CHAPTER – II
MATERIAL AND METHODS

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II.1. Experimental animal :

The chicken (*Gallus gallus* or *G. gallus domesticus*) is a domesticated fowl found in the wild Indian and Southeast Asian Red Jungle Fowl. *Gallus gallus* (murghi) is subspecies derived from the Red Jungle Fowl of India. *Gallus gallus* has high immune power and is sustainable to the village environment along with its high quality flesh and egg laying capacity. Hence it is selected for the present investigation.

Classification :

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Aves
Order	-	Galliformers
Family	-	Phasianidae
Genus	-	Gallus
Species	-	gallus

Experimental animal organ extract :

A. pulmonata is widely spread stylommatophoran molluscs in and around Satara. It is easily available in rainy season and contains large amount of mucins. Mucins are having antiangiogenesis properties.

Classification :

Kingdom - Animalia

Sub-kingdom - Non-chordata

Phylum - Mullusca

Class - Gastropoda

Order - Stylommatophora

Genus - Ariophanta

Species - pulmonata

Salivary gland and digestive glands are rich in mucins hence *A. pulomnata* was selected as ideal material to studies effects of salivary gland and digestive gland extracts on angiogenesis.

II.1.1 Selected developmental stages :

Development stages selected for the study of angiogenesis were 48, 55, 66, 72, 88 and 96 hrs. considering the development of CAM and vitelline veins. The doses were initiated at above selected developmental stages and development was continued upto 144 hrs.

II.1.2 Chemical Used :

1.2.A. Hanks Balanced Salt Solution (HBSS) :

HBSS purchased from Himedia laboratories Pvt. Ltd.

23 Vadhani Ind. Est. LBS Marg, Mumbai 400086, India.

II.1.2.B Acetone :

At designed hrs of incubation, Acetone extract of salivary and digestive gland of *A. pulmonata* doses 0.7 mg/ml, 0.9 mg/ml, 1

mg/ml, 1.2 mg/ml, 1.5 mg/ml were administered by window method in aseptic condition for the mortality study. The dose of 1 mg/ml showed 40% mortality which was further used to study effect of salivary and digestive gland extracts of *A. pulmonata.*

II.1.2.C : Alcohol :

Alcohol extract of salivary and digestive gland of *A. pulmonata* doses, 0.7 mg/ml, 0.9 mg/ml, 1 mg/ml, 1.2 mg/ml, 1.5 mg/ml, were administered at designed hrs of incubation by window method in aseptic condition for mortality study. The dose of 1 mg/ml showed 50% mortality which was further used to study effect of salivary and digestive gland extract of *A. pulmonata*.

II.1.3 Experimental protocol :

Step – I : Egg Incubation :

The fertilized eggs were cleaned and disinfected with 70% alcohol and divided in six groups. 48 hrs, 55 hrs, 72 hrs, 88 hrs and 96 hrs. The eggs were incubated in an aseptic incubator in vertical position such as blunt end of egg faced upward and was maintained at 38^{0}C temperature and humidity at 70%.

Step – II : Dose administrations :

Extract preparation :

For the dose administration Hanks Balanced Salt Solution (HBBS) is used as saline. Salivary gland extract was prepared in acetone and alcohol. 20 mg of salivary gland of *A. pulmonata* was homogenated in 1 ml of distilled water. Then 5 ml cold acetone is

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added in the homogenate and kept it at 10^0C for 5 to 7 hrs., centrifuged and evaporated the supernatant to remove acetone. Dissolved the residue as per the dose in HBSS.

(Same procedure used for preparation of digestive gland for acetone and alcohol extract).

Dose administration :

The incubated eggs (embryos) obtained at different hours of incubations were used for administration of dose. At different developmental stages (48 hrs, 55 hrs, 66 hrs, 72 hrs, 88 hrs, 96 hrs) dose was initiated and development was continued upto 144 hrs. Embryo of each group were administered with 1 mg/ml dose of salivary and digestive gland of *Ariophanta pulmonata,* and they were numbered as (I, II, III, IV, V, VI). Normal and control groups were maintained independently with each of experimental group studied.

Method for dose administration : (Window method)

After designed period of incubation the eggs were cleaned with 70% alcohol. A small window was made at blunt end of each of the egg, under aseptic conditions dose 1 mg/ml of HBSS of salivary and digestive gland extract of *A. pulmonata* were injected. Here precaution was taken that the 1 ml dose was spread on embryonic plate uniformly at different stages of development as mentioned in group I – VI. All treatments were given in final volume of HBSS. The windows were sealed with surgical adhesive tape. All

embryo exposures were conducted in proper sterilized conditions prescribed by window method (Kean & Cramer, 2007).

Treatment of salivary and digestive gland extracts was initiated as a single dose at different hrs of development (groups I to VI) and embryos were observed.

Eggs were opened in plate having 0.9% buffered NaCl saline, to view the chorioallantoic membrane for study.

Table II.1 : Exposure schedule of salivary and digestive gland extracts to different developmental stages of chick embryo in different hrs.

Groups according to developmental stages	Groups according to time of exposure to the treatment						Final development in hrs.
	A	B	C	D	E	F	
	48	55	66	72	88	96	
I							144 hrs
II	✓	✓	✓	✓	✓	✓	
III	✓	✓	✓	✓	✓	✓	
IV	✓	✓	✓	✓	✓	✓	
V	✓	✓	✓	✓	✓	✓	
VI	✓	✓	✓	✓	✓	✓	

Eggs incubated at 37^{0}C for 48 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 48 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 48 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 48 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 48 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 48 hrs of incubation and continued upto 144 hrs.

Eggs incubated at 37^0C for 55 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 55 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 55 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 55 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 55 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 55 hrs of incubation and continued upto 144 hrs.

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Eggs incubated at 37^{0}C for 66 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 66 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 66 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 66 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 66 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 66 hrs of incubation and continued upto 144 hrs.

Eggs incubated at 37^{0}C for 72 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 72 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 72 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 72 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 72 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 72 hrs of incubation and continued upto 144 hrs.

Eggs incubated at 37^{0}C for 88 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 88 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 88 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 88 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 88 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 88 hrs of incubation and continued upto 144 hrs.

Eggs incubated at 37^0C for 96 hrs were administered with following doses and the incubation was carried further for 144 hrs.

Group – I : Normal Eggs were incubated upto 144 hrs without any treatment.

Group – II : HBSS control at 96 hrs of incubation embryos were administered with 1 ml HBSS and development was continued upto 144 hrs.

Group – III : 1 mg/ml Salivary gland acetone extract :

Treatment of 1mg/ml salivary gland acetone extract was initiated at 96 hrs of incubation and incubation was continued upto 144 hrs.

Group – IV :1 mg/ml salivary gland alcohol extract.

Treatment of 1 mg/ml salivary gland at alcohol extract was initiated at 96 hrs of incubation and continued upto 144 hrs.

Group – V : 1 mg/ml Digestive gland acetone extract :

Treatment of 1 mg/ml digestive gland acetone extract was initiated at 96 hrs of incubation and continued upto 144 hrs.

Group – VI : 1 mg/ml Digestive gland alcohol extract :

Treatment of 1 mg/ml digestive gland alcohol extract was initiated at 96 hrs of incubation and continued upto 144 hrs.

Study of Angiogenesis :

Angiogenesis of the CAM was studied. The embryos were first opened and floated in the PBS. The embryos with intact CAM were transferred to Petri dish. With the help of a brush folds if any on CAM were removed in PBS and thus CAM was fully spread for observation. Diameter of CAM was measured in 5 different planes using divider and mean value was noted (From diameter area was deduced mathematically).

From the measurement of diameter the area of CAM was calculated mathematically, Calliper plus software was used to count primary, secondary and tertiary vitelline veins. For measurement of area covered by different veins. Bifureation points were used as initiation and termination markers.

II.1.5 Statistical analysis :

The results were the mean values of 5 embryos in each experimental conditions. The statistical calculations were carried out with the help of XL STAT 7.5 computer programme.

Significance :

The probability *P* obtained 't' and 'Z' values at least as calculated for a given member of degree of freedom is given in Fisher's table (Fisher, 1938). The *P* values are signified according to following conventions.

$P > 0.05$ the difference is said to insignificant.

$P < 0.05$ the difference is said to be almost significant.

$P < 0.02$ the difference is said to be significant.

$P < 0.001$ the difference is said to be highly significant.