SUMMARY

For investigating the effect of benzene fraction of aqueous extract of fruits of *Terminalia bellirica* plant, the present project, “Histological and Electron Microscopic Studies of Testes and Spermatozoa of male albino rats after Treatment with Benzene Fraction of *Terminalia bellirica,*” was undertaken. *Terminalia bellirica* is commonly known as “Baheda” and it’s ripe and dried fruits are ingredients of commonly used Trifala powder. Very little scientific research work is available on this plant, so it was thought worthwhile to conduct a well planned research work on its antifertility potential.

Different doses and durations of treatment were used. With every dose and duration, a control group was also maintained throughout the study. During the present project, it was also tried to study the reversibility of the effects of fraction. At the end of 15 and 30 days of extracts feeding, the experimental as well as control animals were weighed and sacrificed for the purpose of histopathological and ultrastructural studies of testes, epididymis, and spermatozoa. Organ weight was taken at the time of dissection. In blood the levels of follicle stimulating hormone, leuteinizing hormone and testosterone were estimated. Sperm morphology, sperm function tests, and sperm count was also carried out. All these data were subjected to statistical analysis wherever possible.

The specific changes in the target organs such as testes and epididymis were studied and it was also observed that whether these changes are permanent or temporary. The spermatozoa were studied under compound microscope as well as scanning and transmission
electron microscope. The aim of the histopathological and ultrastructural studies is to observe changes in the elements of seminiferous tubules and Leydig cells of testes, and epididymis. After making observations, of the histopathological and ultrstructural changes using different doses and durations of benzene fraction of aqueous extract of fruits of *Terminalia bellirica* plant, our results are compared with the findings of other.

The complete research work has been presented in the form of six chapters:-

1. The **first chapter** is of introduction. In this chapter are given the problems related with population explosion, trends of world population growth and its harmful effects on the environment, nature and ultimately on mankind. The need of the use of contraception, different contraceptive devices have been discussed with their merits and demerits. Then need of development of some contraceptive of plant origin for man is discussed. The plants investigated for their antifertility potential are screened and described. Some important plant preparations investigated for their antifertility activity are discussed.

Population explosion or over population denotes a situation in which the number of people living in a country rapidly exceeds its carrying or sustaining capabilities. There is a steady decline in the population growth rate but the current data shows a continued increase in population in the near future but, with the global population expected to reach between 9.5 to 10.5 billion by 2050, according to International Data Base (IDB) World Population CENSIS growth, June 28, 2010.

Indian demography comprises of nearly 1.22 billion people, about 17.11% of world population which is roughly one-sixth of the world’s
population and second largest in the world next to China, with 19.15% of the world population. Although India occupies only 2.4% of the world’s total land area.

Many methods of contraception are available in the market and some commonly used methods are –

- **Barrier methods** - A physical barrier is used to prevent contact of male and female gametes. Male condoms, female condoms like the diaphragm, cervical cap and the contraceptive sponge are some such methods.
- **Permanent sterilization** - Vasectomy for man, and tubectomy or bilateral occlusion for women.
- **Spermicides** - Designed to be used either alone or in combination with the barrier methods.
- **The intrauterine devices** – currently available are a copper containing T and a progesterone releasing device.
- **Hormonal methods** - Oral contraceptives, injectables, the subdermal implants containing levonorgestrel and morning after pills.
- **Others** - natural family planning, emergency contraception are also available.

From time to time many plants have been investigated for antifertility activities by various agencies. A variety of plants are used for the contraception in villages of India and by tribals. Some such plants mentioned in folklore and then investigated scientifically are *Azardicta indica*, *Acacia concinna*, *Albizia procera*, *A. lebbeck*, *A. odoratissima*, *Aloe barbadines*, *Caltha pallustries*, *Portulaca oleracea*, *Carica papaya*, *Astracantha longifolia*, *Cleistanthus collinus*, *Terminalia bellirica*, *Trigonella fomium greecum*.  

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For the antifertility effect in males many other plants have also been investigated such as *Tripterygium wilfordii*, *Ocimum sanctum*, *Gossypium* sp., *Beaumontia grandiflora*, *Aristolochia tagala*, *Astracantha longifolia*, *Adiantum lunulatum*, *Achrostichera aureum*, *Piper beetle*, *Aegle marmelos*, *Ammania baccifera*, *Rumex steudelii*, *Dendrophthoe falcate*, *Colebrookia oppositifolia*, *Madhuca indica*, *Quassia amara*, *Hibiscus rosa sinesis*, *Chordia di chotoma*, *Casiarea tomentosa*, *Diospyras embryopteris*, *Milletria auriculata* and *Melleiazedarac* and were found to be effective. Many plants have promising results, which need to be investigated.

*Gossypium*, *Azadirachata indica* and *Tripterygium wilfordii* are the three most extensively studied plants for potential male antifertility activity.

Out of a long list of plants evaluated for the male antifertility activity, *Terminalia bellirica* is selected mainly because crude extracts of this plant showed promising results during the previous study conducted in this laboratory and secondly this plant has many other established medicinal properties.

2. In the **second chapter** the materials and methods are described. During the course of the present work, the techniques used for preparing the plant material for extraction, preparing benzene fraction at controlled temperatures and experimental protocols are described.

Using the Soxhelet apparatus, the aqueous extract of ripe fruits of this plant was prepared. Then benzene fraction was prepared using standard techniques. This fraction was fed to adult healthy male albino rats of proven fertility in doses of 1 mg and 2 mg/100 g body weight.
For histopathological studies, standard HE stain was used and for electron microscopy tissues were fixed in glutaraldehyde and further processed and studied in the Anatomy department of AIIMS, New Delhi. Spermatozoa were counted, studied under compound microscope, transmission and scanning electron microscope. FSH, LH and testosterone were measured in blood by RIA. For assessing the functioning of sperms, HOS test, AI test and ND test were conducted.

3. The third chapter consists of historical resume. The literature from 1918 to 2014 is given chronologically. The beginning of research work on plants was with the work of Kartikar and Basu in 1918 and in 1933. At some places all available literature regarding a particular plant is also given.

WHO has screened many plants from time to time and presented these findings in “Traditional Medicine Strategy”. Recently from 2010 to 2014 many other plants such as Andrographis paniculata, Madhuca indica, Aegle marmelos, Maytenus emarginata, Citrus medica, Dioscorea esculenta (L) Schott have been investigated for their antifertility potential. The most thoroughly investigated plants are – Gossypium (gossypol), Azadiracta indica (neem) and Tripterygium wilfordii (triptolide).

4. The fourth chapter consists of the observations made during the present project. The observations are presented heading wise.

The observations on the body weight and testes and epididymis weight are given in the first section.

Gross examinations of all these organs and histology and ultrastructure of normal control animals is described. Similarly values of
different hormones studied, sperm count and results of sperm function tests are given. Observations of general sperm morphology, scanning electron microscopy and transmission electron microscopy are described.

Same observations on body weight, organ weight, organ histology of testes and epididymis are dealt in the second part. The histology and ultrastructure of testes, epididymis and sperm, hormone levels, sperm count and sperm function test have been reported after treatment with benzene fraction of aqueous extract of fruits of *Terminalia bellirica* plant. These findings are described in detail along with tables, bar diagrams and photographic plates, separately for each dose and duration.

- The benzene fraction of aqueous extract of fruits of *Terminalia bellirica* caused increase in body weight of experimental animals equivalent to control group with both 1 mg and 2 mg doses of the fraction but decrease in the weight of testes and epididymis was noted.
- The benzene fraction of aqueous extract of fruits of *Terminalia bellirica* caused interruption of normal spermatogenesis at various levels. Some seminiferous tubules were almost empty with only germinal epithelium and few spermatogonia. Mass destruction of germinal components was observed in some sections. Large vacuoles appeared due to disappearance of cells.
- After 30 days treatment with the benzene fraction complete destruction of some tubular elements was observed in case of both 1 and 2 mg doses experimental duration and in some sections fat like drops were observed that was also confirmed under electron microscope.
When sections of testis and epididymus were studied under electron microscope, spermatogonia were unaffected in all dose groups and durations. Primary spermatocytes were also unaffected after 15 days treatment in many experimental animals. The most affected cell types were secondary spermatocytes and spermatids. Mitochondria were main cell organelles which were affected. Mitochondria showed swelling, disruption of cristae and sometimes loss of mitochondrial sheath. Nucleus also showed signs of damage, pyknosis of nuclei and ultimately disappearance of nuclei were also observed.

The most common observation was swollen mitochondria with disintegrated cristae which are probably the result of disrupted metabolism of the cells.

As the germinal epithelium remained intact in all doses and durations the recovery was observed in most of the experimental animals during reversibility studies. It caused complete regeneration.

No observable changes were observed in the epithelial lining of epididymis with the benzene fraction of aqueous extract of fruits of *Terminalia bellirica* under compound microscope except for the presence of fat like drops but the concentration of sperms was reduced after 15 and 30 days. After 30 days treatment most ductules were empty.

Some minor changes were observed in the wall of epididymus under electron microscope such as presence of fat droplets and dilated endoplasmic reticulum. No nuclear damage was observed at any stage of the treatment.

The three hormones assessed during the present study were– FSH, LH and testosterone. The level of all three showed no remarkable change after administration of benzene fraction of aqueous extract of fruits of *Terminalia bellirica* in any dose for any duration.
After 15 and 30 days with benzene fraction of aqueous extract of fruits of *Terminalia bellirica*, sperm count showed decrease. Almost no sperms were observed in some counting chambers after 30 days treatment. The sperm count was near normal after completion of the reversibility period.

Under the compound microscope, no change was observed in the structure of sperms but under scanning electron microscope sperms showed deformation of head, bifid head and presence of cytoplasmic droplets in the tail. Under transmission electron microscope also various abnormalities were observed such as deformed mitochondria, disrupted plasma membrane and abnormal 9+2 arrangement of microtubules in the tail.

5. In the **fifth chapter** results are discussed. The results of various experiments during the present study are discussed and correlated with the work of other research groups. The results of hormone assay, sperm count, sperm morphology, histopathological and ultrstructural findings of benzene fraction of aqueous extract of fruits of *Terminalia bellirica* plant are compared and correlated with the findings of other researchers with different plants or with the effect of spermicidal chemicals wherever it was necessary.

The spermatogonia were unaffected at the ultrastructure level in all dose groups and durations. In many experimental animals primary spermatocytes were also unaffected. The secondary spermatocytes and spermatids were most affected cell types. Mitochondria were main cell organelles which were affected. Mitochondria showed swelling, disruption of cristae and sometimes loss of mitochondrial sheath. Nucleus also showed signs of damage, pyknosis of nuclei and ultimately disappearance of nuclei were also observed.
By observing all the above mentioned findings, it can be said that in testes meiotically dividing cells were most severely affected, type A and type B spermatogonia were unaffected. Not much marked changes were observed in the epididymis. The level of hormones such as FSH, LH and testosterone was in normal range. Low sperm count was observed with both doses of the fraction, ultrastructural studies of sperms revealed many types of abnormalities.

On the basis of above mentioned histopathological and ultrastructural studies it can be said that benzene fraction of aqueous extract of fruits of *Terminalia bellirica* plant acted as potent spermatogenesis inhibitory agent, without affecting sperm mother cells, Sertoli cells and Leydig cells.

It appears that the benzene fraction of aqueous extracts of fruits of *T. bellirica* acts at the level of testes only. For causing infertility, within the testes either germ cells can be affected or Sertoli cells can be affected, which play a key role in the development of spermatozoa. But during the present study, in the sections studied normal Sertoli cells were observed. So, the target of benzene fraction is in all probability germ cells only. The histological and ultrastructural studies reveal that the fraction has affected selectively the cells of testes only, especially the cells undergoing meiotic division. This is the reason spermatogenesis is arrested and cell destruction is observed during different stages of spermatogenesis.

The probable mode of action is selectively on meiotically dividing cells only because histological and ultrastructural studies reveal that spermatogonia layer was intact in all sections studied, even if all other germinal components were missing. It is a well established fact that if all other cell types have been destroyed and only spermatogonia are intact then these can ultimately give rise to all
other type of spermatogenic cells. This is the reason that after a recovery period of 30 days, normal spermatogenesis was observed.

- We can summarize that the benzene fraction of *T. bellirica* has selective effect on the target male reproductive organs i.e. testes. Leydig cells are intact, Sertoli cells are unaffected and there is no major destruction in the wall of epididymis. In the seminiferous tubules only dividing cells are being affected. All these findings indicate that the effect of benzene fraction of *T. bellirica* has mainly on the meiotically dividing cells and this effect is exerted mainly through changes in the mitochondria.

- The study, as a whole, has however provided interesting, encouraging and positive findings which can be further worked out. Before making any claim for the recommendation of these plant preparations for clinical trials all these studies are to be repeated and then again repeated on primate species.

- The one important feature of this study has been that the benzene fraction of aqueous extract of fruits of the plant *Terminalia bellirica* is very effective as spermatogenesis inhibitory agents is working at target organs only i.e. testes as no effect is observed on the histology of epididymis. It means that benzene fraction of *T. bellirica* selectively destroys spermatogenic elements only.

- Another important aspect of the study has been that the damage caused by the benzene fraction was reversible in as short a period as of 30 days.

- This can be safely concluded that benzene fraction of *T. bellirica* has definite effect on the male reproductive organs and these alterations are mostly reversible after cessation of the fraction feeding. The benzene fraction of aqueous extract of fruits of the plant *Terminalia bellirica* showed much potential as future spermicidal agent.
In the light of these findings, it is further proposed that the study can be taken up at more elaborate scale. The study, as a whole, has however proved interesting, encouraging and full of potential to work upon.

6. The **Sixth Chapter** is summary itself.