CHAPTER 1

INTRODUCTION

1.1 Medicinal Plants: Uses, Diversity

Medicinal plants are basic raw material for the production of Ayurveda, Siddha and Unani medicines and are utilized in manufacturing medicines because they have curative powers and healing properties that is because presence of active ingredients. Apart from this, they are also used as natural flavoring agents, cosmetic ingredients, etc. Nirmal et al. (2013) reported medicinal herbs usage by industry in terms of sales i.e. essential oil (7%), spices and herbs (11%), natural cosmetics (12%) and pharmaceuticals (70%). Moreover herbal material such as gums, fixed oils, essential oils, resins extracts, etc. are also extracted from plants. These medicinal practices used especially in developing countries, for application in pharmaceutical, nutrition, perfumery and cosmetic fields. Medicinal herbs are widely used as diet supplements and treating illness too.

According to the International Union for Conservation of Nature (IUCN) and the World Wild Fund for Nature (WWF), 50,000 - 80,000 flowering plant species are used for medicinal purposes worldwide. More than one-tenth of plant species are used in drugs and health products i.e. > 50,000 species are being used. The number of medicinal plants present in different parts of the world vary, (Huang, 2011; Rafieian-Kopaei, 2013) and China and India have the highest numbers of plants with 11,146 and 7500 species, respectively. This is followed by Colombia, South Africa, United States and other 16 countries with percent of medicinal plants ranging from 7% in Malaysia to 44% in India versus their total numbers of plant species (Marcy et al., 2005; Hamilton, 2008; Srujana et al., 2012; Rafieian-Kopaei, 2013).

As stated by the Government of India (GoI), traditional medicines are being used till date as sole means of health care for about 65% of the population (Srivastava, 2002). It is also reported that almost 85% of traditional medicine formulations utilized plants or plant extracts (Vieira and Skorupa, 1993), and this demand is enhancing rapidly throughout the world (Nalawade et al., 2003; Cole et al., 2007). World Health Organization (WHO) postulated that the requirement of medicinal plants is approximately US $ 14 billion per year which will increase at the rate 15-25% annually, and will reach to more than US $ 5 trillion by 2050 (Sharma and Thokchom, 2014). India has covered 2.4% of worlds' area and is one of the hot spot country among the 12 mega-diversity hot spot countries with 8% of global biodiversity of medicinal plants in all three level viz. species diversity, genetic diversity and habitat diversity (Sharma and Thokchom, 2014). Across the country the forest is estimated to harbor 90% of total medicinal plants and it contains 34 hot spots out of which the two are major-

Eastern Himalayas and Western Ghats. India is reported to have 45,000 plant species among them 15,000-20,000 plants are known to have medicinal properties, around 7500 plant species are being used as medicinal plants with an annual demand of US $ 4 billion/year (Sharma and Thokchom, 2014). The report also states that the US is the biggest importer of medicinal herbs from which manufacturing of important products from India in 2013.

Gujarat is also a major contributor towards the biodiversity, as out of sixteen forest types found in India, four are present in the state. Out of ten biogeographical zones of India four are located in Gujarat. Despite its adverse geo-climatic conditions, the state has a remarkable diversity of plant species, there are ~4,320 plant species and 1,315 species have medicinal value which constitute an important component of the biodiversity. Around 1016 plant species are wild where as 299 species are being under cultivation of plantation. Out of all these 102 species needs immediate conservation strategies and as 76 falls under rare category they need to be protected and propagated. In Gujarat, different zones like South Gujarat, Southeast Gujarat, Central Gujarat, North Gujarat, Saurashtra and Kutch harbors dense population of medicinal plants (Fig. 1) (Vibrant Gujarat, 2017). As Gujarat is an industrially developed state, there are nearly 605 ayurvedic pharma industries are present, which use medicinal plants as raw material.

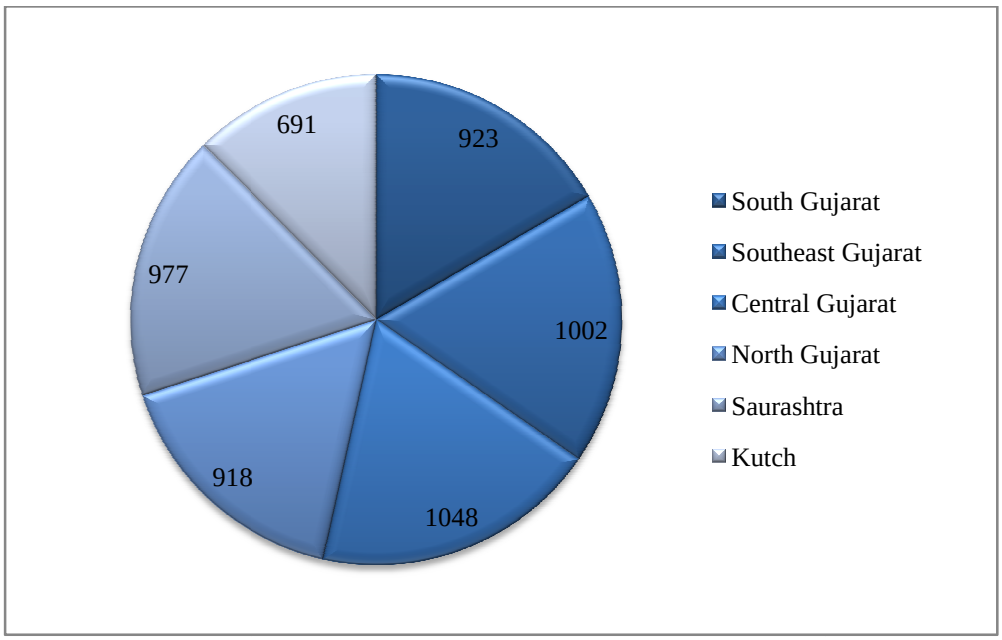

Figure 1. Medicinal plant species availability in different zones of Gujarat.

Thus to cope up with the market demand of herbal formulations, cosmetics and other natural products leads to indiscriminate harvesting of medicinal plants from wild which is one of the main reason for exploitation of wild resources. Along with this, anthropogenic activities like forest degradation, agricultural encroachment, urbanization etc. are other factors due to which natural plant resources are being lost and many of them are now in threatened and endangered categories (Gupta et al., 1998; Tripathi, 2008). Therefore, the sustainable use of medicinal plants should be considered, and good harvesting practices must be formulated.

1.2 Need for Conservation of Medicinal Plants

Medicinal plant resources are being harvested in high volumes from natural habitats and it is nearly increasing by 8-15% per year in different parts of the world in recent decades (Ross, 2005; Bentley, 2010). The flora of the world is being destroyed at an alarming rate. The tropical moist forests and about half the world's plants are in declining stage and estimated 16.8 million ha/annum consistent with UNEP/FAO. Combined with exploitation, this is often pushing many medicinal plants in grave risk of genetic erosion and even extinction. In view of the aforesaid reasons, there is a need to conserve and to propagate important medicinal plants. Conservation aims to support sustainable development of important and useful species which are on the verge of extinction due to over exploitation and habitat destruction. It has been documented that more than 95% of the medicinal plants are collected from the wild, and a number of them are pushed into an endangered category in their natural habitat (Lakshman, 2016). Thus, there is a need to encourage multiplication and cultivation of these plants which can become source for raw material and collection from wild resources can be stopped. In order to achieve this, basically two methods for conservation of plant genetic resources like *in situ* and *ex situ* conservation needs to be undertaken. For welfare of human beings and sustainable development of environment, natural resources of medicinal plants needs to be conserveed by different techniques and various sets of recommendations have been developed, such as providing both *in situ* and *ex situ* conservation (Huang, 2011, Liu et al., 2011).

1.2.1 *In situ* Conservation

In situ conservation focuses on preserving the genetic diversity in the natural habitats either in the wild or in traditional farming system. It includes Gene bank, Biosphere reserve, national park, sacred grooves etc. (Lakshman, 2016). Conservation is cost effective way of

protecting existing biological community in its natural habitat and this approach is ecosystem-oriented, rather than species-oriented. Successful *in situ* conservation depends on rules, regulations, and potential compliance of medicinal plants within growth habitats (Soule et al., 2005; Volis and Blecher, 2010). In addition to this, a wild nursery is established for species-oriented cultivating and domesticating the endangered medicinal plants in a protected area, natural habitats, or a place that is only a short distance from where the plants can naturally grow (Hamilton, 2004; Schippmann et al., 2005; Strandby and Olsen, 2008). Today protecting biodiversity has a central place in all policy decision process at national, international and global level.

1.2.2 *Ex situ* Conservation

Ex situ conservation is the process of cultivating and naturalizing endangered species outside their original habitats (Shinwari and Gilani, 2003; Rita and Silvano, 2006; Barazani et al., 2008). It can be achieved by cultivating and maintaining plant in Botanical gardens, Parks, through long term preservation of plant propagules in gene banks and plant tissue culture techniques. *Ex situ* methods of conservation of medicinal plant is a complementary action to conserve genetic diversity which helps in reducing pressure on wild plants and simultaneously enhancing availability of raw materials (Kadam and Pawar, 2020).

1.3 Plant Tissue Culture Technique

It is one of the most important conservation technique as it offers great potential for rapid cloning from a minimum amount of plant material through organogenesis and somatic embryogenesis. In addition, it plays a key role in the production of plant material required for different purposes such as production of secondary metabolites and their enhancement using different elicitors (Fig. 2). To establish *in vitro* cultures, the selection of a suitable medium is essential (Nagella and Murthy, 2010; Monfort et al., 2018). The commonly used culture medium is Murashige and Skoog (MS, 1962), and it contains the high concentration of total salt and nitrogen content which is an essential element for growth, also it directly affects amino acid and nucleic acid production in the cells (Alvarenga et al., 2015; Grzegorczyk-Karolak et al., 2015; Rahman et al., 2015). However the growth of cultures depends on many factors first is the type of salt strength in the medium, as optimum nutritional requirement is crucial for the growth and development of the explant that considerably mimics the natural growth of the plant. Second is plant growth regulators and their concentrations, because

appropriate blend of hormones are important for the proper growth of plants. Third one is type of explants, as it is gathering of diversity of cells and tissues and only a few of them have the potential for regeneration (Rao and Ravishankar, 2002; Monfort et al., 2018).

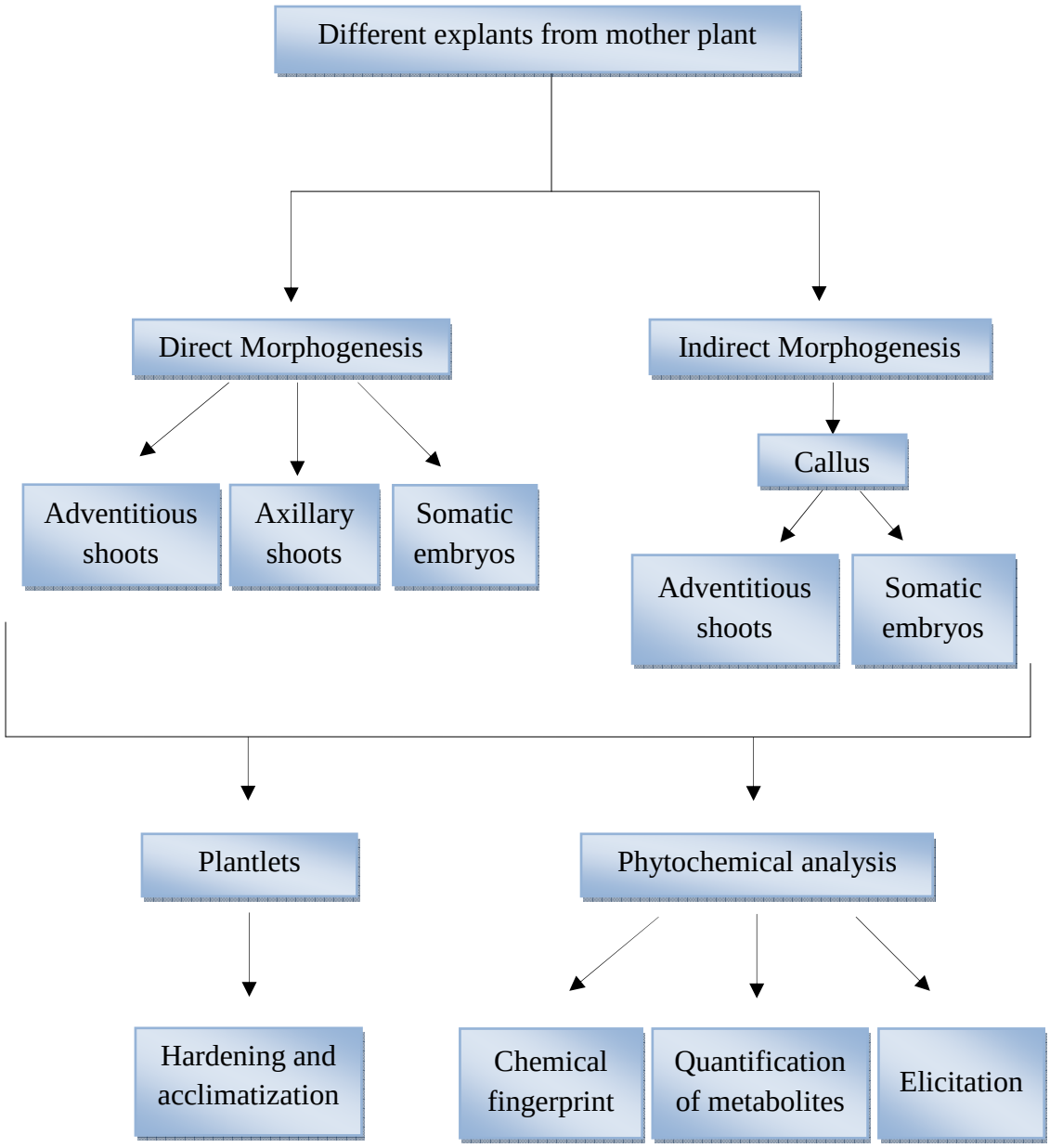

Figure 2. Different aspects of plant tissue culture.

In vitro plants can be obtained through different micropropagation techniques like indirect/direct organogenesis, somatic embryogenesis etc. through which rapid multiplication of plants is achieved throughout the year irrespective of the season. *In vitro* regeneration leads to the development of whole plantlet from a single explant under controlled conditions, and they can be transferred in the field after acclimatization (Rai, 2010).

In vitro cultures offers several distinct advantages:

- Large scale production, no constraints of season and disease free plants
- It may be possible to produce clones of plants that are slow and difficult to propagate vegetatively as well as can be stored over long periods
- The production of plants in the absence of seeds or necessary pollinators to produce seeds
- *In vitro* conservation of germplasm especially of threatened plant species
- Variability in production due to geographical or environmental fluctuations can be nullified

1.3.1 Callogenesis

Callus, a mass of undifferentiated plant cells, have the capacity to regenerate into a whole plant. In culture, this dedifferentiated mass of cells can be maintained indefinitely by repeated sub-culturing into the fresh medium. Stems, leaves, roots, flowers, seeds or any other parts (preferably young explants) of plant species can be used to induce callus tissue, however the successful production of callus mass depends upon plant species, explant type, plant growth regulators, nutrient supply, carbohydrate source and other environmental conditions (Yan et al., 2009; Lee et al., 2011; Prem Kumar et al., 2015; Khan et al., 2016). Dicotyledonous plants are rather amenable for callus induction, as compared to monocotyledonous plants, whereas in woody plants, growth of callus is generally slow. The alteration in carbohydrate source and concentration during cell cultures, especially callus cultures, is a relatively novel approach for elicitation of secondary metabolites (Prem Kumar et al., 2015). Callus cultures can be utilized to establish cell suspension cultures (Mustafa et al., 2011), for plantlet regeneration (Ikeuchi et al., 2013), for induction of somatic embryos (Abbasi et al., 2016), and adventitious root cultures (Sivakumar et al., 2005).

1.3.2 Organogenesis

The process of de novo organ formation is called organogenesis and for the first time shoot organogenesis was documented by White (1939) in tobacco tissue. Organogenesis mainly depends on the ability of the tissue to respond the PGRs during culture. It takes place in three phases: first is dedifferentiation phase, the cells become competent to respond to the organogenic signal, next is induction phase, the cells become determined to enter a

developmental pathway and third is differentiation phase in which organs are formed (Su et al., 2014). It is mainly achieved via indirect and direct organogenesis.

- **Indirect Organogenesis**

Differentiation of organs from the callus is called indirect organogenesis. Induction of plants using this technique does not ensure clonal fidelity, but it could be an ideal system for selecting somaclonal variants of desired characteristics and also for mass multiplication. Induction of plants through the callus phase has been used for the production of transgenic plants in which the callus is transformed and the plant regenerated, or the initial explant is transformed and the callus and shoots are developed from the explants.

- **Direct Organogenesis**

The production of buds or shoots from a tissue with no intervening callus stage is called direct organogenesis. Plants have been propagated by direct organogenesis for improved multiplication rate, production of transgenic plants, and most importantly for clonal propagation (Ara et al., 2000; Saiprasad, 2001). The axillary bud induction/multiple bud initiation technique is the most common means of micropropagation since it ensures the production of uniform planting material without genetic variation.

In vitro regeneration not only depends on the specific balance of cytokinins and auxins, but also on the response of explant tissues to these hormones (Sugiyama, 1999). The plant reaction to plant growth regulators may differ with respect to species, age of plant, environmental conditions, physiological and nutritional status as well as endogenous hormonal balance (Gaspar et al., 1996; Aftab et al., 2010; Idrees et al., 2010; Naeem et al., 2009, 2011). Generally direct shoot regeneration can be achieved via axillary bud proliferation through nodal explant as reported in important medicinal plants like *Holarrhena antidysenterica* (Kumar et al., 2005), *Andrographis paniculata* (Purkayastha et al., 2008), *Hemidesmus indicus* (Shekhawat and Manokari, 2016), *Decalepis salicifolia* (Ahmad et al., 2018) etc., as well as from leaf explant in *Bacopa monnieri* (Joshi et al., 2010), *Jatropha curcas* (Khurana-Kaul et al., 2010), *Digitalis lamarckii* (Verma et al., 2011) and *Aechmea ramose* (Faria et al., 2018). Similarly indirect organogenesis has been reported via leaf derived callus in *Ceropegia bulbosa* (Subbaiyan and Thangapandian, 2017), *H. indicus*

(Pathak and Joshi, 2017), *Abutilon indicum* (Seth and Panigrahi, 2018) and *Vernonia anthelmintica* (Rajan et al., 2020).

1.3.3 Somatic Embryogenesis

Somatic embryogenesis is an *in vitro* method widely used for sustainable clonal propagation. In this process the totipotent cells may directly either embryogenic pathway to form somatic embryos without undergoing the process of fertilization. It is done by zygotic embryos and is considered to be true-to-type in nature which can grow in whole plants (Rani and Raina, 2000; Varshney et al., 2001; Bhattacharyya et al., 2017a). It was first established in carrots (*Daucus carota*), where bipolar embryos developed from single cells (Bhatia and Bera, 2015).

Somatic embryogenesis is a valuable tool in plant biotechnology and can be utilized in a number of ways (Zimmerman, 1993; Bandyopadhyay and Hamill, 2000; Jiménez, 2001; Saiprasad, 2001):

- For large-scale clonal propagation of elite cultivars
- Used to develop synthetic seeds that potentially facilitate direct seeding of elite cultivars or in germ plasm conservation
- During regeneration, root and shoot formation is simultaneously achieved thus eliminating the need for a root induction phase and thus decreases culture period
- As with zygotic embryos, somatic embryos dormancy can be induced, hence long-term storage is also possible
- It offers potential models for studying molecular, regulatory and morphogenetic events in plant embryogenesis

Embryogenesis may be direct or indirect and in the first morphological stage that easily visualized is the globular embryo, followed by the heart, torpedo, and cotyledon stages in dicotyledons. Thus the sequential steps followed during embryogenesis are: initiation of embryogenic callus from vegetative cells, differentiation and maturation of somatic embryo from it and finally germinating into viable plantlets (Zegzouti et al., 2001).

The physiological and molecular mechanisms by which the induction pathway (direct or indirect) of SEs decides and is a crucial step for its manipulation (Grzyb et al., 2018). There are several factors like the type of explant, the genotype of the mother plant, the culture medium, concentrations of PGRs which affect the acquisition of embryogenic potential by

cells (Pencik et al., 2015; Loyola-Vargas and Ochoa-Alejo, 2016) and their differentiation into somatic embryos. For induction of embryos several explants can be utilized; however, the correct development stage of explant determines the progress in initiation of embryogenic callus. In particular, young or juvenile explants formed more somatic embryos than older explants (Woodward and Puonti, 2001; Panaia et al., 2004). One of the difficulties encountered during selection of explants is that the different explant tissues from the same mother plant produced embryogenic callus at different frequencies (Zhang et al., 2001) and required different concentration of growth regulators for the induction. The further enhancement of somatic embryos frequency, it is preferred to add a second round of embryogenesis by transferring globular embryos from the initial induction medium onto cytokinin rich medium.

Cytokinines also known to promote radial over axial growth, when applied to primary embryos leads to numerous secondary embryos budding from the lateral sides of the primary embryo (Eudes et al., 2003). Secondary somatic embryogenesis is a phenomenon where by a new somatic embryos are initiated from the previously formed somatic embryos and this process is also known as repetitive or cyclic embryogenesis. The process begins with the onset of generation of primary somatic embryos from the superficial tissues, of cotyledon or hypocotyl regions of these embryos and the regeneration of secondary and tertiary embryos takes place. For cyclic embryogenesis, need to formulate the medium composition which only focuses on cyclic production of embryos rather than its germination into plantlet.

In vitro techniques are not only indispensable for the rapid multiplication and production of disease-free plants of important and threatened plants, plant transformations, but they can also be employed for production of commercially valuable secondary metabolites (Debnarh et al., 2006; Altpeter et al., 2016).

1.4 Importance of Secondary Metabolite

In plant physiology, the “stress” could be defined as any factor (biotic and abiotic) that modifies (positively or negatively) plant functioning, growth and reproduction (Cheynier et al., 2013). Thus plant synthesizes variety of secondary metabolites as a result of cascade of reactions when higher plants are exposed to multitude of different stresses (Walley et al., 2007). They provide defense against biotic factors such as herbivores, fungi, bacteria, viruses, etc. as well as physical factors like UV radiation, high and low temperature, drought, etc. (Kaur and Pati, 2018). Unlike primary metabolites, secondary metabolites do not have a direct

role in the fundamental life processes, but they are required for plant environmental interaction such as survival, adaptation and competitiveness. Moreover they are generally synthesized at a very low concentration from common precursors as the products of primary metabolism at particular physiological and developmental age of the plant (Namdeo, 2007).

Despite the extensive research into secondary metabolites over such a long period of time, current estimates indicate that only about 6% of higher plants (between 300,000 and 500,000 species) have been systematically studied for their pharmacological potential, and only 15% have been evaluated for phytochemicals in general (Fabricant and Farnsworth, 2001; Cragg and Newman, 2013). These phytochemicals are responsible for various bioactive potentials and major contributors of specific odour, color and taste of plant parts. Generally these secondary metabolites are grouped into three classes based on their biosynthetic pathway including nitrogen-containing compounds (cyanogenic glycosides, alkaloids, and glucosinolates), phenolic compounds (flavonoids and phenylpropanoids), and terpenes (isoprenoids) (Fang et al., 2011).

Some of the important secondary metabolites (given below) are still extracted from plants (Rao and Ravishankar, 2002; Panche et al., 2016):

- Alkaloids: ajmaline (*Rauwolfia serpentina*), berberine (*Coptis japonica*), morphine (*Papaver somniferum*), quinine (*Cinchin ledgeriana*), taxol (*Taxus brevifolia*), vincristine and vinblastine (*Catharantus roseus*)
- Flavonoids: chrysin (*Passiflora caerulea*), naringenin (*Citrus × sinensis*), rutin (*Ruta graveolens*), quercetin and luteolin (*Allium fistulosum*)
- Terpenoids: ajmalicine (*Catharantus roseus*), artemisinin (*Artemisia annua*), azadirachtin (*Azadirachta indica*), ginsenosides (*Panax ginseng*) and linalool (*Lavandula angustifolia*)

Metabolites were extracted from plants using many solvents i.e. hexane, ethay acetate, methanol, toluene, acetone etc. on the bases of polarity. Many a times the extraction of metabolites directly from wild plants is cumbersome due to unavailability of valuable plants and the reasons are, overexploitation of wild plants, difficulties in cultivation, effect of seasonal and geographical variation, tissue or organ specific production and difficulties in purification due to impurities (Halder et al., 2019). These constraints can be overcome by utilizing cultures for extraction as they are known to synthesize similar metabolites present in

the mother plants and hence they can be considered as an excellent source for harnessing of important metabolites.

Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites (DiCosmo and Misawa, 1995; Stockigt et al., 1995; Dörnenburg and Knorr, 1997; Karuppusamy, 2009). The reason is plant cells are biosynthetically totipotent, each cell in culture retains complete genetic information and hence is able to synthesize range of chemicals present in parent plant. This would ensure a continuous supply of products as well as is able to produce novel compounds. Production of secondary metabolites from *in vitro* cultures depends on many factors like type of culture, age of culture and PGRs in the medium which play a crucial role in the synthesis of different compounds as they control the antioxidant potential, fundamental growth and developmental processes (Dörnenburg and Knorr, 1995). According to Zhao et al. (2005), they are active at low concentrations, have specific effects on growth and differentiation, and are also involved in plant secondary metabolism. Few reports showed effect of PGRs on *in vitro* production of secondary metabolites which has been well documented in *Hemidesmus indicus* (Misra et al., 2005; Pathak et al., 2017), *Hypericum hirsutum* and *Hypericum maculatum* (Coste et al., 2011), *Withania coagulans* (Jain et al., 2011) and *Coffea arabica* (Acidri et al., 2020).

1.5 Elicitation of Important Metabolites

Plants are known to synthesize secondary metabolites as a defense mechanism and are often synthesized in low amount which resulted in large amount of harvesting of plant natural resources which affected status of many medicinal plants (Fujita et al., 2006; Sharifzadeh Naeini et al., 2020). This problem can be overcome by treatment of *in vitro* cultures with elicitors which is an effective strategy to enhance secondary metabolite production (Verpoorte et al., 1999; Smetanska, 2008), and the process is known as ‘elicitation’. Plant cell recognize elicitors by microbe associated molecular patterns (MAMPs) and initiates defense responses by altering the expression of transcription factors and/or rate-limiting steps (Mishra et al., 2012). Elicitor, after binding with the receptors, act as signal molecules and switch on signal transduction network inside the cell, this cascade further activates transcription factors regulating the expression of secondary metabolite genes (Pieterse et al., 2006). This ultimately results in increased synthesis and accumulation of desired metabolite(s) (Vasconsuelo and Boland, 2007; Mishra et al., 2012).

Elicitation of cultures has proved to be one of the most effective method to enhance the metabolite of interest using different elicitors (Wang and Wu, 2013). Originally the term 'elicitor' was used for molecules capable of inducing the production of phytoalexins (Keen et al., 1972), but it is now commonly used for stimulating the compounds in any type of plant defense (Montesano et al., 2003). Thus elicitor alters plant cellular activities at biochemical and molecular level and triggers the expression of key genes and transcription factors that control secondary metabolite synthesis (Zhao et al., 2005). Elicitors can be classified by their 'nature' i.e. abiotic or biotic elicitors (Montesano et al., 2003; Namdeo, 2007).

- **Abiotic elicitors** can be considered as substances of non-biological origin, being predominantly inorganic compounds i.e. heavy metals like Cu^{2+}, Cd^{2+} and Ca^{2+} ions, physical factors like UV irradiation and wounding as well as chemicals like methyl jasmonate, salicylic acid etc. (Namdeo, 2007; Gorelick and Bernstein, 2014).
- **Biotic elicitors** are recognized by specific receptors bound to the cell membrane. This stimulus is then transferred to the cell by a signal transduction system, producing changes that ultimately lead to the formation of phytoalexins. They have biological origins like plant (pectin or cellulose) or microorganisms cell walls (chitin or glucans), glycoproteins, G-protein or intracellular proteins, chitosan, fungal homogenate, yeast extract etc. (Veersham, 2004; Vasconsuelo and Boland, 2007).

Elicitation of metabolites in *in vitro* cultures is affected by **s**everal parameters such as elicitor type, its concentration, duration of elicitor exposure, type of culture etc. (Zhao et al., 2010; Sahu et al., 2013). A number of findings have documented that elicitor type and its concentration affected the metabolite synthesis differently e.g. ajmalicine production in *Catharanthus roseus* is reported higher when treated with higher concentration of elicitor as compared to lower concentration (Namdeo et. al., 2002). Further Sahu et al. (2012) documented that not only elicitor type and its concentration, but duration of exposure also affected rosmarinic acid in *Solenostemon scutellarioides* as elicitation with MJ and SA increased the accumulation within day 1, whereas YE increased the content after 3rd day. Similarly, many studies suggested that enhancement of metabolite also depends on type of culture to which elicitor treatment is given e.g. asiaticoside content in *Centella asiatica* is highest in callus culture followed by shoot and cell suspension culture (Krishnan et al., 2019).

Different types of cultures are used for elicitation experiments, but callus and shoot cultures are most commonly used for enhanced production of metabolites. Callus cultures are used for elicitation of naphtodianthrones and phenylpropanoids in *Hypericum perforatum* (Gadzovska et al., 2013) and ursolic and oleanolic acid in *Lepechinia Caulescens* (Vergara Martínez et al., 2017), whereas shoot cultures are reported for enhancement of bacoside A in *Bacopa monnieri* (Sharma et al., 2013) and phenolic acids in *Eryngium planum* (Kikowska et al., 2015).

1.6 Rational for Selection of Species

Asclepiadaceae, also known as milkweed family, has approximately 250 genera and 2000 species which are distributed throughout the world (Sinha and Mondal, 2011) and are widely used for extraction of important metabolites. The second main group of pharmacologically active products are also found in this family (Wiart, 2006). Some of the important species with their constituents from this family are listed below (Table 1).

Table 1. Few Asclepiadaceae family members along with their metabolites and properties.

Plants	Metabolites	Activity/Properties	Reference
Gymnema sylvestre	Gymnemic acids	Antidiabetic, anti-inflammatory, anti-hypercholesterolemic	Bishayee and Chatterjee, 1994; Shaw et al., 2010
	Lupeol	Anticancer, anti-arthritic, antioxidant, hypotensive	Nagaraj et al., 2000; Saleem et al., 2003; Blain et al., 2009; Saleem, 2009
	Stigmasterol	Anti-osteoarthritic, anti-hypercholestrolemic, thyroid inhibitory, antiperoxidative and hypoglycaemic	Batta et al., 2006; Panda et al., 2009; Gabay et al., 2010
	β-amyrin,	Anti-inflammatory, antinociceptive	Soldi et al., 2008; da Silva Júnior et al., 2019

Hemidesmus indicus	2-hydroxy 4-methoxy benzaldehyde	Antimicrobial, antioxidant, anti-aflatoxigenic	Wang et al., 2010; Harohally et al., 2017
	Vanillin	Anti-neuroinflammatory, reduced the frequency of chromosomal aberrations	Bythrow, 2005; Kim et al., 2019
	Rutin	UV-B protective, antioxidant, anticancer, anti-asthamatic	Jung et al., 2007; Choquenet et al., 2008; Lin et al., 2012; Sharma et al., 2013
	Lupeol	Anticancer, anti-arthritic, antioxidant, hypotensive	Nagaraj et al., 2000; Saleem et al., 2003; Blain et al., 2009; Saleem, 2009
Leptadenia reticulata	Stigmasterol	Galactogogue, anti-osteoarthritic, thyroid inhibitory, antiperoxidative and hypoglycaemic	Anjaria et al., 1974; Panda et al., 2009; Gabay et al., 2010
	Lupeol	Anticancer, anti-arthritic, antioxidant, hypotensive	Nagaraj et al., 2000; Saleem et al., 2003; Blain et al., 2009; Saleem, 2009
	p-Coumaric acid	Antioxidant, anti-inflammatory, anticancer, hepatoprotective	Abdel-Wahab et al., 2003; Vetrikumaran et al., 2011; Yoon et al., 2013
	Luteolin	Anticancer, anti-diabetic, neuroprotective	Lin et al., 2008; Nabavi et al., 2015; Sangeetha et al., 2019
	Apigenin	Beneficial effect on endocrine system, cardiovascular diseases,	Gates et al., 2009; Zhang et al., 2016; Zhou et al., 2017

		anticancer, hepatoprotective	
Oxystelma esculentum	Lupeol	Anticancer, anti-arthritic, antioxidant, hypotensive	Nagaraj et al., 2000; Saleem et al., 2003; Blain et al., 2009; Saleem, 2009
	Aesculin	Antioxidant, anti-inflammatory, hepatoprotective	Lin et al., 2000; Biljali et al., 2012; Tianzhu and Shumin, 2015
	Kaempferol	Anti-inflammatory, anticancer, neuroprotective	Li and Pu, 2011; Kim et al., 2016; Nam et al., 2017
	Epicatechin	Anti-atherosclerosis, anticancer, anti-ischemia	Papiez et al., 2010; Leonardo et al., 2013; Morrison et al., 2014
Sarcostemma viminale	β-Sitosterol	Anxiolytic, angiogenic, anti-arthritic	Choi et al., 2003; López-Rubalcava et al., 2006; Tatiya et al., 2011
	Gallic acid	Anticancer, gastrointestinal	Chatterjee et al., 2012; Liao et al., 2012
	Heptadecanoic acid	Anticancer, antioxidant, antifungal,	Sunita et al., 2017; Xu et al., 2019
	Lupeol	Anticancer, anti-arthritic, antioxidant, hypotensive	Nagaraj et al., 2000; Saleem et al., 2003; Blain et al., 2009; Saleem, 2009
Tylophora indica	Tylophorine	Antitumor, antiallergic, anti-inflammatory	Nayampalli and Sheth, 1979; Yang et al., 2006; Wu et al., 2009
	Tylophorinine	Antioxidant, antifungal	Dhiman et al., 2012a, 2012b

	Tylophorinidine	Anticancer, antifeedant, antimicrobial	Rao and Venkatachalam, 2000; Reddy et al. 2009
	β-sitosetrol	Anxiolytic, angiogenic, anti-arthritic	Choi et al., 2003; López-Rubalcava et al., 2006; Tatiya et al., 2011

Many important medicinal plants belong to this family and amongst them *Leptadenia reticulata* and *Tylophora indica* were selected for present study. There are many reasons for the selection, the natural propagation of *L. reticulata* is take place through seeds but low seed setting as well as germination rate of seeds reduces its propagation. On the other hand due to its multipurpose medicinal properties annual demand of this plant increased, which led to overexploitation due to habitat destruction (Shetty and Singh, 1993). This is one factor for poor reproduction which is not enough to cope up with its requirement and the huge demand for this plant by the traditional medicine industries make this plant endangered (Arya et al., 2003; Martin, 2004; Rawat, 2008). Thus many a times it is often adulterated with many other herbs like *Holostemma ada-kodien*, *Dendrobium ovatum*, *D. macraei*, *Flickingeria macraei*, *Cimicifuga foetida* and *Ichnocarpus frutescens* (Kasera and Shukla, 2003; Khare, 2007; Mallikarjuna et al., 2011; Mammen et al., 2011b). Although cultivation of this species is being done utilizing traditional ways by farmers and industries but low percentage of germination, non-availability of genuine plant materials, seasonal availability, restricted distribution and a lack of knowledge about its cultivation practices pose a challenge for its commercial cultivation (Mishra et al., 2009). This plant has great demand in local as well as international markets, and being sold at Rs 211/kg of dry powder and flowers are sold at Rs 80/kg (Shekhawat et al., 2006). Annual demand of *L. reticulata* is 200-500 MT and its cost of cultivation in 2016-2017 is Rs. 36602.5/ha and thus included in list of 95 prioritized plants by NMPB as it is still collected from wild (Goraya and Ved, 2017).

Whereas another plant *T. indica* is essentially accredited for its medicinal properties because of its wide range of alkaloids in the form of bioactive secondary metabolites, such as tylophorine, tylophorinine, and tylophorinidine (Gantait and Kundu, 2017). But the lack of proper cultivation practices and the destruction of plant habitats have led to a rapid decline in its wild populations (Jayanthi and Mandal, 2001). Whereas the plant is usually propagated by

seeds but the viability and germination rate is low in the wild and vegetative propagation through stem cuttings is poorly explored (Dhandapani and Balu, 2002; Chandrasekhar et al. 2006). The annual demand of *T. indica* is 1.80 MT (Goraya and Ved, 2017).

Both these plants are being mentioned in the 'List of prioritized plants for development and cultivation under scheme of NMPB plants which are eligible for subsidy' (https://www.nmpb.nic.in). Hence to conserve these species and to provide an alternative of wild plants for pharmaceutical industry, *ex situ* conservation strategy like plant tissue culture needs to be developed.

1.6.1 *Leptadenia reticulata* (Retz.) Wight and Arn

Leptadenia reticulata (Retz.) Wight and Arn (Fig. 3) is commonly known as jivanti or dodi, is a multipurpose medicinal climber, and according to Athara-Veda (one of the samhitas), it is an Indian origin plant known for its medicinal value from 4500 to 1600 BC and is known due to life and strength providing characteristic (Arya et al., 2003; Kasera and Shukla, 2003). It is distributed in tropical as well as subtropical parts of Asia, Africa, Cambodia, Mauritius, Sri Lanka, Burma, Nepal, Madagascar, Malay Peninsula, Philippines etc. (Arya et al., 2003; Schmelzer and Gurib-Fakim, 2013). In India this is reported in the sub-Himalayan tract of Punjab, Uttar Pradesh, and throughout the Deccan Peninsula up to an altitude of 900 m above sea level and naturally occurs on hedges (Parabia et al., 2007). *L. reticulata* is a branched twiner with watery sap and corky deeply cracked bark. Leaves are ovate-cordate, glabrous above and pubescent beneath. The flowers are in many flowered cymes, greenish yellow, follicles broadly lanceolate and turgid (7.0–8.0 cm in length). Plant flowers seen throughout the year but fruiting is observed during the months of February–April and seeds are brown and are dispersed through air.

- **Medicinal Properties**

The whole plant ameliorates 'tridoshas' (Vatta, Pitta and Kapha) and mentioned as a Rasayana herb in Ayurveda due to its revitalizing and rejuvenating properties (Sivarajan and Balachandran, 1994). Patel (1947) drew attention for the first time by reporting the usefulness of 'Leptaden' (equal proportion of *Leptadenia reticulata* and *Breynia patens*) in preventing habitual abortion and allied conditions in women and later pointed out its lactogenic and galactogogue properties. It is effective in stimulating lactation in short time without any harmful consequence on household animals and women, and thus it is an important ingredient of poultry-feed as well (Dash et al., 1972). The plant is a stimulant and prevents miscarriage

Figure 3. ***Leptadenia reticulata*** **(Retz.) Wight and Arn plant growing in the Botanical Garden of the University.**

(Patel and Dantwala, 1958) and its twigs, tubers and follicles are eaten as a vegetable and are useful in treatment of various skin diseases and inflammation (Singh and Pandey, 1998). Plant is given as tonic for weak debility, commonly given to those suffering from lack of energy in the body to provide strength (Prajapati et al., 2003). It is one of the ingredients of the traditional household remedy 'Chyawanprash' and contributes for its cooling, eye tonic, nutrient and aphrodisiac properties (Parle and Bansal, 2006). Some other known herbal formulations which consist of *L. reticulata* are: 'Siladan'- useful in a mental disorder (Dandiya and Chopra, 1970), 'DLH-3041'- for inhibition of mast cell degranulation (Padmalatha et al., 2002), 'Speman'- useful in oligospermia, increasing sperm motility as well as sperm count (Agrawal and Kulkarni, 2003). *L. reticulata* is known to cure hematopoiesis, emaciation, cough, dyspnea, fever, burning sensation, night blindness and dysentery (Anjaria and Gupta, 1967; Sivarajan and Balachandran, 1994). Some of the important medicinal activities of *L. reticulata* are:

- antifertility (Basu et al., 1961)
- anti-abortificant (Achari and Sinha, 1966)
- anabolic and vasodilator (Anjaria et al., 1975)
- antiepileptic (Pushpa et al., 2010)
- antipyretic, analgesic and anti-inflammatory (Mohanty et al., 2015; Sneha et al., 2016a)
- anti-cancerous (Sathiyanarayanan et al., 2007)
- antioxidant and cardioprotective (Wakade et al., 2007)
- immune booster and rejuvenating (Girishkumar et al., 2010)
- antiulcer activity (Bodhanapu et al., 2011)
- hepatoprotective (Nema et al., 2011)
- diuretic (Mohanraj et al., 2012)
- immunomodulatory (Pravansha et al., 2012)
- antidiabetic (Venkatesan et al., 2014)
- wound healing (Sneha et al., 2016b)

➢ **Phytoconstituents**

Preliminary phytochemical analysis of *L. reticulata* confirmed the presence of terpenoids, alkaloids, flavonoids, sterols, tannin, saponins, glycosides and tocopherols etc. (Verma and Agarwal, 1962; Anjaria et al., 1974; Pal et al., 2012). The multipurpose medicinal properties of this plant is because of the active constituent stigmasterol having

lactogenic/galactagogue effect (Anjaria et al., 1974). It also contains a triterpenoid laptadenol, n-triaconate, sitosterol, amyrin acetate, lupanol-3-O-diglucoside and leptidin whereas the tubers contain fructosan of the insulin type (Kirtikar and Basu, 1975). Krishna et al. (1975) reported hentricontanol, α-amyrin, β-amyrin, stigmasterol, diosmentin and luteolin whereas Srivastav et al. (1994) reported pregnane glycosides viz. reticulin, deniculatin and leptaculatin. It contains other important metabolites like apigenin, rutin, *p*-coumaric acid, lupeol, simiarenol, β-sitosterol quercetin, isoquercetin, meso-inositol and its monomethyl ether (Subramanian and Lakshman, 1977; Sastry et al., 1985; Prashanth et al., 2003; Rajanna et al., 2009; Bawra et al., 2010; Geetha et al., 2011; Pal et al., 2012).

The volatile compounds reported in *L. reticulata* (Rajeswari and Rani, 2014) are: phytol, esters like benzene carboxylic acid, ethyl hydrogen succinate, hexadecanoic acid ethyl ester, 4-oxazolecarboxylic acid, 4,5-dihydro-2-phenyl-, 1-methylethyl ester, 9,12-octadecadienoic acid ethyl ester, 9-octadecenoic acid ethylester, dodecanoic acid, n-hexadecanoic acid, 3-hydroxy-4-methoxybenzoic acid, 6-octadecenoic acid, pentadecanoic acid and tetradecanoic acid. Recently Godara et al. (2019) documented other pharmacologically active volatiles like γ-sitosterol**,** campesterol**,** stigmastan-3,5-diene, cholesteryl myristate, pristane, hexahydrofarnesxol, phytol acetate, stearic acid, arachidic acid, eicosanoic acid, isopropyl myristate, linolenic acid ethyl ester, linolenic acid methyl ester, headecanoic acid methyl ester, dibutyl phthalate, phthalic acid diisobutyl ester, coniferyl alcohol and 2-(2-hydroxypropan-2-yl)-4-methoxy-5-methyl phenol, n-tetracosanol-1, montanol, quercitol, 2-methyl-z,z-3,13-octadecadienol and trans-9-hexadecen-1-ol, ascorbic acid 2,6-dihexadecanoate, heptadecane, pentadecane, hexadecane and E-15-heptadecenal from different parts of the plant.

1.6.2 *Tylophora indica* (Burm. F.) Merrill

Tylophora indica (Burm. F.) Merrill [Synonym- *Tylophora asthmatica* (L.f.) Wight and Arn.] (Fig. 4) is commonly known as antamul and Indian Ipecac. It is distributed throughout southern and eastern part of India in plains, forests and hilly places in India and normally found in Uttar Pradesh, Bengal, Assam, Orissa, Himalayas and sub Himalayas (Anonymous, 1969; 1978a). The plant is a perennial climber and can reach up to 1.5-3.0 m height and grow with short stocky rhizomes (3-4 mm thick) and has fibrous roots (Kirtikar and Basu, 1991; Schmelzer and Gurib-Fakim, 2013). It has simple, opposite 2-10 cm long leaves having entire, ovate or orbicular margins. Flowering and subsequent fruiting generally occur during the months of October to December. Flowers are green-yellow outside with a purple inside form

Figure 4. ***Tylophora indica*** **(Burm. F.) Merrill plant growing in the Botanical Garden of the University.**

on an axillary umbel like cyme. The fruit is 5-10 cm long with number of seeds which is ovate in shape and around 2-2.5 cm long (Gupta, 2003). Roots are widespread with longitudinally fissured corky bark and they have a sweet taste turning acrid, aromatic odor and a brittle fracture.

- **Medicinal Properties**

This plant is widely used in Ayurvedic medicines for the treatment of respiratory diseases like asthma and bronchitis (thus locally known as 'asthma plant'), jaundice, dermatitis, rheumatism, inflammation, whooping cough, ulcer, allergy, dysentery and diarrhoea (Anonymous, 1978b; Nayampalli and Sheth, 1979; Chopra et al., 1986; Kirtikar and Basu, 1991). It seems to be a good medical remedy for psoriasis, seborrhea, anaphylaxis, leucopenia and as an inhibitor of the Schultz–Dale reaction (Sarma and Misra, 1995). It also possesses antihistaminic, antirheumatic, hypotensive and antiamoebic activities (Dhananjayan et al., 1975; Haung et al., 2004). The leaves and roots are reported to have wound healing, antifeedant, bacteriostatic, cathartic, stimulative, diaphoretic, emetic, expectorant and stomachic effects and also prevent myocardial damage (Kirtikar and Basu, 1991; Varrier et al., 1994; Asdaq et al., 2008; Asdaq and Sowmya, 2010). This plant is a good source for the treatment of rheumatic and gouty pains as well as hydrophobia (Joshi, 2000) and is known to inhibit cellular immune responses (Ganguly and Sainis, 2001) and has antibacterial, anti-spasmodic, antiarthritis and lysosomal enzyme inhibiting activities (Gupta et al., 2010). The two alkaloids i.e. tylophorine and 7-methoxycryptopleurine of *T. indica* inhibited N and S protein activity (protease inhibition) as well as viral replication of coronavirus (Yang et al., 2010). Later on it was also reported that tylophorine targets RNA replication of coronavirus and cellular JAK2 mediated dominant NFκB activation (Yang et al., 2017). Some other medicinal properties of different extracts of *T. indica* are:

- immunomodulatory (Ganguly et al., 2001)
- anticancer (Gao et al., 2004; Wu et al., 2009; Bach and Lee, 2019)
- hepatoprotective (Patel et al., 2007; Mujeeb et al., 2009)
- nootropic (Kulkarni and Juvekar, 2009)
- cardioprotective (Asdaq and Sowmya, 2010)
- anticatalepsy (Shyamjith et al., 2012)
- antiangiogenic (Saraswati et al., 2013)
- antivenoum (Sakthivel et al., 2013)

- antioxidant (Bhatia et al., 2013)
- antileukemic and anticandidal (Roychowdhury et al., 2013)
- antidiabetic (Bhatia et al., 2015)
- anti-anxiety (Manikkoth et al., 2012)
- anti-neuroinflammatory (Gupta et al., 2020)

➢ **Phytoconstituents**

T. indica contains numerous bioactive compounds such as flavonoids, saponins, alkaloids, and tannins (Rao et al., 1971; Benjamin and Mulchandani, 1973). But main pharmacological activities of this plant are attributed to major alkaloids like tylophorinidine (Mulchandani et al., 1971), tylophorine (Gellert, 1982) and tylophorinine (Gopalakrishnan et al., 1979). Other alkaloids like tyloindicines A-J (Ali et al., 2001), 3-O-demethyl tylophorinidine (Dhiman et al., 2013) and tylophorinicine along with (+) septicine, isotylocrebrine, sterols, flavanoids, wax, resins, tannins (Govindhari et al., 1975) and septidine (Gupta et al., 2010; Gurav et al., 2011; Kaur and Singh, 2012) was also reported. Some rare alkaloids like desmethyl-tylophorine, desmethyl-tylophorinine, anhydrous tylophorine, γ-fagarine, skimmianine, 14-hydroxyisotylocrebrine and 4,6-desmethylisodroxy-omethyltylophorinindine and non-alkaloidal compounds like coutchone, cetyl alcohol, quercetin, kaempferol, α- and β- amyrins, tetratriacontanol, octaosanyl octacosanoate, stigmasterol, tyloindane, β-sitosterol and *p*-methoxysalicylaldehyde were also present in plant (Ali, 2008).

The present study was framed to cover all the above aspects with following objectives:

1.7 Objectives

- Establishment of callus and shoot cultures for *L. reticulata* and *T. indica*
- Chemical profiling of *in vivo* and *in vitro* cultures
- Quantification of targeted metabolites in selected species
- Elicitation of targeted metabolites in cultures and gene expression analysis using molecular technique