CHAPTER I
INTRODUCTION

1.1 General Introduction
1.1.1 Trends in Phytotherapy

The History of Medicine dates back practically to the existence of human being. The currently accepted modern system of medicine, namely Allopathy, which has contributed significantly in combating many disease conditions, has gradually developed over the years through the scientific and observational efforts of scientists. However, it often has problems of toxicity and resistance. The earlier methods of bioprospecting were based on short duration observations. Such observations based on “Little Traditions” do not give a total picture and are likely to identify only such sources that can produce immediate effects. Retrospective analysis of the pharmaceutical development reveals that many drugs were developed through random screening of chemicals, and also some came in serendipity arising from the sharp-eyed observations of the physicians and scientists (Patwardhan, 2000, 2005). Therefore, the ignorance phase towards botanical medicines did not last long.

Fossil records date human use of plants as medicines at least to the Middle Paleolithic age, some 60,000 years ago (Solecki, 1975) and from that point, the development of traditional medicinal systems incorporating plants as a means of therapy can be traced. The value of such systems is much more than a significant anthropological or an archaeological fact. Recently, considerable attention has been paid to utilize eco-friendly, bio-friendly plant-based products for prevention and cure of different human diseases. In recent years, attention has been refocused on the plant kingdom as a potential source of new compounds for commercial use in a variety of industries including pharmaceuticals, food, cosmetics and agrochemicals. A major contribution to the pharmaceutical industry from plant sources now comprise about 25% of the prescribed drugs (Borris, 1996; Turner, 1996).

India has well-recorded and traditionally well-practiced knowledge of herbal medicines. India is perhaps the largest producer of medicinal herbs. Medicinal herbs have been in use in one form or the other under the three Indigenous systems of medicine, Ayurveda, Siddha and Unani. It is generally believed that the traditional systems of
medicine rely to a great extent on phytotherapy, which causes little side effects. Phytotherapeutics are essentially secondary metabolites of plants, produced as a defense mechanism (Balandrin et al., 1985). Therefore, phytotherapeutics cannot be easily exonerated from side effects. In fact, several plant products are toxins, carcinogens, mitotic spindle poisons, immunosuppressants, etc.

1.1.2 Male Antifertility

One major concern in male reproductive biology, which has attracted the attention of biologists and clinicians, is the deterioration of semen quality and sperm counts. The study of Carlsen et al. (1992), which was a historical analysis of 62 separate sperm count studies, concluded that human sperm counts have decreased to 50% over the past 50 years. Since then, there is a growing body of evidences supporting the idea that there is a declining trend in human sperm counts (Irvine et al., 1996; Auger et al., 1995; Swan et al., 1997; Skakkebaek, 1999; Acacio et al., 2000). Also, the cases of male infertility/subfertility due to azoospermia/oligozoospermia, caused in view of several primary/secondary manifestations, are on the increase as is seen in the long queues in infertility clinics and proliferation of centres practicing assisted reproductive technologies. Hardly any other disease or disorder of body function has such a fundamental impact on a man’s self-appreciation as infertility. In trying to find the basis for the decline in male reproductive health and the deterioration of semen quality, the major causes have been found in the environmental/industrial chemicals, dietary toxins and cytotoxic drugs, which make access into the human system mostly through occupational exposure and/or contamination of drinking water or through the dietary route. Quite a few cytotoxic drugs taken towards cure of ailments, like antibiotics, antimetabolites, immunosuppressive drugs, chemotherapeutics, receptor blockers, microtubule disruptors, etc. when intentionally might cause serious side effects including male infertility/subfertility. As regards the use of drugs, there is little choice.

1.1.3 Male Reproductive Toxicology

In the attempt made by the scientists across the world, the various researchers have established that a putative decline in the semen quality is indeed happening due to various
chemical exposures. This lead to resurgence of interest in male reproductive toxicological studies. A search through the literature for the period from 1995-1997 shows that testis has been the major emphasis in toxicology (nearly 400 papers), whereas the number of papers focused on toxicity associated with the epididymis is fewer than 90. While, historically, the testis has been implicated with compromised sperm quality and quantity, it is important to remember that the epididymis plays pivotal role in the post-testicular sperm maturation consisting of initiation into motility, acrosome stability and shedding of the cytoplasmic droplet (CD) (Bedford, 1976; Amann et al., 1993; Cooper, 2000).

Over the years, reproductive toxicological studies have classified numerous chemicals as reproductive toxicants based on changes in the epididymal sperm number and/or changes in the qualitative measures such as sperm motion and sperm morphology. Still other studies have reported toxicity based on histological and/or biochemical alterations. In many studies toxicant-induced alterations in the quality and/or quantity of epididymal sperm have often been proven secondary to testicular insult. Studies on testicular toxicants as dibromochloropropane (Whorton et al., 1977), dinitrobenzene (Hess et al., 1988; Linder et al., 1988), ethylene glycol monethyl ether (Chapin et al., 1985, 1986) and dibromoacetic acid (Linder et al., 1995) were conducted using exposures that were sufficiently long to identify testicular toxicity. The quantitative and/or qualitative effects on epidymal sperm that were observed in these studies indicated testicular toxicities. Skakkebaek (2002) reported that over the last couple of generations, people all over the world have been exposed to an increasing number of endocrine disrupters in our environment, including dichlorodiphenyltrichloroethane (DDT), PCB, certain pesticides, the phthalate DBP, synthetic steroids in meat and many other agents, which act as agonists or antagonists of sex steroids, thus, contributing to high frequency of reproductive problems.

Indeed, longitudinal evaluations of the effects of dinitrobenzene (Linder et al., 1988) and dibromoacetic acid (Linder et al., 1995), beginning around postexposure day 2 and continuing for at least 70 days revealed that it was not until two weeks post-exposure that compromise in epididymal sperm numbers, epididymal sperm motility and epididymal sperm morphology became apparent. To minimize the likelihood that toxicant-induced testicular alterations might play a role in any observed epididymal toxicity, the structure
and function of the epididymis, including qualitative and quantitative evaluations of the sperm, must be done before the sperm and fluid that are resident in the testis during the exposure period ever gain access to the epididymis. Early dominant lethality studies of methyl chloride revealed that post-implantation losses occurred as early as one week after acute inhalation exposure (Working et al., 1985). Subsequent work by Chapin et al. (1984a, b), proved that in this instance the epididymal toxicity appeared to be independent of the testis. The mutagenic agent cyclophosphamide was shown to produce post-implantation loss within one week of exposure (Qiu et al., 1992). Moreover, this exposure altered the distribution of specific epididymal epithelial cell types (Trasler et al., 1988). One of the chemicals associated with epididymal toxicity is a-chlorohydrin. Early studies demonstrated the antifertility effects occurred within only a few days of dosing (Tsunoda and Chang, 1976).

While the decrease in fertility was initially linked to granuloma formation in proximal segments of the ductus epididymidis, subsequent work indicated direct action via active metabolites on sperm through competitive inhibition of glyceraldehyde-3 phosphate dehydrogenase (Jones and O' Brian, 1990) and alterations in the protein profile (Tsang et al., 1981). However, the possibility that these alterations in sperm protein composition may be secondary to toxicant-induced compromise in secretory function of the epididymal epithelial cells has not been explored adequately. 6-chloro-6-deoxyglucose, a putative contraceptive agent, was reported to inhibit association of secretory proteins with the sperm membrane (Tsang et al., 1981). Both ethane-dimethanesulphonate (EDS) and chloroethylmethanesulphonate (CEMS) were shown to elicit profound structural and functional changes in the epididymis within four days (Klinefelter et al., 1994a, b). These changes appeared to be unrelated to circulating androgen status or testicular fluid and included an epididymal specific reduction in the cunida epididymal sperm, diminution in specific proteins in detergent extracts of sperm and disappearance in the number of clear cells of the cauda epididymidal epithelium.

While the results of many of the experiments mentioned vide supra strongly suggest that these chemicals exert a direct action on the epididymis, no in vivo model could confirm direct action. The possibility remains that toxicant-induced alteration of testis function compromises the composition of the circulation, which may in turn
compromise the epididymis. Almost all aspects of male reproduction, including germ cell differentiation, spermatogenesis, androgenesis, post-testicular mechanisms of sperm maturation, accessory reproductive gland function, endocrine/paracrine regulatory mechanisms, etc., may be affected.

In this connection with a view to overcome the side-effects of allopathic drugs, the herbal curative methodology of use of isolated/purified compound(s) mimicking the allopathic practices might also cause side effects as seen from the experience with vincristine, vinblastine, podophyllotoxins, colchicines, etc. Many such trials involving phytotherapeutics have proved that compounds with such manipulations could be potential candidates for male contraception (Stanley et al., 1993; Akbarsha et al., 1995; Akbarsha et al., 1998; Akbarsha and Murugaian, 2000). WHO has identified plant-based chemical male contraception as one of the thrust areas (WHO, 1990). This is because of the discovery that phytochemicals in several plants can interfere with the aspects of male reproduction. Partial success was made using the phytochemicals gossypol (from cotton seed) and tryptolide (from *Trypterygium wilfordii*) (Qian et al., 1986; Lohiya et al., 1990; Waites et al., 1998; Yu and Chan, 1998). Several more hundreds of plants are being screened for male antifertility property for development into male contraceptives (Handlesman, 1994; Akbarsha et al., 1995; Bai and Shi, 2002 a, b; Parveen et al., 2003; Das et al., 2004).

Plumbagin, a naphthoquinone from several species of plants, is already in use as a therapeutic. It is the aim of the present work to find the extent to which this phytochemical can bring about male reproductive toxicological manifestations, when used as a therapeutic.

1.1.4 Male Contraception

Overpopulation, under the confines of limited food supply, living conditions and health care, is a global problem with grave implications for future (Kamal et al., 2003). The need for a wider range of methods of fertility regulation for men has been a consistent recommendation emanating from a number of international fora over the past few years. This need was reiterated once again, during the Fourth World Conference on Women, held
in Beijing in September 1995. The WHO Task Force has continued to focus its research activities since 1995 on the three main areas viz., i) hormonal methods of inhibiting sperm production, ii) alternative methods of vas occlusion, and iii) novel compounds that inhibit the fertilizing ability of sperm (Griffin, 1995). This last focus has included the funding of several projects in the area of mission-oriented basic research on male reproductive physiology. Calls have increased for a wider availability of family planning facilities, and also for men to have a share in this responsibility. Interference in the female reproductive biology is the method of contraception mostly in practice and it has led to gender discrimination in practicing contraception (von Hertzen and Van look, 1996). Therefore, in the recent times there is emphasis on male contraception as well. It is now generally accepted that the currently available methods of fertility regulation are inadequate to meet the varied and changing personal needs of couples at different times in their reproductive lives, and in the widely different geographical, cultural and religious settings that exist around the world (Wang, 1999).

An ideal male contraceptive should be safe, effective and reversible and not have an effect on the libido. The current methods of contraception result in an unacceptable rate of unintended pregnancies (Henshaw, 1998). The male-specific contraceptive methods currently available are abstinence from sex, withdrawal (coitus interruptus), condoms and vasectomy. Vasectomy, once considered the most viable method, has come under severe criticism because it is not an easily reversible method, and it has led to several secondary manifestations and physiological disturbances. The concerns regarding the side effects and the inconveniences of the existing methods prevent their universal acceptance in view of the psychological perception of human species (Beckman and Harvey, 1996; Moore et al. 1996). Hence, the development of additional male methods of fertility control can provide tremendous social and public health benefits, and considerable research has focused on hormonal male contraceptives analogous to the female pills.

Suppression of spermatogenesis by sex steroids has been considered as a reversible chemical method of male contraception for a long time despite the side effects associated with the hormonal methods. One of the major barriers in development of male hormonal contraceptives has been the unfavorable pharmacokinetic profile of the available testosterone preparations, which are only injectable and require frequent administration.
More recent research on hormonal methods has sought to improve sperm suppression and prevention of the side effects related to androgens. The most studied regimens include androgen-progestin combinations that suppress gonadotrophins, thereby blocking sperm production. Such combinations have been shown to induce azoospermia or clinically significant oligozoospermia more quickly than androgens alone. Furthermore, progestins allow the use of less testosterone and, thereby, reduce androgen-related side effects. Androgens alone or progestogens combined with androgens were effective in inducing azoospermia in Caucasian men (Shearer et al., 1978). The research on hormonal methods of male contraception has resulted in marketable products commonly used for male contraception such as danazol (Dixit et al., 1981), depot medroxy progesterone acetate (DMPA) (Bhiwgade et al., 1991), cyproterone acetate (Wu, 1997), melatonin (Xiao et al., 1995), testosterone buciclate (Behre et al., 1995), androgen esters (WHO, 1996; Wu et al., 1996), levonorgestrel (Bebb et al., 1996), Ru-486 (Sanchez-Criado et al., 1999) and ignortestosterone (Nagata et al., 1999), etc. These are found to be effective in the suppression of spermatogenesis but the handicaps with long-term use relate to the disturbances caused to the normal physiology by the hormonal manipulations.

Currently, there is emphasis on immunological approach in male contraception. Antigens that stimulate the immune system to produce antibodies that are capable of interrupting the reproductive process have been under investigation for long time for their candidature to be promising vaccines. Contraceptive vaccines provide a potential for nonhormonal contraceptives. The most promising is a vaccine intended to inhibit the function of human chorionic gonadotropin (hCG) (Schwartz and Gabelnick, 2002). Several versions of this vaccine have undergone preclinical and clinical trials, but they have been mired in controversies over theoretical safety rules and potential for abuse (Schwartz and Gabelnick, 2002). Work on development of this immunocoontraceptive has, for all intents and purposes, come to a halt because of the lack of interest from pharmaceutical industries and objections from women’s health care activists (Schwartz and Gabelnick, 2002).

The observation that antisperm antibodies (ASA) inhibit sperm function in vitro, induce infertility in experimental models, and are associated with some cases of clinical infertility, provided a basis for male contraceptive development (Jensen, 2002). The
advantages of these vaccines include safety, tolerance, convenience of administration, low cost and potential for relative long lasting, yet reversible effects (Diekman and Herr, 1997). Biological targets for male contraceptive vaccines include a number of sperm-specific proteins and gonadotrophins. The potential male vaccine candidates include sperm surface protein PH-20 (Primakoff et al., 1988; 1997), testis-specific lactate dehydrogenase (LDH-C [4]) and hormone/hormone receptor-based proteins including GnRH (Miller et al., 1997), FSH (Moudgal et al., 1997), inhibin (Vanage et al., 2000), and LH & FSH (Remy et al., 1996). The efforts are only half way through.

The World Health Organization (WHO) has identified phytotherapeutic approach also as a potential male contraceptive approach (Waites, 1993). This is in view of the fact that man has coevolved and lived with plants from time immemorial, and the Natives and Tribals have been practicing several herbs towards male contraception. Unlike the other methods, which are in general not cost-effective, and several others not reversible, the phytotherapeutic approach is considered safe, cost-effective and reversible (Lohiya et al., 1999, 2002; Kamal et al., 2003). Several National Programmes have been initiated to screen the herbs for male antifertility and to identify the phytochemical basis of the antifertility. Since the work of Henshaw in 1953, there has been a steady accumulation of information regarding the screening of plants having antifertility efficacy. Various reviews on medicinal plants and their active principles for fertility regulation have come up (Kamboj and Dhawan, 1982, 1989; Akbarsha et al., 1995; Kamal et al., 2003).

Thus, male antifertility and male contraception are the two sides of the same coin.

1.1.5 Plumbagin

*Plumbago rosea*, *Plumbago europaeae* and *Plumbago zeylanica* (Fam: Plumbaginaceae) are used as herbal remedy for several ailments.

*P. zeylanica* or white lead wort is native to Southeast Asia (Fig.1). It is a much branched, evergreen shrub that grows wild in India and has been used by rural and tribal people for hundreds of years and in the Ayurveda and Siddha systems of Medicine. The leaves and roots of this plant have vesicant and caustic effect on skin (Irvine, 1961; Watt
Plumbago zeylanica L. 1 twig; 2 & 3 ovary, t.s. & l.s.; 4 pistil; 5 calyx; 6 corolla, cut open; 7-9 anthers; 10 flower.
and Breyer-Brandwijk, 1962). The plant has also been used as an abortifacient by introduction of it into the vagina (Burkhill, 1935). Lewis (1922) reported the death of an African woman who was rubbed all over the body with the bark of this plant. Masai girls of Southern Africa and the natives of Hawaii use the irritant effect of the plant to produce postinflammatory hyperpigmentation for cosmetic purposes (Watt and Breyer-Brandwijk, 1962). The leaves of this herb work well for treatment of laryngitis and rheumatism and are also found to act as an aphrodisiac. A tincture of the root bark is used as an ingredient in many products. Root powder of *P. zeylanica* contains protease enzymes, trace quantities of vitamins A, B₁, and C and was found to be a gastrointestinal flora normalizer as it stimulated the proliferation of coliform bacteria in mice (Iyengar and Pendse, 1966).

The root of *P. roseae* (Fig. 2) is used in several caustic preparations, particularly by malingerers (Behl, 1966; Quisumbing, 1951). Waring (1875), referring to *P. rosea*, noted that the fresh bark of the root rubbed into a paste and applied to the skin causes pain and ultimately blisters. The root powder of *P. roseae* produced 100% antifertility effect when administered at a dose of 100mg/kg body weight orally from D1 to D7 of pregnancy in albino rats. The dried roots were less active than the fresh root (Burkhill, 1935).

*P. europeae* has long been used in France to relieve tooth-ache (Grieve, 1995; Itoigawa, 1999; Botanical Dermatology Database, 1999 a, b). The plant reddens and vesicates healthy skin and is used as a counter-irritant (Burkhill, 1935). The plant was formerly used by beggars in Southern Europe to produce sores on the skin with the intention of inciting pity.

Plumbagin (PL) is the active principle in these and related herbs. The most exploited source of PL is the root of *Plumbago* species (*P. rosea, P. zeylanica* and *P. europea*), although it can also be obtained from the roots of tribes belonging to Droséeraceae (Harborne, 1966; Thomson, 1971). Plumbagin (5-hydroxy, 2-methyl, 1,4-naphthoquinone; C₁₁H₈O₃; Mol.Wt =188.17), a yellow naphthoquinone pigment, is soluble in organic solvents and slightly soluble in hot water.
Plumbago indica
The biological activities of the crude plant extracts from different *Plumbago* species as well as PL have been studied extensively. Plumbagin is an important naphthoquinone, which has anticancer (Parimala and Sachidanandam, 1993; Srinivasan et al., 2004), antibacterial, antifungal (Didry et al., 1994), antimutagenic and insecticidal activities (Kubo et al., 1983).

There are evidences to suggest that PL may have potential as a chemotherapeutic or a chemopreventive agent. Pure PL has been used primarily to exploit its property as a superoxide generator, an antibiotic and an antineoplastic agent (US Patent and Trademark Office, 1999). Crude extracts of PL from Plumbaginaceae and Droseraceae have long traditions of use in folk medicines. The antibacterial properties of PL are well documented in traditional medicine. The stem barks of *Pera benesis*, containing PL as the most active compound, are employed by the Chimane Indians in the Bolivian Amazon for treatment of cutaneous leishmaniasis. Plumbagin from sundew, reportedly, has great benefit in treating bronchitis and whooping cough (Itoigawa, 1999; Fournet et al., 1992; Hoffman, 1999). It has been evaluated by the Developmental Therapeutics Programme of National Cancer Institute (NCI) in its screening panel against HIV-1 and for its anticarcinogenic activity (Krishnaswamy and Purushothaman, 1980; NCI, 1999). However, PL is a redox cycling compound that is capable of generating superoxides, damaging various biomolecules (Farr et al., 1988; Babu and Brown, 1995). Very limited information on the toxicity of PL, including evidences that it may be an abortifacient, a mutagenic agent, etc., that is available raises concerns of safety when applied in therapy. No information on the metabolism, distribution and excretion of PL is identified in the available literature.

No reports of occupational exposure to PL during its production or processing are found in the available literature as no listing is found for PL in the National Occupational Exposure Survey (NOES), which was conducted by the National Institute for
Occupational Safety and Health (NIOSH) between 1981 and 1983. Human exposure to PL occurs primarily through consumption of the extracts on use.

The investigations carried out by Krishnaswamy and Purushothaman (1980) proved PL to act as a mitotic inhibitor in onion root tips by arresting cell division as expressed by occurrence of mitotic anomalies like ploidy, micronuclei, anaphase bridges, giant cells and stickiness and lagging of chromosomes. Santhakumari et al. (1980) showed lower concentrations of PL to arrest cell growth, proliferation and decrease in mitotic index with accumulation of cells in metaphase in chick embryo fibroblast cultures. At higher concentrations nuclear and cytoplasmic vacuolization, disintegration of cytoplasm, karyopyknosis and nuclear polymorphism occurred. Thus, at lower concentrations PL behaves as a spindle poison and arrests cell division, but at higher concentrations it also exhibits radiometric nucleotoxic and cytotoxic effects (Santhakumari et al., 1980).

Literature relating to the effect of PL on reproductive system suggest it to be an abortifacient (Kini et al., 1997). Preliminary studies on the effect of PL on the male reproductive system revealed testicular lesions in dogs (Bhargava, 1984), a decrease in the activity of seminal vesicles and prostate gland in gerbils (Bhargava, 1984) and decrease in the number of spermatids and spermatocytes and the diameter of seminiferous tubules and Leydig cell in rats (Bhargava, 1986).

In this background of several reports of potential application of PL as curative for cancer and other ailments, and only few cursorial studies on the male reproductive toxic effects of PL, prompted the present study be taken up to find the responses in the male reproductive system to treatment with PL, with special reference to testis and epididymis so as to project PL as a potential candidate for male contraceptive testing.

1.2 Biology of Male Reproduction

The male reproductive system consists of the primary gonad, namely a pair of testes, the testicular excurrent duct system consisting of rete testis, ductuli efferentes, epididymis, vas deferens and ductus ejaculatorius and the male accessory sex glands seminal vesicles, prostate gland and Cowper's gland.
1.2.1 The Testes

The testes performs two major functions, i) production of spermatozoa and ii) synthesis of steroid hormones. These functions proceed separately, in the neighbouring compartments. Seminiferous tubules (ST) are highly complex. ST constitutes the spermatogenic compartment (Clermont, 1972). The epithelium of the ST is formed of two kinds of cells, namely the somatic element, the Sertoli cells, and the germinal elements, the spermatogonia, spermatocytes, spermatids and spermatozoa. The steroidogenic compartment lies in the interstitium present outside the seminiferous tubules and contains Leydig cells, macrophages, mast cells and capillaries. Spermatogenesis is a continuous process involving the proliferation of spermatogonia, formation of spermatocytes and spermatids and differentiation of spermatids into spermatozoa. Steroid hormones produced by the Leydig cells take part in the control of spermatogenesis and the structure and function of accessory reproductive glands (de Kretser and Kerr, 1994).

1.2.1.1 Sertoli cells

Sertoli cells are the somatic elements of the seminiferous epithelium (Griswold et al., 1988; Grove et al., 1990; Bardin et al., 1994). They are large-sized cells resting on the basement membrane and extending up to the lumen of the tubule. They possess a pleomorphic nucleus, and a well-developed system of cytoplasmic organelles and cytoskeletal elements (Russell and Peterson, 1985; Vogl et al., 1991; Bardin et al., 1994). They develop highly complex intercellular junctions between themselves and between them and the germinal cells. With such highly complex organization, they perform a number of important functions including participation in the formation of the blood–testis barrier, supporting the differentiating germinal elements, facilitating release of spermatozoa, synthesis of a specific protein known as androgen binding protein (ABP), secretion of rete testis fluid and synthesis of specific factors/principles that regulate the differentiation of the germinal elements (Jegou, 1993; Bardin et al., 1994). The tight junctional complexes between Sertoli cells result in partitioning of the seminiferous tubules into the outer ‘basal compartment’, which is in contact with the vascular supply and contains spermatogonia and preleptotene spermatocytes, and the inner ‘adluminal’ compartment containing meiotic germinal cells and spermatids (Pelletier et al., 1997).
Testosterone secreted by the Leydig cells binds to ABP and this facilitates entry of the hormone into the seminiferous tubules where it is required for maintenance of spermatogenesis (Ritzen et al., 1981; Vogl et al., 1991). The Sertoli cell also performs the role of removing the residual body released from the differentiating spermatids, through phagocytosis, thereby cleansing the seminiferous tubules of the cell debris (Eddy and O’Brien, 1994).

1.2.1.2 Germinal cells

The germinal cells, the spermatogonia, spermatocytes, spermatids and sperm are found in an orderly sequence from the basement membrane towards the lumen of the seminiferous tubules, and are closely associated with the Sertoli cell (Chapin, 1988). The events leading from differentiation of spermatogonia towards the development of mature sperm constitute spermatogenesis. Spermatogenesis is a precisely co-ordinated process of cell differentiation. The first step in spermatogenesis is the division of stem cells, which results in a population of proliferating spermatogonia. The latter divide and develop through A1, A2, A3, A4 and intermediate stages into type B spermatogonia (Toppari and Parvinen, 1985; de Kretser and Kerr, 1994). Mitotic division of type B spermatogonia produces primary spermatocytes. The latter enter into the meiotic division and undergo morphological differentiation. This differentiation involves chromosome replication during the leptotene and zygotene stages. Pairing of homologous chromosomes results in synaptonemal complexes. Synthesis of DNA, accompanied by division of the chromosomes, results in each of the homologous chromosome splitting into two sister chromatids. Chiasmata occurs between the paired homologous chromosomes (Toppari et al., 1986; de Kretser and Kerr, 1994). Each one of these events carries tremendous cytological and genetical implications.

The division of the cell at the end of the first meiotic division produces two equivalent secondary spermatocytes. Each of these cells, after a short interphase, divides during the second meiotic division into two spermatids. Thus, during the process of meiosis one spermatocyte gives rise to four spermatids. The spermatids establish a highly complex association with the Sertoli cells.
During mammalian spermatogenesis, DNA synthesis occurs in the spermatogonia and in the leptotene spermatocytes. The rate of synthesis of DNA in spermatogonia increases progressively from A to B spermatogonia. Thus, in the rat the maximum rate of spermatogonial DNA synthesis occurs during Stage V of the seminiferous epithelial cycle. This is referred to as premeiotic DNA synthesis. The second phase of DNA synthesis occurs in the preleptotene spermatocytes. It occurs during the later part of Stage VII and during Stage VIII. Aₕ spermatogonia are the stem cells of spermatogenesis in the rat, mouse, Chinese hamster and ram. The Aₕ spermatogonia divide mitotically and the daughter cells become either two new stem cells or stay together as Aₚᵣ spermatogonia which will divide further to form chains of four, eight or sixteen Aₐᵣ spermatogonia. The Aₕ, Aₚᵣ and Aₐᵣ spermatogonia are together called undifferentiated spermatogonia (de Rooij et al., 1990). With increase in the number of Aₐᵣ spermatogonia, the cells become arrested in G₁ phase. Later, almost all the Aₐᵣ spermatogonia differentiate into A₁ spermatogonia, which form the first generation of the differentiating type of spermatogonia. The differentiating spermatogonia pass through a series of cell divisions, ultimately giving rise to spermatocytes.

The stem cell multiplication and differentiation in the primates follow a slightly different pattern (de Rooij and Vergouwen, 1991; Alder, 1996). Aᵣ and Aᵣₜ spermatogonia are the stem cells in the primate testis. All cells derived from one spermatogonial stem cell belong to what is called a clone. The cells in a clone retain morphological identity. All the cells in a clone are connected by narrow cytoplasmic bridges (de Kretser and Kerr, 1994). Spermatogonial stem cells, being highly clonogenic, are susceptible to damage by irradiation and chemicals.

1.2.1.4 Leydig cells

Leydig cells are clustered around the blood vessels of the interstitial tissue. Leydig cells are endowed with extensive machinery for steroid biosynthesis (de Kretser and Kerr, 1994; Hall, 1994). They secrete androgens and, as more recently reported, certain non-steroidal regulatory substances that regulate the Sertoli cell as well as germinal cells (de Kretser and Kerr, 1994; Hall, 1994). The androgens regulate the accessory reproductive organs. Leydig cells exert considerable control over the vasculature and thereby the entry
of blood-borne products into the testis (Setchell and Galil, 1983). Recently, there has been emphasis on peritubular myoid cells, particularly with special reference to the factor called \( PmodS \), which might regulate Sertoli cell function (Skinner and Fritz, 1986).

1.2.1.5 Endocrine and paracrine control of testis function

The growth, development and maintenance of the mammalian testis are under the control of endocrine and paracrine mechanisms. Luteinizing hormone ((LH), follicle stimulating hormone (FSH) and prolactin, secreted by the adenohypophysis, are the primary regulators of the testicular function. This secretion is in turn under hypothalamic control (di Zerega and Sherins, 1981). Thus, testicular function is under the central control. The paracrine control of the testis lies in different testicular cell types secreting substances, which affect each other. Pituitary FSH is required for initiation of spermatogenesis. Whether the testis requires a continuous support of FSH for maintaining spermatogenesis still remains enigmatic, because it has been claimed that once it has been initiated, androgens are capable of sustaining spermatogenesis even in the absence of FSH (Sharpe, 1984; Santulli et al., 1990; Seethalakshmi et al., 1990a, b). However, FSH has been shown to exert a number of effects on the Sertoli cells so as to increase their size, rate of protein synthesis and production of seminiferous tubule fluid. FSH also stimulates secretion of androgen binding protein (Means et al., 1976; Bremner et al., 1981; Orth, 1984; Barlett et al., 1986; Yoon et al., 1987). Though the involvement of prolactin in the regulation of the testis has been advocated, the precise role of this hormone is not yet known. It has been suggested that prolactin can regulate the number of LH receptors on Leydig cells (Sharpe, 1983; Sheth and Garde, 1992). Prolactin can also regulate cholesterol metabolism in the Leydig cells. Leydig cells are under the control of pituitary LH. Leydig cells secrete androgens, the principal hormone being testosterone. Androgens are synthesized from cholesterol and its ester. Synthesis of androgens from cholesterol involves enzymatic reactions, namely hydroxylation, dehydrogenation, isomerization and C-C cleavage. Cytochrome P-450 enzyme of the mitochondrial membrane is critical in the hydroxylation and C-C cleavage (Hall, 1994).

Paracrine factors produced by the Sertoli cells act on the germinal cells and form the local regulation of the spermatogenic cycle. Seminiferous growth factors are released
by Sertoli cells and form passive regulators, which permit or do not permit germinal cells to enter meiotic stage of differentiation. Plasminogen activators (PA's) are serine proteases produced by the Sertoli cells, and have been suggested to play a role in facilitating the release of mature spermatids by the Sertoli cells. Sertoli cells produce transferrin, ceruloplasmin, lactate and pyruvate in stage-specific manner and are involved in the regulation of spermatogenesis. Paracrine factors secreted by the Sertoli cells have been suggested to affect the development and function of Leydig cells. Germinal cells too have been shown to affect the functioning of Leydig cell and Sertoli cell by paracrine mechanisms (Bardin et al., 1994).

1.2.2 The Epididymis

The epididymis, as an organ, lies adjacent to the testis. It is a single convoluted duct extending from the vasa efferentia to the vas deferens. Its length varies from 3m to 80m in mammals and is surrounded by a connective tissue capsule with septulae that divide the organ into a number of lobes. Investigations on the epididymal histology started in the mid-nineteenth century and, since then, investigators have attempted to subdivide epididymis along its length into different segments basing their judgement on anatomy, histology and cytology. The epididymis was first divided into 3 anatomical segments namely the head lying on the top of the testis, the body lying along the side and the tail lying at its posterior aspect. They were respectively called caput, corpus and cauda of the epididymis (Hermo, 1995). Benoit (1926) added the concept of one more segment namely the initial segment as the earliest segment, lying ahead of the caput. Very recently another segment known as intermediate zone, between the initial segment and caput was discovered in rat (Hermo et al., 1991a, b). However, several investigators have also found more major or subtle differences in the histological organization along the length of the ductus epididymidis and have identified various zones/segments in a variety of mammalian species and presented them in a numerical order (Abe et al., 1984a, b, c; Abou-Haila and Fain-Maurel, 1984; Goyal and Williams, 1991).
1.2.2.1 Cell types in the epididymal epithelium

The contributors to these roles of the epididymis are the various epithelial cell types lining the duct which themselves together are responsible for the histological differentiation and the changing luminal microenvironment of the ductus epididymidis. The epithelium of epididymis is formed of several cell types, principal cell (PC), narrow cell (NC), apical cell (AC), clear cell (CC), basal cell (BC) and intraepithelial leukocyte cell population, named by the various authors as halo cells, intraepithelial lymphocytes (IELs) or intraepithelial macrophages (IEMs). Cell types like mitochondria-rich cells, dark cells, etc., have also been reported but such cell types do not have consistent usage in the literature.

Principal cells

Principal cells are the most abundant cell type in the epididymal lining making up about 80% of the total cells in all zones. They are tall columnar cells, spanning the entire height of the epithelium, which decreases from initial segment to cauda (Robaire and Hermo, 1988). They are densely microvillated towards the apical border with the microvilli decreasing from initial segment to cauda. From the basal portion of the microvilli, coated and uncoated pits of the apical plasma membrane are produced. Laterally, the neighbouring cells establish intercellular association consisting of zonula occludens subjoining the apical border, which constitutes the blood epididymis barrier, followed by a series of zonulae adherentes forming the tight junctions, though there could still be intercellular spaces between the PCs. Basally the cells rest on a basement membrane. The ultrastructural organization of the PC and its variation in relation to the segmentation of the epididymis has been studied extensively by several investigators, and reviewed by Robaire and Hermo (1988) and others (Cyr et al., 1995; Hermo et al., 1998; Hermo and Robaire, 2002).

The diagnostic ultrastructural features relate to the position and organization of the nucleus, the abundance and distribution of the mitochondria, the prolific organization of the Golgi complex, the dense and abundant smooth, granulated and sparsely granulated endoplasmic reticulum, the membrane-bound vesicles, coated as well as uncoated,
containing the material endocytosed from the lumen or from within, the varied
multivesicular bodies, the few to abundant lysosomes, etc., (Robaire and Hermo, 1988;
Goyal and Williams, 1991).

The PC is attributed with the following important roles in respect of processing of
the sperm and modulation of the luminal contents:

- Secretion of proteins, simple as well as conjugated.
- Fluid-phase as well as receptor-mediated endocytotic uptake of materials from the
  lumen.
- Absorption of fluid from and secretion into the lumen thereby modifying the
  composition of the luminal fluid and the concentration of the sperm.
- Contributing to the varying luminal microenvironment, etc.

Apical cells

Apical cells are confined to the initial segment (Clermont and Flannery, 1970; Sun
and Flickinger, 1979) or present even in the intermediate zone (Adamali and Hermo, 1996;
Adamali et al. 1999a). They are similar to principal cells but can be distinguished from the
location of their nuclei, which is in the upper half of the cells and the dome-shaped
luminal border with a few or without microvilli but possess luminal flaps and folds. The
cytoplasm is pale, with numerous mitochondria and vacuoles of varying sizes in the apical
cytoplasm. Pinocytic/coated vesicles are absent (Goyal and Williams, 1991). Apical cells
are intensely reactive for Yf-Subunit of glutathione-S transferase suggesting a role in the
protection of the luminal sperm from the attack of electrophiles (Adamali and Hermo,
1996). In possessing carbonic anhydrase activity, the AC’s may be concerned with luminal
acidification (Cooper, 1999). The β-hexosaminidase and cathepsin D activities indicate
role in lysosomal degradation of endocytosed proteins (Adamali and Hermo, 1996). In the
mouse epididymis these cells are involved in the synthesis of a glycoprotein, which binds
to sperm tail and prevent tail-to-tail sperm agglutination (Feuchter et al., 1987).
Immunocytochemical studies indicate ACs to possess positive sites for estradiol receptors
and cytokeratine. Mitochondrial aggregation in the apical cytoplasm suggests these cells
to play a role in the generation of ATP that is required for the transport of H⁺ and Cl⁻ ions
across the cell membrane (Goyal and Williams, 1991).
Narrow cells

Narrow cells are found only in the initial segment (Serre and Robaire, 1998) or in the intermediate zone also (Adamali and Hermo, 1996) and are identified by their deep-staining cytoplasm, dense elongated nucleus located in the upper half of the cell and an ill-defined narrow base contacting the basement membrane through a peduncle (Sun and Flickinger, 1979; Adamali and Hermo, 1996). Clermont and Flannery (1970) categorised them as non-stereociliated cells. They are characterized by electron-dense cytoplasmic matrix containing numerous cup-shaped vesicles in the most apical cytoplasm and abundant mitochondria and multivesicular bodies in the supranuclear cytoplasm (Sun and Flickinger, 1979). They may be concerned with degradation of proteins within the lysosomes, protecting the spermatozoa from the changing environment of harmful electrophiles, modification of pH in the lumen of the distal end of the ductus epididymidis and maintenance of a quiescent state in the spermatozoa (Adamali and Hermo, 1996; Abarsha and Averal, 1999a). Narrow cells are known to be endocytotic but little is known of what they endocytose but the intense localization of the lysosomal enzyme hexosaminidase which is a marker of NC, is a clear indication of a role for this cell in endocytosis (Adamali and Hermo, 1996, Adamali et al., 1999a, b).

Clear cells

Clear cells are found in the corpus and cauda epididymides. These cells are highly vacuolated in the apical region and possess dense granules above or below the nucleus. The basal region contains pale or moderately dense bodies. Nucleus is variable in position, round, pale-staining and with a prominent nucleolus. According to Abe et al. (1984c) and Abarsha and Averal (1999a) the NCs are stereociliated, whereas according to Cooper (1999) they are not stereociliated. CCs plays a role in the removal of the disintegration products of the cytoplasmic droplets released from the sperm, which are further acted upon by the lysosomal enzymes for their ultimate removal (Hermo et al., 1988; Abarsha and Averal, 1999b). They may also be concerned with fluid- phase endocytosis of proteins and their further processing through the lysosomes (Cooper, 1999). The carbonic anhydrase of the CCs may be concerned with acidification of the lumen (Cooper, 1999).
**Basal cells**

Basal cells are the second largest cell population of the epididymal epithelium along the entire length. They are flat, elongated or triangular in shape and form a network beneath the principal cells along the length of the epididymis (Yeung et al., 1994). Basal cells contain glutathione S-transferase (GST) and Cu-Zn superoxide dismutase (SOD). Therefore, it has been proposed that the basal cells play a role in detoxification mechanisms (Veri et al. 1993; Nonagaki et al., 1992). Lysosomes filled with lipofuscin pigment, in these cells, suggest that these cells have a role in scavenging reactive oxygen species (Yeung et al., 1994). In the murine and human epididymis, the basal cells express macrophage antigens (Seiler et al., 1998; Yeung et al., 1994). In the developing murine epididymis, the increase in the testicular fluid entering the excurrent duct is correlated with a higher expression of macrophage antigen (Seiler et al., 1999). However, in the Brown Norway rat, basal cells identified by an antibody against GST Yf (Veri et al., 1993) did not recognize an antibody against monocyte-macrophage (Serre and Robaire, 1999). Flickinger et al. (1997) also observed that the basal cells of the Lewis rat did not stain with an antibody against macrophages.

**Halo cells (or) Intra-epithelial lymphocytes / intra-epithelial macrophages**

Intra-epithelial lymphocytes / intra-epithelial macrophages are found at all levels of the epididymal epithelium. The nature of these cells has been a subject of controversy since their discovery by Reid and Cleland in 1957. They can be distinguished by the dense nucleus with patches of peripheral condensed chromatin and pale staining cytoplasm (Robaire and Hermo, 1988). They are migratory cells and found at different heights along the epithelium in clear spaces. They are believed to form an immunological barrier (Wang and Holstein 1983; Robaire and Hermo, 1988). Flickinger et al. (1997) proposed that IELs in the epididymal epithelium are CD8\(^+\) leukocytes and those in the interstitium are macrophages. Recently, antibodies labeling the three main types of immunocompetent cells have been used to resolve the distribution of T lymphocytes, B lymphocytes, monocytes and macrophages in the reproductive tract. The ED1 antibody distinguishes an intracytoplasmic antigen in monocytes, tissue macrophages and free macrophages (Damoiseaux, 1994). In the epididymal epithelium of young Brown Norway rats, the
number of cells that stain for antibody against monocyte-macrophages (ED1\(^{+}\)), helper T lymphocytes (CD4\(^{+}\)) and cytotoxic T lymphocytes (CD8\(^{+}\)) is equivalent to the number of halo cells (Serre and Robaire, 1999).

**Quantitative Histology**

Quantitative histological data regarding the relative abundance of the various cell types along the length of the epididymis are available (Robaire and Hermo, 1988). By far, PCs are the most numerous constituting 60 – 80% of the epididymal epithelium, followed by CCs (5% to 30%), BCs (12% to 15%) and the IELs/IEMs (3.8% to 10%). The NCs confined to the initial segment and the intermediate zone alone constitutes about 3% (Robaire and Hermo, 1988).

**1.1.2.2 Epididymal lumen**

The milieu surrounding the spermatozoa is certainly the complex fluid found in any exocrine gland. This composition results from i) continuous and progressive changes in its composition throughout the male excurrent duct, and ii) the presence of components in unusual concentrations, some of which are not found in any other body fluid (Daucheux et al., 1998). The first proof that epididymal cells are able to synthesize and secrete proteins and glycoproteins was obtained by autoradiographic studies (Neutra and Leblond, 1966; Vendrley and Durliat, 1968). The characterization of such proteins was achieved when proteins of rete testis fluid and cauda epididymal fluid were resolved using SDS-polyacrylamide gel electrophoresis (Koskimies and Kormano, 1975). Different cellular secretion pathways have been proposed in several species, but the mode of release into the epididymal lumen remains uncertain, *i.e.*, whether it is apocrine or merocrine (Hermo and Robaire, 2002). However, *in vivo* studies (Turner et al., 1994; 2000) and *in vitro* incubation of isolated epididymal tubules (Syntin et al., 1996) show that most of the proteins are secreted into the lumen. About 60% of the total protein synthesis in the proximal region in rodents is released into the lumen of the duct. In the corpus and cauda epididymides, the proportion decreases to 20-40% (Vreeburg et al., 1990; Turner et al., 1994). In rats, about 205 spots have been detected corresponding to 87 proteins secreted in proximal part of epididymis (Turner et al., 2000).
The lumen of the epididymis contains spermatozoa and a fluid whose composition with respect to ions, small organic molecules, protein and other larger macromolecules has clearly demonstrated that the environment to which spermatozoa are exposed during their epididymal transit is continuously undergoing major changes (Hinton and Palladino, 1995; Hinton et al., 1996; Wong et al., 2002). The region-specific gene expression in the epididymal epithelium may contribute to such continually changing microenvironment (Kirchoff, 1999), which plays a major role in sperm maturation (Hinton and Palladino, 1995; Leung et al., 1998; Jensen et al., 1999; Fouchecourt et al., 1999). There is also a view holding epididymal duct as a protector of maturing spermatozoa (Hinton et al., 1995; Fouchecourt et al., 1999).

1.2.2.3 Roles of the Epididymis

The anatomical/histological segmentation of the epididymis has a bearing on the role of the epididymis in the processing of the sperm. The epididymis performs such roles as sustenance and protection of spermatozoa, contribution to maturation and storage of spermatozoa, and transport of spermatozoa to the distal parts. The initial segment is the most critical part of the epididymis contributing mainly to reabsorption of fluid arriving from the testis through the ductuli efferents, leading to increased concentration of the sperm and considerable change in the ionic composition of the luminal fluid (Leung et al., 1998; Clulow et al., 1998) secretion of several proteins, glycoproteins and small molecular weight compounds (Hinton et al., 1995; Cooper, 1998; Dacheux et al., 1998) and endocytotic uptake of particulate as well as dissolved materials from the lumen (Robaire and Hermo, 1988; Hermo et al., 1991c, d). The sperm thus, concentrated and lying in a medium, modified in ionic composition and containing the secretory products of the initial segment, arrive at the intermediate zone, which is concerned with secretion as well as endocytosis (Hermo, 1995). At the caput, the epithelium further secretes proteins, glycoproteins and small molecular weight compounds. These secretory products interact with the sperm in various fashions, which include addition of new proteins to the sperm surface, deletion of some of the existing proteins and modification of the existing proteins (Cooper et al., 1998). In this process the spermatozoa undergo considerable changes in the surface domains (Eddy and O’Brien, 1994; Cooper, 1998). It is believed that these changes to the sperm are essential for them to acquire motility and fertilizing ability. The
sperm, thus altered, arrive at the corpus where the role of epididymal epithelium is not
despite known, but the interaction between the spermatozoa and the luminal content
towards the physiological maturation of the sperm continues (Yeung et al., 1994). The
cauda is essentially considered as an organ of storage of sperm until ejaculation though it
may also be concerned with cleansing the dead, defective and aged spermatozoa.

Another important event towards the physiological maturation of sperm concerns
the cytoplasmic droplet (CD). At the time of release from the seminiferous epithelium, the
spermatozoa posses a small droplet of cytoplasm, the CD, attached to the neck region.
During the transit of spermatozoa into the caput the CD migrates along the mid-piece of
the flagellum. Wherein the corpus the CD is laterally displaced and shed and, thus, in the
cauda the spermatozoa are generally devoid of the CD. The droplet, thus shed, undergoes
fragmentation in the lumen of the cauda due to enzymatic action of the luminal content
and such fragments are endocytosed by the epithelium (Janulis et al., 1996). The shedding
of CD by the sperm is generally considered as an important requirement for motility of the
sperm to fertilizing the ovum (Janulis et al., 1996; Yeung et al., 2000; Akbarsha et al.,
2000a).

Epididymal processing of the sperm is known to occur over a specific length of
time. It is known that the sperm spend 10-11 days in the ductus epididymidis in mouse and
rat and undergo the various changes towards their physiological maturation. They remain
viable in the cauda in the absence of ejaculation for upto 5 days in the rat and 6 days in the
rabbit (Robaire and Hermo, 1988). Data are also available on the duration for which the
sperm remain in the different segments of the epididymis (Jones and Clulow, 1987). In the
recent times, there is also emphasis on a continuously changing luminal microenvironment
of the ductus epididymidis in relation to the physiological maturation of the sperm. A
number of papers have reported change in the luminal microenvironment including the
fluid, ions, proteins, etc. Such a variation in the luminal microenvironment and the
constancy at the respective segments are critical factors in the epididymal sperm
maturation process (Hinton and Palladino, 1995; Wong et al., 2002).
1.2.2.4 Endocrine Control of Epididymis

Pioneering studies for more than six decades have established epididymis as an important target organ for androgens (Mann, 1964; Robaire and Hermo, 1988). Investigations have produced evidence indicating the 5α-reduced metabolite of testosterone, namely 5α dihydroxytestosterone (DHT), as responsible for maintaining the epididymal structure and function (Gloyna and Wilson, 1969; Robaire et al., 1977). The epididymis receives a dual supply of androgens namely circulating androgens (CA) and luminal androgens (LA), in which the concentration of CA reaching the epididymis is the same at all regions and that of LA differs depending upon the region (Boujard et al., 1982; Brooke, 1983; Turner, 1984). The entry of luminal testosterone, a substrate for 5α-reductase enzyme, turns on the 5α-reductase gene, providing a segment-specific regulation for maintenance of epididymal function (Turner, 1991; Robaire and Viger, 1995).

1.3 Hypothesis and Objectives of the present study

Plumbagin, a secondary metabolite of Plumbago spp., has been primarily exploited for its antibiotic, antineoplastic, antibacterial and anticancer properties. Although only very little information on toxicity of PL, its metabolism, excretion, etc., are available, recent publications on the mechanisms of action of PL on various types of cancer cell lines indicate it to be a phytotherapeutic, with all prospects to be developed into an anticancer drug. It is in this context, it has also been shown that the therapeutic property of PL lies in its capacity to generate electrophiles in excess of what the cell can manage (Munday and Munday, 2000). In the in vivo situation, this can affect the normal cells as well. Thus, it is hypothesized that PL, when used as a therapeutic, can affect the non-target tissues. Among these tissues, the male reproductive tissues can be highly vulnerable targets in view of the clonogenicity of the germinal cells and the complexity of the structure and function of the epididymidal epithelial cell types. In other words, it is hypothesized that PL is a potential male reproductive toxicant. If this hypothesis is proved, then PL could be recommended for male contraceptive testing.
Thus, following are the objective of the present work:

1. To evaluate the male reproductive toxic effects of PL with special reference to testis, epididymis and sperm.

2. To test the efficacy and potential of PL to be a candidate for investigation towards its development into a male contraceptive agent.