MATERIAL & METHODS
EXPERIMENTAL STUDY

Material:

A. Animals:

1) **Rabbits**: Healthy adult albino rabbits of either sex, weighing between 1 to 2 kg of body weight, were used for biochemical and haematological studies.

2) **Rats**: Anti-inflammatory and ulcerogenic activity of drugs was studied in adult albino rats of either sex, weighing between 100 to 200 gms of body weight.

3) **Mice**: Analgesic study on acetic acid-induced writhing and toxicological study was undertaken in albino mice of either sex, weighing between 20-30 gms of body weight.

B. Drugs and chemicals:

(a) **Drugs**:

1) Tromaril (Unichem, Bombay) A suspension of Tromaril was freshly prepared in 2% gum acacia just before use.

2) Indomethacin (I.D.P.L., Hyderabad) A suspension of indomethacin was prepared in 2% gum acacia just before use.

3) Aspirin (Jaychem, Bombay) It was also freshly prepared as a suspension in 2% gum acacia for each experiment.

4) Tolmetin Sodium (McNeil Laboratories, Washington) An aqueous solution was prepared freshly in distilled water just before use.

5) Brufen (Boots) It was used as a suspension in 2% gum acacia freshly prepared just before experiment.
(b) **Chemicals**

For pharmacological studies:

1) Acetic acid - 1% solution of acetic acid was prepared in normal saline.

2) Carrageenan - A 1% suspension of carrageenan in 2% gum acacia in normal saline was prepared just before the experiment.

**Biochemical studies**:

All chemicals of Analar (BDH) grade were used.

(1) **For Blood Sugar**

a) **Standard Glucose Solution**

A stock solution of 15 mg% of glucose was prepared in 0.2% benzoic acid. It was stored in the fridge at the temperature of 4°C. The solution was diluted 20 minutes at the time of estimation.

b) **Alkaline Copper Reagent**

40 gms of anhydrous sodium carbonate was dissolved in 400 ml of distilled water. 7.5 gms of tartaric acid was added to it and then the tartaric acid dissolved, then 4.5 gms of crystalline copper sulphate was added and mixed thoroughly. Finally the volume was made up to 1000 ml.

c) **Phosphomolybdc Acid Solution**

35 gms of molybic acid and 5 gms of sodium tungstate were dissolved in 200 ml of 10% sodium hydroxide. It was boiled vigorously till ammonia
small disappeared and cooled at room temperature. The volume was then made upto 350 ml by adding concentrated (85%) phosphoric acid. The final volume was made upto 500 ml with distilled water.

d) **Sodium Tungstate Solution**

10 gms of sodium tungstate was dissolved in 100 ml of distilled water and was stored in glass stoppered bottle.

ea) **Sulphuric Acid**

0.67 N solution was prepared in distilled water and was kept in glass stoppered bottle.

b) For Serum Uric Acid:

   a) Sodium Tungstate -

      10% solution.

   b) Sulphuric Acid -

      0.67 N solution.

c) **Uric Acid Reagent**

100 gms of sodium tungstate and 20 gms of anhydrous disodium hydrogen phosphate were dissolved by heating in 150 ml of distilled water in a beaker. In another beaker 24 ml of conc. sulphuric acid was diluted with 75 ml of distilled water. Both the solutions were mixed together and poured into a flask while shaking vigorously. This resulting solution was boiled gently for 1 hour under a reflex condenser and the volume was made upto 1000 ml.
d) Sodium Cyanide (J.R. Lab. Chem.) -
   12% of solution was prepared in distilled water.

e) Urea - 
   50% aqueous solution of urea was used.

f) **Stock Standard Uric Acid Solution** -
   60 mg of lithium carbonate (Analar BDH) was dissolved in 15 to 20 ml of distilled water. The solution was heated to 60°C and 100 mg of uric acid (Reidal, Hungary) was added to it and stirred vigorously till it dissolved. Subsequently it was transferred to a 100 ml flask. 20 ml of 40% formalin was added to the solution and then 1 ml of 50% V/V acetic acid was added slowly with continuous stirring. The final volume was then made up to 100 ml with distilled water.

g) **Standard for use** -
   1 ml of stock standard uric acid solution was diluted to 500 ml with distilled water just before use.

For Hematological Studies:

(1) **Platelet Count** -

A solution of 1% formaline was prepared in 34.3 gm/l of trisodium citrate.
b) *Ethylene diamine tetraacide* –

A solution of 10 mg/ml was prepared in distilled water.

c) *Fibrinogen* –

i) Normal saline –

0.9% solution of sodium chloride.

ii) Sodium citrate –

3.8% solution in distilled water.

d) *Calcium chloride* –

2.5% solution of anhydrous calcium chloride in distilled water was used.

e) *Acetone* –

*Prothrombin Clot Lysis Time* :

a) Sodium citrate –

3.8% solution was prepared in distilled water.

b) *Calcium Chloride* –

0.278% solution in distilled water was used.

c) *Acetic acid* –

1% solution of acetic acid was prepared.

d) *Borate solution* –

9 gms of sodium chloride and 1 gm of sodium borate were dissolved in 1 litre of distilled water in order to give a pH of 9.0

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**Methods** :

**Analgesic activity** :

Analgesic effect of anti-inflammatory drugs like aspirin, indomethacin, ibuprofen, tolmetin and tramadol, were
evaluated in albino mice by the method of acetic acid-induced writhing (Collier et al., 1965). In this study 10
ml/kg of 1% acetic acid solution in normal saline was
injected intraperitoneally in six groups consisting of 10
mice each. The animals were observed for writhing which
was characterised by a wave of constriction and elongation
traversing caudally along the abdominal wall often accom-
panied by extension of hind limbs. Mice were pretreated
with drug 30 minutes before. One group of mice was trea-
ted orally with 2 ml/kg of distilled water which served
as control while the other five groups received aqueous
solution of tolanon (20 mg/kg, 50 mg/kg, 100 mg/kg) and
suspension of tromaril (100 mg/kg, 150 mg/kg, 200 mg/kg),
indomethacin (2 mg/kg, 5 mg/kg, 10 mg/kg), aspirin (20 mg/kg,
40 mg/kg, 50 mg/kg), ibuprofen (10 mg/kg, 20 mg/kg, 30 mg/kg),
respectively, in 2% gum acacia. Absence of writhing
indicated the analgesic activity of drug.

Anti-pyretic action:
The antipyretic activity was assessed by the method
of T.A.B. vaccine-induced pyrexia (Saxena, 1979). In
this method six groups consisting of 6 albino rabbits in each
were used. One group serving as control was treated with
2 ml/kg of distilled water ad libitum and other five
groups received aspirin, tromaril, indomethacin, ibuprofen
orally in a volume of 2 ml/kg in doses of 50 mg/kg, 100
mg/kg, 200 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg, 250 mg/kg,
5 mg/kg, 10 mg/kg and 10 mg/kg, 30 mg/kg and 40 mg/kg respec-
tively as a suspension in 2% gum acacia and tolmexitin (25 mg/kg and 30 mg/kg) as a aqueous solution.

The normal rectal temperature of a group of rabbits was recorded by a clinical thermometer by introducing it 2 cm deep into the rectum at hourly intervals for a period of 4 hours. It was observed that in normal healthy rabbits the variations in the body temperature were minimal from 11 AM to 2 PM; therefore, the antipyretic activity of drugs was studied during this period. T.A.B. vaccine was administered intravenously into the marginal ear vein of rabbit in a dose of 0.5 ml/rabbit. The temperature was recorded every 30 minutes until it approached the normal. In the control group of rabbits, it was found that peak pyrexia was attained between 60 minutes and 90 minutes of administration of T.A.B. vaccine and the body temperature returned to normal after 4 hours. In view of this observation, it was decided to inject the drug after 60 minutes of the vaccine administration and likewise the rectal temperature was recorded every 30 minutes till recovery.

**Anti-inflammatory Activity:**

The anti-inflammatory effect was studied by carrageenin-induced rat hind paw oedema (Winter et al., 1962). In order to evaluate the anti-inflammatory activity, three dose levels of each drug was used in each group. The study was undertaken in six groups of albino rats of either sex consisting of 10 rats in each group, weighing
between 150-200 gm. One group treated with 2 ml/kg of distilled water served as control while the remaining five groups were treated with tammaril (100 mg/kg, 200 mg/kg), aspirin (50 mg/kg, 100 mg/kg, 150 mg/kg), indomethacin (1 mg/kg, 2 mg/kg, 5 mg/kg), brufen (10 mg/kg, 150 mg/kg, 20 mg/kg) and aqueous solution of tolmetin (30 mg/kg, 50 mg/kg, 100 mg/kg).

The inflammation was induced by injecting 0.1 ml of 1% carrageeann suspension subcutaneously into the planter sponaeosis of the right hind paw. Marked and measurable oedema developed after three hours of injection. The paw volume was measured by a plethysmometer (Bittle et al., 1957). The difference between the volume before and 3 hours after carrageeann injection was taken as the volume of paw oedema. Drug was administered orally, 1 hours prior to carrageeann injection. The % inhibition of oedema volume (% anti-inflammatory effect) was calculated by the following formula:

\[
(1 - \frac{V_{t}}{V_{c}}) \times 100
\]

where

\(V_t\) = Volume of paw swelling in treated rats.

\(V_c\) = Volume of paw swelling in control rats.

**Ulceregenic Effect**

Ulceregenic activity of the anti-inflammatory drugs were evaluated by three methods. Each method comprised of 6 groups of six rats each. One group served as control and the other five groups were treated with anti-inflammatory
agents. The control group received 2 ml/l of distilled water ad libitum while the other groups received 100 mg/kg, 200 mg/kg, 400 mg/kg suspension in 2% gum acacia of aspirin, tobramycin 100 mg/kg, 200 mg/kg, 400 mg/kg, brufen 50 mg/kg, 100 mg/kg, 200 mg/kg and indomethacin 2 mg/kg, 4 mg/kg, 6 mg/kg and aqueous solution of tolmetin (100, 200, 300 mg/kg).

(a) **Ulcerogenic activity of drugs on chronic administration**

Five groups consisting of 6 rats each, were treated with drugs orally once daily for four consecutive days and one group was treated likewise with distilled water on the fifth day the rats were sacrificed under ether anesthesia. The stomach was removed and opened along the greater curvature. It was examined with the help of a magnifying glass for the presence of ulcers. The ulcer index was calculated according to the method of Dhawan and Sisulal (1973) by the following formula:

\[ UI = \left( \frac{ADU \times \text{Percent RU}}{100} \right) \]

where

- \( UI \) = Ulcer index
- \( \text{Percent RU} \) = percentage of rats with ulceration.
- \( ADU \) = Average degree of single ulceration for each group, which was determined by adding together the degree of all ulceration (DU) for the group divided by number of animals.
(b) **Effect of drugs on experimental ulceration produced by Shay's method** (Shay et al., 1945):

Rats were anaesthetised with ether and laparotomy was done. Gastroduodenal junction was identified and ligated. The abdomen was closed with stitches. The animals were treated with distilled water or drug just after ligation. Four hours after ligation, the rats were sacrificed. Stomach was removed and examined for the presence of ulcers to calculate ulcer index.

(c) **Effect of drugs on stress-induced ulceration** (Rossi et al., 1956):

After 8 hours of fasting the rats were wrapped in the wire gauze to expose them to immobilization stress for a period of 4 hours. The rats were allowed distilled water or drug just before exposing them to stress. After 4 hours of stress, the rats were sacrificed and stomach was examined and ulcer index calculated.

**Biochemical studies:**

Blood sugar and serum uric acid levels were estimated in rabbits to assess the effect of anti-inflammatory drugs on these parameters. Each parameter was studied in 6 groups consisting of 6 rabbits each. One group received distilled water (2 ml/kg) serving as control whereas remaining five groups were treated with drugs.

(a) **Collection of blood samples:**

Blood samples were collected from marginal vein of rabbits in fluoride vials for estimation of blood sugar and in plain vials for serum uric acid estimation. After one hour, the blood samples were centrifuged (at 1000
r.p.m.) and decanted to get clear serum. Blood samples were collected just before and at hourly intervals for 6 hours and 7 days after drug treatment.

(b) Estimation of blood sugar:

The rabbits were treated with tromosal (200 mg/kg, 250 mg/kg), indomethacin (2 mg/kg), aspirin (100 mg/kg) and brufen (10 mg/kg) as a suspension in 2% gum acacia while tolmetin (10 mg/kg) was given as an aqueous solution orally. Blood sugar level was estimated by the method of Folin and Wu (1920). 3.5 ml of distilled water was taken in a centrifuge tube and to it was added 0.1 ml of blood in order to hemolyse the red blood cells. 0.2 ml of 10% sodium tungstate and 0.2 ml of 0.67 N sulphuric acid were subsequently added to precipitate the proteins. After mixing vigorously, it was allowed to settle down for some time and then centrifuged for 10 minutes at 1000 r.p.m. 2 ml of the supernatant was pipetted in a Folin sugar tube. A blank was prepared by taking 2.0 ml of distilled water while standard was prepared by taking 2.0 ml from the solution obtained by diluting the stock solution of standard glucose 20 times in separate test tube.

To all these test tubes, 2.0 ml of alkaline copper solution was added and kept in boiling water bath for 6 minutes and allowed to cool for 2 minutes. 2.0 ml of phosphomolybdic acid was then added to each test tube and again boiled for 5 minutes and cooled for 2 minutes. The final volume was then made up to 12.5 ml with distilled
water and mixed properly. The readings were taken immediately at 650 nm filter of the photocolorimeter. The blood sugar level was calculated by the following formula:

\[
\text{Blood sugar mg} = \frac{\text{Optical density of unknown}}{\text{Optical density of standard}} \times 100
\]

(c) **Estimation of serum uric acid**

The rabbits were treated with drugs in five groups with indometacin (2 mg/kg), tromaril (200 mg/kg), aspirin (100 mg/kg), ibufen (10 mg/kg) as a suspension in 2% gum acacia and tolmetin (10 mg/kg) as aqueous solution orally respectively.

Serum uric acid level was estimated by the method of Brown (1945). 0.1 ml of serum was added to 3.5 ml of water in a centrifuge tube. 0.2 ml of 10% sodium tungstate and 0.2 ml of 0.67 sulphuric acid were added to precipitate the proteins. After mixing vigorously it was allowed to stand for sometime and then centrifuged. 2.0 ml of supernatant was taken in a test tube. The standard and the blank were prepared by taking 2.0 ml of diluted uric acid standard and 2.0 ml of distilled water respectively in the other two tubes.

To each test tube, 2.0 ml of 12% sodium cyanide, 2.0 ml of 50% urea and 1.0 ml of phosphotungstic acid reagents were added, one by one, mixing well after each addition. It was left for 1 hour for the development of colour and finally the volume was made upto 10 ml by
adding 3.0 ml of distilled water. The readings were taken at 520 μm filter of the photometer. Serum uric acid level was estimated by using the following formula:

\[
\text{Serum uric acid (mg%) = \frac{O.D. \text{ of unknown}}{O.D. \text{ of standard}} \times 9}
\]

Haematological studies:

Haematological studies like platelet count, clotting time, estimation of plasma fibrinogen and euglobulin clot lysis time were undertaken in rabbits.

(a) Collection of blood and drug administration for platelet count and clotting time:

Six groups of albino rabbits of 6 rabbits each of either sex were treated orally with 2 ml/kg of distilled water which served as control and remaining groups were administered indomethacin (5 mg/kg), aspirin (100 mg/kg), brufen (10 mg/kg), tramazol (200 mg/kg) as a suspension in 2% gum acacia and an aqueous solution of tolmetin (50 mg/kg).

Blood was collected from the marginal ear vein in capillary tube and postaural pipette for clotting time and platelet count respectively.

(b) For plasma fibrinogen and euglobulin clot lysis time (E.L.T.):

Plasma fibrinogen and euglobulin clot lysis time (E.L.T.) were studied in six groups of albino rabbits of 6 rabbits each of either sex weighing between 1 to 2 kg. Animals were treated with 2.0 ml/kg of distilled water orally for 7 days in one group serving as control and the
other five groups received orally for 7 days trimaril (200 mg/kg), aspirin (100 mg/kg), brufen (10 mg/kg), indomethacin (200 mg/kg) as a suspension in 2% gum acacia and aqueous solution of tolmetin (50 mg/kg) orally respectively.

Ear of the rabbit was shaved, a sharp cut was made on marginal ear vein and blood sample was collected in a centrifuge tube containing 3.8% solution of sodium citrate 1/6 V/V of total blood collected. The blood was centrifuged and the supernatant plasma was used for both estimations. A low temperature was maintained for collection of the samples.

Platelet count:

The platelet count was done by the method of Bacia and Lewis (1975). R.B.C. pipette was rinsed with E.D.T.A. solution and the blood was taken from the marginal ear vein directly in R.B.C. pipette up to mark 1. It was diluted to 100 times by taking normal citrate diluting solution up to mark 101 of the pipette and mixed thoroughly. After discarding first few drops, the neubauer chamber was filled with the solution and was kept in a moist chamber for 20 minutes to settle the platelets. The platelets in central small square and 4 corner small squares (as in case of R.B.C. count) were counted by using 4 mm objective and X10 eye piece.

Calculation:

Platelet count/cu mm of blood = N x 5000
where N = No. of platelets in 5 squares.
Clotting time:

The clotting time was studied by the method of Best and Taylor (1966). The blood from marginal ear vein was allowed to run in capillary tube. The end of the tube were sealed by plasticin and the tubes were immersed in a water bath maintained at 37°C. At every 15 sec. interval, small fragments of tube were broken off and the end point was recorded when a string of clot was observed.

Plasma Fibrinogen:

Plasma fibrinogen was estimated by the method of Saxena et al. (1979). 1.0 ml of citrate plasma was added to 10 ml of physiological saline (0.9%) solution in a small beaker and the mixture was allowed to clot by addition of 1.0 ml of 2.5% calcium chloride and incubated at 37°C in a water bath. When clotting was complete, the clot was tipped into the palm of hand and the fluid was extruded out by gentle pressure until the clot was small enough to be squeezed between the fingers and resulted into a compact ball of fibrin. This fibrin ball was kept for 30 minutes in distilled water and then for 30 minutes in acetone and allowed to dry in a hot air oven. Then after cooling at room temperature it was weighed on a sensitive analytical balance.

Calculation:

Plasma fibrinogen in mg% = Dry weight of fibrin ball × 100

Fibrinolysis clot lysis time (F.L.T.)

Plasma F.L.T. was determined by the method of Buehler
(1958). 0.5 ml of plasma was added in 9.0 ml of distilled water in a glass test tube. The pH was adjusted to 5.3 by adding 0.1 ml of 1% acetic acid. The tubes were kept for 30 minutes in a refrigerator at 4°C for the globulin fraction of the plasma to precipitate and then centrifuged for 5 minutes at 3000 r.p.m. The supernatant was decanted and the tubes were stirred gently until the globulin fraction was completely dissolved in borate solution. 0.5 ml of 0.276% calcium chloride was then added to the solution of globulin in borate and the time, at which mixture clot was observed, was recorded. When the tubes were incubated at 37°C and examined at frequent intervals to see the lysis. When the lysis was almost complete, the clot was observed every five minutes for accurate measurement. Mean time taken between the clot formation and its complete lysis was recorded as the globulin clot lysis time.

**Acute toxicity study (Ghosh, 1971):**

Acute toxicity was studied in 5 groups of albino mice of either sex consisting of 10 mice in each group, weighing between 25 to 40 gm. All the animals were kept on standard laboratory diet. 2% suspension in gum arabic of trimaril, aspirin, indomethacin, bromfen and aqueous solution of tolmetin were administered orally to mice in graded doses. After administration of the drug, clinical signs were observed daily for 24 hours. Values of LD₅₀ were calculated on the basis of the mortality occurring during this period in a particular dose.
**Statistical analysis**:  
The data obtained in the present study was analysed statistically by the student's 't' test. Indices and percentage inhibition were calculated by using standard procedures. Wilcoxon sign rank test was applied in non-parametric data.

**Clinical study**:  
(a) **Patients**:  
Twenty patients, suffering from 'definite' or classical rheumatoid arthritis, were selected for the clinical study and were kept under observation for treatment from July '82 to February '83. 12 female and 8 male patients were included in the study. These patients were divided into two groups - aspirin-treated which served as control group and tromaril-treated as study group. A detailed history regarding classical symptoms of the disease were recorded and patient reporting any symptom like nausea, vomiting, diarrhoea, drowsiness, burning sensation, epigastric pain, headache, insomnia, haematemesis, melaena or rash in either group was excluded from the study. Patient receiving either drug in prescribed doses was assessed daily for development of any side effect.

(b) **Study design**:  
The study was carried out as a comparison between aspirin and tromaril in daily doses of 2400 mg and 1600 mg respectively. All the anti-inflammatory drug being used previously was withdrawn and patients were allocated to two
treatment groups. The response of the therapy was noted after two weeks and four weeks of treatment. Evaluation of digital joint (P.I.P.) circumference duration of morning stiffness, grip strength, degree of pain, walking time, fever and E.S.R. was made before the start of study, after 2 weeks and four weeks treatment period. A record of side-effects reported by the patient during treatment was also maintained. Complete haematological investigations, urine examination and stool examination were also performed initially and after 4 weeks of treatment.

Digital Joint (P.I.P.) Circumference:
P.I.P. circumference was studied with the help of a measuring tape by the method of Mathur et al. (1980). Measurement was recorded initially and after two weeks and four weeks of drug treatment.

Grip strength:
Assessment of grip strength was done by the ability of the patient to raise the mercury level in the sphygmomanometer by squeezing the rubber ball as described by Mathur et al. (1980). The reading was noted before starting the treatment and at the end of two weeks and four weeks of therapy.

Walking time:
It was evaluated by recording the time taken by the patient to walk a distance of 50 feet (Sattur et al., 1980).
Assessment of pain:
Subjective assessment of pain was done by the method of Punjabi et al. (1980). Different scores were given depending upon the degree of severity of pain.

Fever:
The effect of aspirin and tromaril on pyrexia was evaluated by clinical thermometer. Changes in body temperature (oral) were recorded before treatment and at an interval of 1 hour, 3 hours, 6 hours and 8 hours after administration of drug. The pyrometer used for comparison will be:

1- Rate of variation in temperature.
2- Degree in fall of temperature.
3- Duration of fall of temperature.

E.S.R.:
It was evaluated by the method of Wintrobe (1975). Changes in E.S.R. were recorded before initiating the therapy and after 4 weeks of treatment in both aspirin and tromaril-treated groups.

Side effects:
Drug induced side effects were recorded regularly as reported by patients during the period of active drug treatment.