2. Materials and Methods

2.1 MATERIALS :

2.1.1 Selection of Animals.

To study the effect of water soluble part of ethanolic extract of Piper betle wistar strain male albino rats.(Haffkine Biopharmaceuticals Ltd.,Bombay) were selected as experimental animals. The selected rats were 3-4 month's old weighing 180 to 200 gm.

2.1.2 Maintainance of Rat Colony.

The white rat colony was maintained in animal house of the Department. Separate rooms were reserved for record and surgery, cage cleaning and storage of food. The room in which the experimental animals were kept; were maintained at about 24°C. Exaust fans were attached to the rooms to decrease odours and moisture gene rated by rats. All rooms are protected against the entrance of wild rats,cockroaches and any other animals which may carry infection.

The rats were maintained in the animal cages. All the cages were thoroughly cleaned every alternate day with disinfectants, air dried and then rats were transfered to them. The cages were arranged on racks which were also cleaned every alternate day. An accurate weighing balance having capacity to weigh 1 to 500 gm was maintained and rats were weighed at regular intervals. For experimental obeservations hereafter described, rats of a perticular weight were taken. The rats were supplied with water 'ad libitum.' Dry food pellets supplied by Hindustan Lever Co.Ltd.,Bombay were used as food for rats.

2.1.3 Plant Material.

Petiols of Piper betle were collected from pan-shops and pan vendors, cleaned, washed with water,shed dried and then used for the extraction procedure.

2.2 METHODS:

2.2.1 Extraction Procedure.

The shed dried, powdered plant material was mixed with sufficient quantity of Absolute alcohol for 10 to 12 hours. Then the mixture was stirred well in a stirrer. The supernant is collected and the procedure is repeated for three to four times. The pieces and particles remained were filled in Soxlet apparatus for extraction. Thus the active principlesdissolved in alcohol are obtained,this dilute mixture is subjected to vacuum distillation. The alcohol is collected in another flask and the semisolid remnant is then kept for evaporation at room temperature in close trough. The residue is then dissolved in distilled water. The resin is seperated by filtration and the clear solution of extract in water is obtained which is then kept at 40° C. temperature for further evaporation and then the powdered extract is obtained which is then dissolved in physiological saline solution.

2.2.2 Preparation of Extract.

One gram of extract was macerated firstly with 1 ml of saline and then diluted to 8 ml of mammalian saline(0.9%). 0.5 ml of this etract of Piper betle henceforth reffered as extract contains about 60 mg of extract. After every 15 days the extract was prepared freshly and always stored at low temperature(0° C. to 1°

C) in order to maintain the potency of the extract and to avoid the bacterial contamination.

2.2.3 Dose and Duration Effect.

Male albino rats were grouped in to two groups -Control and Experimental. Each group consisted of 30 rats. Each animal from experimental group daily received plant extract intraperitoneally. Each rat was injected with 0.5ml of the extract containing 60 mg of extract. (about 350 mg/Kg/Day) while those of control group received equal quantity of the vehical (0.7 % saline) by the same route. The experiment was conducted for sixty days of duration.

For each parameter to be studied different sets of experiment were planned. Each time after every 10 days of interval five animals from each group were sacrificed by cervical dislocation. All the reproductive organs were dissected out and blotted dry of blood and weighed. Tissues were processed as per requirements of different parameters. Small pieces of tissues were fixed in Bouin's fluid for histology.

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 embeded in paraffin wax. The sections were cut at 5-6 u . These sections were stained with routine Haematoxylene-Eosin (H-E) technique for the study of histological alterations in the testis and other organs of reproduction caused due to induced aspermatogenesis.

The spermatogenesis follows a seminiferous epithelial

wave (Parey et al.,1961), which constitutes a series of cell association. Two main classifications have been established for the spermatogenesis in rat for these cell associations, which are known as LC method (Leblond & Clermount,1952) and RG method (Roosen-Runge and Giesel , 1950), The LC method subdivided the process of spermatogenesis in to fourteen different stages, while RG method subdivided it into eight 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 method and RG method, it appeared more practical to observe the alterations in different types of spermatogenic cells, Sertoli cells and in Leydig cells seperately, instead of observing the changes in seminiferous tubules as such. The detailed discription of alterations in each cell type is given in Chapter 3 which deals with histological alterations in testis.

2.2.5 Bicassay of Enzymes.

Biochemical studies on two enzymes viz. acid phosphatase and alkaline phosphatase were carried out in reproductive tract by employing following methods.

A. Acid Phosphatase : (Orthophosphoric-monoester-phosphohydrolase)(E.C.3.1.3.2)

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

I. Preparation of sample-

The homogenation of the tissues was carried out in glass

mortar and pestle which were previously well washed and rinsed with distilled water, dried and kept in the ice box of a refrigerator for nearly five hours. Such a homogenation has two advantages ,

i. During hompgenation no loss of enzyme activity occurs due to low temperature. The temperature increased due to the friction of the mortar and pestle does not exceed beyond 12°C at the end of homogenation

ii. During homogenation when the tissue is first crushed at the bottom of the mortar, it instantaneously freezes and then gradually thaws which helps in breaking the lysosomes. When the tissue was thawing, 2.00 ml of chilled Sodium citrate buffer (0.05 M, pH 4.8) was added and homogenation was carried out to completion. When a perfectly uniform suspension was formed the homogenate was transferred to a calibrated flask and further dilution was done by adding the necessory quantity of Sodium citrate buffer. Care was taken to see that the final concentration was about 1% (W/V) or even less. Throughout the work concentration of the homogenate of the tissue was kept practically constant.

II. Purification of the Sample.

The partial purification of the enzyme was done by the method of Varute and More (1971) which was a modification of the early steps of purification of acid phosphatase from rat liver, as described by Igarashi and Hollander (1968). The partially purified extract contained about 90% of the total activity of acid phophatase of crude homogenate.

III. Preparation of Reagents.

i. 0.05 M p-nitrophenyl phosphate in Sodium citrate (Sigma 0.1 M, pH 4.8) was prepared and stored at 4 C.

ii. 0.1 N NaOH

IV. 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 equillibrate for 5 - 10 minutes. Control test tube contained 1.0 ml substrate buffer solution. The tubes were shaken gently, stoppered and incubated in water bath, previously adjusted to 37.5° C, for 30 minutes. The incubation interval was kept constant throughout the work.

V. Colour production and spectrophotometric measurement.

At the end of the incubation, the reactions were stopped by adding 4.0 ml of 0.1 N NaOH and tubes were centrifuged for 5 minutes at 2000 g. The optical density was read at 400 nm on Spectronic20 using control as a reference.

VI. Calculations -

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

$$\frac{O.D \times 2.76 \times \text{dilution}}{0.2 \times \text{Wt. of sample (gm)}} = \text{Acid phosphatase activity in p-nitrophenol micromol/gm}$$

B. Alkaline Phosphatase : (Orthophosphoric-monoester - phosphohydrolase) (E.C.3.1.3.1)

The activity of the enzume was determined according to

the method described by Linnhardt and Walter (1965).

I. Preparation of sample -

Homogenation of tissue was carried out in the same manner as described for acid phosphatase except in place of Sodium citrate buffer, Glycine buffer (0.05 M, pH 10.5) was used.

II. Preperation of reagents -

i. 0.05 M p-nitrophenol phosphate (Sigma) in Glycine buffer (5.5 x 10^-3,pH 10.5) : 375 mg Glycine, 10 mg MgCl2 and 16.0mg p-nitrophenol phosphate were dissolved in 42 ml of 0.1 N NaOH and diluted to 100 ml with distilled water and stored in freezer.

ii. 0.02 N NaOH

III. Enzyme reaction -

To each test tube, 1.0 ml of substrate buffer was added and kept for equilibration at 37° C for 10 minutes. Then 0.1 ml sample was added and the tubes were shaken and kept for incubation for 30 minutes at 37° C. After incubation the reaction was terminated by adding 10 ml of 0.02 N NaOH. A control tube contain 1.0 ml substrate. After the addition of NaOH to the control tube, 0.1 ml sample was added. All the tubes were centrifuged and the reading was taken against the control at 400 nm .

IV. Calculations -

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

$$\frac{\text{O.D.} \times 11.82 \times \text{dilution}}{0.1 \times \text{Wt. of tissue (gm)}} = \text{Alkaline phosphatase activity in p-nitrophenol micromol/gm}$$