CHAPTER - TWO

MATERIAL AND METHODS

CHAPTER TWO

2. MATERIAL AND METHODS

2.0	Introduction	12
2.1	MATERIAL	12
2.1.1	Selection of animals	12
2.1.2	Maintenance of rat colony	12
2.1.3	Plant material	13
2.1.4	Preparation and administration of dose	13
2.2	METHODS	13
2.2.1	Body weight	13
2.2.2	Organ weight	14
2.2.3	Fertility study	14
2.2.4	Histology	14
2.2.5	Tubular diameter	15
2.2.6	Enzymes	15
2.2.6.a.	Acid phosphatase	15
2.2.6.b.	Alkaline phosphatase	17

2.0 Introduction

The material and different methods used in the present dissertation are described briefly in this chapter.

2.1 MATERIAL

2.1.1 Selection of animals

To study the effects of Plumbagin, the wistar strain male albino rats (Haffkine Biopharmaceuticals Ltd. Bombay) were selected as experimental animals. The selected rats were 100 days old weighing about 120 to 140 g each.

2.1.2 Maintenance of rat colony

The white rat colony was maintained in the Zoology Department of Willingdon College,Sangli. The room in which the experimental animals were kept was maintained at about $24^{o}C$. An exhaust fan was attached to room to decrease odours and moisture generated by rats. The room was protected against the entrance of wild rats, cats, cockroaches which may carry infections.

The rats were maintained in the rat cages. Before the start of the experiment all the rats were allowed 15 days quarantine period. The rats were equally divided into two groups, control-group A and experimental-group B. Each group was of 12 rats.

All the cages were thoroughly cleaned every alternate day with disinfectants, air dried and then rats were transferred to them. Each rat was kept in a separate cage. The cages were arranged on clean racks which were cleaned every day. Rats were weighed at regular intervals of 12 days. For observations hereafter described, rats of a particular weight were taken. The rats were supplied with water ad libitum. Dry food pellets were used as food for rats.

2.1.3 Plant material

Plumbagin was purchased from Bio-organics, Madras. Puriety of Plumbagin sample was checked from its M.P., TLC and infra-red spectrum.

2.1.4 Preparation and administration of dose

50 mg Plumbagin was dissolved in 5 ml Tween 80 and 45 ml saline. Thus 1 ml of saline contained 1 mg of Plumbagin. It was always stored at 4^{0}C temperature in a freeze. After every 12 days the Plumbagin extact was prepared freshly and stored at low temperature in order to maintain the potency and to avoid the contamination. The experimental rats (Group B) were injected (ip) with Plumbagin, at a dose of 5 mg/kg body wt. on alternate days for a period of 72 days. Control rats (Group A) received the equal quantity of Tween 80 + saline.

For each parameter to be studied different sets of experiment were planned. Each time after every 12 days of interval two animals from each group were sacrificed by cervical dislocation, 24 hours after the last dose of the treatment. Testes, caput epididymides, cauda epididymides, vas deferenses seminal vesicles, prostate glands and Cowper's glands were dissected out, cleared off fat and connective tissue, blotted and weighed accurately on torsion balance to the nearest 0.5 mg. The tissues were fixed in Bouin's fluid for histological examination. Similarly in the second set of the experiment, two animals from each group were sacrificed. Half of the reproductive organs were processed for histology and the remaining were processed for biochemical estimations.

2.2 METHODS

2.2.1 Body weight

The rats of both the groups were weighed at regular intervals of 12 days with the help of one pan balance.These body weights were recorded.

2.2.2 Organ weight

Similarly a separate record of each organ weight was maintained.

2.2.3 Fertility study

With the view to find out infertility induced in the male albino rats by the administration of Plumbagin, fertility test was carried out at all dose intervals. Two male rats of proven fertility were administered with the selected dose of Plumbagin and were employed for fertility tests. Fertility of the control and the treated animals was tested at day first of the experiment and then at an interval of 12 days till the termination of Plumbagin treatment. One normally cycling and previously proven fertile female on the day of vaginal pro-estrous was paired with one experimental male and left for 96 hours. After 96 hours of co-habitation the male and female were separated. After 21 days of gestation, the females were permitted to litter at term. The average number of litters sired by each female was then calculated (WHO,1983).

2.2.4 Histology

Small pieces of tissues were fixed in Bouin's fluid for 24 hours, tissues were well washed in running water, dehydrated through ethanol grades, cleared in xylene and ceder wood oil and embeded in paraffin wax. The sections were cut at 7 µm. These paraffin sections were stained with routine Haematoxylene-Eosine (H-E) technique for histological examinations.

The spermatogenesis follows a seminiferous epithelial wave (Perey et al.,1961) which constitutes a series of cell associations. Two main classifications have been established for the spermatogenesis in rat for these cell associations, which are known as LC method (Leblond and Clermount,1952a ; 1952b) and RG method (Roosen-Runge and Giesel,1950). The LC method

subdivided the process of spermatogenesis into 14 different stages, while RG method subdivided it into 8 stages. A very high resolution optical microscopy is essential to observe clearcut differences in these stages. So instead of the 14 or 8 stages used in LC and RG method, it appeared more practical to describe spermatogenesis with reference to tunica propria, basement membrane, spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid, sperms and sperms in the lumen.

2.2.5 Tubular diameter

Morphological study of seminiferous tubules and epididymal tubules was carried out with the help of occulometer (monocular microscope). Diameter of the tubule of seminiferous and epididymides was calculated. All the measurements were recorded in milimeter and finally they were converted in μm. Tubular diameter of a 100 tubules was recorded and the mean value was calculated.

2.2.6 Enzymes

Biochemical studies on two enzymes viz. acid phosphatase and alkaline phosphatase were crried out by employing following methods.

2.2.6.a Acid phosphatase

(Orthophosphoric-monoester-phosphohydrolase,E.C.3.1.3.2)

I) Biochemical assay

The activity of the enzyme was determined according to the method described by Linnhardt and Walter (1965).

a) Preparation of sample - The homogenation of the tissues was caried out in glass mortar and pestle which were previously well washed, rinsed in distilled water, dried and kept in the ice box of freeze nearly for 5 hours.

Such a cold homogenation has two advantages. (i) During homogenation no loss of enzyme activity occurs due to low temperature and the temperature increases due to friction of the mortar and pestle does not exceed beyond 12°C at the end of the homogenation and (ii) during homogenation when the tissue is first crushed at the bottom of the mortar, it instantaneously freezes and then gradually thaws whcich helps in breaking the lysosomes. When the tissue was thawing, 2.0 ml of chilled acetate buffer (0.05 M, pH 4.8) was added and homogenation was carried out completely. When a perfectly uniform suspension was formed the homogenate was transferred to a calibrated flask and further dilution was done by adding the necessary quantity of the sodium citrate buffer. Care was taken to see that the final concentration was about 1 % (W/V). Throughout the work the concentration of the homogenate of the tissue was kept practically constant.

b) Purification of the Sample - The partial purifiction of the enzyme was done by the method of Varute and More (1971).

c) Reagents -

1) 0.05 m p-nitrophenol phosphate in sodium citrate buffer (Sigma 0.1 M, pH 4.8) was prepared and stored at 4°C.

2) 0.1 N NaOH

d) Enzyme reaction - To each test tube were added 1 ml substrate buffer solution and 0.2 ml aliquot of well suspended tissue homogenate. The mixture was allowed to equilibrate for 5-10 minutes. Control test tube contained 1.0 ml substrate buffer solution. The tubes were shaken gently, stoppered and incubated in the incubator previously adjusted for 37.5^{o}C for 30 minutes. The incubation interval was kept constant throughout the work.

e) Colour production and spectrophotometric measuremens - At the end of the incubation, the reactions were stopped by adding 4 ml of 0.1N NaOH and the tubes were centrifuged for 5 minutes at 2000 g. The optical density was read at 400 m μ on Specol using control as a reference.

f) Calculations - The optical density was converted to micromoles of p-nitrophenol from the formula suggested by Linnhardt and Walter (1965) for tissue sample.

$$\frac{\text{O.D.} \times 2.76 \times \text{dilution}}{0.2 \times \text{Wt.of tissue sample in g.}} = \text{Acid phosphatase activity in p-nitrophenol } \mu \text{ mol/g.}$$

2.2.6.b Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase,E.C. 3.1.3.1)

I) Biochemical assey

The activity of the enzyme was determined according to the method described by Linnhardt and Walter (1965).

a) Preparation of sample - The homogenation of the tissue was carried out in the same manner as described for acid phosphatase except in the place of sodium citrate buffer, glycine buffer (0.05 M,pH 10.5) was used.

b) Reagents -

1) 0.05 M p-nitrophenol phosphate (Sigma) in glycine buffer (5.5×10^{-3}M, pH 10.5) 375 mg glycine, 10 mg $MgCl_2.6H_2O$ and 160 mg p-nitrophenol posphate were dissolved in 42 ml of 0.1 N NaOH and diluted to 100 ml with distilled water stored in a freez.

2) 0.02 N NaOH.

c) Enzyme reaction - To each test tube 1.0 ml of substrate buffer was added and kept for equilibration at 37.5^{o}C for 10 minutes. Then 0.1 ml

of sample was added and the tubes were shaken and kept for incubation for 30 minutes at $37.5^{o}C$. After incubation the reaction was terminated by adding 10 ml 0.02 N NaOH. A control tube contained 1.0 ml substrate. After the addition of the NaOH to the control tube, 0.1 ml sample was added. All the tubes were centrifuged and the reading was taken against control at 400 $m\mu$.

c) Calculations - Alkaline phosphatase activity was directly measured by using the formula suggested by Linnhardt and Walter (1965) for tissue samples.

$$\frac{\text{O.D. x 11.82 x dilution}}{\text{0.1 x Wt. of tissue sample in g.}} = \text{Alkaline phosphatase activity in p-nitrophenol } \mu\text{mol/g.}$$