SUMMARY

The present project, “Histological and Electron Microscopical Studies of Testes and Spermatozoa of male albino rats after Treatment with Chloroform Fraction of Terminalia bellirica,” was under taken for investigating the effect of chloroform fraction of aqueous extract of fruits of Terminalia bellirica plant. It 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 imperative to conduct a well plan research work on its antifertility potential.

During the present project, it was also tried to study the reversibility of the effects of fraction. With every dose and duration, a control group was also maintained throughout the study. 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. Biochemical estimations of total proteins, cholesterol, fructose, alkaline phosphatase, acid phosphatase and were conducted in testes. 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 experimental design was prepared in such a manner so as to evaluate the specific changes in the target organs such as testes and epididymis and to see 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. The biochemical studies were also carried out to see the metabolic state of testes and their correlation with changes in histology and ultrastructure. The histopathological and ultrastructural findings of different doses and durations of chloroform fraction of aqueous extract of fruits of *Terminalia bellirica* plant are compared with each other.

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

1. In the **first chapter** are given the trends of world population growth and problems related with its harmful effects on the environment, nature and ultimately on mankind. A summary of available methods of birth control is given and 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.

2. Current data shows a continued increase in population in the near future but a steady decline in the population growth rate, 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. 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.

3. Although India occupies only 2.4% of the world’s total land area, 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.

4. Medical science has given many methods of contraception; 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 men, and tubectomy or bilateral occlusion for women.

Spermicides - Designed to be used either alone or in combination with the barrier methods.

Hormonal methods - Oral contraceptives, injectables, the sub dermal implants containing levonorgestrel and morning after pills.

Others - The intrauterine device (currently available are a copper containing T and a progesterone releasing device), natural family planning, emergency contraception are also available.

5. A variety of plants are used for the contraception. From time to time many plants have been investigated for antifertility activities by various agencies. Some such plants are Acacia concinna, Albizia procera, A. lebbeck, A. odoratissima, Aloe barbadines, Azardicta indica, Caltha pallustries, Portulaca olaracea, Carica papaya, Astracantha longifolia, Cleistanthus collinus, Terminalia bellirica, Trigonium folium- grecum.

Some other plants investigated for the antifertility effects in male rats are Beaumontia grandiflora, Ocimum sanctum, Tripterygium wilfordii, Aristolochia tagala, Astracantha longifolia, Adiantum lunulatum, Achrosticherm aureum, Piper betle, 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 Melio azedarac and were found to be effective. Unfortunately none of the aforesaid plants have so far been fully investigated. Many plants have promising results, which need to be investigated.

The three most extensively studied plants for potential male antifertility effect are Gossypium, Azadirachata indica and Tripterygium wilfordii.

6. Out of the hundreds of plants evaluated for this purpose, 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.

7. The second chapter deals with the materials and methods. Techniques employed during the course of the present work for preparing the plant material for extraction, preparing chloroform fraction at controlled temperatures and experimental protocols are described.

The aqueous extract of ripe fruits of this plant was prepared. Then chloroform 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 2mg/100g body weight.

Standard HE stain was used for histopathological studies and tissues for electron microscopy were fixed in gluteraldehyde and processed for the studies in AIIMS. Spermatozoa were counted, studied under compound microscope, transmission and scanning electron microscope. For the biochemical estimation of total proteins – Biuret method, for sugar – GOD – POD method, for cholesterol – Enzymatic method, for acid phosphatase King’s method, for alkaline phosphatase – King and King’s method, and for lactate dehydrogenase – UV Kinetic method were used. Sperm count was performed on Neubauer’s counting chamber. For assessing the functioning of sperms, HOS test, AI test and ND test were conducted. FSH, LH and testosterone were measured in blood by RIA.

8. The historical resume is given in the third chapter. The literature is collected from the beginning of research work on plants, of Kartikar and Basu in 1918 and in 1933 to the work of Alagammal et.al. (2013) on whole plant extract of Polygala rosmarinifolia. All this literature is arranged in chronological order. At some places all available literature regarding a particular plant is also given.
Different plants screened by WHO from time to time and their reports on “Traditional Medicine Strategy” are also discussed. Recently in 2010, 2011 and 2012 many plants such as *Andrographis paniculata, Madhuca indica, Aegle marmelos Maytenus emargineta, Citrus medica, Dioscorea esculenta* (L) Schott have been investigated for their antifertility potential. The most thoroughly investigated plants are – *Gossipium* (gossypol), *Azadirachta indica* (neem) and *Tripterygium wilfordii* (triptolide).

9. The observations made during the present project are presented in the **fourth chapter**. The observations are given heading wise.

   In the first section gross observations on the body weight and testes and epididymis weight, gross examinations of all these organs and histology and ultrastructure of normal control animals is described. Similarly values of different biochemical parameters studied, hormones, sperm count and results of sperm function tests are given. Observations of general sperm morphology, scanning electron microscopy and transmission electron microscopy are described.

   The second part deals with the same observations on body weight, organ weight, organ histology of testes and
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epididymis and biochemical levels such as total proteins, cholesterol, fructose, alkaline phosphatase and acid phosphatase in testes, histology and ultrastructure of testes, epididymis and sperm, hormone levels, sperm count and sperm function test have been reported after treatment with chloroform 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.

10. The administration of chloroform fraction of aqueous extract of fruits of *Terminalia bellirica* caused increase in body weight 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.

11. At biochemical level, the level of cholesterol was increased with both doses of chloroform fraction. It was decreased (came back to near normal) after reversibility period in both treatment groups.

12. The level of total proteins was also increased after 15 and 30 days of the treatment. The level came back to the normal level after 30 days of reversibility period.

13. An increase in the level of fructose in the testes was observed with chloroform fraction but a decrease was observed after
the stopping of fraction feeding, i.e. the level was in normal range.

14. In case of alkaline phosphatase, the level was decreased after 15 and 30 days of treatment. The levels of ALP again increased after reversibility experiment and came back to almost normal level.

15. The level of acid phosphatase was increased and came in normal range after discontinuation of the fraction feeding.

16. Normal spermatogenesis was interrupted at various levels by chloroform fraction of aqueous extract of fruits of *Terminalia bellirica* and caused thickening of basement membrane. 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.

17. The chloroform fraction resulted in complete destruction of some tubular elements in case of both 1 and 2 mg doses after 30 days experimental duration and fat drops were observed that was also confirmed under electron microscope.

18. Ultrastructural studies revealed that 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.

19. The swollen mitochondria with disintegrated cristae are probably the result of disrupted metabolism of the cells.

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

21. With the chloroform fraction of aqueous extract of fruits of *T. bellirica*, under compound microscope no observable changes were observed in the epithelial lining of epididymis 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.

22. Under electron microscope some minor changes were observed such as presence of fat droplets and dilated
endoplasmic reticulum. No nuclear damage was observed at any stage of the treatment.

23. The level of all three hormones assessed – FSH, LH and testosterone showed no remarkable change after administration of chloroform fraction of aqueous extract of fruits of *Terminalia bellirica* in any dose for any duration.

24. The sperm count showed decrease after 15 and 30 days with chloroform fraction of aqueous extract of fruits of *T. bellirica*, almost no sperms were observed in some counting chambers. The sperm count was near normal after completion of the reversibility period.

25. When sperms were examined under 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.

26. The fifth chapter consists of the discussion in which the results of various experiments are discussed and correlated with the work of other research groups. The results of biochemical studies, hormone assay, sperm count, sperm
morbidity, histopathological and ultrstructural findings of chloroform 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.

27. The results of various biochemical parameters are substantiated by the histological and ultrastructural findings. Increase in fructose level is supported by the absence of sperms in the testes and epididymis, increase in cholesterol level is accompanied by normal Leydig cells, decreased level of ALP with reduction in spermatogenesis in the seminiferous tubules and increase in ACP activity is correlated with increased catalytic activities in seminiferous tubules and so on.

28. At ultrastructure level spermatogonia were unaffected in all dose groups and durations. Primary spermatocytes were also unaffected in many experimental animals. The most affected cell types were secondary spermatocytes and spermatids. Mitochondria were main cell organelles which were effected. 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.
29. In nut shell, it can be said that in the 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.

30. On the basis of above mentioned histopathological, ultrastructural and biochemical studies it can be said that chloroform 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.

31. Another important aspect of the study has been that the chloroform fraction is working at target organs only i.e. testes. No effect is observed on the histology of epididymis. It means that chloroform fraction of *T. bellirica* selectively destroys spermatogenic elements only. The chloroform fraction also showed no sign of toxicity as was apparent in another study in the present laboratory only, from the study of liver.
32. The probable mode of action of chloroform fraction of aqueous extracts of fruits of *T. bellirica* appears to be at the level of testes only. 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 normal Sertoli cells were observed in the sections studied. So, the remaining target of the chloroform fraction are 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.

33. 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.

34. At this stage it can be concluded that chloroform fraction of *T. bellirica* has definite effect on the male reproductive organs and these alterations are mostly reversible after cessation of the fraction feeding. 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 chloroform fraction of *T. bellirica* has mainly on the meiotically dividing cells and this effect is exerted mainly through changes in the mitochondria.

35. The salient features of this study have been that the chloroform fraction of aqueous extract of fruits of the plant *Terminalia bellirica* is very effective as spermatogenesis inhibitory agents. Further, the damage caused was reversible in as short a period as of 30 days.

36. All these findings are promising and encouraging. In the light of these finding, it is further proposed that the study can be taken up at more elaborate scale. At the end of the experiments it would also be essential to carry out the mating studies on the experimental animals and if every result comes out positive then to extend this study to other animal models e.g., monkeys or other primates.

37. All these studies are to be undertaken before making any claim for the recommendation of these plant preparations for clinical trials. The study, as a whole, has however provided interesting, encouraging and positive findings which can be further worked out.