CHAPTER 1

INTRODUCTION

1.1 MEDICINAL PLANTS

Plants have been an exemplary source of medicine for thousands of years (Roberts 1988). The World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the source of many modern medicines (Pezzuto 1996). It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant materials. The most popular analgesic, aspirin, was originally derived from species of *Salix* and *Spiraea*. Some of the most valuable anticancer agents such as Paclitaxel (*Taxus brevifolia*) and Vinblastine (*Catharanthus roseus*) are derived solely from plant sources.

India is the largest producer of medicinal plants and is rightly called the "Botanical garden of the World" (Govind and Maduri 2006). Around 20,000 medicinal plants have been recorded (Ballabh and Chaurasia 2007) however traditional communities are using only 7,000 to 7,500 plants for curing different diseases (Perumal and Ignacimuthu 2000). These medicinal plants are listed in various indigenous systems such as Siddha, Ayurveda, Amchi, Unani and Allopathy (Rabe and Staden 1997). Plant secondary metabolites such as alkaloids, flavanoids, saponins, phenols, steroids and tannins are the primary active ingredients of ayurvedic drugs. The medicinal plants, besides having natural therapeutic values against various diseases, also
provide high quality food and raw materials for livelihood. Currently the pharmacologically active ingredients of many ayurvedic medicines are being identified and their usefulness as drugs was determined. Hence the traditional knowledge with its holistic and systematic approach supported by scientific documentation can serve as an innovative and powerful discovery engine for newer, safer and affordable medicines (Azaizeh et al 2003).

1.2 ANTIDIABETIC MEDICINAL PLANTS IN INDIA

The Indian system of traditional medicine (Ayurveda) provides a number of medicinal plants to treat diabetes in India (Varier 1995). Traditional knowledge and historic literatures on medicine play an important role in the discovery of novel leads from medicinal plant (Grover et al 2002). It has been estimated that the global burden of diabetes for 2011 would be 285 million which is projected to increase to 438 million in 2030 (WHO 2002). One of the major problems with this herbal formulation is that the active ingredients are not well defined. It is important to know the active component and their molecular interactions, which will help to analyse therapeutic efficacy of the product and also to standardize the drug. Efforts are now being made to scientifically investigate the mechanism of action of natural products (Manisha et al 2007).

There are many active hypoglycemic constituents isolated from the medicinal plants such as dipropyl disulphide oxide (Allium cepa), allicin (Allium sativum), lophenol (Aloe vera), andrographolide (Andrographis paniculata), α-sitosterol (Azadirachta indica), leucodelphinidine (Ficus bengalensis), gymnemic acid (IV) (Gymnema sylvestre), pterostillbene (Petrocarpus marsupium), swerchirin (Swertia chiratia) and trigonellin (Trigonella foenum). Though many of the plants are reputed in the indigenous system of medicine for their hypoglycaemic activities but lack scientific evidence proving their efficacy and safety (Grover et al 2002).
The traditional medicinal practice of Kolli hills of Namakkal district, Tamilnadu, India have revealed the use of Andrographis lineata for various ailments including diabetes, snakebites, cancer and inflammation (Balu and Alagesaboopathi 1995). Although the leaves of A. lineata were very effective in treating diabetes, there was no proper scientific documentation pertaining to the active principle for its antidiabetic effect. In this context an attempt was made to scientifically document the traditional claim in this endemic medicinal plant of South India.

1.3 **ANDROGRAPHIS LINEATA WALL. EX. NEES: AN ENDEMIC MEDICINAL PLANT**

Scientific classification

- **Kingdom**: Plantae
- **Division**: Magnoliophyta
- **Class**: Magnoliopsida
- **Order**: Lamiales
- **Family**: Acanthaceae
- **Genus**: Andrographis
- **Species**: lineata

A.lineata belongs to the family Acanthaceae which is said to be a large tropical family of about 240 genera and over 220 species. It is endemic in distribution namely in the hilly regions of Asia, Africa, Brazil and Mexico (Lawrence 1969). The genus *Andrographis* comprised of annual herbs or small shrubs, including about 40 species distributed in the hilly habitats of tropical Asia (Anonymous 1948). About 18 species of *Andrographis* are reported to occur in India of which *A.lineata* is relatively unexplored (Lawrence 1969). It is an erect perennial herb which is 1.5-2.0 m tall, endemic to Deccan and Carnatic regions of South India (Gamble 1956). This species is herbaceous (Mathew 1983; Fyson 1915) which is known to occur in
the hills above 1440 m (Figure 1.1 a) (Mathew 1983) in moist or dry or rocky soils. It is called as “Periyanangai” local name in Tamil. The root stock is 80-100 cm. The stem and branches are four angled with thick leaves (Figure 1.1 b). Inflorescence are 10.0 to 15.0 cm long and panicles are glandular hairy. The corolla is white in color with purplish blotches. The capsules (seeds) are green in color of oblong shape with glandular hairs (Figure 1.1 c and d). The fruiting period occurs during August to November and the flowering period occurs from July to October.

Figure 1.1 Habitat of *Andrographis lineata* Wall. ex. Nees.

a) Habitat (Kolli hills, Namakkal District)
b) Habit of *A. lineata*
c) Flowers and seeds of *A. lineata*
d) *A. lineata* grown in green house
1.4 NEED FOR CONSERVATION OF MEDICINAL PLANTS

Most of the plants used in traditional medicines are collected from the wild and only a few have been domesticated (Lucy 1999). Hence, there is a real danger of genetic erosion, which in turn calls for the need for conservation by using propagation techniques. Micropropagation is an important tool in plant tissue culture which produces mass multiplication of “true to type” rare, endemic and endangered medicinal plants under disease free conditions which are independent of seasonal constraints (Chaturvedi 1979). Therefore, owing to its poor regeneration efficiency by conventional methods (seed germination or stem cutting) an attempt was made to micropropagate this potentially important endemic medicinal plant (*A. lineata*) through shoot tip and nodal explants.

1.5 CLONAL FIDELITY IN MICROPROPAGATED PLANTS

Extensive reports are available on the occurrence of tissue culture induced variation (Chen et al 1998) which warrants the need to verify clonal fidelity of micropropagated plant. Tissue culture induced variations can be determined at the morphological, cytological, biochemical and molecular levels with several techniques (Barazani et al 2002). Molecular markers suitable for generating DNA profiles have proved to be an effective tool in accessing the genetic stability of the regenerated plants (Bennici et al 2003). These markers are not influenced by environmental factors but can generate reliable and reproducible results. DNA based techniques which include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) are used to study the genetic stability of the *in vitro* regenerated plants. Among these techniques, RAPD is the most preferred in the detection of genetic diversity since it has the advantage of being technically accurate, rapid and requires only small amount of DNA (Shiran et al 2007). In the present study,
the genetic fidelity of *in vitro* regenerated *A.lineata* was analysed using RAPD technique.

### 1.6 PHYTOCHEMICALS AS ANTIOXIDANTS

Phytochemicals are chemical compounds formed during the plant normal metabolic processes. These chemicals are often referred to as “secondary metabolites” of which there are several classes including alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids (Harborne 1973). They are present in a variety of plants and utilized as an important components of food (fruits, seeds, herbs and vegetables) in both human and animals (Okwu 2010). There are several standard methods used for the phytochemical screening of medicinal plants as described for alkaloids, steroids, phenols, flavonoids, saponins, glycosides and tannins (Mohammad et al 2011). Methods for quantitative analysis of phytochemicals such as phenolics, flavonoids, alkaloid, saponins and glycosides were also estimated by Edeoga et al (2005).

Antioxidants are secondary metabolites found to occur naturally in plants such as fruits and vegetables. An antioxidant can be defined in simple terms as a compound that inhibits or prevents oxidation of a susceptible substrate. Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (Alam 2009). Common antioxidant includes vitamin A, vitamin C, vitamin E, carotenoids, lutein and β-carotene (Dahanukar et al 2000). These plant based dietary antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (Fridovich 1998).
During metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Erhola et al 1997). Oxidative stress plays a major role in diabetes (Sian et al 2003). Supplementation of dietary antioxidants reduces the risk of oxidative damage and thus arising a necessity to extract these antioxidants from the plant matrices (Urisni et al 1994).

DPPH assay (1, 1 diphenyl 2, picryl hydrazyl), nitric oxide scavenging activity, superoxide scavenging activity, lipid peroxidation assay, reducing power ability, FRAP method (Ferric Reducing Ability of Plasma), cytochrome C test and xanthin oxidase method are the various in vitro models which are used to evaluate the potency of the plant extract to scavenge free radicals (Wang and Curtis 2006). Hence, in our study phytochemical screening is carried out to confirm the presence and quantify the phytoconstituents in the leaves of A.lineata using various solvents based on polarity. The best solvent which extracted maximum phytochemicals was selected for further in vitro antioxidant studies to evaluate the ability of the extract to scavenge free radicals.

1.7 IN VITRO AND IN VIVO MODEL SYSTEMS FOR ANTIDIABETIC ACTIVITY

α-glucosidase inhibition assay is the widely used in vitro model to screen the antidiabetic efficacy of the plant extract. It works on the principle of preventing the increase of post prandial hyperglycemia. On the other hand in vitro models are also available in cell lines for screening antidiabetic activity are pancreatic (RINm5F, BRIN BD 11, HIT T15), adipocytes (3T3-L1), hepatic (HEP G2, H 4 IIE), muscle (L6, C2C12, CaCO₂). These cell lines are based on the mechanism of impairment of glucose uptake from small intestine, stimulation of insulin secretion, insulin mimetic and insulin
sensitization activity at target tissue such as liver, skeletal muscle or adipocyte and antagonism of glucagons activity (Patel and Mishra 2011).

Prior to the cell line study the plant extracts are subjected to cytotoxic studies (MTT assay) to determine the viability of the cells. This assay works with the principle of reduction of yellow color (MTT) to purple formazan by the enzyme reductase which is present in living cells (Freshney 2000). Based on the cytotoxic studies, the dosage levels of the plant extract to the cell lines were optimized. Hence, the potency of the *A. lineata* extract to inhibit the carbohydrate digesting enzymes (α-glucosidase) by preventing post prandial hyperglycemia was studied. Insulin mimicking and sensitization activities were also evaluated in the extract to study the synergistic effect in 3T3-L1 cell line.

Animal models of diabetes are greatly useful and have distinct advantage in biomedical studies because they offer promise of new insights into diabetic research (Srinivasan and Ramarao 2007). The currently available compounds to induce diabetes in rats are alloxan monohydrate, streptozotocin (STZ), nicotinamide, ferric nitritriacetate, ditizona and antiinsulin serum. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin (Raju and Balaraman 2008).

STZ (2-deoxy-2-(3-(methyl nitrosureido)-D-glycopyranose)) is synthesized by *Streptomyces aachromogenes* and it is well known for its selective β-cell cytotoxicity, less toxic than alloxan and allows the consistent maintenance of *diabetes mellitus* (Balamurugan et al 2003). In the present study, the ameliorative effect of the EtALL extract was analysed in STZ induced rats.
1.8 BIOACTIVITY GUIDED FRACTIONATION

Bioassay guided fractionation is a procedure whereby the extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is checked for its purity in the thin layer chromatography (TLC) and further evaluated in a bioassay system (in vitro model). In vitro bioassays can be divided into two groups, molecular assays and cellular assays (Rees et al 2001). Molecular assays look for activity using isolated systems such as enzymes, receptors and genes, while cellular assays use intact cells. Molecular assays usually deal with a single subcellular target, while cellular assays detect any substance inhibiting cell growth (Atta-ur-Rahman et al 2005).

Lipogenesis is the process by which simple sugars such as glucose are converted into fatty acids, which are subsequently esterified with glycerol to form the triacylglycerols and are packed as very low density lipoproteins which are secreted from the liver (Gregoire et al 1998). The active fraction which exhibits the highest percentage of lipogenesis activity were selected and then subjected to spectral analysis to determine its structure and composition. The three main structure determining techniques used are mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR). These methods have seen vast improvements in sensitivity and technological advances over the past ten years and when taken in tandem they represent very powerful means of solving unknown molecular structures (Mendonca-Filho 2006). High-resolution mass spectrometry in most instances, provide the molecular mass to such a precision that the molecular formula can also be determined. The exact number of hydrogen and carbon atoms may be verified from one dimensional (1D NMR and $^1$H-$^1$H
COSY) data, which gives information about connectivity’s between magnetic nuclei in the sample, elucidating the full structure. IR gives the details of the possible functional groups present in the bioactive compound. Therefore, in the present study the bioactive principle for antidiabetic effect in the leaves of *A. lineata* was isolated using bioassay guided fractionation technique and the structure elucidation of the bioactive compound was carried out using spectral analysis.

1.9 GENE EXPRESSION

Transcriptional profiling is a tool that provides unique data about disease mechanisms, regulatory pathways, and gene function (Welsh et al 2001). This technology not only allows comparison of gene profiles in normal and pathological tissues but also helps to establish interrelationships among genes (Yokomori et al 1999).

Carbohydrate metabolism and differentiation of 3T3-L1 adipocytes are associated with diabetes. Peroxisome proliferators activated receptor (PPAR-γ) and the CCAAT/enhancer binding protein family (C/EBP-α, β, and δ) are critical factors in 3T3-L1preadipocyte differentiation (Po-Jung et al 2005). PPAR-γ is a member of the nuclear receptor super family of transcription factors and it is predominantly expressed in adipose tissue. C/EBP families are basic leucine zipper transcription factors (Wei et al 2005). C/EBP family and PPAR-γ are sequentially expressed during 3T3-L1 preadipocyte differentiation. The promoters of several adipogenic genes are regulated by these transcription factors during adipocyte differentiation. Hence, in the present study the isolated bioactive principle was evaluated for the expression level of PPAR-γ and C/EBP-α genes.
1.10 MOLECULAR DOCKING IN ANTIDIABETIC DRUG DEVELOPMENT

Plant based compounds now serve as the base for drug discovery. Computer-aided drug design (CADD) is an exciting and diverse discipline where various aspects of applied and basic research merge and stimulate each other (Brooijmans and Kuntz 2003). Drug discovery typically starts with an analysis of binding sites in target proteins or an identification of structural motifs common to active compounds. It ends with the generation of small molecule “leads” suitable to further chemical synthetic work (Becker et al 2006).

Molecular-docking methodologies ultimately seek to predict the best mode by which a given compound will fit into a binding site of a macromolecular target (Hongming et al 2006). The currently available docking software’s are GLIDE, GOLD, FLEX-X, and AUTODOCK. Among these software’s GOLD is highly regarded within the molecular modeling community for its accuracy and reliability. Hence, GOLD software was used to dock the isolated bioactive principle with the receptors (PPAR-\(\gamma\) and C/EBP-\(\alpha\)).

There has been an extensive research focused on PPAR-\(\gamma\) belonging to nuclear receptor family and C/EBP-\(\alpha\) CAAT enhancer binding proteins which are ligand-activated transcription factors. PPAR-\(\gamma\) and C/EBP-\(\alpha\) is expressed most abundantly in adipose tissue and mediates the antidiabetic activity of the insulin-sensitizing drugs belonging to the thiazolidindione (Campbell 2005). This key transcriptional factor plays a pivotal role in regulating adipogenesis, insulin sensitivity and glucose homeostasis (Willson et al 2000). A drug molecule is triggered when the binding of small molecule to the receptor protein is perfectly done. Such protein-ligand interaction is comparable to the lock-and-key principle, in which the lock encodes the
protein and the key is ensembled with the ligand. The major driving force for binding appears to be hydrophobic interaction whose specificity is however controlled by hydrogen bonding interactions (Hugo 1998). Therefore, in the present study the binding efficiency is determined to evaluate the best fit molecule of the isolated bioactive compound to the receptors (PPAR-\(\gamma\) and C/EBP-\(\alpha\)).

1.11 MOTIVATION OF THE PRESENT STUDY

*Andrographis lineata* is an erect perineal herb endemic to Deccan and Carnatic regions of South India. The leaves of *Andrographis lineata* have been reported for various ailments including diabetes, snakebites, cancer and inflammation. There are reports on insecticidal activity, antimicrobial, diuretic and hepatoprotective activity. Due to the wide range of medicinal applications, the plant is generally harvested from the wild, a process jeopardizing the natural biodiversity. The uncontrolled harvesting coupled with habitat destruction is the main cause of decline in the population of this plant and hence sustainable harvest of this plant from wild is no longer a viable option. Therefore, some other strategies should be devised to meet the requirement of this plant for traditional medicine, which is considered as an important mode of health care. *In vitro* conservation strategies have several benefits for the quick cultivation of medicinal plants, thereby providing a continuous supply of plant materials from elite germplasm lines, which can make significant contributions to the exploitation of therapeutic properties of these plant species and eliminate the need for harvest from the wild. Hence an attempt was made to formulate a protocol to ecorestore this valuable medicine plant for phytomedicine production.

Diabetes, particularly obesity and metabolic syndrome related type 2 diabetes, is a major health problem in the Western world, and it is becoming an increasing threat in developing countries as wealth accumulates and
lifestyles changes. It may be caused by metabolic syndrome, which is characterized by a reduced sensitivity to insulin signaling and a reduced efficiency of glucose transport, primarily in adipocytes and muscle cells, leading to hyperglycemia and hyperinsulinemia. Glitazones can enhance adipocyte differentiation to increase glucose uptake in cells to decreases blood glucose levels is the mechanism being widely used for the treatment. However, this could result in excessive accumulation of white adipose tissue. Recent studies showed that white fat tissue accumulation plays crucial role in the development of obesity and type 2 diabetes.

Therefore there is a contradiction in using glitazones for the treatment of obesity and T2D. Thus, a novel small active molecule with insulin-like actions, but have no effect on accumulation of white adipose tissue is desirable. Plants have been an exemplary source of medicine for thousands of years. The World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines.

India is the largest producer of medicinal plants and is rightly called the "Botanical garden of the World". The traditional knowledge with its holistic and systematic approach supported by scientific documentation can serve as an innovative and powerful discovery engine for newer, safer and affordable medicines for their hypoglycaemic activities. There is a lack of scientific evidence proving their efficacy and safety.

One of the major problems with this herbal formulation is that the active ingredients are not well defined. It is important to know the active component and their molecular interactions, which will help to analyze therapeutic efficacy of the product and also to standardize the drug. Although the leaves of *A. lineata* were very effective in treating diabetes, there was no proper scientific documentation pertaining to the bioactive molecule for carbohydrate metabolism. Therefore the ethanolic crude extract of
Andrographis lineata was subjected to in vitro and in vivo study. Further the bioactive fraction with the highest lipogenic activity was identified and analysed for m-RNA expression (PPAR-\(\gamma\) and C/EBP-\(\alpha\)). The insilico studies proved the identified bioactive molecule as the best fitted molecule for both the receptors with positive scores.

1.12 OBJECTIVES OF THE PRESENT WORK

- To optimize a reproducible micropropagation protocol and to confirm the genetic stability in the in vitro regenerants.
- Screening, isolation and characterization of active principle for antidiabetic activity.
- Evaluation of active principle by gene expression studies (PPAR-\(\gamma\) and C/EBP-\(\alpha\)).
- To perform molecular docking studies in PPAR-\(\gamma\) and C/EBP-\(\alpha\) for the identified active principle.