Chapter-II

Materials and Methods
1. Materials

1.1 Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH), nicotinamide adenine dinucleotide phosphate oxidized (NADP), nicotinamide adenine dinucleotide reduced (NADH), flavin adenine dinucleotide (FAD), ethylene diamine tetra-acetic acid (EDTA), sodium dithionite, thiobarbituric acid (TBA), pyrogallol, poly-L-lysine, tris-base, tris-HCl, horse radish peroxidase (HRP), xanthine, glucose-6-phosphate, bovine serum albumin (BSA), Mayer’s hematoxylin, dichlorophenolindophenol (DCPIP), 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB), alcian blue 8GX, neutral red, toluidine blue, propidium iodide, methylene blue, N,N′-dimethyl-p-phenylene diamine, N,N′-dimethyl-m-phenylene diamine, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dimethylhydrazine (DMH) and glutathione reductase were obtained from Sigma (Sigma Chemical Co., St Louis, MO). Poly-HRP plus ONE detection System (Thermo Scientific). Cisplatin was purchased from Dr. Reddy’s Laboratories Ltd, Hyderabad, India. Hydrogen peroxide, xylene, ethanol, magnesium chloride, sulphosalicylic acid, perchloric acid, trichloroacetic acid (TCA), tween-20, ferric chloride, folin ciocalteau reagent (FCR), sodium potassium tartrate, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium citrate, acetone, methanol, sodium azide, formaldehyde, xylene, ethanol, acetic acid, hydrochloric acid (HCl) and sodium hydroxide were purchased from E. Merck Limited, India. TNF-α were purchased from e-Bioscience, USA. Caspase-6 and caspase-3 kit were purchased from Invitrogen [supplied with VEID (for caspase-6) and DEVD (for caspase-3) substrates]. Primary antibodies of phospho-p38 (dilution 1:200, Santa Cruz), p53 (dilution1: 100, Santa Cruz), Bak (dilution 1:100, Biolegend), cleaved caspase-3 (dilution 1:400, Cell Signalling), VEGF (dilution 1: 300, Neomarkers, Fremont CA), Caspase-9 (dilution 1: 300, Neomarkers, Fremont CA), i-NOS (dilution 1: 100) Neomarkers, Fremont CA), Connexin-43.
(dilution 1: 400, Santa Cruz), COX-2 (dilution 1: 300, Santa Cruz), NF-kB-p65 (dilution 1:100, Biolegend), Ki-67 (dilution 1: 300, Neomarkers, Fremont CA), PCNA (dilution 1: 500, Neomarkers, Fremont CA), Bcl-2 (dilution 1: 200, Santa Cruz), Survivin (dilution 1: 100, Neomarkers, Fremont CA) were used. Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse secondary antibody (dilution 1: 50, Jackson ImmunoResearch Lab Inc.). All other chemicals and reagents were of the highest purity grade commercially available.

1.2 Natural Chemopreventive Agents: Farnesol, Chrysin, Rutin and Glycyrrhizic acid were purchased from Sigma-aldrich (Sigma Chemical Co., St Louis, MO).

1.3 Animals: Four to six-week-old, male albino rats (120–150 g) of Wistar strain were obtained from Central Animal House of Hamdard University, New Delhi, India. All procedures for using experimental animals were checked and permitted by the “Institutional Animal Ethical Committee (IAEC)” that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Chennai, India. They were housed in polypropylene cages in groups of four rats per cage and were kept in a room maintained at 25±2 °C with a 12 h light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory animal diet and water ad libitum.

2. Methodology

2.1 Preparation of 1,2-dimethylhydrazine (DMH) solution for colon cancer induction

DMH was weighed and dissolved in distilled water containing 1 mM EDTA to ensure the stability of the chemical just prior to use and the pH was adjusted to 6.5 with 1 M NaOH solution.
2.2 Tissue processing

At the end of experiment, all the animals were anaesthetised with mild anaesthesia and sacrificed by cervical dislocation. Colons were removed quickly, cleaned free of irrelevant material and immediately perfused with ice-cold saline (0.85% sodium chloride). A small piece of colon was preserved immediately after washing in 10% neutral buffered formalin for histological and immunohistochemical analysis.

2.3 Homogenization

Colons were homogenised in appropriate buffer (usually, phosphate buffer, 0.1 M, pH 7.4) using polytron homogenizer (Kinematica-AGPT 3000).

2.4 Post-Mitochondrial Supernatant (PMS) and microsome preparation

The homogenate was filtered through muslin cloth, and were centrifuged at 3000 rpm for 10 min at 4°C in a Remi Cooling Centrifuge (C-24 DL) to separate the nuclear debris. The aliquot so obtained was centrifuged at 10,500g for 20 min at 4°C to obtain PMS, which was used as a source of various enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge (Beckman L7-55) at 34,000 rpm at 4°C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%).

2.5 Estimation of Different Parameters

2.5.1 Measurement of lipid peroxidation (LPO)

The assay for membrane lipid peroxidation was done by the method of (Wright et al. 1981) with some modifications. The reaction mixture in a total volume of 3.0 ml contained 1.0 ml tissue homogenate, 1.0 ml of TCA (10%), and 1.0ml TBA (0.67%). All the test tubes were placed in a
boiling water bath for a period of 45 min. The tubes were shifted to ice bath and then centrifuged at 2500×g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/gram tissue by using a molar extinction coefficient of 1.56×10⁵ M⁻¹ cm⁻¹.

2.5.2 Measurement of xanthine oxidase (XO) activity

The activity of xanthine oxidase was assayed by the method of (Stirpe and Della Corte 1969). The reaction mixture consisted of 0.2 ml PMS which was incubated for 5 min at 37 °C with 0.8 ml phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml xanthine (9 mM) and kept at 37 °C for 20 min. The reaction was terminated by the addition of 0.5 ml ice-cold perchloric acid [10% (v/v)]. After 10 min, 2.4 ml of distilled water was added and centrifuged at 4000 rpm for 10 min and µg uric acid formed/min/mg protein was recorded at 290 nm.

2.5.3 Measurement of reduced glutathione (GSH) level

The GSH content in colon was determined by the method of (Jollow et al. 1974) in which 1.0 ml of PMS fraction (10%) was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (10mM) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on spectrophotometer (Milton Roy Model-21 D). The GSH content was calculated as µmol DTNB conjugate formed/gram tissue using molar extinction coefficient of 13.6×10³ M⁻¹ cm⁻¹.

2.5.4 Measurement of glutathione peroxidase (GPx) activity

The GPx activity was calculated by the method of (Mohandas et al. 1984). A total of 2 ml volume consisted of 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 1.44 ml phosphate buffer
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2.5.5 Measurement of glutathione-S-transferase (GST) activity

The GST activity was measured by the method of (Habig et al. 1974). The reaction mixture consisted of 2.4 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1.0 mM), 0.2 ml CDNB (1.0 mM) and 0.2 ml of cytosolic fraction in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as μmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 × 10^3 M⁻¹ cm⁻¹.

2.5.6 Measurement of glutathione reductase (GR) activity

The GR activity was measured by the method of (Carlberg and Mannervik 1975). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1.0 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml of 10% PMS in a total volume of 2.0 ml. The enzyme activity was assessed at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 × 10^3 M⁻¹ cm⁻¹.

2.5.7 Measurement of glucose-6-phosphate dehydrogenase (G6PD) activity

The activity of glucose-6-phosphate dehydrogenase was determined by the method of (Zaheer et al. 1965). The reaction mixture consisted of 0.3 ml Tris–HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose-6-phosphate (0.8 mM), 0.1 ml MgCl₂ (8 mM), 0.3 ml PMS (10%) and 2.1 ml distilled water in a total volume of 3 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of 6.22 × 10^3 M⁻¹ cm⁻¹.
2.5.8 Measurement of superoxide dismutase (SOD) activity

The SOD activity was measured by the method of (Marklund and Marklund 1974). The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24mM in 10mM HCl) and 100 µl PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

2.5.9 Measurement of catalase (CAT) activity

The catalase activity was measured by the method of (Claiborne 1985). In brief, the assay mixture consisted of 2.0 ml phosphate buffer (0.1 M, pH 7.4), 0.95 ml hydrogen peroxide (0.019 M) and 0.05 ml of PMS (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. The catalase activity was calculated in terms of nmol H$_2$O$_2$ consumed/min/mg protein.

2.5.10 Measurement of quinone reductase (QR) activity

The QR activity was determined by the method of (Benson et al. 1980). The 3ml reaction mixture consisted of 2.13 ml Tris–HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM), and 50 µl PMS (10%). The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and the enzyme activity was calculated as µmol of DCPIP reduced/min/mg protein using molar extinction coefficient of 2.1 × 10$^4$ M$^{-1}$ cm$^{-1}$.

2.5.11 Assay for hydrogen peroxide (H$_2$O$_2$)

Hydrogen peroxide (H$_2$O$_2$) was assayed by H$_2$O$_2$-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of (Pick and Keisari 1980). 2.0 ml of supernatant, suspended in 1.0 ml of solution containing phenol red (0.28 nm), horse radish peroxidase (8.5 units), dextrose (5.5 nm) and phosphate buffer (0.05 M, pH 7.0) was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10N) and then centrifuged.
at 800 × g for 5 min. The absorbance of supernatant was recorded at 610 nm against a reagent blank. The quantity of H₂O₂ produced was expressed as nmol H₂O₂/h/gm tissue based on the standard curve of H₂O₂ mediated oxidation of phenol red.

2.5.12 Measurement of cytochrome P450 activity

The cytochrome P450 activity was measured by the method of (Omura and Sato 1980). The assay mixture consisted of 0.2ml microsomal fraction, 0.8ml of phosphate buffer (0.1 M, pH 7.4) and a pinch of sodium dithionite. Then pass carbon monoxide for 30 sec through the reaction mixture and the absorbance was recorded at 450nm and 490nm. The activity of cytochrome P450 was calculated as nmoles CytP450 activity/mg protein using molar extinction coefficient (E₄₅₀₋₄₉₀) = 91 m⁻¹ cm⁻¹.

2.5.13 Immunohistochemistry

Thick sections of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water followed by antigen retrieval in sodium citrate buffer (10mM, pH 6.0). The slides were then allowed to cool for 15 minutes and washed 3 times with tris-buffered saline (TBS) for 5 minutes each. Incubate the slides in 3% H₂O₂ in methanol for 10 min to reduce the endogenous peroxidase activity and then subject to power block (UltraVision Plus Detection System, Thermo Scientific) for 10 min to block non-specific binding. After rinsing the sections in TBS, the slides were incubated overnight at 4°C with primary antibody inside humidified chamber and then were washed in TBS. The sections were incubated with biotinylated goat anti-polyvalent secondary antibody (UltraVision Plus Detection System, Thermo Scientific) for 20 min and then were rinsed in TBS. The sections were again incubated with streptavidin peroxidase plus (UltraVision Plus Detection System, Thermo Scientific) for 30 min. The sections were washed in TBS and developed with 3, 3'
Diaminobenzidine (DAB) solution (UltraVision Plus Detection System, Thermo Scientific) until sections become brown. The sections were counterstained with Mayer’s haematoxylin, mounted by using mounting media and then visualized under the light microscope (Olympus BX51).

2.5.14 Fluorescent Immunohistochemistry

Thick sections of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water followed by antigen retrieval in sodium citrate buffer (10mM, pH 6.0). The slides were then allowed to cool for 15 minutes and washed 3 times with tris-buffered saline (TBS) for 5 minutes each. Incubate the slides in 3% H₂O₂ in methanol for 10 min to reduce the endogenous peroxidase activity and then subject to power block (UltraVision Plus Detection System, Thermo Scientific) for 10 min to block non-specific binding. After rinsing the sections in TBS, the slides were incubated overnight at 4°C with primary antibody inside humidified chamber and then were washed in TBS. The sections were incubated with FITC-conjugated secondary antibody (dilution 1: 50) for 20 min and then were rinsed in TBS. The sections were counterstained with propidium iodide to visualize nuclei, mounted by using mounting media and then observed under the light microscope (Olympus BX51).

2.5.15 Mast Cell Staining: For detection of mast cells, colon was fixed in methacarn solution (methanol: chloroform: glacial acetic acid:: 4:2:1) overnight at 4°C and then fixed in 4% neutral buffered formalin for additional 24 hours. The colonic sections of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water. The sections were stained with 0.1% toluidine blue (pH 2.3) in 1% sodium chloride solution for 5min. The slides were then washed three times in distilled water and dehydrated
quickly in alcohol, clear in xylene and mounted by using mounting media. The slides were then evaluated under the light microscope (Olympus BX51). Staining with toluidine blue permits the identification of mast cells because mast cell granules stain metachromatically, resulting in deep purplish-blue granular cytoplasmic staining.

2.5.16 Aberrant Crypt Foci (ACF) Assay

ACF assay was done by the method of (Bird 1987). Colons were excised from rats immediately, flushed with ice-cold saline (0.85% NaCl), opened longitudinally and fixed flat between two sheets of filter paper in 10% formalin (Sigma chemical, St. Quentin, France). Colons picked up in random order were stained for 6 min in a 0.05% filtered solution of methylene blue. The number of ACF per colon and number of crypts in each ACF were counted under light microscope (Olympus BX51) at x40 magnification.

2.5.17 Mucin Depleted Foci (MDF) Assay:

MDF assay was done by the method of (Caderni et al. 2003). Colons, after being scored for ACF, were stained with high iron diamine-Alcian blue procedure (HID-AB) to evaluate mucin production. Briefly, colons were rinsed in distilled water and left for 18 hours in freshly prepared HID solution (50 mL of distilled water with 120 mg N-N'-dimethyl-m-phenylene diamine, 20 mg N-N'-dimethyl-p-phenylene diamine, and 1.4 mL of 60% ferric chloride). After rinsing, colons were counterstained in 1% alcian blue solution for 30 min. MDF number, and number of crypts per MDF, were counted under light microscope (Olympus BX51) at x40 magnification.

2.5.18 HID-AB staining for sulphomucin and sialomucin

The colonic sections of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water. The colonic sections were stained with high iron diamine (HID) solution for 18 hours at room temperature in the dark and
washed 3 times with distilled water. Stains the sections with 1% alcian blue (dissolved in 3% acetic acid solution) for 30 min and washed 3 times with 80% ethanol. Then washed with distilled water and dehydrated quickly in alcohol, clear in xylene and mounted by using mounting media. The slides were then evaluated under the light microscope (Olympus BX51).

2.5.19 Staining for goblet cells analysis

The colonic sections of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water. The sections were stained with 1% Alcian blue (pH 2.5) in 3% acetic acid solution for 30 min and then rinsed for 1 min in 3% acetic acid solution to prevent non-specific staining. The slides were then washed in distilled water and the sections were then counterstained with neutral red (0.5% aqueous solution) for 20 sec, dehydrated in alcohol and mounted by using mounting media. The slides were then evaluated under the light microscope (Olympus BX51).

2.5.20 Histology

The colon were excised, flushed with saline, cut open longitudinally along the main axis, and then again washed with saline. These colonic sections fixed in 10% buffered formalin for at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol, cleared in benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections were cut from the distal colon. The paraffin embedded colonic tissue sections were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and permeabilized with permeabilization solution (0.1M citrate, 0.1% TritonX-100). These sections stained with haematoxylin and eosin (H&E) and were observed under light microscope (Olympus BX51) at 10x and 40x magnifications to investigate the histoarchitecture of colonic mucosa.
2.5.21 Measurement of protein

The protein concentration in all samples was determined by the method of (Lowry et al. 1951). Peptide bonds form a complex with alkaline copper sulphate reagent, which gives a blue colour with Folin’s reagent. Briefly, 0.1ml (10% w/v) was diluted to 1ml water and protein precipitated with equal volume of TCA (10%), samples were kept overnight 4°C and centrifuged at 800 X g for 5 minutes. The supernatant was decanted and discarded. The pellet was dissolved in 5ml of NaOH (1N). Finally 0.1ml of diluted aliquot was taken for colour development. 0.1ml of aliquot was further diluted to 1ml with water and then 2.5ml of alkaline copper sulphate reagent containing sodium carbonate (2%), copper sulphate (1%), and sodium potassium tartarate (2%) was added. Following 10 minutes after addition of alkaline copper sulphate reagent to allow complex formation 0.25ml of Folin Ciocalteau Reagent (FCR) was added. After 30 minutes blue colour developed that was read at 660 nm for standard Bovine serum albumin (BSA 0.1mg/ml) was used.

2.6 Statistical analysis

The data from individual groups were presented as the mean ± S.D. Differences between groups were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test and minimum criterion for statistical significance was set at p < 0.05 for all comparisons.