CHAPTER III

MATERIAL AND METHODS
CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

3.1.1 Chicks

Healthy day old, Marshal broiler chicks were obtained from M/s. Huma Hatcheries Ltd., Udgir for this experiment.

3.1.2 Fungal culture

Pure culture of Aspergillus parasiticus NRRL – 2999 and Aspergillus ochraceus (allutaceae) NRRL – 3174 available in the Department of Pathology, College of Veterinary & Animal Sciences, Udgir were used to obtain the required quantity of aflatoxin and ochratoxin.

3.1.3 Field feed samples

A total of 28-field broiler feed samples/feed ingredients were randomly collected from different feed manufacturers and broiler farms, in Marathwada region for screening of mycotoxins.

3.1.4 Toxin binder

Commercial mycotoxin binder UTPP Biotech was obtained from M/s. Vetcare Division of Tetragon chemie Ltd., Bangalore. It was added in the feed at the rate of 1.5 kg/tonne, as recommended by the manufacturer.
3.1.5 **Diagnostic kits**

Different diagnostic kits required for estimation of protein, albumin, cholesterol, uric acid, alkaline phosphatase and bilirubin were obtained from M/s. Ezra Brothers Pvt. Ltd., Mumbai (DIALAB, GmbH, Austria).

3.2 **Methods**

3.2.1 **Extraction of mycotoxins**

**Aflatoxin**

Extraction of aflatoxin was done as per the method described by Shotwell *et al.* (1966). Strain of *Aspergillus parasiticus* NRRL – 2999 was grown on Czapeck Dox agar medium for 8 days at room temperature (30°C). In a sterile glass flask (1000 ml capacity), 250 gram of clean unpollished rice was taken and 5ml sterile distill water was added, such 15-20 flasks were autoclaved at 15 lbs/inch² pressure for 15 minute and then allowed to cool. Few loopful of fungal growth from agar was taken in sterile distilled water in 10 ml. test tubes and mixed well and then inoculated into each flask at room temperature. The tightly closed flasks were then kept away from sunlight for a period of 10-18 days with frequent rotating motion. Few ml of distilled water was added every 24 hr for first 3 days. Flasks were shaken vigorously to prevent the formation of clumps of rice at the bottom. In between 10th and 18th day after the change of initial white color to light yellowish brown and then to
dark green, the flasks were taken out. Rice containing released aflatoxin was autoclaved, dried at 100°C in hot air oven and ground to powder form. The powder was stored in sterile glass container till use. Two hundred gms. of this moldy powder was then subjected to HPLC for estimating the mycotoxin contents as per modified Romers method at Animal Feed Analytical and Quality Control Laboratory (FAQCL), Nammakal (Sundaram et al., 2001).

**Ochratoxin**

Extraction of ochratoxin was done as per following procedure.

Laboratory strain of *Aspergillus ochraceus* NRRL-3174 was grown on Czapeck Dox Agar in test tubes for 8 days at room temperature (30°C). For extraction of ochratoxin, culture was grown on a mixture of wheat (10 parts), soybean (40 parts) and groundnut (40 parts) as per the method adopted by Moregacunkar (2002).

These components were selected as clean, undamaged and of good quality after removing uneven, blackened and low quality grains, if any. It was half ground and placed in 500 ml and 1000 ml sterile flasks and soaked with 40 ml of sterile distilled water. The flasks were autoclaved at 121°C for 30 minute and then cooled. Five ml of sterile saline was added in 10 ml test tubes and loopful of *A. ochraceus* was mixed in each tube, which was then added in each flask at room temperature. Tightly closed flasks were kept in the dark to avoid direct exposure to sunlight for a period of 10-15 days with frequent
rotating motion in order to break the mycelial mass. Few ml of sterile distilled water was being added every 24-48 hrs for first 3-4 days. Between 15-30 days, the change in initial white colour to sulphur colour and then to black colour was noted and the flasks were taken out. The fungus culture thus grown was autoclaved, dried at 50°C in hot air oven for overnight and then ground to a fine powder form. The powder was stored in sterile glass container till use. Then 200 gms of this moldy powder was subjected to HPLC for knowing the mycotoxin content as per the modified procedures at Animal Feed Analytical and Quality Control Laboratory, Nammakal (Sundaram et al., 2001).

3.2.2 Survey of mycotoxin in field feed samples

The individual feed ingredients (ground nut expeller, ground maize, fish meal etc.) and the feed samples (starter, finisher etc.) were procured from local feed factories, poultry units or of commercial feed units. These samples were evaluated for presence of several mycotoxins, if any, by subjecting them to HPLC at Animal Feed Analytical and Quality Control Laboratory, Nammakal (Sundaram et al., 2001).

3.2.3 Experimental birds

A total of two hundred forty, day old commercial broilers (Marshal, M/s. Huma Hatcheries, Udgir) were randomly divided into eight groups of 30 chicks each.
All the chicks were immunized for Marek's disease on day 0 and Lasota and IBD vaccine on 7th and 18th day, respectively.

3.2.4 Feeding of toxins

Broiler chick mash (starter and finisher) was procured from M/s Fehad Poultry farms, Parbhani. Aflatoxin B1 (1.5 ppm), ochratoxin A (1.5 ppm) and combination of each of aflatoxin and ochratoxin (0.75 ppm) were fed to experimental birds through feed. UTPP Biotech was added at the rate of 1.5 gm/kg of feed in each intoxicated groups and the control groups. Feeding of the treated diets commenced from day one and continued till the termination of experiment on 42nd day of age. The details of feeding are shown in Table 1.

3.2.5 Parameters studied

Feed intake (FI)

Every day a known quantity of feed was fed and after 24 hrs the feed in balance was recorded. The difference between the feed offered and in balance was worked out to know the actual feed consumed by each group in total. The feed consumption was expressed as gms/day/group.

Body weight gain (BWG)

Chicks from each group were weighed (gms/bird/week) individually at weekly intervals.
Feed conversion ratio (FCR)

On the basis of weekly weight and feed intake, the FCR of each group was calculated at weekly intervals.

Relative organ weights

Six birds on each intervals were randomly sacrificed for the record of their relative organ weights (%) viz liver, spleen, kidney, bursa of fabricious and heart on day 0 (pre treatment interval), 14th, 28th and 42nd day of study.

Serum biochemistry

Biochemical parameters were estimated in six chicks sacrificed at fortnight intervals in each group. The blood samples were collected for separation of serum. The serum samples thus collected were kept at -20°C temperature until analyzed.

The individual serum samples were analyzed for total protein (gms/dl), albumin (gms/dl), uric acid (gms/dl), cholesterol (mg/dl), alkaline phosphatase (U/lit) and bilirubin (mg/dl) using automatic biochemical analyzer (StatFax - 2000, Awareness Technology, INC, Palm City, Florida, USA).

Hematological studies

The blood samples were collected directly from the heart into sterilized vials containing anticoagulant (EDTA @ 1.0 mg/ml) for the above hematological studies except for blood clotting time, for which blood samples
were drawn into nonheparinised capillary tubes directly from the syringe immediately after collection.

The haematological parameters were recorded in six birds randomly selected and sacrificed from each group on 14th, 28th and 42nd day of study.

**Mortality**

The number of dead birds in each group was recorded daily. The carcasses were necropsied to ascertain the cause of death.

### 3.2.6 Pathological studies

**Clinical signs**

All the chicks were observed daily for clinical signs throughout the experimental period and the observations were recorded group wise.

**Gross studies**

The gross pathological lesions, if any in the visceral organs and brain were recorded from dead or sacrificed birds.

**Histopathological studies**

Pieces of heart, lungs, liver, spleen, kidneys, intestine and bursa of suitable thickness were collected from the birds sacrificed or died due to toxicity and preserved in 10 per cent neutral formal saline. The tissues were embedded in paraffin and processed as per the standard procedure. The sections were cut at 3 to 5 μ thickness and stained with Harri’s hematoxyline and eosin for microscopic examination (Culling, 1974).
Statistical analysis

The data collected was analyzed by applying Completely Randomized Design (CRD) as per the method described by Snedecor and Cochran (1968).
<table>
<thead>
<tr>
<th>Sr no</th>
<th>Period of study</th>
<th>Operations / Treatments</th>
<th>Dose of mycotoxin / Toxin binder</th>
<th>Age of birds (Days)</th>
<th>Groups of chicks / birds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CON</td>
<td>TBR</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1-42</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6 weeks</td>
<td>Plain feed</td>
<td>No lesion, No binder</td>
<td>1-42</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>6 weeks</td>
<td>Mice feed mixed with UTPP Biotech</td>
<td>UTPP @0.5 g/mgKg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>6 weeks</td>
<td>Aflatoxin B1 mixed feed</td>
<td>Aflatoxin @1.5 mg/kg feed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6 weeks</td>
<td>Aflatoxin B1, and UTPP Biotech mixed feed</td>
<td>Aflatoxin @1.5 mg/kg and UTPP @0.5 mg/kg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>6 weeks</td>
<td>Ochratoxin A mixed feed</td>
<td>Ochratoxin @1.5 mg/kg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>6 weeks</td>
<td>Ochratoxin A and UTPP Biotech mixed feed</td>
<td>Ochratoxin @1.5 mg/kg and UTPP @0.5 mg/kg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>6 weeks</td>
<td>Aflatoxin B1, and Ochratoxin A mixed feed</td>
<td>Aflatoxin @0.75 mg/kg and Ochratoxin @0.75 mg/kg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>6 weeks</td>
<td>Aflatoxin B1, and Ochratoxin A along with UTPP Biotech mixed feed</td>
<td>Aflatoxin @0.75 mg/kg and Ochratoxin @0.75 mg/kg along with UTPP Biotech @1.5 mg/kg fed</td>
<td>1-42</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Only once</td>
<td>Vaccination – Lasota and IBD (L1=L2)</td>
<td>-</td>
<td>07th and 12th</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Weekly</td>
<td>Body weight / Feed consumption / FCR</td>
<td>-</td>
<td>1-42</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Fortnightly</td>
<td>Blood sacrificed for organ-weight, hematology, sero-biochemistry, and gross and histopathology</td>
<td>-</td>
<td>1-42</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate number of birds sacrificed.

- HS = Highly significant (P < 0.01)
- CON = Untreated control
- AFB = Aflatoxin B1 treated
- OCA = Ochratoxin A treated
- AAO = Aflatoxin and Ochratoxin treated
- TBR = UTPP treated
- AFT = Aflatoxin B1 and UTPP treated
- OCT = Ochratoxin A and UTPP treated
- AOT = Aflatoxin and Ochratoxin and UTPP treated
Plate 3: Stages of development of fungal growth of *Aspergillus parasiticus* from left to right on day 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> respectively on half ground rice.

Plate 4: Stages of development of fungal growth of *Aspergillus ochraceus* from left to right on feed ingredients.
Plate 5: Glass jar containing dried and powdered feed ingredients containing known quantities of aflatoxin and ochratoxin in it.

Plate 6: Brooder arrangement for experimental chicks.