Study Design
The present study aims to deduce the role of COX-2 enzyme in normal and drug mediated ulcer healing along with other potent mechanisms that act in healing process. The study has been designed in light of following objectives:

3.1 Objectives of the present study

a) To study the healing effect of standard anti-ulcer drugs (omeprazole, ranitidine, sucralfate and misoprostol) in acetic acid induced chronic gastric ulcer model with celecoxib as a negative control.

b) To study the extent of healing by histopathology in rats treated with different anti-ulcer drugs.

c) To study the effects of different drugs on lipid peroxidation and level of antioxidant enzyme like catalase, superoxide dismutase.

d) To study the enhanced healing mechanism by COX-2 with following parameter: free and total acidity, pepsin activity, extent of proton pump inhibition, total carbohydrate: protein ratio (sum of hexoses, hexosamine and sialic acid: protein ratio), total DNA content and myeloperoxidase activity.

e) To study the effect of different drugs on PGE₂ level using competitive Enzyme immuno assay (EIA).

f) To study the expression level of COX-2 m-RNA transcript and protein in acetic acid induced chronic gastric ulcer model, treated with different anti-ulcer drugs along with selective COX-2 inhibitor (celecoxib) as a negative control.

g) To study the expression pattern of growth factors like epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in rats treated with different anti-ulcer drugs.
3.2 Experimental design for the study:

The study has been structured into two parts. Initially, a pilot study has been carried out for all the five drugs Omeprazole (OMZ), Ranitidine (RANI), Sucralfate (SUC), Misoprostol (MISO) and Celecoxib (CELE) to deduce the most effective dose and most effective time period. In the second phase different biochemical estimations, expression analysis and other parameters effecting ulcer healing were examined for each category of drug taking the most effective dose and time period respectively.

**Pilot Study:**

Pilot study has been carried out to evaluate the most effective dose by using graded doses of all the above drugs and most effective time period of drug treatment that achieves maximum ulcer healing for four of the drugs namely omeprazole, ranitidine, sucralfate and misoprostol, while similar work was done in case of celecoxib (negative control) to determine the most effective dose and most effective time period for tissue damaging.

**Experimental Study:**

All the experiments have been carried out in seven sets each corresponding to one of the experimental groups- Control (vehicle treated), Control (untreated), OMZ, RANI, SUC, MISO and CELE. At the first step, chronic gastric ulcers were induced by serosal application of acetic acid followed by a drug treatment of the most effective dose of each drugs as deduced from the pilot study. Subsequently, on the last day of the drug treatment, pyloric ligation was done to collect the gastric juice and the animals were sacrificed. Gastric juices and ulcer tissues were then excised for further examinations.

Ulcer healing is measured by comparing the ulcer area and histopathological examinations, revealing different patterns of mucosal reconstruction. Being the primary objective of the study, expression level of COX-2 m-RNA was estimated by reverse transcriptase PCR (RT-PCR), while that of COX-2 protein was estimated by western blot analysis. In addition expression level of different growth factors namely-
EGF, bFGF and VEGF was also measured using the same technique of western blotting. PGE₂ level in both ulcerated and non-ulcerated area was measured by EIA.

Various other factors which have their role either in causation or in healing of ulcers were also evaluated for each category of drug. Free and total acidity, pepsin, total carbohydrate, total protein, and their ratio (TC: TP) as a measure of mucin content has been evaluated from gastric juice. Myeloperoxidase (MPO) activity as a marker of neutrophil infiltration and the enzyme activity of various anti-oxidant enzymes that acts against the free radicals like lipid peroxidation, superoxide dismutase and catalase has been measured from ulcerated tissue.

Finally, an elaborated comparison was done for each of these factors on ulcer healing separately as well as cumulatively for each category using different statistical packages and an inference has been drawn regarding the possibility of COX-2 enzyme being a major determinant in both drug mediated and normal ulcer healing. A broad outline of whole of the study setup has been mentioned in Figure 3.1 (a-d).
Figure 3.1: Scheme of Experimental design in the present study

(a) Induction of ulcers and evaluation of Ulcer healing

1. **Induction of chronic gastric ulcer with acetic acid**
2. **Drug treatment from 3rd day**
3. **Pyloric ligation on last day of drug treatment**
4. **Animals were sacrificed after 4 hrs of pyloric ligation**

- **Gastric Juice**
  - **Biochemical estimation**
  - **Measurement of ulcer area**
  - **Histopathological Examination**
  - **Expression Analysis**

- **Ulcerated Tissue**
  - **Determination of ulcer healing**
Study Design

Figure 3.1: Scheme of Experimental design in the present study

(b) Biochemical estimations

Induction of chronic gastric ulcer with acetic acid

↓

Drug treatment from 3rd day

↓

Pyloric ligation on last day of drug treatment

↓

Animals were sacrificed after 4 hrs of pyloric ligation

Gastric Juice

↓

Ulcerated Tissue

→ Free and total acidity

→ Peptic activity

→ Total proteins

→ Total carbohydrates

→ Hexoses

→ Hexosamines

→ Sialic acid

Proton pump inhibition

Total DNA content

Myeloperoxidase activity

Prostaglandins E2 estimation

Lipid peroxidation

Anti-oxidants

Superoxide dismutase

Catalase
Figure 3.1: Scheme of Experimental design in the present study

(c) Expression analysis

Induction of chronic gastric ulcer with acetic acid

\[ \downarrow \]

Drug treatment from 3rd day

\[ \downarrow \]

Pyloric ligation on last day of drug treatment

\[ \downarrow \]

Animals were sacrificed after 4 hrs of pyloric ligation

Gastric Juice

\[ \downarrow \]

m-RNA expression

\[ \downarrow \]

COX-2

Ulcerated Tissue

\[ \downarrow \]

Protein expression

\[ \downarrow \]

COX-2

Growth factors

\[ \rightarrow \]

Epithelial Growth Factor (EGF)

\[ \rightarrow \]

Basic Fibroblast Growth Factor (bFGF)

\[ \rightarrow \]

Vascular Endothelial Growth Factor (VEGF)
Figure 3.1: Scheme of Experimental design in the present study

(d) Correlation of biochemical estimation, expression analysis and histopathological examination on ulcer healing for each category of drugs

- **Biochemical estimation**
  - **Gastric Juices**
    - Free and total acidity, peptic and Total carbohydrate : protein ratio
  - **Ulcerated tissue**
    - PGE$_2$, MPO activity, Proton pump inhibition, Total DNA content, Lipid peroxidation and anti-oxidant study

- **Expression studies**
  - RNA expression
  - COX-2 mRNA
  - Protein expression
  - COX-2 enzyme and growth factors

- **Histopathological Examination**
  - Mucosal reconstruction, Glandular re-arrangement and migration of epithelial cells