MATERIAL AND METHODS

All the animal experiments were conducted at the animal facility of Poultry Science Department of Anand Agricultural University, Anand. The experimental trials were approved by the Institutional Animal Ethics Committee, according to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. The toxicity test used strictly followed the procedures of the drugs and cosmetics rules, 1945, Appendix – III Animal Care Standard.

Test Chemical

A commercial insecticide formulation of combination insecticide (Ci) which is constituted of chlorpyrifos (50%) and cypermethrin (5%) was used for the study.

Figure 1: Nurelle D 505

1. **Test Article and Manufacturing Company**
   Product Commercial Name: Nurelle D 505 (Figure 1).
   Company Identification: Dow AgroSciences India Pvt. Ltd; Mumbai.

2. **Composition/Information on Ingredients**

Chlorpyrifos	CAS # 002921-88-2	50%
Cypermethrin	CAS# 052315-07-8	5%
Emulsifiers		8%
O-Xylene	CAS# 001330-20-7	q.s.to make 100%

3. **Chemical Name.**
   a. **Chlorpyrifos:** O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
   b. **Cypermethrin:** [(±)-α-Cyano-(3-phenoxyphenyl) methyl (±)-cis/trans-3-(2,2-dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate]

4. **Registration No.**
   CIR-26,393/97/Chlorpyrifos + Cypermethrin (EC)-7

5. **Manufacture License No.**
   0403/0001/M/D/ Date. 10/07/1981

6. **Stability.**
   Stable under normal storage conditions. Unstable at elevated temperatures.

7. Physical And Chemical Properties

Melting Point	<-5°C
Solubility in Water	Forms emulsion
Specific Gravity	(Liq.) 1.398 @ 43.5°C
Density	1.1 g/ml @ 20°C
Appearance	Yellowish clear liquid
Odor	Xylene like

Fertilized RIR Eggs and Insecticide Injection:

The fresh fertilized RIR eggs were collected from Poultry Science Department of Anand Agricultural University, Anand. Eggs were swabbed with povidone-iodine to avoid infection. Each egg was weighed and marked to receive the respective dosage treatment of the combination insecticide for three experimental groups and corn oil for one control group, of six each (n=6). Combination insecticide was diluted in corn oil so as to get doses of 0.01, 0.05 and 0.1 µg/egg based on a prior dose range study followed by probit analysis.

On zero day of incubation, the eggs were candled and the air space positions in the egg shell were located and marked. Marked surfaces were swabbed with 70% alcohol. A sterile and sharp needle was used to make a small hole at the located air space and then appropriate dose was administered in to the air sac of the eggs assigned to experimental and control groups (Blankenship *et al*., 2003) with a sterile syringe attached with 36 G needle. The hole was immediately sealed with molten paraffin wax and the egg was transferred to the incubator (Figure 2). The whole dosage injection procedure was done in a completely aseptic-condition under a laminar flow to avoid infection. The dose volume of Combination insecticide and vehicle control (corn oil) was 25µl/egg.

Figure 2: Treated and non-treated eggs marked and dosed

Egg Weight:

The weight of eggs assigned for experimental groups and control group was recorded prior to incubation on the day of commencement of treatment and at weekly intervals thereafter.

Incubation and Hatching:

A clean, disinfected and fumigated automatic egg incubator (Figure 3) and hatcher (Figure 4) were used. Automated incubator regulates the factors such as temperature of 37.5 ± 0.5 ºC, 75-80% relative humidity, and turns the eggs when necessary. Immediately after treatment, eggs of both the treated and control groups were kept in the incubator with proper marking for 18 days (Figure 5). On 18th day, the egg candling was done and the chick holding eggs were transferred to hatcher till the day of hatch (Figure 6).

Figure 3: Automatic Egg Incubator

Figure 4: Chick Hatcher

Figure 5: Treated and non-treated eggs kept in incubator

Figure 6: Candling of treated and non-treated eggs

Wing Band:

On the hatch out day, the live chicks that hatched out or were culled were wing banded to attain an identity of their respective dosage category (Figure 7) and rest of the eggs were opened manually to collect the data for other parameters.

Figure 7: Chick wing banded

Rate of Hatchability and Mortality:

The initial egg weight, hatchling weight and the subsequent gain in body weight was monitored on weekly basis post hatch till a period of 25 weeks for the first generation and 4 weeks for the second generation at their individual and respective time period. The rate of mortality was also recorded for the analysis of toxicity effect.

$$\text{Hatchability of Fertile Eggs (\%)} = \frac{\text{Number of Hatched Chicks}}{\text{Total Number of Fertile Eggs}} \text{ X } 100$$

$$\text{Embryo Mortality Rate (\%)} = \frac{\text{Number of Dead Embryos}}{\text{Total Number of Fertile Eggs}} \text{ X } 100$$

Growth Monitoring:

The growth of animals was monitored with subsequent body gain. The comparison was made between treated and control group.

Post Hatch and Vaccination:

On day 21 after hatching, the percentage of hatch and mortality was recorded in the experimental as well as control group. The hatchling groups (F1 generation) from the treated and control eggs were vaccinated immediately by Marek's Disease Vaccine followed by other vaccines as per AICRP-S9 Generation schedule in later stages (Table 8). Then the hatchlings were tagged with wing bands (Figure 7) with respect to their dosage treatment to attain an identity and were housed in separate pens with immediate supply of water. To promote the survival of the chick assisted hatching was involved in case of those chicks who fail to progress normally at hatching stage due to deformities or weakness caused by Combination insecticide.

Feed Consumption:

Post hatching, after three hours starter mash feed was provided to the chicks *ad libitum*. This helps chick grow as it contains quality oils and protein sources. From hatch out day till seven weeks chick crumb was given, then chick were transferred to Poultry Grower Pellets. Before changing to poultry grower pellets, it is preferable to mix the chick crumbs with the grower pellets to gradually introduce them over a space of a week. Grower Pellets are formulated for young poultry, designed specifically to help support steady growth and sexual maturity. Layers Pellets should be fed 3 weeks before chicken start to lay, which is approximately 20 - 24 weeks of age. Layers pellets contain optimum levels of protein, lysine, methionine and linoleic acid for good egg size, with generous levels of calcium & phosphorus to help good shell strength. Now the layers should be transferred to complete formulated food layer mash to keep chicken healthy. The nutrient value of the feed for respective stage is as mentioned in the table below (Table 1).

Table 1: Nutrient Value of the Feed

Sr. No.	**Nutrient Name**	Chick Crumbs (1-7 weeks)	Grower Pellets (8 – 11 weeks)	Developer Mash (12 - 14 weeks)	Pre Layer Pellets (15 till 1st egg)	Layer Mash (1st egg-25weeks)	Layer Mash (25-50 weeks)	Layer Mash (51-72 weeks)
1	**M.E. (kcal/kg)**	2970	2850	2650	2650	2600	2400	2480
2	**Protein (%)**	20.5	19	16.5	17.5	18.5	15.5	15.25
3	**Lysine (%)**	1.1	0.9	0.8	0.9	0.9	0.75	0.72
4	**Methionine (%)**	0.48	0.45	0.4	0.45	0.45	0.4	0.38
5	**Calcium (%)**	1.05	1.05	1.0	2.6	3.9	3.5	3.83
6	**Available Phosphorous (%)**	0.48	0.45	0.43	0.46	0.46	0.42	0.4
7	**Sodium (%)**	0.21	0.21	0.2	0.18	0.18	0.18	0.185
8	**Linoleic Acid (%)**	1.4	1.2	1.2	1.35	1.4	1.1	1.1
9	**Crude Fiber (%)**	4.5	5.5	3.0	4.0	4.5	4.7	4.8

Dietary Supplement:

A dietary supplement is intended to provide nutrients that may otherwise not be consumed in sufficient quantities. And we know just like us humans, birds can pick up viruses but with the right supplements, they can be made to resist viral infections. So in addition to feed and water, the birds were provided with mineral mixture, Vimeral and Lactovet Powder. Vimeral is a powerful anti-stress liquid vitamin supplement for poultry feeding with key features to remove stress, act as immunostimulant, and help better growth and better egg production. The nutritional value per ml and suggestive usage for poultry is as shown in Table 2 and Table 3.

Table 2: Liquid supplement of vitamins for poultry feeding

Company Name: Virbac Animal Health (ISO 9001 certified company)	
Brand Name: Vimeral , Batch No: SC 3418s	
NUTRITIONAL VALUE PER mL	
Vitamin A (Palmitate)	12000 I.U.
Vitamin D	6000 I.U.
Vitamin E	48 mg
Vitamin B12	20mg

Table 3: Suggestive usage – Poultry

Chicks	5ml / 100 birds
Growers	7 ml / 100 birds
Layers	10 ml / 100 birds

Lactovet powder is an animal feed supplement with unique combination of effective balanced minerals like calcium, phosphorus, vitamins and minerals with natural elements used during the experiment to resist viruses. It is recommended for all kinds of productive animals to achieve better production and maintain their growth and health. Lactovet acts as health promoter and tonic. It replenishes the daily loss of calcium, improves fertility or breeding efficiency, helps in efficient utilization of absorbed nutrients and improves health & vigour. The nutritional value is shown in Table 4 and suggestive usage for poultry is- mixing rate 2.5% of feed.

Table 4: Lactovet powder - Nutritional value

Company Name: Rakesh Pharmaceuticals	
Brand Name: LACTOVET POWDER	Batch No: SC 3418
Vitamin A (Vitamin A Palmitate)	200000 I.U.
Vitamin D3	70000 I.U.
Calcium	28.00%
Phosphorus	14.50%
Cobalt	0.02%
Copper	0.08%
Iron	0.50%
Iodine	0.03%
Zinc	0.20%
Magnesium	0.50%
Asparagus racemosus	10.00%
Leptadenia reticulate	14.50%

Vaccination:

Vaccination is commonly used in commercial poultry and increasingly used in backyard birds to control disease. Vaccines mimic natural infection, allows the birds to build up immunity to the disease without any harmful effects. This way the RIR birds are prevented from getting the disease. As per AICRP-S9 Generation schedule, the following vaccines were administrated in the RIR chicks at various stages of their growth (Table 5).

Table 5: Vaccine Schedule

Sr. No	Age	Disease	Type of Vaccine	Route of Administration
1	Day Old	Mereks	Marek's Disease Vaccine	Subcutaneous Injection
2	5 – 7 Day	Ranikhet	Ranikhet Disease Vaccine F1 (Lasota Strain)	Intra Ocular
3	14 Days	Infectious Bursal Disease	IBD (Gumboro) Live Intemediate Strain	Ear Drops
4	4th Week	Infectious Bursal Disease	IBD (Booster)	Drinking Water
5	5th Week	Ranikhet	Ranikhet (Lasota Strain)	Drinking Water/Milk
6	7th Week	Fowl Pox	Fowl Pox Vaccine	Wing
7	9th Week	Ranikhet	Ranikhet Disease Vaccine (R_2B/Mukteswar) Strain	Intramuscular
8	12th Week	Avian Infectious Bronchitis	Avian Infectious Bronchitis VaccineMassachusetts Type Strain	Drinking Water
9	16th Week	Avian Influenza	RD – Killed	Under Skin

All the details pertaining to vaccines administered (commercial details, contents, dosage limit and route of administration) are depicted below in a tabular form (Fables 6 to 13 and Figures 8 to 12).

Table 6: Vaccination on day one; Route of application: Subcutaneous injection in the neck

Marek's Disease Vaccine, Live, IP Vet.	(HVT FC-126 Strain)
1000 Doses	For poultry use
Protect from light	Store at +2°C to +8°C
Each dose contains: HVT Strain PFU ≥ 10^3 grown on SPF chick embryo fibroblasts.	
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd. (VentriBiologicals, Vaccine Division) Gat No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Batch No. MD11-003	
Mfg date: 09/02/2011	Expiry date: 08/02/2013
Mfg. Lic. No. PD-10	

STERILE DILUENT- 200ml FOR MAREK'S DISEASE VACCINE, LIVE.		
Store at room temperature		
CONTENTS:		%w/v
N-Z-Amine		0.35
Potassium Dihydrogen Phosphate	IP	0.049
EDTA	IP	0.02
Dipottasium Hydrogen Phosphate	IP	0.119
Refined sugar	IP	6
Sodium Hydroxide	IP	0.013
Phenol Red Indicator	IP	0.003
Water For Injection	IP	q.s.
Dose: 0.2 ml per Chick		

Figure 8: Pictorial depiction of **v**accination on first day

Table 7: Vaccination on day six; Route of application: Intra ocular

Ranikhet Disease Vaccine, Live, IP Vet. (NEWCASTLE Disease Vaccine, Live)	Lentogenic (La Sota) Strain
500 Doses	For poultry use
Lentogenic (La Sota Strain) EID_{50}/Dose≥ 10^6	SPF ORIGIN
Protect from light	Store at +2°C to +8°C
Each dose contains: HVT Strain PFU ≥ 10^3 grown on SPF chick embryo fibroblasts.	
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd. (VentriBiologicals, Vaccine Division) Gat No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: LA11-024	
Expiry date: 04/10/2012	

STERILE DILUENT- 15 ml FOR RD/(ND)/IB/IBD/AE VACCINE, LIVE.		
Store at room temperature		
CONTENTS:		%w/v
Potassium Dihydrogen Phosphate	IP	0.068
Sodium Hydroxide	IP	0.015
Water for Injection	IP	q.s.
Dose: 0.03 ml per chick.		

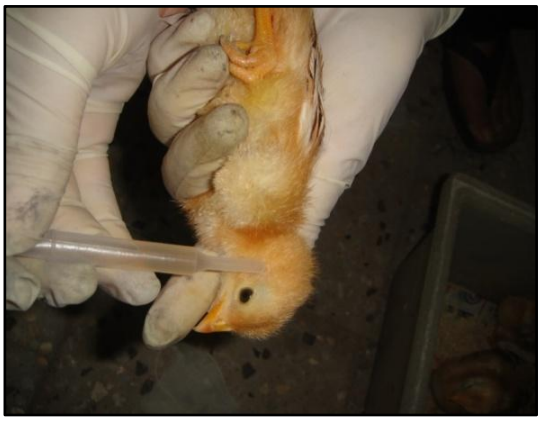

Figure 9: Intraocular vaccination on the 6th day

Table 8: Vaccination on 14th day: Route of application: Ear drop

Infectious bursal disease vaccine, living I.P.vet.	Intermediate strain
200 Doses	For poultry use
Brand name : GUMBORO I	
Lentogenic (La Sota Strain) EID_{50}/Dose≥ 10^6	SPF ORIGIN
Protect from light	Store below 8°C
Each dose contains: NLT $10^{3.5}$ EID_{50} IBD VIRUS	
Manufactured by: Hester biosciences.ltd. Meshana, gujarat-382721	
Mfg. Lic. No. G1016	
Batch No: 6044	
Expiry date: FEB 2012	

STERILE DILUENT- 15 ml
To reconstitute 200 Doses of Live Las, Live B1 (Newcastle Disease Vaccine Living I.P. Vet.)
Live M48 (Avian Infectious Brochitis Vaccine, Living I.P. Vet)
Live LAS-MAS, Live B1-M48 (Newcastle Disease & Avian Infectious Brochitis Vaccine, Living B.P. Vet)
Gumboro I, Gumboro I+ (Infectious Bursal Disease Vaccine, Living I.P. Vet)
Batch no: 56027

Table 9: Vaccination on 4th Week. Route of application: Drinking water

Infectious bursal disease vaccine, living I.P.vet.	Intermediate strain
Gumboro Disease Vaccine, Live	
1000 Doses	For poultry use
Brand name : GUMBORO I	
Lentogenic (La Sota Strain) EID_{50}/Dose≥ 10^6	SPF ORIGIN
Protect from light	Store at +2°C to +8°C
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd. (VentriBiologicals, Vaccine Division) Gate No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: IN11-019	
Expiry date: 28/10/2012	

STERILE DILUENT- 30 ml
For RD/(ND)/IB/IBD/AE Vaccine, Live
Store at room temperature
CONTENTS
Potassium Dihydrogen Phosphate
Sodium Hydroxide
Water for Injection
Dose: 0.03 ml per chick.

Table 10: Vaccination at 5th Week; Route of application: Drinking water/chilled milk

Ranikhet Disease Vaccine, Live, IP Vet. (NEWCASTLE Disease Vaccine, Live)	
Lentogenic (La Sota) Strain	
500 doses	for poultry use
Lentogenic (La Sota Strain) EID_{50}/Dose≥ 10^6	SPF Origin
Protect from light	Store at +2°C to +8°C
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd (VentriBiologicals, Vaccine Division) Gate No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: LA11-024	
Expiry date: 04/10/2012	

Figure 10: Chilled milk laced with vaccine given on 5th week

Table 11: Vaccination on 7th Week; Route of application: Wing web

Fowl Pox Vaccine, Live, I.P.Vet.	
1000 doses	for poultry use
Fowl Pox strain ≥ 10^2 EID_{50} grown on SPF Chick Embryo Fibroblasts.	SPF ORIGIN
Protect from light	Store at +2°C to +8°C
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd (VentriBiologicals, Vaccine Division) Gat No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: FP11-017	
Expiry date: 07/10/2013	

STERILE DILUENT- 500ml For Fowl Pox Vaccine, Live, I.P.Vet		
Newcastle Disease Vaccine, Live		
Mesogenic (R_2B/Mukteswar) Strain		
Store at room temperature		
CONTENTS:		%w/v
Potassium Dihydrogen Phosphate	IP	0.068
Sodium Hydroxide	IP	0.015
Sodium Chloride	IP	0.65
Phenol Red Indicator	IP	0.0001
Water for Injection	IP	q.s
Dose: 0.5 ml per Chick		

Figure 11: Wing web vaccination on 7th Week

Table 12: Vaccination on 9th Week; Route of Application: Intramuscular

Ranikhet Disease Vaccine, Live, IP Vet.	
(NEWCASTLE Disease Vaccine, Live)	
Mesogenic (R_2B /Mukteswar) Strain	
1000 doses	for poultry use
Mesogenic (R_2B/Mukteswar) Strain EID_{50}/Dose$\geq 10^6$	SPF ORIGIN
Protect from light	Store at +2°C to +8°C
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd (VentriBiologicals, Vaccine Division) Gat No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: RB11-010	
Expiry date: 26/12/2012	

STERILE DILUENT- 500 ml
For Fowl Pox Vaccine, Live, I.P.Vet.
Newcastle Disease Vaccine, Live
Mesogenic (R_2B/Mukteswar) Strain
Store at room temperature
CONTENTS:
Potassium Dihydrogen Phosphate
Sodium Hydroxide
Sodium Chloride
Phenol Red Indicator

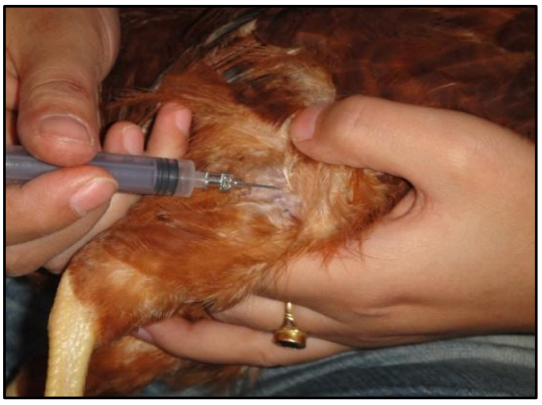

Figure 12: Vaccination through intra muscular route on 9th Week

Table 13: Vaccination on 12th Week Route of application: Drinking water

Avian Infectious Bronchitis Vaccine, Live, IP Vet.	
(Massachusetts Type Strain)	
1000 doses	for poultry use
Massachusetts Type Strain $\geq 10^{3 \cdot 5}$ EID_{50}	SPF Origin
Protect from light	Store at +2°C to +8°C
Manufactured by: Venkateshwara Hatcheries Pvt. Ltd (VentriBiologicals, Vaccine Division) Gat No. 56, 57 & 58 Malkhed, Tai-Haveli, Dist-pune, INDIA.	
Mfg. Lic. No. PD-10	
Batch No: IB11-015	
Mfg. date: 31/05/2011	
Expiry date: 30/11/2012	

STERILE DILUENT- 30ml.		
For RD/(ND)/IB/IBD/AE Vaccine, Live		
Store at room temperature		
CONTENTS:		%w/v
Potassium Dihydrogen Phosphate	IP	0.068
Sodium Hydroxide	IP	0.015
Water for Injection	IP	q.s
Dose: 0.035 ml per Chick		

Chick Malformation:

Malformation observed in chick was recorded and chick malformation rate was calculated using the below mentioned formula:

$$\text{Chick malformation rate (\%)} = \frac{\text{Number of Chick Malformations}}{\text{Total Number of Fertile Eggs}} \text{ X } 100$$

Record of Weight of Liver and Kidney:

Six birds of each group (treated and control) at 25 week for F1 generation and 4 week for F2 generation were euthanized by cervical dislocation. The liver and kidney were removed and the absolute weight and relative weight of the kidney and liver were recorded. The relative weight was calculated according to the formula given below.

$$\text{Relative weight of the kidney} = \frac{\text{Weight of the Kidney}}{\text{Total Body Weight}}$$

$$\text{Relative weight of the liver} = \frac{\text{Weight of the Liver}}{\text{Total Body Weight}}$$

A portion of liver and kidney tissue was homogenized (Tris-HCl: 10 mM, pH 7.4, EDTA: 5 mM, KCL: 10 mM, Sucrose: 250 mM) and was stored in a freezer at −25°C until analysis.

Haematological, Biochemical and Histopathological Assessment:

The experiment was conducted on two generations; F1 generation of 25 week old RIR parent and its F2 generation of 4 week old RIR chicks. Haematological and biochemical analysis were conducted on blood samples collected from F1 generation and F2 generation birds, while histopathological changes were studied by euthanizing the chicks at the end of the experiment and studying the effects on the organs (microscopically).

Haematological Estimations:

Blood samples were drawn from 25 week old parent and 4 week old chick by cardiac puncture using EDTA rinsed 2ml disposable syringes and collected into EDTA coated vials. After collection, the whole blood samples were used immediately or refrigerated (2-4 °C) to be used for hematological estimations.

Hematology analyzer, Mindray (BC-2300) (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) was used to evaluate haematological parameters including total erythrocyte (RBC) count, haemoglobin (Hb) concentration, packed cell volume (PCV), total leukocyte (WBC) count and differential leukocyte counts. Complementary indices of the erythrocytic component including mean corpuscular haemoglobin concentration – MCHC, mean corpuscular haemoglobin – MCH, and mean corpuscular volume – MCV, were also analyzed. Effect of treatment on differential white blood cell counts was enumerated in blood smears prepared and stained panoptically with Microkrom Microscopy Stains (Giemsa Stain) immediately after sampling.

Biochemical Estimations:

Blood samples were drawn from 25 week old parent and 4 week old chick by cardiac puncture using 2ml disposable syringes and collected into vials. Blood was allowed to clot for 30 minutes and blood samples were centrifuged at 2500 rpm. Serum was collected and used immediately for biochemical estimations.

Versatile Bio-Chemistry analyzer, MICRO LAB (RX-50V) (Micro Lab Instruments, India) was used to evaluate biochemical parameters including Serum glucose, Serum albumin, Serum globulin, Serum protein, Urea, Blood Urea Nitrogen (BUN), Creatinine, Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and nucleic acid (DNA, RNA) content.

Aliquot from whole blood collected for hematological estimation was centrifuged at 2500 rpm to collect the plasma. Spectrophotometer, UNICO (Model S-1205) (United Products and & Instruments, Inc., NJ- USA) was used to analyze the Plasma Cholinesterase (ChE) activity.

Histopathological Estimations:

All the chicks from experimental and control groups were terminally euthanized at the end of the treatment period. The chicks were properly weighed and examined carefully for external abnormalities before the necropsy. The thoracic and abdominal cavities were then cut opened and comprehensive investigations of the organs were carried out to detect changes or abnormalities, if any.

Postmortem findings and details were recorded i.e. gross changes in organ size, shape and any visible lesion. Liver and Kidney tissue were collected for histopathological examination. The whole process of histopathology was carried out as per the steps described by (Goyal et al. 2010).The dissected out tissues were cleaned with physiological saline solution (0.89%). The tissues were immediately put in 10% neutral formalin solution for subsequent processing and histopathological studies.

The tissues fixed in formalin were thoroughly washed in running tap water, dehydrated in ascending grades of alcohol and acetone, cleared in benzene, and embedded in paraffin wax at 58°C. Five microns thickness sections from paraffin embedded tissues were stained with haematoxyline and eosine (H&E) stain (John and Bancroft, 1996).These stained slides were examined under Leica DM2500 Microscope and pictures were captured using EC3 Camera (utilizing LEICA LAS EZ (V 1.6.0) software).

Statistical Analysis

Raw data were processed and analyzed to give group means and standard error with significance. All the parameters characterized were subjected to relevant statistical method (*viz*., Bartlett's test, ANOVA, Dunnett's test or Student's t test) using GraphPad Prism version 5, GraphPad Software, San Diego California USA (Motulsky, 1999). A 'p' value less than or equal to 0.05 was considered as statistically significant.