Chapter 2

Materials and methods

2.1. TEST ARTICLE

The test substance used for this study is a commercial combination of chlorpyrifos and cypermethrin. It is manufactured by AIMCO Pesticides Limited, located at Santa Cruz, Mumbai, India. This insecticide is marketed as Anaconda505^{TM}, constituting of chlorpyrifos (50 %) and cypermethrin (5 %). The details of the product are given in the table (Figure 2.1).

Commercial Name	ANACONDA 505^{TM}
Manufactured by	AIMCO Pesticide Pvt. Ltd; Mumbai, India
Chemical name a) Chlorpyrifos b) Cypermethrin	 O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate [(±)-a-Cyano-(3-phenoxyphenyl) methyl (±)cis/trans-3-(2,2dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate]
Registration No.	CIR-26,393/97/Chlorpyrifos + Cypermethrin (EC)-7
Manufacture License No.	0403/0001/M/D/ Date; 10/07/1981
Manufacturing Date	10/04/2014
Expiry Date	09/04/2017
Stability	Stable under normal storage conditions. Unstable at elevated temperatures.
Physical and Chemical Properties	
Appearance	Pale yellow, viscous liquid
Melting Point	< 5°C
Specific Gravity	(Liq.) 1.398 at 43.5 °C
Density	1.1 g/ml at 20 °C
Odour	Xylene like

Figure 2.1: Commercially available Anaconda505^{Tm}

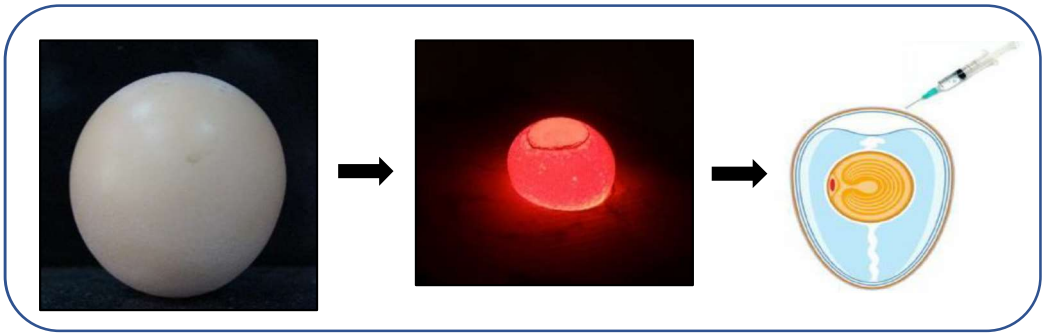
2.2. TEST SYSTEM

The study was focussed on understanding the developmental toxicity potential of the combination insecticide (Ci) Anaconda505 on chick embryos. Fertilized eggs of Rhode Island Red breed of domestic chicken were chosen as a model to study the toxic manifestation of Ci during various stages of embryonic development. The breed is predominantly known for its egg laying capacity and easy availability. However, to avoid discrepancy with the developmental stages, only freshly laid fertile eggs were selected and were procured from Intensive Poultry Development Unit, Vadodara. These eggs were then cleaned with cotton swab soaked with 10 % Povidone iodine solution (Betadine solution) to remove any external contamination and were kept at 4 °C until use. The experiments were conducted as per the guidelines of Drugs and Cosmetics rules 1945, Appendix-III animal care standard and were approved by the Institutional Animal Ethics Committee (IAEC endorsement no:84/08/2014-2) in accordance with the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

2.3. INSECTICIDE INJECTIONS

The eggs weighing 55 ± 5 g were chosen for the study and randomly allotted to the control (which received olive oil) and the treatment groups (which was administered with 0.05 µg per egg of Ci in olive oil). Prior to dosing, the air sac in the eggs were marked by a pencil (occurs mostly on the blunt end side) with the help of traditional candling method. The concentration of sub-lethal dose was decided based on the previous work done in the lab (Uggini et al., 2012). The eggs were dosed in a sterile environment in a laminar air hood and using BD 1 ml insulin syringe 50 µl of Ci (equivalent to 0.05 µg) was injected into the air sac on day "0" of incubation (Figure 2.2). After dosing, the point of injection on the eggs were sealed by molten paraffin wax.

Figure 2.2: Diagrammatic representation of candling and injection of test article into the air cell of the egg.



2.4. INCUBATION

Automated incubator (Scientific equipment works, New Delhi) was set at a temperature of 37.5 °C ± 0.5 and relative humidity of 70–75 % for incubation. The eggs were kept with their broad ends facing upwards and were turned automatically every 1 hour. The eggs were candled once in two days and the dead or unfertilized ones were removed.

Figure 2.3: Eggs kept in Environmental Chamber

2.5. SAMPLE COLLECTION

The Hamburger–Hamilton stages (HH) are a universally accepted series of 46 sequential developmental stages of chick embryo which starts from the laying of the egg up to the chick hatchlings (Hamburger and Hamilton, 1951). This system provides an advantage over other staging series like Carnegie system, as it allows accurate characterisation of the structural changes during the embryonic stages. In this study, the embryos which after incubation had reached Hamilton-Hamburger stages 13 (Day 2), 24 (Day 4), and 36 (Day 10) were collected and processed for further analysis.

2.6. RATE OF MORTALITY

The eggs were regularly candled every 2 days till the 10^{th} day of incubation to cull out the infertile or dead embryos. The rate of mortality was calculated by the formula given below:

$$\text{Embryo mortality rate (\%)} = \frac{\text{Number of dead embryos}}{\text{Total number of fertile eggs}} \times 100$$

2.7. RATE OF MALFORMATION

The chick embryos were visually examined for occurrences of any malformations after opening the eggs. Malformations observed were photo documented. The rate of malformation was calculated by the formula given below:

$$\text{Embryo malformation rate (\%)} = \frac{\text{Number of malformed embryo}}{\text{Total number of live fertile eggs}} \times 100$$

2.8. ROUTINE REAGENTS AND BUFFERS

All the chemicals used were of analytical grade and purchased from manufacturers of lab chemicals *viz.*SD Fine-Chem Ltd. (Mumbai), Sisco Research Laboratories Pvt. Ltd. Mumbai), Qualigens, HiMedia (Mumbai) and Sigma-Aldrich (USA). The following is the details of the reagents and buffers prepared and used throughout the study.

2.8.1. PHOSPHATE BUFFERED SALINE (PBS)

Application: PBS is a buffer frequently used in biological applications, such as washing cells, transportation of tissues, and dilutions. PBS closely mimics the pH, osmolarity, and ion concentrations of the body fluid.

Component	Mass (g)	Molarity (M)
NaCl	8	0.137
KCl	0.2	0.0027
Na_2HPO_4	1.44	0.01
KH_2PO_4	0.24	0.0018

Procedure:

1. Prepared 800 ml of buffer solution by adding all the constituents in the prescribed amounts in a suitable container.
2. Adjusted solution to desired pH (typically pH ~ 7.4).
3. Made up the volume to 1 L with sterile distilled water.

2.8.2. TRIS BUFFERED SALINE (TBS)

Application: TBS is an isotonic and non-toxic buffer used in various biochemical and molecular biology experiments especially solutions containing nucleic acids. It can also be used for

increasing permeability of the cell membranes in animal tissues. Majorly TBS is used in immuno-blotting techniques for both membranes washing and antibody dilution.

Component	Mass (g)	Molarity (M)
NaCl	8	0.137
KCl	0.2	0.0027
Tris base	3	0.0248

Procedure:

1. Added 8 g NaCl to 800 ml of distilled water in a suitable container.
2. The added 200 mg KCl and 3 g of Tris base to this solution.
3. Adjusted the solution to pH ~7.4 with HCl and made up the volume up to 1 L with sterile distilled water.

2.8.3. HANK'S BUFFERED SALT SOLUTION

Application: Maintains physiological pH and osmotic balance for cells of animal origin.

Component	Mass (g)	Molarity (M)
NaCl	8	0.14
KCl	0.4	0.005
$CaCl_2$	0.14	0.001
$MgSO_4$-$7H_2O$	0.1	0.0004
$MgCl_2$-$6H_2O$	0.1	0.0005
Na_2HPO_4-$2H_2O$	0.06	0.0003
KH_2PO_4	0.06	0.0004
Glucose	1	0.006
$NaHCO_3$	0.35	0.004

Procedure:

1. Prepared 800 ml of distilled water with 8 g NaCl, 400 mg KCl, 140 mg $CaCl_2$ in a suitable and cleaned storage container.
2. Added 100 mg $MgSO_4$-$7H_2O$, 100 mg $MgCl_2$-$6H_2O$, 60 mg Na_2HPO_4-$2H_2O$, 60 mg KH_2PO_4 to the solution.
3. Stirred the components vigorously and then added 1 g of glucose and 350 mg $NaHCO_3$ to the solution.
4. Made up the volume by sterile distilled water to 1 L.

2.8.4. HOWARD'S RINGER SOLUTION

Application: Ringer's solution is a solution of several salts dissolved in water for the purpose of creating an isotonic solution relative to the body fluids of an animal.

Components	Mass (g)	Molarity (M)
NaCl	7.2	0.12
$CaCl_2$ (anhydride)	0.17	0.001
KCl	0.37	0.005

Procedure:

1. Poured 800 ml of distilled water in a suitable container.
2. Added 7.2 g NaCl, 0.17 g of $CaCl_2$ and 0.37 g of KCl to the solution.
3. Then slowly added sterile distilled water until volume reached 1 L.

2.8.5. PFA SOLUTION

Application: Formaldehyde (H_2CO) is one of the most conversant and effective fixatives, suitable for nearly all types of tissue. A 4 % formaldehyde solution stabilized with phosphate buffered saline, is commonly used to fix and protect tissue structures of human or animal origin.

Components	Mass (g)	Molarity (M)
Paraformaldehyde	40	1.33

Procedure:

1. Made 800 ml 1X PBS at 60ºC.
2. Added 40 g paraformaldehyde with stirring.
3. The added 5 ml of 1 N NaOH and stirred until solution turned clear.
4. Neutralized with 5 ml of 1 N HCl.
5. Made up the volume to 1 L with 1X sterile PBS.

2.9. RNA EXTRACTION

Principle:

The tissues excised from the embryos were kept in TRIzol reagent containing phenol and chloroform mixture. This typical mixture was then centrifuged to form three distinct layers. According to the polarity of the solution, the aqueous phase is on top because it is less dense than the organic phase formed by phenol:chloroform mixture. The hydrophobic lipids settle in lower organic phase while the proteins and DNA stay at the interphase. As RNA does not

neutralize by acid phenol reaction, it forms hydrogen bond with water, keeping it in the aqueous phase. The upper phase was then carefully pipetted out to extract RNA.

2.9.1. DEPC (DIETHYL PYROCARBONATE) WATER

Components	Concentration
DEPC	0.1 %
Dissolved in 1 L distilled water. The solution was kept overnight on magnetic stirrer and autoclaved at 15 psi for 15 minutes.	

2.9.2. TBE BUFFER (10X)

Components	Concentration
Tris base	10.8 %
Boric acid	5.5 %
0.5 M EDTA	4 %
Volume made up to 1 litre with DEPC water.	

2.9.3. RNA LOADING DYE (5X)

Components	Concentration	Volume (µl)
EDTA	500 mM	8
Formaldehyde	40 %	72
Glycerol	-	200
Formamide	-	30
BPB	2.5 %	30
10X TBE	-	400
Volume made up to 1 ml with DEPC water		

2.9.4. OTHER REAGENTS USED

Components	Concentration
TRIzol reagent (Phenol + GITC)	-
Chloroform	-
2-Propanol	-
Ethanol	75 % (in DEPC water)

Components	Concentration
Agarose	1 % (TBE buffer)
Ethidium bromide	25 mM

All the reagents used in the RNA isolation were strictly prepared in DEPC water and autoclaved at 10 Psi for 20 minutes to protect the RNA from RNase action. All the glassware, plastic wares and other apparatus were autoclaved before use. The bench tops, pipettes and the instruments used were cleaned by RNaseZAP (Sigma-Aldrich, USA) prior to the experiment. The isolation was carried out using a clean pair of gloves.

2.9.5. RNA ISOLATION

Procedure:

1. Tissue was homogenised with TRIzol reagent (1 ml per 100 mg tissue) in sterilized mortar-pestle, followed by centrifugation at 8,000 g for 20 minutes at 4 °C.
2. The supernatant was pipetted out and taken in new Eppendorf tubes. 200 µl of Chloroform was added to it while gently shaking the tubes for at least 15 minutes.
3. The mixture was then centrifuged at 12,000 g for 15 minutes at 4 °C. The organic and aqueous phases were seen separated with a visible white interphase.
4. The upper layer was carefully dispensed into a new microcentrifuge tube (strictly without contamination from the lower layers) containing 500 µl isopropanol.
5. This was gently mixed and kept in -20 °C deep freeze for at least 1 hr to allow precipitation. The tube was then centrifuged at 12,000 g for 15 minutes at 4 °C.
6. The supernatant was discarded and the pellet, which contains RNA, was washed with 200 µl 75 % ethanol and centrifuged again. After a total of two such washes, the tube was opened and left on ice for the pellet to dry.
7. After excess ethanol had dried off, the pellet was dissolved in 30 µl DEPC water.

2.9.6. RNA QUANTIFICATION

Procedure:

RNA was quantified using fluorometric assay.

Qubit assay: RNA solution was diluted 1:40 and assayed on the Qubit 3.0 (Life technologies, USA) using its fluorometric assay kit. The following formula was used to analyse the quantity of RNA.

A ratio of the absorbance at 260/280 was calculated to check for quality of the RNA isolation. A ratio of around 2 indicates good quality of the RNA yield. Further, 3 µl of the RNA solution was electrophoresed on a 1 % agarose gel containing ethidium bromide. Three distinct and sharp bands reflected good integrity of the RNA.

2.10. cDNA SYNTHESIS

Complementary DNA was synthesized from the isolated RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA).

The kit uses random hexamers/primers for reverse transcription of RNA into cDNA by reverse transcription. 2 µg of total RNA was used from each sample for cDNA synthesis.

Components	**Volume (µl)**
10X RT buffer	2.0
10X RT random primers	2.0
25X dNTP mix (100 mM)	0.8
MultiScribe Reverse Transcriptase	1.0
Nuclease-free water	4.2
RNA (diluted by DEPC water)	10

The following program was set on the thermal cycler:

Temperature (°C)	**Time (min)**
5	10
37	120
85	5.0

The cDNA prepared was collected and stored at -20 °C till further use.

2.11. POLYMERASE CHAIN REACTION

Principle:

PCR is based on the ability of DNA polymerase to synthesize complementary strand to the template strand. Small DNA strand of about 18 to 25 nucleotides called as primer is used to add nucleotides on complementary DNA template at 3' end. The primers can bind only when the DNA strands are separated, generally done by heating and called as DNA denaturation step. The primers anneal to the single-stranded DNA template at specific temperature (depending on primer sequence) and then DNA-Polymerase binds to this double stranded DNA produced at

around 72 °C (extension). This starts synthesis of the new DNA strand. After multiple cycles of the PCR reaction, the specific sequence gets accumulated in billions of copies.

2.11.1. TBE BUFFER (10X)

Components	Concentration
Tris base	10.8 %
Boric acid	5.5 %
0.5M EDTA	4 %
Volume made to 1 litre with DI water	

2.11.2. DNA LOADING DYE (6X)

Components	Concentration
Glycerol	30 %
Bromophenol Blue	0.25 %

2.11.3. OTHER REAGENTS USED

Components	Concentration
2X PCR master-mix (Sigma, USA)	-
Primer pairs	-
Nuclease-free water	-
Agarose	2 % in TBE buffer
Ethidium bromide	25 mM

2.11.4. PRIMER DESIGNING FOR PCR

Primers for the current study were designed using the online Primer-BLAST and BLAST tools provided by National Centre for Biotechnology Information. Sequences of the target mRNA with regions of maximum sequence homology were identified in Chick (*Gallus domesticus*) using the BLAST tool and these regions of the mRNA were specified in the Primer-BLAST tool for designing of primers. All primers were designed with GC content close to 50 %, melting temperature between 58-62 °C and minimum internal complementary hair pin loops to produce an amplicon size below 200 base pairs for gene expression studies by Real-Time PCR. A list of the primer sequences used in this study are mentioned in the last section of this chapter.

PCR Procedure

1. For each reaction, a reaction mix was prepared as given below.

Components	**Volume (µl)**
2X Master-mix	5
Forward primer (5 µM)	1
Reverse primer (5 µM)	1
cDNA template	1
Nuclease- free water	2

2. The reaction tubes were run on a Bio-Rad T100 thermal cycler after mixing the reagents thoroughly. The following program was used.

Steps	**Temperature** (^{o}C)	**Time (Sec)**	
Initial denaturation	95	180	
Denaturation	95	10	35 Cycles
Annealing	58-62	30	
Extension	72	30	
Final extension	72	180	

2.12. REAL-TIME POLYMERASE CHAIN REACTION

Principle:

Real-Time PCR (RT-PCR) also known as quantitative PCR or qPCR, allows amplification of DNA in real time using a fluorescent reporter molecule. A generic sequence non-specific double-stranded DNA-binding SYBR green was used in the study. The principle is that SYBR dyes bind to double-stranded DNA and as the amount of double-stranded DNA increases, the fluorescent signal increases correspondingly. RT-PCR can be divided into four stages: linear ground phase (fluorescent signal not detected), early exponential phase (fluorescent signal rises significantly), linear exponential phase (log phase) and plateau phase (fluorescent signal will no longer increased).

For the current study, all quantitative gene expression analyses were carried out by real time PCR using SYBR Green based master-mix (Takara Bio, Japan) on a LightCycler 96 machine (Roche Diagnostics, Switzerland).

Real-time PCR protocol

1. For each reaction mix, following composition was prepared:

Components	**Volume (µl)**
2X SYBR Green master-mix	5.0
Forward primer (0.5 µM)	0.5
Reverse primer (0.5 µM)	0.5
cDNA template	1.0
Nuclease-free water	4.0

2. The samples were taken in triplicates and the reactions were run in 96-well plates (Genaxy, USA) sealed with clear sealing films. The following program was used for amplification:

Steps	**Temperature** (ºC)	**Time (Sec)**	
Initial denaturation	95	100	
Denaturation	95	10	45 Cycles
Annealing (acquisition)	58-62	30	
Extension	72	30	
Final extension	72	180	

3. This was followed by a melt-curve analysis with the following program:

Temperature (°C)	**Time (Sec)**
95	10
65	60
97	1

4. Data analysis: Data was analysed using the LightCycler 96 software version 1.1. Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (18S rRNA) from those of the target gene. Mean of these normalized Cq values were plotted.

$$\Delta\mathrm{Cq} = \mathrm{Cq\ of\ target\ gene} - \mathrm{Cq\ of\ reference\ gene}$$

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001). For fold change of gene expression in sample 2 as compared to that in sample 1: ΔΔCq values were plotted on a graph with vertical axis following a logarithmic scale.

2.13. PROTEIN ISOLATION AND QUANTIFICATION

Principle:

Proteins were extracted from the tissues excised from the embryos and were later used for many biochemical and analytical techniques (PAGE, Western blotting). Efficient disruption and homogenization of animal tissues in the below mentioned buffers ensured a high yield of proteins.

2.13.1. TRIS-TRITON LYSIS BUFFER

Components	Mass (g)	Molarity (mM)	Storage
Tris base	6.1	50	Stored in cold conditions (2-4°C)
NaCl	11.7	200	
$CaCl_2$	1.1	10	
Triton X-100	10	15	
Protease inhibitor (Sigma, USA) was added freshly before use as per manufacturer's instructions.			

2.13.2. RIPA BUFFER

Components	Mass (g)	Molarity (mM)	Storage
Tris base	6.1	50	pH was to 8. Store in cold conditions (2-4 °C)
NaCl	11.7	150	
SDS	1.0	3	
Triton X-100	10	15	
Sodium deoxycholate	5.0	12	
Protease inhibitor (Sigma, USA) was added freshly before use as per manufacturer's instructions.			

2.13.3. BRADFORD REAGENT (Bradford, 1976)

Application: The Bradford assay is the most preferred and reliable colorimetric assay for estimation of proteins. It consists of the dye Coomassie Brilliant Blue G-250 dissolved in phosphoric acid and methanol or ethanol which forms a complex with aromatic and basic content of proteins in solution resulting in a shift in the absorption maximum of the dye from 465 to 595 nm at acidic pH. Moreover, it is compatible with many reducing agents such as dithiothreitol at a very low concentration. The protein concentration of an unknown sample can be determined

with the help of a calibration curve. Bovine serum albumin was used as a standard to make the calibration curve.

Components	Mass or Volume
Coomassie Brilliant Blue G-250	100 mg
Methanol/Ethanol	50 ml
85 % ortho-phosphoric acid	100 ml

Procedure:

1. Mixed 50 mg of Coomassie Brilliant Blue G-250 in 50 ml of methanol/ethanol and allowed to dissolve for two hours.
2. Added 100 ml of 85 % H_3PO_4 to the solution.
3. Poured the solution from step 2 into 500 ml of H_2O and mixed
4. Filtered using Whatman filter paper no. 1 to remove the precipitates
5. Added an additional 750 ml of H_2O and Stored at 4 ^oC in amber coloured bottle.

2.14. SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Principle:

Proteins are mixtures of hydrophobic and hydrophilic amino acids that are assembled in a sequential manner to attain a complex folded form. These folded structures are dominated by hydrophobic residues interiorly and polar residues present outside. The presence of charged moieties and their geometry in the protein structure are known to affect their migration rate under the influence of electric field. Therefore, the proteins are boiled with anionic detergents like sodium dodecyl sulphate (SDS) and 2-mercaptoethanol, imparting a uniform shape and charge-mass ratio. When proteins are separated by electrophoresis through a gel matrix, they get separated solely on its primary amino-acid chain length.

2.14.1. SAMPLE BUFFER/LOADING BUFFER (5X)

Components	Concentration	
Tris base	250 mM	pH set to 6.5
SDS	10 %	
Glycerol	50 %	

Components	Concentration	
Bromophenol blue	0.1 %	
β-Mercaptoethanol (Added freshly)	100 mM	

2.14.2. TANK BUFFER

Components	Concentration
Tris base	25 mM
Glycine	250 mM
SDS	0.2 %

2.14.3. GEL STOCK (30 %)

Components	Concentration
Acrylamide	29 %
Bis-acrylamide	1 %
The solution was filtered through Whatman filter paper no. 1 and was stored for a night in amber coloured bottle at room temperature.	

2.14.4. STACKING GEL COMPOSITION (4 %; 3 ml)

Components	Volume (ml)
30 % Gel stock	0.5
De-ionised Water	2.1
1 M Tris Cl (pH 6.8)	0.360
10 % SDS	0.02
10 % APS (freshly prepared)	0.02
TEMED (added only before pouring the gel)	0.002

2.14.5. RESOLVING GEL BUFFER (12 %; 5 ml)

Components	Volume (ml)
30 % Gel stock	2
De-ionised Water	1.6
1.5 M Tris Cl (pH 8.8)	1.3
10 % SDS	0.05
10 % APS (freshly prepared)	0.05

Components	Volume (ml)
TEMED (added only before pouring the gel)	0.003

2.14.6. FIXATIVE FOR GELS

Components	Concentration (%)
Methanol	50
Acetic acid	10

2.14.7. COOMASSIE STAIN

Components	Concentration (%)
Coomassie Brilliant Blue R250	0.01
Methanol	40
Acetic acid	10

2.14.8. DESTAINING SOLUTION

Components	Concentration (%)
Methanol	30
Acetic acid	5

Procedure:

1. Preparation of Polyacrylamide gel: Vertical gel casting unit was used for moulding of acrylamide gels. First, resolving gel buffer was poured between PAGE plates in the casting assembly and water was overlaid to prevent air which inhibits polymerization. The set up was left undisturbed for 20-30 minutes to fully polymerize the gel. The excess water was pipetted out carefully. The stacking gel acrylamide solution was poured in the casting unit and a comb of appropriate size was inserted immediately to form wells for loading the samples.
2. Sample preparation: The protein samples were then heated with the loading buffer for 20 minutes and then were placed on ice till the samples were loaded onto the gel along with the molecular weight markers. The gel was run at 100 volts and stopped once the sample buffer containing bromophenol blue dye reaches the bottom of the gel.
3. CBB staining: Gel was fixed in fixative for at least 20 minutes and then stained with CBB staining solution.

2.15. WESTERN BLOT

Principle:

This technique is used for the detection of specific proteins in a mixture. Once the proteins are electrophoresed based on their molecular weight by SDS-PAGE, they are transferred on to a hydrophobic membrane, the process known as blotting. The gel is kept next to the nitrocellulose or PVDF membrane and electric field is applied to facilitate the migration of proteins from the gel to the membrane. The protein of interest is first probed on the membrane via primary antibody and the unreacted antibody is washed away using TBS-T buffer. Subsequently, a secondary antibody labelled with an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase is used to detect the target protein and visualized chromogenically.

2.15.1. BJERRUM-SCHAFER-NIELSEN BUFFER FOR SEMI-DRY TRANSFER

Components	Concentration	pH ~9.2; Freshly prepared buffer was kept in cold conditions
Tris base	48 mM	
Glycine	39 mM	

2.15.2. WASHING BUFFER (TBS-T)

Components	Concentration
Triton X-100 (to be made in TBS)	0.1 %

2.15.3. BLOCKING BUFFER (TBS-MT)

Components	Concentration
Skimmed milk powder (dissolved in TBS-T)	5 %

2.15.4. ANTIBODY DILUTION BUFFER

Components		Concentration (%)
Bovine Serum Albumin	Both added in TBS-T solution	2
Sodium azide		0.02

2.15.5. BCIP-NBT STOCK SOLUTION (50X)

Components		Concentration (mg/ml)
BCIP		9.4

NBT	Both the components mixed together in 67 % DMSO Solution	18.75

2.15.6. ALP SUBSTRATE BUFFER

Components	**Molarity**	
Tris base	0.1 M	pH set to 9.5
NaCl	0.1 M	
$MgCl_2$	0.05 M	

Procedure:

1. 6-10 sheets of filter paper and PVDF membrane same size as the gel were cut.
2. PVDF membrane was soaked for 15 seconds in methanol followed by distilled water for 5 minutes and then in Bjerrum-Schafer-Nielsen Buffer for 10 minutes while preparing transfer sandwich.
3. The filter papers were also kept in transfer buffer for 10 minutes to avoid trapping of air bubble.
4. The arrangement was made in the following order: 3-5 sheets filter paper - Gel - Membrane - 3-5 sheets filter paper. Precautions were taken to remove the air bubbles trapped within the sandwich.
5. Transfer was carried out at 100 mA as per the following conditions:

Protein size range	**Transfer time (min)**
Below 40 kDa.	10
40 to 70 kDa	20
Above 70 kDa	25

4. Ponceau stain was used for rapid reversible detection of protein bands on the membranes.
5. The membrane was then processed for antibody probing by placing it in the blocking buffer.
6. The membrane was incubated overnight at 4 °C. On the next day, the membrane was washed three times consecutively with ten minutes intervals.
8. The membrane was then exposed to secondary antibody for more than 30 minutes followed by washing with the buffer three times.
9. Later, it was incubated with streptavidin-ALP conjugate for 15 minutes and followed by three washes. This was succeeded by colour development in ALP substrate.

10. After the development of the western blot, densitometric analysis was done by measuring the band intensity using Doc-ItLS software (GeNei Imaging systems, India). The densitometric values were used to compare the bands of interest and the results were expressed in arbitrary units. The values obtained were normalized with the band intensity of β-actin.

2.16. ALCIAN BLUE AND ALIZARIN RED STAINING

Principle:

For the analysis of structural deformities, the first step is the investigation of skeletal architecture. The whole-mount staining allows assessment of the structural elements in their appropriate locations for detecting changes in skeletal patterning. Differential staining involves usage of alcian blue and alizarin red which stains cartilage and bone respectively. Alcian blue being a cationic dye, binds strongly to sulphated glycosaminoglycans and glycoproteins occurring mostly in the cartilage, while alizarin red, an anionic dye, binds to cationic metals such as calcium and localizes in bone.

The reagents used in the procedure are as follows:

Components	**Concentration (%)**
Paraformaldehyde (PFA)	4
PBS	-
Acetone	-
Ethanol	-
Alcian blue 8GX (A5268, Sigma)	0.1
Alizarin red S (A5533, Sigma)	0.1
Potassium hydroxide (KOH)	1
Sodium borate	2
Glycerol	-

Procedure:

1. The control and treated embryos were collected on day 10 for studying the skeletal deformities if any.
2. For this the samples were first isolated and then washed in chilled PBS solution to remove the debris.

3. After cleansing, the samples were immediately stored in ethanol for 48 hours at 4°C followed by acetone for 2 hours.
4. These samples were further processed in freshly prepared alcian blue and alizarin red stain, for differential staining for 4 hours at room temperature in dark conditions.
5. The stained specimens were rinsed in ethanol for 1hr, washed under running tap water and then cleared in 1 % KOH solution until the skeletons were visible.
6. The destaining procedure was done in a graded sequence of glycerol and 1% KOH and finally stored in 100% glycerol (Mcleod, 1980).

2.17. BIOCHEMICAL ESTIMATION OF HYDROXYPROLINE

Principle:

Collagen is the most abundant protein found mainly in the bone, tendon and skin. Out of 20 essential amino acids, proline is found to be incorporated into collagen along with its hydroxylated form (4-hydroxyproline) in large amounts for the synthesis of the polypeptide chain. Therefore, the chemical methods employed for the detection of collagen are suitably based on the determination of protein-bound hydroxyproline in the tissues. The free imino acid released after the oxidation of hydroxyproline to pyrole due to acid hydrolysis. These acidic moieties were then reacted with p-dimethyl-amino-benzaldehyde to impart a reddish-brown intense colouration to the solution and was immediately read at 560nm.The hydroxyproline assay was performed as described by Edwards and O'Brien (1980).

2.17.1. CHLORAMINE-T SOLUTION

Reagents	**Concentration**	
Chloramine-T reagent (Sigma)	1.41 %	
Aldehyde-perchloric acid reagent		
p-dimethyl-amino-benzaldehyde (Sigma)	15 %	Made up the volume to 100 ml with distilled water
n-propanol	60 %	
perchloric acid (70%, reagent grade)	26 %	

2.17.2. BUFFER SOLUTION

Buffer solution	**Concentration**
Citric acid monohydrate	4.435 %
Glacial acetic acid	1.06 %

Sodium acetate trihydrate	10.66 %
Sodium hydroxide	3.03 %
n-propanol	26.67 %
Made up the volume to 3L with distilled water and adjusted the pH to 6.5 with NaOH solution.	

2.17.3. HYDROXYPROLINE STANDARD PREPARATION

Concentrations of hydroxyproline standards were prepared as follows:

1. Primary standard: 0.5 g stock hydroxyproline (Calbiochem, USA) was dissolved in 1000 ml of the buffer (500 µg hydroxyproline per ml buffer).
2. Secondary standard: 20 ml primary standard brought to a final volume of 100 ml with the buffer (100 µg/ml)

   4 µg/ml: 4 ml of secondary to a final volume of 100 ml

   2 µg/ml: 2 ml of secondary to a final volume of 100 ml

   0 µg/ml: Pure buffer solution

Procedure:

1. The tissue samples of known weights were excised, and acid hydrolysed at 20 psi for 3 hours with 6 M HCl.
2. Upon completion of hydrolysis, hydrolysates and standards were kept in oven (60 °C) for 8hrs for complete evaporation of water.
3. Later, buffer solution (94 µl) and Chloramine-T solution (6 µl) were added in all the tubes, followed by incubation for 20 minutes at room temperature.
4. Subsequently, the tubes were incubated at 60 °C in water bath for 15 minutes. The absorbance of the reaction product was read at 560 nm. Sample concentrations were determined from the standard curve.

2.18 HAEMATOXYLIN AND EOSIN STAINING

Principle:

Haematoxylin is a dark bluish or violet stain carrying a positive charge that binds to negatively charged components in the cell such as DNA and RNA, whereas eosin is negatively charged and reddish-pink stain binding with positively charged moieties within the cell. When the specimen is processed with the stains together, a complex called as hemalum is formed as a product of aluminium ions and oxidised haematoxylin. This dye and metal complex bind to the DNA

residing within the nuclei, imparting blue colour to it. As a counter stain to the nuclei staining, the aqueous solution eosin Y colours eosinophilic structures either in red, pink or orange.

Reagents	**Concentration**
Neutral buffered formalin (fixative)	10 %
Xylene	-
Different grades of alcohol	70 %, 80 %, 95 % and 100 %
Harris's Haematoxylin and eosin	-
DPX (mounting medium)	-

Procedure:

1. The desired tissue was first excised from the embryos, rinsed in PBS and was fixed in 10 % NBF solution.
2. The fixed tissue samples were later dehydrated by escalating the alcohol gradations (50, 70, 90 and 100 %) at room temperature.
3. The tissue samples were kept in xylene for 30 minutes and then infiltrated for an hour in hot molten paraffin wax.
4. These tissues were separated by metal blocks to get solidified and then were sectioned to 5 microns thickness using a rotary microtome (Leica RM 2155).
5. The sections were taken on glass slides previously coated with egg albumin.
6. The sections were rehydrated and stained with Harris's Haematoxylin and eosin for 10 minutes and washed under tap water to remove excess stain.
7. The slides were air dried and dehydrated in series of alcohol concentrations from 30% to 90 % for a few seconds.
8. After dehydration, xylene was used as a clearing agent to remove any extra wax for 10 minutes. These cleared tissue sections were permanently mounted with DPX.
9. The tissue architecture was then visualized using Leica DM2500 microscope and microphotographs were captured using EC3 Camera (utilizing LAS EZ software).

2.19. STATISTICAL ANALYSIS

The data were processed, and the values are expressed either as Mean ± SEM or Mode with range in parenthesis, and the differences between the control and treated groups were considered significant when the 'p' value was less than or equal to 0.05. All the parameters in the given

study were subjected to relevant statistical tests such as Student's t test and Mann-Whitney U test using GraphPad Prism version 6.1, GraphPad Software, San Diego California USA.

2.20. SEQUENCES OF PRIMERS USED IN THE STUDY

Gene	Forward Primer	Reverse Primer
SHH	TGCTAGGGATCGGTGGATAG	ACAAGTCAGCCCAGAGGAGA
PITX2	GCTACCCCGACATGTCCAC	TTCTTGAACCAAACCCGAACC
GLI3	TCTCGTAGCAGTTCGTCAGC	TCAGAGCAGGGCTTATTGCG
GLI2	GGGGATGGCTTTACGGAGAC	CAATGGAGGAGGCCCGTG
SOX9	GAACAAACCCCACGTGAAGC	TCATTCAGCAGCCTCCACAG
RUNX2	CTGGTGCCTTTTGGGTTGTG	TCGACACGTCTTGCTTAGCG
DLX5	GGAATGCGGATGGGGGATTT	CCACAGCTGAGCCGAAAAAC
COL10A1	CTTCACGGTTTAGCTTCACAAG	TTGTGGTCCTGCAACAGAGT
L1CAM	TTCCCCCGGAGTATGGTGC	CTGGGGAAGACCACGAGTTG
HOX10A	GGAGCCCGTAGGCAATTCAAA	ACGCTCACGAGTCAGGTACA
HOX11A	CAGCTCCAGTGGACAACGG	CAGCTCCAGTGGACAACGG
PAX6	AGCAAGGATACAGGTGTGGT	TGTGGGATCGGCTGGTAAAC
CDH1	GAAGACAGCCAAGGGCCTG	TCTGGTACCCCTACCCTCTTG
BMP2	ATGTTGGACCTCTATCGCCTG	CCAAAACTTCTTCGTGGTGG
BMP4	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC
BMP7	ATCTGCCTACAAAATTGGTTCTC	TACTCACAGCGCATTCTCACTT
CL CASPASE3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT
VIMENTIN	GACCAGCTGACCAACGACAA	GAGGCATTGTCAACATCCTGTC
FGF2	ATCCGGGAGAAAAACGACCC	TTGGTCGTCTCGCTCCAAAC
FGF8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
CDH2	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC
BMP1	CCAGCAAAGTGTGTGTGTGG	GAGGCGCTTTTGATGTCGTC
PCNA	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC
WNT11	GACCTGGGTATCGATGGGGA	GGCTTTCAAGACCTGTCTCC
WNT1	AAGTCGGGAAGGAGAGGTGA	GAGCCATCTGAAACTGCCCT
WNT5A	GACACTTGGCAGCACAATGG	CCCTAGAGACCACCAAGAGC
WNT7A	TATCGTCATCGGGGAAGGGT	GCTGCTTCTCTGCTACCCAC
WNT6	TTGGTCATGGACCCCAACAG	CCTCGCTGACGATTTCTGGT
PXN	TCTGACTTTAAGTTCATGGCACAG	TCGCTACCCCCAGTTTGTTC
MYOD1	CGGAATCACCAAATGACCCAA	ATCTGGGCTCCACTGTCACT

Gene	Forward Primer	Reverse Primer
AHR	ACCTGTGCAGAAAATAGTAAAGCC	GCTGAGCCTAAGCACAGACA
CYP1A1	ACCACGACGAGAAGATCTGG	AGATCAGCACCTTGTCAGCC
CYP3A4	AGTGCAATGGGACTCCTTCC	GGCCATATCCCATAGAGCACC
CYP3A5	TGGGTATGAGCCCACCAGTA	CATACGTGAGCGGAGCCTTA
18S RRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGAAC