CHAPTER-III

METHODOLOGICAL CONSIDERATION

The main objectives of the study were:

(1). To select which dose of aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. is equivalent to referenced-drug ranitidine which prevent gastro-duodenal ulceration induced by aspirin.

(2). To see whether alkaline phosphatase (AP) enzyme activity, mucosal thickness (MT) and ulcer index (UI) could be modulated by aspirin induced gastric and duodenal ulcer following pretreatment with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn.

(3). To see whether antioxidant enzymes (SOD, catalase) activity, reduced glutathione (GSH) level and lipid peroxidation (LPO) could be modulated by aspirin induced gastric and duodenal ulcer following pretreatment with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. and ranitidine.

(4). To see whether antioxidant enzymes (SOD, catalase) activity, reduced glutathione (GSH) level and peroxidation (LPO), mucosal thickness (MT) and ulcer index (UI) could be modulated by ethanol induced gastric and duodenal ulcer following pretreatment with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. and ranitidine.

(5). To see whether antioxidant enzymes (SOD, catalase) activity, reduced glutathione (GSH) level and peroxidation (LPO), mucosal thickness (MT) and ulcer index (UI) could be modulated by immobilized-cold stress induced gastric...
and duodenal ulcer following pretreatment with aqueous extract of ripe fruit's pulp of *Cucurbita pepo* Linn. and ranitidine.

(6). To explore whether gastric secretory function (pH, volume of secretion, acidity and mucus content in all its fraction) and combined Alcian blue-PAS stained histological picture of the stomach and duodenum could be altered in cerebellar nodular lesion (CNL) induced gastric and duodenal ulcer following pretreatment with aqueous extract of ripe fruit's pulp of *Cucurbita pepo* Linn. and ranitidine.

(7). To examine whether serotonin (5-HT) content with enterochromaffin (EC) cells count, mucosal thickness (MT) and ulcer index (UI) could be changed in cerebellar nodular lesion (CNL) induced gastric and duodenal ulcer following pretreatment with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. and ranitidine.

The present study requires a comparative evaluation of different parameters of gastric and duodenal function following pre-treatment with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. and ranitidine on different ulcerogenic models. No work on the aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. influences on gastric and duodenal functions have been investigated in this laboratory thus it seemed desirable to study the role of aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. on gastric and duodenal tissues of different ulcerogenic models and simultaneously different parameters related to ulceration (as mentioned above) were observed as a supporting evidences.
THE MATERIALS AND METHODOLOGY IS DESCRIBED AS FOLLOWS:-

To fulfil the above objectives we have chosen the following materials and methods and ulcer models such as:

Collection of plant materials and authentication:

The ripe fruit of *Cucurbita pepo* Linn. (*C. pepo*) was purchased from the local vegetable market and the identity of the fruit was authenticated by the Botanical Survey of India, Shibpur, Howrah, West Bengal, India and kept in normal room temperature.

Preparation of extract:

During extraction, the outer surface of the ripe fruit of *C. pepo* was washed with distilled water, the skin was discarded and seeds were removed. The ripe fruit’s pulp of *Cucurbita pepo* Linn. (1 kg) was cut into pieces, sun dried and ground with the help of an electrical grinder to get a free flowing powder. This powder was subjected to extraction by dissolving in double distilled water (1:3) at normal room temperature for 48 hours then filtered through Whatman No.1 filter paper and vacuum dried in a lyophilizer at 40°C -50°C temperature. A viscous and sticky substance was obtained. The final yield was 29.3% (w/w) and kept in cold (4°C) temperature which was dissolved in double distilled water for future use (Sarkar and Guha, 2008; Tahiliani et al., 2000).

Drugs and doses:

- Sodium pentobarbitone (PB; 40 mg/kg body weight; Abbott, India Ltd)
- Aspirin (500 mg/kg body weight; German, Remidies Ltd)
- Ranitidine (10 mg/kg body weight; German, Remidies Ltd)
- Benzyl penicillin (PCN; 10,000 IU; Alembic India Ltd) as an antibiotic
- Anaesthetic ether (Kabra, Drugs India Ltd)

Animals and maintenance:

Inbred Holtzman strain adult albino rats (180-200g) of either sexes were received from Indian Institute of Chemical Biology (IICB), Jadavpur, Kolkata, West
Bengal, India and housed individually in a normal room (28°C temperature; 60% room humidity with 12:12 hrs light: dark cycle) and both the control and experimental rats were maintained on a daily schedule of standard laboratory diet. Drinking water was supplied ad libitum. Food intake (g/day/rat) and body weight of the rats were recorded daily and maintained throughout the experimental period. Experiments were carried out after the approval by the Animal Ethical Committee (AEC) of the institute.

Grouping of rats:

Rats were divided into 5 groups as follows:-

Group 1: Control rats: Control rats were fed with distilled water of same volume of extract using orogastric cannula.

Group 2: Control group were treated with Cucurbita pepo Linn. extract: Aqueous extract of ripe fruit’s pulp of Cucurbita pepo Linn. at a dose of 400 mg/kg body weight was administered orally using orogastric cannula for 14 consecutive days.

A preliminary experiment was conducted to study the effective dose (ED) of aqueous extract of ripe fruit’s pulp of Cucurbita pepo Linn. against a single dose of aspirin (500 mg/kg body weight) induced gastric and duodenal ulcer in rats model using graded doses of aqueous extract of ripe fruit’s pulp of Cucurbita pepo Linn. i.e., 200 mg/kg, 300 mg/kg, 350 mg/kg, 400 mg/kg, 450 mg/kg, 500 mg/kg body weight. Results showed that aqueous extract of ripe fruit’s pulp of Cucurbita pepo Linn. at a dose of 400 mg/kg body weight exhibited significant protection and hence this effective dose (ED) of Cucurbita pepo Linn. (400 mg/kg body weight) was used throughout the experiments.

Group 3: Different ulcerated rat models e.g.,

1. Aspirin induced ulcerated rat model.
2. Ethanol (Eth-OH) induced ulcerated rat model.
3. Immobilized-cold stress induced ulcerated rat model.
4. Cerebellar nodular lesion induced ulcerated rat model.
Group 4: Different ulcerated rat models were pretreated with aqueous extract of ripe fruit’s pulp of *Cucurbita pepo* Linn. e.g.,

1. Aspirin induced ulcerated rats were pretreated with extract of ripe fruit’s pulp of *C. pepo*.

2. Ethanol (Eth-OH) induced ulcerated rats were pretreated with extract of ripe fruit’s pulp of *C. pepo*.

3. Immobilized-cold stress induced ulcerated rats were pretreated with extract of ripe fruit’s pulp of *C. pepo*.

4. Cerebellar nodular lesion induced ulcerated rats were pretreated with extract of ripe fruit’s pulp of *C. pepo*.

Group 5: Different ulcerated rat models were pretreated with referenced antiulcer drug ranitidine: Ranitidine at a dose of 10 mg/kg body weight was administered orally using orogastric cannula for 14 consecutive days.

A preliminary experiment was conducted to study the effective dose (ED) of ranitidine against a single dose of aspirin (500 mg/kg body weight) induced gastric and duodenal ulcer in rats model using graded doses of ranitidine i.e., 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg body weight. Results showed that ranitidine at a dose of 10 mg/kg body weight exhibited significant protection against a single dose of aspirin (500 mg/kg body weight) and hence this effective dose (ED) of ranitidine (10 mg/kg body weight) was used throughout the experiments in following rat’s model:-

1. Aspirin induced ulcerated rats were pretreated with ranitidine.

2. Ethanol (Eth-OH) induced ulcerated rats were pretreated with ranitidine.

3. Immobilized-cold stress induced ulcerated rats were pretreated with ranitidine.

4. Cerebellar nodular lesion induced ulcerated rats were pretreated with ranitidine.
Preparation of different ulcerogenic rat models:

(I). Aspirin induced gastric and duodenal ulcer:

Rats were previously fasted for 24 hours and given a single dose of aspirin (500 mg/kg body weight) (Szabo et al., 1985; Bose et al., 2003) dissolved in double distilled water and was administered orally by orogastric cannula (Cho and Ogle, 1979). After 4 hours, the rats were anesthetized by ether and then they were sacrificed and their stomach and duodenum were collected. Stomach was opened along the greater curvature to expose the mucosal epithelial surface. The stomach was lavaged with 0.9% saline to remove the food particles and ulcers were scored (Szabo et al., 1985). The length of the longest diameters the lesions were measured and summated to give a total lesions score (in millimeter) for each rat the mean ulcer index (MUI) for each group were calculated.

(II). Ethanol (Eth-OH) induced gastric and duodenal ulcer:

Haltzman strain adult albino rats weighing 200-300g were deprived of food 24 hours prior to the experiments but they were allowed free access to water. The rats were administered 1 ml of 70% ethanol intragastrically (i.g) by orogastric cannula and for 1 hour (Cho and Ogle, 1992). After 1 hour, the rats were euthanized with ether and the stomach and duodenum were excised. The stomach was opened along the greater curvature to expose the mucosal epithelial surface. The stomach was lavaged with 0.9% saline to remove the food particles and ulcers were scored (Szabo et al., 1985). The length of the longest diameters the lesions were measured and summated to give a total lesions score (in millimeter) for each rat the mean ulcer index (MUI) for each group were calculated.

(III). Immobilized-cold stress induced gastric and duodenal ulcer:

Rats were suspended horizontally in restraint cage at dark condition and subjected to immobilized-cold stress by placing them in the refrigerator compartment at 4°C temperature for 3 hours daily and repeated consecutively for 7 days. Immobilized-cold stress was performed as per procedure followed by Senay and Levine (1967). After 7 days, they were sacrified under anesthesia and their stomach and duodenum were collected and stomach(s) were opened along the greater curvature to expose the mucosal epithelial surface. The stomach was lavaged with 0.9% saline to remove the food
particles and ulcer(s) were scored (Szabo et al., 1985). The length of lesions were measured by millimetre scale and summated to give a total lesions score (in millimeter) for each rat the mean ulcer index (MUI) for each group were calculated.

(IV). Cerebellar nodular lesion (CNL) induced gastric and duodenal ulcer by stereotaxic technique:

The rats were anaesthetized with sodium pentobarbital (40 mg/kg body weight; Abbott India Ltd) intraperitoneally (i.p). Each rat was placed in a stereotaxic instrument (INCO, India Ltd.) equipped with a custom-made ear bar, which prevents the damage of the tympanic membrane. Head was fixed in such a position that lambda and bregma suture were in the same horizontal plane by introducing the incisor bar properly attached to the mouth. All surgery was performed under strict aseptic conditions. Care was taken to prevent any damage of tympanic membrane. Surgery was performed by a midline incision on the back of head. The scalp was incised posteriorly in the midline and the adjacent pericranial muscles and fascia were retracted laterally. After retracting the nuchal musculature, the overlying bone was drilled at the specific loci and a burr hole was made on the posterior aspect of the skull as per stereotaxic co-ordinates of the stereotaxic atlas of cerebellar nodular area (AP=12.8, L=0.4, D=6.8 - Pellegrino and Cushman, 1967). Such, that electrode could penetrate the required area of the cerebellum. The bleeding, if any, was controlled by aseptic bone wax.
Fig 3.1: Immobilized-cold stress technique

Fig 3.2: Cerebellar nodular lesion (CNL) by stereotaxic technique
Electrolytic lesion:

Electrolytic lesions were made in the nodular cerebellum by conventional bipolar electrode (insulated by epoxylite with 0.5 mm tip exposed) using 1.5 mA DC (milliampere direct current) for 20 seconds.

Post operative care:

Following recovery from surgery all rats were carefully maintained with all the necessary precautions and aseptic measures as a routine measure. All rats were injected benzyl penicillin (10,000 IU) intramuscular (i.m) for three (3) consecutive days after surgery. For first two days, rats were given glucose saline intraperitoneally (i.p) until the rats became capable of taking either liquid milk or diet.

Implantation of gastric cannula and collection of gastric secretion:

Prior to implant of gastric cannula, all rats were kept overnight fasting, but they were allowed free access to water. Stainless steel gastric cannula was implanted under anesthesia according to the method of Guha et al., 1974. Post operative care was taken for 7 days. After post operative period the rats were placed in perplex glass made restraining cages and gastric secretion was collected at particular time interval under fasting condition through sialistic tube firmly attached with the cannula by opening the screw. Prior to gastric juice collection, stomach was washed with 0.9% saline.

Collection of gastric secretion:

After post operative period of 7 days rats were fasted for overnight but they were allowed free access to water and placed in the restraining cages (No. 51339, Stolting Co, Chicago), Prior to gastric juice collection stomachs were lavaged with 0.9% saline until the efferent was clear of any food particles. Gastric juice was collected through sialistic tube firmly attached with the cannula by opening the screw (Guha et al., 1974).
Fig 3.3: Implantation of gastric cannula

Fig 3.4: Gastric juice collection technique
(V). Parameter studied:

**Morphological studies:**

(1). Ulcers scoring:

The stomach and duodenum were collected and stomach was opened along the greater curvature to expose the mucosal surface and stretched on a flat paraffin bed. The stomach was lavaged with 0.9% saline to remove the food particles and the ulcer scoring was performed (Szabo et al., 1985).

<table>
<thead>
<tr>
<th>Severity score</th>
<th>Ulcer type</th>
<th>Length of ulceration (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no pathology</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>a small ulcer</td>
<td>1-2</td>
</tr>
<tr>
<td>2</td>
<td>a medium ulcer</td>
<td>3-4</td>
</tr>
<tr>
<td>4</td>
<td>a large ulcer</td>
<td>5-6</td>
</tr>
<tr>
<td>8</td>
<td>a larger ulcer</td>
<td>&gt;6</td>
</tr>
</tbody>
</table>

The length of lesions were measured by millimetre scale and summated to give a total lesions score (in millimetre) for each rat ulcer index (UI) for each group were calculated. The sum of the total severity scores in each group of rats divided by the number of rats was expressed as the mean ulcer index (MUI).

(2). Measurement of mucosal thickness (MT) of gastric and duodenal tissues:

For determination of mucosal thickness (MT) the transverse sections (5 μm) of stomach and duodenum tissues were taken. The sections were stained with haematoxylin and eosin (H & E). At least 10 determination of mucosal thickness (MT) was made on at least two sections from each specimen. The mucosal thickness (MT) of both tissues was measured by a stage micrometer. Sections were examined with objective 10X (visual field diameter, 2.5 mm) and eyepiece 5X (with scale bar inserted) (McQueen et al., 1984).
**Biochemical studies:**

Biochemical estimation of serotonin (5-HT) neurotransmitter content from gastroduodenal tissues:

**Biochemical estimation of serotonin (5-HT) content:** Stomach and duodenum tissues were weighted and 5-HT level was estimated following the fluorescence spectrophotometrical method of Amar *et al.*, 1982. Stomach and duodenum were collected in cold condition and homogenized with 5 ml acidified butanol. Then 2 ml homogenate was mixed with 5 ml 10% heptane and 2.5 ml 0.003(N) HCl and then shaken for 5 min and centrifuged at 2000 rpm for 10 min. Acid layer (2.25 ml) was eluted and mixed with 100 mg alumina and 0.5 ml of 2(M) sodium acetate. The mixture was shaken for 5 min and centrifuged at 2000 rpm for 10 min. The precipitate was discarded. The supernatant was mixed with 1.5 ml of 10% isobutanol and shaken with 1 ml salt saturated buffer solution at pH=10. Then 1 ml heptane was added and 2.5 ml 0.1(N) HCl was mixed. This was taken for the estimation of serotonin (5-HT). The fluorescence of 5-HT was measured in the Perkin-Elmer MPF 44B fluorescence spectrophotometer with activation and emission wavelength set at 295 nm and 550 nm respectively.

Biochemical estimation of antioxidant enzymes and lipid peroxidation:

**Tissue preparation:** Rats were sacrificed by over dose ether anaesthesia on 15th day from start of experiment. The stomach and duodenum were collected and opened along the greater curvature to expose the mucosal epithelial surface. To note the distribution of ulcer(s) the stomach and duodenum were washed with 0.9% saline. The tissues were weighed and homogenized in ice-cold phosphate buffer and prepared for biochemical estimation.

(i) **Measurement of superoxide dismutase (SOD):** Superoxide dismutase (SOD) was estimated by the method of Marklund and Marklund, 1974. Stomach and duodenum tissue samples were homogenized with 5 ml of ice-cold 0.1(M) phosphate buffer (pH=7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. Then 0.1 ml of sample was mixed with 0.8 ml of TDB solution (triethyleamine, diethyleamine and
buffer mixture). Reaction started by the addition of 4 μl of NADH. Then 25 μl of EDTA-MnCl₂ mixture was added to it. Thereafter spectrophotometric readings were recorded at 340 nm. After recording of spectrophotometric reading, 0.1 ml of mercaptoethanol was added with those mixture and again spectrophotometric reading were recorded at 340 nm wave length.

(ii) Measurement of catalase (CAT): Catalase activity was estimated by the method of Aebi, 1974. Stomach and duodenum tissue samples were homogenized with 5 ml of ice-cold 0.1(M) phosphate buffer (pH=7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. The precipitate was then stirred with the addition of 15 ml of ice-cold 0.1(M) phosphate buffer and allowed to stand in cold condition with occasional shaking. The shaking procedure was repeated for thrice. 1 ml of sample was added with 9 ml of H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from the changes in absorbance at 350 nm wave length.

(iii) Measurement of reduced glutathione (GSH): Glutathione was measured according to the method of Ellman, 1959. Stomach and duodenum tissue samples were homogenized with 5 ml of ice-cold 0.1 M phosphate buffer (pH=7.4). The equal quantity of homogenate was mixed with 10% trichloroacetic acid (TCA) and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH= 8.4), 0.5 ml of 5' 5- dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm wave length within 15 min.

(iv) Measurement of lipid peroxidation (LPO): Lipid peroxidation (LPO) was measured according to the method of Rehncrona et al., 1980. Stomach and duodenum tissue samples were homogenized with 5 ml of ice-cold 0.1(M) phosphate buffer (pH=7.4). The homogenates was then centrifuged at 3000 rpm for 10 min. 0.5 ml of sample was mixed with 1 ml of TDB solution (triethyleamine, diethyleamine and buffer mixture) and then the mixture was incubated at 37°C for 1 hour. To it, 0.5 ml of trichloroacetic acid (TCA) was added, vortexed and the absorbance was read at 350 nm wave length. After recording of spectrophotometric reading, 1 ml sample
was mixed with 500 µl mercaptoethanol and again the absorbance was read at 350 nm wave length.

Biochemical analysis of gastric juice:

(I). **Acidity**: Acidity was measured with 0.01 (N) NaOH solution by using 0.5% Topper’s reagent and 1% phenolphthalein as an indicator (Guha and Maiti, 1990).

(II). **Estimation of mucus content**: 0.5 ml of gastric juice together with 5 ml of absolute alcohol was centrifuged for 20 minutes. The precipitate obtained was dissolved in 0.5 ml of 0.1 (N) NaOH solutions. Then 0.1 ml from this solution was taken for estimation of total protein and rest 0.4 ml was hydrolyzed for estimation of total hexose, hexosamine, sialic acid and fucose.

**Hydrolysis of gastric juice for estimation of mucous content:**

Mucous is a mucopolysaccharide expressed as CHO, protein ratio or CHO/protein (CHO: P). The carbohydrate portion includes total hexose, hexosamine, fucose and sialic acid; 0.5 ml of gastric juice together with 5 ml of absolute alcohol was centrifuged at 5000 rpm for 20 min. The precipitate was obtained and dissolved in 0.5 ml of 0.1 (N) NaOH solution. Then 0.1 ml from this solution was taken for the estimation of total protein and rest of 0.4 ml was taken in bulb shaped test tube (Folin’s tube) and was mixed with 2 ml of 6(N) hydrochloric acid (HCl) and boiled for 2 hours in boiling water bath and after cooling 1 drop of phenolphthalein was added and titrated against 10(N) NaOH solution. Pink colour, which was obtained and disappeared after titration with 6(N) HCl, then the total volume was made up to 4 ml of distilled water. From this, 1 ml each was taken for total hexose, hexosamine, sialic acid and 0.5 ml of fucose.

(i). **Estimation of total hexose**: 1 ml of hydrolyzed solution was taken for estimation of total hexose spectrophotometrically (Elson and Morgan, 1953). 1ml of hydrolysed solution and 5 ml of acidified orcinol mixture were boiled for 15 min and after cooling reading was taken at 540 nm wavelength spectrometrically (Elson and Morgan, 1933).
(ii) Estimation of hexosamine: 1 ml of hydrolyzed solution was taken for estimation of hexosamine spectrophotometrically (Dische and Borenfreund, 1950). In 1 ml of hydrolyzed solution, 1 ml of acetyl acetone reagent was added and boiled for 2 min. After cooling, 0.5 ml of Ehrlich's reagent and 1.5 ml of absolute alcohol was added and kept for 20 min. After 20 min, the reading was taken at 540 nm wavelength spectrophotometrically (Dische and Borenfreund, 1950).

(iii) Estimation of fucose: 0.5 ml of hydrolyzed solution was taken for estimation of fucose spectrophotometrically (Dische and Shettes, 1948). 0.5 ml of hydrolysed solution and 0.5 ml of distilled water was taken in duplicate tubes, then 4.5 ml of 6:1 sulphuric acid (H₂SO₄) was added and boiled exactly for 3 min in boiling water bath. 0.1 ml of cysteine reagent was added in 1 set of sample and wait for 1 hour. Reading was taken at 396 nm (with cysteine) and 430 nm (without cysteine). The reading was taken by different of OD in different wavelength spectrophotometrically (Dische and Shettles, 1948).

(iv) Estimation of sialic acid: 1 ml of hydrolyzed solution was taken for estimation of sialic acid spectrophotometrically (Warren, 1959). 1 ml of hydrolyzed solution and 0.1 ml sodium periodate was mixed thoroughly and waited for 20 min. To it, 0.1 ml sodium arsenate and 3 ml of thiobarbituric acid was added and boiled for 15 min. After cooling, 5 ml of cyclohexanol was added and shaken for 1 min. The mixture was allowed to separate in layers, the upper layer was taken and reading was realized at 532 nm and 550 nm. The reading was taken by different of OD in different wavelength spectrophotometrically (Warren, 1959).

(v) Estimation of total protein: 0.1 ml of unhydrolyzed precipitate was taken for estimation of total protein spectrophotometrically (Lowry et al., 1951). 0.1 ml of unhydrolysed precipitate solution was mixed with 0.4 ml of distilled water. To this 5 alkaline reagent was added and waited for 10 min. 0.5 ml of phenol reagent was given to this solution and after 10 min reading was taken at 600/610 nm wavelength (Lowry et al, 1951).
**Histochemical studies:**

**Different histological staining of gastric and duodenal tissues:**

The stomach and duodenum were fixed in 4% formaldehyde solution and processed for routine paraffin section.

(i). **Haematoxylin and eosin (H & E) staining:** The stomach and duodenal tissues were routinely stained with haematoxylin and eosin (H & E) (Bancroft *et al.*, 1996).

(ii). **Combined Alcian blue- PAS staining:** The stomach and duodenal tissues were stained with combined Alcian blue-PAS (Mowry, 1956).

(iii). **Determination of alkaline phosphatase (AP) enzyme activity:** The stomach and duodenal tissues were stained for determination of alkaline phosphatase (AP) enzyme activity (Gomori, 1952).

**Staining procedure for alkaline phosphatase (AP) enzyme:**

The section was deparaffinised with xylene and placed in the substrate containing incubating medium which was consisted of 2% Na-β-glycerophosphate 2.5 ml, 2% sodium veronal 2.5 ml, 2% calcium nitrate 5.0 ml, 1% magnesium chloride 0.25 ml and distilled water 1.25 ml. The final pH of the incubating medium was made 9.0-9.4 and incubated at 37°C for 30 min. Then the section was washed in distilled water and treated with 2% cobalt nitrate solution to stop the enzymatic reaction. After that washed with distilled water and immersed in 1% ammonium sulphide solution. Then washed with distilled water and counter stained with 2% methyl green solution and washed in running tap water. Finally it was mounted with glycerine jelly (Gomori, 1952).

(iv). **Enterochromaffin (EC) cells staining:** The stomach and duodenal tissues were stained by silver nitrate method for enterochromaffin (EC) cells (Singh, 1964). Each section was deparaffinised and placed in pre-warmed silver nitrate solution at
60°C for 15-30 min and then the section was washed in 1% sodium thiosulphate solution.

**Statistical analysis:**

The data were expressed as MEAN ± S.E.M. and were analyzed statistically using one way analysis of variance (one way ANOVA), followed by multiple comparison 't' test, was used for statistical evaluation of the data. In addition to this, two-tailed Student 't' test was performed to determine the level of significance between the means. Difference below the probability level 0.05 (p<0.05) was considered statistically significant.