MATERIALS AND METHODS

i. MATERIALS
   - Experimental Animal
   - Aloe vera gel

ii. METHODS
   - Morphological study of duodenum of ulcerated mice.
   - Duodenal ulcer index
   - Histological study:
     - Histological study of duodenum of:
       a. Control
       b. Cysteamine-HCl induced ulcer
       c. Aloe vera gel treated ulcerated duodenum of young, adult and old mice.
   - Histochemical study:
     - Periodic acid Schiff (PAS) reaction to demonstrate presence of glycoproteins in the duodenum of control, cysteamine induced ulcerated and Aloe vera gel treated ulcerated duodenum of young, adult and old mice.
     - Alcian blue staining to demonstrate presence of Acid glycoprotein in the duodenum of control, cysteamine induced ulcerated and Aloe vera gel treated ulcerated duodenum of young, adult and old mice by AB pH 2.5
     - Alcian blue staining to demonstrate presence of Acid glycoprotein in the duodenum of control, cysteamine induced ulcerated and Aloe vera gel treated ulcerated duodenum of young, adult and old mice by AB pH 1.
   - Estimation of Proteins (Lowry, et al., 1958), Hexose (Dubois, et al., 1956), fucose (Dische and Shettles, 1948) and Sialic acid (Warren, 1959) in duodenal mucosa of control, cysteamine induced ulcerated and Aloe vera gel treated ulcerated duodenum of young, adult and old mice.
• **Estimation of total Lipid Peroxidation** (Wills, 1966) in duodenal mucosa of control, cysteamine induced ulcerated and *Aloe vera* gel treated ulcerated duodenum of young, adult and old mice.

• **Study of Antioxidants status of duodenum:**
  - Estimation of superoxide dismutase (Beaucham and Fridovich 1971) in duodenal mucosa of control, cysteamine induced ulcerated and *Aloe vera* gel treated ulcerated duodenum of young, adult and old mice.
  - Estimation of Catalase (Luck, 1974) in duodenal mucosa of control, cysteamine induced ulcerated and *Aloe vera* gel treated ulcerated duodenum of young, adult and old mice.
  - Estimation of Reduced glutathione (Ellman’s Method, 1959) in duodenal mucosa of control, cysteamine induced ulcerated and *Aloe vera* gel treated ulcerated duodenum of young, adult and old mice.

iii. **STATISTICAL ANALYSIS**

iv. **LIST OF CHEMICALS USED**
MATERIALS

i. Collection and authentication of Aloe vera (Aloe vera barbadensis)

Healthy plant samples were collected from Shivaji University botanical garden. Identification was confirmed with the help of herbarium species from the department of Botany Shivaji University, Kolhapur. The Voucher specimens (TAB -1) were deposited. The authetication certificate of the plant is given in plate No.1.

ii. Extraction of Aloe vera gel

Aloe vera leaves were cut from the plant outside in the laboratory they were washed under running tap water and thoroughly in distilled water from the dorsal side of the leaf. The top skin layer was sliced off from thick end towards the thin end. The gel was scrapped with sterilized scraper from dorsal side of the leaf and collected in the sterilized beaker. The gel was subjected to dialysis for 24 hrs to remove water. The extract obtained was stored at 10 to 12°C in refrigerator.

iii. Experimental Animal Mice (Mus musculus linn) Plate No.2

Healthy swiss albino mice Mus musculus of both sexes, young, adult and old were used for the study. The breeding pairs were obtained from Rajarambapu college of Pharmacy, Kasegaon, Tal -Walwa; Dist -Sangli 415 404 (209/CPCSEA, 01st June 2000) and were reared in air-conditioned departmental animal house. Young mice 20 ± 2 to 26 ± 2 gm/BW of 2 month age, Adult mice 35 to 42 ± 2 gm/BW of 5 to 6 month age and old mice 45 to 50 ± 2 gm/BW of 16 to 18 month age were used for present investigation throughout received Amrut mice feed (Pranav Agro Industries, Pvt. Ltd, Sangli) and water ad libitum.

- Control group: The young, adult and old mice were given 0.5 ml of distilled water/ day/ animal for 15 days.

- Duodenal ulcer induced group: The young, adult and old mice was given subcutaneous injection of cysteamine-HCl (40mg/100gm/BW) dissolved in 0.5 ml distilled water (Szabo, 1978).
- *Aloe vera gel treated group*

  The young, adult and old mice was given subcutaneous injection of cysteamine HCl (40mg/100gm/BW) dissolved in 0.5 ml distilled water (Szabo, 1978) after 24 hr of after the dose of cysteamine- HCl administration six mice received orally *Aloe vera* gel 200 mg/kg dissolved in 0.5 ml distilled water/day/mouse for 15 days (Subramanian, *et al.*, 2007). The treatment was once a day and it was continued for next 15 days.

  Three mice from control group were kept along with *Aloe vera* gel treated mice.

**iv. Removal of duodenum:**

The control, cysteamine-HCl administered and cysteamine induced ulcerated *Aloe vera* gel receiving mice were weighed and sacrificed by cervical dislocation, the duodenum were removed, weighed and were proceed for gross morphological, histological, histochemical and biochemical studies (freezed and homogenized in respective homogenization media).

**METHODS**

**i. Determination of body weight of mice:**

Animals were weighed (in gm) before starting experiment, during respective treatment and also after completion of each treatment. The record of these observations was maintained.

**ii. Duodenal ulcer index**

The duodenal ulcer index was evaluated with respect to severity of percentage of ulcers. The severity of ulcers was measured evaluated via scale from 0 to 3 measurements by using stereomicroscopic observation then the severity of ulcer formation confirmed by tissue sectioning and staining of the ulcerated parts of the duodenum.

0 = No Ulcers
1 = Superficial mucosal erosion
2 = Deep Ulcer penetrating to submucosa
3 = penetrating or perforating ulcers.

Formula for the calculation of the ulcer index:
Ulcer Index = Mean Severity + Incidence (i.e. Positive/Total) 2.
The method used was described by Szabo (1978).

iii. **Histology of duodenal structure by Hematoxyline-Eosine (HE) staining method (Gurr, 1962).**

For histological study duodenum were fixed in Calcium acetate formalin (CAF) [2 gm Calcium acetate powder + 10 ml Formalin + 90 ml distilled water] for 24hrs. The duodenal tissue were washed with the help of running tap water for 24 hours; after proper washings tissues were passed gradually through alcoholic grades for dehydration, cleared in xylene and embedded in paraffin wax. The sections were cut at a thickness of 6 μ and stained with Hematoxylene and Eosin (H/E).

a) **Reagents:**
- Alcohol grades (30%, 50%, 70%, 90%, absolute)
- Xylene
- Hematoxylene
- 90% Alcoholic Eosin
- Dextrene Diphenyl Pthalate Xylene (DPX)

b) **Procedure:**

**Staining Procedure:**
- Tissue section slides were deparaffinized in Xylene.
- Sections were passed through the alcohol grades (absolute alcohol, 90%, 70%, 50%, 30% and distilled water) gradually for hydration.
- After hydration tissue section slides were stained with Hematoxylene for 8 minutes.
- After Hematoxylene staining brought to running tap water for 5 minutes.
- Dehydrates the slides passing through an alcoholic grade (30%, 50%, 70%, 90%)
- After dehydration tissue sections stained with 90% alcoholic eosin after proper staining dipped in absolute alcohol for 5 minutes.
• Tissue were cleared in xylene and mounted in Dextrene Diphenyl Pthalate Xylene (DPX).

c) Result:
Nuclei were stained with blue colour and cytoplasm stained with pink.

iv) Histochemistry of duodenum

For histochemical study duodenum were fixed in neutral buffered formalin for 24 hrs. For the glycoprotein histochemistry study of duodenal mucosa and nature of glycoproteins from pyroduodenal junctions the sections were subjected Periodic Acid–Schiff (PAS) reagents staining (Mc Manus, 1964 and Hotchkiss, 1948) technique.

a) Principle: A positive Periodic Acid–Schiff Reaction (PAS) stained all type neutral muco-substances i.e. glycoproteins. In Periodic Acid–Schiff Reaction the periodic acid oxidizes the carbon bond forming aldehydes which react to the fuschin-sulfurous acid which form the magenta color.

b) Reagents:
• Alcohol grades (30%, 50%, 70%, 90%, absolute Alcohol)
• Xylene
• 1% Periodic acid (1 gm. periodic acid + 100 ml of distilled water).
• Schiff Reagent: (1 gm. basic fuchsin powder is dissolved in 100 ml of boiled distilled water continue to boil for 5 minutes, then cool the solution and filtered.After at 50°C add 2 ml 0.1N HCl cool upto 25°C then add 1gm of Sodium meta-bi-sulphate (Na₂S₂O₃) and kept in cool and dark overnight.Add 2 gm activated charcoal shake it well and filter (treatment of this solution with activated charcoal produces straw coloured Schiff's reagent).
• 0.5% Sodium meta-bi-sulphate (Na₂S₂O₃): (500 mg Sodium meta-bi-sulphate (Na₂S₂O₃) dissolved in 100 ml of distilled water).
• Dextrene-Diphenyl-Pthalate Xylene (DPX)
c) **Staining Procedure:**

- After dewaxation and sections were passed through the alcohol grades (absolute alcohol, 90%, 70%, 50%, 30% and distilled water) gradually for hydration.
- Tissue sections oxidized in 1% periodic acid for 10 minutes.
- After oxidation tissue sections brought to running tap water for 5 minutes.
- Treated with Schiff’s reagent for 30 minutes in dark (until deep magenta color developed).
- Gently dipped in 0.5% sodium meta-bi-sulphate for 6 minutes.
- Rinsed in distilled water and proceeds for dehydration passing through alcoholic grade (30%, 50%, 70%, 90% and absolute alcohol).
- Cleared in xylene and tissue sections were mounted in Dextrene-Diphenyl-Pthalate Xylene (DPX).

d) **Results:** Neutral muco-substances were stained with magenta color and nuclei were stained with blue color.

➢ **Demonstration of sulphated acid muco-substances by Alcian blue pH 1 staining** (Mowry, 1956).

a) **Principle:**

One of the histochemical method of distinguishing sulphated acid carboxy mucins in tissue sections involves lowering the pH of the staining bath below the pK of the carboxyl group, to around pH 1.0, at which point the sulphate groups are dissociated and stain selectively with basic dyes at pH 1, and strongly acidic sulphomucins exhibit alcianophilia.

b) **Reagents:**

- Alcohol grades (30%, 50%, 70%, 90%, absolute)
- Xylene
- 0.1 N HCl (8.985mL of conc. HCl + 100 ml of distilled water)
- Alcian Blue (AB) stain at pH 1 (1 gm of Alcian Blue powder + 100 ml 0.1 N HCl).
• Hematoxylene
• Dextrene Diphenyl Pthalate Xylene (DPX)

c) Procedure:
For the study of sulphated acidic mucosubstances staining Alcian blue (AB) at pH 1.0 technique were used.
• Tissue section slides were deparaffinized in Xylene.
• Sections were passed through the alcohol grades for hydration (absolute alcohol, 90%, 70%, 50%, 30% and distilled water) respectively in each grade for 5 minutes.
• Gently dipped in 0.1 N HCl for 10 minutes at room temperature.
• Stained with AB stain at pH 1 for 1 hr. at room temperature.
• Rinsed in 0.1 N HCl.
• Counter stained with hematoxylene for 30 sec- 1 minute at room temperature.
• Washed in distilled water proceed for dehydration with alcohol grades (30%, 50%, 70%, 90%, absolute alcohol).
• Cleared in xylene and tissue sections were mounted in Dextrene-Diphenyl-Pthalate Xylene (DPX).
• Observed under microscope for presence of AB pH 1 positive material (sulphated mucosubstances).

d) Results:
Sulphated muco-substances were stained with light blue color and nuclei were stained with dark blue color.

➢ Demonstration of acidic mucosubstances by Alcian blue pH 2.5 staining (Mowry, 1956).
a) Principle:
Alcian blue complexes with carboxyls and sometimes sulphates. The degree of acidity of the mucosubstances is indicated by the extinction of acidophilia at various pH values. At pH 2.5 sialomucins and weakly acidic sulphonmucins colour blue.
b) Reagents:
- Alcohol grades (30%, 50%, 70%, 90%, absolute)
- Xylene
- 3% Acetic acid (3ml of Acetic acid + 100 ml of distilled water)
- Alcian Blue (AB) stain at pH 2.5 (1 gm. of Alcian Blue powder + 100 ml 3% acetic acid solution)
- Hematoxylene
- Dextrene Diphenyl Pthalate Xylene (DPX).

c) Staining Procedure:
- Tissue section slides were deparaffinized in Xylene.
- Sections were passed through the alcohol grades for hydration (absolute alcohol, 90%, 70%, 50%, 30% and distilled water) respectively in each grade for 5 minutes.
- Treated with 3% Acetic acid for 10 minutes at room temperature.
- Tissue sections stained with AB at pH 2.5 for one hour at room temperature.
- Treated with 3% Acetic acid for 5 minutes.
- Washed in distilled water proceed for dehydration with alcohol grades (30%, 50%, 70%, 90%, absolute alcohol).
- Cleared in xylene tissue sections were mounted in Dextrene-Diphenyl-Pthalate Xylene (DPX), observed for presence of AB pH 2.5 positive materials (acidic sulphated mucosubstances).

d) Results:
Sialomucins stained with light blue color indicating presence of acidic mucosubstances.

v) Biochemistry

➢ Estimation of Protein (Lowry’s et al., 1958)

Tissue homogenized with by using refrigerated glass mortar and pastle. Crushing of tissue was carried out at the bottom of the mortar with instantaneous freezing and gradual thawing by using chilled distilled water. Uniform homogenates
were prepared which was centrifuged at 10°C at 3000 rpm for ten minutes. The supernatant was used for estimation of protein.

**a) Principle:**
Carbamyl group of protein molecules reacts with copper and potassium of the reagent to give blue colored copper-potassium biuret complex. This complex together with tyrosine and phenolic compound present in the protein reduce the phosphomolybdate of the Folin phenol reagent to intensify the colour of the solution. The intensity of color depends upon the amount of aromatic amino acids present in the sample and both intensity as well as concentration of aromatic amino acid will vary for different proteins.

Universally most of the times for the assay of protein estimation technique Bovine Serum Albumin (BSA) used as standard protein, because of its low expensive, with high purity and easily commercially available. The Lowry’s method is sensitive to pH range and hence, the pH should be maintained between at 10-10.5 range.

**b) Reagents:**
- 0.1N NaOH (4 gm of Sodium Hydroxide (NaOH) flakes + 1000 ml of distilled water).
- Reagent A – 2 % Sodium Carbonate (Na₂CO₃) prepared in 0.1N NaOH (2gm of Sodium Carbonate (Na₂CO₃) dissolved in 100 ml of 0.1N NaOH).
- Reagent B - 0.5% CuSO₄ in 1 % Sodium-potassium tartarate (Na/K- tartarate)
- Reagent ‘C’– It was prepared freshly before use (Mix the 50 ml Reagent - A and 1 ml Reagent - B).
- Reagent ‘D’- Folin Ciocalteau-Phenol reagent.
- Standard Protein Solution (BSA) – Bovine serum albumen 25 mg dissolved in 250 ml of distilled water.
- Folin-ciocalteau- Phenol reagent – 100 mg sodium tungastate Na₂SO₄.2H₂O were dissolved in 700 ml distilled water. 100 ml of Hydrochloric acid (HCl) and 50 ml 85 % Phosporic acid (H₃PO₄) were added and the mixtures were refluxed for 10 hours in glass apparatus. After that 150 gm of Lithium Sulfate, 50 ml of distilled water and a few drops of Br₂ were added. The mixture were
boiled for 15 minutes to remove excess bromine, cooled at room temperature and make up to 1000 ml and then filtered. The reagent appears golden yellow in colour which was stored in refrigerator. It is considered as stock solution or reagent and which was diluted with equal volume of water before use.

c) Procedure -

Three test-tube were taken and labeled them as Sample, Standard and blank respectively. 0.5 ml supernatant of duodenum extracted from tissue was taken in test tube labeled as ‘Sample’. 0.5 ml Standard protein solution that is solution of BSA taken in test tube labeled as standard. Made the additions as per the following table:

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Sample</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample</td>
<td>0.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Bovine Serum Albumen</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Reagent ‘C’</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>5.</td>
<td>Reagent D (Phenol reagent)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Wait for 10 minutes at Room Temperature.

Mix the reagent and Kept for 30 minutes at room temperature.

Reading was taken at 660 nm on spectronic -20 colorimeter adjusting to zero with blank.

d) Reaction

The two reactions involved in the final colour production, First one biuret reaction, in which an alkaline medium Cu2+ divalent ions reacts with the protein molecules and forms Cu+ monovalent ions and in second reaction, Cu+ ions and tyrosine, tryptophan present in the proteins reacts with Folin phenol reagent. It reduces phosphomolybdic complex of reagents into sodium molybdate, tungstate and phosphotungstate to forms blue purple colour.
e) Calculation

The concentration of protein 1 mg / duodenal tissue was determined by using std. graph of Bovine - serum albumen.

**Isolation of Glycoprotein from Duodenum (Satakopan and Kurup, 1977):**

The isolated duodenum was homogenized and treated with acetone for 72 hrs. Acetone was changed after every 6 hrs. The acetone dried powder was then extracted with ether acetone (3:1 vol: vol for one hour). In this procedure the tissue gets defatted. The dry defatted powder extracted thrice after every 24 hrs at room temperature with 1M CaCl$_2$. The dialysis was carried out against distilled water to remove CaCl$_2$. The protein was precipitated at 0$^0$C by 5 \% Trichloro-acetic acid (TCA). The protein precipitation was subjected to papain digestion (Crystalline papain 1/3 rd the dry weight of the powder for 72 hrs at 65$^0$C in 0.2 M acetate buffer having pH 7) containing 2 mg Cysteine hydrochloride / ml fresh papain was add up into 5 volume of cold absolute alcohol and allowed to stand at 0$^0$C for 24 hrs to precipitate protein. After centrifugation the moisture of supernatant was dehydrated in vaccum. The residue was dissolved in a small amount of water and applied for the estimation of protein and carbohydrate components.

➢ **Estimation of Hexose (Dubois, *et al.*, 1956).**

a) **Principle:**

It is a rapid and reproducible method used for the estimation of simple sugars and their derivative has been described by Dubois, *et al.*, 1956, which utilizes phenol as the specific organic; colour developing agent. In addition to simplicity and sensitivity this method offers advantage that it was largely unaffected by the presence of proteins. As a consequence it provides a useful technique for estimating the carbohydrate content of glycoproteins.

b) **Reagents:**

Phenol reagent: Phenol reagent was prepared by addition of 20 gm of glass distilled water and 80 gm redistilled phenol (Sp. gravity 1.84).
c) **Procedure:**

For the estimation of Hexose isolated glycoprotein residue from Brunner's gland was dissolved in water (1 mg/ml). D-glucuronic acid was used as standard (2.5 mg D-glucuronic acid in 100 ml Distilled water) and distilled water was taken as blank. Hexose estimation was carried out thrice in triplicate for each sample. The threes test tubes labeled them as standard, blank and sample. Addition was made as per table given below:

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>2 ml</td>
</tr>
<tr>
<td>D-glucuronic Acid</td>
<td>2 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>2 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Reagent</td>
<td>0.05ml</td>
<td>0.05ml</td>
<td>0.05ml</td>
</tr>
<tr>
<td>Conc.H$_2$SO$_4$</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Allow to stand all the test tubes for 30 min. at room temperature.

Readings were taken at 480 nm or 490 nm on spectronic 20 colorimeter by adjusting to zero with blank.

d) **Reaction:**

In this method the heat required for colour development is provided by the exothermic reaction of sulfuric acid and water. Hexoses react with phenol generates orange yellow color.

f) **Calculation:**

\[
\frac{\text{O.D. of sample} \times \text{amount of Standard}}{\text{O.D. of Standard} \times \text{Amount of sample}} = \text{ug/mg glycoprotein}
\]

➢ **Estimation of Fucose: (Dische and Shettles, 1948)**

This method can be employed in the following manner to measure the fucose content of insoluble and soluble glycoprotein.
a) Reagents:

- 0.02 % standard α- D (+) fucose: 0.02 gm standard α- D (+) fucose + 100 ml distilled water.
- Mixture of H$_2$SO$_4$ and distilled water (6:1)
- 3% Cysteine hydrochloride: 3 gm Cysteine hydrochloride + 100 ml distilled water.

b) Procedure:

For the determination of concentration of fucose from duodenal tissue residue dissolved in distilled water (2 mg / ml distilled water). The prepared homogenate was centrifuged at 5000 rpm at 10°C for 10 minutes. Supernatant of centrifuged homogenate was used as sample for estimation of fucose. Estimation was carried out three times in triplicate for each sample. Four test tubes were taken and labeled as Sample, standard, blank and control. Additions were made as per table given below:

<table>
<thead>
<tr>
<th></th>
<th>Standard ( in ml)</th>
<th>Blank ( in ml)</th>
<th>Sample ( in ml)</th>
<th>Control ( in ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.02 % standard α-D (+) fucose</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (D.W.)</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mixture of H$_2$SO$_4$ and water (6:1)</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

- All the test tubes were shake it well and placed in water bath at 20°C - 22°C for 10 minutes.
- Test tubes were tightly packed with glass bulbs placed into boiling water bath definitely for 3 minute.
- Then add 0.1 ml 3% aqueous cysteine hydrochloride reagent in Sample, standard and blank test tubes, except control tubes. All the solution of
test tubs mixed it well and kept for 2 hours at room temperature. The optical density was taken at 396 nm and 427 nm at against blank.

**Reaction:**

Methyl pentoses give a very small tinge of visible color in this reaction whose maximum absorbance near to 396 nm. Neutral hexose, including galactose mannose and glucose gives a yellow color under the conditions of this reaction. It is possible to correct for the contribution of the hexoses to the 396 nm reading by measuring the absorbancy of the sample both at 396 nm and to 427 nm where the hexose absorption because of the symmetrical shape at its spectrum is equal to its absorption at 396 nm. The absorption of the methyl pentoses at the higher wavelength is negligible.

c) **Calculation:**

- Optical density (O.D.) of the control at 400 nm - Optical density (O.D.) of the control at 427 nm = X
- Optical density (O.D.) of the sample at 400 nm - Optical density (O.D.) of the sample at 427 nm = Y
- Optical density (O.D.) of the sample, A = Y – X
- Optical density (O.D.) of standard at 400 nm – Optical density (O.D.) of standard at 427 nm = Z
- Optical density (O.D.) of standard B = Z – X

**Formula:**

\[
\frac{\text{Optical density (O.D.) of sample A}}{\text{Optical density (O.D.) of std. B}} \times \text{Amount of Standard}
\]

\[
\mu g \text{ Fucose / mg proteins} = \frac{X}{\text{Optical density (O.D.) of sample A}}
\]

➢ **Estimation of sialic acid (Warren, 1959).**

a) **Principle:** Sialic acids is oxidized with sodium periodate in concentrated phosphoric acid. The periodate oxidation product is coupled with thiobarbituric acid and the resulting chromophore is extracted into cyclohexanone. The thiobarbituric acid assay is reproducible, sensitive (E = 57,000 for N-acetylneuraminic acid) and considerably more specific than other methods and permits accurate direct analysis for sialic acids in tissue
hydrolysates. The thiobarbituric acid is specific enough to permit accurate
direct measurement of the sialic acid content of tissues. It is the unique
method widely used for only measurement of unbound sialic acid.

b) Reagents:
- N-acetylneuraminic acid (0.001%) (A 9646 Sigma chemicals USA).
- 0.1 N H₂SO₄ (2.77 ml of the conc. H₂SO₄ diluted in 1 litre of distilled water.
- 0.2 M sodium metaperiodate prepared in 9 M phosphoric acid.
- 10% sodium arsenite prepared in 0.5 M sodium sulphate and 0.1N H₂SO₄.
- 0.6% thiobarbituric acid in 0.5M sodium sulphate.

c) Procedure:

For estimation of sialic acid duodenal tissue was homogenized in distilled
water (1mg / ml distilled water). 0.1 N Sulphuric acid (H₂SO₄) was added to
the homogenates and centrifuged at 5000 rpm at 10°C for 10 minutes. Then
heated at 80°C for one hour to release bound sialic acid. Due to mild acid
hydrolysis reaction bound Sialic acid released from glycoproteins. They are
present at terminal position in these molecules and labile nature of their
glycosidic bonds. Complete liberation of sialic acid can be carried out by use of
0.1 N H₂SO₄ at 80°C for 1 hr.

Assay: Additions were made as per table given below:

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample</td>
<td>--</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>2</td>
<td>N-acetylneuraminic acid (0.001 %)</td>
<td>0.2 ml</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>0.1 N H₂SO₄</td>
<td>--</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>4</td>
<td>Periodate Solution</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Mix the solution well and kept at room temperature for 20 mins

| 5      | 10 % Sodium Arsenite | 1.0 ml | 1.0 ml | 1.0 ml |

Shake all test tubes till yellow color disappeared
### Materials and Methods

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.6% Thiobarbituric acid</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td></td>
<td>Shake vigorously after addition of solution by capping and all the test tubes were heated in boiling water bath for 5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cyclohexanone</td>
<td>4.3 ml</td>
<td>4.3 ml</td>
<td>4.3 ml</td>
</tr>
</tbody>
</table>

All the test tubes were shaken two times and then centrifuge at 1000 rpm for 3 minutes. The upper layer of Cyclohexanone phase red transferred to cuvettes. The optical density (O.D.) taken at 549 at nm against blank.

d) Calculation:
The sialic acid concentration per mg protein was determined by standard graph of N-acetylneuraminic acid.

#### Antioxidants enzyme activity

**Determination of total lipid peroxidation (Wills, 1966)**

a) Principle:
Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serve as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (thiobarbituric acid) to give a pink coloured product having absorption maximum at 532 nm.

b) Reagents:
- 75 mM Potassium Phosphate Buffer (pH 7.04)
- 1mM Ascorbic acid
- 1mM Ferric chloride
- 20% Trichloroacetic acid (20 gm Trichloroacetic acid + 100 ml of H₂O).
- 0.67% Thiobarbituric acid (0.67 gm Thiobarbituric acid + 100 ml of H₂O).
- Chlorotetracycline (10ppm)
Preparation of Reaction mixture (Freshly prepared): The reaction mixture was prepared by adding 1mM ascorbic acid and 1mM FeCl₃ in 100 ml of 75 mM Potassium Phosphate buffer (pH 7.0) 10 ppm. Chlorotetracycline was added acts as an antibiotic.

c) Procedure:

Tissue homogenate was prepared in reaction mixture (2mg/ml). Aliquots of 0.2 ml homogenate mixed with 1.8 ml of distilled water and 1ml of 20% Trichloroacetic acid (TCA). Immediately 2 ml of 0.67 % Thiobarbituric acid (TBA) was added and the all test tubes were placed in boiling water bath for 20 min. All tubes were cooled and centrifuged at 1000 rpm for 10 minutes. Absorbance was taken at 532 nm against a control containing all the reagents except the homogenate. 2 ml distilled water used for control, 1 ml 20% Trichloroacetic acid (TCA), 2 ml of 0.67 % Thiobarbituric acid (TBA) Zero absorbance was set by control.

d) Calculations:

Formula for calculation of MDA content:

\[
\text{Lipid peroxidation / mg of tissue} = \frac{\text{Optical density (O.D.) of Sample} \times 3 \times 6}{0.156 \times 0.4}
\]

Where,

- 0.156 = absorbance for 1mM soln. of MDA in a 1cm thick cell at 532 nm.
- 0.4 = concentration of tissue in mg present in 0.2 ml of sample
- 3 = Volume of sample taken for photometric measurement
- 6 = Scaling factor for conversion to per hour

➢ Estimation of Reduced glutathione (Ellman’s method, 1959)

a) Principle: The principle of Ellman’s method was based on the formation of yellow colour after addition of 5, 5'- dithio-bis-2-nitrobenzoic (DTNB) is with sample or compound containing sulphhydril groups. The developed colour intensity was read at 412 nm.

b) Reagents:

• 10% TCA (Trichloro acetic acid) [10 gm in 100 ml D.W.]
• 0.2M Pottassium di-hydrogen Orthophosphate [2.271 gm in 100 ml D.W.]
• 0.2 M NaOH [0.8gm in 100 ml D.W.]

**Phosphate Buffer ( pH 8)** [ 50 ml 0.2M Pottassium di-hydrogen Orthophosphate + 46:1 ml 0.2M NaOH volume made up to 200 ml and adjust pH 8]

• 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) solution [ 0.4 mg 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) in 1ml 1%Sodium citrate solution] i.e. Ellman’s Reagent.

• 1% Sodium citrate solution [1gm of Sodium citrate in 100 ml D.W]

c) Procedure –

1gm of scrapped glandular tissue of duodenum is homogenized in 10 ml of chilled 10 % Trichloacetic acid (TCA) and homogenised tissue sample were centrifuged at 3000 rpm for 10 minute then supernatant were separated. 0.5 ml of supernatant was taken in test tube then added 2 ml of 0.2M Phosphate buffer (pH 8) into it. After that 0.2 ml of DTNB solution added and take the optical density at 412 nm. The amount of glutathionate is calculated using the absorptivity 13,600M⁻¹cm⁻¹.

➢ **Estimation of superoxide dismutase (SOD)** (Beaucham and Fridovich method 1971).

a) Principle:

The substrate Nitro blue tetrazolium (NBT) reduced blue colour pharmazone dye by superoxide radical. Superoxide radical are produced photochemically in the reaction mixture due to addition of the riboflavin. EDTA in assay mixture donates e- to riboflavin in presence of UV light. The amount of dye formed was measured at 560 nm on spectrophotometer.

b) Reagents:

• 0.2 M Phosphate buffer of pH 7.8
• 10 mM Ethylene diamine tetra acetic acid (EDTA)
• 130 mM Methionine
• Nitro blue tetrezolium (NBT, 150 Dm)
• Riboflavin (60 Dm)

c) Procedure:

**Preparation of sample:** The homogenates were prepared in 0.25 M sucrose and 1mM Ethylene diamine tetra acetic acid (EDTA) and centrifuged at 3000 rpm for 15 minutes. Supernatants were obtained and supernatant was centrifuged at 10,000 rpm at 1- 4°C for 10 min. The supernatant after centrifugation were discarded and the pellets were resuspended in 0.2 M phosphate buffer of pH 7.8. They were used as sample for estimation of SOD activity.

Different test tubes were taken for each of the homogenates and labeled accordingly. The following additions were done. A system without homogenate was treated as control.

<table>
<thead>
<tr>
<th>Sample tube</th>
<th>Control tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4ml Phosphate Buffer pH7.4</td>
<td>3.4ml Phosphate Buffer pH7.4</td>
</tr>
<tr>
<td>0.3ml EDTA(10mM)</td>
<td>0.3ml EDTA(10mM)</td>
</tr>
<tr>
<td>1.2ml Methionine (130mM)</td>
<td>1.2ml Methionine (130mM)</td>
</tr>
<tr>
<td>0.6ml Nitro blue tetrazolium (NBT) 750 uM</td>
<td>0.6ml Nitro blue tetrazolium (NBT) 750 uM</td>
</tr>
<tr>
<td>0.1ml Homogenate</td>
<td>Phosphate Buffer pH7.8</td>
</tr>
</tbody>
</table>

The reaction was started by addition of 0.4 ml riboflavin and by placing the tubes in front of two 18W fluorescence tubes for 30 min. After exposure to fluorescence light the tubes were removed and covered with black paper to terminate reaction. The optical density was measured at 560 nm on spectrophotometer. The assay mixture which was not exposed to light considered as control.

d) Calculations:

\[
\text{% inhibition of Farmazone product} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100
\]
1 unit of enzyme activity was taken as quantity of enzyme that reduced absorbance reading to 560 nm in comparison with those lacking enzyme.

- **Estimation of Catalase (CAT) (Luck method, 1974):**

  a) **Principle:** The ultra violet absorption spectra of hydrogen peroxide can be measured at 240 nm, the absorbance of hydrogen peroxide lowered when it degraded by catalase enzyme. From the decrease in optical density, the enzyme activity can be calculated.

  b) **Reagents:**

     - 0.2 M Phosphate buffer solution of pH 7.8.
     - 2 mM Hydrogen peroxide (H₂O₂) in phosphate buffer.

  c) **Procedure: Preparation of sample:**

     For the estimation of catalase enzyme, duodenums were removed, weighed, and freezed. Then the thawed tissues were homogenized separately 15 mg/ml 0.067M phosphate buffer (pH 6.8) in pre cooled glass mortar and pestle. The homogenized sample were centrifuged at 4ºC at10,000 rpm for 15 minutes. The supernatants were used as enzyme source for the estimation of enzyme activity.

  **Enzyme Assay:** 3 ml (0.067M) phosphate buffer containing H₂O₂, 50 ml of enzyme source was added in cuvette, mixed well and absorbance read at zero second at 240 nm on double beam UV spectrophotometer Systronic 220 (room temperature). Record the absorbance at each minute till the reading remains stable.

  d) **Calculations:** One enzyme unit was considered as the amount of enzyme required to decrease absorbance at 240 nm by 0.05 units.
STATISTICAL ANALYSIS: (Fischer, 1936; Snedecor, 1946; Wills, 1949)

Results were interpreted with student ‘t’ test statistical methods. All values are indicated as mean ± S.D. The statistical analysis was carried out by using student ‘t’ test. Calculated probability (P) value: of P < 0.01 considered statistically significant, P < 0.05 considered almost significant and P < 0.001 was considered highly significant.

1. \( \overline{X} \) = Arithmetic mean of X independent variables.

\[
\overline{X} = \frac{\sum X}{N}
\]

Where, \( N \) = number of variables

2. Standard Deviation (SD):

\[
\text{S.D.} = \sqrt{\frac{\sum (X - \overline{X})^2}{N - 1}}
\]

3. Statistical significance:

\[
\text{S.} = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}
\]

Where, \( N_1 \) = Number of observations of first group.
\( N_2 \) = Number of observations of second group.
\( S_1 \) = Standard Deviation (SD) of first group.
\( S_2 \) = Standard Deviation (SD) of second group.

4. Student ‘t’ test:

\[
t = \frac{\overline{X}_1 - \overline{X}_2}{S} \sqrt{\frac{N_1 + N_2}{N_1 \times N_2}}
\]

Where, \( \overline{X}_1 \) = Mean of first group
\( \overline{X}_2 \) = Mean of second group
The probability ‘P’ of obtained ‘t’ values was tailed from the tabulated ‘t’ value from Fischer’s table at the probability level 0.1, 0.02, 0.05, 0.01 and 0.001 at the respective degrees of freedom. In the present work the degree of freedom was eight. If the calculated value was higher than the tabulated value at the probability level, 0.05, 0.01 and 0.001 then the difference was accepted as significant. The ‘P’ value are significant according to the following conversions:

- $P > 0.01$ = the difference is said to be non–significant.
- $P > 0.05$ = the difference is said to be non–significant.
- $P > 0.1$ = the difference is said to be non–significant.
- $P < 0.05$ = the difference is said to be almost significant.
- $P < 0.02$ = the difference is said to be significant.
- $P < 0.01$ = the difference is said to be significant.
- $P < 0.001$ = the difference is said to be highly significant.
## LIST OF CHEMICALS USED

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name of the Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glycerol</td>
</tr>
<tr>
<td>2.</td>
<td>Sodium metabisulphate (Na$_2$S$_2$O$_3$)</td>
</tr>
<tr>
<td>3.</td>
<td>Hydrochloric acid (HCl)</td>
</tr>
<tr>
<td>4.</td>
<td>Cysteamine Hydrochloride (C$_2$H$_7$NS.HCL)</td>
</tr>
<tr>
<td>5.</td>
<td>Acetic acid (CH$_3$COOH)</td>
</tr>
<tr>
<td>6.</td>
<td>Hydrogen Sulphate (H$_2$SO$_4$)</td>
</tr>
<tr>
<td>7.</td>
<td>Sodium Hydroxide (NaOH)</td>
</tr>
<tr>
<td>8.</td>
<td>Di-sodium hydrogen phosphates (Na$_2$HPO$_4$.2H$_2$O)</td>
</tr>
<tr>
<td>9.</td>
<td>Acetone (CH$_3$)$_2$</td>
</tr>
<tr>
<td>10.</td>
<td>Trichloroacetic acid (TCA) (C$_3$HC$_3$O$_2$)</td>
</tr>
<tr>
<td>11.</td>
<td>Cyclohexanone (CH$_2$)$_3$CO</td>
</tr>
<tr>
<td>12.</td>
<td>Cysteine hydrochloride (C$_3$H$_7$NO$_2$S.HCL)</td>
</tr>
<tr>
<td>13.</td>
<td>Bovine Serum albumen (BSA)</td>
</tr>
<tr>
<td>14.</td>
<td>Periodic acid (H$_5$IO$_6$)</td>
</tr>
<tr>
<td>15.</td>
<td>Dextrene Diphenyl Phthalate Xylene (DPX)</td>
</tr>
<tr>
<td>16.</td>
<td>Alcian Blue</td>
</tr>
<tr>
<td>17.</td>
<td>Xylene (C$<em>8$H$</em>{10}$)</td>
</tr>
<tr>
<td>18.</td>
<td>Alcohol(ethanol) (C$_2$H$_5$OH)</td>
</tr>
<tr>
<td>19.</td>
<td>Copper sulphate (CuSO$_4$)</td>
</tr>
<tr>
<td>20.</td>
<td>Sodium-potassium tartarate (Na/K- tartarate )</td>
</tr>
<tr>
<td>21.</td>
<td>Sodium Carbonate (Na$_2$CO$_3$)</td>
</tr>
<tr>
<td>22.</td>
<td>Folinciocalteau-Phenol reagent</td>
</tr>
<tr>
<td>23.</td>
<td>Calcium chloride (CaCl$_2$)</td>
</tr>
<tr>
<td>24.</td>
<td>Papain</td>
</tr>
<tr>
<td>25.</td>
<td>Sulfuric Acid (H$_2$SO$_4$) (01491)</td>
</tr>
<tr>
<td>26.</td>
<td>Sodium Sulfate (Na$_2$SO$_4$)</td>
</tr>
<tr>
<td>27.</td>
<td>Phenol (CH$_3$OH)</td>
</tr>
<tr>
<td>28.</td>
<td>Periodic Acid Schiff’s reagent stain (1158A)</td>
</tr>
<tr>
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</tr>
<tr>
<td>29.</td>
<td>N-acetylneuraminic acid (C₁₁H₁₀NO₄)</td>
</tr>
<tr>
<td>30.</td>
<td>Sodium Metaperiodate (NaIO₄)</td>
</tr>
<tr>
<td>31.</td>
<td>Sodium Arsenite (NaAsO₂) (01348)</td>
</tr>
<tr>
<td>32.</td>
<td>Phosphoric acid (H₃PO₄)</td>
</tr>
<tr>
<td>33.</td>
<td>Calcium Acetate (00372)</td>
</tr>
<tr>
<td>34.</td>
<td>Thiobarbituric acid (TBA)</td>
</tr>
<tr>
<td>35.</td>
<td>Ascorbic acid (C₆H₇O₆)</td>
</tr>
<tr>
<td>36.</td>
<td>Chlorotetracycline (C₂₂H₂₃ClN₂O₈)</td>
</tr>
<tr>
<td>37.</td>
<td>Pottassium di-hydrogen Orthophosphate</td>
</tr>
<tr>
<td>38.</td>
<td>DTNB (5,5'-dithiobis-(2-nitrobenzoic acid))</td>
</tr>
<tr>
<td>39.</td>
<td>Sodium citrate (Na₃CH₅O₇)</td>
</tr>
<tr>
<td>40.</td>
<td>Ethylene diamine tetra acetic acid (EDTA) (C₁₀H₁₆N₂O₈)</td>
</tr>
<tr>
<td>41.</td>
<td>Methionine (C₅₅H₁₁N₂O₃S)</td>
</tr>
<tr>
<td>42.</td>
<td>Nitro blue tetroxolium (NBT)</td>
</tr>
<tr>
<td>43.</td>
<td>Riboflavin (C₁₇H₂ON₄O)</td>
</tr>
<tr>
<td>44.</td>
<td>Hydrogen peroxide (H₂O₂)</td>
</tr>
<tr>
<td>45.</td>
<td>Formaldehyde (HCOH)</td>
</tr>
<tr>
<td>46.</td>
<td>Haematoxyline (07955A)</td>
</tr>
<tr>
<td>47.</td>
<td>Eosin (Powder)</td>
</tr>
<tr>
<td>48.</td>
<td>Basic Fuchsin (Powder)</td>
</tr>
<tr>
<td>49.</td>
<td>Activated Charcoal</td>
</tr>
<tr>
<td>50.</td>
<td>Bromine (Br₂)</td>
</tr>
<tr>
<td>51.</td>
<td>Lithium Sulfate (LiSO₄)</td>
</tr>
<tr>
<td>52.</td>
<td>Sodium tungstate (Na₂SO₄.2H₂O)</td>
</tr>
<tr>
<td>53.</td>
<td>α-D-Fucose</td>
</tr>
<tr>
<td>54.</td>
<td>D-glucoronic acid</td>
</tr>
<tr>
<td>55.</td>
<td>Paraffin Wax</td>
</tr>
<tr>
<td>56.</td>
<td>Ferric Chloride (FeCl₃)</td>
</tr>
</tbody>
</table>