal plants and their purified constituents have beneficial therapeutic potentials. In this context, our work aimed to broaden the understanding of the anticancer potential of  $\beta$ -sitosterol in a DMH-induced experimental colon carcinogenesis model, which could be beneficial in anticancer therapy.

## Materials and methods

### Chemicals

In this study, extraction, separation, and purification of  $\beta$ -sitosterol were done as reported by us previously (18) from *A. curassavica*. DMH, p-nitrophenyl- $\beta$ -D-glucopyranoside, p-nitrophenyl- $\beta$ -D-glucoside, p-nitrophenyl- $\beta$ -D-galactopyranoside, p-nitrocatechol sulfate, and p-nitrobenzoic acid were purchased from Sigma Chemical Company, Bangalore, India. All other chemicals, including solvents, were of high purity and analytical grade, marketed by HiMedia Chemicals, Mumbai, India.

### Animals

Experiments were carried out with male Wistar rats aged 5 weeks, obtained from the Central Animal House, King Institute, Chennai, Tamil Nadu, India. The animals were cared for in compliance with the principles and guidelines of the Ethical Committee for Animal Care and the Institutional Animal Ethical Committee, in accordance with the Indian National

Law on Animal Care and Use (Reg. No. 833/a/2004/CPC SEA). The animals were housed 4 per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk in a specific pathogen-free animal room under controlled conditions of 12 h light/12 h dark, with temperatures of  $24 \pm 2$  °C and relative humidity of  $50 \pm 10\%$  until the end of the experimental period. The rats were held in quarantine for 1 week and had access to food and tap water ad libitum. A commercial pellet diet containing 4.2% fat (Hindustan Lever Ltd, Mumbai, India) was powdered and mixed with 15.8% peanut oil, making a total of 20% fat in the diet. This modified powdered pellet diet was fed to the rats in all groups throughout the experimental period of 16 weeks.

### Experimental design

The experimental animals were divided into 6 groups with 10 animals in each group. The initial body weights of all animals in this study protocol were measured to be between 80 and 120 g. The animals' weights were recorded once a week throughout the experimental period and prior to sacrifice. The animals in groups 3–6 received subcutaneous injections of DMH at a dose of 20 mg/kg body weight once a week for the first 4 consecutive weeks. Prior to subcutaneous injection, DMH was dissolved in 1 mL of EDTA. The pH was adjusted to 6.5 with 1 mL of NaOH to ensure the pH and stability of the chemical, and it was used immediately after preparation.

### Treatment of $\beta$ -sitosterol for the animals

- Group 1: Rats received a modified pellet diet along with intragastric intubation of 0.1% carboxymethyl cellulose (CMC; 1.0 mL) throughout the experimental period.
- Group 2: Rats received a modified pellet diet + 20 mg/kg body weight  $\beta$ -sitosterol suspended in 0.1% CMC (1.0 mL), peroral (p.o.), every day throughout the experimental period.
- Group 3: Rats were administered with 20 mg/kg body weight DMH subcutaneously once a week for 4 consecutive weeks and kept without any treatment for the next 12 weeks.
- Group 4: Rats were treated as in group 3 along with  $\beta$ -sitosterol (5 mg/kg body weight, p.o.)

supplemented daily throughout the entire experimental period of 16 weeks.

Group 5: Rats were treated as in group 3 along with  $\beta$ -sitosterol (10 mg/kg body weight, p.o.) supplemented daily throughout the entire experimental period of 16 weeks.

Group 6: Rats were treated as in group 3 along with  $\beta$ -sitosterol (20 mg/kg body weight, p.o.) supplemented daily throughout the entire experimental period of 16 weeks.

At the end of 16 weeks, all of the animals were sacrificed under anesthesia (intraperitoneal administration of ketamine hydrochloride (30 mg/kg body weight) by cervical dislocation between 0800 and 1000 hours after an overnight fast. Tissue samples were immediately transferred to ice-cold containers, weighed, and homogenized using an appropriate buffer in a tissue homogenizer. At the end of the experimental period, fresh fecal pellets were collected and the activity of hyaluronate lyase was estimated using the method of Shiau and Chang (19). The normal mucosa was compared with the abnormal mucosa. The rest of the tissues and the colon contents of 10 rats of each group were transferred to ice-cold containers to measure the activity of  $\beta$ -glucuronidase,  $\beta$ -glucosidase, and  $\beta$ -galactosidase using the method of Freeman (20). The activity of nitroreductase was

estimated using the method of Bratton and Marshall (21). Sulfatase was measured using the method of Rowland et al. (22). Proteins were estimated using the method of Lowry et al. (23).

### Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance, and significant differences among treatment groups were evaluated by Duncan's multiple range test. The results were considered statistically significant at  $P < 0.05$ . All statistical analyses were made using SPSS 11.0 (SPSS, Tokyo, Japan).

## Results

### Effect of $\beta$ -sitosterol on fecal (bacterial) enzymes

Table 1 summarizes the activities of  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, and nitroreductase in fresh fecal samples. The activities of these enzymes were increased 2–3 times in rats treated with DMH alone (group 3) as compared to the rats in the control group (group 1). The activities were reduced in all  $\beta$ -sitosterol-supplemented rats (groups 4–6) as compared to rats treated with DMH alone (group 3). Furthermore, there was a significantly ( $P < 0.01$ ) greater reduction in  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase,

Table 1. Effect of  $\beta$ -sitosterol supplementation on fecal bacterial  $\beta$ -glucuronidase,  $\beta$ -glucosidase, and  $\beta$ -galactosidase enzyme activities in control and experimental rats.

Groups	$\beta$ -Glucuronidase	$\beta$ -Glucosidase	$\beta$ -Galactosidase
	mg of <i>p</i> -nitrophenol liberated/min/g protein		
Control	19 $\pm$ 1 <sup>a</sup>	48 $\pm$ 3 <sup>a</sup>	28 $\pm$ 2 <sup>a</sup>
Control + $\beta$ -sitosterol (20 mg/kg)	20 $\pm$ 1 <sup>ab</sup>	50 $\pm$ 3 <sup>a</sup>	29 $\pm$ 2 <sup>a</sup>
DMH	27 $\pm$ 2 <sup>c</sup>	108 $\pm$ 8 <sup>b</sup>	71 $\pm$ 5 <sup>b</sup>
DMH + $\beta$ -sitosterol (5 mg/kg)	25 $\pm$ 1 <sup>cd</sup>	103 $\pm$ 7 <sup>bc</sup>	68 $\pm$ 5 <sup>bc</sup>
DMH + $\beta$ -sitosterol (10 mg/kg)	24 $\pm$ 1 <sup>d</sup>	100 $\pm$ 7 <sup>c</sup>	65 $\pm$ 5 <sup>c</sup>
DMH + $\beta$ -sitosterol (20 mg/kg)	21 $\pm$ 1 <sup>b*</sup>	70 $\pm$ 5 <sup>d*</sup>	44 $\pm$ 3 <sup>d*</sup>

Data are presented as means  $\pm$  SD of 10 rats in each group. <sup>a-d</sup>: values not sharing a common superscript letter are significantly different at  $P < 0.05$ . \*: values are significantly different at  $P < 0.01$  as compared to the group treated with DMH alone.

hyaluronate lyase, and nitroreductase (21%, 45%, 37%, 41%, and 26%, respectively) activities in animals supplemented with high dose of  $\beta$ -sitosterol for the entire period (group 6) as compared to the other  $\beta$ -sitosterol-supplemented groups.

Table 2 shows the fecal nitroreductase, hyaluronate lyase, and sulfatase activity following  $\beta$ -sitosterol supplementation in the different treatment regimens. In carcinogen-treated rats (group 3), mean activities of the enzymes were elevated 2-fold compared to rats in the control group (group 1). In addition, there was a significant ( $P < 0.05$ ) reduction in the fecal enzyme activity in animals supplemented with medium

dose of  $\beta$ -sitosterol; the animals supplemented with  $\beta$ -sitosterol at a high dose (group 6) showed significantly ( $P < 0.01$ ) greater reduction in the enzyme activity.

#### Effect of $\beta$ -sitosterol on colonic (mucosal) biotransforming enzyme activities

Table 3 represents the activities of colonic mucosal enzymes. After the 16 weeks of the experimental period, the colonic mucosal enzymes, such as  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, and nitroreductase, had activity that was 2–3 times

Table 2. Effect of  $\beta$ -sitosterol supplementation on fecal bacterial hyaluronate lyase, sulfatase, and nitroreductase activities in control and experimental rats.

Groups	Hyaluronate lyase †	Sulfatase#	Nitroreductase®
Control	2.7 ± 0.2 <sup>a</sup>	26 ± 2 <sup>a</sup>	21 ± 1 <sup>a</sup>
Control + $\beta$ -sitosterol (20 mg/kg)	2.8 ± 0.2 <sup>a</sup>	26 ± 2 <sup>a</sup>	21 ± 1 <sup>a</sup>
DMH	5.5 ± 0.5 <sup>b</sup>	61 ± 4 <sup>b</sup>	36 ± 2 <sup>b</sup>
DMH + $\beta$ -sitosterol (5 mg/kg)	5.2 ± 0.3 <sup>bc</sup>	58 ± 4 <sup>bc</sup>	34 ± 2 <sup>bc</sup>
DMH + $\beta$ -sitosterol (10 mg/kg)	5.1 ± 0.3 <sup>c</sup>	51 ± 3 <sup>c</sup>	33 ± 2 <sup>c</sup>
DMH + $\beta$ -sitosterol (20 mg/kg)	3.3 ± 0.2 <sup>d†</sup>	34 ± 2 <sup>d†</sup>	27 ± 2 <sup>d†</sup>

†: mg of glucose liberated/min/mg protein, #:  $\mu$ mol of *p*-nitrocatechol liberated/min/g protein, ®:  $\mu$ mol of *p*-aminobenzoic liberated/min/g protein.

Data are presented as means ± SD of 10 rats in each group. <sup>a-d</sup>: values not sharing a common superscript letter are significantly different at  $P < 0.05$ . <sup>†</sup>: values are significantly different at  $P < 0.01$  as compared to the group treated with DMH alone.

Table 3. Effect of  $\beta$ -sitosterol supplementation on colonic biotransformation enzymes of control and experimental rats.

Groups	b-Glucuronidase	b-Glucosidase	b-Galactosidase	Nitroreductase
	$\mu$ g of <i>p</i> -nitrophenol liberated/h/g protein			$\mu$ mol of <i>p</i> -aminobenzoic liberated/min/g protein
Control	8 ± 0.6 <sup>a</sup>	25 ± 1 <sup>a</sup>	22 ± 1 <sup>a</sup>	12 ± 0.9 <sup>a</sup>
Control + $\beta$ -sitosterol (20 mg/kg)	9 ± 0.6 <sup>a</sup>	26 ± 2 <sup>a</sup>	23 ± 1 <sup>a</sup>	13 ± 1 <sup>a</sup>
DMH	16 ± 1.2 <sup>b</sup>	67 ± 5 <sup>b</sup>	44 ± 3 <sup>b</sup>	24 ± 1 <sup>b</sup>
DMH + $\beta$ -sitosterol (5 mg/kg)	15 ± 1.1 <sup>bc</sup>	65 ± 4 <sup>bc</sup>	41 ± 3 <sup>bc</sup>	23 ± 1 <sup>bc</sup>
DMH + $\beta$ -sitosterol (10 mg/kg)	11 ± 0.8 <sup>c</sup>	62 ± 4 <sup>c</sup>	42 ± 3 <sup>c</sup>	22 ± 1 <sup>c</sup>
DMH + $\beta$ -sitosterol (20 mg/kg)	12 ± 0.9 <sup>d†</sup>	40 ± 3 <sup>d†</sup>	28 ± 2 <sup>d†</sup>	15 ± 1 <sup>d†</sup>

Data are presented as means ± SD of 10 rats in each group. <sup>a-d</sup>: values not sharing a common superscript letter are significantly different at  $P < 0.05$ . <sup>†</sup>: values are significantly different  $P < 0.01$  as compared to group treated with DMH alone.

higher ( $P < 0.05$ ) in rats treated with DMH alone (group 3) than in the control rats (group 1), whereas the activities of these enzymes were significantly ( $P < 0.05$ ) reduced in DMH-administered rats supplemented with  $\beta$ -sitosterol (groups 5 and 6) when compared to the unsupplemented DMH-treated rats (group 3). Significantly ( $P < 0.01$ ) greater reduction in the percentage of  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\beta$ -galactosidase, and nitroreductase activities (35%, 42%, 36%, and 39%, respectively) was observed in rats supplemented with a high dose of  $\beta$ -sitosterol during the entire period (group 6) of the study.

## Discussion

In rats exposed to DMH alone, the body weight gain and growth rate were reduced compared to the control throughout the experimental period. The reduction in body weight gain and growth rate observed in rats treated with DMH alone may be an integral part of the carcinogenic process. Cachexia (catabolic clinical state) may be caused by a combination of endocrine and immunological disturbances resulting from host-tumor interactions. The body weight gain and growth rate were significantly improved in rats with  $\beta$ -sitosterol supplementation. Although the mechanisms of body weight gain during  $\beta$ -sitosterol supplementation are unknown, the relationship appears to be complex, since body weight gain and growth rate are thought to be regulated by a variety of interactions involving food intake, carcinogenicity, and the consequent tumor burden.

A number of studies have suggested a key role played by certain resident gut bacteria in the development of large bowel cancer. Epidemiological studies and laboratory research have indicated a strong association between the metabolic activity of the intestinal microflora and cancer of the large bowel (24). The activation of procarcinogens could be mediated enzymatically by intestinal bacteria, and the activities of colonic bacterial enzymes are increased by dietary fat (25,26). On DMH administration we observed increased colonic and fecal activities of carcinogen-retoxyfying enzymes such as  $\beta$ -glucuronidase,  $\beta$ -glucosidase, and  $\beta$ -galactosidase. Increased expression of intestinal mucosal  $\beta$ -glucuronidase,  $\beta$ -glucosidase, and  $\beta$ -galactosidase in relation to a high risk of colon

cancer is well documented (24). In addition, it has been reported that *E. coli*, a  $\beta$ -glucuronidase-positive bacteria, increases the production of active carcinogenic metabolites in the colon and is thus responsible for colon carcinogenesis (27).

Epithelial mucins are a family of secretory cell surface glycoproteins expressed by epithelial tissues. Many human mucin genes appear to encode mucin to protect and lubricate epithelial tissues by forming a viscoelastic gel layer (28). Hyaluronate lyase, a hydrolytic enzyme secreted by the intestinal microflora, degrades the protective mucous layer of the colon (24). A change in hyaluronate lyase activity is accompanied by a change in the rate of mucin degradation, leading to a shift in the balance between mucin secretion and degradation (29). Enhanced degradation of the mucosal lining of colonic epithelial cells (mucin) ensures a greater contact of the toxic carcinogen with the colonocytes. This may be accompanied by an increased susceptibility of the colonic cells to being transformed (20).

Measurements of colonic bacterial and fecal biotransforming enzyme activities in rats exposed to DMH were found to be several times higher when compared to the control rats. The influence of diet on tumor development and its relationship to DMH-retoxyfying enzymes have been used extensively to test the influence of diet on colon carcinogenesis (30). The activities of these bacterial enzymes were significantly decreased following  $\beta$ -sitosterol supplementation, especially when 20 mg/kg body weight DMH was supplemented throughout the study period (group 6). Reduction of bacterial enzyme activity in the large bowel is paralleled by a decrease in the frequency of colonic aberrant crypt foci (27).

We previously reported the cancer chemopreventive potential of  $\beta$ -sitosterol in DMH-induced experimental colon carcinogenesis by virtue of its radical quenching ability with minimal toxic effects to the normal cells (VERO: monkey kidney cell line). The antioxidant potential of  $\beta$ -sitosterol may be responsible for the chemopreventive potential of DMH-induced experimental carcinogenesis (18). In our present study,  $\beta$ -sitosterol supplementation to DMH-induced animals decreased the levels of the fecal bacterial and colonic biotransformation enzymes in a dose-dependent manner. The antibacterial activity of

$\beta$ -sitosterol may be responsible for the reduction in the enzyme levels by controlling the gut microflora to near-normal activity.

The present study demonstrates that  $\beta$ -sitosterol offers protection to the colon from DMH-induced experimental colon carcinogenesis by restoring the activities of fecal bacterial and colonic biotransformation enzymes in a dose-dependent manner. Hence,  $\beta$ -sitosterol can be a potential chemopreventive agent against colon carcinogenesis.

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