CHAPTER 1

INTRODUCTION

Medicinal Plants have been widely used in traditional medicine for several generations (Matthews et al., 1999). The art of using traditional medicine has employed several plant products. In India, Latin America and several Asian countries alternate medicine has flourished for generations.

Medicinal plants play a major role in the primary health care of about 80% of the world's inhabitants especially in developing countries (Farnsworth et al., 1985). In these countries, using local traditions and beliefs, is still the mainstay of health care. The application of modern scientific technologies in this area will increase the output for the discovery of new drugs.

The World Health Organization (WHO) estimates that 4 billion people, i.e. 80 percent of the world population, presently use plant medicine for primary healthcare (Akerele, 1983). Natural products are suited for random screening approaches to drug discovery. The structural diversity provided by natural products is unsurpassed by synthetic chemistry; natural products frequently have drug like properties so that they are absorbed, distributed and metabolised in the body. Natural products provide starting points that can be optimised by complimentary techniques of combinatorial chemistry. Consequently, natural products can be an economical source of novel compounds for discovering activities against challenging biological targets.

Only in the recent years, traditional forms of medicine did open out and allow the training of individuals in a scientific manner. There is a vast sea
of knowledge and the interplay between modern sciences and traditional medicine help in developing of new molecules as drugs.

The main objective of this thesis is to establish in vitro screens to investigate the anti-diabetic potential of these traditional plants already in use for diabetes treatment for hundreds of years in India. Advanced analytical techniques are employed to identify and characterize the nature of the anti-diabetic compounds present in these traditional Indian plants. Specific cellular targets have been identified and the interaction of these molecules on such targets are used to develop bioactivity based screenings.

Traditional plants have been used for diabetes and several hundred plant species with reputed remedial efficacy. However, very few of these plants have been investigated and await adequate scientific and medical evaluation. Accordingly World Health Organisation (WHO) recommended that traditional plant treatment for diabetes warrant further evaluation (WHO report, 1980).

From the traditional medicine's point of view, the application of modern scientific tools to validate the medicinal properties of plants will help in improving the quality control of the preparations of traditional medicine. They also help to confirm the specific bioactivity and the mechanism of their interaction on host cells.

The potential impact of this technology can serve us in three ways:

(a) Used directly as pharmaceuticals as an alternate form of medicine.
(b) Used as template for chemical synthesis of related medicinal compounds.
(c) Research tools in drug development namely identifying active chemical compounds.
1.1 SYSTEMS OF MEDICINE

1.1.1 Traditional Medicine

Traditional medicine is defined in the WHO Eighth General Programme of Work as "Traditional medicine is widespread throughout the world. As its name implies, it is part of the tradition of each country and employs practices that are handed down from generation to generation of healer. Its acceptance by people receiving care is also inherited from generation to generation". Atleast 80% of population in developing countries depend on traditional medicine for primary health care (Bodeker, 1996).

It is the sum total of all the knowledge and practices used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation verbally or in writing. Traditional medicine might also be considered as a solid amalgamation of dynamic medical know-how and ancestral experience.

Traditional plant medicines have played an important role in biomedicine. Medicinal plants have been widely used in traditional forms of medicine for several generations. The art of healing in traditional medicine has employed the use of different plant products. These products are either used as extracts or prepared into mixtures and have been successful in treatment of diseases. In India and several Asian countries like China traditional medicine flourished over generations. Unconventional medicine is used even in developed countries (Eisenberg et al., 1993).
Three most popularly used systems - Ayurveda, Siddha and Unani, which together account for most of the present day uses of Indian Systems in the country and form an essential part of Indian culture.

1.1.2 Indian System of Medicine (ISM)

The term Indian System of Medicine (ISM) covers both the systems, which originated in India and outside but were adopted in India in course of time. These systems are Ayurveda, Siddha, Unani etc. Among these, Ayurveda and Siddha are the two oldest systems and Unani-Tibb (10th century AD) came much later. These systems have become a part of the culture and traditions of our country.

It is only in India that at present all these systems of medicine are currently in a high degree of usage. Even today, when Allopathy dominates the scene, there is a vast majority of people in our country, for whom these indigenous systems of medicine are the only easily available, affordable and acceptable health care and cure resources.

India's traditional medical systems are part of a time-honoured and time-tested culture that still intrigues people today. A culture that has successfully used nature to treat primary and complex ailments for over 3,000 years obviously has a contemporary relevance. In an age when toxic drugs are increasingly unwelcome and when people are inclined in using viable alternatives, India's medical heritage must be documented, saved and used. These traditions are passed from generation to generation verbally and no systematic and scientific documentation has been done.
In recent years there is a resurgence of interest and usage of indigenous systems of medicine, because of people's belief that these medicines are generally free from harmful reactions and after-effects.

1.1.2.1 Ayurveda

The oldest Indian system of medicine 'Ayurveda', originated between 2500 and 500 BC. The word Ayurveda derived from Ayus(r), meaning life and veda means knowledge. Thus Ayurveda means 'Science of Life'. It is the ancient Indian system of health-care and longevity. Ayurveda takes a holistic view of man, his health and illness (Pietroni, 1987). Plant materials are the dominant part of Ayurveda. Charka Samhita (~900 BC) is the first recorded treatise fully devoted to the concepts and practice of Ayurveda. It lists about 341 plant materials for use in medicine (Mehata, 1979; Sharma, 1981). The next mark in the Ayurvedic literature is Sushruta Samhita (~600 BC). It describes about 395 medicinal plants (Singhal, 1979; Majumdar, 1971; Krishnamurthy, 1991).

1.1.2.2 Siddha

Siddha System is one of the oldest systems of medicine in India. The term 'Siddha' means achievement and 'Siddhars' were saintly figures who achieved results in medicine through the practice of Yoga. Eighteen 'Siddhars' are said to have contributed towards the development of this medical system. Siddha literature is in Tamil and it is practiced in Tamil speaking parts of India. The Siddha System is largely therapeutic in nature. About 500 medicinal herbs have been listed in the Siddha Materia Medica and have been in use in
Tamilnadu for years. *Adhatoda zeylanica* (or *Adhathodai* in Tamil), is used for coughs, colds, wheezing, fevers etc.

Siddha system studied thousands of plants for medicinal and health problems which are relevant at this day and age. Siddha is a systematic medical system with an accurately defined, documented symptomatology with well researched remedies making it an ideal bridge between modern medicine and other complimentary medical systems (Subbarayappa, 1997).

### 1.1.2.3 Unani system of medicine

Unani System of medicine originated in Greece (460 BC-377 BC). Arabs and Persians brought it to India. Nan is the Arabic name for Greece which denotes the origin of the system. Hippocrates established his philosophy of health on the word 'Physis' which meant simply 'Organism' and he postulated that life comprised a reciprocal relationship between organism and environment. He explained that disease was a normal process and its symptoms were the reaction of the body to the disease. The main function of the physician was to aid the natural forces of the body. He held that the body has four humours that keep up the balance. He also laid emphasis on diet and drugs for curing diseases (Concon, 1983).

### 1.1.3 Modern Medicine

#### 1.1.3.1 Allopathy

Conventional medicine is sometimes referred to as allopathic medicine. The term Allopathy is derived from the Greek allo, meaning opposite, and pathos, meaning suffering. In general, conventional medicine
tends to focus on the disease and employs techniques to oppose it. Allopathy is a term coined by Samuel Christian Hahnemann 300 years ago to refer to the mainstream medicine of the time, and to be distinguished from his new discipline of homeopathy (Widakowich, 2000). Therefore, Allopathy, referred to the contrary philosophy of treating a symptom or disease with an agent which produces the opposite effect. Today, the term allopathy is used to refer Western science-based medicine.

1.1.4 Traditional Medicine and Allopathy

Over the years allopathic medicine has dominated on the other forms of medicine. The reason for this may be due to the fact that allopathy developed self adapting modern science and that it promoted the training of individuals in specific fields of interest. This may have been the lacuna in the wide spread development of traditional forms of medicine. Documentation of data and treatment procedures were not streamlined and were kept secret.

The major difference between allopathy and other forms of medicine is that, allopathy uses purified structurally elucidated compounds. In most of the other forms of medicine, the preparations administered are crude extracts. The belief is that no one compound is effective and it is the synergism between the various compounds is the one that produces the effect. This appears to be the difference between allopathic and traditional medicine.

1.1.5 Traditional Medicine and Diabetes

Physicians of the ancient world utilised plant medicines to treat diabetes. Ancient Egyptian Medical Text books, the Ebers Papyrus described
the pathology of a clinical condition resembling diabetes (Massengill, 1943). In India, during the period 700-200 BC, Physicians diagnosed diabetes by tasting the urine of patients for sweetness. The Sushruta Samhita, an Ayurvedic text book written between the fourth and fifth centuries BC, describes two types of diabetes. The author distinguished between a genetic disorder and the other type due to dietary indiscretion. Even 2500 years ago, Ayurvedic physicians recognised clinical entities of insulin dependent diabetes mellitus (IDDM) and non insulin dependent diabetes mellitus (NIDDM). Historic accounts reveal that plant materials have been used for millennia to treat diabetes (Ackerknecht, 1982).

1.2 NEW MOLECULES FROM PLANTS AND THEIR ROLE IN DRUG DEVELOPMENT

Plants are the source for many drugs. Natural products served as a major source of drugs for centuries (Clark, 1996). Plant materials have played a dominant role in the introduction of new molecules as therapeutic agents (Foye, 1981; Sukhdev, 1989). Several new therapeutic agents from plant materials have been derived such as Reserpine (Mc Gahren et al., 1969), Deserpidine, Rescinnamine, Vinblastine and Vincristine (Cox, 1990). Some of the most important chemotherapeutic agents currently in use for the treatment of certain types of cancer are plant based eg. Vinblastine and Vincristine both isolated from Catharanthus roseus. A diterpinoid constituent of several Texas sps. is effective in the treatment of metastatic ovarian cancer and for lung cancer, metastatic breast cancer and malignant melanoma (George et al., 1995).
Healthcare remedies used in many parts of the world to treat a variety of illnesses have a common beginning from wild plants. Aspirin, for instance, owes its origin to the willow tree. Hippocrates used powder extracted from willow bark to treat pain and reduce fever in the fifth century BC. Over 2000 years later, the chemical compound salicin was isolated from willow bark, the precursor to the active compound found in modern aspirin (Budavari, 1996).

Plants form the source of new compounds which constitute the base for new pharmaceutical products and a major component of the burgeoning markets for herbal health care remedies and natural products. They also provide a source of income for growers, traders, collectors and manufacturers of plant-based medicines. In this dual role as a source of healthcare and income, medicinal plants make an important contribution to the larger development process of the country.

1.2.1 Strategies for drug development

Several strategies have been employed to discover and invent new therapeutic agents. Screening of compounds of natural origin has been the source of innumerable therapeutic agents. Random screening as a tool in discovering new biologically active molecules has been most productive. Advances in cell & molecular biology and genetic engineering have enabled biologists to design target specific screens on cell based mechanisms.

1.2.2 Isolation of active components

Isolation and purification of the active ingredients of medicinal plants for valuable medicines was started in the 19th century. In 1815, F. W. Sertürner isolated morphine from opium extract (Sertürner, 1817) (Fig 1.1). Papaverin was
in 1848, but its antispasmodic properties were not discovered until 1917 (Sneader, 1985).

The isolation of an active compound is the first stage in the development of a new agent which might be developed as a drug for advancement to clinical trials and possibly to commercial use (Grever et al., 1992). While the initial plant sample (0.3-1.0 kg) collected generally yields enough extract (10-40 g) to permit the isolation and structural elucidation of the pure, active constituent. Subsequent secondary testing and preclinical development might require gram or even kilogram quantities. Approval of an agent for clinical development could require multi-kilogram quantities. In order to isolate sufficient quantities of an active agent for preclinical development, re-collections of 5 to 200 kg of the dried plant material might be necessary, preferably from the original collection site.

After extraction of the drug is then studied through *in vitro* screens on cell lines or primary cultures, in animal models then in humans, until a physician prescribing the drug. The medicine can be used rationally after full
information is available about its primary effects, side effects, duration of action, route of elimination, contraindications and interactions with other drugs. The new drug development process takes about a decade.

1.2.3 **Drugs from plants**

Over 120 pharmaceutical products currently in use are plant-derived and some 75% of these were discovered by examining the use of these plants in traditional medicine (Farnsworth, 1988). Plants have long been a very important source of drugs. Examples are digoxin extracted from foxgloves, *Digitalis lanata* (Hollman, 1996) used to treat heart failure and paclitaxel (Pandey, 1998) a new and promising anticancer agent. The first tranquiliser, reserpine, came from the Indian plant *Rauvolfia serpentina* used in traditional Ayurvedic medicine for mental disturbances (Dev, 1999). Clinically useful drugs from plants are given in Table 1.1.

1.2.4 **Drug development process**

After the isolation of bioactive compound, *in vitro* screening and animal model studies (*in vivo*), only five in 5,000 compounds that enter preclinical testing make it to human testing. And only one of those five is approved for sale. Once a new compound has been identified in the laboratory, medicines are developed as follows (Fig 1.2 & Table 1.2):

Preclinical testing: The compound is studied in laboratory and conduct animal studies to show its biological activity against the targeted disease, and the compound is evaluated for safety (Wierenga *et al.*, 1999).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant Source</th>
<th>Therapeutic Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajmalicine</td>
<td>Rauvolfia serpentina (L.) Benth. Ex Kurz (Apocynaceae) (Indian snakeroot)</td>
<td>Circulatory stimulant</td>
</tr>
<tr>
<td>Arecoline</td>
<td>Areca catechu L. (Palmae) (Betel-nut palm)</td>
<td>Anthelmintic</td>
</tr>
<tr>
<td>Asiaticoside</td>
<td>Centella asiatica (L.) Urban(Umbelliferae)</td>
<td>Vulnerary</td>
</tr>
<tr>
<td>Atropine</td>
<td>Atropa belladona (belladona)</td>
<td>Anticholinergic</td>
</tr>
<tr>
<td>Bromelain</td>
<td>Ananas comosus (L.) Merrill (Bromeliaceae)</td>
<td>Anti-inflammatory;</td>
</tr>
<tr>
<td>Chymopapain</td>
<td>Carica papaya L. (Caricaceae) (Papaya)</td>
<td>Proteolytic;Mucolytic</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Curcuma longa L. (Zingiberaceae) (Turmeric)</td>
<td>Choleretic</td>
</tr>
<tr>
<td>Deserpine</td>
<td>Rauvolfia tetraphylla L. (Apocynaceae)</td>
<td>Antihypertensive;</td>
</tr>
<tr>
<td>Digitalis</td>
<td>Digitalis purpurea (Fox gloves)</td>
<td>Cardiotoxic</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Mucuna deeringiana (Bort.) Merrill(Leguminosae)</td>
<td>Antiparkinsonism;</td>
</tr>
<tr>
<td>Emetine</td>
<td>Cephaelis ipecacuaha (Brot.) Richard(Rubiaceae)</td>
<td>Amoebicide;</td>
</tr>
<tr>
<td>Glauarubin</td>
<td>Simarouba glauca DC. (Simaroubaceae)</td>
<td>Amebicidal</td>
</tr>
<tr>
<td>Glaziovine</td>
<td>Ocotea glazioyii Mez (laurecaceae)</td>
<td>Antidepressant</td>
</tr>
<tr>
<td>Gossypol</td>
<td>Gossypium spp. (Malvaceae) (Cotton)</td>
<td>Male contraceptive</td>
</tr>
<tr>
<td>Hyoscyamine</td>
<td>Duboisia Myoporoides R.Br. (Solanaeae)</td>
<td>Anticholinergic</td>
</tr>
<tr>
<td>Kawaina</td>
<td>Piper methysticum Forst. f. (Piperaceae)</td>
<td>Tranquilizer</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>Crotalaria spectabilis Roth (Leguminosae)</td>
<td>Antitumor agent</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Strophanthus gratus (Hook.) Baill.(Apocynaceae)</td>
<td>Cardiotoxic</td>
</tr>
<tr>
<td>Papain</td>
<td>Carica papaya L. (Caricaceae) (Papaya)</td>
<td>Proteolytic;Mucolytic</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>Physostigma venenosum Balf. (Leguminosae)</td>
<td>Anticholinesterase</td>
</tr>
<tr>
<td>Picrotoxin</td>
<td>Anamirta cocculus (L.) Wright &amp; Arn (Fish berry)</td>
<td>Analeptic</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Pilocarpus jaborandi Holmes (Rutaceae)</td>
<td>Parasympathomimetic</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Cinchona ledgeriana Moens (Rubiaceae)</td>
<td>Antiarhythmic</td>
</tr>
<tr>
<td>Quinine</td>
<td>Cinchona ledgeriana Moens (Rubiaceae)</td>
<td>Antimalarial; Antipyretic</td>
</tr>
<tr>
<td>Quisqualic acid</td>
<td>Quisqualis indica L. (Combretaceae)</td>
<td>Antihistaminic</td>
</tr>
<tr>
<td>Rescinnami</td>
<td>Rauvolfia serpentina (L.) Benth.(Apocynaceae)</td>
<td>Antihypertensive;</td>
</tr>
<tr>
<td>Reserpine</td>
<td>Rauvolfia serpentina (L.) Benth.(Apocynaceae)</td>
<td>Antihypertensive;</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Lonchocarpus nicou (Aubl.) DC.(Leguminosae)</td>
<td>Piscicide</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>Datura metel L. (Solanaeae)(Recurved thorapple)</td>
<td>Sedative</td>
</tr>
<tr>
<td>Strychnine</td>
<td>Strychnos nux-vomica L. (Loganiaceae)</td>
<td>CNS stimulant</td>
</tr>
<tr>
<td>Taxol</td>
<td>Taxus brevifoliat Nut(Pacific yew)</td>
<td>Anticancer</td>
</tr>
<tr>
<td>Theobromine</td>
<td>Theobroma cacao L. (Sterculiaceae) (Cocoa)</td>
<td>Diuretic; Vasodilator</td>
</tr>
<tr>
<td>Vasicine</td>
<td>Adhatoda vasica Nees (Acanthaceae)</td>
<td>Oxytocic</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>Catharanthus roseus (L.) G.Don (Apocynaceae) (Madagascal rosy periwinkle)</td>
<td>Antitumor agent</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Catharanthus roseus (L.) G.Don (Apocynaceae) (Madagascal rosy periwinkle)</td>
<td>Antitumor agent</td>
</tr>
</tbody>
</table>
Table 1.2 Drug development process from plants

| Isolation of bioactive compound from plants |
| Structural elucidation and changes on parent molecule |
| Animal models and clinical trials |

Fig. 1.2 Drug Discovery Process
Clinical Trials, Phase I: These tests involve about 20 to 80 normal, healthy volunteers to study a drug’s safety profile and the safe dosage range. The studies also determine how a drug is absorbed, distributed, metabolised, and excreted as well as the duration of its action (Wierenga et al., 1999).

Clinical Trials, Phase II: In this phase, controlled trials of approximately 100 to 300 volunteer patients (people with the disease) assess a drug’s effectiveness (Wierenga et al., 1999)

Clinical Trials, Phase III: This phase usually involves 1,000 to 3,000 patients in clinics and hospitals. Physicians monitor patients closely to confirm efficacy and identify adverse events.

Approval: After the FDA approval the new medicine becomes available for physicians to prescribe. For some medicines, FDA requires additional trials (Phase IV) to evaluate long-term effects. Discovering and developing safe and effective new medicines takes about 15 years (Table.1.3 & Fig.1.3).

1.2.5 Plant compound classes

Active plant compounds include flavonoids, saponins, lignans, and tannins. Many of these compounds are bitter or astringent. There are many classes of plant compounds but mostly plant derived drugs are alkaloids. Among the classes of chemical compounds isolated having biological activity are alkaloids (Noble, 1990), flavonoids, steroids, glycopeptides, terpinoids (Craig, 1997) etc. The plant compound classes, active component, source and activity are given in Table.1.4.
Fig. 1.3 Complete drug development process
### Table 1.3 Complete Drug Development Process

<table>
<thead>
<tr>
<th>Early Research/Preclinical Testing</th>
<th>Clinical Trials</th>
<th>FDA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years</td>
<td>Phase I</td>
<td>Phase II</td>
<td>Phase III</td>
</tr>
<tr>
<td>Test Population</td>
<td>Laboratory and animal studies</td>
<td>20 to 80 healthy volunteers</td>
<td>100 to 300 patient volunteers</td>
</tr>
<tr>
<td>Purpose</td>
<td>Assess Safety and biological activity</td>
<td>Determine safety and dosage</td>
<td>Evaluate effectiveness, look for side effects</td>
</tr>
<tr>
<td>Success Rate</td>
<td>5,000 compounds evaluated</td>
<td>5 compounds enter trials</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2.6 Plant based anti-diabetic drugs

More than 400 traditional treatments for diabetes have been reported all over the world. But a few only have been scientifically analysed to determine their hypoglycemic properties (Bailey and Day, 1989; Perez *et al.*, 1984; Winkleman, 1989). One of the scientific investigations led to the development of *metformin*, the widely used commercial anti-diabetic drug from Goat's rue (*Galega officinalis*) also known as French Lilac (Bailey, 1992).
More than 1000 species of plants reported for diabetes treatment. According to reports out of 1,123 plants used to treat diabetes, 295 plants have been screened (Marles and Farnsworth, 1994).

Hypoglycemic activity of the plants available in the country has been scientifically documented in a more detailed way (Jahodav, 1993). The mechanism of blood sugar lowering by the crude/impure swerchin (SWI) is isolated from the hexane fraction of *Swertia chirayita* was investigated using animal experiments (Saxena *et al.*, 1993). The hypoglycemic effect of orally administered extracts of leaves and roots of *Coccinia indica* has been reported earlier (Hossain *et al.*, 1992).

**Table 1.4 Plant compound classes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Active component</th>
<th>Plant source</th>
<th>Type of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Vincristine and Vinblastine</td>
<td><em>Catharanthus roseus</em></td>
<td>Anticancer</td>
</tr>
<tr>
<td>Steroids</td>
<td>Digoxin</td>
<td><em>Digitalis purpurea</em></td>
<td>Cardiotonic</td>
</tr>
<tr>
<td></td>
<td>Digitoxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Vernolepin &amp; Taxol</td>
<td><em>Veronia</em> species &amp; pacific yew tree</td>
<td>Anticancer</td>
</tr>
<tr>
<td>Quinones</td>
<td>Shikonin</td>
<td><em>Rauvolfia serpentina</em></td>
<td>Antimalarial, Antibacterial</td>
</tr>
<tr>
<td>Lignans</td>
<td>Etoposide</td>
<td><em>Podophyllus</em> sp.</td>
<td>Anticancer</td>
</tr>
<tr>
<td></td>
<td>Teniposide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Puerarin</td>
<td><em>Pueraria lobata</em></td>
<td>Antihepatotoxic, Antimicrobial</td>
</tr>
</tbody>
</table>
Recent international studies showed that aqueous extracts of *Agrimony eupatoria* (agrimony), *Medicago sativa* (lucerne), *Coriandrum sativum* (coriander) *Eucalyptus globulus* (eucalyptus), *Agaricus ampestris* (edible mushroom) and *Viscum album* (mistletoe) enhanced insulin secretion and mimicked the effect of insulin on glucose metabolism *in vitro* (Gray and Flatt 1997a,b,c; Gray and Flatt 1998a, b, c; Gray and Flatt 1999a,b).

Such dual pancreatic and extrapancreatic actions would prove to be an important advance on existing therapies used to treat and control diabetes, such as oral hypoglycemic drugs (which act either by enhancing insulin secretion or by improving the action of insulin). These combined findings illustrate the enormous potential of plants for use as drugs and the discovery of natural products for diabetes therapy.

Many plants have been used for the treatment of diabetes mellitus in traditional systems of medicine throughout the world. Indeed, along with dietary measures, plant preparations formed the basis of the treatment of the disease until the introduction of insulin in 1922.

A number of review articles have been published on the traditional use of plants in diabetes (Swanston-Flatt *et al.*, 1991; Bailey and Day, 1989) and on plants and phytochemicals whose reputed hypoglycemic effects have been scientifically investigated (Perl, 1988; Handa *et al.*, 1989; Day, 1990; Marles and Farnsworth, 1994).
1.3 IN VITRO BIOSCREENS

1.3.1 What are in vitro screens?

In vitro literally means in glass; referring to a process or reaction carried out in a culture dish or test tube. Whereas in vivo denotes to test in living organism. To achieve rapid drug development, active candidate compounds can be appraised via an integrated Programme of in vitro studies. A number of in vitro models have been used for the detection and characterisation of the compound. The recent approach of using functional bioassays as screening templates is the best way to determine specific biological activity of the compound.

Animals and human patients can-not be used routinely for in vitro tests because of the amount of material, which would be needed to satisfy all the aspects of drug discovery Programme. The use of animals and living organisms are minimised due to ethical laws. Alternate materials like cell lines and primary culture are encouraged to detect the drug activity.

1.3.2 On what basis they developed?

In vitro screens are based on creating conditions in a culture plate or micro tube as existing inside the cell or organism. These are useful to test the cellular and molecular markers whose levels are elevated or decreased in a particular cell or organ in diseased conditions. These in vitro screens are used to study the cellular markers and help in new drug development process for specific diseases.
1.3.3 Screening of plants

There is an estimation that out of 2,50,000 higher plants less than 10% have been screened (Harvey, 2000) as it is time consuming and slow process. Thus it is needed to develop high throughput screens. Plant extracts and compounds are to be tested at a rapid rate. In vitro screens help to identify potential and novel therapeutic agents. At present bioassays are carried out on animal models only, which is a time consuming process. The availability of high throughput screens (HTS) will provide quick and reliable information of specific bioactivity.

1.3.4 Conventional assays

Conventional assays are mostly carried out on living animals by employing biochemical and pharmacological methods. There is a limit to the number of parameters that can be measured in a single experiment. The results are also variable depending upon the route of administration of the compound to be tested. The test compound is required in a large quantity. These are slow, time consuming and expensive. (Farnsworth, 1990)

1.3.5 In vitro Screening

The recent screening techniques called 'High-throughput screening' (HTS) introduced in drug discovery (Cox et al., 2000). The plant extracts and compounds are tested at a rapid rate. Then 'HTS' can be evaluated further through in vitro screens. As the name indicates it is defined to screen 'more than 100,000 samples per week'. High throughput primary screening can produce many thousands of compounds of different kinds. Recent approach of
using functional bioassays as screening templates may be the best way to determine specific biological activity and then proceed to rapid isolation of the active molecule leading to structural elucidation. This requires a variety of rapid, relatively straightforward, relevant and reliable systems that provide physicochemical and biological information of the compound. *In vitro* approaches have the potential to provide information about the biological activity on specific primary culture or cell line. Additionally, it is possible to study the effects of agents on different cell types from multiple species, including humans.

### 1.3.6 Advantages over conventional screens

Conventional screens take longer time even years to get the information on the biological activity of a compound where as *in vitro* screens yield quick results in a matter of few hours and days. Conventional screens require large amounts of compounds in milligrams and grams. *In vitro* screens require small amounts of compound in nanograms or micrograms. *In vitro* screens utilise culture of cells, tissues, and organs, including those of human origin. Advantages of this technique are that cells and tissues can be maintained in a defined, controlled environment.

### 1.3.7 *In vitro* screens for detecting anti-diabetic activity

Conventional anti-diabetic screens are mostly carried out on animal models especially on rats and mice by inducing diabetes artificially with chemicals like alloxan (Dunn *et al.*, 1943) and streptozotocin (Rerup, 1970). There are commercial insulin secreting β-cells lines like MIN-6, HIT-T15,
RINm5F available from ATCC, USA and used for testing the insulin secretion activity after treating cells with the test compounds.

*In vitro* glucose uptake and glucose metabolism, and on insulin secretion by BRIN-BD11 cells were investigated (Gray and Flatt, 1998a). A glucose-responsive clonal insulin-secreting cell line, BRIN-BD11, produced by electrofusion of immortal RINm5F cell with New England Deaconess Hospital rat pancreatic B-cell was used to evaluate insulin secretion (McClenaghan *et al.*, 1996a, Gray and Flatt 1997a,b). This cell line responds to a wide variety of insulinotrophic stimuli including glucose, amino acids, hormones, neurotransmitters and drugs (McClenaghan *et al.*, 1996a, b, 1998, McClenaghan *et al.*, 1998). The appropriateness of BRIN-BD11 cells for screening of anti-diabetic plant materials and characterisation of novel insulin-releasing natural products has been described (Gray and Flatt 1997a,b, 1998b).

### 1.3.8 Glucose uptake assay

Peripheral glucose uptake into the cells is a major problem in diabetes. Most of the glucose uptake assays are by using radiolabeled (L-[\(^{14}\)C] or \(^{3}\)H - D glucose) glucose (Soengas and Moon, 1998; Vedavanam *et al.*, 1999) added to the medium at the rate of 0.1\(\mu\)Ci/ml and incubated. At different time points glucose uptake was measured in an automated scintillation counter. A simple and non radioactive enzymatic glucose uptake assay is developed (Ueyama *et al.*, 2000). Glucose transport activity in mammalian cells has been monitored by radiolabeled tracers such as \([^{14}\)C] 2-deoxy-D-glucose (Sokoloff *et al.*, 1977), \([^{18}\)F] fluoro-2-deoxy-D-glucose (Turkheimer *et al.*, 1994) and \([^{14}\)C] or \([^{3}\)H] 3-O-methyl-D-glucose (Dienel *et al.*, 1997 and Axelrod and Pilch, 1983). Recently developed a fluorescent D-glucose derivative, 2- [N-(7-
Fig. 1. 4 Glucose homeostasis and insulin action
24-nitrobenz-2-oxa-1,3-diazol-4-y1amino] -2-deoxy-D-glucose (2-NBDG)', allows a more sensitive measurement of glucose uptake in real time in single, living cell (Yoshioka et al., 1996) (Fig 1.4).

1.4 GLUCOSE TRANSPORTERS

1.4.1 RT-PCR: Expression of Glucose Transporters (GLUTs)

Glucose is transported into the cells through specific transporters. In diabetes the transport of glucose is reduced. There are no reports available on the expression of glucose transporters with the treatment of plant extracts. However, with commercial anti-diabetic drugs like Troglitazone treatment, the expression level of glucose transporters was studied (Park et al., 1998). In this study standardization was done for RT-PCR technique with cells and tissues treated with plant extracts.

1.4.2 Glucose transport into cells

Glucose transport into cells is dependent on the actions of insulin in target tissues like muscle and adipose. The main peripheral binding sights for insulin are located on fat and muscle cells, which includes cardiac muscle (Garvey, 1992). Overweight people have an increased amount of adipose tissue
compared to lean people and cause decreased insulin action and glucose transport (Torres, 1999).

When glucose levels rise in blood, the pancreas secretes the hormone insulin. Insulin signals the tissues to metabolise glucose for energy. It also triggers glucose to move into skeletal muscle and adipocytes, where some of it is used for energy. However, the body does not use all the glucose right away but stored as glycogen in the liver, muscles and fat cells. When blood glucose levels fall, the liver and kidney synthesise glucose and release it into the blood stream. The glycogen pools formed earlier are also broken down to provide glucose for energy (Stryer, 1988).

1.4.3 Types of Transporters (GLUTs)

Two classes of glucose carriers are described in mammalian cells: a) the Na(+)‐glucose co-transporters and b) the facilitative glucose transporters.

1.4.3.1 Sodium dependent glucose transporters

The Na(+)‐glucose co-transporter transports glucose against its concentration gradient by coupling its uptake with the uptake of Na+ that is being transported down its concentration gradient. The Na(+)‐glucose co-transporter is expressed by absorptive epithelial cells of the small intestine and involved in the dietary uptake of glucose. The same or a related protein may be responsible for the reabsorption of glucose by the kidney. These transport glucose depending on Na⁺ concentration. They actively transport glucose across membranes while Na⁺ flows in the opposite direction.
1.4.3.2 Facilitative glucose transporters

Mammalian cells also express a second family of facilitative glucose transporters. Facilitative glucose carriers are expressed by most of the cells. The facilitative glucose-transporter isoforms have distinct tissue distributions and biochemical properties and contribute to the precise disposal of glucose under varying physiological conditions. Facilitative glucose transport is mediated by members of the Glut family. Six members of the Glut family have been described thus far.

The facilitative glucose transporters are GLUT-1 (erythrocyte type), GLUT2 (liver type), GLUT3 (brain type), GLUT-4 (muscle/fat type), and GLUT5 (small intestine type). Facilitative glucose transporters (GLUTs) have shown to be multifunctional. They transport water and ring compounds as large as nitrobenzene-diazole-aminoglucose and sugar. GLUT-1 is abundant in human red cells and also found in many fetal and adult tissues. Its basic function is to increase supply for growing and dividing cells, and to transport glucose across blood brain barrier and other barrier tissues (Lin et al., 1990).

GLUT-4 is present in skeletal muscle, heart and adipocytes. It increases transportation in response to higher blood insulin. The subcellular localisation of the GLUT-4 (muscle/fat) isoform changes in response to insulin, and this isoform is responsible for most of the insulin-stimulated uptake of glucose that occurs in muscle and adipose tissue. The GLUT-5 (small intestine) facilitative glucose-transporter isoform is expressed at highest levels in the small intestine and may be involved in the transcellular transport of glucose by absorptive epithelial cells (Bell et al., 1990).
Adipocytes and muscle contain primarily the GLUT-4 glucose transporter isomer and small amounts of GLUT-1 also present. GLUT-4 is present only in insulin dependent tissues (Assimacopoulos et al., 1991). whereas GLUT-1 is widely distributed. GLUT-7 is the transporter present in the endoplasmic reticulum membrane that allows the flux of free glucose out of the lumen of this organelle after the action of glucose-6-phosphatase on glucose 6-phosphate (Mueckler 1994). The presence of various glucose transporters in different tissues is shown in Table 1.5.

Table 1.5 Glucose transport into the cells

<table>
<thead>
<tr>
<th>TYPE OF GLUCOSE TRANSPORT</th>
<th>TRANSPORTER</th>
<th>TARGETS</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Sodium dependent</td>
<td>-</td>
<td>Intestinal tract, kidney</td>
</tr>
<tr>
<td>b. Facilitative</td>
<td>GLUT 1</td>
<td>Blood, brain, muscle, RBC, colon, Kidney, placenta, adipose tissue</td>
</tr>
<tr>
<td></td>
<td>GLUT 2</td>
<td>Liver, kidney, intestine, pancreas</td>
</tr>
<tr>
<td></td>
<td>GLUT 3</td>
<td>Neurons, brain, placenta, kidney</td>
</tr>
<tr>
<td></td>
<td>GLUT 4</td>
<td>Adipose tissue, skeletal and Cardiac muscles</td>
</tr>
<tr>
<td></td>
<td>GLUT 5</td>
<td>Small intestine and Spermatozoa.</td>
</tr>
<tr>
<td></td>
<td>GLUT 7</td>
<td>Endoplasmic reticulum</td>
</tr>
</tbody>
</table>
1.5 GLUCOSE METABOLISM

1.5.1 Metabolism to Glucose-6-Phosphate (G6P)

Glucose after entering the cell is rapidly phosphorylated to Glucose-6-Phosphate (G-6-P) in the presence of ATP. This ensures that the concentration gradient across the plasma membrane is maintained and glucose does not leak back out of the cell. This is catalysed by hexokinase in muscle and glucokinase in liver (Fig. 1.5).

The cytosolic concentration of glucose -6- phosphate is dependent on the glucose uptake by the tissues. Muscle and adipose tissue have glucose transporter, GLUT 4, which is translocated from cytoplasmic vesicles to the cell membrane in the presence of insulin. This results in an increased uptake and phosphorylation of glucose and in elevated glucose-6-phosphate intracellular levels.

The rates of whole-body glucose metabolism and muscle glycogen synthesis and the glucose-6-phosphate concentrations in muscle were approximately 80 percent lower in the patients with diabetes than in the normal subjects (Cline et al., 1999).

1.5.2 Glucose metabolism assay (GS and G-6-P)

Diabetes is a metabolic disorder. It can be improved by enhancing the levels of certain enzymes and metabolites. Glycogen synthase (GS) is a rate limiting enzyme of glycogen synthesis. The activity of GS is decreased in diabetes. RT-PCR technique is employed to detect the level of GS. Glucose
Muscle

Liver

Glucose

Insulin

Plasma
membrane

Glucose

GLUT4

GLUT2

Glucose

Glucose

Hexokinase

Glucokinase

Glucose-6-Phosphate

Glucose-6-Phosphate

Fig. 1.5 Scheme showing glucose metabolism
Fig. 1.5 Scheme showing glucose metabolism
after entering the cell is phosphorylated into Glucose-6-Phosphate and can be measured by colorimetric assays.

1.5.3 Activity of Glycogen Synthase (GS)

Glycogen Synthase (GS) is the key insulin regulated enzyme that controls muscle glycogen formation (Yki-jarvinen, 1987; Kruszynska YT. 1986). Alteration in insulin action causes reduced muscle glycogen synthase (GS) activity (Ortmeyer, 1998). In diabetes the ability of insulin to stimulate glycogen synthase in muscle and adipocytes is impaired. Glycogen synthase exists in an active or dephosphorylated form and an inactive or phosphorylated form. The inter conversion of these two forms is regulated by insulin.

In diabetes, activation of glycogen synthase, glucose transport and phosphorylation are impaired. The main effect of insulin on the activation of glycogen synthase in muscle and adipose tissue is due to the elevated intracellular glucose 6-phosphate levels resulting from the stimulated glucose transport. Glucose is the biological fuel to the cells in many organisms. Glucose is synthesised in the liver by glycogen synthase to form glycogen, which is the storage form of glucose (Zierler, 1999).

In diabetes, glucose transport and phosphorylation are impaired, as is the activation of glycogen synthase. In other tissues, such as liver, which have no insulin-stimulated glucose transporters, an elevation in extracellular levels of glucose results in increased cytoplasmic levels of glucose 6-phosphate and activation of glycogen synthase. Insulin has essentially no effect in glycogen synthesis in normal liver.
The main effect of insulin on the activation of glycogen synthase in muscle and adipose tissue is due to the elevated intracellular glucose-6-phosphate levels resulting from the stimulated glucose transport.

1.6 DIABETES AND ITS PROBLEMS IN HEALTH CARE

1.6.1 Diabetes History

Over 2000 years ago, the ancients noticed that some people excreted copious amounts of sweet-tasting urine that attracted ants. (Tasting urine was a diagnostic tool in many cultures). They named the condition *diabetes mellitus*, from the Greek for "fountain" and the Latin for "honey."

Diabetes is a metabolic disorder and over 250 million people are affected worldwide (Seidell, 2000). It is a continuously growing problem and cause many health problems like retinopathy, nepropathy, hypertension etc.

1.6.2 WHO definition

The word *diabetes* means 'to flow through' was coined by Greek Physician 'Aeretaeus' in first century (Satoshkar and Bhandarkar, 1988), *mellitus* refers to the high level of glucose in the urine. As per World Health Organisation (WHO) it is defined as "A metabolic disorder characterised by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both" (WHO report WHO/NCD/NCS/99.2, 1999). It is due to relative or complete destruction of insulin secreting β-cells in pancreas.
1.6.3 Symptoms

Diabetes mellitus presents characteristic symptoms like thirst, hunger, polyuria, blurring of vision, tiredness, sleepiness and weight loss. Often symptoms are not severe or absent. Diabetes may be present in patients for a long time before the diagnosis is made.

The long term effects include specific complications are
i) Retinopathy causes blindness ii) Nephropathy causes renal failure iii) Neuropathy with risks of foot ulcers, amputation and autonomic dysfunction including sexual dysfunction. It also leads to cardiovascular complications.

1.6.4 Classification

There are two types of diabetes: Type -1 or Insulin Dependent Diabetes mellitus (IDDM). This is also called Juvenile diabetes (JD) as it develops in the early stage of life. The second one: Type- 2 or Non Insulin dependent Diabetes Mellitus (NIDDM), also called mature onset diabetes. NIDDM results from an imbalance between insulin sensitivity and insulin secretion. There is an impairment in body's ability to respond to insulin. Out of the two types of diabetes more than 95% are NIDDM cases. In general diabetes denotes Type-2 (NIDDM) only. Non-insulin dependent diabetes mellitus (NIDDM), or Type-2 diabetes is one of the largest health problems in the world. NIDDM currently affects some 6-12 million persons in the US, and incidence is increasing despite continual advances in medical care. Approximately 95% of diabetics are Type-2, presenting with symptoms well into adulthood (Broadhurst, 1997).
1.6.5 Anti-diabetic drugs used in allopathy

Diabetics are mostly dependent on two classes of oral synthetic compounds, i.e. sulfonylureas and biguanides (metformin). In addition to these two, other anti-diabetic agents available for the treatment of type 2 diabetes, are the glucosidase inhibitor (acarbose) and the new class drugs, thiazolidinedione (troglitazone, rosiglitazone).

1.6.5.1 Sulfonyl ureas

Sulfonylureas work predominantly through pancreatic mechanisms by stimulating the release of insulin from pancreatic β-cells. The mechanism by which sulfonylureas stimulate insulin secretion appears to be receptor-mediated (Hribal et al., 2001). Insulin secretion is initiated when the drug binds to a cell surface receptor on the pancreatic β-cell. This interaction inhibits the efflux of potassium ions due to depolarisation that causes an influx of calcium, leading to the release of insulin.

1.6.5.2 Biguanides (Metformin)

Metformin is a unique drug that acts by increasing glucose uptake in muscle and peripheral tissues and by decreasing the production of glucose in the liver. Unlike the sulfonylureas, which are classified as hypoglycemic agents, metformin is more accurately described as an antihyperglycemic agent because it does not cause hypoglycemia when used alone in Type-2 diabetic patients. Metformin lowers plasma glucose levels in Type-2 diabetes by reducing insulin resistance and inhibiting gluconeogenesis.
The administration of metformin 0.5–3 g/day for up to 3 months increased peripheral glucose utilisation by 18–29% relative to baseline or placebo. This improvement in glucose utilisation occurs in skeletal muscle (Borst et al., 2000) in fat and intestinal tissue, and possibly in erythrocytes.

1.6.5.3 New class of drugs (Thiazolidinedione)

In 1997 a new drug, Troglitazone belonging to the class thiazolidinediones was approved and marketed. It increases the peripheral insulin sensitivity. Thus it lowers the levels of glucose and insulin. The usual requirement is 200 to 600 mg/day. The second drug in this class is Pioglitazone that is ten times more effective than Troglitazone in lowering glucose level. Most recently, other drugs such as rosiglitazone are developed. Rosiglitazone is in a class of drugs which enhance peripheral tissue sensitivity to insulin, thereby increasing glucose uptake.

1.6.6 Anti-diabetic drugs used in traditional medicine

Many traditional plants like Trigonella foenum gracum (Ventryam), Syzygium cumini (Naval), Azadiracta indica (Neem), Aegle marmelos (Vilvam) and many other plants are in use as anti-diabetic drugs in India. Gymnema sylvestre (Sirukuringi) is widely used today all over India for treating diabetes mellitus. In layman’s language, it is called GUR MAR. ‘Gur’ means sugar and ‘Mar’ means kill. It is a powerful herbal sugar killer for diabetes mellitus patients. Dried leaves are used in various forms such as hot tea in daily doses of 3 to 4 grams continuously. The anti-diabetic activity of Agrimony eupatoria has been documented as a traditional treatment of diabetes (Gray and Flatt, 1998a).

Most of these traditional plant extracts act as insulin mimics and increase glucose uptake. They also stimulate the pancreatic β-cells to release insulin. Some inhibits gluconeogenesis and reduces production of glucose.

1.6.7 Side effects of allopathic drugs

Sulfonylureas induce cardiovascular diseases besides having other possible adverse reactions. Chlorpropamide and glibenclamide (glyburide) cause hypoglycemia. Biguanides are weak hypoglycemics and have limited clinical use. The most common adverse effects of metformin are gastrointestinal symptoms that may be relieved by dosage reduction. Troglitazone is generally well tolerated. The most serious adverse event
reported with short- and long-term troglitazone therapy is idiosyncratic hepatocellular injury. Although these drugs are widely accepted as being efficacious in treating some diabetics, they are ineffective in many others.

Sulphonylureas and biguanides have been employed for over 4 decades as oral anti-diabetic agents, but they have a limited capacity to provide long term glycemic control and can cause serious adverse effects. Thus, more efficacious and tolerable anti-diabetic agents are required. Recent years have witnessed the introduction of agents with novel modes of action, that is, the alpha-glucosidase inhibitors acarbose and miglitol (which reduce postprandial hyperglycaemia) and the first of the thiazolidinedione insulin sensitising drugs-troglitazone and rosiglitazone. Troglitazone has been withdrawn in some countries due to adverse effects (Evans and Krentz 1999).

In March, 2000 at the behest of the Food and Drug Administration (FDA), Warner-Lambert Company agreed to stop selling troglitazone, or Rezulin, in the United States due to severe liver toxicity. Consequently, testing of plant extracts has continued. The search of an acceptable cheap and blood sugar lowering agents from plant sources has been rightly considered as an alternative.

1.7 INSULIN AND CONTROL OF BLOOD SUGAR

1.7.1 Insulin in diabetes

Insulin was discovered by Sir Fredrick Banting, a Canadian Physician in 1922. For this he was awarded the Nobel prize along with his colleague Best. Insulin is secreted by β-cells of islets of langerhans in pancreas. A shortage of insulin in the body results in diabetes. The word insulin comes
from the Latin word for island. Canadians Frederick Grant Banting and Charles Herbert Best were the first to obtain, from extracts of pancreas, a preparation of insulin that could replace a deficiency of the hormone in the human body.

In mammals, insulin is the principal hormone controlling blood glucose and acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by liver (White and Kahn, 1994). The insulin secretion decreases or is absent in diabetics due to destruction of islets of langerhans in pancreas. Some times insulin is insufficient to act on the tissues due to insulin resistance.

1.7.2 Insulin and glucose uptake

Insulin stimulates glucose uptake in muscle and adipose cells primarily by recruiting GLUT-4 from an intracellular storage pool (Fig. 1.6) to the plasma membrane (Haney et al., 1995).

Dysfunction of this process known as insulin resistance causes hyperglycemia, a hallmark of diabetes and obesity. Insulin promotes transfer of glucose into the cells of most tissues. Notable exceptions are the brain, liver and pancreas where glucose transport is insulin independent. Insulin increases skeletal muscle glucose uptake over 3 folds. Serum glucose is modulated by pancreatic hormone, insulin. The homeostatic objective is to keep a fairly constant serum glucose of 80-100 milligrams/deciliter (mg/dl).

Insulin is most commonly associated with levels of blood glucose. As blood glucose increases, insulin increases in tandem; and it is the insulin, which signals or transduces the uptake of glucose, predominantly into muscle and
Fig. 1.6  Insulin action and translocation of Glucose Transporter-4 (GLUT-4)
adipose cells. Cells of the nervous system and the liver do not require insulin for glucose transport. Wortmannin prevents the insulin-induced increase in glucose uptake in various cells and tissues.

1.7.3 Insulin resistance

Insulin resistance is a major pathophysiological abnormality in diabetes (De Fronzo et al., 1992). Peripheral insulin resistance is defined as inhibition of normal insulin stimulation of whole body glucose uptake. Hepatic insulin resistance is defined as inhibition of normal insulin suppression of hepatic glucose production.

Insulin resistance is due to an impaired biological response to either exogenous or endogenous insulin. The measured biological responses could reflect, metabolic processes (changes in carbohydrate, lipid or protein metabolism) and mitogenic processes (alterations in growth, differentiation, DNA synthesis, regulation of gene transcription). In vivo biological responses to insulin vary according to insulin concentration, exposure time, tissue delivery, and pulsatility. The primary mechanism is characterised by reduced efficiency of translocation of the GLUT-4 in muscle cells.

1.8 IMMUNE RESPONSE AND CELLULAR EVENTS

1.8.1 Immunity implications in diabetes

Diabetes, especially type 1 (IDDM) is an auto immune disease caused by the infiltration of T-lymphocytes in the islets of langerhans. They destruct the insulin secreting β-cells. Th1 cells produce IL-2, IFN-γ, and TNF-α (lymphotoxin); Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. The
balance between the two responses appears to regulate the pathogenesis of several diseases in addition to diabetes. There is evidence that crude cytokine preparations can either stimulate insulin secretion or cause inhibition of secretion and islet cell death.

1.8.2. Cytokines involved in diabetes

The levels of protective cytokines IL-4 and IL-13 are low in diabetes whereas TNF-α and IL-1β increases. IFN-γ appears to be important in the initiation and expression of β-cell damage. Cytokine regulation may be of central importance in the pathogenesis of autoimmune diabetes (Karlsson and Ludvigsson, 1998a).

Interferon-γ plays an important role in the development of type I diabetes. In RT-PCR experiments IFN-γ mRNAs was expressed only in the liver, and the mean concentration of IFN-γ in the serum was high (3533 pg/ml). The results suggest that pancreas specific expression of IFN-γ is important for the development of diabetes.

1.9. ANIMAL MODEL STUDIES

There are many advantages of using animal models in research work on diabetes as it helps in the development and evaluation of newer agents for the treatment of diabetes (Chattopadhyay et al., 1997). Induction of diabetes in animals can be carried out by various ways – using different chemical diabetogens (Dunn et al., 1943), surgically by partial pancreatectomy and genetic manipulation by selective inbreeding (Karasik and Hattori, 1986).
Induction of diabetes by various chemical diabetogens is also dependent on the species, the strain, sex and the diet of the animals. There are variations in the susceptibility to diabetes amongst different strains of mice. C57 BL/KSJ mice were found to be more susceptible than C 57 BL/6, A/J, AKR/J, C3H/HeJ counterparts (Karasik and Hattori., 1986). Variations in susceptibility also observed amongst male and female mice of the same strain, males being more susceptible to insulin dependent diabetes mellitus (IDDM) than females (Karasik and Hattori, 1986). Male rats showing greater susceptibility to the diabetic state. Diabetic animal models are divided into two categories:

(a) Naturally occurring models.
(b) Chemically induced models.

1.9.1 Natural diabetic animals

Naturally diabetic animal models are by inducing gene mutation. Examples in mice are ob/ob and KK whereas in rat is Zucker fatty fa/fa strain. KK mice are an inbred strain established from Japanese native mice by Kondo (Iwatsuka H. et al, 1974). After identifying the KK mice as a spontaneous diabetic model, Nishimura transferred the yellow obese gene (A') into KK mice and developed a new strain KK-A' diabetic model. This strain has been considered as insulin resistant model (Nishimura, 1969; Takada Y. et al., 1996).

1.9.2 Chemical induction of diabetes

Diabetes can be induced by the administration of different chemicals like streptozotocin (STZ) or alloxan to a variety of animals, but most often rats and mice are the experimental models.
1.9.2.1 Alloxan induced

Chemically alloxan is 2,4,5,6 tetra-oxo-hexahydropyrimidine. Alloxan was one of the most widely used chemical diabetogens during initial research work on experimental diabetes. It is freely soluble in water and slightly acidic with a pKa of 6.63. Alloxan acts as a diabetogen in rats, mice, rabbits, dogs, hamster, sheep and monkey, but guinea pigs are resistant to alloxan induced diabetes (Rerup et al., 1970).

Diabetes is induced by intraperitoneal injection of alloxan monohydrate at a dose of 40-150 mg/kg body weight in rats previously starved for overnight. Alloxan is dissolved in normal saline or sodium citrate of pH 4.5. The fasting glucose level in blood was measured before inducing.

1.9.2.2 Streptozotocin induced

Streptozotocin (STZ) is a broad spectrum antibiotic isolated from Streptomyces archromogenes in 1959 (Herr et al., 1967). Chemically STZ, is 1-methy P.1-in nitrosurea linked to position C2 of D-Glucose. It is soluble in water. STZ induces diabetes in almost all the species. Diabetogenic dose varies with the species and the optimal dose required to produce diabetes in various species was found to be 50-60 mg/kg, in rats through intraperitoneal or intravenous.

A single intravenous injection of streptozotocin at a dose of 60 mg/Kg body weight of animal can induce diabetes. The chemical is dissolved in 0.05 citrate buffer (50mg/ml) having pH 4.5. (Like and Rossini 1976).
1.9.3 Establishment of diabetes

Diabetes is established after one to two weeks of induction of chemical. When the blood glucose level is increased to 200-300 mg/dl, the animals are ready for experiments. The glucose level in blood is tested by GOD-POD (Glucose Oxidase Peroxidase) method. After induction of diabetes the test compound is dissolved in normal saline or phosphate buffered saline (PBS) and administered, and monitored reduction in blood glucose.

1.10 PURIFICATION AND STRUCTURAL ELUCIDATION

1.10.1 Extraction

One of the most important and time-consuming activities involves isolating, separating, and purifying bioactive compounds. Extraction (literally, "taking out by force") is a useful technique for separating compounds having different polarities. The compounds to be separated are treated with a mixture of a polar solvent (H₂O, Lactic acid, Acetonitrile, Methanol, etc.) and a non-polar solvent (Chloroform, DCM, Benzene, Hexane etc). Some compounds dissolve in nonpolar and some in polar solvents. By separating these phases and allowing the solvents to evaporate, compounds can clearly be separated.

1.10.1.1 Small scale extraction

Small scale extraction is carried out in microtubes for initial experiments. Ground material is sequentially extracted at room temperature. 100 milligram samples of each powder are weighed out into small vials and dissolved in 1 ml solvent to give aliquots suitable for screening, while the remaining materials are kept as bulk samples suitable for subsequent fractionation and isolation studies.
1.10.1.2 Large scale extraction

Large scale extraction is carried out in flasks of several liters volume. The powdered material is weighed in grams or kilograms and dissolved in different solvent systems until the total material is submerged. Successive extraction is done for three times with each solvent and rotaevaporated.

1.10.2 Chromatography

A Russian botanist named Mikhail Tsvet in 1906 developed one of the fastest-growing methods of separating compounds. This technique was called chromatography (literally, "writing with color") because it was first used to separate the colored pigments in plants. The basic principle behind chromatography is simple. A gas or liquid is allowed to flow over a solid support. Compounds having high affinity for the solvent are carried faster on the stationary support. Compounds that have a higher affinity for the solid move more slowly.

1.10.3 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) makes use of a flat, relatively thin layer of material such as alumina (Al₂O₃) or silica (SiO₂) coated on glass plate or plastic support. Small samples of the compounds to be separated are then placed on the silica or alumina with a micro-capillary and the TLC plate is immersed in a solvent until the solvent line is just below the point where the samples were applied. The solvent slowly moves up the plate, carrying the components of the mixture with it. Some of these components have a high affinity for the Al₂O₃ or SiO₂ support, and they move very slowly. Others have
a high affinity for the solvent, and they move more rapidly. It results in a separation of the mixture into its components based on their relative affinities for the stationary solid phase and the mobile liquid phase.

In thin layer chromatography (TLC) (a) The sample is applied near one end of a plate coated with silica or alumina. (b) The plate is then immersed in a solvent, which rises up the plate by capillary action. After the solvent is traveled to more than two third height, the plate is removed from the container, dried and developed with iodine or sulphuric acid or ninhydrin by dipping or spraying. It can also be observed under UV light for fluorescent compounds. The retardation factor or $R_F$ factor is calculated by $R_F = \frac{d_R}{d_M}$, where $d_R$ is distance travelled by the component from line of origin and $d_M$ is distance traveled by solvent. The $R_F$ value should be below 1.

1.10.4. Column Chromatography

This process of fractionation and testing is continued until the pure active component(s) is isolated. The initial plant collection sample (0.3 to 1.0 kg) will generally yield enough extract (10 to 40 g) to permit isolation of the pure, active constituent in sufficient quantity for complete structural elucidation which will be sufficient preliminary screening. Secondary testing and preclinical studies
(pharmacology, formulation, toxicology), however, might require gram or even kilogram quantities, depending on the degree of activity

This technique involves filling a glass column with a solid support, applying up to several grams of the mixture to the top of the column, and then slowly washing the column with solvent mixtures. The fraction are collected in large scale and evaporated through rotaevaporator or by passing nitrogen gas.

1.10.5 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a separation technique utilising differences in distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has a different distribution equilibrium depending on solubility in the phases and/or molecular size. The components move at different speeds over the stationary phase and are thereby separated from each other. This is the principle behind HPLC.

The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phases. Compounds move in the column only when they are in the mobile phase. Compounds that tend to be distributed in the mobile phase therefore migrate faster through the column.
When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of that compound. Retention time is therefore used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination. The retention time of the target compounds and the concentration for each unit of peak area are based on data obtained in advance by analyzing a sample with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds. A HPLC system is basically composed of 1) a pump, 2) an injector, 3) a column, 4) a column oven and 5) detector.

High Performance Liquid Chromatography (HPLC) is a term describing the separation of a mixture based upon the ability of the components of that mixture to partition between a stationary phase contained in a column and a moving liquid phase flowing through that column. Based upon the differing partition coefficients of the components of the mixture, the various compounds appear, or elute from the column at different times. This technique can be used for quantitation, for purification and recovery of valuable compounds, or for analytical determinations.

1.10.6 Nuclear Magnetic Resonance (NMR)

Traditionally, X-ray crystallography is used for establishing the structure of compounds. However, it is useful only for those that can be coaxed into a crystalline state. The development of multidimensional NMR and more powerful instruments (e.g., the 500-MHz NMR) opened the door for solving the
structure of compounds in an aqueous environment, as they exist in biological systems (Miller, 2000).

In NMR many nuclei have a spin with a spin state. The spin state determines whether a spinning nucleus will be able to generate an NMR signal. The most common isotope of the first element Hydrogen sometimes called Protium possess a nuclear spin and capable of generating an NMR signal. However, Deuterium does not have a favorable nuclear spin state and did not generate an NMR signal. Carbon-13 does have a spin state that will generate an NMR signal.

Its applications within drug discovery are enormous by the combination of NMR with high-performance liquid chromatography (HPLC), and in the ability to obtain spectra of compounds using solid-phase. It provides structural information, increasing the quality of structures through NMR. The diversity of applications of NMR to drug design is a testament to the contributions that NMR can make to the drug discovery process (Keifer, 1999).

For the last half-century since its discovery, nuclear magnetic resonance (NMR) has become the single most powerful form of spectroscopy in both chemistry and structural biology. The dramatic technical advances over the past 10–15 years, which continue apace, have markedly increased the range of applications for NMR in drug discovery process. It helps in identification of a compound or a component of a mixture and to determine in the study of protein–ligand interactions. These form the basis for its most exciting uses in the full three-dimensional structure of the complex, with all the information this yields for structure-based drug design (Gordon, 2000).