CHAPTER TWO

MATERIAL AND METHODS

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2.1 MATERIAL

2.1.1 Selection of Animals

Male albino rats (Wistar Strain, procured from Hindustan Antibiotics, Pune) were selected as experimental animals to study the effects of *Syzygium cumini*. The selected rats were 3-4 months old weighing 160-180 grams each.

2.1.2 Maintenance of rat colony

The white rat colony was maintained in the animal house of the department. For record and surgery, cage cleaning and storage of food, separate rooms were reserved. The room in which experimental animals were kept, was maintained at about 24°C. Exhaust fans were attached to the room to decrease odours and moisture generated by rats. The room was well protected against the entrance of wild rats, cockraches and any other animal which may carry infections.

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 transferred to them. The cages were arranged on clean racks which were also cleaned every alternate day. Each rat was kept in separate cage. Rats were weighed at regular intervals of 10 days. Rats of a particular weight were taken for experimental observations hereafter described.

The rats were supplied with water ad libitum. Dry food pellets, supplied by Lipton India Ltd., Bangalore, were used as food for rats.

The rats were divided into two groups -control and experimental. All the rats were allowed for 15 days quarantine period, before the start of the experiment.

2.1.3 Plant material

Shed dried powder of Syzygium cumini seeds was procured from Sheth Sakharam Nemchand Ayurved Rasashala, Solapur, Maharashtra, India.

2.2 METHODS

2.2.1 Extraction method :

The shed dried powder of Syzygium cumini was subjected to soxlation and extracted with adequate amount of petroleum ether at 40°-$60^{o}C$ for 8-10 hours. The substance thus obtained was concentrated by vacuum distillation under reduced pressure and controlled temperature of 55°- $58^{o}C$. This concentrate was dried in a porceline dish in a vacuum desiccator.

2.2.2 Preparation and administration of dose :

10 gm. of petroleum ether extract was mecerated with 20 ml. of Tween-80; into which added 80 ml. of mammalian saline. Thus a 10% solution of extract was prepared. This was kept in freeze when not in use. At the time of administration, it was allowed to come to room temperature. After every fifteen days the extract was prepared freshly in order to maintain the potency of the extract and to avoid contamination.

2.2.3 DOSE AND DURATION :

Experimental male albino rats of proven fertility were injected with 0.5 ml of the prepared extracted intra-peritoneally (about 200-250 mg/kg body weight/day) while control rats were injected with equal quantity of Tween-80 and saline. The experiment was conducted for 60 days.

Different sets of experiments were planned for each parameter to be studied. Each time after every 10 days of interval 5 animals from each group were sacrificed by cervical dislocation; 24 hours after the last dose of the treatment. All the reproductive organs were dissected out, blotted dry of blood, cleared off fat and weighed. Tissues were processed as per the requirement of the paramenters.

2.2.4 WEIGHT :

The rats of both the groups were weighed at regular intervals of 10 days; these body weights were recorded. Similarly weights of various reproductive organs, from each animal, after dissection were recorded.

2.2.5 HISTOLOGY :

Small pieces of tissues were fixed in Bouin's fluid dor 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 6-7 μm. These paraffin sections were stained

with Haematoxylene - Eosin (HE) technique for histological examinations in testes and accessory reproductive organs.

The spermatogenesis follows a seminferous 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 RG method (Roosen-Runge and Giesel, 1950) and LC method (Leblond and Clermont, 1952 a; 1952 b). The RG method subdivided the process of spermatogensis into eight stages while LC method subdivided into fourteen stages. A very high resolution optical microscopy is essential to observe clearcut differences in these stages, so instead of 8 or 14 stages used in RG method and LC method, it appeared more practical to study spermatogensis and plant extract induced alteration with reference to different types of spermatogenic cells, sertoli cells as well as in Leydig cells.

2.2.6 Tubular diameter :

Morphological study of seminiferous tubules was carried out with the help of oculometer (monocular microscope) Diameters of these tubules were calculated. The measurements were recorded in mm and finally they were converted into μm-Tubular diameter of 100 tubules was recorded and the mean value was calculated.

2.2.7 ENZYMES (BIOASSAY) :

Biochemical studies of two enzymes viz. acid phosphatase and alkaline phosphatase were carried out 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 Linnhard and Walter (1965).

I) Preparation of sample :

The homogenete 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 5 hours. Such homogenization has two advantages -

1) During homogenization no loss of enzyme activity occurs due to low temperature. The temperature increase due to friction of the morter and pastle does not exceed beyond 12°C at the end of homogenization.

2) During homogenization when the tissue is first crushed at the bottom of the mortar, it instantaneously freezes and then gradually thaw, which helps in breaking the lysosmes. When the tissue was thawing, 2.0 ml of chilled sodium citrate buffer (0.05 M, pH 4.8) was added and homogenization 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 necessary quantity of sodium citrate buffer. Care was taken to see that the final concentration of the homogenate of the tissue was about 1% (w/v) or even less. Through out the work concentration of the homogenate of the tissue was kept practically constant.

II) PURIFICATION OF SAMPLE :

The partial purification of the enzyme was done by the method of Vartue and More (1971) which was a modification of the early steps of purification, described by Igarashi and Hollander (1968).

III) REAGENTS :

(i) 0.05 M P-nitrophenol phosphate in sodium citrate buffer (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 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°C for 30 minutes. The incubation interval was kept constant throughout the work.

At the end of the incubation, the reactions were stopped by adding 4 ml of 0.1 N NaOH and the tubes were centrifuged for 5 minutes at 2000 g. The optical density was read at 400 mμ on specol using control as a reference.

V) CALCULATIONS :

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

b) ALKALINE PHOSPHATASE :

(Orthophosphoric monoester phosphohydrolase E.C.3.1.3.1)

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

I) PREPARATION OF SAMPLE :

The homogenate 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.

II) REAGENTS :

i) 0.05 M p-nitrophenol phosphatase (Sigma) in glycine buffer (5.5×10^{-3} M, pH 10.5) : 375 mg glycine 10 mg $MgCl_2 \cdot 6 H_2O$ and 165 mg P-nitrophenol phosphate were dissolved in 42 ml of 0.1 N NaOH and diluted to 100 ml with distilled water and stored in a freeze.

ii) 0.02 N NaOH.

III) ENZYME REACTION :

To each test tube 1.0 ml of substrate buffer was added and kept for equilibrium at 37.5°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°C. After incubation the reactions were stopped by adding 10.0 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 control at 400 mμ.

IV) CALCULATIONS :

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

$$\frac{\text{O.D.} \times 11.82 \times \text{dilution}}{0.1 \times \text{wt. of tissue (g)}} = \text{Alkaline phosphatase activity in p-nitrophenol } \mu \text{ mole/g.}$$