Chapter I

Introduction
Medicinal plants are one of the precious blessings of nature for the humans, especially for livelihood of poor communities and tribal races among all over the world. Generally medicinal plants are phanerogams and more than 10 percent of higher plants are of medicinal value (Prance, 2001). In spite of human beings, animals also use medicinal plants for their self medication (Zoopharmacognosy). It is found that people suffering from side effects caused by synthetic drugs move towards natural herbal products during search of alternative modes of treatment. An adverse drug reaction (ADRs) causes 3% death and 12% hospitalization in Sweden and 5% death in United States (Ambasta et al, 2016). However Fatal Adverse Drug Reactions (FADRs) in the plants medicine are very low thus provide scientific explanation for utilization of medicinal plants. FADRs are regarded as seventh most common death cause in Sweden. It is widely reported that there is presence of disease inhibitory substances in the herbal medicine, which supports the use of medicinal plants in traditional practices (Shinwari et al, 2009).

India accounts for 8% of the total global biodiversity with an estimate of 49,000 species of plants, among which 4900 are endemic. Due to tremendous rising of global population and anthropogenic activities, there
is excessive eroding of natural ecosystem so many of them are facing extinction. There is no reliable figure for total number of medicinal plants on earth and number and percentage for country and region vary greatly. The number of species used medicinally includes 35000-70000 or 53000 worldwide (Schippmann et al., 2002) and 7500 in India (Shiva, 1996).

Though, India has rich biodiversity but growing demand causes heavy strain on existing resources leading to a number of species in the category of either threatened or endangered. Over 70% of medicinal plants collection involves destructive harvesting due to use of plant parts like roots, barks, wood, stem or whole plants in case of herbs. This possesses a definite threat to the genetic stock or to the diversity of medicinal plants. There is rapid loss of traditional medical knowledge and practices due to their dependency on verbal transformation, impacts of modern cultural transformation and rapid land degradation (Manandhar, 1990; Caniago and Siebert, 1998; Joshi and Joshi, 2000). At the same time there is depletion of resources due to over exploitation and lack of management system (Malla et al, 1995).

Globally, the IUCN has estimated about 12.5% of total world vascular plants, totaling about 34,000 species are under varying degree of threat (Phartyal et al., 2002). IUCN recognizes the following categories: Extinct, Extinct in wild, critically endangered, endangered, vulnerable, near
threatened, least concern, data deficient and not evaluated, while critically endangered, endangered and vulnerable together constitute threatened category. Species with small population, at present not endangered or vulnerable but are at risk; due to localized and restricted geographical area or thinly scattered (Singh et al., 2006). A species may become threatened and vulnerable with extinction due to natural and manmade causes (Singh and Choudhary, 2002). According to WHO 80% of world population uses herbs for their treatment, resulting increased demand for medicinal plants (Kala, 2005).

Herbal medicines are currently in demand and their popularity are increasing day by day (Verma and Singh, 2008). The plant kingdom represents an enormous reservoir of chemical compounds. India possesses an extremely rich biodiversity and these provide numerous plants with medicinal value. It is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world (Ahmedulla and Nayar, 1999). India is one of the 12 mega biodiversity centres having about 10% of the world’s biodiversity wealth which is distributed across 16 agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes. Out of 17,000 species of higher plants reported to occur within India, 7500 are known to have medicinal uses (Shiva, 1996). This proportion of medicinal plants is the highest known in any other country against the existing flora of that country (Kala, 2006). The interest in
nature as a source of potential chemotherapeutic agents is continuing. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world today. Higher plants contribute no less than 25% of the total (Farnsworth and Soejarto, 1985; Cragg and Newman., 2005). Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (Balick and Cox, 1997). Later on chemical principles were established and chemicals were isolated for clinical trials.

Ayurveda is the oldest medicinal system in India. It is derived from the Indian words ‘Ayar’ (life) and ‘veda’ (knowledge or science) and hence means the science of life. It is similar to Galenical medicine in that it is based on body humours (dosas) and the inner life force (prana) that is believed to maintain digestion and mental activity. The living and the non-living environment including humans are considered to be elements: earth, water, fire, air and space. For an understanding of these traditions, the concept of impurity and cleansing is also essential. Illness is the consequence of imbalance between the various elements and it is the goal of the treatment to restore this balance. The ayurvedic system of medicine uses about 700, Siddha uses about 600, Amchi uses 600 and modern medicine about 30 species (Joy et al., 2001). About 8000 herbal
remedies have been codefined in Ayurveda. The Rigveda has recorded 67 medicinal plants, Yajurveda 81 species and Atharvaveda 290 species.

The Charak Samhita, an age-old written document on herbal therapy, reports on the production of 340 herbal drugs for curing various diseases (Prajapati et al., 2003). Charaksamhita and Sushrut Samhita had described properties and uses of 1100 and 1270 species respectively in compounding of drugs and these are still used in the classical formulations. Approximately 25% of drugs are derived from plants and many others are synthetic analogues build on prototype compounds isolated from plant species in modern pharmacopoeias (Rao et al., 2004).

Withania somnifera (L.) Dunal commonly referred to as Indian ginseng. It is an erect, evergreen, perennial shrub and a member of family Solanaceae. It is a widely used medicinal plant in the treatment of inflammations and as anti-tumour agent (Naidu et al., 2003). It is well known for years as an important drug in Ayurvedic literature. Roots of the plant Withania somnifera (Ashwagandha) reportedly exhibit antioxidant, immunomodulatory and haematopoietic properties (Mishra et al., 2000). Roots are prescribed as medicines for hiccups, several female disorders, bronchitis, rheumatism, dropsy, stomach and lung inflammation, and skin diseases. The ingredients in medicines are prescribed for curing disability and sexual weakness in males (Joshi et al.,
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2010). According to red list of threatened species, 44 plant species are critically endangered, 113 endangered and 87 vulnerable. *W. somnifera* proved to be 99.75% of the endangered medicinal plant (Siddique et al., 2005; Rahman, 2001). As over harvesting of *W. somnifera* that plant root is going to be endangered in the Southern India (Manickam et al., 2000). The active pharmacological components of *Withania somnifera* constituents are withanolides (Steroidal lactones with ergostane skeleton) and alkaloids (Elsakka et al., 1990). The active content of Indian *Withania somnifera* are withaterin-A and withanolide-D, both are present in leaves and roots of the plant. Total alkaloid content in the root of the Indian type has been reported to be between 0.13 to 0.31% of this plant and showed antitumor and radio sensitizing effects in animal models (Sharma et al., 2009). It also possesses anti-stress, immunomodulatory, anti-oxidant and antibacterial activity (Kupchan et al., 1965; Devi et al., 1992; Devi et al., 1993).

Cultivation of medicinal plants especially of high value is creating new dimension in the field of agriculture. Indian herbal industry is at blooming stage. However, cultivation of medicinal plant is not easy. It is a challenging task due to very little knowledge of seed biology. Many efforts have not been made to search elite specimen and their propagation. *Withania somnifera* generally grows on lower as well as higher altitude in rare thus needs conservation. It should be propagated on
lower altitudes to have its commercially significant products like secondary metabolites. Secondary metabolites of *Withania somnifera* have got potential application as therapeutics. As germination of seed in this plant is very poor, it is better to grow the plant, *in vitro* conditions from various explants. The technique of micropropagation is applied with the objective of enhancing the rate of multiplication. Through the culture, over a million of plants can be grown from a small piece of plant tissue within 12 months. Such proliferative rate of multiplication can not be expected by any *in vivo* methods. The natural habitats for medicinal and aromatic plants are disappearing fast and together with environmental and geographical disabilities to get plant derived components have become difficult. Industries and scientists should consider the possibilities of investigation of *in vitro* culture as an alternative supply additional agriculture. The provision of alternative sources of *Withania somnifera* by encouraging its cultivation will go a long way in reducing their heavy dependence on the wild populations. Conventional propagation methods have proved to be inadequate to meet this challenge. Large scale production through plant *in vitro* regeneration will provide a means of putting the plant onto the market at lower prices. In addition, the technique is cost effective, relatively simple and can be performed by semi-skilled persons.
1.1 Tissue culture

Tissue culture is the growth of tissues or cells separated from the organism. This is typically facilitated via use of a liquid, semi-solid, or solid growth medium, such as broth or agar. Tissue culture commonly refers to the culture of animal cells and tissues, with the more specific term plant tissue culture being used for plants. The term "tissue culture" was coined by American pathologist Montrose Thomas Burrows, M.D (Carrel and Burrows, 1911).

In plant tissue culture explants are used to initiate their growth in culture. The none dividing, differentiated and quiescent cells of the explants when grown on a nutrient medium first undergo changes to achieve the meristematic status. The phenomenon of matured cells reverting to a meristematic and forming undifferentiated callus tissue is termed as dedifferentiation. The ability of the component cells of the callus to differentiate into a whole plant or a plant organ is termed as redifferentiation.

The role of medicinal and aromatic plants in pharmaceuticals, perfumery, flavour and cosmetic industry is strengthening India’s economy needs no elaboration. Because on increased demands, over exploitation and ruthless collection of these plants from natural resources have resulted in their rapid depletion resulting in endangered status and extinction of
various species. Therefore, utmost important of today is to expedite the pace of propagation of fast depleting species and also promising strategies for their conservation.

Plant tissue culture has stepped in as a promising tool to complete this task because it aims at the production of plants at large scale from small pieces of stock plants in relatively short periods of time in small area without any physiological barriers and seasonal interruption which results in biomass increase. It has a good means not only for the propagation and commercialization of existing germplasm but also for the conservation of genetic resources. Moreover, it will also help in the production of genetically upgraded plant population which can fruitfully be employed in plantation crop, aromatic, medicinal plants and condiments most of which are vegetatively propagated (Paramageetham, 2000). The science of plant tissue culture takes its roots from path breaking research in Botany like discovery of cells followed by propounding of cell theory that cell is the basic unit of organisms. Based on this principle a German physiologist developed the concept of *in vitro* cell culture. He isolated single fully differentiated individual plant cells from different plant species like palisade cells from leaves of *Laminum purpureum*, glandular hair of *Pulmonaria* and pith cells from petioles of *Eichhornia crassipes* etc. He was first to culture them in Knop’s salt solution enriched with
In his cultures, cells increased in size, accumulated starch but failed to divide. Therefore, Haberlandt’s prediction failed that the cultured plant cells could grow, divide, develop into embryo and then to whole plant. A term coined for the potential of a cell is known as totipotency (Steward, 1968). Despite lack of success, Haberlandt made several predictions about the requirements of media in experimental conditions which could possibly induce cell division, proliferation and embryo induction. Haberlandt is thus regarded as father of tissue culture.

Taking cue from Haberlandt’s failure, Hannig chose embryogenic tissue to culture (Hannan et al., 2007). He excised nearly mature embryos from seeds of several species of crucifers and successfully grew them to maturity on mineral salts and sugar solution. For about next 30 years upto 1934, there was very little further progress in cell culture research. Within this period, an innovative approach to tissue culture using meristematic cells like root and stem tips were reported independently (Robbins, 1922). All these research attempts involving culture of isolated cells, root tips or stem tips ended in development of calluses.

There were two objectives to be achieved before putting Haberlandt’s prediction to completion. First, to make the callus obtained from the explants to proliferate endlessly and second to induce these regenerated calluses to undergo organogenesis and form whole plants. It was in
1930s, when progress in plant tissue culture accelerated rapidly owing to an important discovery that vitamin-B and natural auxins were necessary for the growth of isolated tissues containing meristems. This breakthrough came by reporting that not only could cultured tomato root tips grow but could be repeatedly subcultured to fresh medium of inorganic salts supplemented with yeast extract (White, 1934). He later used vitamin B namely pyridoxine, thiamine and proved their growth promoting effect (White, 1937). First plant growth regulator (PGR) was discovered indoleacetic acid (IAA). IAA is a naturally occurring member of a class of PGRs termed ‘auxins’. The successful culture of cambium cells of several tree species to produce callus and addition of auxins enhanced the proliferation of his cambial cultures (Gautheret, 1983). Further research made possible the continuous callus cultures of carrot slices and tumour tissues of hybrid *Nicotiana glauca* x *N. langsdorffii* (White, 1939). Thus, the possibility of cultivating plant tissues for an unlimited period was independently endorsed.

Adding to the ongoing improvements in the culture media, growth of seedlings from heart shaped embryos by enriching culture media with coconut milk besides the usual salts, vitamins and other nutrients was achieved. This provided tremendous impetus for further work in embryo
culture. Stem tip cultures yielded success when the exact part of shoot meristem identified gave rise to whole plant (Ernst, 1976).

After 1950, there was an immense advancement in knowledge about effect of PGRs on plant development. The fact that coconut milk is nutritional requirement for tobacco callus besides auxin indicated the non auxinic nature of milk. This prompted further research and so other classes of PGRs were recognized like induction of cell division and bud formation in tobacco by adenine (Skoog and Tsui, 1948). This led to further investigations of ‘kinetin’- a derivative of adenine (6-furyl aminopurine) (Skoog et al., 1965). Kinetin and many other similar compounds which show bud promoting activities are collectively called cytokinins, a cell division promoter in cells of highly mature and differentiated tissues. They worked further to propose the concept of hormonal control of organ formation (Skoog and Miller, 1957). Their experiment on tobacco pith cultures showed that high concentration of auxin promoted rooting and high kinetin induces bud formation or shooting. However, now the concept is altered to multiple factors like source of plant tissue, environmental factors, composition of media, polarity and growth substances being responsible for determination of organogenesis. Besides PGRs, scientists tried to improve culture media by differing essentially in mineral content. In this direction, they prepared
a medium by increasing the concentration of salts twenty-five times higher than Knops (Murashige and Skoog, 1962).

Next step for realization of Haberlandt’s objectives was development of whole plant from the proliferated tissue of these cells. In 1966, the classical work of Steward on induction of somatic embryos from free cells in carrot suspension cultures brought an important breakthrough by finally demonstrating totipotency of somatic cells, thereby validating the ideas of Haberlandt (Steward et al., 1964). This ability of regenerating plants from single somatic cell through normal developmental process had great applications in both plant propagation and also addition of auxin enhanced the proliferation of his cambial cultures. Anther culture of *Datura* and raised embryos which developed into haploid plants initiated androgenesis (Guha and Maheshwari, 1966).

This discovery received significant attention since plants recovered from doubled haploid cells are homozygous and express all recessive genes thus making them ideal for pure breeding lines. Traditionally, plants have been collected for medicinal use from wild areas. In natural product research, the presence of large amount of plant biomass is necessary to provide enough bioactive compounds from the plant tissue. This presents an unfeasible solution due to the lack of reliable and abundant supply of the plant material. Natural habitats for medicinal plant are disappearing
fast and together with environmental instabilities, it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of using cell cultures as an alternative supply for the production of plant natural products (Dicosmo and Misawa, 1995). Plant cell cultures have the potential of providing a low cost route to numerous plant derived natural products that are very expensive to synthesize chemically or that occur naturally at very low concentration. Propagation of plants through tissue culture has become an important and popular technique. The continuous supply of plantlets will overcome the contamination problem and reduce the time for sterilization process. Tissues from various organs such as stem and leaf of the axenic plantlets can be induced to form callus. Callus tissue can serve as an experimental system to investigate the biological activities using specific bioassays. However, many factors contributed to the ability of a specific tissue to form callus such as medium and plant growth regulators.

In vitro propagation of medicinal plants with bioactive principles and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants.
During the past few decades, tissue culture techniques have been manipulated for many purposes such as for the improvement of plants growth, secondary metabolites production, biological activities and transformation. A significant advance in plant tissue culture techniques have led to the use of callus and cell suspension culture (undifferentiated cells) of some plant species for the study of biological activities and production of valuable secondary metabolites (Mulabagal et al., 2004; Bestoso et al., 2006). Cell suspension cultures have been sought to deal with problems of low concentrations secondary metabolites in whole plants, like artemisinin (Basile et al., 1993) and paclitaxel (Luo et al., 1998).

*In vitro* propagation of different plant species have shown that the tissue culture technique may be a solution for rapid propagation of such selected useful plant species and their subsequent exploitation. It has also been found that the explants of an alkaloid producing plant cultured *in vitro* retain the capacity to synthesize alkaloids to the same extent to that of intact plant (Bhatt et al., 2008). Technology for large-scale plant cell cultures has been demonstrated in Japan with the production of shikonin by Mitsui Chemicals Industry Ltd., and production of ginseng, saponins by Nitto Denko Co. (Ushiyama, 1991). In the present work, tissue culture of *Withania somnifera* has been done.
1.2 Reasons for choosing *Withania somnifera* for the studies:

These plants have been sources of medicine right from the ancient time. So, many books and articles have been written so far on the medicinal and other values of this plant. They are most popular herbs related to herbal conservation in all discussions, symposia, seminars, etc. These glorious herbs were found in abundance once upon a time in the world. Now a day, over-exploitation of these plants by local people will make them rare and endangered in near future.

This work aimed at development of a more efficient common protocol for callus induction, whole plant production, transfer of *in vitro* grown plant to soil and further studies by optimizing the growth regulators such as auxins and cytokinins.

Due to these reasons, the said plant was selected for the study and made them an important issue so that conservationists, botanists, entrepreneurs and NGOs come forward to rescue and save it for future uses.

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