Chapter II

LITERATURE REVIEW
1. Diabetes Mellitus

Diabetes mellitus is an endocrine disorder, with a characteristic rise in blood glucose levels and glycosuria. The glycosuria is a primary characteristic attributed to the failure of the kidney to reclaim glucose, which is then released into the urine. The above condition results in an increase in the volume of the urine because of the osmotic effect of glucose (Diabetes mellitus, http://en.wikipedia.org/wiki/diabetes). 

Diabetes mellitus is distinct from diabetes insipidus. They both result in the production of large amounts of urine (diabetes), but in the former the urine is sweet and in the latter it is not. Diabetes insipidus is caused by the deficiency of Antidiuretic Hormone (ADH) (Medline plus medical encyclopedia, http://www.nlm.nih.gov). Based on the distinctive features, diabetes mellitus can be categorized into several categories:

1) Insulin-Dependent Diabetes Mellitus (Type 1 diabetes).
2) Non Insulin-Dependent Diabetes Mellitus (Type 2 diabetes).
3) Malnutrition-related Diabetes Mellitus.
4) Gestational Diabetes Mellitus.
5) Impaired Glucose Tolerance (Inherited form of Diabetes).
6) Secondary Diabetes.

Of the above categories, Insulin-Dependent Diabetes Mellitus (IDDM or Type-1) and Non-insulin Dependent Diabetes Mellitus (NIDDM) comprise the two major types. A form of IDDM called the Juvenile-onset diabetes, usually develops in children or young adults, but can occur at any age when the body stops producing insulin, a pancreatic hormone that enables the body to regulate the utilization of glucose found in foods for energy purposes. NIDDM is also called adult-onset or Type-2 diabetes, which results when the body doesn’t synthesize enough insulin or is unable to utilize the insulin produced, which is also termed as insulin resistance (Joslin, 1921). This form of diabetes usually occurs in people who are over 40, overweight, and have a family history of diabetes. It is a lifestyle related disease (Zimmet, 2001).
1.1 Common Symptoms Associated with Diabetes Mellitus

There are several symptoms that a diabetic exhibits. The symptoms include excessive thirst (polydipsia), frequent urination (polyuria), weight loss, increased hunger (polyphagia), blurred vision, irritability, fatigue, tingling or numbness in the hands or feet, frequent skin, bladder or gum infections, wounds that don't heal, dry mouth, dry or itchy skin, impotence (males).

There are very rare cases in which there are no symptoms and these patients are mainly of the Type 2 diabetes. In such cases, the disease remains undetected leading to complications. The onset can be so sudden that the symptoms may not even be recognized. The classic symptom of being hungry frequently stems from the fact that the diabetic cannot utilize glucose well as an energy source within cells. The glucose circulates in the blood, but the cells are unable to absorb it to use as a fuel. The excess blood sugar, therefore, is not reabsorbed in the kidney and hence enters the urine. The excess sugar due to osmotic effect increases the volume of urine leading to polyuria (Diabetes symptoms, http://www.diabetes-guide.org).

1.2 Diagnosis of Diabetes

The American Diabetes Association (ADA) recommends that all individuals aged 45 and above, particularly those with a Body Mass Index (BMI) equal to or greater than 25, should be tested for diabetes, the tests being repeated every two to three years. Testing is recommended to be frequent in individuals having any of the following diabetes risk factors:

i) BMI equal to or greater than 25 (overweight).
ii) Family History of Diabetes (first degree relative i.e. American, Hispanic American, Native American, Asian American or Pacific Islanders).
iii) Members of high-risk ethnic population (African American, Hispanic American, Native American, Asian American or Pacific Islanders).
iv) Gestational diabetes or pregnant with a baby weighing more than 9 pounds.
v) High HDL-cholesterol levels (HDL-cholesterol equal to or less than 35 mg/dl, or Triglyceride level equal to or greater than 250 mg/dl.)
vi) High Blood Pressure.

vii) Impaired Glucose Tolerance or Impaired Fasting Glucose.

The ADA recommendations for diagnosing diabetes are
1) Fasting plasma glucose is equal to or greater than 126mg/dl;
2) Diabetes symptoms exist and casual plasma glucose is equal to or above 200 mg/dl; or
3) Plasma glucose is equal to or greater than 200mg/dl during an oral glucose tolerance test.

The ADA is also planning to recommend that patients be considered pre-diabetic if their fasting blood glucose level is above 100mg/dl but less than 125mg/dl and whose glucose levels are at least 140mg/dl but less than 200mg/dl following an oral glucose tolerance test (OGTT) (OGTT, http://web.indstate.edu).

**Figure 1 Glucose tolerance between a normal person and a diabetic person**

Glucose tolerance curve for a normal person and one with insulin-dependent diabetes mellitus. The dotted lines indicate the range of glucose concentration expected in a normal individual.

**1.3 Assessment of Control of Diabetes**

To check whether the treatment prescribed is controlling diabetes, generally two methods are employed that include (1) Frequent measurements of Blood Glucose, and (2) Measurement of Glycohemoglobin.

**1.3.1 Measurement of Blood Sugar**

Blood glucose can be measured randomly at any time, referring to the same as Random Blood Sugar (RBS) or it could be measured after a fasting period of over
8 hours, which is referred to as Fasting Blood Sugar (FBS). In non-diabetic individuals, the blood sugar levels fall back to fasting levels within 3 hours of eating. Therefore, blood glucose levels checked after 2 hours of eating is known as Post-Prandial Sugar (PPS) test (Burrin, 1990). In diabetics, the glucose in the blood persists even after 2 hours of a meal. The normal fasting blood glucose level is between 70 and 110mg/dl (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

1.3.2 Measurement of Glycosylated Hemoglobin (HbA1c)
This test is a periodic monitoring system. The level of glycosylated hemoglobin correlates with a person's recent overall blood sugar levels. Hemoglobin A1c levels depicts the blood sugar levels of the past 2-3 months. If the blood sugar levels have been running high during the previous months, then the HbA1c levels will be high, while they will be in normal range if the blood sugar levels have been normal in the previous few months (Colman, 1997).

1.4 Types of Diabetes Mellitus
Diabetes Mellitus is a heterogeneous clinical disorder with numerous causes. Two main classifications of diabetes mellitus exist, idiopathic and secondary.

1.4.1 Idiopathic diabetes
It is divided into two main types: insulin dependent and non-insulin dependent. IDDM or Type 1 diabetes is defined by the development of ketoacidosis in the absence of insulin therapy. NIDDM or Type 2 diabetes is characterized by persistent hyperglycemia but rarely leads to ketoacidosis. Type 2 diabetes generally manifests after 40 years of age and therefore, is also known as adult onset-type diabetes. Type 2 diabetes could also be a genetic disorder, the defects causing both insulin resistance and insulin deficiency (Idiopathic diabetes, http://web.indstate.edu). Type 2 diabetes can be of two forms: Late onset associated with obesity, and late onset not associated with obesity.

The onset of Type 2 diabetes is correlated strongly with obesity and its associated insulin resistance, also known as Syndrome X (Reaven, 1988). This metabolic syndrome arising out of obesity includes a cluster of atherosclerotic cardiovascular
disease risk factors, one of which involves insulin resistance. Insulin resistance is however, not always associated with obesity.

1.4.2 Secondary Diabetes

These specific types of diabetes are due to several causative factors:

i) Maturity onset type diabetes of the young (MODY). This is caused due to specific mutations. It is characterized by onset prior to age 25, showing impaired cell function and insulin resistance. There are evidences that indicate mutations in 10-12 different genes. Mutations in 6 genes have been clearly correlated with MODY (Frayling, 2001).
   a. MODY 1: It is a transcription factor identified as hepatic nuclear factor-4 (HNF-4).
   b. MODY 2: It is pancreatic glucokinase (Hattersley, 1992).
   c. MODY 3: It is the transcription factor HNF-1. This gene is also known as hepatocyte transcription factor-1 (TCF-1).
   d. MODY 4: It is a homeodomain transcription factor insulin promoter factor-1 (IPF-1). This gene is more commonly called PDX 1 derived from pancreas duodenum homeobox-1.
   e. MODY 5: This is also transcription factor HNF-1, also called hepatocyte transcription factor-2 (TCF-2).
   f. MODY 6: This is bHLH transcription factor NeuroD1. It was first identified as a neural fate-inducing gene, and is identical to hamster beta-2 gene, that regulates insulin transcription. The latter is therefore, also known as NeuroD2.

   (Secondary diabetes, http://www.holistic-online.com)

ii) Pancreatic disease: Pancreatectomy i.e. Removal of pancreas also leads to secondary diabetes. Cystic fibrosis and pancreatitis can also lead to destruction on of the pancreas (Pancreatectomy, http://en.wikipedia.org).

iii) Endocrine disease: Some tumors can produce counter-regulatory hormones that oppose the action of insulin or inhibit insulin secretion. These counter-regulatory hormones are glucagon, epinephrine, growth hormone and cortisol.
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a) Glucagonomas are pancreatic cancers that secrete glucagon.
b) Pheochromocytomas secrete epinephrine.
c) Cushing syndrome results in excess cortisol secretion.
d) Acromegaly results in excess growth hormone production.
e) Drug induced diabetes; treatment with glucocorticoids and diuretics can interfere with insulin function.
f) Anti-insulin receptor autoantibodies (Type B insulin resistance).
g) Mutations in the insulin gene.
h) Mutations in insulin receptor gene which lead to the syndromes like Leprachaunism, Rabson-Mendenhall syndrome, Type A insulin resistance, gestational diabetes and many other genetic syndromes.

(Secondary diabetes, http://www.holistic-online.com)

1.4.3 Insulin-Dependent Diabetes Mellitus (IDDM)

1.4.3.1 Etiology of IDDM
Type 1 diabetes is known to be the result of an autoimmune reaction to antigens of the islet cells of the pancreas. There is a strong association between IDDM and other endocrine autoimmunities, like Addison disease. Additionally, there is an increased prevalence of autoimmune disease in family members of IDDM patients.

Types of Autoantibodies
1. Islet cell cytoplasmic antibodies: The primary antibodies found in 90% of type 1 diabetics are against islet cell cytoplasmic proteins (termed ICCA, islet cell cytoplasmic antibodies). In non-diabetics ICCA frequency is only 0.5% - 4%. The presence of ICCA is a highly accurate predictor of development of IDDM. They are not specific for just the B-cells, but also recognize antigens in other cell types in the islet. However, the autoimmune attack appears to selectively destroy β-cells. There are two possibilities: the destruction of the islet cells due to antibodies, or the production of
antibodies due to destruction of the islet cells. The consequence is the 
decline of the titer of the ICCA over time (Myers, 1995).

2. Islet cell surface antibodies: In 80% cases of Type 1 diabetes there is 
presence of autoantibodies against cell-surface antigens (ICSA). The ICSA 
titer declines like the ICCA over time (Van De Winkel, 1982).

3. Specific antigenic targets of islet cells: Antibodies to glutamic acid 
decarboxylase (GAD) have been identified in over 80% patients newly 
diagnosed with IDDM. Like ICCA, anti-GAD antibodies decline overtime 
in Type 1 diabetics (Myers, 1995). The presence of anti-GAD is also a 
strong predictor of future IDDM development. Anti insulin antibodies 
(IAA) have been identified in both IDDM patients and in relatives at risk to 
develop IDDM. These IAA are detectable before the onset of insulin 
therapy in Type1 diabetics and in around 40% of young children with 
IDDM.

1.4.3.2 Pathophysiology of IDDM

The autoimmune destruction of pancreatic β-cells leads to a deficiency of insulin 
secretion that in turn leads to the metabolic derangements associated with IDDM. 
In addition to the loss of insulin secretion, the function of the pancreatic β-cells is 
also abnormal. There is a characteristic excess secretion of glucagon in IDDM 
patients, while hyperglycemia generally leads to reduced glucagon secretion. This 
increased glucagon levels accentuates the metabolic defects due to insulin 
deficiency. One such metabolic disruption is that patients with IDDM rapidly 
develop diabetic ketoacidosis in the absence of insulin therapy. Somatostatin can 
be administered to suppress glucagon secretion, thereby giving rise to a 
concomitant suppression in the rise of glucose and ketone bodies. The IDDM 
patients are unable to secrete glucagon in response to hypoglycemia, and this 
therefore, leads to potentially fatal hypoglycemia in response to insulin treatment 

In patients with poorly controlled IDDM, there is a defect in the ability of target 
tissues to respond to the administration of insulin. This impairment could be due to 
multiple biochemical mechanisms. Insulin deficiency leads to elevated levels of
free fatty acids in the plasma due to uncontrolled lipolysis in adipose tissue. Free fatty acids suppress glucose metabolism in peripheral tissues such as skeletal muscle. This impairs the action of insulin in these tissues. Additionally insulin deficiency decreases the expression of a number of genes necessary for target tissues to respond normally to insulin – such as glucokinase in liver and the GLUT 4 class of glucose transporter in adipose tissue (Watson, 2001).

The major metabolic defects, which result from insulin deficiency in IDDM, are impaired glucose, lipid and protein metabolism.

(1) Glucose metabolism
Uncontrolled IDDM leads to increased hepatic glucose output. The liver glycogen stores are mobilized followed by gluconeogenesis to produce glucose. Insulin stimulates glucose uptake in liver, adipose tissues and the glucose uptake is accomplished by insulin-mediated movement of glucose transporter proteins to the plasma membrane of these tissues. A reduced glucose uptake by peripheral tissues further leads to a reduced rate of glucose metabolism. Insulin regulates the levels of hepatic glucokinase leading to a reduced rate of glucose phosphorylation in hepatocytes, which in turn increases delivery of glucose to the blood. Insulin also affects several anabolic enzymes through covalent modifications. Thus, elevated glucose levels in blood are a combined effect of enhanced glucose production in hepatocytes and reduced peripheral tissue metabolism. Glycosuria ensues when capacity to reabsorb glucose by the kidneys is surpassed, followed by polyuria leading to loss of water and electrolytes. The loss of the water leads to polydipsia. The negative caloric balance owing to glycosuria and tissue catabolism leads to an increase in appetite and food intake, i.e. Polyphagia (Caprio, 1990).

(2) Lipid Metabolism
Insulin stimulates hepatocytes to synthesize triglycerides and storage of triglycerides in adipose tissue. Insulin moreover inhibits lipolysis. In uncontrolled IDDM there is a rapid mobilization of triglycerides leading to increased levels of plasma free fatty acids. The free fatty acids are taken up by numerous tissues, including liver and metabolized to provide energy. In presence of insulin, the levels of malonyl CoA rise, which inhibits carnitine palmitoyl transferase I, the
enzyme required for the transport of fatty acyl-CoAs into the mitochondria. Therefore, in absence of insulin, the reverse occurs whereby fatty acyl CoAs are mobilized into the mitochondria for β-oxidation leading to formation of acetyl CoAs. These acetyl CoAs get further oxidized by the TCA cycle. In hepatocytes, majority of the acetyl CoA is metabolized into the ketone bodies, acetoacetate and β-hydroxybutyrate. These ketone bodies are used for energy production by the brain, heart and skeletal muscle. The increased availability of free fatty acids and ketone bodies exacerbates the reduced utilization of glucose furthering the ensuing hyperglycemia. Production of excess ketone bodies leads to ketoacidosis, which in diabetics can be diagnosed by smelling the breath (Caprio, 1990).

Lipoprotein lipase, an enzyme found on the surface of the endothelial cells lining the vessels act on plasma triglycerides to glycerol and fatty acids, which are taken up by adipocytes for storage. The activity of lipoprotein lipase requires insulin and therefore, in IDDM patients, hypertriglyceridemia is a common phenomenon (Caprio, 1990).

(3) Protein metabolism
Insulin regulates the synthesis of many genes, either positively or negatively, which then affect overall metabolism. The main consequence is increased synthesis of protein and reduced rate of protein degradation. Therefore, insulin deficiency leads to increased protein catabolism, which in turn leads to elevated concentrations in plasma amino acids. These amino acids serve as precursors for hepatic and renal gluconeogenesis. In liver, the increased gluconeogenesis contributes to hyperglycemia in IDDM patients (Charlton, 1998).

1.4.4 Non-Insulin-Dependent Diabetes Mellitus (NIDDM)

1.4.4.1 Etiology of NIDDM
NIDDM is characterized by a lack of the need for insulin to prevent ketoacidosis. It is an idiopathic non-autoimmune disorder with strong genetic susceptibility. These susceptible genes are yet to be identified due to their heterogeneity. Obesity is a major risk factor that predisposes to NIDDM. Genetic studies in mice and rats have
demonstrated a link between genes responsible for obesity and those that cause diabetes mellitus (Etiology of NIDDM, http://web.indstate.edu).

1.4.4.2 Pathophysiology of NIDDM
NIDDM have detectable levels of circulating insulin. On the basis of oral glucose tolerance testing, the essential elements of NIDDM can be divided into 4 distinct groups; those with normal glucose tolerance, chemical diabetes (impaired glucose tolerance), diabetes with minimal fasting hyperglycemia (fasting plasma glucose $<140\text{mg/dl}$), and diabetes mellitus in association with overt fasting hyperglycemia (fasting plasma glucose $>140\text{mg/dl}$). In many NIDDM patients despite high levels of insulin, the plasma glucose levels are elevated, indicating insulin resistance. In the progression from impaired glucose tolerance to diabetes mellitus the level of insulin declines indicating that patients with NIDDM have decreased insulin secretion (Pathophysiology of NIDDM, http://web.indstate.edu). Therefore, insulin resistance and insulin deficiency are the primary cause of NIDDM.

Several genes have been studied that play a major role in causing NIDDM. Some of the genes studied are pancreatic glucokinase (MODY 2), GLUT-2 (Glucose transporter), glucagon receptor, glucagon-like-protein-1 (GLIP-1), glucokinase regulatory protein and hexokinase-1. Recent studies have shown one of the members of the nuclear hormone receptor superfamily of proteins in the etiology of Type 2 diabetes. Thiazolidinedione are a new class of drugs that are being used to increase the sensitivity of the body to insulin. These drugs bind to and alter the function of the peroxisome proliferator-activated receptor-$\alpha$ (PPAR-$\alpha$). PPAR-$\alpha$ is also a transcription factor and when activated, binds to another transcription factor known as the retinoid X receptor (RXR). When these two proteins are complexed a specific set of genes become activated. PPAR-$\alpha$ is a key regulator of adipocyte differentiation; it can induce the differentiation of fibroblasts or other undifferentiated cells into mature fat cells. PPAR-$\alpha$ is also involved in the synthesis of biologically active compounds from vascular endothelial cells and immune cells. Mutations in the PPAR-$\alpha$ have been correlated with insulin resistance (PPAR, http://www.en.wikipedia.org).
1.5 The “Metabolic Syndrome” or Syndrome X

Syndrome X is not directly associated to NIDDM and the associated insulin resistance, but it has a very close association with obesity, which is one of the major causes of Type 2 diabetes. The metabolic syndrome is defined as a clustering of atherosclerotic cardiovascular disease risk factors that include visceral adiposity (obesity), insulin resistance, low levels of HDLs and a system proinflammatory state (Metabolic syndrome, http://www.en.wikipedia.org).

The role of adipose tissue stems from the fact that the organ is active at secretion of cytokines, termed adipocytokines, which include tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), leptin, adiponectin and resistin. Plasma leptin levels are associated with obesity and predictive potential for cardiovascular pathology. Insulin resistance has a significant role to play in cardiovascular pathology as insulin is involved in fat homeostasis, to induce the storage of fuel. This storage can be as fat (triacylglycerols) in adipose tissues or as carbohydrate in the form of glycogen in liver and skeletal muscle. The effect of insulin resistance at the level of fat homeostasis is an increase in circulating triacylglycerols, referred to as dyslipidemia. Due to insulin resistance, there is an increase in the delivery of peripheral fatty acids to the liver, which in turn drives hepatic triglyceride synthesis. These triglycerides are then packaged into lipoprotein particles termed VLDLs (very low density lipoproteins), which are returned to circulation. Therefore, the insulin resistance with its associated negative effects, like increased triacylglycerols, decreased HDLs and hypertension contribute to the progression of atherosclerosis (Metabolic syndrome, http://www.en.wikipedia.org).

1.6 Regulation of Blood Glucose

Blood glucose levels are maintained within a very narrow range by insulin and glucagon, hormones secreted by the pancreas. Both the hormones are in response to the blood sugar levels. Insulin is secreted by the β-cells in response to high levels of blood glucose. Insulin, in turn has an effect on a number of cells, including muscle cells, red blood cells, and fat cells. In response to insulin, these cells absorb glucose out of the blood thereby bringing down the blood glucose levels to normal. On the other hand, glucagon is secreted by the alpha cells of the
pancreatic islets in response to low levels of blood glucose. Glucagon induces some cells, especially hepatocytes to release glucose into the blood after glycogenolysis and gluconeogenesis. The normal blood glucose is between 70mg/dl and 100mg/dl. Levels below 70mg/dl are termed as hypoglycemia, while levels beyond 180mg/l are termed hyperglycemia (Blood sugar, http://www.biology-online.org).

1.7 Insulin

Insulin, a peptide hormone secreted by the β-cells of the Islets of Langerhans, is a key player in the control of intermediary metabolism.

1.7.1 Structure of insulin

Insulin is composed of two peptide chains referred to as the A chain and B chain. A and B chains are linked together by two disulfide bonds, and an additional disulphide is formed within the A chain. In most species, A chain consists of 21 amino acids and the B chain of 30 amino acids.

Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulphide bonds, both ends of the A chain and C terminal residues of the B chain. Insulin molecules have a tendency to form dimers in solution due to hydrogen bonding between the C-termini of B chains. In the presence of zinc ions, these insulin dimers form hexamers. As a monomer, insulin is a small protein, with a molecular weight of ~6kd. It is synthesized in significant quantities only in β-cells in the pancreas. The insulin mRNA is translated as a single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin. This proinsulin consists of three domains: an amino-terminal B-chain, a carboxy-terminal A chain and a connecting peptide known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases, which excise the C peptide, thereby generating the mature form of insulin. Insulin and the free C peptide are packaged in the Golgi into secretory granules, which accumulate in the cytoplasm. When the β-cells is appropriately stimulated, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood. The C peptide is also secreted into blood,
but has no known biological activity. The secretion of insulin is controlled primarily by elevated blood glucose but is also promoted by some neural stimuli like site and taste of food, increased blood concentrations of other fuel molecules like amino acids and fatty acids (Insulin, http://www.enocrineweb.com).

The amino acid sequence being highly conserved among vertebrates, insulin from one mammal is biologically active in another mammal, which is important in preparing commercial therapeutic insulin. A number of recombinant insulin analogs have been developed to counter certain problems like hexamer formation delaying its diffusion and absorption into blood, e.g. Insulin lispro, which is engineered such that lysine and proline residues on the C-terminal end of the B-chain are reversed, which does not alter receptor binding, but minimizes the tendency to form dimers and hexamers (Insulin, http://www.wikipedia.org).

1.7.2 The Insulin Receptor and Mechanism of Action
Insulin receptor composed of two alpha subunits and two beta subunits linked by disulphide bonds is embedded in the plasma membrane. The alpha chains are entirely extracellular and house insulin binding domains, while the linked beta chains penetrate through the plasma membrane. The receptor is a tyrosine kinase, transferring phosphate groups from ATP to tyrosine residues on intracellular target proteins. Binding of insulin to the alpha subunits causes the beta subunits to autophosphorylate, thereby activating the catalytic activity of the receptor. The activated receptor in turn phosphorylates a number of intracellular proteins, activating or inactivating them and generating a biological response. One of such intracellular proteins is insulin receptor substrate 1 or IRS-1, which serves as a type of docking center for recruitment and activation of other enzymes that ultimately mediate insulin's effects (Eisenberg, 2005).

1.7.3 Effect of insulin on Carbohydrate Metabolism
On digestion of carbohydrates, glucose is absorbed into the blood in the small intestine. This elevates levels of glucose in the blood, which in turn stimulates the release of insulin. The released insulin acts on the cells throughout the body to stimulate uptake, utilization and storage of glucose. There are two ways by which insulin affects glucose metabolism:
i) Insulin facilitates the entry of glucose into muscle, adipose and several other tissues through a family of hexose transporters, known as GLUT. The major transporter of glucose is GLUT4; levels in the plasma membrane are elevated through the action of insulin (up regulation). In the absence of insulin, GLUT4 glucose transporters are present in cytoplasmic vesicles. On binding of insulin to receptors, the vesicles containing the glucose transporters fuse with the plasma membrane such that the GLUTs get inserted there, giving the cell an ability to efficiently take up glucose. When blood levels of insulin decrease, there is down regulation of the insulin receptors by recycling the glucose transporters back into the cytoplasm (Watson, 2001).

ii) Insulin stimulates the liver to store glucose in the form of glycogen. Glucose absorbed from the small intestine is immediately taken up by hepatocytes and either is converted to glycogen or is oxidized to produce ATPs. Insulin stimulates glycogen synthesis by activating hexokinase, which phosphorylates glucose, trapping it within the cell. On the other hand, at the same time insulin inhibits glucose-6-phosphatase, and activates phosphofructokinase and glycogen synthase (Insulin, http://www.en.wikipedia.org)

1.7.4 Effect of insulin on Fat Metabolism
Insulin promotes synthesis of fatty acids in the liver, when the liver is saturated with glycogen, all the excess glucose taken up by hepatocytes is shunted into pathways leading to synthesis of fatty acids, which are exported from the liver as lipoproteins. Insulin inhibits breakdown of fat in adipose tissue by inhibiting the intracellular lipase that hydrolyzes triglycerides to release fatty acids. Insulin facilitates entry of glucose into adipocytes, and within those cells, glucose can be used to synthesize glycerol. This glycerol, along with the fatty acids delivered from the liver, is used to synthesize triglyceride within the adipocyte (Physiological effects of Insulin, http://www.vivo.colostate.edu).

1.7.5 Other Effects of Insulin
Insulin stimulates the uptake of amino acids, contributing to its overall anabolic effect. Insulin levels are low; there is intracellular protein degradation. Insulin also increases the permeability of many cells to potassium, magnesium and phosphate
ions. Insulin activates sodium-potassium ATPases in many cells, causing a flux of potassium into cells (Physiological effects of Insulin, http://www.vivo.colostate.edu).

1.8 Therapeutic Considerations for Hyperglycemia

1.8.1 Type 1 Diabetes or IDDM
In Type 1 diabetes, the cause of hyperglycemia is because of insufficient secretion or no secretion of insulin from the pancreas. This could be a result of autoantibodies or due to defects in the β-cells of the pancreas. Thus, the main therapeutic consideration in Type 1 diabetes is to administer insulin exogenously in doses dependent on the severity of the condition. The dose of insulin is critical as excess amount of insulin could lead to hypoglycemia, which in turn could lead to other complications (Diabetes treatment: Using insulin to manage your blood sugar http://www.mayoclinic.com).

1.8.2 Type 2 Diabetes or NIDDM
In Type 2 diabetes, the major goal of any therapeutic intervention is to reduce circulating glucose levels, for which several pharmacological strategies have been developed.

1. The alpha-Glucosidase inhibitors: Acarbose and miglitol are two types of alpha-glucosidase inhibitors. They interfere with the action of the alpha-glucosidases present in the villi of the small intestine. This reduces the absorption of glucose into the systemic circulation, which in turn enables the pancreatic β-cells to more effectively regulate insulin secretion. The advantage of this drug is that they function locally and have no major systemic action. Alpha-glucosidase inhibitors are not hypoglycemic but are effective at reducing fasting plasma glucose (FPG) levels and levels of HbA1c. The common adverse side effects of these inhibitors are abdominal bloating and discomfort, diarrhoea and flatulence (Ketz, 2001).

2. The sulfonylureas: The sulfonylurea and meglitinide classes of oral hypoglycemic drugs are referred to as endogenous insulin secretagogues because they induce the pancreatic release of endogenous insulin. Tolbutamide, acetohexamide, chlorpropamide and tolazamide are first
generation of sulfonylureas while Glipizide (Glucotrol), Glimepiride (Amaryl) and Glyburide (Diabeta, Micronase, Glynase) are second generation sulfonylureas. These are drugs that induce pronounced hypoglycemia. They function by binding to and inhibiting the pancreatic ATP-dependent potassium channel that is normally involved in glucose-mediated insulin secretion. Sulfonylureas have no significant effects on circulating triglycerides, lipoproteins or cholesterol (Veitch, 2004).

3. The Meglitinides: The meglitinides repaglinide (Prandin) and nateglinide (Starlix) are non-sulfonylurea insulin secretagogues that are both fast acting and of short duration. Meglitinides bring about significant reduction in fasting plasma glucose as well as HbA1c. Its mechanism of action is by binding to a receptor on the pancreatic β-cells, which is distinct from the receptors for the sulfonylureas. They also exert effects on potassium conductance. Like sulfonylureas, the meglitimidides have no effects on the circulating levels of plasma lipids (Saloranta, 2002).

4. The Biguanides: The biguanides are a class of drugs that function to lower serum glucose levels by enhancing insulin mediated suppression of hepatic glucose production and enhancing insulin stimulated glucose uptake by skeletal muscle. Metformin (Glucophage) is a member of this class. Metformin does not increase insulin release from the pancreas and the risk of hypoglycemia is minimal (Horton, 2000).

The major site of action of metformin is the liver and therefore its use can be contraindicated in patients with liver dysfunction. The drug is ideal for obese patients and for younger type 2 diabetics. Research has shown that metformin improves insulin sensitivity by increasing insulin receptor tyrosine kinase activity, enhancing glycogen synthesis and increasing recruitment and transport of GLUT 4 transporters to the plasma membrane. Metformin has also been shown to affect mitochondrial activities, like having a mild, inhibitory effect on complex-I of oxidative phosphorylation, having antioxidant properties. Metformin activates both glucose 6-phosphate dehydorgenase (G6PDH) and AMP-activated protein kinase, AMPK, which is involved in regulation of both lipid and carbohydrate
metabolism. In adipose tissue, metformin inhibits lipolysis while enhancing re-esterification of fatty acids.

In adolescent females with type 2 diabetes, the use of metformin is recommended to reduce the incidence as well as the potential for polycystic ovarian syndrome, PCOS, which is brought on by the hyperinsulinemia of type 2 diabetes. Insulin effects on the ovary drive conversion of progesterone to testosterone and a reduction in serum hormone binding globulin (SHBG). The effect of hyperinsulinemia leads to a hyperandrogenic state in the ovary resulting in follicular atresis and ovulatory dysfunction.

5. The Thiazolidinediones (TZDs): The TZDs, such as troglitazone, rosiglitazone and pioglitazone have been proven to be effective hypoglycemic agents in insulin resistance (Murray, 1995). The TZDs function as agonists for the nuclear receptor peroxisome proliferator activated receptor alpha PPAR, which is a member of a superfamily of transcription factors that include PPAR-β and PPAR-γ. PPAR-α exists as a heterodimer with the nuclear retinoids X receptor (RXR). The heterodimer binds to PPAR response elements (PPREs) in a number of target genes. Without ligand bound the heterodimer is associated with a co-repressor complex that includes a histone deacetylase. Deacetylated histone keeps DNA in a transcriptionally repressed state. When ligand binds to PPAR-α, the co-repressor complex dissociates and a co-activator complex containing histone acetylase associates resulting chromatin structural changes and transcriptional activation. The net effect of the TZDs is a potentiation of the actions of insulin in liver, adipose tissue and skeletal muscle, increased peripheral glucose disposal and a decrease in glucose output by the liver. Genes affected by PPAR-α action include those encoding glucokinase, GLUT 4, malic enzyme, lipoprotein lipase, fatty acyl-CoA synthase and adipocyte fatty acid binding protein. PPAR-α is predominantly expressed in adipose tissue and its effects as agonists in the liver and skeletal muscle may be exerted via endocrine signaling from adipocytes (Murray, 1995).

6. Targeting glucagon-like peptide-1 (GLP-1): The primary metabolic responses to GLP-1 release from the enteroendocrine L-cells of the gut are
inhibition of glucagon secretion and enhancement of glucose-dependent insulin release from the pancreas, both effects leading to decreased glycemic index (Zander, 2002; Nauck, 1996). The hormonal action of GLP-1 is rapidly terminated as a consequence of enzymatic cleavage by DPP IV. Recent studies have shown that either infusion of GLP-1 or inhibition of DPP IV can result in reductions in plasma glucose concentrations, reductions in HbA1c and improvement in pancreatic β-cell function (Ahren, 2002). Thus, both represent potential targets for the prevention of the hyperglycemia associated with diabetes and impaired insulin function.

DPP IV was originally identified as the lymphocyte cell surface antigen CD26. In humans CD26 functions in many pathways that are not directly related to its peptidase activity. It harbors adenosine deaminase binding (ADA) properties and is involved in extracellular matrix binding. CD26 expression and activity are enhanced upon T-cell activation. CD26 interacts with other lymphocyte cell surface antigens including ADA, CD45 and the chemokine receptor CXCR4. It has been seen that in knockout mice lacking CD26 there is enhanced insulin secretion and improved glucose tolerance. The major clinical advantage of the use of DPP IV inhibitors is its use orally (Boonacker, 2002).

1.9 Complementary and Alternative Medicine (CAM)
In the past decade, there has been an increasing demand and awareness in the general public of the use of alternative medicines. In response to this increasing use, the American Diabetes Association had issued a Position Statement in 2001 on “Unproven Therapies” that encouraged health care providers to seek a response from patients on their acceptance and awareness of alternative therapies, and practices, its effectiveness, potential harm and other practical observations (CAM, http://www.noah-health.org).

Two major surveys: (1) 1996 Medical Expenditure Panel Survey and (2) AHRQ Evidence Reports have reported that ~10% of patients visited CAM professionals for diabetes treatment. Although the scientific literature on the efficacy of CAM in
the treatment of diabetes is relatively sparse and heterogeneous, there are several studies and reports on mind-body techniques, massage therapy, Yoga, Ayurveda, alternative dietary/lifestyle modifications, aromatherapy, acupuncture, traditional Chinese Medicine (TCM). Majority of the literature has focused on herbs or other dietary supplements. Plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world (CAM, http://www.cam.org.nz). Many modern pharmaceuticals used in conventional medicine today also have natural plant origins. Example: Metformin has been derived from the flowering plant, *Glaega officinalis* (Goat’s Rue or French Lilac) (Witters, 2001).

1.9.1 Ayurveda
Ayurveda is an age-old system being practiced in India along with other systems like Siddha, Unani and Yoga. The Siddha system, a variant of Ayurveda, is prevalent primarily in Tamil Nadu, in the south of the country. The Unani Tibb was originally derived from Greek and Arabic medicine. Both the systems are very similar to Ayurveda while therapeutic aspects of Yoga are merely one facet of Ayurveda. Herbal therapy has been the best-studied aspect of Ayurveda.

Ayurveda comes from Ayur and Veda; Ayur meaning life or longevity and Veda meaning knowledge. Therefore, Ayurveda is knowledge of life or longevity. It is a comprehensive system of traditional health care that emphasizes the relationship among body, mind and spirit. Originating in India roughly three thousand years ago, it seeks to restore an individual’s innate harmony. Primary Ayurvedic treatments include diet, exercise, meditation, herbs, massage, exposure to sunlight, controlled breathing and detoxification treatments. There are three treatise of Ayurveda: Charaka Samhita on medicine; the Susruta Samhita on surgery; and the Ashttanga Hridaya Samhita (Ayurveda, http://en.wikipedia.org).

1.9.1.1 Ayurvedic Diagnosis & Treatment of Diabetes
The Indian word for diabetes is madhumeha, “madhu” meaning sweet/sweetness and “meha” excessive urination. The earliest description of madhumeha is found in the Atharveda, one of the four sacred Vedas, that dates to around 1500 to 1000 BC. Caraka defined madhumeha as the disease in which the patient passes urine
characterized as astringent, sweet, and rough. It was later modified to ascertain that the sweetness prevailed in the whole body (Mukherjee, 2002).

Madhumeha is classified in the group of urination disorders known as prameha. Even as early as Susruta’s time, two types of vataja pramehas are described for diabetes. The first, Sahaja, is thought to be due to a genetic defect and hence corresponds to juvenile-onset diabetes. The second, apathyanimittaja, is believed to be acquired later in life due to excessive habits, such as overindulgence in food or sweets (Mukherjee, 2002; AHRQ reports).

Vagbhata and Susruta, in their writings have described the symptoms of diabetes as a honey-like sweetness of urine as well as thirst, polyphagia, lassitude, tiredness, obesity, looseness of limbs, non-relishing of food, burning sensation of the skin, epileptic fits, insomnia, numbness of body, and constipation. Exercise and diet are important adjuncts to the primary diabetes treatment (AHRQ reports, http://www.ncbi.nlm.nih.gov).

1.9.2 Herbal Therapy for Diabetes
Ethnobotanical studies of traditional herb remedies used for diabetes around the world have identified more than 1200 species of plants with hypoglycemic activity. The pharmacopoeia of India is especially rich in herbal treatments for diabetes. Eighty five percent of the 20 antidiabetic plants most widely used around the world are prescribed in India (Marles and Farnsworth, 1995).

1.9.2.1 Common Herbs Used to Treat Diabetes
Coccinia indica: Coccinia indica, also known, as Ivy gourd is a creeping plant that grows wildly in many parts of the Indian subcontinent. The mechanism of action of Coccinia indica is not well understood, but the herb appears to have insulin-mimetic properties (Hossain, 1991). The one randomized controlled trial of this herb, conducted in India, reported significant changes in glycemic control following 6 weeks use of powder from crushed dried leaves in poorly controlled type-2 diabetes (Khan, 1979). There are other reports also with the evidence that the herb’s effect was comparable to a conventional drug (Brahmachari, 1963).
Ginseng species: These include Chinese or Korean ginseng (*Panax ginseng*), Siberian ginseng (*Eleutherococcus senticosus*), American ginseng (*P. quiquefolius*), and Japanese ginseng (*P. japonicus*). The roots of the herb have been principally used in the treatment. The principal components are believed to be the triterpenoid saponin glycosides (ginsenosides or panaxosides). Hypoglycemic effects have been shown in streptozotocin rat models. Reported mechanisms of action include decreased rate of carbohydrate absorption into the portal hepatic circulation, increased glucose transport and uptake mediated by nitric oxide, increased glycogen storage, and modulation of insulin secretion. Several randomized controlled trials have been carried out using American ginseng with reported decreases in fasting blood glucose and HbA1c, suggestive of possible hypoglycemic effect (Konno, 1985).

*Allium cepa*: *Allium cepa* (onion) is known to contain allylpropyl disulphide, which has hypoglycemic properties. Reported mechanisms of allium species include increased secretion or slowed degradation of insulin, increased glutathione peroxidase activity, and improved liver glycogen storage. Significant decrease in fasting serum glucose has been reported. S-Methyl cysteine sulfoxide isolated from onion exhibited antidiabetic and antihyperlipidaemic activity in diabetic rats. The effects were comparable to those of glibenclamide and insulin (Kumari, 1995).

*Allium Sativum*: *Allium sativum* of the Allium species, commonly known as garlic, is generally used as a flavouring condiment in dishes. Much of the clinical literature on garlic has focused on its potential antioxidant activity and microcirculatory effects (e.g., allicin and ajoene for use in hypertension and hyperlipidemia). There are comparatively fewer studies on its effects on insulin and hypoglycemic activity. It, like *Allium cepa*, also has allyl propyl disulphide, a volatile oil, and S-allyl-cysteine sulfoxide, a sulfur containing amino acid. Experiments on alloxan-induced animal models have shown moderate reductions in blood glucose. The same effect was however not seen in pancreactectomized animals (*Allium sativum*, http://www.naturaldatabase.com).

*Ocimum sanctum*: *Ocimum sanctum* (holy basil, Tulsi), a commonly used herb in Ayurveda, when studied in diabetic animal models has shown to have hypoglycemic effect. The mechanism of action is unclear with hypothetical
suggestions indicating enhanced β-cells function and insulin secretion (Yeh, 2003). Clinical trials of *Ocimum sanctum* have shown positive effects on both fasting and post prandial sugar levels in patients with type 2 diabetes (Agrawal, 1996). Local preparation of fresh leaf powder mixed with water was administered for 4 weeks with no adverse effects. Oral administration of alcohol extract of leaf lowered the blood sugar level in normal, glucose fed hyperglycaemia and streptozotocin induced diabetic rats. The extract potentiated the action of exogenous insulin in normal rats. The activity of the extract was 91.55 and 70.43 percent of that of tolbutamide in normal and diabetic rats (Chattopadhyay, 1993).

*Trigonella foenum graecum*: *Trigonella foenum graecum* (fenugreek) is a legume extensively cultivated in India, North Africa, and the Mediterranean. It is a common condiment used in Indian cooking and commonly used herb in Ayurveda. Defatted seeds of fenugreek, which are rich in fiber, saponins, and protein, have been described in early Greek and Latin pharmacopoeias for hyperglycemia (Blumenthal, 2000). Purported mechanisms include delay of gastric emptying, showing carbohydrate absorption, and inhibition of glucose transport from the fiber content, as well as increased erythrocyte insulin receptors and modulation of peripheral glucose utilization. Many studies in alloxan-rat models have shown modulated exocrine pancreatic secretion. Ingestion of an experimental diet containing 25 g fenugreek seed powder by 60 non-insulin dependent diabetic patients reduced total cholesterol, LDL, VDL and triglyceride levels significantly (Sharma, R D. 1996). Diet containing raw or germinated seeds (12.5g/day) reduced the fasting blood sugar of NIDDM patients (Neerja A 1996).

*Bauhinia forficata*: *Bauhinia forficata*, indigenous to rainforests and tropical areas of South America, has been used traditionally as a treatment of diabetes. It has generally been used as tea infusions. Reports however, have not shown significant decrease in glucose or HbA1c, contrary to belief (Fuentes, 2004). There is a contra indication of the extract having insulin like effect (Vasconcelos, 2004).

*Myrcia uniflora*: *Myrcia uniflora* is also a commonly used South American herb used for the treatment of diabetes. Reports, however, have not shown significant hypoglycemic effect (Pepato, 1993).
**Ficus Carica**: *Ficus carica* is a popular plant used for diabetics in Spain and in other South Western European areas. Several studies have purported their potential hypolipidemic effect in diabetic rats. Its mode of action on glucose effect is not known, however, some studies suggest facilitation of glucose uptake peripherally. Fig leaf tea infusions for a period of 4 weeks in type 1 diabetics have shown to bring a decrease in postprandial glucose and insulin requirements. There, however, was no effect on fasting glucose. No effect was seen in C-peptide levels, thereby supporting non-insulin mediated effect (Yeh, 2003).

**Opuntia streptacantha**: *Opuntia streptacantha* (nopal) or the prickly pear cactus can be found in arid regions throughout the Western Hemisphere, and is commonly used for glucose control by Mexicans. It has a high soluble fiber and pectin content, which may affect intestinal glucose uptake, partially accounting for its hypoglycemic actions. Reports have shown decreased postprandial glucose and HbA1c with synergistic effects with insulin in diabetic models, while in pancreactectomized animals, there is no relation of its hypoglycemic effect with insulin. The hypoglycemic effects are more profound in type 2 diabetics with no adverse effects (Yeh, 2003).

**Siliburn marianum**: *Siliburn marianum* (milk thistle), a member of the aster family, has been studied more for its effects on alcoholics and viral hepatitis than for its glycemic control. It is known to be rich in flavonoids, potent antioxidants. Its mechanism is based on its antioxidant activity and effects on hepatocyte stabilization with decreased glutathione oxidation, as well as on restoration of normal malondialdehyde concentrations (Yeh, 2003).

**Gymnema sylvestre**: *Gymnema sylvestre*, commonly known as Gurmar, is a commonly used herb in Ayurveda. It is a woody climber growing in tropical forests of Central and Southern India. Chewing the leaves is known to cause a loss of sweet taste. Studies of an ethanol leaf extract, GS4, in diabetic rat and rabbit models have reported regeneration of β-cells of islets of Langerhans, decreases in blood glucose, and increases of serum insulin (Shanmugasundaram, 1990). Although the mechanism of action is unknown, there are theories suggesting an
increase in glucose uptake and utilization, increase in insulin release through cell permeability, increase in β-cell number, and stimulation of β-cell function. There are trials that have shown improved glycemic control in both Type 1 and Type 2 diabetics (Chattopadhyay, 1998).

**Momordica charantia:** *Momordica charantia* is a vegetable indigenous to tropical areas, including India, Asia, South America, and Africa. It is also known as balsam pear, karela, and bitter gourd. Its active components are thought to be charantin, vicine, and polypeptide-p (an insulin-like protein similar to bovine insulin) (Marles, 1995). Theoretical mechanisms include increased insulin secretion, tissue glucose uptake, liver muscle glycogen synthesis, glucose oxidation, and decreased hepatic gluconeogenesis. Studies in alloxan-induced diabetic rabbits have shown hypoglycemic effects. The aqueous extract of fruit was found to be more effective in diabetes than the powder of the dried fruit (Srivastava, 1993). Fresh fruit juice was found to be a potent scavenger of superoxide radical. The antidiabetic activity has been suggested to be mediated through the scavenging of toxic oxygen radicals (Rao, 1991).

**Aloe vera:** *Aloe vera,* which is popularly used to treat burns and promote wound healing, has been also used to treat diabetes. Its dry sap as well as gel has been used. The gel contains glucomannan, a hydrosoluble fibre, which may be responsible for its hypoglycemic activity (Yeh, 2003).

**Pterocarpus marsupium Roxb. and Pterocarpus santalinus Linn.:** These two closely related species are used as folk remedies for diabetes in Southern India. *P. marsupium* is commonly known as false teak, bijasal, vijayasara or pitasala, and is a large deciduous tree. *P. santalinus,* or red sandalwood, is a smaller tree and is known as rakta chandana or lal chandan. The wood and bark of the trees are used as decoctions. The sap from *P. marsupium* yields a reddish gum, called kino, which is high in tannic acids. Epicatechins have been isolated from bark and heartwood. These flavonoids have demonstrated an ATP-dependent enhancement of glucose-stimulated insulin release from isolated pancreatic islet cells *in vitro* (Manickam, 1997). Phenolic constituents isolated from *P. marsupium,* identified as
marsupin, ptersupin, and pterostilbene, have demonstrated hypoglycemic activity in rats (Nagaraju, 1991). A 95% alcohol extract of wood powder of *P. santalinum* has also showed hypoglycemic effect in experimental streptozotocin treated rats.

**Ficus bengalensis Linn.:** *Ficus bengalensis* Linn., belonging to the Family Moraceae, is also commonly known as Vad or Bor. It grows all over India and the parts studied for their medicinal values are the stem bark, fruit and root. It has been mentioned in the Caraka Samhita for use in excessive urination, fever toxicosis etc. Aqueous and ethanolic extracts of the bark have been found to hinder glucose absorption significantly in mice, reduce blood sugar level in normal and alloxanized rabbits (Gujral, 1954; Joglekar, 1962).

A glycoside and flavonoids isolated from the bark caused hypoglycemia (Deshmukh, 1960; Brahmachari, 1964). Augusti (1975) demonstrated that bengalanoside isolated from bark caused hypoglycemia. Phytosteroline isolated from the roots also have been observed to cause maximum fall of blood sugar level equivalent of 81% of tolbutamide after 4 hours, given per oral to fasting rabbits (Ambika & Rao, 1967). The dimethoxyether of leucopelargonidin from *Ficus bengalensis* administered orally (100-250 mg/kg) to normal and diabetic rats caused significant hypoglycemia associated with rise in serum insulin in both normal and moderately diabetic rats and dogs (alloxanised) (Augusti, 1994; Geetha, 1994).

A single dose of a glycoside of pelargonidin, 3-0-alpha-L-rhamnoside isolated from the bark, decreased fasting blood glucose tolerance by 29 percent (Cheriyan, 1993). Water extract treatment (50mg/kg body wt/day) brought down the level of total serum cholesterol in diabetic rabbits (Shukla, 1995). There are several studies on its toxicity status indicating extended use to have reversible hepatic damage (Joglekar, 1962).

**Swertia chirayita Roxb. ex. Flam.Karsti:** *Swertia chirayita* belonging to the Family Gentianaceae is also commonly known as Chiraita. It has not been mentioned in the Caraka Samhita and Susruta Sanhita as an antidiabetic plant. Entire plant has been used as decoction in fever, for purification of blood and breast milk. The
earliest reports showed that 50% ethanolic extract of *S.chirayita* lowers blood sugar in albino rats (Dhar, 1968; Mukherjee, 1968). A xanthone isolated from the hexane fraction of *S.chirayita* plant, identified as 1,8-dihydroxy-3-5-dimethoxyxanthone (Swerchirin), has a very significant blood sugar lowering effect in fasted, fed glucose loaded and tolbutamide pretreated albino rat models (Madhubala, 1991; Saxena, 1996). *In vitro* glucose uptake and glycogen synthesis by muscle was significantly enhanced by serum of Swerchirin treated rats. Swerchirin also seems to greatly enhance glucose stimulated insulin release from isolated islets (Saxena, 1996).

**Syzygium cumini Linn. Skeels:** *Syzygium cumini* Linn. Skeels., belonging to Family Myrtaceae, also is known by the synonym *Eugenia jambolana* L.E. Jambolanum D.C.; and its common name is Jamun. It is commonly used in diarrhoea, nausea and is native to India. Studies have indicated the ethanolic extract of the seeds to be effective in lowering blood sugar of alloxan diabetic rats and rabbits (Mukherjee, 1963). The aqueous extract of seeds and fruits administered in different doses per oral caused significant hypoglycaemia in rabbits (Indira, 1979; Jain, 1967). The decrease in blood sugar in alloxanized rats and rabbits suggested that the drug acted by extra pancreatic effect (Bhaskaran, 1986; Giri, 1985). Clinical studies have shown that the jamun seeds produced good symptomatic relief along with regulation of blood sugar and were also considered to be more effective than *Pterocarpus marsupium* when administered per oral to diabetic individuals.

**Acacia arabica:** *Acacia arabica* Willd, belonging to Family Mimosaceae is commonly known as Babool. *Acacia arabica* contains alkaloids of phenylethylamine-tryptamine and betacarboline classes. A flavonoid obtained from ethanolic extract of *A.arabica, A.benthami, A.modesta* reduced the blood sugar level of normal and diabetic rats after one week feeding (Singh, 1975; Singh, 1977). A protein isolated from *A.melanoxylon, A.bauhinia* and *A.retusa* when exchanged for casein in rat diet caused significant lowering of blood sugar, both in normal and diabetic rats (Singh, 1977).
Adiantum capillusveneris Linn.: Adiantum capillusveneris L., belonging to the Family Adiantaceae, is commonly known as Hansraj. It is found throughout India. It has been observed that 10-mg/kg dose administered to fasted rabbits caused 10-15 mg blood sugar lowering from 2 to 5 hours upto 24 hours (Jain & Sharma, 1967).

Adiantum caudatum: Adiantum caudatum Linn., belonging to the Family Adiantaceae, is commonly known as Mayurshikha. This plant is distributed throughout India and the whole plant has been studied. It has been used as a substitute of A. capillusveneris juice in the treatment of diabetes (Kapur, 1977; Mukherjee, 1981).

Aegle marmelos: Aegle marmelos Linn., Coorea, belonging to Family Rutaceae is commonly known as Bel or Bilvapatra. Its use has been mentioned in Susruta Samhita for urinary diseases. It is found throughout India. No hypoglycemic activity has been found in fruit pulps (Gupta, 1967), however the leaves and roots have hypoglycemic activity (Dhar, 1968). There are reports suggestive of the leaf extracts having improved effect on functional state of pancreatic cells in diabetic rats (Dar, 1996). Aqueous extract of the leaves exhibited hypoglycaemic activity in normal and diabetic rabbits (Rao, 1995) and rats (Sharma, 1996); an increase in plasma insulin levels being its mechanism of action. A significant decrease in liver glycogen, blood urea and cholesterol were observed in diabetic rats (Ponnachan, 1993), and the activity has been attributed to alkaloidal constituents of the extract.

Albizia chinensis: Albizia chinensis (Osbrook). Merr., belonging to Family Mimosaceae, is also known by its synonyms A. stipulata and A. moluccana (B.Roxb.) and has common names like Kanujera and Udala. It has been mentioned in Susruta Samhita for urethral discharges. It is found throughout India. Seeds administered to young rats caused insignificant lowering of blood sugar level of alloximised rats after a week. However, blood sugar was lowered by ~20% (Singh, 1975; Shrothi, 1963).

Azadirachta indica: Azadirachta indica A. Juss., belonging to Family Meliaceae, is commonly known as Neem. It is available throughout India. Decoction of tender
leaves, oil from seeds and nimbidin a triterpenoid, when fed to young rabbits exhibited significant hypoglycaemia (Pillai, 1981). Aqueous extracts of leaves administered to diabetic dogs also lowered blood sugar significantly after 30 minutes (Satyanarayana, 1977). There are reports that have shown lowering of fasting blood sugar of normal rats on administration of oil obtained from the seeds of *A. indica* and *Melia azadirachta* (Sharma, 1983). Intraperitoneal injection (20mg) of petroleum ether extract of neem seed oil in streptozotocin induced diabetic mice had caused blood sugar lowering by 40.39% four hours after and 51.72% after 8 hours in normal mice whereas in diabetic mice, 54.28% and 67.22% respectively (Purohit, 1990). There are clinical studies also which have shown the neem oil to reduce general symptoms of diabetes (Bhargava, 1987). Decoction of neem leaves, however, has been found to be hepatotoxic in rats.

*Benincasa hispida* (Thumb.) Cogn.: *Benincasa hispida* (Thumb.) Cogn., belonging to the Family Curcurbitaceae, is commonly known as Petha, Raksa. It has been mentioned that it can be used in the treatment of Madhumeha (Kirtikar, 1933; Nadkami, 1954; Chopra, 1958). There are no reports suggesting its hypoglycemic activity and its mode of action.

*Bambusa dendrocalamus*: *Bambusa dendrocalamus* belonging to Family Poaceae is also commonly known as Ruslogi. Pharmacological reports of aqueous extract of leaves administered per oral have shown lowered blood glucose in overnight fasting rabbits (Bapat, 1969). A propylene glycol soluble compound isolated from aqueous extract of leaves caused significant blood sugar lowering when injected intraperitoneally in alloxanized and normal rabbits (Bapat, 1970).

*Bougainvillea spectabilis*: *Bougainvillea spectabilis* Willd, belonging to Family Nyctaginaceae, is found throughout India. Alcoholic extract of the leaves (0.4 gm/kg) caused significant blood sugar lowering in normal and alloxanised mice (Narayanan, 1984). Water-soluble fraction of the same also has exhibited blood sugar lowering in diabetic rats (Shankar, 1986). Pinitol (D-chiro(+)-O-methylinositol) when administered orally to normal fasted mice, caused maximum blood sugar lowering by 2 hours, whereas treatment with pinitol (5 doses) in 72 hours induced fall in blood sugar in alloxanised mice (Narayanan, 1987).
Caesalpinia crista: Caesalpinia crista Linn., belonging to Family Caesalpiniaceae, is commonly known as Karanju. Dry seed material (0.51 and 1.5g per oral) caused blood sugar lowering in normal rabbits (Rao, 1994). The fruit-shell aqueous extract has also shown to lower blood sugar in normal and alloxanised/streptozoticinised rats (Banerjee, 1998). Several clinical studies have also shown the blood sugar lowering capacity of C. crista (Chopra, 1956; Jain, 1967).

Casearia esculenta: Casearia esculenta Roxb., belonging to Family Flacourtiaceae, is commonly known as Mori, Bokra. Decoction of the roots (25 mg/kg) has significantly lowered blood sugar in rats. Two crude resin substances and water-soluble crystalline materials caused hypoglycaemia in rabbits (Choudhary, 1967). The effect of C. esculenta root extract in doses of 25, 50 and 100g/oral to human volunteers and diabetics was that of mild hypoglycemia (Ahuja, 1968).

Cassia sophera: Cassia sophera Linn, belonging to Family Caesalpiniaceae, is commonly known as Bas-ki-Kasunda. Aqueous extract of seeds, leaves, flowers caused 25% blood sugar lowering in normal and alloxanised rabbits and dogs but C. fistula seeds exhibited marked hypoglycaemia in normal albino rats without any effect on alloxanised rabbits (Shrotri, 1963; Singh, 1975). It has been observed that a decoction of C. amiculata, which was administered to 8 diabetic cases, caused lowering of fasting blood glucose.

Ceiba pentandra L.: Ceiba pentandra L. Gaertn, belonging to Family Bombaceae, is also known by its synonym Eriodendron anfractuosum D.C., with common names like Shvetasalmali and Safed sevara. The plant is known to contain phytochemicals like Quercetin and Kaempferol glucosides and flavonosides. The juices from roots can control/cure diabetes (Jain, 1967).

Cinnamomum tamala: Cinnamomum tamala (Hamm.) Nees. & Ebern., belonging to Family Lauraceae, is also known commonly as Tejpata. The leaves of the plant yield an essential oil, which resembles cinnamon oil and contains d-α phellandrene and Eugenol. There are several studies that have shown the dried leaves to lower
blood sugar (Chandola, 1990). *C. tamala* lowers blood sugar in adrenaline-induced hyperglycaemia in albino rats (Tripathi, 1990). The hypoglycemic fraction of the same when tested on patients significantly increased glucose tolerance and a definite rise in plasma insulin and fall in blood glucose (Tripathi, 1990).

**Clerodendrum phlomidis:** *Clerodendrum phlomidis* L.F., belonging to Family Verbenaceae, is also commonly known as Arani or Urani and is distributed throughout India. Alcoholic extract of the whole plant was made into a decoction and fed to normal rats in doses of 1g and 2g of crude material/day. The blood sugar lowering observed was equal to that of tolbutamide. In adrenaline hyperglycaemia in rabbits, the alcoholic extract was more powerful than decoction. It has been reported that crude extract of *C. phlomidis* in clinical trials have shown reduction in blood sugar (Bhattacharya, 1975) and improvement in glucose tolerance (Chaturvedi, 1983).

**Cyamopsis tetragonoloba:** *Cyamopsis tetragonoloba* Taub., belonging to Family Fabaceae, is also commonly known as Gowar. A high molecular weight, hydrocolloidal polysaccharide galactomannan has been isolated. Hot aqueous extract of fruit and seeds (20 mg/kg/oral) showed significant hypoglycaemia in normal as well as alloxanized rabbits (Pillai, 1980). Along with blood sugar, total lipids, free and esterified cholesterol, triglycerides and phospholipids were also lowered significantly (Srivastava, 1978). Clinical studies have shown significant sustained lowering of blood sugar in both type 1 and type 2 diabetics (Pillai, 1980) along with reduced intestinal glucose absorption by inhibiting mixing action of intestine (Paranjothy, 1986) as well as lowering of HbA1c (Gupta, 1989).

**Ficus racemosa:** *Ficus racemosa* Linn., belonging to Family Moraceae, is also known by its synonym *Ficus glomerata*. It is commonly known as Gular. The root bark has been mentioned for use in eyeache, earache, dental pain etc. It is distributed throughout India. A tetracyclic triterpene glucanoacetate has been isolated from the leaves, bark and heartwood. The alcoholic extract of bark produces hyperglycaemic response in anti-pituitary extract treated rats (Gupta, 1964). Blood sugar lowering in normal and alloxanised rabbits after administration of aqueous extract of bark per oral has also been noticed (Shrotri, 1960).
**Ficus religosa:** *Ficus religosa* Linn., belonging to Family Moraceae, is commonly known as Pipal. The plant is distributed throughout India and the tender root or bark is mentioned in Caraka Samhita for use in skin infection, wounds, as purgative etc. The sitosterol glucoside obtained from bark has been observed to lower blood sugar in treated rabbits (Ambike, 1967). Spasmolytic anticytchaline activity of *Ficus religiosa* extract has been reported (Malhotra, 1960) and has LD 50 p.o of 2.24 g/kg in albino rats (Malhotra, 1960b).

**Inula racemosa:** *Inula racemosa* Hook. f, belonging to Family Asteraceae, is distributed throughout India. A decoction of boiled roots was found to lower fasting blood sugar and also showed protection of rabbits against glucose-induced hyperglycaemia (Tripathi, 1979). *Inula racemosa* is a better quick acting (2-4 hours) blood glucose lowering agent compared to *Sanskirra lappa* when alcoholic extract of the drug are administered (400 mg/kg) to albino rats.

**Mucuna pruriens:** *Mucuna pruriens* Hook; Baker, belonging to Family Fabaceae is distributed throughout India. A seed diet has been shown to lower blood sugar levels in healthy albino rats (Pant, 1968), however, there was no significant decrease in glycemic index in alloxanised rats (Joshi, 1970). There were reports of hypoglycemic activity during general screening of the plant (Dhawan, 1977; Sharma, 1978).

**Musa paradisiacal Linn.:** *Musa paradisiacal Linn.*, belonging to Family Musaceae, is commonly known as Kela. The pith, bulb and roots are used in dermatosis, leucoderma, piles, urinary disease, abdominal diseases and blood vomiting. The plant is distributed throughout India. It has been found that liquid extract of flower, a non-saponifiable portion is hypoglycaemic (Jain, 1968). The dried unripe fruit of the plant administered per oral in a single dose (0.51 and 1.5 mg/kg) to normal and alloxanised rabbits induced (~32%) blood sugar lowering after 4 hours (Rao, 1994).

**Phyllanthus fraternus:** *Phyllanthus fraternus* Linn., belonging to Family Euphorbiaceae is commonly known as bhuyamli. It is present throughout warmer parts of India. Decoction of the plant is used in blood poisoning, jaundice, herpes
etc. The aqueous extract of leaves is reported to induce blood sugar lowering in normal and alloxan diabetic rats (Ramakrishna, 1982) and its hypoglycemic activity was greater than that of tolbutamide. Flavonoid compounds extracted from the plant in 100mg/kg p.o. induced hypoglycemia in alloxanized rats (Hukeri, 1988). However, no blood sugar lowering was observed in normal rats. It has been also observed that at a dose of 1 g thrice daily for a period of 3 months, the extract lowered the blood sugar of severe and moderate diabetic patients (Sivaprakasan, 1995).

**Phaseolus vulgaris:** *Phaseolus vulgaris* Linn., belonging to the Family Fabaceae is commonly known as Lobia, Rajmah. It is present throughout India and has been mentioned in Caraka Samhita for treatment of fever, haemothermia. The *P. mungo* is used against suppuration, rheumatism etc. The green pod shell of *P. vulgaris* is used as diuretic and adjuvant in minor diarrhoea. (Kamath, 1982) had studied the effect of legumes like Bengal gram (*Cicer arietinum*) and red kidney beans (*P. vulgaris*) against blood sugar rise and insulin response stimulated by wheat, rice, potatoes and found that the former were more effective in reducing blood glucose level as compared to the latter. The effect is not due to insulin release as the insulin response was significantly decreased.

**Rivea cuneata:** *Rivea cuneata* Wight, belonging to Family Convulvulaceae, has a synonym in *Argyreia cuneata* Kerr-Gawl., and is found throughout India. The leaves of the plant yield a glucoside soluble in water and alcohol but insoluble in other organic solvents. On acid hydrolysis it yields glucose and a water insoluble aglycone. The effect of the glucoside when studied in alloxan induced diabetic rats was found to revert the effect of alloxan with a suggestive hypertrophy and hyperplasia of the Islets of Langerhans. Oral administration of milk extract of the leaves for 3 to 5 days was observed to bring about a significant remission of the symptoms of diabetes (Mukherjee, 1957). The glucoside when administered orally did not lower fasting blood sugar in man (Mukherjee, 1964).

**Securigera securidaca:** *Securigera securidaca* Linn., belonging to Family Leguminoseae, has a synonym in *S. coronilla* D.C. and is found mainly in Eastern India. Several phytochemicals have been isolated from the plant including a
nonglucosidal bitter principle from the seeds, a crystalline glycoside called securigerin, two bitter crystalline substances sercurin and coroniltin, a bland olive green fixed oil, a sterol and a bitter resin. Therefore, the extract of the seeds administered in 0.5 mg/kg doses were found to lower blood sugar in cats and rabbits for 4 hours (Chatterjee, 1965).

**Salacia dulcis**: *Salacia dulcis* occurs in two varieties, yellow and brown. The yellow variety is called *S. prinoides* D.C., a vine belonging to Hippocrataceae family and the brown variety constitutes an allied species *S. macrosperma* Wight (Mehta & Handa, 1969). It is distributed throughout India. Administration per oral of decoction from leaves and roots in 1gm/kg doses, caused significant blood lowering in rabbits (Arora, 1973). The decoction and infusion of root bark (0.5 and 1.0gm/kg) showed moderate degree of hypoglycaemia in fasting and glucose fed rabbits, with the latter dose giving a blood sugar lowering activity comparable to tolbutamide.

**Scoparia dulcis**: *Scoparia dulcis* Linn., belonging to Family Scrophulariaceae, has common names like Chinibuta, Chinisakam and Gurara. It is a native of America but is widely grown in different parts of India. The active principle isolated from its alcoholic-aqueous extract is known as amellin. Addition of amellin (5g/100g) to high carbohydrate diet prevented deposition of glycogen in liver and prevented rise of lactic acid in liver and muscles (Nath, 1949). Amellin when injected daily in acetoacetate induced hyperglycaemia, blood sugar was lowered to normal level and amellin also prevented elimination of excessive urea and inorganic phosphate in the urine (Nath, 1951).

**Tinospora cordifolia**: *Tinospora cordifolia* (Willd), belonging to Family Menispermaceae, is commonly known as Giloe, Gulancha and is distributed throughout India. The extract of *Tinospora cordifolia* administered per oral (20mg/100gm body weight) to normal and diabetic rats, not only lowered blood sugar but also inhibited glucose induced hyperglycaemia, the effect being more significant in moderate alloxan diabetes (Prasad, 1992).
**Tecoma stans:** *Tecoma stans* (Linn.) H.B. & K., belonging to Family Bignoniaceae, grows throughout India. Glucose tolerance was observed to increase in rats after use of aqueous decoction and alcoholic extract and the same was observed in rabbits as well (Gupta, 1967).

**Tricosanthes dioica:** *Tricosanthes dioica* Roxb., belonging to Family Cucurbitaceae, has a synonym *Momordica dioica* Roxb and is commonly known as Gol Kandra, Patal. It has been mentioned in Caraka Samhita for fever, pruritus, pimples, diarrhoea, as an astringent and appetizer. It is a creeper, usually available in Eastern and Northern part of India. 2% gum acacia suspension of 95% ethanolic extract of the whole plant fed by oral intubation to male albino rats showed significant lowering of fasting blood sugar levels (Chandrashekar, 1988). The effect of feeding *T. dioica* seeds on glucose tolerance in the normal human subjects and mild diabetic patients was studied and a marked significant fall was observed in the glucose level at every interval of Glucose Tolerance Test exhibiting significant hypoglycemic effect (Sharma, 1989).

### 2. Plant Tissue Culture

Plant Tissue Culture is the technique of growing plant cells, tissues and organs in an artificially prepared nutrient medium, static or liquid, under aseptic conditions. The term ‘micropropagation’ is a more accurate way of citing the primary application of plant tissue culture.

The technique has developed around the concept that a cell is totipotent. Totipotency is a phenomenon in which a single cell has the capacity and ability to develop into a whole organism. Meristematic cells are located at the tips of stems and roots, in leaf axils, in stems as cambium, on leaf margins, and in callus tissue (Bhojwani, 2001 rev. ed.). In plants, even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus. When non-dividing, quiescent cells from the differentiated tissues are grown on a nutrient medium the cells first undergo certain changes to achieve meristematic state. These include replacement of non-functional cellular components, which are damaged by lysosomal activity during the process of cytoquiescence (Bomman, 1974).
The phenomenon of a mature cell reverting to the meristematic stage and forming undifferentiated callus tissue is termed dedifferentiation. As a multicellular explant generally comprises different types of cells, the callus derived from it would be heterogeneous. It has the ability of its own component cell to form a whole plant or plant organs. This phenomenon is called redifferentiation. Dedifferentiation mostly involves embryonization of cells leading to callus formation. However, embryonic explants often exhibit differentiation of roots, shoots or embryos without an intervening callus phase. Tissue culture techniques are useful to study the factors that elicit the totipotentiality of cells as well as investigation of factors controlling cytological and histological differentiation (Bhojwani, 2001 rev. ed.).

Under the influence of genetic make-up, location, light, temperature, nutrients, hormones and many other factors, meristematic cells differentiate into leaves, stems, roots etc. in an organized fashion. In the nineteenth century, Harberlandt (1902) tried to isolate and culture single cells from the leaves of flowering plants. He had found that such a system would provide an excellent opportunity for investigating the properties and potential of plant cells and also to understand the interrelationships and complementary influence of cells in multicellular organs. To date it is possible not only to culture free cells but also to induce divisions in a cultured cell and to raise whole plant from it. The cloning of single cells permits crop improvement through the extension of the techniques of microbial genetics to higher plants. Large-scale cultivation of plant cells in vitro provides a viable alternative for the production of large number of commercially important phytochemicals (Mulabagal, 2004).

The word “callus” associated with plant tissue culture is a characteristic growth on a wound on plants, or on a culture medium. It is an amorphous aggregate of loose parenchyma cells, which proliferate from the mother cells that can differentiate into roots, shoots, embryoids, thereby developing into plantlets. Callus may be hard or brittle or could be soft and friable. When there is organogenesis directly from a mother cell, then it is termed as direct organogenesis but if it is from callus tissue, then it is termed as indirect organogenesis. The type of organogenesis is mainly dependent on the balance between various plant growth regulators especially auxins and cytokinins. When adventitious shoot bud initiation takes
place in the callus tissue, it is specifically termed as caulogenesis; on the other hand, when it is applicable to roots, it is termed as rhizogenesis (Bhojwani, 2001 rev. ed.).

2.1 Cytodifferentiation
Vascular differentiation, especially the tracheary elements (TES) has been extensively studied in the area of in vitro and in vivo cytodifferentiation. In an intact plant tissue differentiation goes on in a fixed manner, which is characteristic of the species and the organ.

2.1.1 Factors affecting vascular tissue differentiation
Auxins and sucrose have a profound effect on vascular tissue differentiation. They qualitatively as well as quantitatively affect vascular differentiation. Cytokinins and gibberelic acid also affect xylogenesis to a certain extent. In mesophyl cell cultures of Zinnia, it was observed that the absolute concentration of the two hormones i.e. Auxins and cytokinins in the culture medium is more important than auxin/cytokinin ratio (Lin. 1990). The effect of auxin on vascular tissue differentiation seems to be closely dependent on the presence of sugar (Fosket, 1964). Studies using Zinnia system have highlighted the importance of calcium in TE differentiation. Calcium deprivation or application of calcium channel blockers or calmodulin antagonists inhibited TE differentiation (Roberts & Haigler, 1990). An increase in temperature is also known to enhance xylem formation. Light is also reported to stimulate wound vessel differentiation. Stress is also an important factor essential for the induction of tracheary elements. Use of inhibitors of ethylene synthesis caused blockage of TE differentiation.

2.1.2 Organogenic Differentiation
Most plants provided with appropriate conditions would differentiate shoot buds and roots from somatic as well as reproductive tissues. Whole plant regeneration from cultured cells may occur either through shoot-bud differentiation or somatic embryogenesis. There are distinct morphological differences between shoot buds and embryos, in which, the former is a monopolar structure that develops, procambial strands establishing a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explant. The embryo, on the other hand
is a bipolar structure with a closed radicular end. It has no vascular connection with the maternal callus tissue or the cultured explant. Plant regeneration from isolated cells, protoplasts or unorganized callus is generally more difficult than that from intact explants such as cotyledons, hypocotyls and immature embryos. Genes can be inserted into cells of intact explants to bring about variation. The regenerates obtained through de novo differentiation of shoot buds or somatic embryos directly from the explants also exhibit genetic variability suitable for somaclonal variant selection. Therefore, during the last decade considerable attention has been paid to optimize protocols for in vitro organogenic and embryogenic differentiation directly from immature embryos and seedling explants (Bhojwani, 2001 rev. ed.).

There are several factors that affect shoot-bud differentiation. It was particularly observed in various studies that cytokinins like adenine and kinetin promote shoot bud differentiation and development. On the other hand, auxins inhibit bud formation, even negating the effect of cytokinin. In combination, a relatively higher concentration of auxin favours cell proliferation and root differentiation, whereas relatively higher levels of cytokinin promote bud differentiation. Thus, quantitative interaction between auxin and cytokinin brings about root-shoot differentiation. It has been observed that when cereal callus tissue is transferred from 2,4-D supplemented medium to a medium lacking it or having cytokinin in its place, organogenesis is profound. In tobacco, gibberelin inhibits shoot-bud differentiation. It has been observed that gibberelin is most effective at the stage of meristemoid foundation. During shoot formation in cultures, a significant elevation in levels of ethylene and carbon dioxide has been observed. Abscissic acid also has been observed to induce shoot formation in certain recalcitrant explants. Thus, exogenous hormones play a direct role in organogenesis (Bhojwani, 2001 rev. ed.).

Electric stimulation or application of weak (1µA) electric current markedly enhances organogenic and embryogenic differentiation in tissue culture. The electric treatment on the callusing medium, which normally does not favour any caulogenesis, results in some greening and shoot-bud differentiation. The application of microampere current to the callus on shoot differentiation medium caused five-fold stimulation of shoot-bud differentiation as compared to the control.
The electric stimulation of caulogenesis affects both the numbers of cultures forming shoot buds and buds per culture.

In some plants almost all parts are capable of *in vitro* plant regeneration whereas in others this potential is restricted to only certain tissues. In plants where different explants respond, some may be more regenerative than the others. Several factors influence the regenerability of an explant. These factors are the organ from which it is derived, the physiological state of the explant and its size. Orientation of the explant on the medium and the inoculation density may also affect shoot bud differentiation. The physiological status of an explant is affected by the age of the donor plant, which has a direct bearing on the regenerability of the explant. The young and meristematic tissue give rise to raising of regenerated cultures while mature and differentiated explants fail to show such a response. This is especially true for cereals and tree species (Kumar, 2000).

### 2.1.3 Genotype

It is well established that for *in vitro* differentiation, the genotype of the plant plays an equally critical role as the growth regulators. Genotype specificity to regeneration has been reported in a number of plants and genetic variation for regeneration occurs between varieties and in out breeding species, even within varieties for e.g. Strawberries (Randi, 2005).

### 2.1.4 Physical factors

There are several reports suggesting that cultures in a solid medium grew in completely unorganized state whereas in the liquid medium with an identical composition there was organized growth. Studies on tobacco cultures grown on medium solidified with 1% agar showed extensive bud formation but negligible differentiation on the surface of liquid medium thereby suggesting that with lowering the agar concentration there was a striking alteration in the morphogenic patterns. It is important to maintain the osmotic pressure in media by addition of components like NaCl, sorbitol or mannitol or any other carbon source.

The photoperiod also plays an important role in differentiation. Callus maintained under continuous light remained whitish and did not exhibit organogenesis but
alternating light and dark periods proved beneficial (Pillai, 1968). The quality of light also influences organogenic differentiation. Blue light seems to promote shoot and bud differentiation whereas red light stimulates rooting (Bagga, 1986). Raised temperatures increased growth of the callus but inhibited shoot bud differentiation.

Organogenic differentiation in cell and tissue cultures, in response to hormonal manipulation of the culture medium is a multistep process. A series of intra cellular events, collectively called induction, occur before the appearance of the morphologically distinguishable organ. Induction leads to irreversible commitment of cells to follow a particular developmental pathway.

Under the conditions favouring unorganized growth, the meristems in a callus are random and scattered. When the tissue is transferred to conditions supporting organized growth, localized clusters of cambium like cells arise. These localized clusters or meristemoids are termed as nodules or growing centers. These nodules may become vascularized due to the appearance of tracheidal cells in the center, which then become the site for organ formation in the callus. The center of the nodules gives rise to roots and on transfer to a semisolid medium a shoot bud arises at a locus different from the root. A vascular connection gradually develops between the root and the shoot forming a whole plant. There are several histochemical studies evaluating the changing status of nucleic acid, protein and carbohydrate in differentiating and non-differentiating calli. Significant rise in RNA and protein contents was observed in shoot forming regions of the calli while there wasn’t much detectable difference in the levels of DNA. The intracellular accumulations of starch has been ascribed a positive role in the process of shoot bud differentiation (Bhojwani, 2001 rev. ed.).

2.1.5 Somatic Embryogenesis

A zygote divides and develops into an embryo after fertilization in a process called embryogenesis. However fertilization is not always essential to stimulate an egg to undergo embryogenesis. During the last 3 decades considerable information has accumulated to establish the embryogenic potential of somatic plant cells leading to an explosion in a number of species that form somatic embryos. Any cell, in which differentiation is not irreversible, will, if placed in an appropriate medium, develops in an embryo like way and produces a complete plant. Conventionally
somatic embryogenesis is regarded as a two-step process: Induction of embryogenesis and embryo development followed by embryo maturation (Bhojwani, 2001 rev. ed.).

2.1.5.1 Factors affecting somatic embryogenesis

**Explant:** Explant selection plays a major role in obtaining regenerating cultures of many recalcitrant plants. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures of these plants. The somatic embryos may arise directly or after slight callusing. Fully expanded leaves are also suitable for induction of somatic embryogenesis especially the basal portion of leaves.

**Genotype:** Genotype effect on somatic embryogenesis occurs as for regeneration via shoot bud differentiation. Genotypic variations could be due to endogenous levels of hormones (Carman, 1990). Transfer of cultures from media containing high auxins levels to media devoid of auxins increased embryogenic response. Cytoplasmic factors as well as genetic disposition are implicated in the control of somatic embryogenesis (Willman, 1989).

**Growth Regulators:** A general procedure followed for the induction of somatic embryogenesis is growing cultures on media containing a synthetic auxin followed by transfer to an auxins free medium for embryo differentiation. 2,4-D has been the most commonly used auxin for the induction of somatic embryogenesis. There are several studies that also have shown use of media without hormones but with high concentrations of sucrose to induce somatic embryogenesis (Kamada, 1989). On a proliferation medium, callus differentiates localized groups of meristematic cells called ‘Proembryogenic Masses’ (PEM). In subsequent subcultures on the proliferation medium the embryogenic cells continue to multiply without the appearance of embryos. The PEMs on transfer to a medium containing a very low level of auxin or no auxin develop into embryos. Such a medium is referred to as embryo development medium. The presence of an auxin in the proliferation medium seems essential for the tissue to develop embryos in the embryo development media. The tissue maintained continuously in auxins free medium did not form embryos. Therefore the proliferation medium is regarded as the induction
medium for somatic embryogenesis and each PEM and unorganized embryo (Bhojwani, 2001 rev. ed.).

Among the large number of growth regulators tested, 2,4-D is by far the most effective for producing embryogenic cultures. A high level of endogenous auxins could decrease the embryogenic potential of a culture. Prolonged duration of subculture and sucrose starvation in preceding passage also is seen to promote embryo formation. 2-Chloro phosphonic acid, which releases ethylene in plant tissue, suppresses the development of mature somatic embryos without an appreciable reduction in the growth and multiplication of PEMs. There are mixed reports of the presence of cytokinin in the induction of embryogenic cultures. Gibberelin also seems to inhibit somatic embryogenesis (Bhojwani, 2001).

**Nitrogen source:** The form of nitrogen in the medium significantly affects *in vitro* embryogenesis. Reduced nitrogen has proved beneficial for embryo development. In many cases addition of casein hydrolysate over individual amino acids stimulated rapid development of somatic embryos from maturation to germinable stage. Proline, alanine, arginine and glutamine when added to the callus maintenance medium resulted in a 100-fold increase in embryo production. Organic acids such as potassium citrate, potassium malate or potassium tartrate also increased the number of embryos formed, improved the quality of somatic embryos in terms of conversion frequencies and enhanced accumulation of seed storage proteins.

**Polyamines:** There is some evidence to suggest that polyamines are required for embryo development *in vivo* and *in vitro* (Altman, 1990; Mengoli, 1992). Increase in the endogenous level of polyamines and the enzymes for their biosynthesis is concomitant with the induction of somatic embryogenesis. Inhibitors of polyamine biosynthesis suppress somatic embryogenesis suggesting the involvement of polyamine in the same (Altman, 1990).

**Oxygen concentration:** Oxygen tension has been shown to promote embryogenic development in cultures (Kaman, 1990). Incubation in a low oxygen environment reduced the amount of 2,4-D required to initiate embryogenic callus. Low oxygen
level also significantly decreased precocious germination of somatic embryos and the frequency of abnormal scutellar enlargement.

**Electrical Stimulation:** Exposure to mild electric field considerably promotes the embryogenic response increasing the number of embryo. The electric stimulus seems to promote the differentiation of organized cultures (shoots or embryos) by affecting cell polarity through changes in the organization of microtubules (De Jong, 1993). Another striking effect of electric treatment was the induction of asymmetric first division coupled with a relatively short period of cell expansions resulting in spherical structures composed of many small irregular shaped cells and a few large ones.

**Selective Subculture:** Multicellular explants are generally heterogeneous in terms of the morphogenic potential of its constituent cells. Only a small proportion of these cells are able to express then cellular totipotency under a set of culture conditions. Therefore, the calli derived from such explants are also heterogenous. Sometimes the embryogenic/organogenic portions of the callus are distinct from the non-morphogenic tissue on the basis of their morphological appearance and it is essential to make selective subcultures to establish regenerating tissue cultures. Two types of calli are generally formed: (1) White or off white or pale yellow, compact and often nodular callus. (2) Soft, granular and transluscent callus. Of these the first type of calli exhibit embryogenic differentiation (Vasil, 1991).

**Other Factors:** High potassium has been observed to be necessary for embryogenesis in certain species (Brown, 1976). Cefotaxime, an antibiotic, when added in media, increased embryogenic response in embryo cultures of wheat (Mathias, 1986) and in barley (Mathias, 1987).

### 2.1.5.2 Large Scale Production of Somatic Embryos

As the multiplication of embryogenic cells and the subsequent development of SEs can occur in liquid medium, somatic embryogenesis offers a potential system for large scale plant propagation in automated bioreactors. For mass production of somatic embryos in bioreactors, callus is initiated on a semi-solid medium. Pieces of undifferentiated or embryogenic callus are transferred to liquid medium in small
flasks and agitated on a shaker. After a few cycles of multiplication in flasks, the embryogenic suspension may be filtered through a sieve of suitable pore size and PEMs or globular embryos transferred to the bioreactor flask. Most of the modern bioreactors are fitted with probes for measurement and control of temperature, agitator speed, pH, pO₂ and pCO₂, which not only allows cultivation of cells under highly controlled conditions but also enables precise analysis of interacting factors for cell growth and embryo development (Preil, 1991; Denchev, 1992).

Table 1 Natural products of industrial importance

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>Alkaloids</th>
<th>Ajmalicine, atropine, berberine, codeine, reserpine, vincristine, vinblastine.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroids</td>
<td>Diosgenin</td>
<td></td>
</tr>
<tr>
<td>Cardenolides</td>
<td>Digetoxin, digoxin</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food and flavours</th>
<th>Sweeteners</th>
<th>Stevioside, thaumatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bittering agent</td>
<td>Quinine</td>
<td></td>
</tr>
<tr>
<td>Pigment</td>
<td>Crocin</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pigments and Perfumes</th>
<th>Pigments</th>
<th>Shikonin, anthocyanins, betalins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragrances</td>
<td>Rose oil, jasmine oil, lavender oil.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agrochemicals and fine chemicals</th>
<th>Agrochemicals</th>
<th>Pyrethrins, salannin, azadirachtin.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine chemicals</td>
<td>Proteases, vitamins, lipids, latex, oil.</td>
<td></td>
</tr>
</tbody>
</table>


2.2 Production of Secondary Metabolites

Higher plants produce a great variety of secondary products. These secondary products seem to play a minor role in the basic life processes of the plant but often have an ecological role, such as attractant of pollinators and chemical defense against microorganisms, insects and higher predators (Wink, 1988). Many of these natural products have been used as sources of a large number of industrial products, including agricultural chemicals, pharmaceuticals and food additives (Table 1). Several of these natural products have been replaced by synthetic substitutes owing to cost considerations, yet a number of commercially important high value chemicals are extracted from plants.
Table 2  Some examples where cell cultures have produced natural compounds in amounts equal to or higher than whole plants

<table>
<thead>
<tr>
<th>Product</th>
<th>Plant Species</th>
<th>Yield (% Dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole Plant</td>
</tr>
<tr>
<td>Ajmalicine</td>
<td>Catharanthus roseus</td>
<td>0.3</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Morinda citrifolia</td>
<td>2.2</td>
</tr>
<tr>
<td>Berberine</td>
<td>Coptis japonica</td>
<td>2.4</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Coffea arabica</td>
<td>1.6</td>
</tr>
<tr>
<td>Catharantine</td>
<td>Catharanthus roseus</td>
<td>0.0017</td>
</tr>
<tr>
<td>Diosgenin</td>
<td>Dioscorea deltoridea</td>
<td>2.4</td>
</tr>
<tr>
<td>Ginsenoside</td>
<td>Tanax ginseng</td>
<td>4.5</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>Coleus blumei</td>
<td>3</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Peganam harmala</td>
<td>2</td>
</tr>
<tr>
<td>Serpentine</td>
<td>Catharanthus roseus</td>
<td>0.26</td>
</tr>
<tr>
<td>Shikimic acid</td>
<td>Galium mollugo</td>
<td>2-3</td>
</tr>
<tr>
<td>Shikonin</td>
<td>Lithospermum erythrorhizon</td>
<td>1-2</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>Trigonella foenumgraceum</td>
<td>0.4</td>
</tr>
<tr>
<td>Tripodiolide</td>
<td>Tripterygium wilfordii</td>
<td>0.01</td>
</tr>
<tr>
<td>Vomilenine</td>
<td>Rauwolfia serpentina</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Source. Mulabagal, 2004

Many important compounds are difficult to synthesize due to their structural complexity and hence the dependence on extraction from the plant source. Besides their direct application, the natural plant products serve as model compounds for the chemical synthesis of new, more potent analogues. The secondary metabolites could also serve as the backbone for further chemicals modifications. For example, podophyllotoxin obtained from *Podophyllum sp* is used for the synthesis of the clinically applied antitumor agents etoposide and teniposide (Holthuis, 1988). There has been an increasing interest among scientists to produce high value natural plant products by cell culture, which can overcome many of the problems associated with industrial production of these phytochemicals by extraction from field grown plants (mass cultivated or natural populations). In cultures, large-scale production of these natural compounds can be carried out throughout the year unaffected by the season (Mulabagal, 2004).

Of the various plant products produced by plant tissue culture, pharmaceuticals have received maximum attention (Fig.2).
Cell cultures provide means for de novo synthesis of natural products as well as serve as ‘factories’ for bioconversion of low value compounds into high value products. Some novel compounds produced in cell cultures are not produced in intact plants. At least 85 novel compounds including 23 alkaloids, 19 terpenoids, 30 quinones and 11 phenyl compounds have been isolated from some 30 different plant culture systems (Phillipson, 1990). With improvement in technology, there have been several cases in which cell cultures have been shown to produce higher amounts of the products than the intact plants from which they are derived (Table 2).
2.2.1 Plants, secondary metabolites and the market

The pharmaceutical and healthcare sector focuses on all kinds of research ranging from animals to plants, recombinant proteins, plant-derived vaccines, etc. with the sole aim of intensifying the approach of disease prevention and treatment. Taking a look at some of the facts and figures about the position of plants or their natural products in this area of industry, it is observed that globally the market of medicinally useful formulations, drugs and healthcare products comprises of more than 60% products derived from plant origin. More stress is being laid on medicinal plant products, which play a twofold role as traditional medicines and also as trade commodities, meeting the demand of distant markets (Raskin, 2002).

Almost 70% modern medicines in India are derived from natural products. Sales of medicinal plants have grown by nearly 25% in India, one of the largest users of medicinal plants in the past ten years (1987-1996) and this is the highest rate of growth in the world (Wakdikar, 2004). Drug products derived from higher plants have a good commercial value. It is interesting to know that in the U.S.A. 25% of all the drugs dispensed contained compounds derived from flowering plants in the 1970s (Raskin, 2002). In 1980, American consumers paid about $8 billion for prescription drugs derived solely from higher plants (Balandrin, 1985). Plants continue to be important sources of new drugs, as evidenced by the approvals in the United States in 1983 of two new plant-derived drugs. India, with its rich biodiversity ranks first in percent flora, which contain active medicinal ingredient. World Health Organization (WHO) has estimated that nearly 80% of the populations of developing countries rely on plant drugs both as traditional medicine and also for their primary health care needs (Wakdikar, 2004). There is an accretion in consumer interest in the “natural” therapies in the U.S. and also in other countries (Nolan, 2004).

Primary metabolites are also referred to as ‘high volume-low value bulk chemicals’. These primary metabolites obtained from higher plants are generally valued at less than $1 to $2 per pound ($2.20 to $4.40 per kilogram) and are readily available in large quantities in the market. However there are some exceptions, two of them being mannitol and β-carotene which cannot be extracted, isolated and purified very easily, hence making them expensive. Compared to
primary metabolites, the secondary metabolites are ‘higher value-lower volume’
products. They are often valued at several dollars to several thousand dollars per
pound on account of their smaller quantities and the difficulty of extraction and
isolation. For example, purified opium alkaloids codeine and morphine are valued
in the range of $400 to $600 per pound ($650 to $1250 per kilogram). Rare volatile
oils such as rose oil are often valued at over $1000 per pound ($2000 to $3000 per
kilogram). Another excellent example is that of the anticancer Vinca alkaloids,
having a wholesale value of about $1,11,00,000 per pound ($50,00,000 per
kilogram), and their retail value may be as high as $4,40,00,000 per pound
($2,00,00,000 per kilogram). Also on account of their highly complex structures,
secondary metabolites cannot be synthesized economically (Balandrin, 1985).

According to an online press release dated back to 1998, about 50% of the total
plant sales came from single entities whereas the remaining 50% came from herbal
remedies. In this report it was estimated that secondary metabolites such as
atropine, digoxin, morphine, paclitaxel, pilocarpine, reserpine, scopolamine,
topotecan and vincristine, etc. are expected to increase their market share of the
combined total future dollar sales, mainly due to novel drug developments. These
are single entity plant drugs (Magnani, 1998).

The total market for plant-derived drugs was valued at $22,608 million in 1997 and
was projected to reach $30,688.5 million in 2002, representing an average annual
growth rate (AAGR) of 6.3%. Terpenes were predicted to contribute the most to
the dollar sales of plant-derived drugs. It was also speculated that the terpene
category should increase its share in the years to come. Terpenes were valued at
$7,660 million in 1997 and were expected to reach $12,400 million in 2002, at an
AAGR of 10.1%. The glycoside category comprising of flavanoids, saponins,
anthraglycosides and digitalis compounds were valued at $7,300 million in 1997
and were estimated to reach $9,230 million just in the next 5 years, with an AAGR
of 4.8%. BCC (Business Communications Company, Inc.) pointed out that the
category of alkaloids comprising of the Rauwolfia, Vinca, etc. alkaloids were
valued at $3,600 million in 1997. They were envisaged to reach $4,045 million in
2002 representing an AAGR of 2.4% (Magnani, 1998).
The statistics presented above clearly indicate that plant secondary metabolites have a great potential in the market and an escalation in their sales value can be expected in the future. Also, India has enormous scope to reach the acme of global market of plant based medicines and products, with several private sector and government Research and Development institutions in India being involved in plant and natural plant products based research. Industry oriented R&D institutes such as Dabur Research Foundation, Himalaya Health Care, Zandu Pharmaceuticals, Avestha Gengaine Technologies, Reliance Life Sciences, Hamdard, etc. are already involved or are emerging in the area of plant natural product based research. For example, Reliance Life Sciences is an emerging company focusing on selected species for research to enhance both the quality and quantities of products of secondary metabolites using Metabolic Engineering (Wakdikar, 2004).

### 2.2.2 Strategies used to optimize product yield

The productivity of cell lines is greatly influenced by the culture conditions, of which culture medium is the most important. In general, growth and production of secondary metabolites generally occurs in the late stationary phase when the medium gets depleted of some of its important constituents. Growth inhibition is often associated with cytodifferentiation and the induction of enzymes for secondary metabolism. A dual culture system is used which involves Biomass production in a medium optimum for cell production, which is then transferred to a different medium that does not support good growth of the cells but is favorable for product yield (Bhojwani, 2001 rev. ed.). The most useful modifications needed in the growth medium for use in secondary metabolite production are:

a) Reduction or elimination of 2,4-D or other phytohormones;

b) Reduction of phosphate level; and

c) Increase in sucrose level or alternation of Carbohydrate (C) by Nitrogen (N) ratio.

The optimum media for the production of different metabolites by a cell line are likely to be different. Moreover, various constituents within the media composition can be varied for an efficient output. The two-stage bioreactor is appropriate for increased secondary metabolite production but a single step process would be more
cost-effective. Hence, the possibility of combining growth and production steps in
the same medium has been tried in production of several compounds (Wakdikar,
2004).

Plant growth regulators affect growth and differentiation and, thus, affect
secondary metabolite production by cultured cells. In general an increase of auxin
level, such as 2,4-D, which stimulates dedifferentiation and proliferation of cells,
reduces the level of secondary metabolites. Therefore, generally auxins are added
to the growth medium but omitted or used at a lower level in the production
medium. Gibberelin also generally inhibits the secondary metabolite production
(Yoshikawa, 1986). Enhanced berberine production by cell cultures of Captis
japonica in response to GA₃ (10⁻⁸-10⁻⁵M) is a rare example of the promotive effect
of these phytohormones on secondary metabolite production. This enhancement is
ascribed to GA₃ induced inhibition of starch synthesis, decrease in nitrogen uptake
and increased uptake and turnover of sucrose (Hara, 1994).

It has been shown that the pH of the media is able to enhance cell membrane
permeability, thus, helping in the release of intracellular alkaloids (Jardin, 1991).
Light is another regulatory factor in the production of alkaloids in plant cell
cultures. The importance of light for the optimal expression of some pathways in
cultured cells has been demonstrated, including for flavonoids (Hahlbrock, 1979),
cardenolides (Ohlsson, 1983) and betacyanins (Berlin, 1986). Light affected both
the production and the site of product accumulation. The gaseous environment,
mainly the availability of oxygen and carbon dioxide, also plays an important role
in the production of secondary metabolites by cell cultures. It has been reported
that high-density cultures of C.roseus, when cultivated at high dissolved oxygen
concentration had a higher ajmalicine production. This was attributed to increased
availability of dissolved oxygen being able to stimulate the oxidative metabolism
responsible for the conversion of ajmalicine into serpentine (Schlatmann, 1994).
The type of closure can significantly affect the headspace gas composition of shake
flask cultures. Depending on the permeability of the closure, gaseous compounds
produced by the culture can accumulate in the headspace of the flasks.
The explants used to initiate tissue cultures are highly heterogeneous with regard to the metabolic productivity of its constituent cells. The heterogeneity is expected to increase under culture conditions, which are known to induce genetic and epigenetic changes. These cells and cell clusters exhibit considerable variation for the accumulation of secondary metabolites (Ellis, 1985). Heterogenous cultures productivity would be an average of its high and low yielding cells. Selection and cloning of high yielding cells from such cultures is, therefore, regarded as an effective method to improve in vitro production of secondary metabolites. The cultures are initiated from selected high yielding genotypes, which are screened to obtain the best producing variants. From established cultures of these lines, selection can be made for better yielding sub clones (Songstad, 1990).

Cultured cells being prone to spontaneous genetic changes need periodic selections to maintain high productivity of the cultures. Selection should be made under conditions that are suitable for product formations. It is important that the selected cell lines be stable. Metabolite synthesis by plant cells is highly influenced by the physiological state of the cells hence the high yield by the selected lines may be due to altered gene expression. For the isolation of stable lines, repeated selections should be made and only if product of a cell line remains stable over several selection cycles will a true variant be identified. In nature, a number of compounds (Phytoalexins) are synthesized by plant cells in response to chemical or microbial attacks. Increased production of secondary metabolites in media supporting poor growth of cells is also regarded as a stressed response (Dicosmo and Touers, 1984). Consequently both biotic and abiotic factors have been tested as elicitor and shown to improve the production of secondary metabolites in plant cell and organ cultures (Eilert, 1987).

Elicitor induced products are frequently released into the medium (Brodelius, 1990). Simple organic and inorganic molecules can induce product accumulation in cultured cells. Enhanced accumulation of catharantins in the cells of C.roseus is in response to NaCl, KCl and sorbitol. Addition of vanidine sulphate to all cell suspension cultures of C. roseus resulted in the productions of catharanthin, serpentine and tryptomine (Tallevi, 1988). The production of dimeric alkaloids by shoot cultures could be induced by irradiation with near ultraviolet light (Hirata.
1992). The time of application of elicitor is critical for the yield of secondary metabolites by cultured cells. Most of the cultures generally respond to an elicitor only during the growth phase. The time of elicitor application may affect not only the quantity of product but also the production pattern (Eilert, 1986). Elicitor treatment after the culture has already started to accumulate the inducible compound does not enhance or accelerate its production (Komburik, 1985).

### 2.2.3 Immobilization of Cells

One of the major problems in commercialization of cell culture based products for secondary metabolite production is the high production cost due to slow growth of plant cells, low product yield, genetic instability of the selected lines, low shear, resistance of cells and intracellular accumulation of the products. Some of these problems can be reduced by immobilized cell culture. In this technique, cells are confined within a reactor system preventing their entry into the mobile phase, which carries a substrate and the products. *C. roseus* and *Daucus carota* cells were the first immobilized cells, entrapped in alginate beads (Brodelius, 1985; Scragg, 1991).

Immobilization is only relevant where the production process involves two stages; the first optimized for biomass production by suspension culture and the second optimized for product formation by immobilized cells. The potential advantages of immobilized cell cultures are:

1. It may enable prolonged use of biomass;
2. By immobilization of cells, the cell density in a bioreactor can be increased 2-4 times that in suspension cultures and this enables the use of small reactors reducing the cost of medium, equipment installation and downstream processes;
3. The entrapped cells as a simple bioreactor design may be used;
4. It separates the cells from the medium and, therefore, if the product is extracellular, it can simplify downstream processing;
5. It uncouples growth and product formation which allows product optimization without affecting the growth;
6. The non-dividing immobilized cells are less prone to genetic changes and, therefore, provide a stable production rate;
(7) It minimizes fluid viscosity, which in cell suspensions cause mixing and aeration problems; and
(8) It promotes secondary metabolite secretion in some cases.

Immobilization of cells on the surface of an inert support, such as fiberglass mats and unwoven short fiber polyester, has also been examined for \textit{in vitro} production of secondary metabolites (Tyler, 1995). Surface immobilization promotes the natural tendency of plant cells to aggregate, which may improve the synthesis and accumulation of secondary metabolites.

Apart from production of secondary metabolites, plants and plant cell cultures have been also used for production of recombinant proteins and antibodies (Hiatt, A., 1989). Using plant-cell suspension cultures as bioreactors are a promising molecular farming option for secondary metabolites like vincristine, vinblastine, ajmalacine, reserpene etc. (Fischer, R., 1999). Transformed plant cell lines using \textit{Agrobacterium}, particle bombardment, electroporation or viral vectors, are being established as model systems for higher production levels.

\subsection*{2.2.4 Biotransformators and Addition of Precursors}

Addition of Precursors: Secondary metabolite production may be stimulated by addition of appropriate precursors or related compounds in the culture medium e.g. amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids and ephedrine. Phenylalanine, a biosynthetic precursor of rosemarinic acid is added to suspension cultures of \textit{Salvia officinalis} to increase the production of the same. Addition of phenylalanine into the agar medium of \textit{Taxus cuspidata} cells stimulated the biosynthesis of the anticancer compound, taxol (Bhojwani, 2001 rev. ed.).

Biotransformation: Instead of the addition of a particular compound as a precursor into the culture medium of plant cells, a suitable substrate compound may be biotransformed to a desired product using plant cells e.g. biotransformation of β-methyl digitoxin to β-methyl digoxin using \textit{D. lanata} cells. The precursors used should be easily available and inexpensive (Bhojwani, 2001 rev. ed.).
2.2.5 Natural products from plants
Phytochemicals as the word implies are the individual chemicals from which plants are made. The general categories of plant natural products are organized very broadly in terms of increasing oxidation state. This begins with the lipids, including the simple and functional hydrocarbons, as well as the terpenes. The unsaturated natural products include the polyacetylene and aromatic compounds. The primarily hydrophilic molecules include the sugars, the alkaloids, the amino acids and the nucleosides.

Lipids: Lipids are water insoluble biomolecules that are soluble in nonpolar solvents and contain a large hydrocarbon region in this structure. Lipids form the main structural compounds of membranes, are sources of fuel for storage and transport and form protective surface coatings. They may also be involved in cell recognition, species specificity, and tissue immunity. Hydrocarbons, the least polar organic natural products may be either saturated or unsaturated. Saturated hydrocarbons are the simplest and least polar natural products for e.g. turpentines consist of simple hydrocarbons particularly n-heptane, which can be derived from conifers like *Pinus jeffreyi* and *P. sabiniana*. In living plants, saturated hydrocarbons are universally distributed as the waxy coatings on leaves, and as cuticle waxes on the surfaces of the fruits. The simplest unsaturated hydrocarbon is Ethylene, an important plant hormone.

Larger unsaturated hydrocarbons are also common as plant waxes. As the chain length and degree of unsaturation increases, the hydrocarbons become waxy and then solid at room temperature. Waxes may be either long chain hydrocarbons or esters of fatty acids. The polyacetylenes are a unique group of naturally occurring hydrocarbon derivates characterized by one or more acetylenic groups in their structures. These have been found to have a fairly specific distribution in plant families including Campanulacae, Asteraceae, Araliaceae Cittosporacae and Apiaceae. Biosynthetically the polyacetylenes are likely to be derived while enzymatic dehydrogenation from the corresponding olefins for e.g. Safynol in Safflower oil from *Carthamous tintorius* have been shown to act as natural phyto alexins, helping to detect the microorganisms which attract these plants (Bhojwani, 2001 rev. ed.).
There are many hydrocarbons that are halogenated like laurinterol that have been obtained from red algae. A large variety of volatile alcohols e.g. Aldehydes, Ketones and Esters occur in small concentration in plants and have been classically referred to as essential oils. Their role may be related to their often-strong odours, attracting them to insect pollinators and animal seed disseminators. All of the straight chain alcohol (C$_1$ to C$_{10}$) has been found in plants in either free or esterified form. Hydrocarbon sulphides, found in relatively few plants are recognizable by their obnoxious odours. Sulphides are common in the species of Allium, many of which are lachrymators and have pungent odours. The glucosinolates or mustard oil glucosides help to create the flavours of the mustard, raddish, onion and garlic. The most common sulphur containing natural products are the amino acids Cysteine and Methionine (Bhojwani, 2001).

Fatty acids are the simplest lipids characterized by a polar hydrophilic head region connected to a long hydrophilic tail. The most common fatty acids found in plants are oleic and palmitic acid. The fatty acids are utilized as building block components of the saponifiable lipids. Unsaturated fatty acids predominate in higher plants.

The terpenes are among the most widespread and chemically diverse groups of natural products. Despite their structural diversity they have a simple unifying feature by which they are defined and easily classified. Terpenes are a unique group of hydrocarbons based natural products, whose structure may be derived from isoprene, giving rise to structures, which may be divided in isopentane units. The number of five carbon units they contain thus classifies terpenes: Hemiterpenes (C$_5$), Monoterpenes (C$_{10}$) Sesquiterpene (C$_{15}$), Diterpene (C$_{20}$), Triterpene (C$_{30}$), and Tetraterpenes (C$_{40}$). The terpenes are of a similar biogenetic origin, in which isopentenyl pyrophosphate and dimethylallyl pyrophosphate combine to yield geranyl pyrophosphate leading to mono terpenes. Similarly, compounds derived from pharnesyl pyrophosphate lead to sesquiterpene and triterpenes (Daniel, 1990). Thus various combinations and oxidations give rise to a large variety of terpenes. The function of terpenes in plants is generally considered to be both ecological and psychological. Many of them inhibit the growth of competing plants (allelopathy). Some are known to be insecticidal; some are found
to attract insect pollinator. The plant hormones, abscissic and gibberellic acid belong to this family. Practically all plants steroids are hydroxylated at C₃ and infact steroids have profound importance as hormones, co-enzymes and pro vitamins. Saponins are high molecular weight triterpene glycosides containing a sugar group attached to a sterol or other triterpene. They are widely distributed in the plant kingdom and composed of two parts: glycone (sugar) and aglycone or genin (triterpene). Typically, they have detergent properties readily form foams in water, have a bitter taste, and are piscicidal (toxic to fish) (Bhojwani, 2001).

Saponins are constituents of many plant drugs and folk medicines, especially among Asian people. Saponins may be mono or polydesmodic depending on the number of attached sugar moieties. Biosynthetically, the saponins are comprised of six isoprene units and are derived from squalene. Commercially important preparations based on saponins include sarsaparilla root (Sarsaparilla spp.) licorice (glycyrrhiza spp.), ivy leaves (Hedera spp.), primula root (Primula spp.) as well as ginseng (Panax spp.) (Konno, 1985).

Many different plant species synthesize saponins as a part of their normal programme of growth and development (Haralampidis, 2002). By 1927, Kofler had listed some 472 plants containing saponins. Now it has been found that 90 families of plants contain saponins. In 1970, Gubanov found that 76% of the families contain saponins, from the systematic investigation of 1730 central Asian plant species (Hostettmann, 1995). Interest in these molecules stems from their medicinal properties, antimicrobial activity, and their likely role as determinants of plant disease resistance (Haralampidis, 2002). Saponins, from a variety of sources, have been shown to have hypcholesterolemic, anti-coagulant, anticarcinogenic, hepatoprotective, hypoglycemic, immunomodulatory, neuroprotective, anti-inflammatory and anti-oxidant activity (Rao, 2002). Saponins occur in plants that are used as human foods eg. Soya bean, Chick-pea, sugar beet, kidney bean, broad bean etc.

Saponins are also present in numerous herbal remedies. The saponin content depends on factors such as age, cultivar, the physiological state and geographical condition of the plant. There can be considerable variation in composition and
quantity of the saponins in the vegetable material from various places. Saponin distribution among the organs of plants varies considerably, eg. Ginsenoside level in *Panax ginseng* (Araliceae) is lowest in leafstalks and stem (0.77 %), intermediate in the main root (1.3%), but highest in leaves and root hairs (5.2% and 6.1% respectively). Saponins are not simply ballast materials but they maybe important regulatory substances in metabolism and development of an organism. That is, they may be physiologically significant constituents. This is implied by the fact that they are localized in organelles, which have a high metabolite turnover rate (Hostettmann, 1995).

**Triterpenoid saponins:** Triterpenoid saponins, or sapogenins, are plant glycosides, which form lather in water and are used in detergents, or as foaming agents or emulsifiers, and have enormous medical implications due to their antifungal, antimicrobial, and adaptogenic properties. Glycyrrhizin, from licorice root, is an example of a saponin used for antiinflammatory purposes in place of cortisone (Batchelder, 1995).

This division of triterpenoid saponins forms by far the most extensive collection of saponins. To date, it comprises over 750 triterpene glycosides with 360 sapogenins. The triterpenoid saponins can be mono-, bi- or even tridesmosidic. One sugar chain is often attached at the C3 and a second is frequently found to be esterified to carbohydrate group at C17 of the aglycone (Hostettmann, 1995). Plant material often contains triterpenoid saponins in considerable amounts. Eg. Primula root contains about 5-10% saponins, licorice root between 2-12% glycyrrhizin and quillaia bark upto 10% of saponin mixture. So, the concentration of saponins in plants is high compared to other secondary metabolites. Oleanane triterpenoids, also have been the subject of an update (Ibid). The number of triterpenoids is very large and only a small proportion of it has been characterized in glycosidic form. Triterpenoids are frequently isolated only after hydrolysis of plant extract and it is not always easy to ascertain from a published work whether they actually occur in free or glycosidic form in plant material (Ibid).

**Aromatics:** Almost all plants contain variety of natural products, which include an aromatic ring containing one or more hydroxyl substitutes. The vivid colours that
light up the plants are generally derived from three sources, the tetrapyrroles, the terpene based carotenes and the aromatics, also referred to as the acetogene. Many aromatics are already known and new structures are continuous by being discovered. In some cases their functions are well known for e.g. the polyphenolic lignins are structural components of the cell wall. Aromatic compounds are formed by several biosynthetic rules such as polyketide and shikimate pathways from terpenoid origins. Phenolic substances are water-soluble and form ether linkages with carbohydrate residues because of the acidity of the phenol functionality (Bhojwani, 2001).

**Non-phenolic Aromatics:** These include amino acids, tryptophan and phenylalanine, the inner alkaloids and auxin (Indole-3-acetic acid).

**Tetrapyrroles:** The most common tetrapyrroles are the chlorophylls followed by the cytochromes the linear tetrapyrroles including phytochrome, phycoerythrin and Phycocyanin are believed to be critical compounds for plant morphogenesis.

**Phenols:** The vast majority of the plant based natural products are phenols. Its numerous categories include the simple phenols, phenyl propanoids, flavonoids, tannins and quinones.

Most of the simple phenols are monomeric components of the polymeric polyphenols and acids, which make a plant tissue including lignin, melanin flavonoids and tannins. These individual components are obtained by acid hydrolysis of plant tissues. The compounds include p-hydroxyl benzoic acid vanillic, sallillic and gallic acids. Many of the phenols also exist as their methyl ethers. Apiol is a major constituent of the essential oil of parsley seed and is a powerful diuretic. The phenyl propaloids contain a three-carbon side chain attached to a phenol; common examples include the hydroxycoumarins, phenyl propones, and the lignans. Coumarin is common to numerous plants and is a sweet smelling volatile material, which is released from nearly moored hay. The phenyl propones are not phenols since they lack the hydroxyl functionality. Thus they are not water-soluble but rather are essential oils and include euginol, the major principle oil of clones (Bhojwani, 2001).
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The flavonoids have two benzene rings separated by a propane unit and are derived from flavole. They are generally water-soluble compounds and their more conjugated compounds often are brightly coloured. They are generally found in plants and they are glucosides, which can complicate structure determinations. The different classes within the group are distinguished by additional oxygen containing heterocyclic rings and hydroxyl groups. These include the catechins, leucoanthocyanidins, flavonones, flavonols, flavones, anthocyanidins, flavonols, chalcones, aurones and isoflavons. Other common groups include the xanthones and the condensed tannins (Daniel, 1990).

The tannins are common to vascular plants existing primarily within woody tissues. Tannins consist of various phenolic compounds that react with protein to form water insoluble co-polymers. Plant tissues that are high in tannin content have a highly bitter taste and may be either condensed or hydrolysable. The quinines form strongly coloured pigments covering the entire visible spectra. They are however found in the internal regions of the plant, thereby not imparting a colour to the extension of the plant. Generally quinines are derived from benzo-quinone, naphtha-quinone or antro-quinone structures (Daniel, 1990).

Carbohydrates: Sugars or carbohydrates are the primary products of photosynthesis and are essential as a source of energy to plants. They are stored as starch or functions, used as sucrose and polymerized to form cellulose, the main cell wall structural material of plants. Finally they combine to form glycosides of many fundamental groups of natural products, including terpenes, saponins, phenols and alkaloids. Most of the carbohydrates found in plants occur as polysaccharides of high molecular weight. The various structural polysaccharides include cellulose, polygalactoorannans (pectin), xylans, glucomannans, chitins and the glycosaminoglycans (Bhojwani, 2001).

Amines, Amino acids, and Proteins: The common plant amines can be subdivided into aliphatic monoamines, aliphatic polyamines and aromatic amines. Occasionally these materials are classified as alkaloids rather than amines. Simple aliphatic amines like methylamine, hexylamine have typically strong, fish-like aromas. For e.g. Heracleum sphondylium has such molecules, which act as insect
attractants. Common polyamines include putrescine, agmatine, spermidine and spermine. These polyamines are thought to have many functions and are invariably found complexed with nucleic acids. There are several physiologically active aromatic amines. Mescaline, the active principle of the peyole cactus, *Lophophora williamsii*, is an aromatic amine that is a potent hallucinogen. Amino acids and non-protein amino acids are found in plants abundantly (Bhojwani, 2001).

**Alkaloids:** The alkaloids include those natural products that contain nitrogen, usually as part of a cyclic system. Compounds of this type are numerous among plants and are known to have potent pharmacological properties. Example: caffeine, quinine, nicotine, cocaine, morphine and strychnine. Biosynthetically, they may be derived from amino acids, terpenes or aromatics. Because of this diversity, they are often derived from the plant source rather than being produced synthetically. They are grouped based on their ring systems, which include pyridine, tropane, isoquinoline, nicotine, morphine etc. The alkaloids, because of their amine functionality, are basic in nature. The free amines are relatively polar lipophilic substances, whereas treatment with acid forms water-soluble salts (Daniel, 1990).

**Nucleosides, nucleotides and Nucleic acids:** Apart from the normal bases, several unusual bases with closely related structures are also found in plants. 5-methylcytosine is found in the DNA of wheat germ. The pyrimidine glycosides, vicine and convicine have been found in certain legume seeds. The methylated purines, theobromine and caffeine also known for their stimulant effects, occur regularly in plants (Daniel, 1990).

### 2.2.6 Secondary Metabolites and Bioreactor

Facilities for the large-scale production of secondary metabolites from plant cells have been developed, but further research is needed to improve their economic efficiency. One of the production methods includes the use of fermentation vats similar to those used for the commercial culturing of microorganisms but with gentler mixing procedures to avoid disruption of plant cells. This method offers the advantage that batch cultures can be used to harvest nonexcreted metabolites (Balandrin, 1985).
Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Bioreactors have several advantages for mass cultivation of plant cells (Mulabagal, 2004):

i. It gives better control for scale up of cell suspension cultures under defined parameters for the production of bioactive compounds.

ii. Constant regulation of conditions at various stages of bioreactor operation is possible.

iii. Handling of culture such as inoculation or harvest is easy and saves time.

iv. Nutrient uptake is enhanced by submerged culture conditions, which stimulate multiplication rate and higher yield of bioactive compounds, and large numbers of plantlets are easily produced and can be scaled up.

Since the biosynthetic efficiency of populations varies, a high yielding variety should be selected as a starting material. The fundamental requirement in all this is a good yield of the compound, and reduced cost compared to the natural synthesis by the plants (Tripathi, 2003). In order to cultivate plant cells on a large-scale, fermentors with different sizes are useful. Many researchers have designed various types of fermentors since the end of 1950's. The simplest vessel is a carboy system described by Tulecke and Nickell in 1959 that consists of a rubber-stoppered 20 L carboy fitted with four tubes (air-in, air-out, medium-in and sample-out). Filtered compressed air is employed for oxygen supply, aeration and agitation of the medium. Lamport used a roller-bottled system using a round flask in 1964. Veliky and Martin proposed a V-shape fermentor. It is an inverted flask carrying two teflon-coated stirring bars on a glass pin situated at the bottom of the flask. A drain/sample port is also located at the bottom. The top of the flask is fitted with four standard taper penetrations (Misawa, 1998).

However, the most common types of system on the bench are a stirred-jar fermentor, which is used for microbial cultivation although some minor alteration is made. For example Martin increased the size of each impeller blade to 1 inch with a commercially available 7.5 L New Brunswick Microferm Fermentor. Some factors such as cell-type and the run time are also important, when using a bioreactor. The sensitivity to shear is due both to the large size of the cells and to the relatively inflexible cellulose cell wall. Thus, with normal blade impellers the
cells may twist which will inhibit mitosis and, for this reason, airlift fermentors are recommended by some researchers. The large size of the plant cell contributes to its comparatively high doubling time (12 h - several days), which thus prolongs the time required for a successful fermentation run (Ibid).

The bioreactor system has been applied for embryogenic and organogenic cultures of several plant species. Significant amounts of sanguinarine were produced in cell suspension cultures of *Papaver somniferum* using bioreactors. Ginseng root tissue cultures in a 20 ton bioreactor produced 500 mg/L/day; of the saponin that is considered as a very good yield. Jeong have established the mass production of transformed *Panax ginseng* hairy roots in bioreactor. Hahn has observed the production of ginsenoside from adventitious root cultures of *Panax ginseng* through large-scale bioreactor system (1-10 ton) (Tripathi, 2003).

Bioreactors offer optimal conditions for large-scale plant production for commercial manufacture. Much progress has been achieved in the recent past on optimization of these systems for the production and extraction of valuable medicinal plant ingredients such as ginsenosides and shikonin. Roots cultivated in bioreactors have been found to release medicinally active compounds, including the anticancer drug isolated from various *Taxus* species, into the liquid media of the bioreactor which may then be continuously extracted for pharmaceutical preparations. Conventional practices require the harvest of the bark of trees, all approximately 100 years old, to obtain 1 kg of the active compound taxol. Research over the last two decades has established efficient protocols for isolated cell cultures and a large-scale bioreactor system. The acceptance of this process for the industrial production of this invaluable compound has recently been established and will significantly impact the production of the tumor-inhibiting pharmaceutical (Ibid).

The most detailed assessment available in the literature for the production of a specific product in plant cell cultures is concerned with the production of ajmalicine from *Catharanthus roseus* cell cultures. In this case, a 20% market penetration was assumed, ie. 800 kg/yr and production was based on the use of a two-stage batch culture process. Stage two parameters include specific productivity
of 0.26 mg/g/day (based on final dry weight), final product concentration of 0.06% dry weight and a maximum fresh weight concentration of 160 g/L. It was estimated that the cost of production would be $3,215/kg ajmalicine ($7.30/lb dry biomass) and this compares with $619/kg ($0.70/lb dry biomass) from the intact plant. Clearly, this 5-fold increase in costs is unacceptable from a commercial point of view. This example serves to illustrate the need to choose products that are particularly expensive to produce in the field. Shikonin is a good example, in that the long growing period (3-5 years) and strict climatic requirements mean that the cost of the plant raw material is high $6.80/lb. It has been suggested that a quick way to assess the attractiveness of a cell culture method vs. conventional agriculture, is to calculate a specific biosynthesis rate "based on the final dry weight and the total time of fermentation or land occupation". With shikonin there is an 830-fold increase in plant cell culture, whilst with ajmalicine from *C. roseus* there was only a 24-fold improvement (Misawa, 1994).

Cost analysis indicate that by means of current technology, production of a secondary metabolite in plant cell culture is economical for cultures producing more than 1 gram of compound per liter of cell culture for compounds with a value exceeding $500 to $1000 per kilogram. The total market for the compound must be sufficiently large to warrant the capital expenditures needed to develop a tissue culture system. In view of such economic constraints, candidates for commercial production by plant tissue culture are limited to a few types of high-value, plantspecific compounds. These include diosgenin-derived steroid hormone precursors, *Digitalis* glycosides, opium alkaloids (codeine and morphine), the *Catharanthus* alkaloids, and, possibly, complex mixtures such as essential oils (for example, rose oil). Five to twenty times more *Catharanthus* alkaloids (potential anticancer agents) were obtained by tissue culture methods than from whole plants. Accumulation of *Catharanthus* alkaloids at about 75 percent of the accumulation from small-scale cultures (0.07 liter) has been achieved from cells grown in a 750-liter production fermentor (Balandrin, 1985).

A German pharmaceutical company is going ahead with plans to produce *Digitalis* glycosides in tissue culture systems within the next few years. Mitsui Petrochemical Industries is already marketing tissue culture-derived shikonin, a
red-colored naphthoquinone compound used as a dye and an astringent. Valuable pharmaceutical intermediates and drugs could in the future be produced in 1000-liter (and larger) vats of plant cells, a situation analogous to the current industrial production of antibiotics and other chemicals by continuous fermentation of microbial cultures (Balandrin, 1985).

Scrugg has calculated the effect of run time on productivity. Assuming a product yield of 1% dry weight, biomass yield of 20 g dry weight/L. and a bioreactor volume of 1,000 liters a 300 day year was estimated, since time would be required for maintenance, contaminated runs, etc. Similarly, as the yield of product increases the reactor volume required decreases exponentially. Under the conditions described above, a 100,000 L fermentor would be necessary to produce 300 kg/yr (Misawa, 1994).

For commercialization, it is necessary to progress through several stages increasing the volume at each stage until the requisite bioreactor size is attained. In theory, it is anticipated that such large-scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation. Generally speaking, the culture period in plant cell cultures is longer than that in microbial cultures, and it is crucial to protect against microbial contamination (Ibid).


Classification
Kingdom: Plantae
Division: Angiospermae
Class: Dicotyledoneae
Order: Contortae
Family: Asclepiadeaceae
Genus: Gymnema
Species: sylvestre R.Br. (Source: Plants Database, USDA)
Botanical synonyms

_Asclepias germinata_ Roxb., _Periploca sylvestris_ Retz.

Vernacular names

English: Periploca of the wood; Hindi: Gudmar; Sanskrit: Mesasringi; Kannada: Kadhasinge; Malayalam: Chakkarakkolli, Madhunasini; Tamil: Sirukurunkay, Sakkaraikolli; Telugu: Podapatra (Oudhia, 2002).

3.1 Botanical description

It is a large woody climber. Its leaves are elliptic, acuminate, base acute to acuminate, glabrous above sparsely or densely tomentose beneath; flowers are small, inaxillary and lateral umbel like cymes, pedicels long; calyx has long lobes, ovate, obtuse, pubescent; Corolla is pale yellow campanulate, valvate, corona single, with five fleshy scales. The scales are adnate to throat of corolla tube between lobes; anther is connectively produced into a membranous tip, with 2 erect pollinia, 2 carpels, unilocular with locules having many ovules; Follicles are long and fusiform (Gudmar, National Ministry of Plants Board, India). The flowering and fruiting season is from September to December (Pullaiah, 2002).

3.2 Geographical distribution

It is found throughout India, especially Central and Southern India. It is also distributed in Asia, Tropical Africa, Malaysia and Srilanka (Minas, 2002; Gudmar, National Ministry of Plants Board, India).

3.3 Cultivation of Gymnema

_Gymnema_ can be propagated by stem cuttings and by seeds. It is under cultivation as a medicinal crop in fairly large areas, in different parts of India. Standard cultivation practices for the commercial cultivation have not yet been developed (Oudhia, 2002). The plant grows in a variety of soil and agro-climatic conditions in tropical and sub-tropical regions up to 600 m. Mature seeds are collected between October-December and sown in polyboxes/bags or small plots as nursery. The seedlings raised are transplanted in field during February-March. The plant grows well with the onset of rainy season. The climber is given proper support for its better growth and development. The plant can also be propagated through cuttings
and planted during rainy season. After one-year leaves are ready for harvesting. Since leaves and roots are the main parts that are used, the leaves are usually collected during October-February, cleaned and dried in shade. The roots are collected during summer and are cleaned, washed and cut into pieces and dried (Gudmar, National Ministry of Plants Board, India). The leaves have been widely used in herbal medicine preparations. It has been used for thousands of years within the Indian Ayurvedic tradition for adult-onset diabetes (Herb information, www.holistic-online.com).

3.4 Traditional uses
Susruta has described Gymnema sylvestre R.Br., as a destroyer of madhumeha (glycosuria) and of the urinary disorders. The plant is also reported to be bitter, astringent, acrid, thermogenic, anti-inflammatory, anodyne, digestive, liver tonic emetic, diuretic, stomachic, stimulant, anthelmintic, laxative, cardiotonic, expectorant, antipyretic and uterine tonic. It is useful in dyspepsia, constipation, jaundice, haemorrhoids, renal and vesical calculi, cardiopathy, asthma, bronchitis, amenorrhoea, conjunctivitis and leucoderma (Oudhia, 2002, Minas, 2002).

3.4.1 Clinical Applications
i) Antidiabetic effect:
   a. Type 1 (Insulin-dependent) Diabetes Mellitus: Prolonged oral Gymnema therapy has been demonstrated to reduce insulin requirements, improve blood glucose homeostasis by reducing HbA1c and glycosylated serum proteins, improves diabetes-related hyperlipidemia. It has also been observed to reduce serum amylase activity. Use of Gymnema leaves has also increased serum C peptide levels, indicative of an increased β-cell activity.
   b. Type 2 (Non-insulin) Diabetes Mellitus: In Type 2 diabetes mellitus also, Gymnema has been observed to reduce blood glucose, glycosylated hemoglobin and plasma proteins. It has been used as a supplement along with the dosage of conventional oral hypoglycemics. In this case as well the β-cells of the pancreas function normally (Tominaga, 1995).
ii) Obesity: Its ability to anesthesize the taste of sweetness for several hours has been a principle behind its use in dieting and weight control (Maeda, 1989).

iii) Anti-inflammatory activity: A significant anti-inflammatory activity has been observed on using aqueous extract of Gymnema leaves (Oudhia, 2002).

3.4.2 Effects of Gymnema

i) On insulin: Several in vitro studies have revealed the plant to be insulinotropic in nature, by inducing an increase in release of insulin from the β-cells. The mechanism seems to involve an increase in cell membrane permeability (Persaud, 1991). Gymnema extracts have been observed to increase insulin secretion in streptozotocin induced diabetic rats as well as alloxan induced diabetic rats. Both streptozotocin and alloxan induce diabetes by damaging β-cells of the pancreatic islet tissues. Histological studies have revealed the repair or regeneration of damaged β-cells by prolonged treatment with gymnema extracts (Shanmugasundaram, 1990).

ii) On hyperglycemia: On injection of anterior pituitary extracts into rat, the blood glucose levels get elevated due to increased gluconeogenesis and the inhibition of peripheral glucose utilization. It was observed that administration of gymnema orally to these animals significantly reduced blood sugar, with little effect on blood sugar in normal controls. It is also observed that gymnema could attenuate hyperglycemia in normal and mildly diabetic rats when given before the oral administration of glucose. The gymnema extracts ameliorated moderate hyperglycemia in rats chemically induced to be moderately diabetic. Moreover, it also significantly increased longevity in moderate or severely diabetic rats. In glucose fed hypoglycemic rats, on administration of exogenous insulin and gymnema extract, there was a significant reduction in the glycogen content of the tissues. Based on several studies with chemically induced diabetic rabbits, gymnema has shown to enhance the activity of several enzymes involved in the utilization of glucose.
along insulin dependent pathways (Chattopadhyay, 1993). The following results have been observed with *gymnema* treatment: a) Decreased fasting glucose levels, b) Increased glycogen storage in the liver and muscle tissue, c) Decreased lipid deposition in tissues, d) Increased protein content in tissues typically laden with lipids, and e) Increased glyceraldehydes-3-phosphate dehydrogenase activity, glycogen synthesis and glucose-6-phosphate dehydrogenase activity (Chattopadhyay, 1998).

The leaf extract and gymnemic acids have been observed to interact with receptors that inhibit the release of gastric inhibitory polypeptide (GIP) in rats. GIP is a hormone released by the duodenal mucosa to control the dumping of food from the stomach into the small intestine and to stimulate an anticipatory release of insulin into the blood stream. The release of GIP is stimulated by glucose reaching duodenal receptors that can be inhibited by *gymnema*.

iii) On hypoglycemia: In humans and animals Beryllium compounds’ toxicity induces decrease in blood glucose and damage to hepatocyte. Gymnema has demonstrated a modulating effect on Beryllium caused hypoglycemia in rat (Prakash, 1986).

iv) On taste sense for sweetness: The gurmarin constituent in gymnema is known to selectively suppress the sucrose responses of the chorda tympani nerve in rats and mice. In addition, saliva contains gurmarin-binding proteins. Reduced preference for sucrose in animals on gymnemic acid regimes is probably caused by gurmarin. Extracts applied to taste buds on the tongue effectively neutralize the gustatory stimuli to the brain for the taste quality of sweetness. The anti sweet principles of *Gymnema* include Gymnemic acids and *Gymnema* saponins apart from Gurmarin. *Gymnema* saponins completely inhibited the perceptions of sweetness induced by 0.1M sucrose solutions, which is about half the activity exhibited by the gymnemic acids. It is observed that this structure of saponins affects the magnitude of the sweetness inhibition. Acylation of the saponin in the gymnemic acids produces greater inhibition. The reduced sensitivity to sweet substances
produced by *gymnema* might result from the competition of the receptor sites between glycosides and the sweet substances. An electrophysiological study on taste responses in rats suggests that gurmarin acts on the apical side of the taste cell, possibly by binding to the sweet taste receptor protein. In humans and chimpanzees, gymnemic acids suppress the sweet taste of all sweetness but had no effect on taste receptors sensitive to bitter or salty substances in chimpanzees. In experimental models the degree of suppression produced by gymnemic acid (measured by nerve response) varied from complete abolishment (aspartame, saccharin) to about 50% reduction (xylitol) (Maeda, 1995).

v) On cholesterol: The saponins of Gymnema have the ability to increase fecal excretion of cholesterol and other neutral steroids and bile acids in rats in a dose-dependent manner. Three kinds of extracts from *Gymnema sylvestre* leaves, extract (GSE) acid precipitate (GSA) and column fractionate (GSF), of which the gymnemagenin, an aglycone of gymnemic acids, whose concentrations of 58.87, 161.6 and 363.3 mg/g respectively were used for the experiments. These were administered to rats orally at the dose of 0.05-1.0 g/kg for 22 days. Rats were given free access to water and nonpurified diet without cholesterol, and the differences in fecal excretion of steroids and gymnemic acids were investigated. The results of the experiment demonstrated that a high dose of gymnemic acids increased fecal cholesterol and cholic acid-derived bile acid excretion (Nakamura, 1990).

vi) On lipid absorption: Gymnemic acid strongly inhibits the absorption of oleic acid from the intestines of rats in a dose-dependent, reversible manner, which is similar to *Gymnema*’s effect on glucose absorption.

vii) Antimicrobial activity: The ethanolic extract of *Gymnema sylvestre* leaves demonstrated antimicrobial activity against *Bacillus pumilis*, *B. subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and inactivity against *Proteus vulgaris* and *Escherichia coli* (Satdive, 2003).
3.5 Clinical Trials
In a clinical trial conducted on Type 1 (IDDM) diabetics undergoing insulin therapy, the subjects were given GS4, a water-soluble extract of the leaves of *Gymnema sylvestre* at a dose of 400 mg/day. The treatment brought down insulin requirements, the fasting blood glucose and HBA1c and glycosylated plasma protein levels. GS4 therapy also brought down serum lipids. GS4 therapy appeared to enhance endogenous insulin, possibly by regeneration/revitalization of the residual beta cells. GS4 supplementation to Type 2 (NIDDM) patients on conventional oral hypoglycaemic agents for 18-20 months, showed a significant reduction in blood glucose, glycosylated haemoglobin and glycosylated plasma protein along with reduction in dosage of the conventional drugs. A few of the diabetic patients were also able to discontinue their conventional drug and maintain their blood glucose homeostasis with GS4 alone. Thus, in NIDDM patients as well beta-cell regeneration was suggested supported by the appearance of raised insulin levels in the serum of patients. There were trials in which the subjects were administered with leaf powder 10g/day for 7 days. The results indicated that the powder has a hypoglycaemic effect comparable to tolbutamide. When compared to normal subjects in the diabetic patients, serum triacylglycerol, free fatty acids and cholesterol levels were significantly decreased. Ascorbic acid and iron levels were elevated significantly in both groups, whereas excretion of creatine decreased in diabetic patients (Balasubramanian, 1992).

3.6 Toxicity studies
The LD50 of ethanol and water extract of *Gymnema* administered intraperitoneally in mice was found to be 375mg/kg (Bhakuni, 1971).

3.7 Phytochemistry
The leaves of *Gymnema* contain pentriacontane, hentriacontane, phytin, a and b chlorophylls, resin, tartaric acid, formic acid, butyric acid, mucilage, inositol and antheraquinone and alkaloids (Oudhia, 2002). A study indicated that *Gymnema* also contained saponins, flavonoid compounds including kaempferol and quercetin, stigmasterol, and amino acid derivatives betaine, choline, and trimethylamine (Shane-McWhorter, 2001; Sahelian).
The major constituents of *Gymnema sylvestris* are a group of oleanane type triterpenoid saponins known as "gymnemic acids." The latter contains several acylated (tigloyl, methylbutyroyl etc) derivatives of deacetylgymnemic acid (DAGA), which is 3-O-β-glucuronide of gymnemagenin (3β, 16β, 21β, 22α, 23, 28-hexahydroxy-olean-12-ene) (Ye, 2000). The individual gymnemic acids (saponins) include gymnemic acids I-VII, gymnemosides A-F, *gymnema* saponins etc. (Kuzuko, 1989; Hong-Min, 1992, Masayuki, 1997). These gymnemic acids have the capacity to obtund taste for several hours for sweet, but not bitter, sour, astringent, nor pungent substances (Dateo, 1973).

### 3.7.1 Marker constituents

<table>
<thead>
<tr>
<th>Gymnemic acids</th>
<th>R1</th>
<th>R2</th>
<th>M.F.</th>
<th>M.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnemic acid I: tga</td>
<td>Ac</td>
<td>C</td>
<td>C_{44}H_{66}O_{15}</td>
<td>834</td>
</tr>
<tr>
<td>Gymnemic acid II: mba</td>
<td>Ac</td>
<td>C</td>
<td>C_{46}H_{68}O_{15}</td>
<td>836</td>
</tr>
<tr>
<td>Gymnemic acid III: mba</td>
<td>H</td>
<td>C</td>
<td>C_{41}H_{66}O_{14}</td>
<td>782</td>
</tr>
<tr>
<td>Gymnemic acid IV: tga</td>
<td>H</td>
<td>C</td>
<td>C_{41}H_{64}O_{14}</td>
<td>780</td>
</tr>
</tbody>
</table>

The potential importance of the antisweet effect of *Gymnema sylvestre* leaves was appreciated as early as 1848. Gymnemic acid (Fig.3), the anti-saccharine principle of the leaves of *Gymnema sylvestre* is a mixture of triterpene saponins. The isolation and heterogeneity of gymnemic acid A₁ has been studied. It has been found that the major constituents are β-D-glucuronides of differently acylated Gymnemagenins. The structure 3-β, 16-β, 21-β, 22-α, 23, 28 hexahydroxy-olean-12-ene has been assigned to Gymnemagenin (Fig.3). Gymnemic acid A₁ has been described as the major active component. An aqueous ethanol extract of dried leaves was subjected to solvent extraction and gymnemate salt fraction was isolated. Gymnemic acid A₁ rich fraction was obtained by chromatography. Preparative thin layer chromatography also gave gymnemic acid A₁. This was further consolidated by the fact that the sample obtained from preparative thin layer chromatography was identical in chromatographic behaviour with the samples obtained from other laboratories. The fractions A₁₁ and A₁₂ which were obtained by resolving A₁ by thin layer chromatography were found to be active and were distinguished on the basis of their NMR and UV spectral properties (Dateo, 1973).
The structure of gymnemagenin (3-β, 16-β, 21-β, 22-α, 23, 28 hexahydroxy-olean-12-ene), the sapogenin of the antisweet principles of *Gymnema sylvestre*, was established by X-ray analysis of the 3β, 23;21β, 22 α-di-O-isopropylidene derivative. On the basis of this result, the structure of deacylgymnemic acid (Fig.3) was elucidated as the 3-O-β-glucuronide from the carbon-13 nuclear magnetic resonance spectra. Five antisweet principles, gymnemic acid-III, -IV, -V, -VIII, and -IX, were isolated in pure states from the hot water extract of leaves of *Gymnema sylvestre*. Of these, three (GA-III, -IV, and -V) were known, while two (GA-VIII and -IX) were new compounds. The structures of GA (gymnemic acid)-VIII and -IX were elucidated as 3'-O-β-D-arabino-2-hexulopyranosyl gymnemic acid-III and -IV, respectively (Liu, 1992).

Further investigation of hypoglycemic activity of major saponin constituents from "gymnemic acid", a crude saponin fraction of *G. sylvestre*, exposed not only two new saponins, gymnemosides a (1) and b (2), but also gymnemoside b and gymnemic acid V as active principles. Furthermore, an acetyl group linked 16- or 22-hydroxy group in 1 and 2 was found to migrate easily to primary 28-hydroxyl group, while acyl migration from 28-hydroxy group in 3 was little observed (Murakami, 1996). Following the characterization of gymnemosides-a and -b, new triterpene glycosides, gymnemosides-c, -d, -e, and -f, were isolated from the leaves of *Gymnema* (G.) *sylvestre* R. Br and their structures elucidated.

The inhibitory effects of gymnemosides-c, -d, -e, and -f and principal triterpene glycosides from *G. sylvestre* on glucose uptake in rat small intestinal fragments...
were examined, and gymnemic acids II, III, and IV, gymnemasaponin V, and gymnemoside-f were found to exhibit the inhibitory activity (Yoshikawa, 1997).

**Figure 4 Structure of Deacylgymnemic acid**

![Image](molecular_structure.png)


Phytochemical analysis of crude extract of *Gymnema* has also been carried out by some companies. For example, Natural Remedies Pvt. Ltd. found that the major bioactive constituents of *G. sylvestre* are a group of oleanane type triterpenoid saponins namely 'gymnemic acids' 1 and 2 as previously mentioned. They have also elucidated their structure and the individual gymnemic acids, which are saponins, include gymnemic acids I-VII, gymnemosides A-F, gymnemasaponins1-3 etc (Fig.3). Also, the crude extract was found to be odourless and extremely bitter in taste. Identification of the crude extract of leaves was done using thin layer chromatography. The sample was prepared using 50% ethanol and KOH. Vanillin sulphuric acid was used as a spraying reagent. 75% w/w of total Gymnemic acid was obtained using gravimetry and 25% using HPLC (*Ibid*).

### 3.8 Dosage and administration

The dosage used in Ayurvedic medicine ranges from 6 to 60g of dry or powdered leaf per day as an infusion. The adult dosage for *Gymnema* 1:1 liquid extract is 25 to 75 ml per week. Some cases of diabetes show a good response quickly but the results are optimum on continuous use for 6 to 12 month. It is also prescribed in tablet form, in which 8-12g per day of leaf equivalent is recommended, which is variable depending on the intensity of the disease. For sweet craving and sweet taste depression 1-2ml per day of liquid extract is all that is necessary.
Gymnema is also known to combine well with fenugreek, goat’s rue or neem leaf for diabetes; with globe artichoke or blue flag for weight loss; with turmeric, hawthorn, silybum, globe artichoke or garlic for hypercholesterolaemia. As with all saponin-containing herbs, oral use may cause irritation of the gastric mucous membranes and reflux, which is why enteric-coated tablets are advised. Extracts, orally to rats at 0.05-1.0g/kg for 22 days, increased fecal excretion of neutral steroids and bile acids dose-dependently, correlated with fecal gymnemagenin, and significantly reduced food intake and body weight (Nakamura, 1999).

3.9 Gymnema: Active Ingredients of Drugs
The extract of Gymnema sylvestre is used as one of the active ingredients in several diabetic, cholesterol lowering and weight reducing drugs. The following table 4 denotes some of the drugs based on Gymnema sylvestre and their use.

Table 4 Some drugs having extracts of Gymnema sylvestre as one of the active ingredients.

<table>
<thead>
<tr>
<th>Diabetes Control</th>
<th>Cholesterol-lowering drugs*</th>
<th>Weight Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actos</td>
<td>Crestor</td>
<td>AyurSlim (Himalaya drugs)</td>
</tr>
<tr>
<td>Amaryl</td>
<td>lovastatin (Mevacor)</td>
<td></td>
</tr>
<tr>
<td>Avandia</td>
<td>Lipitor</td>
<td></td>
</tr>
<tr>
<td>Diabecon (GlucoCare) (Himalaya drugs)</td>
<td>pravastatin (Pravachol)</td>
<td></td>
</tr>
<tr>
<td>glypizide (Glucotrol XL)</td>
<td>Zocor</td>
<td></td>
</tr>
<tr>
<td>glyburide (Glynase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meshashringi (Himalaya drugs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metformin (Glucophage)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prandin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Gymnema sylvestre when taken along with these drugs increases cholesterol-lowering effect of these drugs (Drug Digest, www.drugdigest.org; Himalaya’s Herbs and Minerals, www.himalayahealthcare.com).

4. Catharanthus roseus Linn

Taxonomy

Alternative Botanical Names: Vinca rosea, Lochnera rosea, Ammocallis rosea.

Common Names: Periwinkle, Madagascar periwinkle, Cape periwinkle, Old Maid, Sadabahar
Classification: *Catharanthus roseus* (L.) G.Don
Kingdom: Plantae-Plants
Subkingdom: Trachobionta-Vascular plants
Super division: Spermatophyta-Seed plants
Division: Magnoliophyta-Flowering plants
Class: Magnoliopsida-Dicotyledons
Subclass: Asteridae
Order: Gentianales
Family: Apocynaceae-Dogbane Family
Genus: Catharanthus G.Don-Periwinkle.
Species: *Catharanthus roseus* (L.) G.Don-Madagascar Periwinkle (National Plant Database-2003/Wealth of India)

4.1 Description
*Catharanthus roseus* is a fleshy perennial growing to 32 in (80cm) high. It has glossy, dark green, oval leaves (1-2 inches long) and flowers all summer long. The blooms of the natural wild plants are a pale pink with a purple "eye" in their centers. The flowers are usually white. The long seedpods are cylindrical and have a downy texture. The whole plant is used for therapeutic purpose.

4.2 Geographic distribution
*Catharanthus roseus* is native to the Indian Ocean island of Madagascar. This herb is now common in many tropical and subtropical regions worldwide, including the southern United States (O'Reilly, 2003).

4.3 Pharmacological studies
An aqueous decoction of the leaves and/or the whole plant is used as household remedy for diabetes in several countries (Grover, 2002). It has been reported that in India, seven flowers/leaves are used in the decoction while in the Cook Islands, 18 leaves are boiled to make a decoction (Sastry, 1953). There are many reports indicating that the leaves of *C. roseus* have significant blood glucose lowering activity. The glycaemic effects of the leaves of *C. roseus* have been studied in normal alloxan-induced and streptozotocin diabetic animal models (Swanston-Flatt, 1989).
The leaf juice of *Catharanthus roseus* was tested for their hypoglycemic action individually and in combination with the seed powder of fenugreek on normal and alloxan-induced diabetic rats. Different doses at 0.5, 0.75 and 1.0 ml/kg and 50, 100 and 150 mg/kg, respectively were used at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h after being fasted for 18 h, and it was found that the effect was dose-dependent but the combination treatment produced a synergistic action (Satyanarayana, 2003). The juice of fresh leaves of *C. roseus* Linn. also reduces blood glucose in normal and alloxan diabetic rabbits and the reduction was dose-dependent (Narmanu, 2003). The results were indicative of a prolonged action in reduction of blood glucose by *C. roseus* and the mode of action of the active compound(s) is probably mediated through enhanced secretion of insulin from the \( \beta \)-cells of Langerhans or through extrapancreatic mechanism.

*C. roseus* is one amongst few plants that have generated a lot of interest among the scientific and medical communities not only for its reported anti-diabetic properties but also for its two anticancer alkaloids-vincristine and vinblastine. Vincristine has proved most effective in treating childhood leukemia, while vinblastin has been effective in treating testicular cancer and Hodgkin’s disease (cancer of the lymphatic system). It was in the 1950s in Jamaica, where periwinkle tea was brewed for treating diabetes. The effects of four *Vinca* alkaloids (Vinblastine, Vincristine, vindesine and vinorelbine) on three neoplastic cello lines (the MXT mouse mammary- cell line and the T24 and J82 bladder cell lines) were studied at three biological levels, i.e., cell proliferation, cell cycle kinetics and morphonuclear characteristics. The results showed that these alkaloids inhibited the cell proliferation of neoplastic cell lines at a concentration of \(10^{-8}\text{M}\), except in the case of the J82 cell line, for which only a showing down of cell proliferation was observed. The decrease in cell proliferation was attributed to alkaloids being able to accumulate cells in the mitotic phase (Pauwels, 2000).

There are over 70 alkaloids that have been isolated from the plant in addition to vinblastine and vincristine. Synthetic vincristine, used to treat leukemia, is only 20% as effective as the natural product derived from *C. roseus*. Further research is needed especially on bioactive compounds, means of preparation, and effectiveness of plants & herbal remedies (Duke, 1985).
Chapter II  Literature Review

The yields of ajmalicine and serpentine produced by *C. roseus* were also increased by addition of XAD-7, a resin, and the ratio between both alkaloids produced was changed. Also, Murashige-Skoog’s (MS) formulation was the most suitable for the production of serpentine by *C. roseus* suspension (Pauwels, 2000).


Taxonomy

*Synonym:* *Ervatamia coronaria, Nerium divaricatum* L. *T. alternifolic* L.

*Classification:*
- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Gentianales
- Family: Apocynaceae
- Germs: Tabernaemontana
- Species: divaricata

*Vernacular names:* Hindi: Tagar; Uchu-sanango

5.1 Botanical description

It is an evergreen shrub that grows to a height of 1.5m with a spread of 1m. The stem is erect and branching; the leaves are opposite, thick, smooth, 10-15cm long and ovate-acuminative; the flowers are white and 5-lobed. ‘Flore Pleno’ is the variety with double flowers. It is a native of India, China and Thailand and prefers well-composted, well-drained, sandy soils in a protected sunny or partly shady position, and is drought and frost tender. Roots are prone to rot in poorly draining soil.

Propagation is by fresh seed or by cuttings. Seed should be planted within a few months of harvest as this rainforest species has a fairly short viability. Germination is low even under ideal conditions, but is greatly assisted by natural or chemical fungicides. They should be sown into sand or sandy soil, kept warm and moist, but not wet.
5.2 Traditional uses

Bark scrapings are used to treat pain after childbirth. Decoctions of the bark are used to alleviate stomach and rheumatic pains, as well as diarrhoea.

Tagar is widely used in India and Sri Lanka. It has been used as an aphrodisiac and is known to increase fertility in women. The latex of the plant has a cooling effect and is applied to wounds to prevent inflammation. Latex mixed with oil is applied to the head to relieve headaches, eye pressure and corneal inflammation. The juice from the flower is used in the treatment of opthalmia. The root when chewed is known to relieve toothache. A decoction with oil on application to the head has been observed to relieve all indispositions, pains of the head. A water decoction of the root is an efficient wormicide and is used to treat respiratory problems such as asthma. In Malaysia, the leaves are pounded with sugar candy and water to prepare syrup for curing coughs. Yunani practitioners have used the flowers as analgesic. In Indonesia, the leaves, bark and twigs may form the main components of an arrow poison used on the Mentawei Islands. The root rubbed up with water and on drinking kills intestinal worms. An infusion of root is believed to have febrifugal properties, while an infusion of both bark and root is used against dysentery. In clarified butter and boiled in water together with other ingredients, it cures coughs, asthma, catarrh, fevers, mania, ulceration, morbid secretion of urine, leprosy, hiccough, vomiting, and swellings.

5.3 Pharmacology

*Tabernaemontana divaricata* contains several alkaloids of the complex indole type, many closely related to ibogaine and voacangine. The ethanol extract of the leaves (double flower variety) comprised a total of 23 alkaloids, including aspidosperma alkaloids, taberhamine, voafinine, N-methyl voafinine, voafmidine, voalenine and the new bisindole alkaloid, conophyllinine in addition to the existing bisindole alkaloid, conophylline and its congener, conofilene (Kam, 2003).

Six new alkaloids, viz, (3S)-3-cyanocoronaridine (2), (3S)-3-cyomoisovoacangine (3), conolobine A(5), conolobine B(6), conolidine(7), and (3R/3S)-3-ethoxyvocangine were obtained from the stem-bark extract of the Malayan *Tabernaemontana divaricata*. The structures were determined by NMR and MS.
analysis. The CN-substituted alkaloids showed appreciable cytotoxicity towards the KB human oral epidermoid carcinoma cell-line (Kam, 2004). Ethanolic extracts of roots, stems, leaves and flowers of *T. divaricata* and *T. pandacaqui* were studied by an observational (Hippocratic) screening method in rat models and were observed to cause sedation, decreased respiration and decreased skeletal muscle tone. The leaf extract of *T. divaricata* also caused vasodilation of ear vasculature. Lethal doses of the extracts were observed to be fatal as they caused respiratory paralysis. Intensity of pharmacological activities was greater with the root and stem extracts. The effects of *T. pandacaqui* were observed to be more potent than *T. divaricata* (Taesotikul, 1989). Crude extracts had anticancer activity. Alkaloids from the seeds, roots and pod depressed bone-marrow activity in rats, resulting in temporary leukopenia. Coronaridine has observed to show autonomic and CNS activity. In mice, it produced analgesia and was effective in suppressing rage caused by foot-shock. Ibogaine has a transient hypotensive effect. In mice, it has weak but definite anticonvulsant properties. It acts as a true hallucinogenic agent and can be used as a stimulant to overcome fatigue and sleepiness. When administered intravenous to anesthetized guinea pigs, the alkaloids produced bradycardia that was resistant to vagotomy and atropine sulfate (4mg/kg i.m.). Blood pressure was lowered, but there was no screening, voacangine exhibited a slight central stimulating effect. The LD$_{50}$ i.v. in the mouse was 54mg/kg. Voacangine had no effect on the heart. A new alkaloid, ervatine, was isolated from the fruit of *Tabernaemontana heyneana*. The ethanol extract containing the alkaloids heyneamine and voacistine prevented pregnancy in experimental rats, although, they were found to possess significant uterotrophic activity (Srivastava, 2001).

**5.4 Tissue Culture Studies**

*Tabernaemontana* species has been explored for its various alkaloids through tissue culture. *In vitro* multiplication of *Tabernaemontana fuchsiaefolia* has been successfully done using apical and hypocotyl explants in MS medium containing different concentrations of the cytokinins benzylaminopurine and kinetin, supplemented with phloroglucinol (Oliviera, 2003). *Tabernaemontana divaricata* cell suspension cultures when fed with early precursors, such as tryptamine and loganin, 57% of the precursors was converted into indole alkaloids such as
strictosidine, vallesamine, O-acetylvallesamine and voaphylline (Lucumi, 1991). The phenolics coniferyl alcohol and sinapyl alcohol and the sterols campestral and stigmasterol have been identified by Capillary Gas Chromatography in cell suspension cultures of *Tabernaemontana divaricata* (Dagmino, 1991).

### 6. *Ocimum Basilicum* var *Thai Queen*

**Taxonomy**

Classification:
- Family: Lamiaceae
- Genus: Ocimum
- Species: Basilicum
- Variety: Thai Queen

**Common Names:** English: Basil; Hindi: Tulsi

(Dr. Duke’s Phytochemical and Ethnobotanical Databases. http://www.ars-grin.gov/duke/)

**6.1 Plant Description**

The genus, *Ocimum*, includes over sixty species of annuals, non-woody perennials and shrubs native to Africa and other tropical and sub-tropical regions of the Old and New World. Basils are characterized by square, branching stems, opposite leaves, brown or black seeds (also called mutles) and flower spikes, but flower colour and the size, shape, and texture of the leaves vary by species. Leaf textures range from smooth and shiny to curled and hairy, and flower are white to lavender/purple. Leaf colour can also vary, from green to blue/purple, and the plants can grow to form 1 to 10 feet in height, depending on the species. A very characteristic smell (spicy) emanates from the plant when crushed. It is in flower from August to September, and the seeds ripen in September. The scented flowers are hermaphrodites (have both male and female organs) and are pollinated by insects like bees. The plant prefers light (sandy) and medium (loamy) soils and requires well-drained soil. The plant prefers acid, neutral and alkaline soils *(Ibid)*.
6.2 Nutritive value
Sweet basil is low in calories, has almost no fat, and is a good source of Vitamin A. Five fresh leaves (2.5g) has less than 1 calorie, 96.6 I.U of Vitamin A, 3.85mg of calcium, 11.55mg of potassium plus smaller amounts of Vitamin C and other vitamins, minerals, protein and fiber. Basil seeds are high in dietary fiber and also include flavonoids and antioxidants. *O. basilicium* is on the USDA's GRAS (Generally Recognized as Safe) list at 2-680 ppm for the leaf and .01-50 ppm for the oil, but there have been suggestions of excessive doses having a carcinogenic effect. The basil essential oil is not recommended to be taken internally (*Ibid*).

6.3 History & Folklores
Basil has a long and interesting history probably originating in Asia and Africa, and there in turn it was thought to be brought by Alexander The Great. Basil’s folklore is as complex as its flavor and aromas. In terms of its legends and symbolism, it has been associated with polar opposites such as love and hate, danger and protection, and life and death. ‘*Ocimum*’ derives its name from the ancient Greek word, Okimom, meaning smell. ‘*Basilicum*’, is Latin for basilikon, which means kingly/royal in Greek. Many authors suggest that basil’s negative associations stems from the similarity of its Latin basilicum to the name of basilisk (or basilicus), the mythical serpent with the lethal gaze (*Ibid*).

6.4 Chemical Compounds
The *Ocimum* species is rich in phytochemicals. Its essential oil has linalool and methyl-chavicol as its main constituents. Eugenol is another chemical, which is found in the essential oil. The amount of each of these constituents varies from species to species. Methylchavicol provides a sweet flavour that is comparable to Anise seeds and French tarragon, linalool produces a floral scent while eugenol gives a characteristic clove like essence. Apart from these, the other constituents include caffeic acid, cineol, p-coumaric acid, p-cymene, Limonene, methylcinnamate, Myrcene, alpha-Pinene, B-pinene, Quercetin, Rutin, Safrole, alpha-Terpinene, Tryptophan (*Chemical Compounds, http://www.ansci.cornell.edu*).
6.5 Medicinal and Ethnobotanical uses

Of the various ocimum species, *O. basilicum* itself has over 50 medicinal activities. In traditional Chinese medicine, it has been used for kidney problems, gum ulcers and as a hemostyptic in childbirth, and for problems as vivid as earache, anorexia, skin diseases, rheumatoid arthritis, menstrual irregularities, and malaria in India (Medicinal Uses, http://www.anisci.cornell.edu).

Both *Ocimum basilicum*, as well as *Ocimum sanctum* is reported to be assisting in detoxifying the body, lowering blood pressure, lowering high blood sugar, lowering cholesterol, easing tension and stress. They are known to be anti-spasmodic, anti-inflammatory, and as an adaptogen (*Ibid*).

In the Ayurveda, it has been used in various decoctions for various ailments. The main uses have been in coughs, colds, fevers, headaches, lung problems, abdominal distention, absorption, arthritis, colon (air excess), memory, nasal congestion, nerve tissue strengthening, purifies the air; sinus congestion, clears the lungs, heart tonic. It is known to free ozone from sun’s rays and also oxygenate the body. It helps relieving depression by cleansing and clearing the brain and nerves. It is known to relieve the effects of poison and difficult urination. It prevents the accumulation of fat in the body (especially for women after menopause), obstinate skin diseases, arthritis, rheumatism, first stages of many cancers possibly by building up the immune system. Tulsi contains traces of mineral copper in organic form, which, in turn is needed for iron absorption. It is believed to purify air by discharging negative ions. It is believed to possess spiritual powers. For example, it somehow is able to open hearts and minds, give love, devotion, faith, compassion and clarity. It cleanses the aura and it is believed to give Divine Protection in the Hindu Vedas. It is one of the two most sacred plants in India (*Ibid*).

In India, the *Ocimum* species has been used to treat alcoholism, as an aphrodisiac, to treat collapses, convulsions and delirium, kidney problems, earache, deafness, fever, headache, labour, parlorituation, spasms. In Algeria, China, Cambodia, Europe, the plant has been used to cure several stomach ailments like stomach pain, cancer, diaphragm tumors, and colic. It is used as a carminative. Apart from its medicinal
uses, an essential oil obtained from the whole plant is used as a food flavouring and in perfumery, dental applications etc. It also makes a good repellent (Ibid).

6.6 Pharmacology

Preliminary studies on holy basil and hairy basil have shown that the leaf and seed may help people with type 2 diabetes control their blood sugar levels. While the action-mechanism of leaf is not understood, the seed is presumed to work as dietary fiber, which in turn helps prevent rapid blood sugar elevations after meals. Moreover, the seed has been found to relieve constipation by acting as a bulk-forming laxative in one uncontrolled human study.

The volatile oil of basil has been shown to have antibacterial, antifungal and antiviral activity in test tube studies. The essential oil in vitro has been shown to have antibacterial activity against Staphylococcus aureus, Salmonella enteritidis and Escherichia coli, antiseptic activity against Proteus vulgaris, Bacillus subtilis, and Salmonella paralyph, and antifungal activity against Candida albicans, Penicillium notatum and Microsporeum gysecum. Its carminative effect has been observed to relieve intestinal gas. There are reports stating its mild diuretic effect. Animal studies for O. suave/O. gratissimum have revealed its anti-ulcer effects (Tan, 2002). A similar study of O. canum suggested its role in lowering blood sugar levels by aid of insulin release in rats (Nyarko, 2002). O. temflorum has been reported to have anti-inflammatory effects in animal studies. The ethanol extracts of Canum basilicum has also been reported to have cardiac stimulant activity on frog-heart in situ preparations (Muralidharan, 2004).

6.7 Toxicity

There are several reports that have claimed certain toxic effects of the Basil family. O.basilicum, which has been studied extensively for its medicinal properties, has been reported to have several potentially dangerous compounds like safrole, rutin, caffeic acid, quercetin and Tryptophan. Acute bovine pulmonary emphysema (ABPE), a respiratory disease in cattle, is caused by absorbed metabolites of tryptophan. Tryptophan is toxic at a dose of 0.25-0.35g/kg-body weight. P-oumaric acid and caffeic acid (phenolic acids) can inhibit digestion of plant cell walls in ruminants, because of their antimicrobial activity. When rumen microbes
metabolize these phenolic acids, benzoic acid, 3-phenylpropionic acid (PPA) and cinnamic acid may be formed. When these compounds are detoxified, hippuric acid is formed. PPA can decrease metabolic efficiency. Detoxifying the compounds costs the animal nitrogen, which also can decrease productivity. Quercetin may be a co-carcinogen in bracken fern (*Pteridium aquilinum*). It has been suggested that it may interact with Bovine papilloma virus type 4, leading to malignant epithelial papillomas in the upper alimentary tracts. However, its adverse effects with respect to *Ocimum* have not been studied. Safrole has been reported to cause cancer in rats, but there are contradictory reports of *Ocimum* having anti-carcinogenic effects as well (Toxicity, http://www.ansci.cornell.edu).

6.8 Tissue Culture

Plant regeneration protocols have been successfully developed for basil, *Ocimum basilicum* L., particularly three varieties (Sweet Dani; methylcinnamate; Green purple ruffles). Callus and shoot induction was initiated on Murashige and Skoog basal medium supplemented with thidiazuron. They were rooted on media devoid of thidiazuron and then acclimatized (Phippen, 2000). Another protocol for micropropagation of *O. basilicum* used cotyledonary leaves from *in vitro* germinated plants as explants in MS medium supplemented with 0.2mg/l NAA in combination with 0.5mg/l BAP. It was observed that higher BAP concentrations induced an increase in the number of explants with shoots (Dode, 2003).

A rapid *in vitro* clonal propagation with nodal explants in MS media supplemented with BAP or Kinetin, followed by rooting in half-strength MS media supplemented with different auxins (Begum, 2002). There are studies that have reported ultra-high CO₂ levels to enhance *in vitro* shoot growth and morphogenesis in Labiatae (Tisserat, 2000).