DISCUSSION

Although hundreds of plants have been evaluated for anti fertility activity but still there is scope for more. Many types of contraceptives are available in the market for population control but there is still scope for herbal products.

During 1970s and 1980s, a lot of work was done on medicinal plants but for about twenty years, interest of scientific community was lost. From late 90s onwards this interest was revived again. A lot of plants and plant products have been successfully tested for their anticancer, antidiabetic, antifertility and many more such activities.

*Terminalia belirica* plant commonly known as “Baheda” was selected for the present project. It is a commonly used plant by vaidhyas mainly for stomach ailments but some local villagers also uses it for antifertility activity. So it was thought worthwhile to conduct a well planed research work on its antifertility potential. Except for one or two research papers, no other research work is available on the antifertility potential of this plant, so it is very difficult to compare our findings with the research work on this plant. Due to this reason we are left with no other choice than to compare our findings with the findings of other researchers of other plants tested for male antifertility activity.

We have already written observations, now we will discuss the topic under following headings.

- Discussion on the body weight and organ weight of control and treated animals of all four groups.
Discussion on the histology and electron microscopic study of testes and epididymis.

Discussion on the sperm morphology and sperm count.

Discussion on the level of hormones.

Discussion on the Effects of Benzene Fraction on Body Weight

The physiology and metabolism of any animal is directly effected by any drug or extract taken, so the body weight of the experimental animals is direct indication of its effect. To clear the picture of the effect of fraction, this gain or loss in body weight is compared with the gain or loss in body weight of control animals. Any substance, either food or drug or plant product which is fed or administered through any route to the experimental animals, it has a definite effect on the physiology and metabolism of that animal.

An increase in the final body weight of experimental animals was observed, when benzene fraction of aqueous extract of fruits of *T. bellirica* was administered to male albino rats. When this gain in body weight was compared with that of control animals of the same group and duration, no significant difference was observed in the gain of body weight. On the other hand, Sharangouda J. Patil et. al. (2010) observed no change in the body weight with *T. bellirica* bark extract. This difference might be due to the fact that fruit powder was used in the present study and bark was used by Sharangouda J. Patil et. al.

As no other work on fruits of *T. bellirica* is available, now we will be comparing our observations with the findings of other workers on other plants. Verma et. al. (1980) while working with *Malvaviscus conzattii* greenm flower extract (sc) on male albino mice observed no
significant loss in body weight after 30 & 40 days treatment. Sinha et. al. (1990) with Abrus precatorius, Chatterjee et.al. (1993) working with Piper betle observed no change

Sharma and Jacob (2000) with Mentha arvensis observed no change in the body weight of albino rats after 20, 40 & 60 days of treatment. Renata Mazaro et. al. (2000) observed no difference statistically, between treated and controlled groups after treatment with Austroplenckia populnea for 7 and 14 days.

Rita de cassia da Silveira esa et. al. (2002) with Mikania glomerates extract observed no significant change in body weight of male Wistar rats after 52 days treatment. Mohammad Hossein Dehghan et. al. (2005) working with Azadirachta indica alcoholic seed extract,.R K Mishra et. al. (2005) and Sathiyaraj et. al. (2010) working with aqueous leaf extract of Azadirachta indica on reproductive organs in male mice observed no significant difference between initial and final body weight after 28 days.

Shajeela et. al. (2011) also observed no change in the body weight of rats after 15 and 30 days with Dioscorea esculenta ethanol extract. Thejashwini et. al. (2012) while working with Cyamopsis Psoralioides observed no significant change in the body weight in ethanolic pod extract groups when compared with respective control groups. Hemad Haddad Kashani et. al. (2012) also observed no change in the body weight after treatment with Dactylorrhiza maculate for a week.

Nidhi Sharma (2001) with Mentha arvensis leaves (methanol extract) observed inhibitory effect on body weight of mice after 40 and 60
days treatment. Similarly Salhesh et. al. (2011) with *Aegle marmelose* observed decrease in body weight.

These studies are not in agreement with our observations but this difference in findings might be due to the fact that different plants have been used by different researchers.

However, our studies are in accordance with the work of Heywood et. al. (1986) who worked with 0.5mg/1kg dose of gossypol and Adhikari et. al. (1989) experimenting with *Piper bealle* alcoholic extract reported increase in the body weight of experimental animals. Mali et. al. (2002) while working with *Martynia annua* roots also observed that final body weight increased markedly of albino rats in 60 days.

During the present study, with benzene fraction the body weight showed moderate gain. This fact indicates that benzene fraction of aqueous extract of *T. bellirica* fruits do not adversely effect the metabolism of the experimental animals. So we may safely conclude that this treatment has no adverse effect.

**Discussion on the Effects of Benzene Fraction on Testes and Epididymis Weight**

As the main target organs for control of male fertility are testes and epididymis as one by the inhibition of spermatogenesis and other by inhibition of sperm maturation. If a particular plant product is showing antifertility activity, it will have direct effect on the weight on these two target organs.

The weight of testes and epididymis of experimental animals was carefully taken after completion of the experiment duration of each group.
and then minutely examined for any morphological change. For the conclusion to be drawn and final assessment, however the histological picture and electron microscopic results will be thoroughly studied, compared, and corroborated.

The loss in the weight of testes and epididymis was observed with the various doses of benzene fraction of the aqueous extract of *T. bellirica* fruits. The damage to the germinal components or arrest of spermatogenesis are most probable cause of the reduction in the weight of testes and epididymis.

Slight decrease was observed in the weight of testes and epididymis after 15 days treatment but significant decrease in the weight of testes and epididymis was observed after 30 days treatment. Not much difference was observed in the testes and epididymis weight in 1 mg and 2 mg dose groups when we compared the percentage decrease of lower and higher dose groups.

When fraction feeding was discontinued for 30 days, after 30 days continuous feeding (i.e. the reversibility group) then slight gain was observed in the weight of testes and epididymis. In some experimental animals the percent weight of testes was equivalent to normal testes weight.

Almost all researchers, working with any plant having potential antifertility activity have measured the weight of testes and epididymis. Sharangouda Patil et. al. (2010) using benzene extract of *Terminalia bellirica* bark in albino rats have reported significant decrease in testes and epididymis weight with 1 and 2 mg doses after 50 days treatment. No
parallel work is available on *Terminalia bellirca* fruit so we will be making more comparisons with the work on other plants.

Tyagi et. al. (1989, 1990) while working with aqueous and acetone extracts of seeds of *Trigonella foenum graecum* (Linn) reported decrease in the testes and epididymis weight of male albino rats after 10, 20 and 30 days. Verma et. al. (1990) working with *Malvaviscus conzatti greenum* (Achania) flower extract also reported decrease in the testes and epididymis weight of male albino rats. Adhikari et. al. (1989) and Sarkar et. al. (2000) both worked with crude extract of stalk of *Piper betle* on male rats and observed decreased testes and epididymis weight. Nidhi Sharma et. al. (2001) with *Mentha arvensis* leaves (methanol extract) also reported significant decrease in epididymis weight after 40 and 60 days. Seetharam et. al. (2003) observed decrease in the testes and epididymis weight of male albino rats with Amalakyadi churna.

In 2005 Raghav Kumar et al studied the effect of aqueous leaf extract of neem *Azadirachta indica* on male reproductive organs of Parkes strain mice and reported no effect on body weight and the reproductive organ weight after feeding 50, 100 mg and 200 mg/kg body weight/day for 28 days.

In 2010, Nidhi Mathur et. al. with *Tecoma stans* leaves observed significant reduction in the weight of testes after 60 days treatment. In 2010 only, Sathiyaraj et. al. with *Azadirachta indica* and Rajesh Chaudhary et. al. with ethanolic extract of *Maytenus emargineta* observed highly significant decrease in the testes weight in albino rats when treated for 30 days and 60 days respectively.
Shajeela et. al. (2011) with ethanol extract of tuber of *Dioscorea esculenta* observed significant decrease in the weight of testes in 14 days. Aladakatti et. al. (2011) observed no change in 24 days after treated with *Azadirachta indica* in albino rats, but when treatment period was increased, a significant decrease in testes was observed. Thejashwini et. al. (2012) while working with *Cyamposis Psoralioides* observed significant decrease in testes weight when compared with control group.

Al-Sanabra et.al. (2013), after a week’s treatment with ethanolic seed extract of Celery (*Apium graveolens* L.) in male albino rats reported significant decrease in testes and epididymis weight. Alagammal et.al. (2013) reported significant decrease in testes and epididymis weight after 14 days treatment with whole plant extract of *Polygala rosmarinifolia*.

But no change in testes weight was observed after 60 days treatment with methanol subfraction of *Carica papaya* seeds by Boomi Manivannan et. al. (2009). Kashani et. al. (2012) with *Dactylorhiza maculate* observed no change in the testes weight after the treatment for one week.

The decreased weight of testes and epididymis, in the experimental animals treated with benzene fraction of *T. bellirica* fruits, indicate probable damage of the germinal components or arrest of spermatogenesis in the testes and absence of sperms in the epididymis. Later on these observations will be corroborated with findings of the histological examinations.
Discussion on the Effects of Benzene Fraction on Histology of Testes

The varying degree of changes in the histology of testes was observed with the benzene fraction of aqueous extract of fruits of *Terminalia bellirica*.

With 1 mg dose, not many changes were observed in the histology of testes of some experimental animals but in others very few secondary spermatocytes and spermatids remained after 15 days treatment. After 30 days treatment mass destruction of germinal components was observed. In 2 mg group of 15 days few changes were observed but after 30 days of treatment more degenerative changes were seen. In some animals only spermatogonia and primary spermatocytes were visible. All other cell types were lacking.

Most experimental animals showed full recovery when fraction feeding was discontinued. All stages of spermatogenesis were observed in most seminiferous tubules and lumen was full of developing spermatozoa. One or two experimental animal showed no recovery and vacuolization was observed in germinal epithelium layer of one experimental animal in reversibility group.

As already mentioned, no similar literature is available on the effect of *T. bellirica* fruit so we will be making comparison with the work of other research groups on other plants.

Some impairment of spermatogenesis was observed in the experimental animals after treatment with *Ocimum sanctum* leaves. Kasinathan et. al. (1972) using *Ocimum sanctum* leaves on male albino rats reported that the spermatogonial cells adhered together in some places of the tubular lumina. The spermatid bundles were not properly
developed. Sperms were scattered all through the tubular lumen and interstitial cells were very sparse and in degenerated condition.

Pakrashi and Pakrashi (1977) using plant extract of *Aristolochia indica* reported arrest of spermatogenesis at different stages and nuclear degeneration in different germinal cell types. Most of the tubules were devoid of spermatids and spermatozoa.

Chronic administration of *M. conzattii* flower extract by Verma et al. (1980) caused a partial degeneration of testicular elements and the severity of effects increased with increase in the number of doses. It produced the complete arrest of spermatogenesis and damage in the germinal elements of mice testes. The tubular lumina were either devoid of spermatozoa or contained debris.

Rao (1988) using alcoholic extract of *Solaum xanthocarpum* seeds for 60 days observed destruction of spermatogenic elements with nuclear pyknosis, degeneration of basement membrane of seminiferous tubules and atrophy of Leydig cells but during present study no degeneration of basement membrane or Leydig cells was observed. S. D. Tyagi et al. (89, 90 and 94) reported arrest of spermatogenesis with crude, aqueous and acetone extract of seeds of *Trigonela foenum graecum* in male albino rats.

Sinha et al. (1990) using the steroidal fraction of seeds of *Abrus precatorius* Linn. on male albino rats observed the spermatozoa were lesser in number and lumina of the tubules contained debris and the residual sperms at 200 mg dose. At 300 mg dose the tubules showed normal spermatogonia but primary spermatocytes, secondary spermatocyte and spermatids showed damage and complete disappearance at some places. Tunica was filled with debris.
Seminiferous tubule and Leydig cell nuclear diameters were reduced in a dose dependent manner.

The aqueous extract of *Vinca rosea* (Linn) leaves by Murugavel and Akbarsha (1991) for 24 days in the male Swiss albino mice caused complete arrest of spermatogenesis, the lumen was obliterated, sperms were not discernible, degenerating germinal elements filled the lumen and only single layer of spermatogenic elements was present. The regression and degeneration of Leydig cells were observed and the cells had a compact rosette appearance with the highly compact nuclei.

The aqueous extract of leaves of *Azadirachta indica* caused complete arrest of spermatogenesis, mass atrophy of the spermatogenic cells and atrophied Leydig cells in male albino rats (Joshi et. al., 1996). Significant reduction in the number of spermatogenic elements like spermatogonia, spermatocytes and spermatids with different extracts of *Hibiscus rosa sinesis* was proposed by Reddy et. al. (1996). This was attributed to non availability of gonadotrophins. But our observations indicate normal structure of Leydig cells. Normal structure of Leydig cells can be maintained only when proper amount of gonadotrophins is available. As the Leydig cells are the source of androgens, the normal Leydig cells in both treated groups indicate normal synthesis of testosterone and availability of gonadotrophins.

Munjal et al, (2002) studied effect of oral administration of finely ground fruits of *S. trifoliatus* at two dose levels 15 mg/100 mg gm body weight and 20 mg/gm body weight for 30 consecutive days. It induced dose dependent changes in testes. The doses resulted in spermatogenic arrest and degeneration of germinal cell types leading to decline in seminiferous tubular diameter.
Our findings are in agreement with Munjal. During the present study also dose dependent changes were observed in the testes and degeneration of germinal components was also observed.

The antifertility activity of methanol extract of *Alstonia macrophylla* was attributed to the embolisation of spermatozoa (Chatopadhyae et. al., 2005). Mishra and Singh (2005) studied the effect of aqueous extract of leaves of *Azadirachta indica* on male mice for 28 days and observed seminiferous tubules showing intraepithelial vacuolation, and loosening of germinal epithelium, occurrence of giant cells, mixing of germ cell in stages of spermatogenesis and degenerated appearance of germ cells. They also reported that the effect of neem treatment on the testes was not uniform as both affected and normal seminiferous tubules were observed in the same sections. During the present experiment vacuolation in germinal components, arrest of spermatogenesis and disturbance of cell arrangement is observed but no giant cells are observed. It appears that some common factor or mode of destruction is present in all plants showing antifertility activity.


With aqueous extract of *Baugainvillea spectabilis* leaves for 30 days, the reduction in diameter of seminiferous tubules, thickness of germinal epithelium and absence of sperms in the lumen of tubules in treated animals was reported by Mishra et. al. (2009). Besides this some of the interstitial cells of Leydig and germinal epithelium were found to be hypertrophied. During the present studies also, the thickness of
basement membrane and absence of sperms in the lumen of tubules in treated animals were observed.

The alcoholic and decoction extract of *Adiantum lunulatum* Burm for 30 days resulted in the arrest of spermatogenesis at secondary spermatocytes stage (Bhatia et al., 2010). The tiered arrangement of the germ cells was disturbed and most of them were seen to have migrated into the lumen. Sloughing of the dead cells occurred into the lumen of the tubules. However upon treatment with same dose for 60 days, the seminiferous tubules were filled with oedematous fluid and cellular debris.

Regressed seminiferous tubules and less number of sperms in the lumen of tubules were reported by Shivabasavaiah et al. (2011) with alcoholic extract of *Madhuca indica* leaves.

Chaudhary et al. (2011) also observed the arrest of spermatogenesis after 30 days treatment with ethanolic extract of *Maytenus emarginata*. The size of seminiferous tubules appeared reduced and vacuolization was observed in the Sertoli cells, spermatogonia and spermatocytes. Germ cells proliferation beyond the level of spermatocyte was also affected. The lumen contained sloughed debris and few germ cells. Leydig cells nuclei diameter area and seminiferous tubular diameter were significantly reduced. The significant reduction in the diameter of seminiferous tubules and Leydig cells of treated animals was observed by Thejashwini et al. (2012), after 40 days treatment with *Cyamopsis psoralioides*.

The effect of benzene fraction appears to be dose independent during the present study. Vacuolization was also observed in the
seminiferous tubules of some experimental animals and degeneration of germ cells was also apparent but no giant cells were observed during any dose or duration of the treatment in any animal.

**Discussion on the Effects of Benzene Fraction on Ultrastructure of Testes**

An important role of basement membrane is maintaining the integrity of tissues. It can stabilize the structure of tissue and send signals to cell. Therefore, alteration of basement membrane structure can impair the severe function of testis but during the present study no change in the structure of basement membrane was observed.

During the present study, spermatogonia were unaffected in all dose groups and durations at ultrastructural level. In some experimental animals primary spermatocytes also remained unaffected. The secondary spermatocytes and spermatids were most affected cell types. The main cell organelles which were affected were mitochondria. Mitochondria showed swelling, disruption of cristae and sometimes loss of mitochondrial sheath. Many signs of damage of nucleus such as pyknosis of nuclei and ultimately disappearance of nuclei were also observed.

Cytoplasmic vacuolization and mitochondrial damage were reported by Oko and co-workers (1982) with gossypol treatment. The Sertoli cells were also damaged so ultrastructural defects in spermatocytes and spermatids, particularly in those that interfere with the spermiation process were attributed to the fact of disturbances in the microenvironment of the Sertoli cells.

It has been reported in the case of gossypol-induced spermatogenic arrest in laboratory animals, that the specific targets of gossypol's action
on spermatogenesis are the mid and late stages of spermatids and spermatocytes, with accompanying degenerative changes in the Sertoli cells. The subcellular target for gossypol is the mitochondria of the spermatogenic cells, and the spermatozoa. The spermatozoa derived from the testes of the animals treated with gossypol show remarkable damage in the mitochondrial sheaths. Inhibition of mitochondrial ATP production in isolated hamster spermatids has also been well documented, suggesting that the mitochondria and their energy production are the specific targets of the action of gossypol on spermatogenic cells.

During the present study also with benzene fraction of *T. bellirica*, mitochondria are the cell organelles that were most affected in the spermatogenic cells but no sign of damage was observed in the Sertoli cells.

Focal degeneration in seminiferous tubules characterized by different shape and size, absence of spermatogenic stages and pyknotic nucleus in the germ cells and cell debris and phagocytes in the lumen were observed in longur monkeys, with styrene maleic anhydride at 540 days by Lohiya *et al.* (2005). The Sertoli cell showed vacuolization and degenerative features of the cytoplasmic organelles. Round spermatids showed vacuolization in the pro-acrosomal Golgi vesicles and membrane damage in the nucleus.

Treatment with different parts of the plants such as leaf powder of *Azadirachta indica* by Aladakatti *et al.* (2005), crude garlic by Z. W. Yang *et al.* (2006) and benzene extract of *O. sanctum* leaves by Aladakatti, *et al.* (2005) on ultrastructure of the rat testis revealed several changes and it can be summarized in three categories such as: (i) vacuolization in the Sertoli cells and germ cells; (ii) degeneration of
mitochondria followed by vacuolization in spermatocytes and spermatids; and (iii) a decrease in nuclear density and ultimately rupture of plasmatic membranes.

The methanol subfraction of *Carica papaya* seeds was used by Manivannan *et. al.* (2009) and they reported vacuolization in the cytoplasm and secretary granules, pro-secretary granules, mitochondria and Golgi bodies were scattered throughout the cytoplasm after 120 days treatment. The vacuolization of the Sertoli cells was also observed after 240 days treatment with methanol subfraction. However the nuclei showed deep indentations, with patchy chromatin material and there were fewer cytoplasmic organelles. Mitochondria were few and vacuolated.

All above researchers have reported vacuolization in Sertoli cells but during the present study no such change was observed in the Sertoli cells. But more or less similar changes were observed in other spermatogenic cells. The most severely affected cell organelle appeared to be mitochondria, which were either swollen or cristae were disrupted. The swollen mitochondria with disintegrated cristae are probably the result of disturbed metabolism of the cell. This finally ends with decrease in the generation of adenosine triphosphate (ATP).

The presence of agranular endoplasmic reticulum and stacks of granular endoplasmic reticulum, numerous mitochondria and multiple Golgi bodies in the basal part of the cytoplasm are characteristics of Sertoli cells, which are metabolically active, producing protein and steroids (J. Pudney, 1986). Proteins necessary for the differentiation of germ cells are secreted at their highest rate in the testes during spermatid elongation and spermiation. Physiologically, Sertoli cells are responsible
for protein secretion, which aids in specific steps of germ cell maturation (K. Boekelheide, 1993).

Normal Sertoli cells were observed with all regular features in all treatment groups during the present study.

During the present study, abnormal spermatozoa were also observed and their number was depleted. At this stage it is difficult to state definitely that whether the abnormal sperms are result of the disturbance of cell function in testes or due to direct effect of the benzene fraction on sperm morphology.

With degeneration of spermatogenic cells it is clear that the process of spermatogenesis was inhibited. The arrest of spermatogenesis can be attributed either to the estrogenic property of the fraction or anti-androgenic property of fraction. Anti-androgenic property can be ruled out by two facts i) Leydig cells appeared normal, ii) Level of testosterone was also normal. Estrogenic property cannot be ruled out with definite certainty but it appears quiet improbable because no visible changes were observed in morphology or behaviour of experimental animals.

Leydig cells are known to have receptors for LH that stimulates these cells to produce testosterone. Both LH and testosterone are responsible for normal spermatogenesis in male rats. The normal structure of Leydig cells indicates that these are not the target organs for action of benzene fraction.

As in the case of chloroform fraction, the benzene fraction also appears to affect meiotically dividing cells only as secondary spermatocytes and spermatids were the cells, most severally affected. It
appears natural because some common alkaloids may be present in both chloroform and benzene fractions.

As spermatogonia remained unaffected, when fraction feeding was discontinued, most experimental animals recovered gradually. It is a well established fact that if all other cell types have been destroyed and only speramtoagonia are intact then these can ultimately give rise to all other type of spermatogenic cells.

**Discussion on the Effects of Benzene Fraction on Ultrastructure of Epididymis**

Epididymis plays an important role in the process of sperm maturity as it is the site of high metabolic activity, ranging from sperm maturity to storage and the importance of androgen in the maintenance of these activities has already been implicated. During the present study it was found that except for the presence of fat droplets, the benzene fraction may have no influence on the histology of the epididymis. Only some minor changes were observed under electron microscope.

While working with gossypol, Bhiwgade and Nair (1989) observed dilated endoplasmic reticulum, hypertrophied mitochondria and different stages of sperm disintegration is seen in the lumen of the epididymis. They also observed cauda epididymis with dense bodies and auto-phagosomes.

Sharma and Jacob (2001) proposed that inhibition of male fertility after administration of petroleum ether extract of the leaves of *Mentha arvensis* is related to decreased spermatozoa density. In the present study also sperm count was quiet low after 30 days treatment and most ductules of epididymis were empty.
No effect on epididymal epithelium with triptolide isolated from *Tripterygium wilfordii* was reported by Huynh *et. al.* (2000). Lohiya *et. al.* (2002) had also reported that benzene extract of seeds of *Carica papaya* alter the testicular histology but had no effect on the epididymal histology. The ultrastructure of epididymis also showed normal configuration. Our results are also parallel to these results and same facts suggest that the fraction acts upon the testicular germ cells only.

In 2004, M.G. Ghodosawar along with his co-researchers studied *Azadirachta indica* leaf extract and reported decreased number of coated micropinocytotic vesicles and invagination on the luminal surface, loss of apical microvilli, disruption of mitochondrial cristae and Golgi apparatus. In nucleus, the chromatin material was pushed to one side. The nucleus membrane was bulged and there was karyokinesis. The size of the cell, nucleus and the cytoplasmic granules were increased. The chromatin material was less, nuclear envelope was bulged, and mitochondria, Golgi apparatus and endoplasmic reticulum were disrupted. Basal cells appeared decreased.

In langur monkeys, Lohiya and co-workers (2005) observed supra nuclear region of the principal cells with characteristic pattern of secretory activity with numerous mitochondria, Golgi bodies and vesicles, while working with styrene maleic anhydride.

The findings these researchers are not similar with our findings. During the present study except for the absence of spermatozoa in the lumen of ductules, only some minor changes were observed in the epithelium of epididymis. Only in occasional sections dilated endoplasmic reticulum was observed. Nucleus was never damaged with any dose or the duration.
Discussion on the Effects of Benzene Fraction on Ultrastructure of Spermatozoa

For optimum male fertility, the development of normal and mature sperms is the key. For male contraception it is not necessary to stop the process of spermatogenesis but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in function of the sperm.

Each spermatozoa consists of head, middle piece and tail and every part is essential for the process of fertilization. If decondensation of head occurs then proper fertilization cannot take place, if acrosome is not properly formed then sperm will not be able to penetrate the ovum. On the other hand if mitochondria are defective then energy will not be provided for the locomotion of spermatozoa, if tail is deformed or arrangement of fibrils is disturb then also locomotion of spermatozoa will be affective.

The ultra structure of spermatozoa has been studied by several researchers after treatment with different plants such as Azadirachta indica, Carica papaya, Ocimum sanctum, Tripterygium wilfordii etc.

The effect of Azadirachta indica leaves on rat spermatozoa was studied by in 1999 by Ravindranath et al. and they reported morphological changes in the head of the spermatozoa in general and the acrosome in particular. In the treated rats change in the shape and size of the sperm head, with a dorso-ventral construction of the middle region of the sperm head i.e. between the anterior and posterior regions were observed. It was rather difficult to differentiate the outer acrosomal and outer plasma membranes. The authors have
attributed these effects to androgen deficiency and general disturbances in carbohydrates located in sperm head. During the present work also morphological changes were observed in the spermatozoa but the level of hormones was normal so these changes are not due to androgen deficiency but due to the effect of fraction feeding.

Some abnormalities in sperm morphology such as head agglutination and irregular and detached heads were reported by Aladakatti et. al. (1999) in rats treated with crude leaf extract of *Azadirachta indica*. Change in the shape and size of sperm head and increase in size of the acrosomal membrane and outer plasma membrane were observed after 14 days treatment with 500 mg/1kg body weight dose of neem leaf powder. They suggested that morphological changes in the head of spermatozoa in general and the acrosome in particular may have resulted from an alteration in the epididymal milieu of rats treated with crude leaf extract of *Azadirachta indica*.

The treatment with *Triptorygium wilfordi* caused complete absence of plasma membrane over the entire middle piece and principal piece and decondensation of the sperm nuclei (Hikim et. al. 2000). Lohiya and co-researchers in 2004 while working with styrene maleic anhydride (SMA) in langur monkeys observed acrosomal disorders, coiled tail and bent mid piece.

In 2005, Mishra et. al., noticed the cytoplasmic droplets attached to the tail of spermatozoa, sometimes, headless and microcephalic spermatozoa were also encountered with aqueous leaf extract of *Azadirachta indica*. Dehghan et.al. (2005) working with alcoholic extract
of Iranian neem seed also observed abnormal head morphology and bent middle piece.

Similarly, Girini et al., (2005) also observed many morphological changes in the spermatozoa with alcoholic seed extract of *Momordica charantia* and suggested that these changes are due to a general disturbance of carbohydrates or polysaccharides present in the acrosome of the sperm head.

Sharangounda J. Patil (2010), noticed abnormalities in the sperms such as their head region was reduced and the tail was wrinkled or coiled with bark extract of *Terminalia bellirica*. During the present study also many abnormalities such as bifid head, bent head, disrupted mitochondria and retention of cytoplasmic droplets in the tail region were observed with both doses and durations of the benzene fraction of *T. bellirica* fruits.

Mukhtar Ahmed et al. (2011), while working with benzene extract of *Ocimum sanctum* observed disruption or loss of plasma membrane and acrosome in the anterior portion of sperm heads. Middle part showed disruption and degeneration of mitochondrial sheath and loss of plasma membrane. Disorganization or commencement of degeneration of the mitochondria was observed in the mitochondrial sheath. Most of the tail sections showed retention of cytoplasmic droplets around the middle part on one side.

The origin of abnormality in the sperm morphology can be its either in the testes or epididymis. During the present study different digrees of degenerative changes were observed in the testes only so the origin of sperm abnormalities is definitely in the testes. No significant
alteration were observed in the epididymal epithelium except for the presence of fat droplets in the cytoplasm or dilated endoplasmic reticulum.

**Discussion on the Effects of Benzene Fraction on Sperm Count**

Although single sperm is required for the fertilization of the egg and the formation of a viable zygote but still large number of sperms is necessary for the process of fertilization. If the number of sperms is not sufficient then fertilization will not take place.

During the present studies, treatment of benzene fraction of aqueous extract of T. bellirica caused decreased sperm count in some experimental animals and in others almost nil sperm count was also observed after 30 days treatment. When no sperms were observed, severe damage in the seminiferous tubules was also observed and epididymis was devoid of sperms. According to the studies conducted by Bedford, (1983) and Raji et. al. (2006), the reduction in sperm count and motility in cauda epididymis is of importance with regard of fertilization.

Several abnormalities were also observed in the sperm morphology besides damage to the seminiferous tubules and thus arrest of spermatogenesis. As sperm count was low, of the remaining sperms some showed deformities of head, middle piece and tail.

The sperm count has been conducted by almost all research groups working on antifertility potential of any plant.

Sharangounda J. Patil, 2010 while working with bark extract of *Terminalia bellirica* reported significant reduction (P<0.05) in the sperm count of cauda epididymis with 10 and 25 mg dose of the benzene
extract and highly significant (P<0.001) reduction with 10 and 25 mg doses of ethanol extract was observed. The decreased sperm count of cauda epididymis may be due to the inhibition of the spermatogenesis due to treatment with the extract of plant. Our findings on sperm count are also in agreement with them.

Kantak and Gogate (1992) observed the reduction in sperm count after short term administration of Tulsi (*Ocimum sanctum*) to male albino rats. Chinoy and Padmann in 1996 reported decrease in sperm count while working with benzene extract of *C. papaya* seeds on male reproductive system. Decreased number of spermatozoa in cauda epididymis was reported by Naseem et. al. (1998) with *Momordica charantia*.

Mazaro et. al. (2000) reported decrease in the sperm number after treatment of rats with aqueous extract of *Austroplenckia populnea*. Similarly decreased sperm count in epididymis of male albino rats and normal count in reversibility experiments were observed by Sarkar et. al. (2000) with crude extract of *Piper betle*.

Nidhi Sharma and D. Jacob (2001) while working with methanol extract of *Mentha arvensis* in male albino mice reported statistically significant decrease in sperm count and vitality.

Bandivdekar et. al. (2002) tested the spermicidal activity of seed oil of *Pongamia glabra* in human and no viable sperms were present in the semen sample of human. Significant decrease in the sperm count from cauda epididymis in all treatment groups was observed by Mali *et. al.* (2002) with *Martynia annua* root extract. K Sandhyakumary et al. in 2002 studied impact of feeding ethanolic
extracts of *Achyranthus aspera* Linn on reproductive function in male rats and reported decrease in sperm number in male rats. Y.N. Seetharam (2003) studied the effect of ethanolic extract of Amalakyudi churna in male albino mice and reported significant reduction in sperm count of treated animals in epididymis.

The aqueous leaf extract of *Azadirachta indica* decreased sperm count in male albino rats as reported by Mishra et. al. (2005). Antifertility potential of aqueous extract of *Bougainvillea sperctabilis* leaves was evaluated in Swiss albino male mice by Mishra et. al. (2009), and total count of sperm was decreased. The decreased sperm count was also observed during the present study.

Significant reduction in sperm concentration after 120-180 days of treatment with methanol subfraction of *Carica papaya* seeds was noted by Manivannan *et. al.* (2009). Akinloye *et. al.* (2010) also observed significant decrease in sperm count with aqueous extract of *Carica papaya* leaf in Wistar rat.

Reduced fertility of male rats with *Tecoma stans* leaves ethanolic extract was reported by Mathur *et. al.* (2010) and significantly reduced sperm count was observed in cauda epididymis. Sathiyaraj *et. al.* (2010) using aqueous extract of *Azadirachta indica* reported significantly decreased sperm count in male albino rats. Depletion of sperm count in the drug treated animals suggests alteration in sperm production in the testes. Change in sperm count resulted in complete infertility within 30 days which ultimately gave rise to complete male sterility. Almost nil sperm count was also observed during the present study after 30 days treatment with benzene fraction of *T. bellirica*. 
Decreased sperm count in the cauda epididymis after the treatment with *Andrograpis paniculata* aqueous leaf extract was noted by K. Sathiyaraj (2011). The reduction in sperm motility in cauda epididymis is of importance with regard to fertilization. Inadequate concentration and immobility of the spermatozoa means they cannot penetrate the cervical mucus and thus fail to fertilize the ova.

Ethanolic extract of *Maytenus emargineta* significantly diminished cauda epididymal sperm motility in male albino rats (Chaudhary et. al. 2011). The dose regimens also produced a significant reduction in cauda epididymal sperm density, but after 30 days withdrawal of the treatment no significant changes were observed in recovery group. Our studies are not in agreement with this work because complete recovery was observed after 30 days discontinuation of the treatment with benzene fraction.

In 2011, Shajeela et. al. reported significant decrease in the caudal epididymal sperm count with ethanol extract of the tuber of *Dioscorea esculenta* (L.) Schott, which has been related to interference with the spermatogenetic process in the seminiferous tubules as already reported.

Mukhtar Ahmed *et.al.*, (2011) conducted SEM studies after treatment with benzene extract of *O. sanctum* leaves and it was revealed that most of the sperms showed deformities including dorsoventrally constricted head with bulged sub acrosomal material in the mid region of sperm heads and disrupted plasma membrane and acrosomal membrane particularly at the anterior region. It was suggested that these changes are probably due to the general disturbance in the protein production or metabolism.
Statistically significant reduction in the caudal epididymal sperm count was reported by Thejashwini et. al. (2012) while working with *Cyamposis psoralioides* in male Swiss albino mice. Whereas in the reversibility groups the level was increased.

During the present study also depletion of sperm count is observed in animals after feeding of the benzene fraction of aqueous extract of *Terminalia bellirica* fruits. The decreased number of sperms suggests alteration in the sperm production in testes mainly due to disturbances in the process of spermatogenesis. Arrest of spermatogenesis is in agreement with the histopathological and ultrastructural findings of the testes, which showed atrophy of germinal components and replacement of many cells by hyaline tissue. Our studies during the present project are in confirmation with the studies of other research groups with other plant preparations.

**Discussion on the Effects of Benzene Fraction on Sperm Functions**

Hypo-Osmotic Swelling (HOS) test, Acrosome Intactness (AI) test and The sperm Nuclear Chromatin Decondensation (NCD) test were conducted for assessing the function of sperms.

For assessing the membrane integrity Hypo-Osmotic Swelling test was conducted. Electron microscopy revealed disruption of plasma membrane. In HOS test also many sperms were observed with straight tail indicating unhealthy sperms.

In 2002 Bandivdekar et al. studied the effect of seed oil of pongamia and reported the hypo-osmotic swelling of the sperm and 80% of the sperm were in coiled form in the sample of human semen.
For the assessment of acrosome of the sperm the Acrosome Intactness (AI) test was performed. If acrosome is normal and is secreting desirable amount of lytic enzymes then only sperms will be able to penetrate the oocyte. During the present study many sperms were observed with abnormal head so, in AI test also no halos were observed around more than 50% sperms, indicating inability of the sperms for penetration.

For the assessment of chromatin material the sperm Nuclear Chromatin Decondensation (NCD) test was conducted. During fertilization chromatin decondenses and then fertilization takes places.

Many spermatozoa were found with already decondensed head during the present investigation.

**Discussion on the Effects of Benzene Fraction on Hormone Levels**

The process of Spermatogenesis depends upon gonadotropin hormones and testosterone. Spermatogenesis is a complex process in which germ cells supported by Sertoli cells undergo mitotic and meiotic divisions to produce elongated spermatids. Androgens produced by the interstitium cells play an important role in maintenance of spermatogenesis in all animals.

During the present study levels of testosterone, FSH and LH were measure in the blood. These levels remained unaffected with all doses and durations of the treatment. Many research groups have also studied hormone levels and some have observed no change in the level of gonadal hormones whereas others have reported reduced levels of these hormones.
The low level of testosterone was observed by Dua and Vaidya (1996), Ganong (1997) after treatment with *C. papaya* extract. They are of the opinion that papain, a biologically active compound in *C. papaya* probably crosses the blood testes barrier and interferes with interstitial cells, hence reduced level of testosterone.

Yang et. al (2006) associated decreased testosterone levels with alterations in Sertoli and Leydig cells. During the present study, the level of testosterone as well as FSH and LH were in normal range and no alteration was observed in the ultrastructure of Leydig cells.

According to the study conducted by Mishra et. al. (2009) for evaluation of antifertility potential of aqueous extract of *Bougainvillea spectabilis* leaves in Swiss albino male mice, total count of sperm decreased along with a decrease in titer of testosterone. During the present study, although decreased sperm count was observed but the level of testosterone was observed normal.

Decline in serum testosterone levels and LH levels were observed by Mali *et. al.* (2002) while working with *Martynia annua* root extract on male rats. They noticed no change in FSH level after treatment.

With methanol subfraction of *Carica papaya* seeds Manivannan *et. al.* (2009) observed that mean serum testosterone level showed fluctuations in the level of testosterone with in control range. Akinloye *et. al.* (2010) observed significant decrease in the serum testosterone concentration. Similarly, M.S. Thejashwini, *et. al.* (2012) in male Swiss albino mice reported significant reduction in the testosterone level, while the reversibility groups did not show any statistically significant difference when compared to the control groups.
Our findings are not in accordance with the work of these researchers as during the present investigation we have observed normal levels of LH, FSH and testosteron in the blood serum of experimental albino rats.

With triptolide isolated from *Tripterygium wilfordi*, Hikim et. al. (2000) reported normal levels of LH, FSH and testosteron in the blood serum of treated albino rats.

Lohiya *et. al.* (2000) while working with benzene extract of *C. papaya* seeds in langur monkey reported testosterone level within control range throughout the study period.

while working with aqueous extract of salep prepared from root-tubers of *Dactylorhiza-maculate* Kashani *et. al.* (2012) also observed no significant difference in the FSH level in control and treated groups while level of LH and testosteron hormone was significant in the experimental group rather than in the placebo and control groups.

Normal levels of testosterone, FSH and LH with both doses and duration of benzene fraction during the present study. Our results are in accordance with the finding of Lohiya and co-workers (2000), Hikim et. al. (2000) and Kashani and co-workers (2012).

For the process of spermatogenesis testosterone has shown to be essential, because it stimulates the conversion of round spermatids into elongated spermatids of the spermatogenetic cycle. Androgen deficiency disturbs spermiation process by altering spermatid-Sertoli cell junctions; which results in premature detachment of round spermatids from Sertoli cells and seminal epithelium. Decreased testosterone levels have been associated with alterations in Sertoli and Leydig cells.
During the present study the plasma levels of testosterone, FSH and LH were not significantly different in control and benzene fraction treated rats, which suggests that benzene fraction of *T. bellirica* fruits has no adverse effect on endocrine cells of the testes. This fact is supported by the histological and electron microscopical studies of Leydig cells, which showed no change in their structure.

**CONCLUSION**

Many of the antifertility compounds identified in plants to date perturb spermatogenesis by causing testicular dysfunction. Reduction in weight of the testis, epididymis, seminal vesicle and ventral prostate; suppression of spermatogenesis; degeneration of epididymal epithelium; and regression or absence of secretory activity of the seminal vesicle and ventral prostate are some of the common outcomes of the use of these plants, as well as several hundred plants undergoing preliminary screening for antifertility activity.

During the present study also reduction in the weight of testis and epididymis, and suppression of spermatogenesis due to degeneration of the cells of seminiferous tubules and abnormalities in the sperm structure were observed.

Depletion of sperm count and abnormal sperm morphology in the drug treated rats suggests alteration in sperm production in the testes and maturation in the epididymis. Changes in both sperm count and morphology will result in infertility. The abnormal sperm functions will also ultimately gave rise to complete male sterility.

Among the plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased
spermatozoa density. For male contraception, it is not necessary to stop spermatogenesis, but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in the function of the sperm.

In mammals spermatogenesis is a continuous process that results in the production of spermatozoa throughout the year. Spermatogenesis is a complex process in which germ cells supported by Sertoli cells undergo mitotic and meiotic divisions to produce elongated spermatids. The interstitial cells i.e. Leydig cells produce androgens which play an important role in maintenance of spermatogenesis in all animals.

Regarding ultrastructural changes, all categories of changes described by other researchers such as vacuolization in the germ cells such as spermatocytes and spermatids, decrease in nuclear density and disappearance of plasma membranes, degeneration of mitochondria followed by swelling and dilation of endoplasmic reticulum have been observed but Sertoli cells appeared to be unaffected.

The Sertoli cells are largely responsible for orchestrating the germ cells through sequential phases of mitosis, meiosis, and differentiation. The Sertoli cells accomplish this task by providing hormonal, nutritional, and physical support. Some drugs and toxicants injure or disrupt the function of Sertoli cells and can effectively reduce their supportive role, resulting in an increase in the elimination of the germ cell numbers via apoptosis. But during the present study normal Sertoli cells were observed indicating some different mode of action of the benzene fraction of *T. bellirica*. 
Leydig cells are known to have receptors for LH that stimulates these cells to produce testosterone. Both LH and testosterone are responsible for normal spermatogenesis in male rats. Any alteration in the plasma membrane of Leydig cells can be associated with the change in membrane receptors, hence changing the testosterone production ultimately. But during the present project no changes were observed in the ultrastructure of Leydig cells in both doses and durations of experimental animals, the fact substantiated by normal levels of testosterone measured.

Besides stimulating the process of spermatogenesis, testosterone also stimulates the conversion of round spermatids into elongated spermatids of the spermatogenetic cycle hence essential for the spermatogenesis. But during the present study, normal Leydig cells were observed along with normal level of testosterone as well as other gonadotropic hormones such as FSH and LH. So sperm deficiency and abnormal structure of sperms is of testes origin only.

During the present study, the level of testosterone as well as FSH and LH were in normal range and no alteration was observed in the ultrastucture of Leydig cells. So andorogen factor can be ruled out completely as working mode of benzene fraction of aqueous extract of T. bellirica fruits.

An important role is played by basement membrane in maintaining the integrity of tissues. It can stable the structure of tissue and send signals to the cell, therefore, alteration of basement membrane structure can impair the several functions of testes. The normal basement membranes during the present study indicate towards normal function of testes at this level. The essential interactions for spermatogenesis are
thought to be between Sertoli cells, Leydig cells, and germ cells. These cells must interact together by ECM of the basement membrane. So thickening of the basement membrane can also be one of the contributory factor for selective destruction of spermatogenic elements after treatment with benzene fraction of *T. bellirica* fruits.

Many of the antifertility compounds identified in plants to date perturb spermatogenesis by causing testicular dysfunction. Reduction in weight of the testis, epididymis, seminal vesicle and ventral prostate; suppression of spermatogenesis; degeneration of epididymal epithelium; and regression or absence of secretory activity of the seminal vesicle and ventral prostate are some of the common outcomes of the use of these plants, as well as several hundred plants undergoing preliminary screening for antifertility activity.

The arrest of spermatogenesis, decrease in sperm count and abnormal sperm morphology along these lines were also observed with the treatment with benzene fraction of aqueous extract of *T. bellirica* fruits but no changes were observed in the Sertoli cells, Leydig cells and hormone levels.

We may conclude that the benzene fraction of aqueous extract of fruits of the plant *Terminalia bellirica* is very effective as spermatogenesis inhibitory agent. It can be further added, that the damage caused by the benzene fraction was reversible in as short a period as of 30 days.

The treatment with benzene fraction of aqueous extract of fruits of the plant *T. bellirica* caused destruction in the seminiferous tubules by causing detachment of germinal components from the germinal
epithelial layer, pyknosis and clumping of nuclei. Shrinkage of tubules was also observed at some places. Thickening of basement membrane was observed. After 30 days of treatment in some experimental animals some tubules showed only thickened basement membrane and germinal epithelium. No other cellular components were present.

The destruction in primary spermatocytes, secondary spermatocytes and spermatids such as pyknosis of nuclei, disappearance of nuclear membrane, swollen mitochondria, dilated endoplasmic reticulum and disrupted Golgi bodies were observed. At ultrastructural level, type A and type B spermatogonia were unaffected. At some places developing spermatozoa were also affected and showed abnormal development.

Decrease in the weight of testes and epididymis is also in confirmation with the degeneration of spermatogenic elements in the testes and absence of spermatozoa in the epididymis. Due to the degeneration of spermatogenic elements in the testes, the decreased number of sperms were observed. The absence of sperms in the epididymis suggests an acute effect on the process of spermatogenesis. Earlier studies have been reported that morphological changes in the head of spermatozoa in general and the acrosome in particular may have resulted from an alteration in the epididymal milieu of rats treated with crude leaf extract of *Azadirachta indica* and alcohol seed extract of *Momordica charantia* suggested that these changes are due to a general disturbance of carbohydrates or polysaccharides present in the acrosome of the sperm head.
The observations of present study revealed that in the SEM studies, many sperms showed deformities including deformed and bifid head, dorsoventral constriction in the head and disrupted plasma membrane and disturbance in mitochondria, bulging of cytoplasm towards one side and presence of cytoplasmic droplets in the tail after the treatment with benzene fraction of *T. bellirica* fruits. These changes are probably due to the disturbance in general metabolism in the spermatogenic cells.

In the drug treated rats, depletion of sperm count and sperm motility suggests alteration in sperm production in the testes and maturation in the epididymis. Changes in both sperm count and motility resulted in partial infertility within seven days. This resulted in abnormal sperm functions which ultimately gave rise to complete male sterility.

Among the plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased spermatozoa density (Watche *et al.*, 2001). For male contraception, it is not necessary to stop spermatogeneisis, but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in the function of the sperm (Dwivedi *et al.*, 1990).

There can be three working modes for any antifertility agent. It might work either by preventing the formation of sperms in testes, or prevent the sperms from becoming mature or influence the sperms in such a way so that they would not be able to fertilize an egg.

If the pituitary gland is not releasing the hormones which are responsible for inducing spermatogenesis or there is degeneration in testes, in both conditions, sperm production can be prevented. It is now known that the release of these pituitary hormones is itself controlled by a
number of ‘releasing factors’ - comparatively simple chemical substances which are produced in the hypothalamus of brain and are transmitted to the pituitary. So we may say a releasing factor given to a man might stop sperm formation.

If the maturation of sperms can be influenced then also sterility can be achieved. So it will be a different approach towards the development of a male pill. After a sperm is formed, it has to undergo a maturing process within the epididymis. Some drugs might selectively affect cauda epididymal sperm maturation without interfering with the endocrine and spermatogenic status of the animal.

No such changes were observed in the epididymis at ultrastructural level which can affect the sperm maturation. The sperm morphology is also affected, but most probably it is at the level of testes as abnormal development of spermatozoa was observed in the testes only. During the hormone assay, normal levels of testosterone, follicle stimulating hormone and luteinizing hormone indicate that infertility is not caused due to disruption of hormone production. It leaves only one possible mode of action i.e. on testes.

The benzene fraction of aqueous extract of fruits of *T. bellirica* appears to work 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 left target of benzene 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.

It is a well established fact that if all other cell types have been destroyed and only speramtgonia are intact then these can ultimately give rise to all other type of spermatogenic cells. Studies at histological and ultrastructural levels reveal that spermatogonia layer was intact in all sections studied, even if all other germinal components were missing. This is the reason that after a recovery period of 30 days, normal speramtogenesis was observed.

This conclusion can be drawn 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. Leydig cells are intact, Sertoli cells are unaffected and there is no major destruction in the wall of epididymis. In the semniferous 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.

In the light of these findings, 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.

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.