MATERIALS AND METHOD

The present project involves preparation of benzene fraction of aqueous extract of fruits of *Terminalia bellirica* and testing of this fraction on male albino rats for antifertility activity.

**Plant Material:** For the present project *Terminalia bellirica* (Linn) plant was selected. It belongs to family Combretaceae and is commonly known as ‘Baheda’ or ‘Bhaira’ in Hindi and as ‘Belliric myrobalan’ in English. It is a moderate to large sized tree growing up to 30 meters in height. It is found throughout India growing wild in deciduous forests.

During this project, for the purpose of proposed study, fresh samples of ripe fruits of *T. bellirica* were collected in season and, in around Meerut (U.P). The plant was authenticated by the Botany Department of Meerut College, Meerut.

**Extraction:** The ripe fruits of *T. bellirica* were dried in shade and powdered. Aqueous extract of the dried fruit powder was prepared with the help of soxhlet apparatus with thermostatically controlled heating mantle. For the aqueous extract the solvent used was double distilled water. Then benzene fraction was prepared. The aqueous extract was subjected to silica gel column chromatography and eluted with benzene. The benzene fraction of aqueous extract was concentrated under reduced pressure and the fraction was used in the investigation (Manivannan et.al. 2009). Benzene fraction was stored in refrigerator in separate air tight container with a crystal of thymol to prevent fungal growth.
**Experimental Animals:**- Male albino rats were selected as experimental animals for the present project. The male albino rats (*Rattus rattus albino*, (Wistar strain)) of known breed were procured from the animal division of Dabur institute, Ghaziabad and Hamdard University, New Delhi. Male animals usually weighing between 150-200 gm. were selected for the experiments. Animals were acclimatized for laboratory conditions for 15 days before starting the experiments and were kept under observation. Balanced diet containing carbohydrates (69%), protein (16%), fat (7%), fibers (6%), mineral and salt mixture (2%), enriched with vitamins was provided. (Cuthbertson, 1957, Joubert, 1967).

During the experiment, all possible precautions were taken to keep the animals in a uniform environment. In summers animals were kept in animal house with water coolers and in winters animal house was heated with the room heaters. All the necessary precautions were taken to keep the animals free from any type of infection. The study was approved by the Institutional Animals Ethics Committee (IAEC) and all animals were cared for according to the guidelines of CPCSEA.

**Experimental Design:**- The experimental animals were randomly divided into two groups. Group 1 was control group, The animals of group 2 were given benzene fraction of T. bellirica at dose 1mg/100 gm and of group 2 were given a dose of 2mg/100 gm body weight dose of the fraction.

Each group was further subdivided into six subgroups The subgroup served as control group for 15 days, subgroup (2) received fraction for 15 days, subgroup (3) served as control group of 30 days, subgroup (4) received fraction for 30 days, subgroup (5) served as control
Set I

Group I
Benzene fraction 1mg/100 g dose

15 days duration
Control Treated

30 days duration
Control Treated

30 days+ 30days reversibility duration
Control Treated

Group 2
Benzene fraction 2mg/100 g dose

15 days duration
Control Treated

30 days duration
Control Treated

30 days+ 30days reversibility duration
Control Treated
group for 60 days and subgroup (6) was reversibility group in which animals were kept for 30 days on normal diet after receiving fraction feeding for 30 days, thus making the total duration of the experiment of 60 days. Animals of group 2 were also further subdivided in the similar manner.

In the first phase of experiments, four animals were taken in each subgroup. After completion of phase 1 experiments, during phase 2, two animals were taken in each subgroup, thus making a total of six animals for each subgroup.

After completion of the experiments, the animals were weighed and dissected and the half portion of testes and epididymis were fixed in Bouin’s Hollande fluid for histopathological studies. The remaining half portion was fixed for electron microscopy, in Glutaraldehyde fixative.

**Histopathological Studies:** For the histopathological studies, the animals were anaesthetized by ether and sacrificed and testes, epididymis, were removed and then weighed. One testis was fixed in Glutaraldehyde fixative for electron microscopy. Whereas the other testis was put in physiological saline for washing blood, then cut into small pieces. Fifty % of the pieces of testes and epididymis were immediately transferred to freshly prepared Bouin’s Hollande solution (Humason 1979), in which they were left for 24 hours for the proper fixation.

After the fixing of tissues, for 24 hours they were thoroughly washed in running water to remove even the last traces of picric acid. These tissues were then gradually dehydrated in alcoholic grades and cleared in sulphar free xylene, then tissues were transferred to already melted mixture of paraffin wax with cerasin of 56 to 58°C melting point.
in winter and 60 to 62°C in summer. After giving necessary changes in paraffin wax, tissue blocks were prepared. The required blocks of the tissues were sectioned at 6µ thickness on a Waswax rotatory microtome.

The sections of testes, epididymis, were stained in heamatoxylene and eosin and then examined under binocular microscope (Carl Zeyiss).

**Ultrastructure Studies:** For ultrastructural studies, tissue, which was cut in to small pieces (1 mm) was fixed immediately in 2.5% glutaraldehyde for 6 hours, washed thrice in phosphate buffer (0.2 mol L-1., pH 7.4), post-fixed in 1% OsO4 for 4 hours, washed in phosphate buffer again, dehydrated in acetone, infiltrated and embedded in low-viscosity spur media and polymerized at 60°C for 48 hours Semithin sections (1µm) were stained with toluidine blue, and ultra thin sections were double stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope.

**Sperm Function tests:** For evaluating the sperm function, the washed spermatozoa were used for physical characteristics like Hypo Osmotic Swelling, Acrosome Intactness and Nuclear chromatin Decondensation. Scores below 50% in the acrosome intactness and mitochondrial activity index tests and below 60% in the hypo-osmotic swelling test were considered sub fertile or infertile (Lohiya, et. al, 2002.).

**Sperm Morphology:** Sperm morphology was studied by preparing the smear of semen from epididymis homogenate. The sperms were examined under compound microscope, in low and high powers.

For ultrastructure study, sperms were examined by scanning electron microscope (SEM) as well as transmission electron microscope.
For the purpose of study, spermatozoa were washed with phosphate buffer (0.01 mol/L, pH 7.2) and pelleted by centrifugation. The sperm pellets were fixed in 2.5% glutaraldehyde for 30 minutes and washed thrice in phosphate buffer followed by distilled water. A thin film of spermatozoa was smeared on a clean glass slide, air dried and mounted on SEM stub with silver paint, sputter coated with gold and observed under SEM.

**Sperm Count:-** For the sperm count 100 mg of epididymis was taken and crushed with 1 ml of physiological saline and then homogenate was kept in incubator for 1 hour at 32-35°C temperature. After that, the specimen was sucked up to 0.5 mark of a white cell diluting pipette (haemocytometer) and then diluted up to 1.1 mark. One drop of evenly mixed sample was applied to Neubauer’s counting chamber under a cover slip. Now the complete spermatozoa i.e with heads and tails had been counted present in the four corner squares covering 4 sq.mm under the low power objective (the microscopic field covers 1sq m m).

Sperm count per ml = $\sqrt{\frac{\text{Sperms counted} \times 20 \times 100}{2 \times 0.}}$

$=\text{sperms counted} \times 100000$

**Hormone Assay:-** Blood samples were also collected for the estimation of gonadotropins i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH) and testosterone by radioimmunoassay (RIA). The concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured in duplicate by double-
antibody RIA method. Serum testosterone was assayed in duplicate using RIA method (WHO 1987).

**Statistical Analysis:** An attempt has been made for a comprehensive analysis of the data collected for this research project. To access the significance of the results obtained, Student’s “t” test has been used. The “t” test is the statistical test of choice for testing the significance of differences in this kind of studies.

The SD has been used to determine the variations in the sample observation and have been calculated with the help of the following formula

The formula for SD is as follows:

\[
SD = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n-1}}
\]

- \( n = \) no. of samples
- \( n-1 = \) degree of freedom
- \( X_i = \) \( i^{th} \) reading on \( X_i \) variable
- \( \bar{X} = \) mean of the observations

The Student’s ‘t’ test was calculated with the help of following formula and the values arrived at were compared with the table values given by Fisher and Yates (1976) to find out the level of significance.

\[
t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{\sigma_{X_1}^2}{n_1(n_1-1)} + \frac{\sigma_{X_2}^2}{n_2(n_2-1)}}} \sqrt{n_1n_2} \\
\sqrt{n_1+n_2 - 2}
\]
Where

\[ x_1^\ast - x_2^\ast = \text{SD of the means} \]

\[ n_1 = \text{number of samples for } x_1 \]

\[ n_2 = \text{number of samples for } x_2 \]

\[ \sigma_{x_1}^2 \sigma_{x_2}^2 = \text{SD of the means under the low power objective (the microscopic field covers } 1\text{sq m m).} \]