CHAPTER 3
CHEMICALS

Chemicals and biochemicals used in this study were either of analytical grade or of highest purity grade available commercially.

Sigma Chemical Co., St. Louis, USA: Bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate (reduced) (NADP), phenylmethylsulfonyl fluoride (PMSF), aloin, hesperidin, benzo(a)pyrene (B(a)P), pyridoxal-5-phosphate, 2-mercaptoethanol, dithiothreitol (DTT), glutathione reductase, thiobarbituric acid (TBA), 5,5'-dithio-bis (2-nitrobenzoic acid), hydrogen peroxide, Tween 80, brij 35, L-ornithine, xanthine oxidase, dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoyl phorbol 13, acetate (TPA)

Naphthalene, sodium azide, chloroform, methanol, acetone, hydrochloric acid, acetaldehyde, methoxethanol, formaldehyde, nitric acid were obtained from S.D. Fine Chemicals, India. 1,4-Dioxane, toluene, acetic acid, orthophosphoric acid, sulphuric acid, sodium chloride, potassium chloride, ascorbic acid, ferric chloride, sodium tungstate, diphenylamine, trichloroacetic acid, citric acid, folin ciocalteu's phenol reagent, 1,4-bis (5-phenyl-2-oxazolyl) benzene, picric acid, benzene, acetone, ethylenediaminetetraacetic acid (EDTA), xanthine, ascorbic acid were purchased from CDH, India. Ethyl acetate, perchloric acid, ethanolamine, magnesium chloride, copper sulphate, sodium potassium tartarate, potassium dihydrogen phosphate, sodium dihydrogen phosphate, sodium acetate were procured from E. Merck. 2,5-diphenyloxazole, formic acid, 2,4-dinitrophenylhydrazine were obtained from Spectrochem, India. Oxidized and reduced glutathione, tris-HCl, 1-chloro-2,4-dinitrobenzene, sulfosalicylic acid, were imported from Ameresco, USA. [14C]Ornithine (specific activity, 56 mCi/mmol), [3H]thymidine (specific activity 82 Ci/mmol) and [3H] dimethylbenz(a)anthracene specific activity mCi/mmol) were purchased from Amersham Corporation, U.K. Anne french hair
removing cream was purchased from Geoffrey Manners & Co. Ltd., Bombay, India.

INSTRUMENTS
The following instruments available in our laboratory or in the central instrumentation facility of Jamia Hamdard were used.
- Cold centrifuge (Remi C 24)
- Spectrophotometer (Perkin Elmer Lamda bio 40)
- Boiling water bath (Yorco)
- Deep freezer (-20°C to -70°C)
- Hot plate (Yorco)
- Incubator 37°C (Yorco)
- Metier balance (Delta Range, AT 261)
- Ultracentrifuge (Beckman L7-55)
- Shaker water bath (Tempo)
- Potter-Elvehjem Teflon: Glass Homogenizer (Remi)
- Polytron (Kinematica PT 3100)
- β-scintillation counter (LKB-Wallace-1410)
- pH meter (Toshniwal)
- Oven (Adair Dutt and Co)

ANIMALS
Swiss albino mice and male wistar rats pathogen free were procured from Central Animal House Colony of Jamia Hamdard and used for all biological experimental work. All animals were housed in an air-conditioned room in polypropylene cages usually in groups of six mice each unless mentioned otherwise. They had free access to pellet diet (Hindustan Lever Ltd., Bombay, India) and water ad-libitum. The animals were kept at room temperature of 24°C (±2°C) and were exposed to alternate cycles of 12 hours light and darkness.

SHAVING PROCEDURE
The dorsal skin of the mouse was shaved with an electric clipper (Oster, model A2) followed by the application of hair removing cream at
least two days prior to the treatment. Only mice that did not show signs of hair re-growth were used.

**TREATMENT PROTOCOL**

Treatment protocols that include selection of dose regimen, mode of administration, time period at which animals were sacrificed etc have been described in the individual chapters.

**DIAGNOSIS OF TUMORS**

Skin: The criteria for the diagnosis of various tumors as reported by Athar et al. 1990 were adopted. Papillomas were identified by their cauliflower, exophytic tumorous growth with narrow or broad base consisting of a series of connective tissue folds covered by stratified squamous epithelium, usually without cellular atypia. Squamous cell carcinomas are composed of very irregular endophytic epithelial growth with frequent cellular atypia. They are usually very differentiated, with the formation of keratinised layers of horny pearls.

Liver: The rats were killed, livers were excised, blotted dry, weighed and examined on the surface for visible macroscopic liver lesions (neoplastic nodules) Bhattacharya and Chatterji 1998.

Forestomach: The mice were killed, forestomach were excised then it was opened and washed thoroughly so as to clean all the undigested food remaining in the stomach. The lesions were counted and the forestomach were stored for the histopathological examination.

**METHODS**

**TISSUE PREPARATION**

The following standard procedure modified from Mohandas et al. (1984), was adopted for preparation of tissue for all the biochemical estimations described in this thesis. After the desired time period, control and treated animals were sacrificed by cervical dislocation. The animals were immediately dissected to remove their skin, the subcutaneous layers were completely scrapped off with the help of a scalpel blade to separate the epidermal layer by keeping the skin sample upside down on
a petridish containing crushed ice. The washed tissue was blotted between the folds of a filter paper and weighed on a mettler balance. For biochemical studies, a known amount of tissue was taken, minced properly and homogenized in appropriate buffer in phosphate buffer (0.1 M, pH 7.4). For soft tissue homogenization was carried out in motor driven teflon coated homogenizer (Remi RU56-3)/polytron (kinematica PT 3100).

SUBCELLULAR FRACTIONATION

The homogenate was filtered through a muslin cloth, and was centrifuged at 800 x g for 5 minutes at 4°C to separate the nuclear debris. The aliquot so obtained was centrifuged in an Eltek refrigerated centrifuge (Model RC 4100D) at 105,00 x g for 20 minutes at 4°C to get post mitochondrial supernatant (PMS) which was used as a source of enzyme. A portion of the PMS was centrifuged in an ultracentrifuge (Beckman, L7-55) at 105,000 x g for 60 minutes at 4°C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%). This pellet was considered to be the microsomal fraction and was suspended in phosphate buffer (0.1 M, pH 7.4) containing phenylmethyl- sulfonyl fluoride (PMSF) and glycerol. The microsomes were stored in liquid nitrogen when not used immediately.

SERUM PREPARATION

Serum was prepared according to the routine method. Briefly, blood was taken out from the retroorbital sinus using heparinized capillary tubes in dried test tubes, which were kept in tilted position for ten minutes at room temperature and then for an hour at 4°C to get serum separated from cellular clot. To remove cellular contamination from the serum, it was centrifuged for 2-3 minutes at 800-x g.

PREPARATION OF SCINTILLATION FLUID

Naphthalene (52 gm), 2,5-diphenyloxazole (3.25 gm), 1,4-bis (5-phenyl-2-oxazolyl) benzene, (0.065 gm) were dissolved in a mixture
containing 1,4-dioxane (250 ml), toluene (aldehyde free, 250 ml) and methanol (150 ml), and stored in dark bottle. Usually scintillation fluid was prepared immediately before its use.

**BIOCHEMICAL ESTIMATION**

For all biochemical estimation, either post mitochondrial supernatant, cytosol or microsomes were used. All biochemical estimations were completed on the same day when animals were sacrificed.

**ESTIMATION OF REDUCED GLUTATHIONE**

Reduced glutathione in the kidney was determined by the method of Jollow et al. (1974). 1.0 ml of PMS (10% w/v) was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for at least one hour and then subjected to centrifugation at 1200 x g for 15 minutes at 4°C. The assay mixture contained 0.1 ml of filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer, 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21D).

**ASSAY OF LIPID PEROXIDATION**

The assay for microsomal lipid peroxidation was done following the method of Wright et al. (1981). The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome obtained from the homogenate (10% w/v), 0.2 ml ascorbic acid (100 mM), and 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for one hour. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml of TBA (0.67%), all the tubes were placed in a boiling water bath for a period of twenty minutes. In the end, tubes were shifted to crushed ice bath and then centrifuged at 2500 x g for 10 minutes. The amount of malondialdehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm.
using a spectrophotometer (Milton Roy Model-21D) against a reagent blank. The results were expressed as nmol MDA formed/hour/gm of tissue at 37°C by using molar extinction coefficient of 1.56x10^5 M^-1 cm^-1.

**[3H]THYMIDINE INCORPORATION ASSAY**

The isolation of renal DNA and incorporation of [3H] thymidine into DNA was done by the method of Smart et al. (1986), as described by Iqbal et al. (1995). Briefly, the control and treated animals received [3H] thymidine (30 mCi/animal) an i.p. injection, eighteen hours after the first treatment of saline or Fe-NTA. All these animals were sacrificed twenty hours after the first treatment by cervical dislocation. Their kidneys were quickly removed, cleaned free of extraneous material and homogenate (10% w/v) prepared in ice-cold water and TCA (10%) was added. The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4°C for overnight. After incubation, it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%) followed by incubation in boiling water bath for 30 minutes. It was then filtered through Whatman-50 filter paper. The filtrate was used for [3H] thymidine counting in liquid scintillation counter (LKB-Wallace-1410) by adding the scintillation fluid. The amount of DNA in the filtrate was estimated by the diphenylamine method of Giles and Myers (1965). The final procedure is, therefore, to add 2 ml of diphenylamine (4%) in glacial acetic acid to 0.2 ml of test solution of DNA in perchloric acid (10%) followed by 0.1 ml of aqueous acetaldehyde (1.6 mg/ml). After incubation at 30°C overnight, the optical density difference at 595-700 nm is read against 595-700 nm blank. The amount of [3H] thymidine incorporated was expressed as DPM/mg DNA.

**ORNITHINE DECARBOXYLASE ACTIVITY**

ODC activity was determined utilizing 0.4 ml renal 105,000 x g cytosolic supernatant fraction (20% w/v) per assay tube by measuring the release of 14CO2 from DL [1-14C] ornithine by the method of O'Brien et al. (1975), as described by Athar et al. (1990). The kidneys were
homogenized in tris-HCl buffer (50 mM, pH 7.5) containing EDTA (0.1 mM), pyridoxal-5-phosphate (0.1 mM), phenylmethylsulfonyl fluoride (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and tween 80 (0.1%) at 4°C. In brief, the reaction mixture contained 400 ml enzyme and 0.095 ml co-factor mixture containing pyridoxal-5-phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), brij 35 (0.02%) and 14Cornithine (0.05 mCi) in a total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and other test tubes, the tubes were covered immediately with a rubber cork containing 0.2 ml ethanolamine and methoxyethanol mixture (2:1) in the central well and kept in water bath at 37°C. After one hour of incubation, the enzyme activity was arrested by injecting 1.0 ml of citric acid (2.0 M) solution along the sides of glass tubes and the incubation was continued for one hour to ensure complete absorption of 14CO2. Finally, the central well was transferred to a vial containing 2 ml of ethanol and 10 ml toluene based scintillation fluid was added to it followed by counting of the radioactivity in a liquid scintillation counter (LKB-Wallace-1410). ODC activity was expressed as pmol 14CO2 released/hour/mg protein.

GLUTATHIONE PEROXIDASE ACTIVITY

Glutathione peroxidase activity was measured according to the procedure described by Mohandas et al. (1984). The reaction mixture consisted of 1.44 ml phosphate buffer (0.05 M, pH 7.0), 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 0.05 ml of glutathione reductase (1 eu/ml), 0.10 ml of glutathione (1 mM), 0.10 ml of NADPH (0.2 mM), 0.01 ml of hydrogen peroxide (0.25 mM) and 0.10 ml of PMS (10% w/v) in a final volume of 2.0 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein by using molar extinction coefficient 6.22x103M-1cm-1.

GLUTATHIONE REDUCTASE ACTIVITY

Glutathione reductase activity was assayed by the method of Carlberg and Mannervik (1975), as modified by Mohandas et al. (1984).
The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml NADPH (0.1 mM), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM) and 0.1 ml PMS (10% w/v) in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22x10^-3 M^-1 cm^-1.

**CATALASE ACTIVITY**

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M), and 0.05 ml PMS (10% w/v) in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H2O2 consumed/min/mg protein.

**GLUTATHIONE-S-TRANSFERASE ACTIVITY**

Glutathione-S-transferase activity was measured by the method of Habiq et al. (1974), as described by Athar et al. (1989). The reaction mixture consisted of 1.425 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM) and 0.30 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6x10^3 M^-1 cm^-1.

**SERUM GLUTAMIC OXALOACETIC TRANSAMINASE (SGOT) AND GLUTAMIC PYRUVIC TRANSAMINASE (SGPT) ACTIVITY**

Serum glutamic oxaloacetic transaminase (sGOT) and glutamic pyruvic transaminase (sGPT) were determined by the method of Reitman and Frankel (1957). Each substrate (0.5 ml) (either 2 mM α-keto glutarate or 200 mM L-alanine or L-aspartate) was incubated for 5 minutes at 37°C in a water bath. Serum (0.1 ml) was then added and the
volume was adjusted to 1 ml with sodium phosphate buffer. The reaction mixture was incubated at 37°C for exactly 20 minutes. The reaction was terminated with an equal volume of TCA (10% W/V), stored in ice and then centrifuged at 4000-x g for 5 minutes. To the supernatant, 0.5 ml of 2,4-dinitrophenylhydrazine (1nM) was added the reaction mixture was left for another 30 minutes at room temperature. Finally, the colour was developed by addition of 5 ml of NaOH (0.4 N) and product read at 505 nm.

LACTATE DEHYDROGENE (LDH) ACTIVITY

The lactate dehydrogense activity was estimated by the method of Wrobleski and La Duc (1955). Briefly the reaction mixture containing 2.7 ml. of phosphate buffer (0.1M, pH), 0.01 ml. serum and 0.1 ml. of reduced nicotinamide adenine dinucleotide was incubated for 20 minutes. To this 0.1 ml. of sodium pyruvate (2.5 mg/ml) was added. The absorbance was read for five minutes at 30 seconds interval. The activity was expressed as milli.l.U.

XANTHINE OXIDASE (XOD)

XOD catalyzes the conversion of xanthine to uric acid, which has a characteristic absorption peak around 290nm. The spectrophotometric method based on the modified procedure as described by Striee and Corta, 1969, will be used.

HISTOPATHOLOGICAL STUDIES

Animals were sacrificed by cervical dislocation and livers and forestomach of each animal were removed immediately. Tissues were fixed with Bouin's solution by the method of Luna, 1968, overnight immersed sequentially for 24 hr in 50% and 70% ethanol to remove picric acid, dehydrated, embedded in paraffin, sectioned at 5mm and mounted on glass slides for Hematoxylin/Eosin staining.
ESTIMATION OF PROTEIN

Protein was estimated 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 0.1 ml of the sample tissue (10% w/v) was diluted to 1 ml with water and the protein precipitated with equal volume of TCA (10%). Samples were kept overnight at 4°C and centrifuged at 800 x g for 5 minutes. The supernatant was decanted and discarded. The pellet was dissolved in 5 ml of NaOH (1 N). Finally, 0.1 ml of the diluted aliquot was taken for colour development.

0.1 ml of aliquot was further diluted to 1 ml with water and then 2.5 ml of alkaline copper sulphate reagent containing sodium carbonate (2%), CuSO₄ (1%) and sodium potassium tartrate (2%) was added. Following 10 minutes after addition of alkaline copper sulphate reagent to allow complex formation, 0.25 ml of Folin's reagent was added. Exactly after 30 minutes the blue colour developed and was read at 660 nm. For standard bovine serum albumin (0.1 mg/ml) was used.

STATISTICAL ANALYSIS

The level of significance between different groups is based on Dunnett t-test followed by analysis of variance test. The level of statistical significance was chosen p<0.05.