Chapter 3

MATERIALS AND METHODS

3.1. ANIMALS

Male Sprague-Dawley albino rats were bred in the Animal House of School of Biosciences, M. G. University with standard facilities having CPCEA approval (No.732/1999). Animals were housed in pathogen free polycarbonate cages with 12 hour day-night cycle at 25 ± 2°C and humidity 55 – 65 %. They were fed with the balanced laboratory diet (Amrut Laboratory Animal Feed manufactured by Nav, Maharashtra Chakan Oil Mills Ltd, Pune). Water was given *at libitum*. The sterile bedding was changed weekly. Male rats weighing 120-150 g. were used as experimental animals. Ethical clearance for handling the animals were obtained from the committee constituted for the purpose.

3.2. CHEMICALS AND BIOLOGICALS

Chemicals like Chicken Type II Collagen, Glucosamine, Galactose, Mannose, Fucose, N-Acetyl Neuraminic Acid (Sialic acid) and the different p-Nitrophenyl glucosides as substrates for glycosidase assay were purchased from Sigma. Incomplete Freund’s Adjuvant (IFA) was obtained from Calbiochem, Aqua E from Troikka Pharmaceuticals Ltd., Sallaki from Gufic Biosciences Ltd. and the spices Ginger and Turmeric from Eastern Condiments. All other chemicals were procured from SRL, India.
3.3. INDUCTION OF ARTHRITIS - Chen and Wei, 2003 [297]

Native Chicken Type II Collagen (Sigma Product No. C 9301) was dissolved overnight at 4 °C in 0.1 M acetic acid and emulsified with equal volume of Incomplete Freund’s Adjuvant (IFA). Rats were given subcutaneous injection of 0.1 ml of emulsified collagen (100 µg.) at the plantar surface of the left foot. After 7 days, a booster dose of similar quantity was injected at the base of the tail. On 14th day, animals with swelling on the paw and limb joints were screened for the experiment and called CIA rats.

3.4. EXPERIMENTAL DESIGN

Altogether 48 rats were used. They were divided into 8 groups:

Group I - Rats received normal laboratory diet and water \textit{ad libitum}. (Control)

Group II - Arthritic (CIA) rats as disease control on normal diet and water.

Group III - CIA rats treated with Vitamin C (100 mg Ascorbic acid in 50 ml drinking water / day / rat).

Group IV - CIA rats treated with Vitamin E (100 IU in 50 ml drinking water / day / rat). Dose prepared by dissolving Aqua-E 200 IU (Troikka Pharmaceuticals Ltd) in 100 ml drinking water.

Group V - CIA rats treated with Glucosamine (100 mg GlcNAc HCl in 50 ml drinking water / day / rat).

Group VI - CIA rats treated with the spice Ginger (100 mg dry, fibre-free ginger powder along with 15 g balanced diet / day / rat in the form of dry cakes).
Materials and Methods

Group VII  - CIA rats treated with the spice Turmeric (100 mg dry along with 15 g balanced diet a day / rat in the form of dry cakes). Treatments were continued for 30 days for all the Groups.

Group VIII  - CIA rats treated with Sallaki (100 mg extract of *Boswellia serrata* along with 15 g balanced diet / day / rat).

Cakes of feed were prepared by drying the dough of 60 g rat feed and one tablet of Sallaki (manufactured by Gufic Biosciences Ltd) containing 400 mg plant extract.

3.5. EVALUATION OF ARTHRITIS

The severity of the disease was monitored and evaluated by measuring the thickness of the swollen paw with a Vernier calipers on 8th, 15th, 22nd and 30th days. Measurements were made for all rats and the mean value was calculated for each group. The biochemical parameters were also studied as described in section 3.7 to 3.11.

3.6. COLLECTION OF SAMPLES

At the end of treatments, animals were deprived of food and water overnight and sacrificed by cervical dislocation. Blood was collected in test tubes by cutting jugular vein. Anticoagulated blood was maintained in EDTA treated tubes. For obtaining serum, blood was allowed to coagulate under room temperature for about 30 minutes. It was then centrifuged at 1500 rpm for 15 minutes avoiding haemolysis. The supernatant was collected and kept in ice cold condition until required. Tissues like Liver, Heart, Kidney, Testis and Brain were excised and placed in ice cold saline. They were washed in cold saline to remove blood, packed in aluminium foil and stored in ice until processed.
3.7. ANALYSIS OF CARBOHYDRATE MOITIES IN GLYCOPROTEINS

3.7.1. Preparation of extracts for carbohydrate analysis

Weighed quantities of wet tissues were homogenized in ice cold condition and each homogenate was dissolved in 2ml of distilled water. Extracts were transferred into graduated tubes and the volume made upto 10ml with distilled water. They were sonicated in ice cold condition for 30 - 45 seconds’ duration each. Suspension was centrifuged at 10000 rpm for 30 min. at 0 - 4 °C and decanted. Supernatants were collected and used for estimations.

3.7.2. Preparation of dry, defatted tissue - Folch et al., 1957 [298]

1 ml. of serum or 5 ml. of sonicated tissue extract was mixed with 5 times the volume of acetone and kept at 0 °C for 72 hrs. Acetone was changed every 24 hrs. After 72 hrs., the precipitate was centrifuged and treated with ether-acetone mixture ( 3:1 v/v ) at 37 °C for 1h followed by ether alone for another 1h. The defatted tissue was dried under vacuum for constant weight and stored at 0 °C until used further.

3.7.3. Papain digestion of defatted tissue - Wagh et al., 1973 [299]

Weighed quantities of dry, defatted tissue were mixed with one-third its weight of crystalline Papain and suspended in a few ml of 0.2 M acetate buffer (pH 7.0) containing 2.0 mg cysteine hydrochloride per ml. The mixture was kept in water bath at 65 °C for 72 hrs. with constant stirring. Fresh Papain was added every 24 hrs. The final digest was then cooled to room temperature. About 4 – 5 volumes of ethanol was added to the digest and kept at 0 °C for 24 hrs. It was then centrifuged at 5000 rpm for 5 min. The supernatant was evaporated to dryness in the cold in vacuum. The residue was dissolved in a known volume of
Materials and Methods
distilled water and the aliquots were used for the analysis of Protein bound Hexoses, Fucoses and Sialic acids.

3.7.4. Estimation of monosaccharides - Winzler, 1972 [300]

3.7.4.1. Protein Bound Hexoses

Protein bound Hexoses were determined by the Orcinol-H$_2$SO$_4$ method of Weimer and Moshin as detailed by Winzler [300] with modifications.

Reagents

- Orcinol-H$_2$SO$_4$ reagent:- Reagent A: 60 ml of concentrated H$_2$SO$_4$ and 40 ml of distilled water. Reagent B: 1.6 g of Orcinol (recrystallized from benzene) dissolved in 100 ml of water.
  
  7.5 volumes of Reagent A was mixed fresh with 1 volume of Reagent B every time.

- Galactose – Mannose Standard (0.2 mg / ml): 100 mg each of galactose and mannose dissolved in distilled water and made up to 100 ml.

Procedure

1. To 1 ml aliquot in a test tube marked ‘Test’ was added 8.5 ml of Orcinol-H$_2$SO$_4$ reagent and mixed well using a cyclomixer.

2. A ‘Blank’ and a ‘Standard’ were prepared similarly by mixing 1 ml distilled water and 1 ml of galactose-mannose standard respectively with 8.5 ml Orcinol-H$_2$SO$_4$ reagent.

3. The tubes were capped with glass balls to minimize evaporation and placed in a water bath at 80 °C for exactly 15 minutes.
4. The tubes were cooled under tap water and the absorbance was measured spectrophotometrically at 540 nm.

**Calculation**

The hexose content was calculated using the formula

\[
\text{Protein Bound Hexoses} = \frac{(\text{OD test} \times \text{con. std})}{\text{OD std}} \div \text{weight of protein powder} \quad (\text{mg/g dry protein})
\]

**3.7.4.2. Fucoses**

Fucoses were estimated by the method of Dische and Shettles as detailed by Winzler, 1972 [300]

**Reagents**

- H$_2$SO$_4$ – H$_2$O Mixture: 6 volumes of concentrated H$_2$SO$_4$ and 1 volume of distilled water were mixed carefully and kept in cold.
- Cysteine Reagent: 3 g of cysteine hydrochloride was dissolved in 100 ml water.
- Fucose Standard (20 µg / ml Fucose): Dissolved 10 mg fucose in distilled water and made up to 100 ml. This stock solution was diluted 5 times accurately.

**Procedure**

1. Three test tubes were marked ‘Test’, ‘Blank’ and ‘Std’. 1 ml of aliquot, 1 ml of distilled water and 1 ml of fucose standard were placed respectively in these tubes. A similar duplicate set was also prepared.
2. Added 4.5 ml of ice cold H₂SO₄ - H₂O mixture to each tube and mixed well maintaining the solutions cold in an ice bath.

3. All the tubes were heated exactly for 3 minutes in a boiling water bath and then cooled in tap water.

4. To the first set, added 0.1 ml of Cysteine Reagent and mixed immediately. These tubes were marked \( \text{cys}^+ \). (The other set prepared to correct for nonspecific colour development was treated as \( \text{cys}^- \))

5. The tubes were left at room temperature for 60 to 90 minutes. The optical density was measured for all the samples at 396 nm and 430 nm using spectrophotometer.

**Calculation**

The absorbance due to methyl pentose in a test sample was determined by subtracting the difference in ODs at 396 and 430 nm of the sample analysed without cysteine from the difference in ODs at 396 and 430 nm of the respective sample containing cysteine. That is

\[
\text{OD test} = (\text{OD}_{396} - \text{OD}_{430})_{\text{cys}^+} - (\text{OD}_{396} - \text{OD}_{430})_{\text{cys}^-}
\]

Similarly,

\[
\text{OD std} = (\text{OD}_{396} - \text{OD}_{430})_{\text{cys}^+} - (\text{OD}_{396} - \text{OD}_{430})_{\text{cys}^-}
\]

\[
\text{Fucose content (mg/g dry protein)} = \frac{(\text{OD test} \times \text{con. std})}{\text{OD std}} \div \text{weight of protein powder}
\]

**3.7.4.3. Hexosamines**

Hexosamines were detected by the method of Elson and Morgan as detailed by Winzler, 1972 [300]
Reagents

- Acetylacetone Reagent: 1 ml of Acetylacetone mixed with 50 ml of 0.5 N Na₂CO₃ (freshly prepared)

- Ehrlich’s Reagent: 0.8 g of p-Dimethylaminobenzaldehyde (recrystallized as the hydrochloride) was dissolved in 30 ml of methanol and 30 ml of con. HCl added to it.

- Glucosamine Standard: 0.05 mg / ml of free glucosamine (0.06 mg / ml of glucosamine hydrochloride) in distilled water.

Procedure

10 mg of defatted protein powder was added to 2 ml of 3 N HCl and hydrolysed in a boiling water bath with an air condenser for 4 hours. After cooling, the hydrolyzate was neutralized with 3 N NaOH until it was barely alkaline to litmus. It was then diluted to 10 ml with water. The resultant solution was tested for Hexosamines by the following steps.

1. To 1 ml aliquots (and to 1 ml of distilled water for blank and 1 ml of glucosamine for standard), added 1 ml of acetylacetone reagent and mixed well. Tubes were capped with marbles to prevent evaporation and placed in boiling water bath for 15 minutes.

2. The tubes were cooled under tap, 5 ml of 95 % ethanol was added to each tube and mixed.

3. Added 1 ml of Ehrlich’s reagent, mixed well and diluted to 10 ml with 95 % ethanol. The optical density was measured after 30 minutes at 530 nm.
Calculation

The hexosamine content was calculated using the formula

\[
\text{Hexosamines (mg/g dry protein)} = \frac{(\text{OD test} \times \text{con. std})}{\text{OD std}} \div \text{weight of protein powder}
\]

3.7.4.4. Sialic acids

Sialic acid was determined by Diphenylamine reaction of Dische as detailed by Winzler, 1972 [300]

Reagents

- Diphenylamine Reagent: 1 g of Diphenylamine (recrystallized from ethanol) was dissolved in a mixture of 90 ml of glacial acetic acid and 10 ml of concentrated sulphuric acid.
- Acid Mixture: Mixed 90 ml of glacial acetic acid and 10 ml of concentrated sulphuric acid.
- Sialic acid Standard: Prepared 0.04 mg/ml concentration by dissolving 1 mg of Sialic acid (N-Acetyl Neuraminic acid – Sigma) in 25 ml distilled water.

Procedure

1. Pipetted 2 ml of aliquot into each of two 15 x 150 mm test tubes.

2. Added 4 ml of DPA reagent into one of the two tubes and marked DPA\(^+\). 4 ml of the acid mixture containing no DPA was added to the other tube and marked as DPA\(^-\).

3. Similarly, a set of blank (2 ml of distilled water + 4 ml of DPA reagent and 2 ml water + 4 ml acid mixture without DPA) and a set of standard
(2 ml standard + 4 ml DPA reagent and 2 ml standard + 4 ml acid mixture) were also prepared.

4. The contents were mixed, the tubes capped with glass marbles and immersed in boiling water bath for exactly 30 minutes.

5. Tubes were then cooled and the OD measured spectrophotometrically at 530 nm with the reagent blank set at zero.

**Calculation**

The Sialic acid concentration was calculated using the formula

\[
\text{Sialic acid (mg} \text{/ g protein)} = \frac{(\text{OD test } \text{DPA}^+ - \text{OD test } \text{DPA}^-) \times \text{con.std.}}{(\text{OD std DPA}^+ - \text{OD test } \text{DPA}^-) + \text{wt. of protein powder}}
\]

**3.8. ASSAY OF GLYCOSIDASES**

**3.8.1. Preparation of extracts for enzyme studies**

Weighed quantities of wet tissues like liver, heart, kidney, testis and brain were homogenized in ice cold condition using mortar and pestle kept in ice bath. The homogenate was dissolved in appropriate buffers as detailed under each enzyme and made up to a fixed volume (10 ml) using the same buffer. It was sonicated in ice cold condition for 30 - 45 seconds' duration each. Suspension was centrifuged at 10000 rpm for 30 min. at 0 - 4 °C and decanted. Supernatants were collected and used for protein estimation and enzyme assay.

**3.8.2. Estimation of Enzyme activity**

The serum and tissue extracts were used for studying the activity of the following glycosidases using exogenous substrates as shown against each.
1. α-D-Mannosidase by using p-Nitrophenyl α-D-mannopyranoside as substrate.
2. β-D-Glucosidase by using p-Nitrophenyl β-D-glucopyranoside as substrate.
3. β-D-Galactosidase by using p-Nitrophenyl β-D-galactopyranoside as substrate.
4. α-L-Fucosidase by using p-Nitrophenyl α-L-fucopyranoside as substrate.
5. β-N-acetyl Hexosaminidase by using p-Nitrophenyl β-N-acetylglucosaminide as substrate.

The enzyme activity was estimated by measuring the hydrolysis of the concerned substrate, the p-Nitrophenol released being determined by its absorbance at 400 nm at pH 10.7. Activity of enzyme was calculated and expressed as unit of enzyme. One unit of enzyme corresponds to the release of 1.0 µmol of p-Nitrophenol per minute at 37 °C and expressed as Unit/ml/minute. Specific activity is defined as Units per milligram of protein.

3.8.2.1. α-D Mannosidase Activity - Ram et al., 1978 [301]

α – D Mannosidase was assayed by measuring the hydrolysis of p-Nitrophenyl α-D- mannopyranoside at an optimum pH of 4.5

Reagents

- p-Nitrophenyl α-D- mannopyranoside : 10 mM
- Sodium acetate buffer, 0.5 M, pH 4.5: Dissolved 4.10 g anhydrous sodium acetate in 100 ml distilled water and added to 2.86 ml glacial acetic acid with constant stirring. Adjusted the pH to 4.5 using a pH meter.
- p-Nitrophenol Standard : 10 µg / ml in water
Glycine buffer, pH 10.7: Dissolved 4.992 g Glycine, 1.958 g Sod. chloride and 4.40 g Sod. carbonate in distilled water and made up to 500 ml. Adjusted the pH to 10.7 with 1 N Sod. hydroxide.

Procedure

Reaction mixture consisting of 0.05 ml of Sodium acetate buffer, 0.1 ml of p-Nitrophenyl α-D- mannopyranoside, 0.025 ml of enzyme and water (0.325 ml made up to a final volume of 0.5 ml) was incubated for 30 minutes at 37 °C. A control avoiding the substrate (final volume 0.4 ml), a reagent blank consisting of 0.05 ml of Sodium acetate buffer, 0.1 ml substrate and 0.350 ml of water (final volume 0.5 ml) and the standard with 0.05 ml of Sodium acetate buffer, 0.1 ml of p-Nitrophenol standard and 0.35 ml of water (final volume 0.5 ml) were also incubated simultaneously. Reaction was terminated by adding 1 ml of Glycine buffer to all the tubes. 0.1 ml of the substrate was added to the control after termination of reaction.

The absorbance of the solution was measured at 400 nm in cells with 1 cm light path and zero set by using the reagent blank. The difference in OD between the test and control was used for calculation of the amount of p-Nitrophenol released.

Calculation

Units of enzyme in terms of µmoles of p-Nitrophenol was calculated using the formula

\[
\mu \text{moles of } p-\text{Nitrophenol} = \frac{[(\Delta OD \text{ test } \times \text{con.std.x1000}) \div 139]}{\text{OD std.xTime}} \div 25
\]
Materials and Methods

where,

\[ \Delta \text{OD test} = \text{Difference in OD between test and control tubes}, \]
\[ \text{con. of std.} = 10 \mu g / ml, \]
\[ \text{Time} = 30 \text{ minutes}, \]
\[ 139 = \text{M.W. of p-Nitrophenol} \]
\[ 25 = \text{Volume of enzyme in } \mu l. \]

1000 for conversion into 1 L,

3.8.2.2. \( \beta \)-D Glucosidase Activity - Hebraud and Ferre, 1988 [302]

\( \beta \)-D Glucosidase was assayed by measuring the hydrolysis of p-Nitrophenyl \( \beta \)-D- glucopyranoside at an optimum \( p^H \) of 4.3 by a modified method of Hebrand and Ferre, 1988.

Reagents

- p-Nitrophenyl \( \beta \)-D- glucopyranoside : 20 mM
- Citrate phosphate buffer, \( p^H \) 4.3: 10 volumes of 0.1 M Citric acid adjusted to \( p^H \) 4.3 using a \( p^H \) meter with approximately 7 volumes of 0.2 M Na₂HPO₄.
- p-Nitrophenol Standard : 10 \mu g / ml in water
- Glycine buffer, \( p^H \) 10.7 : As described under section 3.8.2.1.

Procedure

Reaction mixture consisting of 0.1 ml of Citrate phosphate buffer, 0.1 ml of p-Nitrophenyl \( \beta \)-D- glucopyranoside, 0.025 ml of enzyme and water (0.175 ml) and made up to a final volume of 0.4 ml was incubated for 30 minutes at 37 °C. A control avoiding the substrate (final volume 0.3ml), a reagent blank consisting of 0.1 ml of Citrate phosphate buffer, 0.1 ml substrate
and 0.2 ml of water (final volume 0.4 ml) and the standard with 0.1 ml of Citrate phosphate buffer, 0.1 ml of p-Nitrophenol standard and 0.2 ml of water (final volume 0.4 ml) were also incubated simultaneously. Reaction was terminated by adding 1 ml of Glycine buffer to all the tubes. 0.1 ml of the substrate was added to the control after termination of reaction.

The absorbance of the solution was measured at 400 nm in cells with 1 cm light path and zero set by using the reagent blank. The difference in OD between the test and control was used for calculation of the amount of p-Nitrophenol released.

**Calculation**

Units of enzyme in terms of µmoles of p-Nitrophenol was calculated using the formula as given in section 3.8.2.1.

### 3.8.2.3. β-D Galactosidase Activity - Distler and Jourdian, 1978 [303]

β-D Galactosidase was assayed by measuring the hydrolysis of p-Nitrophenyl β-D- galactopyranoside at an optimum pH of 4.3

**Reagents**

- p-Nitrophenyl β-D- galactopyranoside : 20 mM
- Citrate phosphate buffer, pH 4.3: As described under 3.8.2.2.
- Bovine serum albumin (BSA) : 1 %
- p-Nitrophenol Standard : 10 µg / ml in water
- Glycine buffer, pH 10.7: As described under section 3.8.2.1.
**Materials and Methods**

**Procedure**

Reaction mixture consisting of 0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.1 ml of p-Nitrophenyl β-D-galactopyranoside, 0.025 ml of enzyme and water (0.135 ml) made up to a final volume of 0.4 ml was incubated for 30 minutes at 37 °C. A control avoiding the substrate (final volume 0.3 ml), a reagent blank consisting of 0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.1 ml substrate and 0.16 ml of water (final volume 0.4 ml) and the standard with 0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.1 ml of p-Nitrophenol standard and 0.16 ml of water (final volume 0.4 ml) were also incubated simultaneously. Reaction was terminated by adding 1 ml of Glycine buffer to all the tubes. 0.1 ml of the substrate was added to the control after termination of reaction.

The absorbance of the solution was measured at 400 nm in cells with 1 cm light path and zero set by using the reagent blank. The difference in OD between the test and control was used for calculation of the amount of p-Nitrophenol released.

**Calculation**

Units of enzyme in terms of µmoles of p-Nitrophenol was calculated using the formula as given in section 3.8.2.1.

3.8.2.4. α-L Fucosidase Activity - Opheim and Touster, 1978 [304]

α-L Fucosidase was assayed by measuring the hydrolysis of p-Nitrophenyl α-L-fucopyranoside at an optimum pH of 6.0.

**Reagents**

- p-Nitrophenyl α-L-fucopyranoside : 10 mM
Sodium acetate buffer, 0.25 M, pH 6.0: Dissolved 2.05 g anhydrous Sodium acetate in 100 ml distilled water and added to 1.43 ml glacial acetic acid with constant stirring. Adjusted the pH to 6.0 using a pH meter.

Bovine serum albumin (BSA) : 0.5 mg / ml

p-Nitrophenol Standard : 10 µg / ml in water

Glycine buffer, pH 10.7: As given under section 3.8.2.1.

**Procedure**

Reaction mixture consisting of 0.1 ml of Sodium acetate buffer, 0.1 ml of BSA, 0.1 ml of p-Nitrophenyl α-L-fucopyranoside, 0.025 ml of enzyme and water (0.175 ml) made up to a final volume of 0.5 ml was incubated for 15 minutes at 37 °C. A control avoiding the substrate (final volume 0.4 ml), a reagent blank consisting of 0.1 ml of Sodium acetate buffer, 0.1 ml of BSA, 0.1 ml substrate and 0.2 ml of water (final volume 0.5 ml) and the standard with 0.1 ml of Sodium acetate buffer, 0.1 ml of BSA, 0.1 ml of p-Nitrophenol standard and 0.2 ml of water (final volume 0.5 ml) were also incubated simultaneously. Reaction was terminated by adding 1 ml of Glycine buffer to all the tubes. 0.1 ml of the substrate was added to the control after termination of reaction.

The absorbance of the solution was measured at 400 nm in cells with 1 cm light path and zero set by using the reagent blank. The difference in OD between the test and control was used for calculation of the amount of p-Nitrophenol released.
Calculation

Units of enzyme in terms of μmoles of p-Nitrophenol was calculated using the formula as given in section 3.8.2.1. except for Time which is 15 minutes here.

3.8.2.5. β-N Acetyhexosaminidase Activity - Sarber et al., 1978 [305]

β-N Acetyhexosaminidase was assayed by measuring the p-Nitrophenol released by the hydrolysis of p-Nitrophenyl β-D- glucosaminide at an optimum pH of 4.3.

- p-Nitrophenyl β-D- glucosaminide : 20 mM
- p-Nitrophenyl β-D- galactosaminide : 10 mM
- Citrate phosphate buffer (McIlvaine buffer), pH 4.5: 10 volumes of 0.1 M Citric acid adjusted to pH 4.5 using a pH meter with approximately 7 volumes of 0.2 M Na2HPO4.
- Bovine serum albumin (BSA) : 1 %
- p-Nitrophenol Standard : 10 μg / ml in water
- Glycine buffer, pH 9.8: Dissolved 4.992 g Glycine, 1.958 g Sod. chloride and 4.40 g Sod. carbonate in distilled water and made up to 500 ml. Adjusted the pH to 9.8 with 1 N Sod. hydroxide.

Procedure

Reaction mixture consisting of 0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.08 ml each of p-Nitrophenyl β-D- glucosaminide and p-Nitrophenyl β-D- galactosaminide, 0.025 ml of enzyme and water (0.175 ml) made up to a final volume of 0.5 ml was incubated for 30 minutes at 37 °C. A control avoiding the substrate (final volume 0.34 ml), a reagent blank consisting of...
0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.16 ml of both the substrates and 0.2 ml of water (final volume 0.5 ml) and the standard with 0.1 ml of Citrate phosphate buffer, 0.04 ml of BSA, 0.16 ml of p-Nitrophenol standard and 0.2 ml of water (final volume 0.5 ml) were also incubated simultaneously. Reaction was terminated by adding 1 ml of Glycine buffer to all the tubes. 0.08 ml of each substrate was added to the control after termination of reaction.

The absorbance of the solution was measured at 400 nm in cells with 1 cm light path and zero set by using the reagent blank. The difference in OD between the test and control was used for calculation of the amount of p-Nitrophenol released.

**Calculation**

Units of enzyme in terms of µmoles of p-Nitrophenol was calculated using the formula as given in section 3.8.2.1.

### 3.9. ESTIMATION OF ANTIOXIDANT ACTIVITY

#### 3.9.1. Preparation of 1:20 haemolysate

1 ml of anticoagulated blood taken in a microcentrifuge tube was centrifuged at 3500 rpm for 10 minutes. The plasma and buffy coat (seen as a very thin white layer on the surface of erythrocytes) was removed. Sedimented cells were mixed with cold saline (0.9 %) in a cyclomixer and centrifuged again at 3500 rpm for 10 minutes. The supernatant was decanted. Residue was washed in cold saline and centrifuged repeatedly for 5 or 6 times. Then upper saline was removed. The residue was suspended in an equal volume of cold saline and mixed well.
0.2 ml of the suspension was taken in a plastic tube fitted with stopper. 1.8 ml of haemolysing solution (1 g EDTA dissolved in 10 ml water and 50 µl Mercaptoethanol added to it and finally made up to 100 ml – Store at 4°C, stable for 2 weeks) was added to it and closed tightly. This tube was immersed in freezing mixture (equal amounts of common salt and crushed ice) for a few minutes and then thawed. This was repeated twice. The contents gave 1:20 haemolyzate which was used for the assay of Superoxide dismutase.

### 3.9.2. Preparation of tissue extracts for antioxidant activity studies

Tissues like liver, heart, kidney, testis and brain were washed repeatedly with saline to remove blood and wiped. Weighed quantities of wet tissues were homogenized in known volumes of ice cold phosphate buffer (0.1 M, pH 7.4) and sonicated under ice bath. The suspension was centrifuged at 1500 rpm for 10 minutes. The supernatant was used for protein estimation and assay of catalase, SOD, GSH as well as for the estimation of lipid peroxidation as indicated by levels of Malondialdehyde and Conjugated Dienes.

### 3.9.3. Estimation of Antioxidant activity

#### 3.9.3.1. Assay of Catalase activity

Catalase activity was estimated by the method of Calibrone, 1985 [306]

**Reagents**

- Phosphate buffer, 0.1 M, pH 7.0
- Hydrogen peroxide (H₂O₂), 30 mM : Dilute 3 ml H₂O₂ to 100 ml

**Procedure**

1. 2 ml of buffer, 50 µl tissue extract and 0.5 ml of H₂O₂ were mixed well in a test tube.
2. A control containing 50 µl of distilled water in place of tissue extract was also prepared.

3. Absorbance at 0 time, after 30 seconds and 60 seconds was measured at 240 nm for both test and control using the buffer to set zero.

**Calculation**

The average OD was calculated as

$$T = \frac{2y + z}{2}$$

where

- \( y \) = Difference in OD between 0 time and 30 seconds.
- \( z \) = Difference in OD between 0 time and 60 seconds.
- \( T \) = Average OD for 1 minute.

Enzyme activity is calculated using the formula,

$$\text{Units of activity} = \left( \frac{\mu\text{mol} \text{H}_2\text{O}_2 \text{ / min / mg protein}}{\Sigma x 1 x \text{ vol. of aliquot}} + \frac{\text{mg protein}}{\text{ml extract}} \right) \times 1 \times \text{mg protein} / \text{ml}$

\( \Sigma = \) Molar extinction coefficient (0.71)

1 = Enzyme turn over

**3.9.3.2. Assay of Superoxide dismutase (SOD)**

SOD activity was determined by the method of Paoletti and Mocali, 1990 [307]

**Reagents**

- Phosphate buffer, 0.1 M, pH 7.4
- Ethanol-Chloroform mixture (2:1, v/v)
- Triethanolamine - diethanolamine (100mM each)-HCl Buffer (TDB): Prepared by dissolving 14.9 g Triethanolamine, 10.5 g Diethanolamine and approximately 13.8 ml of con. HCl in water and made up to 1 liter. The final pH was 7.4.
Materials and Methods

- **NAD (P)H, 7.5 mM**: Dissolved 20 mg of either NADH or NADPH, di-sodium salt in 4 ml of water. (Stable at 4°C for a week)
- **EDTA – MnCl₂, 100 mM / 50 mM**: Prepared a stock solution of 200 mM EDTA by dissolving 11.69 g EDTA – acid in 200 ml water and adjusting the pH to around 7 with 1 M NaOH and another stock solution of 100 mM MnCl₂ by dissolving 3.95 g MnCl₂·4H₂O in 200 ml water. Mixed the EDTA and MnCl₂ stock solutions in a ratio 1:1 (v/v) and adjusted the pH of the mixture to 7 by dropwise addition of 10 M NaOH (approximately 0.14 ml per 25 ml of reagent).
- **Mercaptoethanol, 10 mM**: Diluted 50 ml of concentrated thiol (14.2 M) with 71 ml of water.

(TDB, Mercaptoethanol, both EDTA and MnCl₂ stock solutions and mixture were stable at room temperature for a month)

**Sample preparation for SOD**

The blood haemolysate and tissue homogenates were dialyzed against phosphate buffer, 0.1 M, pH 7.4 to remove low molecular weight compounds that might have nonenzymatic SOD-like activity. After dialysis, 2.5 ml of the sample was pre-warmed at 37°C for a few minutes and treated with 1 ml of ethanol-chloroform mixture (2:1, v/v) thoroughly to obtain a thick precipitate. Added 2 ml of distilled water and mixed again using a vortex mixer. Incubated at 37°C for about 15 minutes with occasional stirring. The precipitate was separated using a bench top centrifuge. The supernatant was assayed after proper dilution.
Assay for SOD activity

1. The following solutions were subsequently pipetted into the cuvette: 
   800µl of TDB, 40µl of NAD(P)H, 25µl of EDTA-MnCl₂ and 100µl of 
   the sample (or sample solvent for the control).

2. Mixed thoroughly and the OD was read at 340 nm against air for a 
   stable baseline recorded over a 5-minutes period.

3. Added 100 µl mercaptoethanol, mixed well and monitored the decrease 
   in absorbance for about 8 minutes. The OD was recorded thereafter.

Calculation

The difference in OD (ΔOD₃₄₀) with the sample over 8 minute period was 
calculated and considered as Sample rate. Similar Δ OD₃₄₀ for control was 
accepted as Control rate. The percentage of inhibition was calculated.

Percentage inhibition = \( \frac{\text{Sample rate} \times 100}{\text{Control rate}} \)

This percentage is converted into Units referring to the conversion table 
published [307].

3.9.3.3. Estimation of Glutathione (GSH)

Glutathione level was estimated by the method of Bentler and Kelly, 1963[308]

Reagents

- Phosphate buffer, 0.2 M, \( p^H \) 8.0.
- TCA 5 %.
- Ellman’s reagent.
Materials and Methods

- Standard glutathione solution (100 µg / ml): Dissolved 10 mg of glutathione in 100 ml water.

Procedure

1. From the tissue extract, 0.5 ml was pipetted out and precipitated with 2 ml of 5 % TCA. The mixture was shaken well and centrifuged.
2. To 1 ml of the supernatant, added 0.5 ml of Ellman’s reagent followed by 3 ml of phosphate buffer.
3. The yellow colour developed was read at 412 nm using a reagent blank of 4 ml buffer and 0.5 ml Ellman’s reagent.

Calculation

The amount of GSH was calculated using the formula

\[
\text{GSH in } \mu \text{g / mg protein} = \frac{[\text{OD test} \times \text{con. std} \times \text{D.F.}(10)]}{\text{mg protein / ml}} \times \frac{\text{OD std}}{\text{mg protein / ml}}
\]

3.9.3.4. Estimation of Conjugated Dienes (CD)

Conjugated Dienes were estimated by the method of Lunec et al., 1981 [310]

Reagents

- Chloroform – Methanol reagent (2:1 v/v)
- Cyclohexane

Procedure

1. To 1 ml of tissue homogenate / blood, added 5 ml of chloroform methanol reagent and mixed thoroughly.
2. Centrifuged the mixture for 5 minutes. 3 ml of the lower layer was pipetted out into a tube and evaporated to dryness.
3. To the dry tube was added 1.5 ml of cyclohexane, mixed well and its absorbance read at 233 nm against a cyclohexane blank.

**Calculation**

OD of 1 ml aliquot (extract/serum) at 233 nm is expressed as mM/100 mg CD.

CD concentration was calculated by the formula,

\[
\text{OD} / 100 \text{ mg tissue (mM/100 mg)} = \frac{\text{OD test} \times \text{vol. of homogenate} \times 100 \text{ mg}}{\text{vol. of aliquot} \times \text{Wt. of tissue (mg)}}
\]

3.9.3.5. *Estimation of Malondialdehydes (MDA)*

Malondialdehyde formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for determining the extent of peroxidation reaction. MDA was determined by the method of Buege and Aust, 1978 [309] by a reaction with Thiobarbituric acid giving a red species absorbing at 535 nm.

**Reagents**

- TCA – TBA – HCl reagent: 15 % w/v TCA, 0.375 % w/v Thiobarbituric acid in 0.25 N HCl.

**Procedure**

1. Combined 0.5 ml of blood / tissue extract with 1 ml of the TCA-TBA-HCl mixture and mixed thoroughly.

2. The mixture was heated in a boiling water bath for 15 minutes and then cooled.

3. It was centrifuged at 1000 rpm for 10 minutes and the flocculent precipitate was removed.
4. Absorbance of the supernatant was measured at 535 nm against a reagent blank that contained 0.5 ml of water in place of the sample.

**Calculation**

Malondialdehyde concentration of the sample was calculated using the formula

$$\text{MDA concentration (nmoles / 100 mg tissue)} = \frac{\text{OD test} \times 10^9 \times \text{vol. of homogenate x 100 mg}}{1.56 \times 10^5 \times \text{vol. of aliquot} \times \text{Wt.of tissue (mg)}}$$

where,

- $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$ = Molar extinction coefficient
- $10^9$ for conversion into nano moles.
- Vol. of homogenate = Final volume into which the homogenate was made up.
- Vol. of aliquot = 0.5ml

**3.10. ESTIMATION OF HAEMOGLOBIN**

Haemoglobin was estimated by cyanmethemoglobin method, Beutler, 1975 [311]

In the presence of Potassium ferricyanide at alkaline pH, haemoglobin and its derivatives are oxidized to methemoglobin. Methemoglobin so formed reacts with Potassium cyanide to form cyanmethemoglobin, a red coloured complex which is measured colourimetrically. The colour intensity is proportional to the haemoglobin concentration of blood sample.

**Reagents**

- Drabkin’s solution : Dissolved 200 mg of Potassium ferricyanide ($K_3Fe(CN)_6$), 50 mg of Potassium cyanide (KCN), 1 ml of 1 M Potassium dihydrogen phosphate ($KH_2PO_4$) and 1 ml of Nonidet P 40 and made up to 1 liter with distilled water and adjusted the pH between
7.0 and 7.4. This could be kept for several months in dark polythene bottle between 4 and 20 °C. Hb. standards were diluted in Drabkin’s solution with a range of concentration from 5 mg% to 45 mg%.

**Procedure**

Poured 0.2 ml of 1: 20 haemolyzate to 10 ml of Drabkin’s reagent and mixed thoroughly. It was kept at room temperature for 4 minutes. The absorbance was read at 540 nm against a blank that contained only Drabkin’s solution.

**Calculation**

\[
\text{Blood Haemoglobin (g / dl)} = \frac{\text{OD test} \times \text{Con. std.} \times \text{Dilution Factor}}{\text{OD std}}
\]

**3.11. ESTIMATION OF PROTEINS - Lowry et al., 1951 [312]**

The blue colour developed by the reduction of phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with alkaline cupric tartarate are measured in the Lowry’s method.

**Reagents**

- Reagent-A : 2 % Sodium carbonate in 0.1 N NaOH
- Reagent-B : 0.5 % Copper sulphate ( CuSO₄·5H₂O ) in 1 % Pot. sodium tartarate
- Alkaline copper reagent : Prepared by mixing 50 ml of reagent-A with 1 ml of reagent-B just before use.
- Folin phenol reagent : Commercial Folin-Ciocalteau reagent was diluted 1:1 with distilled water.
Materials and Methods

- Standard Protein stock solution (1mg / ml): Dissolved 100 mg of BSA in 100 ml 0.1 N NaOH carefully with gentle mixing to avoid froth formation.
- Protein working standard (0.1mg / ml): Stock solution was dilute 10 times with 0.1 N NaOH.

Procedure

1. To 1 ml of the test solution (diluted serum / tissue extract) added 5 ml of alkaline copper reagent. Mixed well and allowed to stand at room temperature for 10 minutes or longer.
2. Prepared a reagent blank by substituting test solution with 1 ml of 0.1 N NaOH and a standard by replacing the test solution with 1 ml of protein working standard in separate test tubes as in step 1 above.
3. Added 0.5 ml of diluted Folin-Ciocalteau reagent rapidly with stirring.
4. The colour developed was read after 30 minutes at 660 nm.

Calculation

\[
\text{Amount of protein (mg/ml)} = \frac{\text{OD test} \times \text{Con. std} \times \text{Dilution Factor}}{\text{OD std}}
\]

3.12. STATISTICAL ANALYSIS

Data were analyzed using Analysis of Variance (ANOVA) and the group means were compared by Duncan’s Multiple Range Test (DMRT) using Sigmastat (SPSS version 10.0.5). Values were considered statistically significant at \( P < 0.01 \). The significance in the variation of each parameter between control and arthritic as well as between arthritic and each treatment was analyzed using paired samples t test (2-tailed). The significance of progression/regression of paw oedema in each group towards the 30\(^{th}\) day was analysed using Levene’s test (2-tailed).