ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2013; 4(5): 937 - 950

ORIGINAL RESEARCH ARTICLE

Inhibitory Effect of Gallic Acid on 1,2-Dimethyl Hydrazine Induced Biochemical Alterations, Colonic Bacterial Enzymes and Xenobiotic Metabolizing Enzymes in Rats

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Received 11 Jun 2013; Revised 01 Oct 2013; Accepted 10 Oct 2013

ABSTRACT

Colorectal cancer is the third most common malignant neoplasm in the world. We determined the effect of gallic acid (GA) on xenobiotic metabolizing enzymes, colonic bacterial enzymes, lipids and glycoconjugates in 1,2-dimethylhydrazine (DMH) induced colon carcinogenesis. Group 1 served as control, group 2 rats were given GA, 50mg/kg body weight by intragastric intubation everyday; groups 3-6 were injected DMH (s.c. injections, 20mg/kg body weight) once a week for the first 15 weeks. Group 4 rats received GA as in group 2 starting 1 week before DMH injections and continued till the final exposure [DMH + GA (initiation-I)]. Group 5 rats received GA as in group 2 after the cessation of DMH injections and continued till the end [DMH + GA (post-initiation - PI)] of the experimental period. The rats in group 6 were supplemented with GA as in group 2 from the day of carcinogen treatment and continued till the end of the entire experimental period of 32 weeks [DMH+GA (Entire period-EP)]. The activities of the colonic phase I enzymes and bacterial enzymes were significantly elevated in DMH treated rats, whereas, phase II enzymes activities were significantly reduced. Altered levels of glycoconjugates were observed in liver and colon tissues. Supplementation with GA significantly reduced the activities of phase I enzymes and increased the activities of phase II enzymes in the liver and colonic mucosa; lowered the activities/ levels of biotransforming enzymes, elevated tissue lipids, cholesterol–phospholipid ratio, glycoconjugates and also the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). Our findings suggest that GA could be a possible chemopreventive agent against colon cancer.

Key words: Chemoprevention; Colon cancer; Gallic acid; 1,2-Dimethyl hydrazine.

1. INTRODUCTION

Cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality. Colon cancer is frequently a pathological consequence of persistent oxidative stress, leading to DNA damage and mutations in cancer-related genes, a cycle of cell death and regeneration (Bartsch and Nair, 2002) where cellular overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated. Colon cancer is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum (Fearon and Vogelstein, 1990). Relentless cell proliferation and rapid invasion into surrounding tissues are fundamental characteristics of malignant tumour (Herlyn and Malkowicz, 1991). The progressive introduction of Western dietary habits, especially, increasing the fat intake and decreasing carbohydrate intake, has been paralled by an increase in colon cancer incidence (Tajima *et al*., 1985).

The induction of colorectal cancer with chemical carcinogens in rats is currently a valid experimental model, in its macroscopic, microscopic and biological behavior (Weisburger, 2000). DMH (1,2-dimethyl hydrazine) is a procarcinogen that requires metabolic activation to form DNA-reactive products. The procarcinogen, is an alkylating agent, when injected subcutaneously, it slowly reaches the liver and gets metabolized. Metabolism of these

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compounds involve multiple xenobiotic metabolic enzymes which proceed through several N-oxidations and hydroxylations. The first step in the metabolism of DMH is oxidation, where DMH is converted into azomethane (AOM) by oxidation reaction. Formation of azoxymethane (AOM) from azomethane is catalysed by the second oxidative reaction which is then hydroxylated to methyl azoxy methanol (MAM). At body temperature MAM is further converted into water, nitrogen and methyldiazonium ions by spontaneous decomposition (Fiala *et al*., 1976) which can alkylate macromolecules in the liver and colon especially guanine nucleotides.

DMH or its metabolites reach the intestine through bile, directly into the intestinal lumen and also via circulation as glucuronides, glucosides and to some extent as sulphatides. They are cleaved by β-glucuoronidases, β-glucosidases and sulphatases which are present both in the enterocytes as well as in the intestinal microflora to liberate free methyl carbonium ion, which in turn alkylates the enterocyte ncleotides and proteins. Alkylation of the oxygen atoms of the purine and pyrimidine bases of DNA has been suggested to be critical in mutagenesis and carcinogenesis, because of the possibility of mispairing at these sites during DNA replication (Hawks and Magee, 1974).

Bacterial β-glucosidase hydrolyses plant glycosides to release aglycones, many of which are mutagenic, although some also have anticarcinogenic activity (Rowland *et al*., 1995). Nitroreductase participates in the conversion of aromatic compounds such as dinitrotoluene, nitrobenzenes and nitropyrenes to amines which often exhibit toxic, mutagenic or carcinogenic activities (Facchini and Griffiths, 1981). The excessive activities of these enzymes may be a primary factor in the etiology of colon cancer.

The cytochrome p450 superfamily (officially abbreviated as CYP) is a large and diverse group of enzymes responsible for various phase I metabolic reactions. The function of most CYP enzymes is to catalyze the oxidation of organic substances. The substrates for CYP enzymes include metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotics such as drugs and other toxic chemicals. CYP2E1, an enzyme involved in the metabolic activation of procarcinogens into reactive intermediates capable of forming adducts and damaging DNA is believed to play an essential role in chemical carcinogenesis (Poulsen *et al*., 1993).

In subsequent phase II reactions, these activated xenobiotic metabolites are conjugated with charged species such as reduced glutathione (GSH), sulphate, glycine, or glucuronic acid. These reactions are catalysed by a large group of broad-specificity transferases, which in combination can metabolise almost any hydrophobic compound that contains nucleophilic or electrophilic groups. UDP-glucuronyltransferase (UDPGT) plays an important role in hormonal homeostasis, energy metabolism, bilirubin clearance and xenobiotic detoxification. Though the molecular regulation of UDPGT is not fully understood, various transcription factors play a regulatory role. Reduction of quinines and nitrogen oxides might also make them available for conjugation with UDP-glucuronic acid, facilitating their excretion. UDPGT is ultimately associated with the structure of the membrane to which it is tightly bound.

Glycoconjugates are ubiquitous in mammalian cells and are major components of cellular membranes. Glycopeptides that are present in the plasma membrane of erythrocytes andfibroblasts have diverse immunological properties, participate in cell to cell contact, growth regulation and act as a binding sites for hormones (Gold *et al*., 1968). The carbohydrate content of glycoproteins and glycolipids are known to be altered by DMH (Freeman *et al*., 1978) due to the increased activity of glycosyltransferases. Furthermore, after transformation, the cell membrane glycoproteins are modified both qualitatively and quantitatively (Kim *et al*., 1974).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) (**Fig 1**), a naturally occurring plant phenol, is found in various other natural products like gallnuts, tea leaves, oak bark, apple peels, grapes, strawberries, pine apples, bananas and lemons. GA possesses cytotoxic to cancer cells, and has anti-inflammatory and antimutagenic properties (Gali *et al*., 1991). GA was described as an excellent free radical scavenger and an inducer of differentiation and programmed cell death in a number of tumour cell lines (Jagan *et al*., 2008).

The aim of the study was to investigate the effects of orally administered GA on the bacterial enzymes, xenobiotic metabolizing enzymes and also on the levels of lipids and protein bound

sugars in rats treated with the colon specific carcinogen DMH.

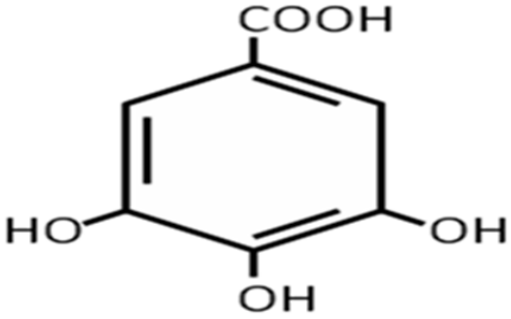

Figure 1: Structure of Gallic acid (3,4,5- trihydroxy benzoic acid)

MATERIAL AND METHODS

Chemicals:

Gallic acid, DMH, bovine serum albumin, p-nitrobenzoic acid, p-nitrophenol β-D-galactopyranoside, p-nitrophenyl β-D-glucoside and p-nitrocatechol sulphate were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals and solvents used were of analytical grade.

Tumor induction:

DMH was dissolved in 1 mM EDTA just prior to use and the pH adjusted to 6.5 with 1 mM NaOH to ensure the stability of the carcinogen. The rats were given subcutaneous injections of DMH for 4 consecutive weeks at the dose of 20 mg/kg body weight.

Preparation of gallic acid:

Gallic acid (GA) was solubilized in water just before treatment and was administered everyday orally at the dose of 50mg/kg body weight [13] for 32 weeks.

Preparation of modified diet:

Peanut oil was used as a promoter of colon carcinogenesis.

Animals:

Four-week old male Wistar rats, weighing approximately 150 g, were obtained from the Central Animal House, Raja Muthiah Medical College and Hospital (RMMCH). They were kept in a plastic cage with bedding and maintained under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%), 12-h light/dark cycle and provided with modified pellet diet and tap water ad libitum. The animals used and the experimental design had the prior ethical approval by the Animal Care and Use Committee of Annamalai University (Reg. No.160/1999/CPCSEA/842).

Experimental design:

Group 1 served as control, group 2 rats were given GA, 50mg/kg body weight by intragastric intubation everyday; groups 3-6 were injected DMH (s.c. injections, 20mg/kg body weight) once a week for the first 15 weeks. Group 4 rats received GA as in group 2 starting 1 week before DMH injections and continued till the final exposure [DMH + GA (initiation-I)]. Group 5 rats received GA as in group 2 after the cessation of DMH injections and continued till the end of the experimental period [DMH + GA (post-initiation - PI)]. The rats in group 6 were supplemented with GA as in group 2 from the day of carcinogen treatment and continued till the end of the entire experimental period of 32 weeks [DMH+GA (Entire period-EP)]. For more clarity experimental protocol is shown in (**Fig 2**).

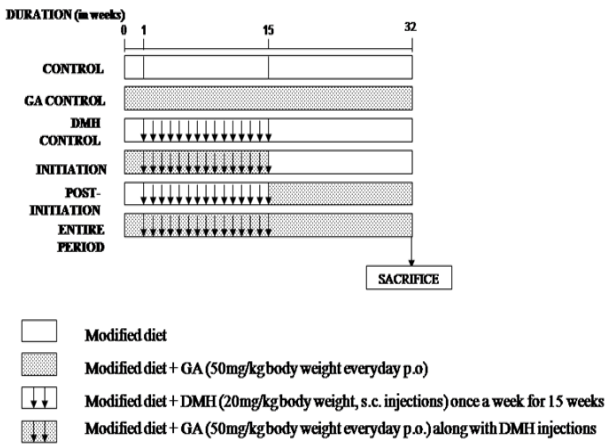

Figure 2: Experimental design

Preparation of tissue homogenates:

Rats were anesthetized by ketamine hydrochloride (20 mg/kg body weight) and sacrificed by decapitation. The liver and the colon were quickly excised, rinsed with saline, blotted dry on a filter paper, weighed and then 10% (w/v) homogenates of different tissues were prepared in appropriate buffer using a tissue homogenizer.

Assay of bacterial enzymes:

The colon was flushed gently with saline, cut open longitudinally and placed on a flat surface. Mucosal samples were collected by gently scraping the colonic mucosal layer using microscopic slides. The collected samples were transferred into preweighed tubes containing 0.1 M phosphate-buffered saline (pH 7.0) and sonicated for 30 s. Faecal and mucosal samples were centrifuged at 2000g for 5 min, and aliquots of supernatant were used immediately.

β-glucosidase activity (EC 3.2.1.21) was assayed by the method of (Freeman, 1986). The mixture of samples and substrate (p-nitrophenyl-β-D-

glucoside) were incubated with 37˚C for 60 min. After incubation, 0.2 M Na_2CO_3, was added to arrest the reaction. The released p-nitrophenol was measured at 400 nm. All reactions were linear with respect to the concentration and incubation time 60 min. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve.

β-galactosidase activity (EC 3.2.1.23) was assayed by the method of (Freeman, 1986). The mixture contained 3 mM p-nitrophenyl-β-D-galactopyranoside, a known volume of sample and incubated at 37˚C for 15 min. Then the reaction was terminated by the addition of 0.25 M Na_2CO_3. Release of p-nitrophenol was measured spectrophotometrically at 405 nm. The amount of p-nitrophenol liberated was determined by comparison with a standard nitrophenol curve.

Nitroreductase activity (E.C.1.5.1.34) was measured by the method of (Bratton and Marshall, 1999). The assay mixture contained 1.5 mM p-nitro benzoic acid, 0.1 M of phosphate buffer and known amount of sample. The reaction was arrested by the addition of 20% TCA and centrifuged. The amount of p-aminobenzoic acid released was measured at 550 nm. The amount of transformed substrate was determined by comparison with a standard curve.

Sulphatase activity (EC 3.1.6) was assayed by the method of (Rowland, 1983). The reaction mixture contained 0.02 M phosphate buffered saline, 1 mM EDTA, 1 mM p-nitrocatechol sulphate and a known amount of enzyme suspension. The reaction was arrested with 0.01 M NaOH, and the amount of p-nitrocatechol liberated was read spectrophotometrically at 492 nm. All the reactions were linear with respect to concentration and incubation time to 60 min.

Preparation of cytosolic and microsomal fractions:

Cytosolic and microsomal fractions were prepared from individual tissues (liver and colonic mucosa), homogenized in 0.25 M sucrose, centrifuged at 9000 x g for 20 min and the supernatant was collected, to this 0.2 vol. of 0.1 M $CaCl_2$ in 0.25 M sucrose were added and the samples were kept on ice for 30 min, centrifuged at 27,000 x g for 20 min, and the clear cytosolic fraction obtained were promptly assayed for phase II enzymes.

Microsomal pellets were washed twice by suspending in 7 ml of 10 mM Tris- HCl (pH 7.4), 0.25 M sucrose and centrifuged at 9000 x g for 20 min, to obtain microsomal fractions, which were promptly assayed for phase I enzymes.

Assay of microsomal enzymes:

NADPH-cytochrome b5 reductase (EC 1.6.2.4) was assayed by the method of (Omura and Takesue, 1970). by measuring the rate of oxidation of NADPH at 340 nm. The reaction mixture contained 0.3 M potassium phosphate buffer (pH 7.5), 0.1 mM NADPH, 0.2 mM potassium ferricyanide and the microsomal preparation in a final volume of 1 ml. The reaction was started at 25˚ C with the addition of NADPH. The enzyme activity was calculated using the extinction coefficient of 6.33 $mM^{-1}cm^{-1}$. One unit of enzyme activity is defined as that causing the oxidation of one mole of NADPH per minute.

Cytochrome P4502E1 (CYP4502E1) activity was assayed by the method of (Watt, 1997). The assay mixture contained 100 µg microsomal protein, 40 mM p-nitrophenol and 0.1 M phosphate buffer. The reaction was initiated by the addition of 10 mM of NADPH and incubated at 37˚C for 60 min. The reaction was stopped with 20% TCA and centrifuged at 1000 rpm for 5 min, 10 mM NaOH was added to the supernatant and the absorbance was measured at 450 nm.

NADH-cytochrome P450 reductase (EC 1.6.2.2) activity was assayed by the method of (Mihara and Sato, 1972). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 0.1 mM NADH, 1 mM potassium ferricyanide and microsomal preparation in a final volume of 1 ml. The reaction was started by the addition of NADH, and the rate of reduction of potassium ferricyanide by NADH was measured at 420 nm. The enzyme activity was calculated using the extinction coefficient of 1.02 $mM^{-1}cm^{-1}$. One unit of enzyme activity is defined as that causing the reduction of one mole of ferric cyanide per minute.

Assay of cytosolic enzymes:

UDP-Glucronyl transferase (UDP-GT, EC. 2.4.1.17) was assayed the method of (Jemal *et al*., 2005).

Biochemical investigations

HMG CoA reductase activity was assayed by the method of (Rao and Ramakrishnan, 1975). Fresh tissue homogenate was mixed with dilute perchloric acid and centrifuged. To the filtrate freshly prepared hydroxylamine reagent (acid hydroxylamine in the case of mevalonate) was

added and shaken well. The absorbance was measured at 540 nm against a saline-aresenite blank.

The tissue lipids were extracted by the method of (Folch, 1957). The rat colon was cut opened longitudinally and scrapped gently to collect the mucosa with an ice-cold microscopic slide. Tissue samples and mucosal scrapings were homogenized using choloroform:methanol mixture (2:1) and the contents were evaporated. Organic layer was used for the various estimations.

Total cholesterol was estimated by the method of (Zletkis, 1953). The samples were treated with ferric chloride-acetic acid reagent, mixed well, and conc.H_2SO_4 was added. After 20 min the absorbance was measured at 560 nm.

Phospholipids were estimated by the method of Zilversmit and Davis, 1950). 4 and few drops of conc. HNO_3.The samples were evaporated to dryness using a Kjeldhal flask. 5N H_2SO_4 were added for digestion, continued till it became colourless, then added 2.5% ammonium molybdate solution. Amino napthol sulphonic acid was added to form a stable blue colour. The absorbance was measured at 660 nm within 10 min.

Free fatty acids were estimated by the method of (Falholt , 1973). Extraction solution was added to the sample, shaken well and kept aside for 15 min. Centrifuged and the supernatant was vortexed with copper reagent. Again centrifuged, the upper phase was transferred to a tube containing 0.4% 1,5-diphenylcarbazide solution and mixed well. After 15 min the absorbance was measured at 550 nm.

Defatted tissue sample was hydrolysed by using 6N HCl and neutralized with 5N NaOH using phenolphthalein as indicator and used for the estimation of glycoconjugates.

Hexoses were estimated by the method of (Niebes, 1972). To the defatted sample 95% ethanol was added, centrifuged and 0.1N NaOH was added to dissolve the precipitate. Orcinol sulphuric acid reagent was added to all tubes and heated. The colour developed was measured at 540 nm. Galactose and mannose were used as standards.

Protein bound hexosamine was estimated by the method of (Wagner, 1972). To the defatted sample HCl was added and kept for 6 h in a boiling water bath. 6N NaOH was used to neutralize the sample. Acetylacetone reagent was added and heated. After cooling Ehrlich's reagent was added and the colour developed was measured at 540 nm.

Sialic acid in tissues was estimated by the method of (Aminoff, 1961). Defatted tissue was dissolved in 0.1N H_2SO_4 and then hydrolysed in a water bath at 100^0C for 1 h. This hydrolysate was treated with 25 mM periodate reagent, and the excess periodate was reduced with 2% sodium arsenite. 0.1 M thiobarbituric acid reagent was added and shaken with butanol containing 5% of 12N HCl. The colour developed in the butanol layer was measured at 549 nm.

Fucose was estimated by the method of (Dische and Shettles, 1948). The sample was mixed with 95% ethanol and centrifuged. 0.1N NaOH was used to dissolve the precipitate. Then H_2SO_4: water mixture was added and heated in a boiling water bath. Subsequently cysteine reagent was added and the colour developed was measured at 396 nm.

Total proteins were estimated by the method of (Lowry, 1951).

Statistical analysis

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

3. RESULTS

The results obtained from various experiments conducted in the study are depicted in tables. The data from various treatment groups have been compared with the normal control animals.

Effect of GA on gut microbial enzymes

(**Table 1**) represents the mucosal and faecal activities of the hydrolytic enzymes β-glucosidase and β-galactosidase. These enzymes were significantly elevated in the rats treated with DMH alone (group 3) at the end of 32 weeks as compared to the control and the rats treated with GA alone (groups 1 and 2). Supplementation with GA to DMH-treated rats significantly ($P < 0.05$) decreased their activities as compared to the rats treated with DMH alone. The remarkable suppression of the activities of these hydrolytic enzymes was found to be greater in rats supplemented with GA throughout the study

period (group 6) as compared to the other GA supplemented groups (groups 4 and 5).

The activities of the colonic mucosal and faecal nitroreductase and sulphatases of control, DMH (group 3) and GA supplemented rats (groups 4–6) at different treatment regimens (initiation, post initiation and entire period) are shown in (**Table 2**). At the end of 32 weeks, in the rats treated with DMH alone (group 3), the activities of these enzymes were significantly increased ($P < 0.05$) as compared to the normal control and GA control rats (groups 1 and 2). Supplementation with GA during the post initiation and the entire period treatment regimens remarkably decreased their activities, which were statistically different ($P < 0.05$) from those of the unsupplemented DMH-treated rats.

Effect of GA and DMH on xenobiotic metabolizing enzymes of phase I enzymes

(**Table 3**) illustrate the effect of GA treatment on the activities of phase I enzymes. DMH treatment enhanced the activities of the liver and colonic mucosal enzymes such as cytochrome P4502E1, NADPH-cytochrome b5 reductase and NADH-cytochrome P450 reductase as compared to the control rats. Supplementation with GA during the entire experimental period significantly reduced the activities of the liver and colonic cytochrome P4502E1, NADPH cytochrome b5 reductase and NADH cytochrome P450 reductase as compared to the DMH alone treated rats.

Effect of GA and DMH on xenobiotic metabolizing of phase II enzymes

Data on the effect of GA and DMH on phase II biotransformation enzyme UDP-glucuronyl transferase (UDP-GT) in the liver and colonic mucosa are presented in (**Table 4**). DMH caused a significant reduction in UDP-GT activities in group 3 (DMH) rats as compared to the control. By contrast, treatment with GA to DMH treated rats produced significantly increase in UDP-GT activities in group 4 (initiation), group 5 (post-initiation) and group 6 (entire period) rats. The effect of GA on the activities of UDP-GT was more pronounced in the group 6 (entire period) rats.

Effect of DMH and GA on HMG CoA reductase activity and tissue lipids

(**Table 5**) shows the HMG CoA reductase activity of control and experimental rats. The activity of HMG CoA reductase in DMH- treated rats (group 3) was significantly ($p < 0.05$) increased as compared to the control rats (group 1), whereas supplementation with GA to DMH-treated (group 4,5 and 6) animals showed significant reduction ($p < 0.05$) in the HMG CoA reductase activity as compared to the DMH alone treated (group 3) rats. These results reveal that GA inhibits the activity of HMG CoA reductase.

(**Table 6**) summarizes the levels of cholesterol in the tissues from different groups of animals. In DMH-treated rats (group 3), the cholesterol levels were significantly ($p < 0.05$) elevated, as compared to the control (group 1). Supplementation with GA to DMH-treated rats (group 4,5 and 6) decreased the tissue cholesterol levels as compared to the DMH alone treated rats (group 3). The cholesterol levels of control rats (group 1) did not differ significantly from control + GA rats (group 2). Thus supplementation with the reduced the cholesterol levels in DMH-treated rats to near normal levels.

The levels of free fatty acids in animals from different groups are shown in (**Table 7**). The tissue free fatty acid content were significantly ($p < 0.05$) increased in DMH (group 3) administered animals as compared to the control animals (group 1). On supplementation with GA to DMH administered (group 4, 5 and 6) animals the free fatty acid content was significantly ($p < 0.05$) decreased as compared to DMH alone treated (group 3) rats. Thus supplementation with GA reduced the free fatty acid levels in DMH-treated rats to near normal levels. Free fatty acid content did not differ significantly between the control (group 1) and control + GA rats (group 2).

(**Table 8**) represents the tissue phospholipid levels of control and experimental animals. Tissue phospholipid content was depleted in rats given DMH (group 3) as compared to the control rats (group 1) whereas in rats supplemented with GA along with DMH (group 4, 5and 6), the levels were significantly ($p < 0.05$) elevated as compared to the DMH alone treated rats (group 3). Thus GA supplementation with the increased the phospholipid levels in DMH-treated rats to near normal levels.

(**Table 9**) shows the cholesterol/phospholipid ratio of control and experimental groups. In DMH alone treated rats (group 3), the cholesterol/phospholipid ratio was increased significantly ($p < 0.05$) as compared to the control rats (group 1). Supplementation with GA to DMH-treated rats (group 4,5 and 6) significantly ($p < 0.05$) decreased the tissue

cholesterol/phospholipid ratio. Thus supplementation with GA reduced the cholesterol/phospholipid ratio in DMH-treated rats to near those of the control.

Effect of DMH and GA on total hexoses and fucose content

The concentration of total hexoses and fucose were significantly ($p < 0.05$) elevated in the liver and colon of DMH-alone treated rats (group 3) as compared to the control. In all the DMH + GA supplemented rats (groups 4–6) the levels were significantly lowered in the liver and colon as compared to the DMH-alone treated groups. This effect was more pronounced in the entire-period GA supplemented rats (group 6) as compared to the DMH-alone exposed rats (**Fig 3 & 4**).

Effect of DMH and GA on hexosamine and sialic acid content

The levels of hexosamine and sialic acid were significantly ($p < 0.05$) higher in the liver and lower in the intestine and colon of DMH-alone (group 3) exposed rats compared to the control (group 1). On supplementation with GA to DMH treated rats during three different time periods, the levels were significantly ($p< 0.05$) reversed in the post-initiation regimen (group 5) and the entire period treatment regimen (group 6) as compared to the DMH-alone (group 3) treated rats (**Fig 5 & 6**).

Table 1: Effect of GA and DMH on bacterial enzymes

Groups	β - Glucosidase[a]		β - Galactosidase[a]	
	Colonic mucosa	Feacal contents	Colonic mucosa	Feacal contents
Control	21.67±2.08[a]	48.75±4.69[a]	22.40±2.2[a]	24.91±2.39[a]
Control +GA (50mg/kg body weight)	23.59±2.27[a]	51.29±4.93[a]	25.24±2.4[a]	25.73±2.47[a]
DMH	64.34±6.19[b]	106.2±10.21[b]	45.33±4.36[b]	67.78±6.52[b]
DMH+GA (50mg/kg body weight) (I)	62.20±5.98[bc]	101.22±9.74[b]	42.1±4.1[b]	62.33±6.00[bc]
DMH+GA (50mg/kg body weight)(PI)	57.14±5.50[c]	97.02±9.33[c]	36.89±3.5[c]	58.16±5.59[c]
DMH+GA (50mg/kg body weight)(EP)	36.87±3.54[d]	52.23±5.02[a]	29.80±2.8[d]	40.83±3.93[d]

Data are presented as the means ± SD of eight rats in each group. [a-d] $P<0.05$, Values not sharing a common superscript letter are significantly different.

[a] mgs of p-nitrophenol liberated/min/g protein

Table 2: Effect of GA and DMH on bacterial enzymes

Groups	Nitroreductase[a]		Sulfatase[b]	
	Colonic mucosa	Feacal contents	Colonic mucosa	Feacal contents
Control	10.22±0.98[a]	12.95±1.24[a]	33.04±3.18[a]	38.87±3.74[a]
Control +GA (50mg/kg body weight)	11.63±1.12[ab]	14.57±1.40[a]	35.38±3.40[a]	39.62±3.81[a]
DMH	18.37±1.76[c]	23.49±2.26[b]	63.35±6.09[b]	71.66±6.89[b]
DMH+GA (50mg/kg body weight) (I)	18.03±1.73[c]	22.07±2.12[b]	61.02±5.87[b]	69.70±6.71[b]
DMH+GA (50mg/kg body weight)(PI)	14.47±1.39[d]	93.08±8.96[c]	53.06±5.10[c]	59.25±5.70[c]
DMH+GA (50mg/kg body weight)(EP)	12.44±1.19[b]	15.28±1.47[a]	42.92±4.13[d]	44.91±4.32[a]

Data are presented as the means ± SD of eight rats in each group. [a-d] $P<0.05$, Values not sharing a common superscript letter are significantly different.

[a]µmoles of p-aminobenzoic acid formed/min/g/protein. [b]µmoles of p-nitrocatechol liberated/min/g/protein

Table 3: Effect of GA and DMH on phase I enzymes

Group	Control	Control+ GA	DMH	DMH + GA (I)	DMH + GA(PI)	DMH + GA(EP)
Cytochrome P4502E1[a]						
Liver	6.12 ± 0.58[a]	6.32 ± 0.6[a]	10.7 ± 1.0[b]	8.97 ± 0.8[c]	7.85 ± 0.7[d]	6.63 ± 0.6[a]
Colonic mucosa	0.86 ± 0.08[ab]	0.76 ± 0.07[a]	2.65 ± 0.2[c]	1.63 ± 0.1[d]	0.99 ± 0.09[b]	0.83 ± 0.08[ab]
Cytochrome b5[b]						
Liver	3.51 ± 0.33[a]	3.11 ± 0.29[a]	5.14 ± 0.49[b]	4.33 ± 0.41[c]	3.91± 0.37[d]	3.31 ± 0.31[a]
Colonic mucosa	0.88 ± 0.08[a]	0.93 ±0.09[a]	3.68 ± 0.35[b]	2.15 ± 0.20[c]	0.97 ± 0.09[a]	0.78 ± 0.07[a]
NADPH- cytochrome p450 reductase[c]						
Liver	60.38±5.81[a]	62.01 ± 5.96[a]	74.66 ± 7.18[b]	71.09 ± 6.84[c]	68.95 ± 6.63[d]	63.03 ± 6.91[a]
Colonic mucosa	13.77 ± 1.32[a]	18.36 ± 1.76[b]	29.17 ± 2.80[c]	19.89 ± 1.91[d]	14.28 ± 1.37[a]	13.15 ± 1.26[a]
NADH –cytochrome b5 reductase[d]						
Liver	21.11 ± 2.03[a]	20.91 ± 2.01[a]	28.66 ± 2.71[b]	22.34 ± 2.10[c]	19.27 ± 1.85[a]	19.58 ± 1.85[a]
Colonic mucosa	7.65 ± 0.73[a]	7.85 ± 0.75[a]	19.07 ± 1.83[b]	11.73 ± 1.12[c]	10.40 ± 1.00[d]	8.26 ± 0.76[a]

Values are expressed as means ±S.D. of each group. Values not sharing a common letters (a–d) differ significantly at $P < 0.05$ (analysis of variance followed by DMRT).* $P < 0.001$ DMH+ GA (EP) versus DMH-treated rats. a. £-mmoles of p-nitrocatechol liberated/min/mg

protein. b. µmoles/mg protein c. One unit of enzyme activity is defined as that causing the oxidation of one mole of NADPH per minute. d. One unit of enzyme activity is defined as that causing the reduction of one mole of ferricyanide per minute.

Table 4: Effect of GA and DMH on phase II enzymes

Group	Control	Control+ GA	DMH	DMH + GA (I)	DMH + GA(PI)	DMH + GA(EP)
UDP-glucuronyl transferase¤						
Liver Colonic mucosa	7.82 ± 0.71 [a] 5.68 ± 0.52 [a]	7.84 ± 0.73 [a] 5.72 ± 0.33 [a]	5.75 ± 0.56 [b] 3.67 ± 0.31 [b]	6.26 ± 0.65 [c] 4.21 ± 0.41 [c]	6.73 ± 0.64 [c] 4.83 ± 0.46[d]	7.34 ± 0.73[a] 5.26 ± 0.52[a]

Data are presented as the means ± SD of eight rats in each group. [a-d] $P<0.05$, Values not sharing a common superscript letter are significantly different.

¤ - µmoles of DCPIP reduced/min/mg protein

Table 5: Effect of GA on tissue HMG CoA reductase activity of control and experimental animals

Groups	Liver	Proximal colon	Distal colon
Control	4.2±0.41 [a]	2.8±0.27[a]	3.0±0.29[a]
Control +GA (50mg/kg body weight)	3.9±0.38 [a]	3.5±0.34 [a]	3.2±0.31[a]
DMH	2.0±0.19[b]	10.2±1.0[b]	2.3±0.22[b]
DMH+GA (50mg/kg body weight) (I)	1.83±0.17[b]	9.38±0.90[b]	1.63±0.57[b]
DMH+GA (50mg/kg body weight)(PI)	3.57±0.34[c]	2.14±0.20[c]	1.53±0.14[c]
DMH+GA (50mg/kg body weight)(EP)	4.5±0.44 [bc]	2.5±0.24 [b]	2.9±0.28 [b]

Values are means ±S.D. of eight rats from each group. Means in the same column not sharing a common letters (a–c) are significantly different ($p < 0.05$) between groups. [a]Values are expressed as a ratio of HMG CoA to mevalonate. Lower ratio indicates higher enzyme activity and vice versa.*$p < 0.01$, values are signficantly different as compared to DMH alone treated group.

Table 6: Effect of GA on tissue cholesterol of control and experimental animals

Groups	Liver	Proximal colon	Distal colon
Control	255.2±24.5 [a]	489.6±47.1[a]	91.6±56.9[a]
Control +GA (50mg/kg body weight)	266.2±25.6 [a]	462.0±44.4 [a]	30.4±51.0[a]
DMH	385.5±37.1[b]	553.8±53.3 [b]	22.2±59.8[b]
DMH+GA (50mg/kg body weight) (I)	3.46±0.33[b]	5.10±0.49[b]	6.13±0.59[b]
DMH+GA (50mg/kg body weight)(PI)	2.14±0.20[c]	4.28±0.41[c]	4.99±0.48[c]
DMH+GA (50mg/kg body weight)(EP)	232.7±22.4 [bc]	447.0±43.1 [b]	510.0±49.0 [b]

Values are means ±S.D. of eight rats from each group. Means in the same column not sharing a common letters (a–c) are significantly different ($p < 0.05$) between groups. *$p < 0.01$, values are signficantly different as compared to DMH alone treated group.

Table 7: Effect of GA on tissue free fatty acid of control and experimental animals

Groups	Liver	Proximal colon	Distal colon
Control	479.4±46.1 [a]	744.6±71.6[a]	61.0±54.0[a]
Control +GA (50mg/kg body weight)	472.2±45.4 [a]	758.8±73.0 [a]	81.4±55.9[a]
DMH	565.0±54.3[b]	838.4±80.7 [b]	33.4±60.9[b]
DMH+GA (50mg/kg body weight) (I)	5.40±0.52[b]	8.18±0.78[b]	6.24±0.60[b]
DMH+GA (50mg/kg body weight)(PI)	4.08±0.39[c]	7.24±0.69[c]	5.40±0.52[c]
DMH+GA (50mg/kg body weight)(EP)	442.6±42.6 [bc]	734.4±70.6 [b]	502.8±48.4 [b]

Values are means ±S.D. of eight rats from each group. Means in the same column not sharing a common letters (a-c) are significantly different ($p < 0.05$) between groups. *$p < 0.01$, values are signficantly different as compared to DMH alone treated group.

Table 8: Effect of GA on phospholipids of control and experimental animals

Groups	Liver	Proximal colon	Distal colon
Control	683.4±65.7 [a]	765.0±73.6[a]	15.1±49.5[a]
Control +GA (50mg/kg body weight)	665.0±64.0 [a]	735.4±70.7 [a]	01.8±48.3[a]
DMH	408.0±39.3[b]	409.0±39.3[b]	92.7±28.1[b]
DMH+GA (50mg/kg body weight) (I)	3.97±0.38[b]	7.52±0.72[b]	2.79±0.26[b]
DMH+GA (50mg/kg body weight)(PI)	6.17±0.59[c]	7.29±0.70[c]	4.28±0.41[c]
DMH+GA (50mg/kg body weight)(EP)	637.5±61.3 [bc]	720.1±69.3 [b]	461.0±44.3 [b]

Values are means ±S.D. of eight rats from each group. Means in the same column not sharing a common letters (a and b) are significantly different ($p < 0.05$) between groups.*$p < 0.01$, values are signficantly different as compared to DMH alone treated group.

Table 9: Effect of GA on cholesterol/phospholipids ratio of control and experimental animals

Groups	Liver	Proximal colon	Distal colon
Control	0.30±0.29 [a]	0.86±0.08[a]	0.49±0.04[a]
Control +GA (50mg/kg body weight)	0.32±0.03[a]	0.81±0.07 [a]	0.59±0.05[a]
DMH	2.85±0.27[b]	2.14±0.20[b]	2.38±0.23[b]
DMH+GA (50mg/kg body weight) (I)	1.53±0.14[c]	1.22±0.11[c]	2.04±0.19[c]
DMH+GA (50mg/kg body weight)(PI)	0.34±0.03[c]	0.85±0.08[c]	0.56±0.05[c]
DMH+GA (50mg/kg body weight)(EP)	0.39±0.03 [bc]	0.88±0.08 [b]	0.54±0.05 [b]

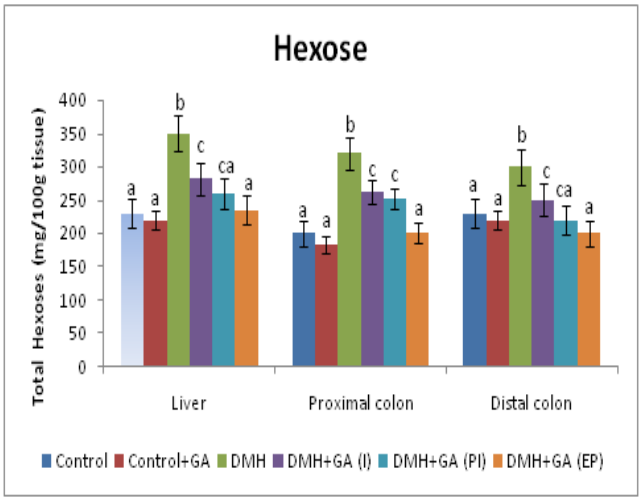

Figure 3: Effect of GA on tissue total hexoses of control and experimental animals

The column heights are the means ±S.D. of the values of 8 rats. Standard deviation is shown as bars. Groups not sharing a common letter (a–c) differ signficantly at $p < 0.05$.*$p < 0.01$, values are significantly different as compared to DMH alone treated group.

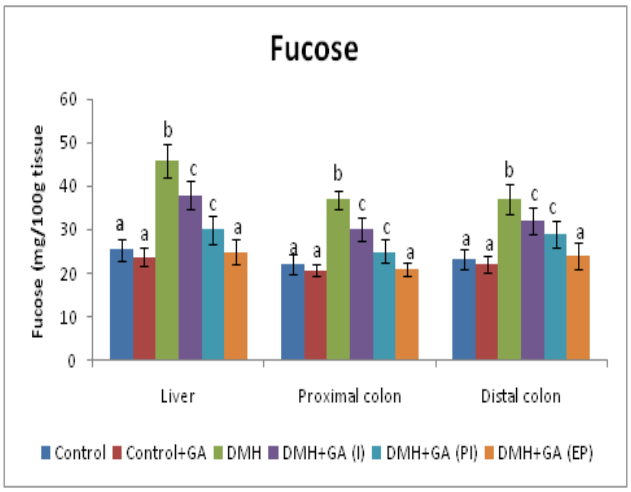

Figure 4: Effect of GA on tissue fucose of control and experimental animals

The column heights are the means ±S.D. of the values of 8 rats. Standard deviation is shown as bars. Groups not sharing a common letter (a–c) differ signficantly at $p < 0.05$.*$p < 0.01$, values are significantly different as compared to DMH alone treated group.

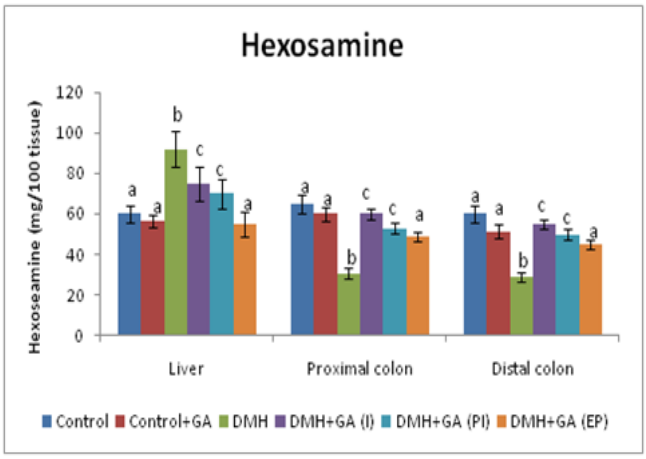

Figure 5: Effect of GA on tissue hexosamine of control and experimental animals

The column heights are the means ±S.D. of the values of 8 rats. Standard deviation is shown as bars. Groups not sharing a common letter (a–c) differ signficantly at $p < 0.05$.*$p < 0.01$, values are significantly different as compared to DMH alone treated group.

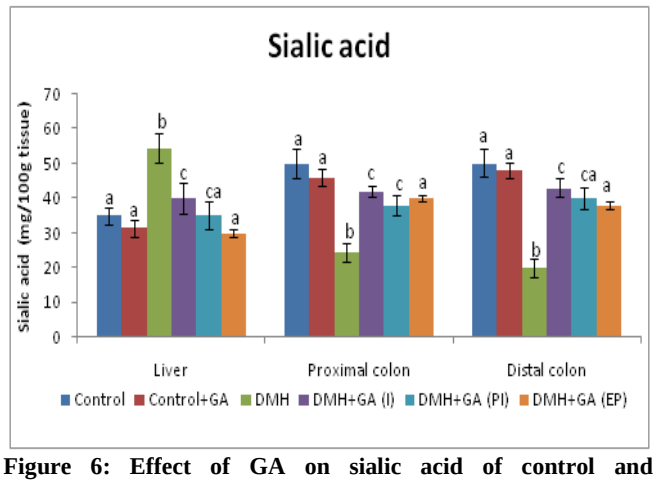

Figure 6: Effect of GA on sialic acid of control and experimental animals

The column heights are the means ±S.D. of the values of 8 rats. Standard deviation is shown as bars. Groups not sharing a common letter (a–c) differ signficantly at $p < 0.05$.*$p < 0.01$, values are significantly different as compared to DMH alone treated group.

4. DISCUSSION

Colon cancer is the third most common cancer worldwide and has become one of the major causes of cancer mortality. Western type-high fat, high protein, low carbohydrate and low fiber diet is thought to be an important factor for the increase in colon cancer incidence in India. In the present study, the effect of GA on biochemical changes, xenobiotic metabolic enzymes of phase I and II as well as on the bacterial enzymes activities in DMH induced rat colon cancer was evaluated.

When high fat diet, rich in ω-6 polyunsaturated fatty acids was fed during the carcinogenic stage, there was a signficant in crease in colon tumour incidence. High fat diet depending on the type of fat and their fatty acid composition can act as a promoter of colon cancer. In general, dietary fat that contains linoleic acid, a precursor of prostaglandin, is effective in promoting tumorigenesis in animals (Carrol, *et al*., 1986) .It is possible that the high incidence of colon tumors in rats fed a high fat diet along with DMH observed in our present study, could be due to the excretion of elevated amounts of bile acids, which act as colon tumor promoters (Kamaleeswari, *et al*., 2006).

Cooper *et al*., 1989 have observed that administration of carcinogen results in doubling of biological membrane cholesterol and gross distortion of cell shape, with changes in the lipid composition, membrane fluidity and morphology. DMH administration is also known to increase the levels of cholesterol in the colon and intestines. Our present results also shows a similar pattern. The increased cholesterol content in the tissues of DMH alone treated (group 3) animals could be attributed to the increased activity of the rate-limiting enzyme of cholesterol synthesis (HMG CoA reductase). Moreover increased cholesterol accumulation may be associated with one of the properties of malignant cells, such as cellular pleomorphism.

Alterations in the lipid fluidity and composition of plasma membranes of colonocytes may play a role in the early stage(s) of the malignant transformation (Garder and Brennes, 1985). The composition of lipids in biomembranes determine lipid fluidity. Besides any alterations in the

phospholipid metabolism will affect the properties and function of membrane proteins, as phospholipids are vital components of biomembranes. The deleterious effect on the membrane is decreased due to lowered phospholipid content in the experimental group thus, leading to its dysfunction. Moreover, the cholesterol/phospholipid ratio is closely related to membrane fluidity, a lower ratio indicating membrane stability. The cholesterol/phospholipids ratio was increased to an appreciable extent in the DMH-induced rats indicating less fluidity, which correlates with other reports (Nalini *et al*., 1997). Supplementation with GA to carcinogen exposed rats showed decreased levels of cholesterol/phospholipids ratio in the tissues and thus increased membrane stability.

The intestinal flora is comprised of a metabolically active group of organisms that produce enzymes catalysing the metabolism of procarcinogens and formation of tumour promoters, which in turn plays a significant role in the pathogenesis of colon cancer (McBain and Macfarlane, 1998). High-fat diet a known risk factor for colon cancer, alters bacterial enzymes and is responsible for the conversion of procarcinogens to proximal carcinogens [35]. Thus the bacterial enzymes become important to determine the chemopreventive efficacy of a potent lead in colon carcinogenesis (Morimitsu *et al*., 2004).

β-glucosidase and β-galactosidase are microbial hydrolytic enzymes responsible for the generation of aglycones from plant glycosides. Aglycones have a variety of biological activities including toxicity, mutagenicity and carcinogenicity. Significant inhibition on the activities of β-glucosidase and β-galactosidase by GA was observed in DMH treated rats. In this context Hughes and Rowland, 2000 have suggested that the changes in β-glucosidase activity can be explained by substrate-induced modulation of bacterial metabolism.

Increased activity of sulphatases and nitroreductases are also known to elevate colon cancer risk. There have been reports showing that certain polyphenols non-competitively inhibit p-form of phenol sulphotransferases. Being a polyphenol, GA is also considered to inhibit sulpho-conjugations leading to decreased activities of nitroreductases and sulphatases. Goldin and Gorbach, 1980 have also reported that decreased faecal activities of nitroreductase are accompanied by a reduction in the incidence of DMH induced colon carcinoma.

The colon specific carcinogen DMH used in our study gets metabolically activated to a proximate metabolite methylazoxymethanol (MAM) by a form of cytochrome P450IIEI mainly in the liver and colon (Sohn, 1991). It is evident that the rate of liver metabolism of DMH is important in determining colon carcinogenicity. Thus any compound that can directly alter cytochrome P450IIEI activity, in general may play a significant role in colon carcinogenesis. A substantial increase in the activities of hepatic and colonic mucosal phase I enzymes was observed in the carcinogen treated animals that was reversed following supplementation with GA. The effect of GA on cytochrome P450 activity may be by altering the activites of microsomal monoxygenases and enhancing the interaction of NADPH-cytochrome P450 reductase with cytochrome P450 binding to the catalytic site. GA actively reduces the activities of NADH cytochrome b5 reductase and cytochrome b5 in cancer bearing animals, indicating its overall inhibitory effect on phase I enzymes.

Induction of phase II enzymes results in the detoxification and accelerated excretion of carcinogens (Letelier *et al*., 2005). Glucuronidation, catalyzed by the UDP-glucuronyl transferase (UDPGT) family of enzymes, is a metabolic pathway involving solublization and detoxification of endogenous steroids, bile acids, drugs and carcinogen (Steinkellner *et al*., 2001). Furthermore UDPGT is tightly bound to the phospholipids of the microsomal membrane (Yang, 2000) and hence the observed decrease in the activity of this enzyme in carcinogen exposed animals may be due to the peroxidative damage to the microsomal lipids in cancer condition.

Thus, it is apparent that in the present study, the activities of phase I enzymes were elevated whereas the activities of the phase II enzyme, UDP-GT was found to be lowered on exposure to the colon specific carcinogen, DMH. Supplementation with GA to DMH treated rats restored the activities of the phase I and phase II enzymes to near those of the control rats. Dual acting agents are generally recognized to be more promising as cancer chemopreventive agents because they inhibit metabolic activation of

carcinogens while promoting detoxification and excretion. From the present study, we found that GA acts as dual-acting agent by suppressing phase-I enzymes and enhancing the activities of phase II enzymes. In this context, green and black tea phenols (Talalay , 1989) are also known to modulate the activities of phase I and II drug metabolizing enzymes. Moreover we had already observed that GA is a potent antioxidant (data not shown). Thus, on the whole, GA has a great potential in colon cancer chemoprevention by virtue of its modulating effects on the phase I and II xenobiotic metabolizing enzymes.

Over the past few years glycoproteins and their relationship to the process of malignant transformation have received considerable attention (Kim, 1975). Alterations in glycoproteins have been noted in various malignancies including human colorectal tumors. Glycoconjugates play a major role in cell–cell communication and they are found on the surface of all cells, blood stream and other body fluids. In many pathological conditions, the carbohydrate structures of glycoproteins are altered. Hexoses and fucose are components of glycoproteins and glycolipids on biomembranes of cells (Staudacher *et al*., 1999). Increased density (levels) of hexoses and fucose at the cell surface of malignant or transformed cell has been reported from studies on both the animal models and human systems. Similarly, in our study carcinogen exposed animals showed elevated levels of tissue total hexoses. The lowering of the levels of total hexoses and fucose on supplementation with GA could be due to the variations in the activities of the enzymes involved in glycoconjugate metabolism directly or indirectly, resulting in the altered severity of malignant transformation. In this context, oncogenes are known to induce the expression of golgi β-1,6 N-acetyl glucosaminyl transferase in many cell types, leading to increased cellular motility and decreased substratum adhesion (Harvey, 1981). The elevated levels of hexoses observed in DMH treated rats may be due to the action of golgi β-1,6 N-acetyl glucosaminyl transferase.

Sialic acid and hexosamine are components of all glycoproteins and glycolipids and in particular, are terminally located on the chains of membrane glycoproteins (Alhadeff, 1989). The data presented here clearly indicate that the levels of sialic acid and hexosamine were markedly lowered in the colon and elevated in the liver of carcinogen-exposed rats than their normal counterparts. These results correlate with our previous report (Nalini *et al*., 1998). Supplementation with GA normalized the membrane bound sialic acid and hexosamine content in the colon and liver of the DMH treated rats, which could result in membrane stabilization and further strengthening of its binding to the extracellular matrix. This mechanism may provide the basis for attenuation and reduction in multilayer growth.

The striking chemopreventive potential of GA against DMH induced colon carcinogenesis may be attributed to the (i) modulation of phase I and phase II xenobiotic metabolizing enzymes (ii) suppression of bacterial biotransforming enzymes (iii) prevention of lipid accumulation (iv) modulation of the metabolism of glycoconjugates.

ACKNOWLEDGEMENT

Financial assistance by the University Grant Commissions, New Delhi, India, is gratefully acknowledged.

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