III. MATERIALS AND METHODS

3.1 Selection of experimental animals

Wistar albino rats procured from the Laboratory Animal Facility of Veterinary College, Hebbal, Bangalore, were used in the present study. They were aged between six to seven weeks and weighed 130-150g.

They were acclimatized to the experimental conditions for one week. After acclimatization, animals were grouped and housed in standard polypropylene rat cages during the experiment. They were maintained under standard laboratory hygienic conditions, providing standard laboratory animal feed and water *ad libitum*. The approval of the Institutional Animal Ethics Committee was obtained prior to start of the experiment.

3.1.1 Selection of urine source

Holstein-Friesion (HF) cross-bred, milking cows aged about 2-3 years were selected for urine collection. Animals were hygienically maintained in Karnataka Veterinary, Animal and Fisheries Sciences University regional campus, Department of Livestock Production and Management, Veterinary College, Hebbal, Bangalore. All the cows were fed with the concentrate prepared at the dairy farm and were provided clean drinking water *ad libitum* and daily animals were also taken outside for grazing in the university grazing land. All the animals were monitored carefully throughout the experimental period.

3.1.2 Collection of urine

Natural voiding mid-stream urine was collected in a sterile glass container. Collected urine was stored in refrigerator at 4°C and utilized for the experiment within two hours of collection. Precaution was taken to avoid any external contamination. Urine was collected from the same cows and used in each
experiment. Immediately after collection physical, chemical and microscopic 
examination were done. Urine specimens were collected and stored as part of 
standard protocols in cohort studies designed to assess exposure to non persistent 
environmental contaminants (Gunter, 1997; Landi and Caporaso, 1997).

3.1.3 Selection of doses for pharmacological assays

The doses corresponding to the dose of 15 ml, 30 ml, 60 ml and 75 ml per 
day in a human being weighing around 60 kg. The doses administered were 0.05 
ml, 0.1 ml, 0.2 ml and 0.3 ml/100 gm bodyweight in rats.

3.1.4 Administration of doses

The fresh urine at 0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml/100 gm was selected 
and was made up to 2 ml by adding distilled water and was gavaged using a 
stomach tube daily for a period of 28 days in sub-acute oral safety study and for a 
period of 90 days in sub-chronic oral safety study. The same dosing pattern was 
used for other pharmacological and immunological studies.

3.2 Evaluation of analgesic effect of cow urine.

3.2.1 Analgesic activity

The hot plate latency assay was based on the method of (Eddy et al., 1950). 
The study was conducted to assess the analgesic effect of urine.
3.2.2 Experimental design

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>MALE</th>
<th>DOSE</th>
<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
</tr>
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<tbody>
<tr>
<td>I</td>
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<td>VII</td>
<td>6</td>
<td>Control</td>
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<tr>
<td>II</td>
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<td>0.05 ml</td>
<td>VIII</td>
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</tr>
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<td>IX</td>
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<tr>
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</tr>
<tr>
<td>V</td>
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<td>0.3 ml</td>
<td>XI</td>
<td>6</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>VI</td>
<td>6</td>
<td>30 mg/kg Ibuprofen</td>
<td>XII</td>
<td>6</td>
<td>30 mg/kg Ibuprofen</td>
</tr>
</tbody>
</table>

3.2.3 Procedure

Thirty six prescreened wistar albino rats of each sex were grouped in six (n = 6). Each group received one dose of the urine (0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml/100gm). At 0, 30 min, 1, 2, 3 and 4 h after urine administration, animals were lowered onto the surface of a hot plate (55 ± 10 °C) (Eddy’s analgesiometer, INCO) enclosed with cylindrical glass and the time for the animal to raise or lick the fore limb was noted as the reaction time (RT). Animals group VI and XII were administered Ibuprofen® (GSK) tabs available in 200 mg tabs as reference drug as a standard control. The male and female albino rats showing reaction time of 10 sec to thermal stimulus of 55 ± 10 °C were selected.

3.3 Evaluation of antipyretic effect of cow urine.

The study was conducted to assess the effect of urine on antipyretic effect.

3.3.1 Procedure

Six groups of rats each consisting of 6 males and 6 females were used for the study. A single dose of urine was administered after induction of fever by using Brewer’s yeast. The groups and doses employed were as follows.
Prior to the experiment, six male and six female in each group were maintained in separate cages for 7 days and the animals with approximately constant rectal temperature were selected for the study.

### 3.3.2 Induction of fever

Commercial brewer’s yeast was procured locally. Pyrexia was induced by subcutaneous injection of granular form of 20 % (w/v) brewer’s yeast suspension at the dose rate of 10 ml/kg into the animals’ dorsal region (Smith and Hambourger, 1935). Initial rectal temperature was recorded. Temperature was recorded up to 18 hours to confirm pyrexia. After 18 hours animals that showed a minimum an increase of 2-3°C in rectal temperature were selected.

The cow urine (0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml) was administered to four groups. Paracetamol (10 mg/kg orally) (Crocin®) available in 500 mg tabs (GSK) were used as reference drug.

The rectal temperature of each rat was measured 18 hours after the injection, using a digital thermometer (SK-1250MC, Sato Keiryoki Mfg. Co., Ltd., Japan). The rectal temperature was determined on hourly bases up to six hours after administration of urine as well as Paracetamol in all treated groups and compared with control.
3.4 Evaluation of acute anti-inflammatory effect of cow urine.

3.4.1 Experimental design

<table>
<thead>
<tr>
<th>GROUPS</th>
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<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
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<td>VII</td>
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<td>Control</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>Diclofenac 10 mg/kg</td>
<td>VIII</td>
<td>6</td>
<td>Diclofenac 10 mg/kg</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>0.05 ml</td>
<td>IX</td>
<td>6</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>0.1 ml</td>
<td>X</td>
<td>6</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>V</td>
<td>6</td>
<td>0.2 ml</td>
<td>XI</td>
<td>6</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>VI</td>
<td>6</td>
<td>0.3 ml</td>
<td>XII</td>
<td>6</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>

3.4.1.2 Formalin-induced oedema in rat hind paw

Oedema was induced by injecting 0.1 ml of 2% (w/v) formalin into the sub plantar region of the right hind paw of the rats according to the method described by Chau, 1989. The rats of test groups (III-VI and IX-XII) were treated orally with 0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml/100 gm body weight cow urine respectively, 1 h before formalin injection. The group (I) was control and the reference group (II and VIII) received Diclofenac at the dose of 10 mg/kg body weight (Diclofam® MAX) orally.

Measurement of paw size was carried out by wrapping a piece of cotton thread round the paw and the length of the thread corresponding to the paw circumference was determined using a meter ruler (Hess and Milonig, 1972; Olajide et al., 2000). Measurement was done immediately before and 3 h following formalin injections.

The percentage thickness was calculated according to the following formula (Olajide et al., 2000).
\[(C_t - C_o) \text{ control} \div (C_t - C_o) \text{ treated} \times 100\]

Percentage thickness = \[
\frac{(C_t - C_o) \text{ control} \div (C_t - C_o) \text{ treated}}{(C_t - C_o) \text{ control}} \times 100
\]

Where \(C_t\) was the paw circumference at time \(t\),
\(C_o\) was the paw circumference before formalin injection,
\(C_t - C_o\) was oedema,
\((C_t - C_o) \text{ control}\) was oedema or paw size after formalin injection to control rats at time \(t\).

3.4.2 Evaluation of chronic anti-inflammatory effect of cow urine

Same procedure was adopted as mentioned in acute anti-inflammatory activity to evaluate the chronic anti-inflammatory activity of cow urine. But oedema was observed up to six days.

3.5 Evaluation of Hepatoprotective activity of cow urine

3.5.1 Drugs

Silymarin was purchased from Micro labs, Hosur, Tamilnadu, India. Carbon tetrachloride (CCl\(_4\)), 1-Chloro-2-4-dinitrobenzene (CDNB), Dithio bis-2-nitrobenzoic acid (DTNB) and trichloroacetic acid (TCA) were purchased from SISCO Research, Pune.

3.5.2 Experimental design

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MAL</th>
<th>DOSE</th>
<th>GROUP</th>
<th>FEMAL</th>
<th>DOSE</th>
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<td>S</td>
<td>E</td>
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<tr>
<td>I</td>
<td>6</td>
<td>Control</td>
<td>VIII</td>
<td>6</td>
<td>Control</td>
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<tr>
<td>II</td>
<td>6</td>
<td>CCl(_4) treated</td>
<td>IX</td>
<td>6</td>
<td>CCl(_4) treated</td>
</tr>
</tbody>
</table>
The experiment was carried out following the method of Manoj and Aqueed (2003) with some modification. The rats were divided into 14 groups (n=6). Animals in group I and VIII served as a vehicle control. CCl₄ in liquid paraffin (1:2) at the dose of 1.0 ml/kg intraperitonially once in every 72 h for 10 days was administered in animals from group II – VII in male rats and IX – XIV in female rats. Group II and IX served as CCl₄ control and were not treated with any drug. Cow urine at the dose of 0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml once daily was administered orally to the animals in group III and VI in male rats and in group X – XIV in female rats respectively for ten days.

Standard drug silymarin at the dose of 25 mg/kg was administered similarly to the animals in group III and X. After 24 h of the last dose, blood was collected from retro-orbital plexus under ether anesthesia.

The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm at 37 °C and used for the assay of biochemical marker enzymes (AST, ALT, and alkaline phosphatase), bilirubin and total protein. Immediately after collecting blood, the animals were sacrificed and liver was dissected out for histopathological studies. AST, ALT, serum alkaline phosphatase

| Group | Dose (ml) | Compound
|-------|----------|----------------|
| III   | 0.05    | Silymarin + CCl₄
| IV    | 0.1     | 0.05ml + CCl₄
| V     | 0.2     | 0.1ml + CCl₄
| VI    | 0.3     | 0.2ml + CCl₄
| VII   | 0.4     | 0.3ml + CCl₄

| Group | Dose (ml) | Compound
|-------|----------|----------------|
| X     | 0.5     | Silymarin + CCl₄
| XI    | 0.6     | 0.05ml + CCl₄
| XII   | 0.7     | 0.1ml + CCl₄
| XIII  | 0.8     | 0.2ml + CCl₄
| XIV   | 0.9     | 0.3ml + CCl₄

| Group | Dose (ml) | Compound
|-------|----------|----------------|
| V     | 1.0     | 0.1ml + CCl₄
| VI    | 1.1     | 0.2ml + CCl₄
| VII   | 1.2     | 0.3ml + CCl₄
| VIII  | 1.3     | 25 mg/kg

The experiment was carried out following the method of Manoj and Aqueed (2003) with some modification. The rats were divided into 14 groups (n=6). Animals in group I and VIII served as a vehicle control. CCl₄ in liquid paraffin (1:2) at the dose of 1.0 ml/kg intraperitonially once in every 72 h for 10 days was administered in animals from group II – VII in male rats and IX – XIV in female rats. Group II and IX served as CCl₄ control and were not treated with any drug. Cow urine at the dose of 0.05 ml, 0.1 ml, 0.2 ml and 0.3 ml once daily was administered orally to the animals in group III and VI in male rats and in group X – XIV in female rats respectively for ten days.

Standard drug silymarin at the dose of 25 mg/kg was administered similarly to the animals in group III and X. After 24 h of the last dose, blood was collected from retro-orbital plexus under ether anesthesia.

The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm at 37 °C and used for the assay of biochemical marker enzymes (AST, ALT, and alkaline phosphatase), bilirubin and total protein. Immediately after collecting blood, the animals were sacrificed and liver was dissected out for histopathological studies. AST, ALT, serum alkaline phosphatase
(ALP), bilirubin and total protein were determined by using commercially available kits (Span Diagnostic Ltd., Surat, India). Serum total protein was measured according to the method of Lowry et al. (1951).

3.6 Evaluation of wound healing activity of cow urine

3.6.1 Experimental design

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>MALE</th>
<th>DOSE</th>
<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
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<td>Control</td>
<td>VIII</td>
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<td>Control</td>
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<tr>
<td>II</td>
<td>6</td>
<td>Nitrofurazone ointment</td>
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<td>Nitrofurazone ointment</td>
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<tr>
<td>III</td>
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<td>X</td>
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<td>0.05ml</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>0.1ml</td>
<td>XI</td>
<td>6</td>
<td>0.1ml</td>
</tr>
<tr>
<td>V</td>
<td>6</td>
<td>0.2ml</td>
<td>XII</td>
<td>6</td>
<td>0.2ml</td>
</tr>
<tr>
<td>VI</td>
<td>6</td>
<td>0.3ml</td>
<td>XIII</td>
<td>6</td>
<td>0.3ml</td>
</tr>
<tr>
<td>VII</td>
<td>6</td>
<td>External application</td>
<td>XIV</td>
<td>6</td>
<td>External application</td>
</tr>
</tbody>
</table>

The animals were divided into six groups of six each. The group I and VIII was considered as the control in male and female rats, the group II and IX served as reference standard and treated with 1% (w/w) Furacin® ointment, the group III – VI and X- XII animals were treated with different doses of cow urine in male and female rats, respectively.
The urine gavaged once in a day, till the healing was complete. The parameter studied was wound closure time. Per cent wound closure was calculated.

3.6.2 Procedure

The rats were inflicted with excision wounds as described by Morton and Malon (1972). Excision wound model were used to evaluate the wound healing activity. Circular wounds of approximately 10 mm diameter were inflicted on the dorsal area skin under mild ether anaesthesia (Jalalpure et al., 2003). The entire wound left open (Patil and Kulkarni, 1984). The areas of the wound were measured (sq. mm) immediately by placing a transparent polythene graph paper over the wound and then tracing the area of the wound on it. This was taken as the initial wound area reading. Group-I and VIII served as negative control. Group-II and IX served as positive control to which nitrofurazone (0.2 % w/w in simple ointment I.P.) was applied topically. Group-III-VI and IX – XII animals were treated with urine. Group VII and XIV animals were treated by externally applying cow urine. All the test samples were applied once daily. The wound area of each animal was measured on 2nd, 4th, 6th, 8th, 10th, 12th and 14th post wounding day. The percentage of wound contraction was calculated from the days of measurements of wound area.

3.7 Evaluation of immunoprotective activity of cow urine.

3.7.1 Immunoprotective study

Immunoprotective study for cow urine was conducted in both male and female rats to generate information on the possible health effects on immunoresponse which might occur as a result of repeated administration of cow urine against a known antigen.
Animal preparation, selection of doses, animal groups, number of animals, administration of doses and observations were done as it was carried out in repeated dose 28-day oral toxicity in rats.

3.7.2 Experimental animal preparation

The study was done for a period of 28 days and different doses were administered daily for the whole period. The groups and doses employed are as follows.

**Experimental design**

<table>
<thead>
<tr>
<th>GROUPS</th>
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<th>DOSE</th>
<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
</tr>
</thead>
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<tr>
<td>I</td>
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<td>0.05 ml</td>
<td>VI</td>
<td>6</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>0.1 ml</td>
<td>VII</td>
<td>6</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>0.2 ml</td>
<td>VIII</td>
<td>6</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>0.3 ml</td>
<td>IX</td>
<td>6</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>V</td>
<td>6</td>
<td>0 (control)</td>
<td>X</td>
<td>6</td>
<td>0 (control)</td>
</tr>
</tbody>
</table>

The antigen was administered on Day 14 and 20. Sheep red blood cells (SRBC) were used as the antigen. Sheep blood was collected in Alsever’s solution and stored at 4°C for one week. SRBC’s were washed three times in pyrogen free sterile normal saline and 2 per cent SRBC suspension was prepared.

3.7.3 Observations
The rats were observed twice a day for health conditions, morbidity and mortality.

3.7.4 Parameters of Humoral immune response

3.7.4.1 Total serum protein concentration (TSP)

The total serum protein concentrations were estimated using ARTOS Semi-Automatic Biochemical Analyzer. The commercially available diagnostic kits from M/s. Swemed diagnostics, Bangalore, were employed for estimation.

3.7.4.2 Haemagglutination test (HAT)

The titers of the agglutinating antibody in the serum were measured by haemagglutination test (Hudson and Hay, 1989).

3.7.4.3 Procedure

In micro haemagglutination plates, 50 μl phosphate buffer saline (PBS) was added to all the wells. A 50 μl serum was added to the first well and mixed. After mixing, 50 μl from the first well was serially transferred to the succeeding wells and the last 50 μl was discarded. A 50 μl of 0.5 per cent SRBC was added to all the wells. The contents were mixed well and incubated at 37°C for one hour. A positive control was kept consisting of 50 μl serum and 50 μl 0.5 per cent SRBC and a negative control consisting of 50 μl PBS and 50 μl 0.5 per cent SRBC suspension.

The highest dilution showing complete agglutination of erythrocytes was taken as an antibody titer of the serum samples.

3.7.4.4 Total serum immunoglobulin concentration (TIG)
Total immunoglobulin concentration (TIG) in serum was estimated according to the procedure described by Mullen (1975).

**Solutions used:**

1. Zinc sulphate solution: Freshly prepared solution was used everyday. The solution was prepared by dissolving 208 mg zinc sulphate (ZnSO$_4$. 7H$_2$O) in one liter carbon-dioxide free distilled water. The distilled water was boiled for 10 to 15 minutes to remove the dissolved carbon-dioxide.

2. Barium chloride solution: The solution was prepared by dissolving 1.15 g of barium chloride (BaCl$_2$. H$_2$O) in 100 ml distilled water. A 3 ml of solution was made up to 100 ml using 0.2 N Sulphuric acid. This solution gave 20 units turbidity reading under standard laboratory conditions.

**3.7.4.5 Procedure**

To each of the test solution tubes containing 6 ml zinc sulphate solution, 0.1 ml serum was added. To the control tube that contained 6 ml distilled water, 0.1 ml serum was added. The tubes were vortexed and incubated for one hour at room temperature for the development of turbidity.

The turbidity measurements were made at a wavelength of 498 nm using a calorimeter. The difference in the turbidity readings obtained between the control tube and the test solution tube was multiplied by 10. The reading obtained was equivalent to the turbidity of 20 units given by barium chloride standard solution treated similarly.

**3.7.5 Parameters of cell mediated immune response**

**3.7.5.1 Total leukocyte count**
The blood was collected on Day 14, 20 and 28 by retro-orbital plexus puncture method using microhaematocrit capillary tubes containing the anticoagulant disodium EDTA. The total leukocyte count was determined using haemocytometer following standard procedure (Jain, 1990).

3.7.5.2 Absolute lymphocyte count

Differential leukocyte count was determined following standard procedure (Jain, 1990).

Absolute lymphocyte count was calculated from the total leukocyte count and differential leukocyte count.

3.7.5.3 DNCB skin sensitivity test

The delayed type of hypersensitivity was measured using dinitrochlorobenzene (DNCB) skin sensitivity. The test was conducted to assess the cell mediated immunity according to the procedure described by Brummerstedt and Basse (1973).

3.7.5.4 Procedure

An area of 3 cm diameter was marked on the left flank of the rat and the hair around the site was clipped. A 0.4 ml of 2 per cent solution of 2, 4-dinitrochlorobenzene in acetone was applied drop by drop on the marked area for primary sensitization on Day 14. The solution was allowed to evaporate quickly by blowing gently. The sensitizing dose was applied only once. After 14 days of the primary sensitization i.e., on day 28, a challenge dose of 0.05 ml was applied at the same site.

The skin thickness was measured using slide calipers before challenge dosing at 0, 24 and 48 hours intervals after the challenge dose.
3.8 Safety evaluation of cow urine

3.8.1 Acute toxicity study

Acute toxicity study was conducted to determine the median lethal dose for both male and female rats separately. Five groups of male rats and five groups of female rats each consisting of six animals were used for estimating LD$_{50}$ value.

3.8.2 Procedure

The rats were fasted overnight prior to administration of the urine. A graded dose of urine was administered as a single dose to rats by gavage using a stomach tube. The rats were observed for a period of 14 days for clinical signs. An attempt was made to arrive at the median lethal dose using the method of Finney (1971).

The group details and doses administered per animal were as follows:

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>MALE</th>
<th>DOSE</th>
<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6</td>
<td>0.06 ml</td>
<td>VI</td>
<td>6</td>
<td>0.06 ml</td>
</tr>
<tr>
<td>II</td>
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<td>VII</td>
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</tr>
<tr>
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<td>IV</td>
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<td>IX</td>
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</tr>
<tr>
<td>V</td>
<td>6</td>
<td>0 (control)</td>
<td>X</td>
<td>6</td>
<td>0 (control)</td>
</tr>
</tbody>
</table>

3.8.3 Selection of doses for safety evaluation.

The doses were corresponding to the highest dose 5 ml / kg body weight.

3.8.4 Administration of doses
The fresh urine at 0.06 ml, 0.125 ml, 0.25 ml and 0.5 ml was selected and was made up to 2 ml quantity by adding distilled water and was gavaged using a stomach tube.

3.8.5 Observations

General clinical observations were made at least once a day and considering the peak period of anticipated effect after dosing. The health condition of the animals was recorded. At least twice daily, all animals were observed for morbidity and mortality.

3.9 Sub-acute oral toxicity study (Repeated dose 28 day oral toxicity study) and Sub-chronic oral toxicity study (Repeated dose 90 day oral toxicity study)

The study was conducted as per the OECD guidelines

3.9.1 Procedure

Healthy young adult male and female rats (female rats which were nulliparous and nonpregnant) were randomly assigned to the control and treatment groups. Five groups of rats consisting of six males and six females in each group were used for the sub-acute oral toxicity study and four groups of rats consisting of six males and six females in each group were used for the sub-chronic oral toxicity study. The groups and the dose employed in both the studies were as follows.

<table>
<thead>
<tr>
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<th>DOSE</th>
<th>GROUPS</th>
<th>FEMALE</th>
<th>DOSE</th>
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</thead>
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<tr>
<td>I</td>
<td>6</td>
<td>0.05 ml</td>
<td>VI</td>
<td>6</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>0.1 ml</td>
<td>VII</td>
<td>6</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>0.2 ml</td>
<td>VIII</td>
<td>6</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>
### 3.9.2 Selection of dosage

The doses correspond to be selected on the basis of human dose 15 ml, 30 ml, 60 ml and 75 ml per day who is weighing around 60 kg.

### 3.9.2.1 Administration of doses

The rats received cow urine daily for a period of 28 days in sub-acute oral toxicity study and for a period of 90 days in sub-chronic oral toxicity study by oral gavage using a stomach tube.

### 3.9.3 Observations

General clinical observations were made at least once a day and considering the peak period of anticipated effect after dosing. The health condition of the animals was recorded. At least twice daily, all animals were observed for morbidity and mortality.

### 3.9.4 Body weight

Each rat was weighed at the beginning of the study and at weekly intervals thereafter till the end of the study.

### 3.9.5 Hematology

Hematological parameters were estimated using blood samples collected on day 0, 14 and 28 in sub-acute toxicity study and on day 0, 30, 60 and 90 in sub-chronic toxicity study by retro-orbital plexus puncture method using microhaematocrit capillary tubes. Disodium EDTA was used as an anticoagulant.
The following hematological parameters were determined following standard methods (Jain, 1990).

1. Haemoglobin concentration (Hb)
2. Haematocrit (Hct)
3. Total erythrocyte count (TEC)
4. Total leukocyte count (TLC)
5. Differential leukocyte count (DLC)
6. Mean corpuscular volume (MCV)
7. Mean corpuscular haemoglobin (MCH)
8. Mean corpuscular haemoglobin concentration (MCHC)

3.9.6 Clinical biochemistry
Serum biochemical parameters were estimated from the serum samples collected from the animals on day 0, 14 and 28 in sub-acute toxicity study and on day 0, 30, 60 and 90 in sub-chronic toxicity study to investigate the toxic effect on organs and tissues specifically on liver and kidney using ARTOS Semi-Automatic Biochemical Analyzer. The commercially available diagnostic kits from M/s. Swemed diagnostics, Bangalore, were employed in the estimation of the following parameters:

1. Aspartate aminotransferase (AST) activity
2. Alanine aminotransferase (ALT) activity
3. Alkaline phosphatase (ALP) activity
4. Blood urea nitrogen (BUN) concentration
5. Creatinine (Creat) concentration
6. Bilirubin concentration
7. Total serum protein (TSP) concentration

3.9.7 Pathology
At the end of the test period i.e., on day 28 and on day 90 the rats were sacrificed and the organs collected for histopathological study.

3.9.8 Collection of organs
After overnight fasting, the rats were weighed and sacrificed on day 28 and day 90 under ether anesthesia and the necropsy was conducted on each carcass to note down gross pathological changes.

The following organs namely liver, kidney, heart, lung and spleen were collected. The organs were cleared off from the adnexal tissues using saline and placed on a blotting paper and gently pressed to remove excess saline adhering to the organ. The representative tissue samples were collected in neutral buffered formalin and processed for histopathology by cutting sections of 5 micron thickness and staining with haematoxylin and eosin (Luna, 1968)

3.10 Evaluation of physical, chemical and microscopic characteristics of cattle urine
3.10.1 Breeds Selection
Different indigenous breeds like Amruthmahal, Saahiwal, Hallikar, Gir, Malnad Gidda, Ongole, Deoni, Kankrej, and cross breeds like Holstein Friesian and Jersy were selected for urine collection. All these animals were selected from ‘Goloka’ a private dairy farm maintained by Ramachandrapura math, Hosanagar, Shivamogga District and at Kaggalipura, Bangalore. Animals were observed for health conditions during collection of urine.
Urine was collected in a sterile container in the early morning daily from the same healthy cow from each breed for 10 days. Immediately after collection urine was examined for physical characteristics and later chemical and microscopic properties were estimated by using reagent strips such as Multistix.

3.10.2 Physical examination

Physical examination was done for pH, specific gravity, appearance, colour, odour and presence of occult blood and any contamination from external source.

3.10.3 Method

Dipping Chemstrip® strip in the test urine and change in colour was compared. (Roche-Boehringer Mannheim Corp reagent strips).

3.10.4 Chemical examination

Urine analysis was used to detect and measure the level of a variety of substances in the urine, including protein, glucose (sugar), ketone bodies, blood, and other substances. For analysis, a thin strip of plastic (dipstick) impregnated with chemicals was used and change in color was observed. Ketone bodies and glucose were estimated by Ketodiastic® manufactured by BAYER Company.

3.10.5 Microscopic Examination

The urine was examined under a microscope for the presence of red and white blood cells, crystals and casts.

3.11 Statistical analysis

The data were analyzed by One-way ANOVA with Dunnett’s post test, using Graph Pad Prism statistical software, version 4.01 (2004). The results were presented as mean ± S.E.M.