III. MATERIALS AND METHODS

The present work was taken up to evaluate the safety of NSAIDs viz. aspirin, paracetamol, ketoprofen, nimesulide, meloxicam and celecoxib in birds. The potential toxic effects of NSAIDs in birds were studied by performing various parameters like hematology, serum biochemistry, gross pathology and histopathology.

3.1 Drugs

In the present study, the NSAIDs such as diclofenac sodium IP, paracetamol IP, nimesulide BP and meloxicam BP were received as gift sample from Mahindra Labs Pvt. Ltd., Peenya Industrial Area, Bangalore 560 058. Ketoprofen USP was received as gift sample from BEC Chemicals Pvt. Ltd., 24/MIDC, Raigad and celecoxib USP was received as gift sample from Cadila Pharmaceuticals Ltd., GIDC, Industrial Estate, Ankleshwar, Gujarat. Aspirin (Acetyl Salicylic Acid) was purchased from Sigma (Sigma Aldrich, St. Louis, Missouri, USA). The chemical name (IUPAC), molecular formula, molecular weight and structure of each NSAID are detailed below.

3.1.1 Diclofenac sodium

The earlier studies conducted in our department had proven that diclofenac is toxic to broiler chickens (Swetha et al., 2005; Mohan et al., 2008a). Besides, it was also proven that broiler chickens could serve as a model for diclofenac toxicity to avian species (Jayakumar et al., 2005). Therefore, in the present study, diclofenac was used as a standard control. The primary mechanism described for its anti-inflammatory, antipyretic and analgesic action is by inhibition of prostaglandin synthesis mediated by enzyme cyclo-oxygenase (COX).
Chemical name: 2-[(2, 6-Dichlorophenyl)amino]benzeneacetic acid sodium salt

Molecular formula: C_{14}H_{10}Cl_{2}NNaO_{2}

Molecular weight: 318.13

Structure:

![Structure Image]

### 3.1.2 Aspirin

Chemical name: 2-(acetyloxy)benzoic acid

Molecular formula: C_{9}H_{8}O_{4}

Molecular weight: 180.15

Structure:

![Structure Image]

### 3.1.3 Paracetamol

Chemical name: N-Acetyl-p-aminophenol

Molecular formula: C_{8}H_{9}NO_{2}

Molecular weight: 151.2

Structure:

![Structure Image]
3.1.4 Ketoprofen

Chemical name : \(2\)-[3-(benzoyl)phenyl]propanoic acid
Molecular formula : \(\text{C}_{16}\text{H}_{14}\text{O}_3\)
Molecular weight : 254.281
Structure :

3.1.5 Nimesulide

Chemical name : \(N\)-(4-Nitro-2-phenoxyphenyl)methanesulfonamide
Molecular formula : \(\text{C}_{13}\text{H}_{12}\text{N}_2\text{O}_5\text{S}\)
Molecular weight : 308.311
Structure :

3.1.6 Meloxicam

Chemical name : 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide.
Molecular formula : \(\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_5\text{S}_2\)
Molecular weight : 351.403
3.1.7 Celecoxib

Chemical name: 4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide

Molecular formula: $C_{17}H_{14}F_3N_3O_2S$

Molecular weight: 381.373

3.2 Spectroscopic and chromatographic characterization of NSAIDs

The genuineness of various NSAIDs used in the present study was confirmed by analytical procedures such as Ultra Violet (UV) visible (vis) spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR) as well as the purity of the compounds was tested and confirmed by Thin Layer Chromatography (TLC).

3.2.1 Chemicals

All chemicals or reagents used for UV-spectroscopy, FTIR and TLC were of analytical grade procured from Merck, India unless and otherwise specified.
3.2.2 Ultraviolet spectroscopy

The UV visible absorption spectra (200 to 400 nm) of different NSAIDs such as diclofenac, aspirin, paracetamol, nimesulide, ketoprofen, meloxicam, and celecoxib were recorded using a double beam UV-visible spectrophotometer (Owen, 2000), Evolution 300 (Thermo Fisher Scientific, Madison, WI, USA) operated by Vision Pro software. Individual stock (1mg/ml) solutions of diclofenac, paracetamol, ketoprofen, celecoxib and acetyl salicylic acid in methanol, nimesulide in acetone and meloxicam in chloroform were prepared. These stock solutions were diluted in ethanol, HCl (0.1 N) and NaOH (0.1 N) and UV-vis spectra were recorded.

3.2.3 Infrared spectroscopy

The procedure involving sample preparation and spectral recordings was carried out according to Stuart (2004). The infrared spectra of all NSAIDs were recorded using FTIR Nicolet 6700 (Thermo Fisher Scientific, Madison, WI, USA) operated by Omnic software 8.1.11. The spectra were obtained in two different modes: 1. A direct transmission mode using a KBr disk method; 2. By attenuated total reflectance (ATR) method using smart orbit diamond ATR accessory.

Approximately one mg each of diclofenac, paracetamol, ketoprofen, celecoxib, nimesulide, meloxicam and aspirin was grounded in potassium bromide (Spectroscopic grade) using mortar and pestle and pressed to a transparent disk using an econopress. The KBr disks were placed in the sample holder to record infrared (IR) spectra. Similarly, few mg of aforesaid NSAIDs were placed individually on the sample plate of the smart orbit and screwed tightly to record IR spectra in ATR mode.
3.2.4 TLC analysis of NSAIDs

TLC analysis of NSAIDs was performed following standard procedure described by Moffat (1986). A Camag HPTLC system comprising of Linnomate 5 automatic sample applicator, Twin-trough chamber (10×10 cm), TLC Scanner 3 and a TLC visualizer photo documentation system operated by WinCATS software was used to test the purity and characterization of NSAIDs. Separate stock solutions (1 mg/ml) of diclofenac, paracetamol, ketoprofen, celecoxib and acetylsalicylic acid in methanol, nimesulide in acetone, meloxicam in chloroform were prepared. From these stock solutions a separate sub-stock (20 ng/µl) was prepared using appropriate diluents. These sub-stocks were used for spotting on TLC plates.

Pre-coated silica gel 60 F254 aluminium TLC plates (layer thickness 0.2 mm, E-Merck, Germany) (10×10 cm) were pre-washed (by developing TLC plates in methanol using Twin trough chamber) and dried in an hot air oven at 100 °C for 30 min. The samples were spotted on the preconditioned TLC plates as a narrow band of 8 mm length at 10 mm from bottom of the TLC plate using automatic sample applicator.

For determination of suitable mobile phase, different chromatography systems were tested and analyzed according to the classification proposed by Moffat (1986). The spotted plate containing samples of diclofenac, paracetamol, ketoprofen, celecoxib, nimesulide and meloxicam was developed using mobile phase isopropyl alcohol: n-hexane (4.9: 5.1 v/v) in a twin trough chamber.

A 10 µl sample from respective sub-stock of each NSAIDs diclofenac, paracetamol, ketoprofen, celecoxib, nimesulide and meloxicam was transformed to a
separate tube. Later this mixture was vortexed. From this mixture, a 10 μl of sample was spotted on TLC plate (2.5×10 cm).

The spotted plate containing mixture of NSAIDs diclofenac, paracetamol, ketoprofen, celecoxib nimesulide and meloxicam was developed using mobile phase isopropyl alcohol: n-hexane (4.9: 5.1 v/v) in a twin trough chamber.

Similar procedure (sample preparation and spotting) was followed for aspirin except for the mobile phase methanol: toluene (1: 1 v/v).

The twin trough chamber containing the mobile phase and spotted plate was allowed to saturate for 10 min and then plates were developed up to 8 cm. The developed plates were dried in the hot air oven at 60 °C for 5 min and were placed in the TLC vizualizer and observed under short uv (254 nm) illumination and used for photo documentation.

These plates were then placed in a TLC Scanner 3 and were scanned at 254 nm to detect the bands separated and to obtain the chromatogram. After obtaining the chromatogram, the peaks were assigned and again scanned at 200 - 500 nm range to record the characteristic uv-absorbance or remission spectra of the each NSAID.

3.3 Experimental birds

Forty eight apparently healthy, unsexed broiler chickens, aged five weeks with body weight ranging from 1.4 to 1.6 kg were procured from commercial poultry farm. The birds were caged individually in experimental animal house maintained under standard laboratory conditions. Medication free feed (free from any antibiotics or coccidiostats) procured from the university poultry farm was fed ad libitum and free
access to potable water was provided. All birds were housed in the experimental animal house for a period of one week for acclimatization.

The birds were maintained as per the protocol outlined in publication of the committee for the purpose of control and supervision of experiments on animal’s standard guidelines (CPCSEA, 2003).

The experimental protocol including number of birds and various procedures involved was approved by the Institutional Animal Ethics Committee (IAEC) bearing approval No. 29/LPM/IAEC/2009 dated 16-05-2009 Veterinary College, Hebbal, Bangalore, India. All birds were acclimatized to the laboratory housing condition for a period of seven days.

3.4 Design of experiment

3.4.1 Experimental procedure

Forty eight broiler chickens were randomly divided into 8 groups consisting 6 birds in each group. The details regarding the groups, drugs and dose of administration are given in Table 1.

3.4.2 Dose selections

Although NSAIDs mainly act by inhibition of cyclo-oxygenase enzyme and thereby reduces the synthesis of prostaglandins, the information on their effectiveness and optimum dose in avian species is limited or not available. Therefore, the selection of dose for NSAIDs such as diclofenac, aspirin, paracetamol, ketoprofen, nimesulide and meloxicam was based on the therapeutic doses as used for treatment of different ailments in domestic animals (Thompson, 2008), whereas, for celecoxib, dose selection was based
on human dose (Reynolds, 2005). The dose administered to individual birds during the experimental period was based on the body weight recorded on the respective day.

**Table 1**: Experimental protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Details of administration</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>Distilled water</td>
<td>1.5 ml P.O.</td>
</tr>
<tr>
<td>Group II</td>
<td>Diclofenac (Standard Control)</td>
<td>Diclofenac solution</td>
<td>2.5 mg/kg P.O.</td>
</tr>
<tr>
<td>Group III</td>
<td>Aspirin</td>
<td>Aspirin solution</td>
<td>10 mg/kg P.O.</td>
</tr>
<tr>
<td>Group IV</td>
<td>Paracetamol</td>
<td>Paracetamol solution</td>
<td>10 mg/kg P.O.</td>
</tr>
<tr>
<td>Group V</td>
<td>Nimesulide</td>
<td>Nimesulide suspension</td>
<td>2 mg/kg P.O.</td>
</tr>
<tr>
<td>Group VI</td>
<td>Ketoprofen</td>
<td>Ketoprofen suspension</td>
<td>4 mg/kg P.O.</td>
</tr>
<tr>
<td>Group VII</td>
<td>Meloxicam</td>
<td>Meloxicam suspension</td>
<td>0.5 mg/kg P.O.</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Celecoxib</td>
<td>Celecoxib suspension</td>
<td>3.5 mg/kg P.O.</td>
</tr>
</tbody>
</table>

**3.4.3 Drug formulations**

During the experimental period, fresh drug solutions or suspensions were prepared daily. The amount of drug used to prepare solutions or suspensions was based on the body weight of birds. The amount of diclofenac required was weighed and transferred to 20 ml test tubes. To this, a known amount of distilled water was added. Later, it was dissolved using cyclomixer. Aspirin was dissolved in distilled water maintained at 40 °C. Ketoprofen, nimesulide, meloxicam and celecoxib were water insoluble. Hence, uniform suspensions for these drugs were prepared separately by transferring a required amount of drugs into 20 ml test tubes containing a known amount of distilled water. These tubes were then kept in ultrasonicator (Powersonic-410, Daichen...
Labtech Co. Ltd., Korea) for 10 min or until a uniform suspension was obtained. The individual drug solutions or suspensions were vortexed using cyclomixer just before dosing.

3.4.4 Administration of drugs

All NSAIDs were orally administered daily directly into the crop of each bird assigned to particular group. Administration of drugs was made by using gavaging tube fixed to two ml syringe. The gavaging tubes of 10 cm length prepared from infant feeding tube (No. 8) were used in the present study.

3.4.5 Observations

General clinical observations were made thrice a day throughout the study period after dosing. All the birds were observed for health condition, feed consumption, morbidity and mortality, if any were recorded.

3.4.6 Blood sampling

The blood samples were collected from cutaneous ulnar vein or right jugular vein by using two ml disposable syringe and needle (22 G, 1.5” length). The blood samplings were carried out before treatment and daily on subsequent days for the period of five days. From each bird, approximately 1.5 ml of blood was collected. From this, 0.2 ml of blood was transferred to one ml storage vials containing 500 μg of Na₂EDTA. The left over blood in the syringe was transferred to clean, dry 3 ml glass tubes. The blood in the glass tubes was allowed to clot. Serum was separated from the clotted blood following centrifugation at 2500 rpm for 10 min at room temperature. The serum was transferred to storage vials of one ml capacity. Then serum samples were stored at -20 °C until it was used for further analysis.
3.4.7 Hematological parameters

Hematological parameters were estimated using blood samples collected from all the birds on day 1 (before treatment) and on subsequent days for the period of five days. The following hematological parameters were determined by using fully automatic blood cell counter (Model PCE-210, Erma Inc., Tokyo, Japan).

1. Total erythrocyte count (TEC)
2. Hemoglobin concentration (Hb)
3. Packed cell volume (PCV)
4. Platelet count

3.4.8 Serum biochemical parameters

The serum biochemical parameters were estimated from serum samples collected from the birds to evaluate toxic effect on organs and tissues specifically on liver and kidney using clinical chemistry analyzer - Microlab 300 (Vitalab Scientific, The Netherlands). The following parameters were estimated using commercially available diagnostic kits from Merck (Ecoline®, Merck Specialties Limited, Kalyan Badlapur Road, M. I. D. C Area, Ambernath) by following the manufacturer instructions furnished in the leaflet supplied along with the diagnostic kit.

1. Aspartate aminotransferase (AST)
2. Alanine aminotransferase (ALT)
3. Alkaline phosphatase (ALP)
4. Blood urea nitrogen (BUN)
5. Creatinine
6. Uric acid
7. Albumin
8. Total serum protein

3.4.9 Estimation of sodium and potassium concentration in serum

The sodium and potassium concentrations in control as well as in all the treatment groups were estimated using serum samples collected on day 1 (before treatment) and on subsequent days for the period of five days by employing flame photometer 128 equipped with FPM compressor 126 (Systronics, India) (Marti and Munoz, 1957).

3.4.10 Pathology

Detailed post mortem examination of the birds was carried out which died during the course of experiment and gross lesions, if any, were recorded. In addition, at the end of study period on day 6, all the birds were humanely sacrificed and subjected to detailed gross necropsy including examination of the external surface of the body, thoracic and abdominal cavities and other contents. The organs were collected for histopathology.

3.4.10a Specimen collection and processing

The birds which died during the course of experiment and all the surviving birds were weighed and sacrificed on day 6. Necropsy was conducted on each bird to observe any gross pathological changes. The liver, spleen, kidneys, heart, lungs, crop, intestine were separated from the adhering tissues using saline. The organs were placed on the blotting paper and gently pressed to remove excess of saline. Liver, kidney, heart and spleen were weighed on an analytical balance and the weight of each organ was recorded. Organ to body weight ratio i.e., organ weight/body weight was calculated.

For histological examination, a representative tissue samples from, liver, lung, heart, kidney and intestine were collected in 10% neutral buffered formalin as well as in
absolute alcohol. The tissues fixed in 10% neutral buffered formalin were processed by routine paraffin embedding technique and sections of 5μ thickness were cut and stained by hematoxylin and eosin (Luna, 1968). The tissues fixed in absolute alcohol were directly cleared and infiltrated with paraffin and sections were stained by DeGalantha’s method to demonstrate urate crystals (Luna, 1968).

For electron microscopic studies, tissue samples (liver and kidneys) collected were transferred to vials and fixed in 3% Gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C and post fixed with 2% aqueous Osmium tetroxide in the same buffer for 2 h. After fixation, the samples were dehydrated in a series of graded alcohols and infiltrated and embedded in Araldite 6005 resin or spur resin (Spur,1969). Ultra thin sections (50-70 nm) were cut with a glass knife on a Leica Ultra cut (UCT-GA-D/E-1/00) microtome. Ultra thin sections were mounted on cooper grids and stained with saturated aqueous Uranyl acetate and counter stained with Reunols lead citrate. The sections were observed under transmission electron microscope (Hitachi H-7500, Japan) at required magnifications and photographs were taken at RUSKA Labs, College of Veterinary Science, SVVU, Rajendra Nagar, Hyderabad, India.

3.4.11 Statistical analysis

The data obtained from the present study were subjected to statistical analysis. Mean values and standard error of mean were calculated and all the values were expressed as Mean ± SEM The data were analyzed by one-way ANOVA with Bonferroni post test (GraphPad Prism, 2004) and P value <0.05 was considered to be significant.