Materials and Methods
In the present chapter, we have described the details about the animals, chemicals and drugs equipments used in the study along with discussing the experimental setup, methodologies followed and technique wise protocols carried out while achieving the proposed objectives according to the scheme described in the section of "Study design"

4.1. Materials

Animals used in the study

All animal experiments were performed according to the Institutional guidelines of Central Drug Research Institute, Lucknow, after obtaining the permission from Laboratory Animal Ethical Committee. Naïve Sprague-Dawley rats of either sex, weighing 180-200 gm were used. Animals were kept in raised mesh bottom cages to prevent coprophagy and were housed three to four animals per cage at temperature 22±2 °C and 12h light/dark (8.00 A.M. to 8.00 P.M.) under controlled environment. Animals were fed with standard laboratory food (Pellet) and water was given ad libitum. The rats were fasted overnight before subjecting them to ulcerogens.

Experimental groups

In order to achieve the proposed objectives, present study has been designed in such a manner that 7 experimental groups have been used in all the experiments. 8 rats were allocated for each of the groups.

Group 1 = Normal animals

Group 2 = Control (vehicle treated animals)

Group 3 = Animals treated with Omeprazole (OMZ)

Group 4 = Animals treated with Ranitidine (RANI)

Group 5 = Animals treated with Sucralfate (SUC)

Group 6 = Animals treated with Misoprostol (MISO)

Group 7 = Animals treated with negative control Celecoxib (CELE)
Chemicals

Chemicals used in different experiments and their manufacturing companies are as follows: Acridine orange, Acrylamide, Agarose, Ammonium per sulphate, Aprotinin, Bis-acrylamide, β-actin primer, Brilliant blue R dye, Bromo phenol blue dye, Calf thymus DNA, Carboxymethyl cellulose, Chloroform-Isoamyl alcohol, COX-2 primer, Epinephrine, Ethidium bromide, Formamide, Hexadecyltrimethylammonium bromide, Glucose, Leupeptin, Mercaptoethanol, Nonidet-40, Phenylmethyl sulfonyl fluoride (PMSF), TEMED and valinomycin from Sigma Chemicals, Topfer’s reagent (S D Fine Chemicals), Calcium chloride (Qualigens Chemicals), Isopropanol, Phenolphthalein, Sodium deoxycholate and Tween-20 (Merck Chemicals), Trizol® reagent (Invitrogen), dNTPs, Taq polymerase (Roche Chemicals), Reverse transcriptase enzyme (Qiagen), Ovine COX-2 protein, Goat polyclonal COX-2 primary antibody, Goat polyclonal FGF-2 primary antibody, Goat polyclonal EGF primary antibody, Mouse monoclonal VEGF primary antibody, Mouse anti-goat IgG-HRP secondary antibody, Goat anti-mouse IgG-HRP secondary antibody from Santa Cruz Biotechnology. All other chemicals were purchased from SRL chemicals.

Drugs

Five drugs have been evaluated out of which four are anti-ulcerogenic agents: Omeprazole and misoprostol (Sigma chemicals), ranitidine (Zydus-Cadila), sucralfate (Menarini Raunaq) while the remaining one is COX-2 specific NSAIDs- Celecoxib (Dr. Reddy’s Lab).

Drugs administration

Drugs were administered daily once in a day as aqueous suspension using gum acacia (0.5%) as surfactant to different group of animals. The control group was given only 0.5% gum acacia as vehicle. Drugs were prepared fresh and were administered from 3rd day of surgery till the day of sacrifice. Dose of the drug and total time duration was determined on the basis of the results of pilot study.
Kits used

cDNA synthesis kit (Qiagen), ECL plus western blotting detection kit (Amersham Biosciences) Enzyme immuno assay kit for prostaglandin estimation (Cayman chemicals) and Low Molecular Weight (LMW) calibration kit for SDS electrophoresis (Amersham Biosciences) have been used.

Instruments

Instruments used for various experiments in the present study are: Hot air oven (SEW), pH meter (Systronics), Spectrophotometer (Shimadzu), Spectrofluorometer (Varian), Cold centrifuge (Heraeus), Polytron homogeniser (Ultra Turrax T-25), Electronic balance (Chyo), High speed ultracentrifuge (Beckmann), Deep freezer (New Brunswick), -20°C freezer, Table top centrifuge, Vortex and Water bath (Remi), Block making station and Automated Microtome (Leica), Gel electrophoresis apparatus and Western blotter (Hoefer), Trinocular zoom stereomicroscope along with image acquisition and analysis system (Olympus), ELISA plate reader (Molecular devices), Horizontal gel electrophoresis system (Banglore-Genei), Thermocycler (PTC 100 MJ-Research) and Video-image analyzer system (Alpha imager).

4.2 Pilot Study

Pilot study was carried out to determine the most effective dose and most effective time duration of drug treatment for each of the drug. Before commencing for various parameters that can lead to ulcer healing, we have carried out ulcer-healing studies for logarithmic graded doses for each of the drug. Ulcer induction was done by application of acetic acid on the serosal layer of mucosa, followed by the drug treatment at different doses for different time periods. On last day of drug treatment, gastric juice was collected and gastric mucosa of ulcerated tissue was scrapped. Ulcer area was measured under stereomicroscope with the help of Biovis image analysis software and healing effect of each of the drug was evaluated from evaluation of ulcer area. Detailed methodology for ulcer induction, drug treatment, gastric juice collection, mucosal scrapping and ulcer healing analysis has been mentioned in the later sections of this chapter.
Materials & Methods

Three logarithmic graded doses and three different time duration of drug treatment were selected for each of the five drugs in the pilot study. The selection was based on the available literature on study of these drugs in rat models (Penney et al., 1994; Tsukimi and Okabe, 1994; Guo et al., 2002; Konturek et al., 2003). These studies have suggested that each of the drugs has an optimal dose and treatment time duration, at which it exerts its maximum efficacy. Any dose below this effective dose is under-effective, while higher doses are often associated with tissue toxicity and other related problems. Different doses and time duration for which treatment of the drugs has been given are mentioned in Table 4.1

Table 4.1: Different doses and time period for which drugs were administered in pilot study

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Studied Drugs</th>
<th>Dose/kg body weight</th>
<th>Duration of drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>OMZ</td>
<td>5, 10 and 20 mg</td>
<td>5, 10 and 14 days</td>
</tr>
<tr>
<td>2.</td>
<td>RANI</td>
<td>15, 30 and 60 mg</td>
<td>5, 10 and 14 days</td>
</tr>
<tr>
<td>3.</td>
<td>SUC</td>
<td>250, 500 and 1000 mg</td>
<td>5, 10 and 14 days</td>
</tr>
<tr>
<td>4.</td>
<td>MISO</td>
<td>50, 100 and 200 µg</td>
<td>5, 10 and 14 days</td>
</tr>
<tr>
<td>5.</td>
<td>CELE</td>
<td>5, 10 and 20 mg</td>
<td>5, 10 and 14 days</td>
</tr>
</tbody>
</table>

The ulcer index and ulcer healing analysis of the pilot study has revealed that the most effective dose was 10 mg/kg body weight for OMZ, 30 mg/kg body weight for RANI, 500 mg/kg body weight for SUC, 100 µg/kg body weight for MISO and 10 mg/kg body weight for CELE respectively. The most effective time duration was found to be of 14 days for all the drugs. Based on these results, all further analysis – biochemical estimations, anti-oxidant studies, expression analysis and histopathological examinations were carried out for the most effective dose in each category of drugs.
4.3 Methods

1. Induction of ulcer

Acetic acid induced chronic gastric ulcer model

Chronic gastric ulcers were induced experimentally in rats according to the method of Konturek et al. (2003), which follows the technique of Okabe et al. (1987) with few modifications. We selected this particular methodology to induce chronic ulcer as it uses low concentration (40%) of acetic acid in comparison to other studies, where very high concentrations of acetic acid have been used. This model provides the luxury of production of well-characterized ulcers and a benign spontaneous ulcer healing.

Under light ether anesthesia, the abdomen of the animals was opened and the stomach was exposed. A plastic tube of 8 mm, opened at both ends was applied tightly to the serosal surface of the anterior wall of the stomach just proximal to the antral gland area. 60 μl of 40% acetic acid was poured through the tube onto the surface of the stomach for 90 sec. Acetic acid remaining on the surface was wiped away with a filter and opened abdomen was closed.

In the preliminary studies, it is observed that this procedure produces histologically well characteristic ulcers in 3 days after acetic acid exposure. Therefore, drug treatment of various drugs: OMZ, RANI, SUC, MISO and CELE were started from the 3\textsuperscript{rd} day of surgery. In one set of experiment animals were treated with different drugs for the next 14 days and were sacrificed, whereas in another set of experiment after 13 days of drug treatment, animals were fasted overnight and on 14\textsuperscript{th} day after 45 min of drug treatment, abdomen was opened under ether anesthesia below xiphoid process; the pyloric portion of the stomach was slightly lifted and ligated avoiding any damage to the adjacent blood vessels. The animals were sacrificed after 4 hrs of the pyloric ligation. Gastric juice was collected in a centrifuge tube and ulcerated tissue was excised for further biochemical estimations. Figure 4.1 shows an excised stomach with gastric ulcer induced by application of acetic acid on 3\textsuperscript{rd} day. After determination of ulcer area, stomachs were fixed with 40% formalin for
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histological studies in one set of experiment; while in other gastric mucosa was scrapped for the biochemical estimations, RNA and protein expression studies.

Figure 4.1: The ulcerated portion of stomach induced by acetic acid ulcer model

![Image of ulcerated tissue]

2. Measurement of ulcer size and percentage ulcer healing

Rat stomach was excised after the completion of experiment. Stomach was rinsed with chilled normal saline and was cut along the greater curvature. Ulcer area was measured with the help of Biovis image analysis software attached with Trinocular stereo zoom microscope and was expressed in mm². Percentage healing was calculated as

\[
\text{Percentage healing} = \frac{C - T \times 100}{C}
\]

Where C = ulcer area in control group; T = ulcer area in treated group.

3. Histopathological analysis of healing

Histological studies were performed according to the method of Ogihara and Okabe, 1993. The most effective means of studying normal and ulcerated tissue involves fixing of the tissue, embedding it in a wax media, cutting into thin sections, mounting it on slides, staining and studying them under a microscope. Histopathological examination involves:

1. Fixation of the ulcerated tissue.
2. Tissue processing, which involves dehydration, clearing, and embedding.
3. Microtomy/sectioning of the blocks.
4. Staining of the sections.
5. Mounting of the stained slides.
6. Microscopic examination of the slides.

Details about each of these steps involved are as follows:

(i) **Fixation**

The ulcerated as well as the normal tissue after removing from the stomach was immediately fixed in 40% formaldehyde solution (Formalin) which act as fixative. The use of fixative is to stop the process of autolysis and putrefaction, which starts immediately after the cell death. The main purpose of the fixative is to give optimal conditions to the tissue such as providing optical differentiation and must harden the tissue.

(ii) **Tissue Processing**

Processing of the tissue involves dehydration, clearing and embedding.

(a) **Dehydration**

Following fixation and washing of fixative with dH₂O, the tissue was passed through a series of graded alcohols (70%-100% alcohol) for 1 hr each time for the dehydration of the tissue.

(b) **Clearing**

After removing the ulcerated tissue from the last bath of absolute alcohol, the tissue was placed in two changes of benzene for 30 min. each. Benzene used as clearing agent removes alcohol and as it has high refractive index (1.5) so it also makes tissue transparent. After two changes of benzene, tissue was transferred to paraffin wax for impregnation.

(c) **Impregnation**

Paraffin wax used for impregnation was of high quality and was free from dust, dirt and water. It was melted and filtered. Paraffin was kept in embedding oven whose temperature was maintained 2°C above the melting point of the wax (45-60°C). The amount of wax taken was approximately 25-30 times of the volume of the tissue. After clearing, ulcerated tissue was kept in melted paraffin wax at 58-60°C for 3-4 hrs. This step is an important step in histopathology as infiltration with a supporting
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medium (paraffin wax) replaces clearing agent. This supporting medium makes tissue firm, facilitates easy sectioning and keeps various components in proper relation.

(c) Embedding or blocking

Embedding is the process of placing the impregnated tissue in a precisely arranged position into a mould containing the embedding medium and causing medium to solidify. The plastic mould was kept on a metal piece. Fresh molten wax was poured into the mould; tissue was lifted with the help of pre-heated forceps and pressed to the bottom, with the cutting surface facing downwards. The label bearing the number of the tissue specimen was fixed to the corner of the solidifying wax. Block was cooled and after that moulds were removed. The block was kept at 4°C for overnight before sectioning.

(iii) Sectioning / Microtomy

Sectioning of the blocks was done with the help of automated microtome (Leica), consisting of the adjustable steel knife. First of all, blocks were trimmed to remove surplus paraffin on the surface and the sides. The cutting surface was made even and parallel. This block was clamped in a clamp holder of the microtome. Steel knife was fixed in the knife holder with slight tilt to produce an angle of 5-8° C between the cutting edge and the block. Gauge was set to 6μm in order to get the serial sections of the same size. The sectioned ribbons of the tissue were placed in a waterbath (43-47°C). Pre-coated albuminized glass slide was immersed in that waterbath in such a way so that it can lift the ribbons on it. Slide was kept vertically till all the water was drained. Glass slide was marked with the help of diamond pencil. Slides were placed on the slide warmer.

(iv) Staining

Haematoxylin and eosin staining was done in order to stain various components of the tissue enabling a good morphological orientation. Haematoxylin stain is a cationic dye and is used to stain the anionic tissue compartment (nuclear portion). In contrast to nuclear staining, eosin is employed as counter stain to haematoxylin. Preparation of the stains is mentioned in Appendix I. Briefly, in staining procedure, slides were initially placed in Kopling jar carrying xylene for 5 min each twice to remove any wax, followed by dehydration with series of alcohol (100, 70, and 50 %) for 5 min. each. The slides were washed with running tap water
for 2 min. and then kept in haematoxylin for 90-120 sec. Again, the slides were washed with running tap water for 5 min so that extra stain can get removed. For counter staining, slides were immersed in Koping jars carrying eosin for 2 min. Slides were immediately dipped in acetone for 2-5 times and at last slides were kept in benzene plus acetone mixture before mounting.

(v) **Mounting**

Mounting procedure involves placing of the mounting media on the stained tissue section and covering it with cover slips. The most commonly used mounting media is Dibutylphthalate xylol (DPX, refractive index 1.52). A single drop of DPX was placed over the stained slide. The coverslip was placed in such a manner so that the DPX gets spread evenly and was pressed slightly to remove air bubbles.

(vi) **Microscopy**

The mounted slides were examined under stereomicroscope having different magnification of 10, 40, 100 and 200X of magnification. Examination of the stained slides was done by adjusting the eye lens and objective lens. Tissue contraction, regeneration of the ulcerated mucosa, formation of granulation tissue, glandular arrangement and inflammatory exudates were observed to study the extent of healing in the ulcerated tissue treated with different anti-ulcer drugs.

4. **Biochemical estimations**

Estimation of various biochemical parameters that either constitutes an offensive or a defensive factor was carried out both in gastric juice and mucosal scraping. Offensive factors estimated are free and total acidity and peptic activity while the defensive factors analyzed are various muco-protective substances of mucin. Other categories of biochemical parameters include anti-oxidant studies, total DNA content, PGE₂ and MPO activity.

**Collection of gastric juice and gastric scraping**

After 4 hrs of pyloric ligation, animals were sacrificed to collect the gastric juice, which was centrifuged for 5 min at 2000 rpm. Volume of the gastric juice was expressed as ml/100gm body weight. The mucosal scraping was taken from the
glandular portion of the stomach and was homogenized in normal saline (10%, w/v) to be used for various biochemical estimations.

**Biochemical estimations in gastric juice**

From the collected gastric juice, free and total acidity, total carbohydrate:protein ratio as a marker of mucin and peptic activity were measured. Preparations of all the chemicals used for the estimation of different biochemical parameter from gastric juice are mentioned in Appendix II

(a) **Free and total acidity**

Free and total acidity was estimated according to the method of Anoop and Jegadeesan, (2003). Briefly, 1 ml of gastric juice was pipetted into a 10 ml conical flask, 2-3 drops of Topfer’s reagent were added and titrated with 0.01 N NaOH (which was previously standardized with 0.01 N of oxalic acid) until all traces of the red color disappears and the color of solution becomes yellowish orange. The volume of alkali added was noted. This volume corresponds to free acidity. Then 2-3 drops of phenolphthalein solution was added and titration was continued until a definite red tinge reappears. Again the total volume of the alkali added was noted. This volume corresponds to total acidity. Acidity was calculated by using the formula

\[
\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{Normality of NaOH} \times 100 \text{ (meq/lit)}}{0.1}
\]

(b) **Peptic activity**

Peptic activity in gastric juice was carried out according to the method of Debnath et al. (1974) with few modifications. Briefly, for each determination, four tubes were taken. Tube (1) and (2) containing 5 ml of substrate i.e. 2% hemoglobin, while (3) and (4) containing 10 ml of TCA were placed in the water bath at 37°C. The gastric juice was mixed with an equal volume of HCl at pH 2.1, warmed to 37°C and 1 ml of mixture was added to each of the tubes - (1) and (4), incubated for 15 min. At the end, contents of tube (1) and (3) were mixed and allowed to stand in the bath for about 4 min. Contents of tube (1) and (3) constitutes “test” and contents of tube (2) and (4) gives “blank”. Both the contents were filtered after 25-30 min, 2 ml of filtrate
was pippeted into 10 ml of NaOH, mixed by gentle rotation. Then, 1 ml of phenol was added and again mixed by gentle rotation. After 30 min, the intensity of color was measured at 680 nm in spectrophotometer. The difference between test and blank gives a measure of peptic activity. As standard, 2 ml of freshly prepared phenol solution containing 50 μg/ml were mixed with 10 ml of NaOH and 1 ml of phenol reagent. After 5-10 min, the color intensity was measured at 680 nm and has been expressed in terms of IU.

(c) Mucoprotective activity

Mucosubstances activity considered as one of the most important defensive factor has been expressed as ratio of total carbohydrates to total proteins. Total carbohydrates were measured in terms of total hexoses, hexosamines and sialic acid.

Total carbohydrates

Method of Goel et al. (1985) was followed to estimate dissolved mucosubstances of the gastric juice in the alcoholic precipitate obtained by adding 90% alcohol in gastric juice in a ratio of 9:1. Briefly, the method consists of taking two aliquots of gastric juice, one of which was used to estimate concentration of total hexoses and hexosamine with the other one for estimating concentration of sialic acid.

In the first aliquot carrying 1 ml of gastric juice, 9 ml of 90% alcohol was added. The mixture was kept for 10 min before it was centrifuged. The supernatant was discarded. The precipitate was dissolved in 0.5 ml of 0.1 N NaOH. Then, 1.8 ml of 6 N HCl was added and the mixture was hydrolyzed in water bath at 100°C for 2 hr. The hydrolysate was neutralized by 5 N NaOH using phenolphthalein as indicator and the volume was made up to 4.5 ml with distilled water, which was used for the estimation of total hexoses and hexosamine as described below. To the other aliquot carrying 0.5 ml of gastric juice, 4.5 ml of alcohol was added. The mixture was shaken for 10 min and centrifuged to obtain the precipitate, which was then dissolved in 0.5 ml of 0.1 N H₂SO₄. This reconstituted solution was transferred to glass-stoppered tubes and then hydrolyzed in a water bath at 100°C for 1 hr. After hydrolysis, the volume was restored to 0.5 ml; 0.2 ml of this hydrolysate was used for the estimation of sialic acid.
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(i) **Hexoses**

Hexoses were estimated by the method of Winzler, 1950. According to this method, 3.4 ml of orcinol-H$_2$SO$_4$ reagent was added to 0.4 ml of hydrolysate and the mixture was then heated in the boiling water bath at 100$^\circ$C for 15 min. The mixture was cooled under running tap water and intensity of the color was recorded in spectrophotometer at 540 nm against the blank, where distilled water was used instead of hydrolysate. Total hexoses content was determined from the standard curve of D-galactose-mannose and has been expressed as µg/ml of gastric juice.

(ii) **Hexosamine**

Hexosamines were estimated by the method of Dische and Borentrend, 1950. About 0.5 ml of the hydrolysate fraction was taken in a glass-stoppered tube, to which 0.5 ml of acetyl acetone reagent was added. The mixture was heated in boiling water bath at 100$^\circ$C for 20 min, and then cooled under running tap water. About 1.5 ml of 95% ethanol and 0.5 ml of Ehrlich’s reagent was added and waited for 30 min. The color intensity was measured in spectrophotometer at 530 nm against blank prepared by using distilled water instead of hydrolysate. Hexosamine content was determined from the standard curve prepared by using D(+-)-glucosamine hydrochloride and concentration has been expressed in µg/ml of gastric juice.

(iii) **Sialic acid**

Sialic acid was measured according to the method of Warren (1959). About 0.5 ml of the hydrolysate in 0.1 N H$_2$SO$_4$ was taken, to which 0.2 ml of sodium periodate was added and mixed thoroughly by shaking. A time of 20 min was allowed to elapse before addition of 1 ml of sodium arsenite solution to this mixture. The brown color produced disappeared after shaking. Then, 3 ml of thiobarbituric acid was added and the mixture was heated in boiling water bath for 15 min. After cooling the tubes, 4.5 ml of cyclohexanone was added and thorough shaking was done for 15s till all the color was taken up by the cyclohexanone supernatant. The mixture was centrifuged at 2000 rpm for 10 min to get a clear pink layer of cyclohexanone. This supernatant was pippeted out and intensity of color was measured in spectrophotometer at 550 nm. The sialic acid content of the sample was determined
from the standard curve of N acetyleneuraminic acid and has been expressed in terms of μg/ml of gastric juice.

After obtaining the concentration (μg/ml) of individual carbohydrates namely hexoses, hexosamine and sialic acid, the total carbohydrate content was calculated by adding the concentration of individual carbohydrates.

**Total proteins**

The dissolved protein in gastric juice was estimated in the alcoholic precipitate obtained by adding 90% alcohol with gastric juice in 9:1 ratio as described by Lowry et al. (1951). According to this method, 0.1 ml of alcoholic precipitate of gastric juice was dissolved in 1 ml of 0.1 N NaOH and from this volume, 0.05 ml was taken in another test tube. 4 ml of alkaline mixture was further added and kept for 15 min. Finally 0.4 ml of phenol reagent was added and again kept for 30 min to allow color development. Reading was taken for the intensity of color in spectrophotometer at 750 nm against blank prepared with distilled water. The protein content was calculated from standard curve prepared with bovine serum albumin and was expressed in terms of μg/ml of gastric juice.

Mucoprotective activity was then measured by taking the ratio of combined concentration of carbohydrates (hexoses, hexosamine and sialic acid) and total dissolved proteins.

**Biochemical estimation in gastric scrapping**

Scraping of gastric mucosa were analyzed for different biochemical estimations like total DNA content as an indicator of cell shedding, MPO activity to determine the neutrophil infiltration, anti-oxidant activities of various enzymes and PGE2 level. Chemical preparations for various biochemical estimations carried out in gastric scrapping are mentioned in Appendix III

(a) **Total DNA content**

**Sample preparation**

Gastric mucosa was homogenized in 0.6 N perchloric acid (PCA) as per the method of Goel et al. (1986). Homogenate was centrifuged to collect the precipitate,
which was washed twice with 0.2 N PCA. Then, 2 ml of 0.3 N KOH was added to precipitate and incubated for 30 min at 37°C. After repeating this step, 2.5 ml of 1.2 N ice cold PCA was added and centrifuged. Precipitate was washed twice with 5 ml of ice cold 0.2 N PCA and again centrifuged to collect the precipitate, to which 1 ml of 0.5 N PCA was added. The mixture was kept at 90°C for 30 min and centrifuged. Supernatant was collected and DNA was estimated from this supernatant.

**Estimation of total DNA content**

1 ml of supernatant obtained was mixed with 2 ml of diphenylamine solution. The mixture was boiled at 100°C for 10 min. Intensity of color was determined by taking reading in spectrophotometer at 595 and 700 nm. DNA concentration was determined from a standard curve prepared with calf thymus DNA and was expressed as μgm/100mg tissue.

(b) **Myeloperoxidase (MPO) estimation**

**Sample preparation**

The ulcerated tissue of the treated group was homogenized in 5 volumes of hexadecyl trimethyl ammonium bromide buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0). The homogenate was subjected to sonication for 15s followed by centrifugation at 2400 rpm at 4°C. The supernatant was shock freezeed and thawed. Protein was estimated in the supernatant by the method of Lowry et al. (1951). Myeloperoxidase (MPO) activity was assessed as a marker of neutrophil infiltration by the method of Gilbert et al. (1974).

**Estimation of MPO activity**

A sample of 100 μl was mixed with 1 ml of 0.22% aqueous guaiacol and 2 ml of 10 mM phosphate buffer (pH 6.0). 20 μl of 0.3% hydrogen peroxide was added to start the reaction. Absorbance at 470 nm was recorded with a Beckman spectrophotometer at 15 sec intervals for 2 min. As a standard, 100 μl of horseradish peroxidase (0.975 U/ml) was combined with 1ml of guaiacol, 2 ml of 10mM phosphate buffer (pH 6) and 5, 10 and 20 ul of 0.3% hydrogen peroxide was added to start the reaction. The absorbance change at 470 nm for 1μmole peroxide/ min was
calculated from the standard curve, which equals 1 unit of MPO activity. Results were expressed as MPO/mg tissue protein.

(c) Free radical generation study

Sample Preparation

The fundic part of the stomach was homogenized (5% w/v) in ice cooled 0.9% saline with a glass homogenizer for 30 s. The homogenate was centrifuged for 800 g for 10 min and the supernatant was again centrifuged at 12,000 g for 15 min to get the mitochondrial fraction. The post mitochondrial supernatant was centrifuged at 1,00,000 g for 1 hr to get the microsomal fraction. Anti oxidation reaction of lipid peroxidation (LPO) and levels of various antioxidant enzymes namely superoxide dismutase (SOD) and catalase (CAT) were estimated in this fraction.

(i) Lipid peroxidation (LPO)

Lipid peroxidation product malondialdehyde (MDA) was measured by the method of Okhawa et al. (1979). The method involves the production of thiobarbituric acid reactive substances (TBARS) at pH 3.5. In each tube, 0.5 ml of standard, 0.2 ml SDS (4%), 1.5 ml acetic acid (20% made in 0.27 M HCl) and 1 ml of thiobarbituric acid (0.8%) were added and volume was made upto 5 ml with distilled water. The tube was heated in water bath at 80°C for 1 hr. when pink color gets developed. The tube was centrifuged and the absorbance was taken at 532 nm. The amount of MDA was expressed in nmole/mg using 1,1,3,3 tetraethoxypropane as an external standard.

(ii) Superoxide dismutase (SOD)

SOD activity was determined according to the method of Misra and Fridovich (1972). Tissue extract (100 µl) was added to 880 µl carbonate buffer of 0.05 M, pH 10.2, 0.1 mM EDTA. Then, 20 µl of 30mM epinephrine in 0.05% acetic acid was added to the mixture and absorbance was recorded for 4 min at 480 nm in a spectrophotometer. The amount of enzyme that results in 50% inhibition of epinephrine auto oxidation is defined as one unit (U) of SOD.
(iii) **Catalase (CAT)**

CAT activity was determined by the method of Aebl et al. (1984). In a cuvette containing 200 µL of phosphate buffer and 50 µL of tissue extract, 250 µL of 0.066 M H$_2$O$_2$ in phosphate buffer was added and decrease in absorbance was recorded at 240 nm for 30 sec in a spectrophotometer. A molar absorbitivity of 43.6 M/cm was used to determine CAT activity, one unit (U) of which is equal to the 1 mole of H$_2$O$_2$ degraded per minute per mg protein.

(c) **Prostaglandin (PGE$_2$) estimation**

Prostaglandin (PGE$_2$) estimation was carried out to determine the levels of PGE$_2$ in normal and ulcerated mucosa of different treated groups. Enzyme immuno assay kit for prostaglandin estimation (Cayman chemicals), based on Competitive Enzyme linked Immuno assay (EIA) was used to estimate the levels of PGE$_2$

(i) **Basic principle involved in EIA**

Competitive enzyme immuno assay (EIA) involves estimation of a free PGE$_2$ based on the competition between PGE$_2$ and PGE$_2$-acetylcholinesterase (AchE) conjugate (PGE$_2$ tracer) for a limited amount of PGE$_2$ monoclonal antibody. Because the concentration of the PGE$_2$ tracer is held constant while the concentration of PGE$_2$ varies, the amount of PGE$_2$ tracer that is able to bind to the PGE$_2$ monoclonal antibody will be inversely proportional to the concentration of PGE$_2$ in the wells. This antibody-PGE$_2$ complex binds to goat polyclonal antimouse IgG that has been previously attached to the wells. When a substrate of AchE (Ellman's reagent in Cayman's kit) is added to the wells, the enzymatic reaction produces a product that has a distinct yellow color, which absorbs strongly at 412 nm. The intensity of this color determined spectrophotometrically and is proportional to the amount of PGE$_2$ tracer bound to the well, which is inversely proportional to the amount of free PGE$_2$ present in the wells during the incubation (**Figure 4.2**).
Figure 4.2: Basic principle involved in competitive enzyme immuno assay (EIA)

(a) Wells precoated with Goat polyclonal antibody; (b) Incubation of tracer, antiserum, and samples carrying free \( \text{PGE}_2 \); (c) Bounded reagent with antibody; (d) Developed well with substrate of \( \text{AchE} \) producing yellow color.

(ii) Sample preparations

The gastric mucosa of different treated group was excised and homogenized in an ice-cold Tris/HCl buffer containing 50mM Tris/HCl (pH 7.4), 100mM NaCl, 1 mM CaCl\(_2\), 1mg/ml D-glucose and 28\( \mu \)M indomethacin according to the method of Guo et al. (2002). The protein concentration of homogenate was measured by the method of Lowry et al. (1951). Homogenate was centrifuged at 12,000g for 30 min at 4\(^\circ\)C for the determination of \( \text{PGE}_2 \) concentration. The concentration of \( \text{PGE}_2 \) present in the supernatant was measured with \( \text{PGE}_2 \) EIA kit. Results are expressed as nanograms of \( \text{PGE}_2 \) per mg of protein.
(iii) **Preparation EIA specific reagents**

Reagents required for EIA namely, PGE₂ standards, PGE₂ AchE tracer, PGE₂ monoclonal antibody, wash buffers and EIA buffers were freshly prepared according to the manufacturer’s instructions. Details of reagent preparations are mentioned in Appendix- III.

(iv) **Plate setup**

A 96 well antibody coated plate was supplied with the EIA kit. The wells are loaded with different samples and reagents according to the scheme provided manufacturers that includes wells for samples, blanks (Blk), standard (S1-8), total activity (TA), non-specific binding (NSB) and maximum binding (Bₒ). Figure 4.3 shows the sample plate format.

**Figure 4.3:** Sample plate format for EIA based PGE₂ estimation

(v) **Estimation of PGE₂ in samples**

Different reagents and samples were added in each of the well of the EIA plate shown in Figure 4.3. The specific reagent and its quantity added in respective wells are shown in Table 4.2. After addition of the reagents and the samples, plate was covered with a plastic film and was incubated at 4°C for 18 hrs.
**Materials & Methods**

Table 4.2: Reagents added in different wells of EIA plate

<table>
<thead>
<tr>
<th>Wells</th>
<th>Reagents/samples</th>
<th>EIA buffer</th>
<th>Standard or sample</th>
<th>Tracer</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blk</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA</td>
<td>-</td>
<td>-</td>
<td>5 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NSB</td>
<td>100 μl</td>
<td>-</td>
<td>50 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B₀</td>
<td>50 μl</td>
<td>-</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>Standard/sample</td>
<td>-</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

(vi) Development of the plate

Wells were emptied and rinsed with wash buffer for five times. 200 μl of Ellman’s reagents was added to each well and plate was covered with plastic film. For development of the color, plate was kept in dark on orbital shaker for 60-90 mins. Plates were read at 412 nm in ELISA plate reader.

(vii) Calculation of the results

Calculations for the free the free PGE₂ in the samples was carried out according to following procedure:

1. Absorbance reading from NSB wells were averaged (NSBavg)
2. Absorbance reading from B₀ wells were averaged
3. NSB average was subtracted from B₀ average. This gives corrected B₀
4. Percent B/B₀ (% sample or standard bound/Maximum bound) was calculated for the remaining wells according to the following formula:

\[
\%B/B₀ = \frac{\text{Absorbance of } S1 - \text{NSB avg}}{\text{Corrected } B₀} \times 100
\]

%B/B₀ was plotted for standards S1-S8 versus PGE₂ concentration (in pg/ml). PGE₂ in the samples was calculated from the plotted standard curve.

(d) Proton pump estimation

**Microsomal preparation**

Gastric mucosa was scrapped off with the help of glass slide on the ice. Mucosal scrapping was used for the preparation of microsomes by differential (density
Materials & Methods

gradient) centrifugation by the method of Zuo et al. (1991). Gastric mucosal scrapping were homogenized using ultra Turrax homogenizer in chilled HEPES/tris buffer (2 mM, pH 7.4) containing 0.25 M sucrose, EDTA (1 mM), PMSF (5 μg/ml) and leupeptin (2 μg/ml). Homogenate was centrifuged at 800 g for 10 min at 4°C and the supernatant thus obtained was re-centrifuged at 10,000 g for 30 min at 4°C. This supernatant (10 ml) was layered over 1 ml of 1 M sucrose in HEPES/tris buffer and centrifuged at 1,00,000 g for 30 min at 4°C using swing bucket rotor of ultra centrifuge. Microsomal pellet thus obtained was washed with HEPES/tris buffer. The microsomes obtained were used for evaluating H⁺ transport assay.

Vesicular H⁺ transport assay

Intact gastric microsomal vesicles were used for vesicular H⁺ transport as described by Lee and Forte (1978) with few modifications. Microsomal pellet was homogenized with the help of glass homogenizer in acidification buffer containing 20 mM HEPES, 150 mM KCl and 5 mM MgSO₄ (pH 7.4) and was subsequently passed 3-4 times through a 26-gauge needle to form homogenous suspension. 1 ml of the assay system contained 10-15 μg protein (enzyme suspension) in acidification buffer (pH 7.4), 10 μM valinomycin and 2.5 μM acridine orange. Alteration in acridine orange fluorescence at excitation wavelength 493 nm and emission wavelength 530 nm (slit width λex = 5 nm; λem = 3 nm) were continuously monitored for 10 min on spectrofluorometer following addition of ATP (5 mM). Effect of drugs has been reported as percent inhibition of enzyme activity in comparison to control taken as 100%.

5. Expression studies

Differential expression level of mRNA and protein COX-2 enzyme were analyzed for each category of drug. RNA expression analysis was carried out by reverse transcriptase PCR (RT-PCR) while expression levels of COX-2 protein was estimated by western blotting. At the same time, expression level of various growth factors namely EGF, bFGF and VEGF was also evaluated by western blot analysis.
Materials & Methods

(a) **m-RNA expression analysis of COX-2 transcript**

Expression analysis of COX-2 transcript was carried out using Reverse-transcriptase PCR (RT-PCR), in which a housekeeping gene - β-actin was used as a control. m-RNA expression analysis involves following steps:

1. RNA isolation by TRIZOL™ reagent method
2. Gel electrophoresis of RNA for quality check
3. Two step RT-PCR:
   (i) Synthesis of single stranded complementary DNA (cDNA) from total cellular RNA using reverse transcriptase COX-2 gene expression was analyzed using COX-2 RNA specific primers.
   (ii) Amplification of cDNA COX-2 and β-actin gene (control) using their specific primers
4. Size fractionation of the amplicons on Agarose gel electrophoresis
5. Intensity of amplicons for COX-2 and β-actin gene mRNA were measured.
6. Ratio of the intensity of COX-2/ β-actin amplicons was calculated based on the densitometry data of their band analysis respectively

Details of the steps involve in RNA isolation and RT-PCR for COX-2 gene and β-actin gene are as follows:

(iii) **RNA isolation (from “TRIZOL™” reagent)**

Total cellular RNA was extracted from ulcerated mucosa using TRIZOL™ reagents and Di-ethyl pyrocarbonate (DEPC) water, which was used as an inhibitor of RNAse (Appendix IV). First of all, homogenized tissue (50 – 100 mg) was taken in a 2 ml DEPC treated eppendorf tube and 1 ml of TRIZOL reagent was added. Repetitive pipetting was done to lyse the tissue. Tube was then incubated for 5 min at 15-30° C, after which 200 µl chloroform (0.2 ml / 1ml Trizol) was added. Tube was shaken vigorously for 15-30 sec and left for 2-3 min at 15-30° C. Reaction mixture was centrifuged at 12,000 rpm for 15 min at 4° C and upper layer (colorless aqueous phase) was taken in a new eppendorf tube, to which 500 µl of iso-propanol was added and was incubated for 10 min at 15-30° C. Contents were centrifuged at 12,000 rpm for 10 min at 4° C, supernatant was discarded and 1 ml of 75% ethanol was added. After centrifuging at 7500 rpm for 5 min, pellet was briefly air dried for 5-10
min and was dissolved in RNase free water. Dissolved RNA was incubated at 55-60°C for 10 min. and stored at -70°C till further analysis was carried out.

(iv) Agarose gel electrophoresis of RNA molecule

Quality check of the extracted RNA molecule was carried out on 1% formaldehyde agarose gel. At first, gel casting tray, combs and electrophoresis tank were washed with DEPC treated water. Composition of gel and RNA sample is shown in Table 4.3.

Table 4.3 Composition of agarose gel and RNA sample for agarose

<table>
<thead>
<tr>
<th>1% Formaldehyde agarose gel</th>
<th>RNA sample</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>2 gms RNA (up to 20 μg)</td>
<td>4.7 μl</td>
</tr>
<tr>
<td>DdH2O</td>
<td>170 ml 5X MOPS buffer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>10X MOPS buffer</td>
<td>20 ml 37% formaldehyde</td>
<td>3.3 μl</td>
</tr>
<tr>
<td>Pre warmed 37% formaldehyde</td>
<td>11 ml Formamide</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

All the gel constituents were mixed and heated to mix the agarose. Once the gel solution was prepared, it was kept at 55-65°C until gel was casted. After casting the gel in 7.5x10 cm long casting tray with 8 wells comb, RNA sample was prepared as shown in table 4.4 and was denatured at 55°C for 5 min. To this sample, 2 μl of 10X loading buffer (Appendix IV) was added and the sample was loaded in respective wells. Hind III λ digest ladder was also run as a DNA marker. Electrophoresis was carried out in 0.5X TBE buffer at 5V/cm for 1 hr. Gel was then stained by ethidium bromide and visualized under UV transilluminator. Two sharp bands corresponding to 28S RNA (4.7kb) and 18S RNA (1.9 kb) along with a faint band of tRNA were appeared in the gel. (Figure 4.4)

Figure 4.4 RNA molecule electrophoresed on 1% formaldehyde agarose gel

Lane1: Hind III λ digest ladder
Lane 2: degraded RNA sample
Lane 3: Intact RNA molecule comprising of 28S RNA (4.7kb) and 18S RNA (1.9 kb) bands
(iii) **Two step RT-PCR**

Two-step RT PCR was carried out to estimate the expression of COX-2 transcript. In the first step, single-stranded complementary DNA (cDNA) was prepared using reverse transcriptase enzyme and oligo DT primers from QIAGEN cDNA synthesis kit. PCR reaction mix and PCR conditions for the first PCR are mentioned in Table 4.4. The reverse transcriptase enzyme used was Moloney murine leukemia virus reverse transcriptase. RNA molecule was first uncoiled at 65°C for 5 min followed by snap cool in ice. Then, all other PCR reagents were added and PCR was carried out in MJ-Research automated thermo-cycler as described by Konturek et al. (2003).

**Table 4.4: PCR reaction mix and PCR condition for 1st PCR of cDNA synthesis**

<table>
<thead>
<tr>
<th>Reaction Mix (30ul)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT buffer (10 mM Tris/HCl, pH 8.3, 50 mM)</td>
<td>2 µl</td>
</tr>
<tr>
<td>KCl, 5 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td>Oligo DT primer mix (0.3 µg/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTPs mix (5 mM each)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Reverse transcriptase (4U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNAse (40 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNA (50 ng-2 µg)</td>
<td>14 µl</td>
</tr>
<tr>
<td>HPLC grade water</td>
<td>9 µl</td>
</tr>
</tbody>
</table>

In the 2nd PCR 2µl product of the 1st PCR was taken as template. Amplification was carried out by normal PCR using Taq polymerase and flanking primers specific for COX-2 gene and β-actin gene used a control for transcript expression. Table 4.5 shows the primer sequences for COX-2 and β-actin genes as mentioned in the reports of Shigeta et al. (1998) and Brzozowski et al. (1999).

**Table 4.5 Primer sequence for COX-2 gene and β-actin gene for mRNA based RT-PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COX-2 gene</strong></td>
<td></td>
</tr>
<tr>
<td>Sense primer</td>
<td>5'- TGA TGA CTG CCC AAC TCC CAT G 3'</td>
</tr>
<tr>
<td>Anti sense primer</td>
<td>5'- AAT GTT GAA GGT GTC CGG CAG C 3'</td>
</tr>
<tr>
<td><strong>β-actin gene</strong></td>
<td></td>
</tr>
<tr>
<td>Sense primer</td>
<td>5'- TTG TAA CCA ACT GGG ACG ATA TGG 3'</td>
</tr>
<tr>
<td>Anti sense primer</td>
<td>5'- GAT CTT GAT CTT CAT GGT GCT AGG 3'</td>
</tr>
</tbody>
</table>
Materials & Methods

PCR reaction mix and PCR conditions for 2nd PCR are mentioned Table 4.6. Primers for both COX-2 gene and β-actin gene were added in the same tube along with other PCR constituents.

Table 4.6: PCR reaction mix and PCR condition for 2nd PCR of RT-PCR analysis

<table>
<thead>
<tr>
<th>Reaction Mix (20ul)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq PCR buffer (10 mM Tris/ HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂)</td>
<td><strong>Initial Denaturation</strong> 94°C for 1 min</td>
</tr>
<tr>
<td>Sense and anti sense primer (0.3 μg/μl)</td>
<td>40 cycles of</td>
</tr>
<tr>
<td>dNTPs ( dGTP, dATP, dTTP, dCTP -10 mM each)</td>
<td>94°C for 30 sec</td>
</tr>
<tr>
<td></td>
<td>55°C for 45 sec</td>
</tr>
<tr>
<td></td>
<td>72°C for 1 min</td>
</tr>
<tr>
<td>Taq Polymerase (3 U/μl)</td>
<td><strong>Final extension</strong></td>
</tr>
<tr>
<td>c-DNA (50 ng-2μg)</td>
<td></td>
</tr>
<tr>
<td>HPLC grade water</td>
<td></td>
</tr>
<tr>
<td>2 μl</td>
<td>72°C for 10 min</td>
</tr>
<tr>
<td>0.5μl each</td>
<td></td>
</tr>
<tr>
<td>0.25μl each</td>
<td></td>
</tr>
<tr>
<td>1 μl</td>
<td></td>
</tr>
<tr>
<td>2 μl</td>
<td></td>
</tr>
<tr>
<td>9 μl</td>
<td></td>
</tr>
</tbody>
</table>

(iv) Visualization of RT-PCR products

Amplicons of 2nd PCR were detected by electrophoresis on 2% agarose gel containing ethidium bromide. Location of predicted products- 702 bp for COX-2 gene and 764 bp for β-actin gene were confirmed by using 100 bp ladder (Invitrogen). The gel was then photographed under UV trans-illumination.

(v) Estimation of COX-2 mRNA expression

The intensity of both the amplicons of 2nd PCR was measured using video image analyzer system (Alphamager v 2.4). The signals for examined COX-2 mRNAs were standardized against that of β-actin mRNA from each sample and the results were expressed as COX-2 mRNA/β-actin mRNA ratio.

(b) Protein expression analysis

Analysis of differential expression of proteins- COX-2, EGF, bFGF and VEGF during healing of ulcers was performed on Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) followed by western blot analysis. Chemical preparations for protein expression studies are mentioned in Appendix IV. The protein expression studies based on western blotting involves the following steps:
Materials & Methods

1. Protein estimation by the method of Lowry et al. (1951) in the normal and ulcerated samples.
2. Polyacrylamide gel electrophoresis (PAGE) of the protein.
3. Western blotting (transfer of bands from gel to nitrocellulose membrane (NCM)).
5. Hybridization of transferred protein with specific antibody.
6. Detection of hybridized protein with enhanced chemiluminescence system.
7. Interpretation of signals.

The detailed procedures of each step carried out in protein expression analysis of COX-2 protein are discussed below. Basic outline of protein expression analysis is shown in Figure 4.5.

Figure 4.5: Western blot analysis of protein fragments
A- Western blot analysis of COX-2

(i) Sample preparation and protein estimation

Tissue from ulcer area, including ulcer margin and the remaining region i.e. non-ulcerated mucosa were scrapped with a glass slide and frozen immediately in liquid nitrogen as described by Konturek et al. (2003). Samples of the stomach were taken from all treated groups and were homogenized in phosphate buffer saline (pH 7.4) containing 1% nonidet-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. Homogenized sample were spun at 12,000 g at 4°C for 15 min and the total protein concentration in the supernatant was measured with the method of Lowry et al. (1951).

(ii) Polyacrylamide gel electrophoresis (PAGE)

Proteins were size fractionated on SDS-PAGE. Gels of 1.5 mm thickness were casted by adding 5% stacking and 12% resolving polyacrylamide in a vertical mini protein electrophoresis apparatus (Amersham Biosciences). Table 4.7 describes the composition of stacking and resolving gel (12 and 15%) casted for protein fractionation. 60 μgm of protein in supernatants of different samples was mixed with loading buffer and were denatured in boiling water bath for 8 min. The samples were quickly spun at 10,000 g to settle down, if any debris. Each sample having 60 μgm of protein was loaded into separate lanes and run at constant voltage (80 V) for two and half hours. Figure 4.6 shows different proteins size fractionated on PAGE.

Table 4.7: Composition of the stacking and resolving gels used for COX-2 expression analysis

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Composition</th>
<th>5% Stacking gel (5 ml)</th>
<th>12% Resolving gel (10 ml)</th>
<th>15% Resolving gel (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30% acrylamide solution</td>
<td>0.83 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>2</td>
<td>Separating/stacking gel buffer</td>
<td>0.63 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>3</td>
<td>Distilled water</td>
<td>3.4 ml</td>
<td>3.3 ml</td>
<td>2.3 ml</td>
</tr>
<tr>
<td>4</td>
<td>Ammonium per sulphate (10%)</td>
<td>0.05 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>5</td>
<td>TEMED</td>
<td>0.005 ml</td>
<td>0.004 ml</td>
<td>0.004 ml</td>
</tr>
<tr>
<td>6</td>
<td>SDS (10%)</td>
<td>0.05 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>
Figure 4.6: Gel picture showing mixture of protein size fractionated on SDS-PAGE with LMW protein marker

Lane 1: Low molecular weight protein marker
Lane 2-6: Protein samples from stomach

Standard low molecular weight (LMW) marker protein mixture was also run in SDS-PAGE along with sample proteins to determine the relative molecular weights of corresponding bands in samples. For assessment of COX-2 bands in western blot analysis, pure ovine COX-2 protein was also run along with the samples.

(iii) Western blotting

The electrophorased proteins were transferred to NCM using a semi-dry transfer cell (Hofer). A NCM piece of 8 cm X 8 cm. in size was cut. Membrane was pre-wetted in ddH₂O for 10 min. followed by 10 min in Towbin buffer (transfer buffer). Six filter papers (3 mm) of 8.5 X 8.5 cm were also pre-wetted in Towbin buffer for 10 min. Three Whatman filters were placed in transfer unit of western blotting, on which NCM was placed by draining excess of buffer. Membrane was handled with blunt ended forceps. Air bubble was gently removed by roller. Gel was kept exactly above the NCM and above it three more filter papers were kept. The whole sandwich is kept in between the anode and the cathode of the transfer unit. Process of transfer was started at constant current, setting voltage as 0.8 V per cm² of the NCM and was ended after 2 hrs. The transferred proteins were confirmed by using Ponceau- S stain. This stain gets removed by washing the NCM with dH₂O.
Materials & Methods

(iv) Blocking of the NCM

The membrane was blocked with blocking solution containing PBS, 5% non-fat milk and 0.1% tween-20 for overnight at 4°C. Blocking was done to block non-specific binding sites.

(v) Hybridization

The conditions of hybridization i.e. the dilution of primary and secondary antibody, incubation time and washing of hybridized product were optimized for COX-2 protein. NCM after blocking was then rinsed with two changes of wash buffer (PBST) in order to remove excess of blocking solution. Membrane was then incubated with primary antibody (1:200) for COX-2 (in PBST) for 2 hrs at room temperature, which is then washed with PBST for 4 times of 15 min each. The horse reddish peroxidase linked mouse anti goat IgG antibody was used as secondary antibody (1:8000), which is again thoroughly washed with PBST for 4 times of 15 min each.

(vi) Detection of Hybridized protein

The membrane was than exposed to luminol reagent in ECL kit (Solution A: Solution B in 40: 1 ratio) according to size of membrane (0.1 ml/cm²). Membrane was incubated with ECL solution for 5 min. Excess of detection reagent was drain off by holding the membrane gently with forceps and touching the edge against a tissue. The membrane was wrapped in a saran wrap and was exposed to X-ray film in a hypercassette for 2 min. Exposed X-ray film was developed with Kodak developer and was fixed in fixer.

(vii) Interpretation of signals

For every blot, known amount of COX-2 positive control was included. Quantitation of density of protein band was carried out by a video densitometer.

B- Western blot analysis of Growth factors

Differential expression analysis was carried out for three growth factors namely EGF, bFGF and VEGF using western blot analysis.

(i) Sample preparation and protein estimation

Expression pattern of different growth factors were studied as described by Luo et al. (2004). All treated stomach samples i.e. ulcerated tissue area and non-
Materials & Methods

Ulcerated mucosa were scrapped with a glass slide and frozen immediately in liquid nitrogen. Sample of the stomachs were taken from all treated groups and were homogenized in a polytron homogenizer with radio immuno precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% α-cholate, 2 mM EDTA, 0.1% Triton-X, 10 % glycerol) with specific concentration of protease inhibitors (100 μg/ml of PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin). Homogenized sample were spun at 15,000 g at 4°C for 15 min and the total protein conc in the supernatant was measured with the method of Lowry et al. (1951).

(ii) Gel electrophoresis

Gel electrophoresis for growth factors was performed as mentioned earlier, but with 15% resolving gel. 60 μgm of the protein was used for the expression studies of growth factors. The composition for the gel used is shown in Table 4.7

(iii) Western blotting

The transfer process of protein band to NCM is exactly similar to that carried out in COX-2 western blotting. After blocking, the respective membranes were probed with antibodies against EGF (1:200), bFGF (1:200) and VEGF (1:200) in PBST for 2 hrs. The membranes were incubated with respective secondary antibodies (1:6000), linked with peroxidase. Immunodetection was performed with ECL system as described in detection of COX-2 blots. Quantitation of the protein blots was carried out by a video densitometer.

4.4 Statistical analysis

All the statistical calculation was performed using Instat Graph-pad version 3.05 and Prism version 3.1 statistical software packages. All values are expressed as mean ± S.E.M. Data was evaluated by One-Way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. One-way analysis of variance (ANOVA) was performed to test whether the mean of a variable differs among groups. Dunnett's test was performed to analyze to compare control data with all other columns but not to each other. "P" value <0.05 was considered significant.