2. 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 Picrorhiza kurroa. The selected rats were 3-4 months old, weighing 160-180 gm. each.

2.1.2 MAINTENANCE OF RAT COLONY :

The white rat colony was maintained in 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, cockroaches and any other animal which may carry infections. The rats were maintained in the animal cages. All the cages were throughly 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 8 days. Rats of a particular weight were taken for experimental observations hereafter described. The rats were supplied

with water ad-libitum. Dry food pallets, supplied by Lipton India Ltd. (A.F.S. Division) Banglore, were used as food for rats.

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

2.1.3 PLANT MATERIAL :

Shade-dried powder of Picrorhiza kurroa was procured from Seth Sakharam Nemchand Ayurvedic Rasa-Shala, Solapur, Maharashtra, India.

2.2 METHODS

2.2.1 METHODS OF EXTRACTION :

The Shade-dried powder of Picrorhiza kurroa was subjected to Soxlation and extracted with adequate amount of petroleum ether at 40° - 60°C for 8-10 hours. The substance thus obtained was concentrated by vacuum distillation under reduced pressure and controlled temperature of 55° - 58° C. This concentrate was dried in a porcelin dish in a vacuum desicator.

2.2.2 PREPARATION AND ADMINISTRATION OF DOSE :

10 gm. of petroleum ether extract was macerated with 20 ml of Tween 80, into which was 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 the

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 extract intraperitoneally (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 48 days.

Different sets of experiments were planned for each parameter to be studied. Each time, after every 8 days of interval 3 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 parameters.

2.2.4 WEIGHT :

The rats of both the groups were weighed at regular intervals of 8 days, their 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 for 24 hours. Tissus were well washed in running water, dehydrated through ethanol grades, cleared in xylene and embeded in paraffin wax. The sections were cut 6-7 μ. These paraffin sections were stained with Haematoxylene-Eosin (HE) technique for histological examinations of testes and accessory reproductive organs.

Spermatogenesis follows a seminiferous epithelial wave (Perey et al; 1961) which constitutes a series of cell associations. Two main classificatons have been established for the spermatogenesis in rat for these cell associations, which are known as RG method (Roosen-Runge and Giesel, 1950) and L-C method (Leblond and Clermont, 1952a, 1952b). The RG method subdivided the process of spermatogenesis into eight stages while LC method subdivided into fourteen stages. A very high resolution optical microscopy is essential to observe clearcut difference in these stages, so instead of 8 or 14 stages used in RG and LC method, it appeared more practical to study spermatogenesis and plant extract induced alteration with reference to different types of spermatogenic cells, Sertoli cells as well in Leydig cells.

TUBULAR DIAMETER :

Morphological study of seminiferous tubules was carried out with the help of oculometer (monocular microscope). Diameter 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.

2.2.6 BIOASSAY OF ENZYMES :

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 Linnhardt and Walter (1965).

1. 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 5 hours. Such homogenation has two advantages :

i) During homogenation no loss of enzyme activity occurs due to low temperature. The temperature increase due to friction of the mortar and pastel 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, 2.0 ml of chilled sodium citrate buffer (0.05M, 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 flack 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. Throughout the work concentration of the homogenate of the tissue was kept practicallly constant.

2. Purification of Sample :

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

3. 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^{o} C.

ii) 0.1 N NaOH.

4. 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~~ was 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 ~~S~~pecol (what?) using control as a reference. ?

5. Calcualtions :

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

b) Alkaline phosphatase : (orthosphosphoric 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).

1. 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.

2. Reagents :

i) 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 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.

3. Enzyme reaction :

To each test tube 1.0 ml of substrate buffer was added and kept for equilibration 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 reaction was stopped by adding 10.0 ml of 0.02 N NaOH. A control tube contained 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.

4. Calculations :

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

$$\frac{\text{O.D.} \times 11.82 \times \text{dilution}}{0.1 \times \text{Wt. of tissue in g}} = \text{Alkaline phosphatase acitvity in p-nitrophenol } \mu\text{ moles/g}$$