REVIEW OF LITERATURE
CHAPTER 2

REVIEW OF LITERATURE

"Genetics loads the Gun, Life style pulls the trigger". There are many diseases that are caused due to genetical disorders and is one of the cause for diabetes mellitus (Elsas Longo, 1992).

DIABETES MELLITUS

Diabetes mellitus (DM) was recognized as early as 1500 B.C. by Egyptian physicians who described it as a disease associated with "The passage of much urine". The term "diabetes" was coined by the Greek physician Aretaeus, who noticed that patients with diabetes had a disease that caused the siphoning of the structural components of the body into the urine (White and Campbell, 1996).

DM afflicts about 5% of the general population. Diabetes is a mysterious illness, a statement made in antiquity by the physician Aerates of Cappadocia (81-138 AD) is still valid today. At first Galen suspected that this illness was caused by a kidney complaint. Avicenna alone has been credited with two additional discoveries, first, the mention of further symptoms besides the triad (polydypsia, polyuria and marasmus) known to antiquity namely physical, mental, sexual weakness, occurrence of carbuncles (Alam et al., 2009), gangrene and secondly the alleged discovery of the sweetness of diabetic urine. The study suggest that for the world as a whole, between the years 1995 and 2025, the adult population will increase by 64%, prevalence of diabetes in adults will increase by 35% and the number of people with diabetes will increase by 122%. For the developed countries, there will be an 11% increase in the adult population, a 27% increase in the prevalence of adult diabetes and a 42% increase in the number of people with diabetes. For the developing countries, there will be an 82% increase in the adult population, a 48% increase in the prevalence of adult diabetes and a 170% increase in the number of people with diabetes (King et al., 1998).

In recent years, developed nations have witnessed an explosive increase in the prevalence of DM predominantly related to lifestyle changes and the resulting
surge in obesity. The metabolic consequences of prolonged hyperglycemia and dyslipidemia, including accelerated atherosclerosis, chronic kidney disease and blindness, pose an enormous burden on patients with diabetes mellitus and on the public health system (American diabetes association, 2009). The number of patients with DM is markedly increasing worldwide. DM is associated with impaired glucose metabolism that leads to an increase in free radical production and increase in triglyceride and lipoprotein levels. Oxygen free radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of antioxidant enzymes and play a role in the long-term complications of diabetes. Therefore, among the various therapeutic strategies, combination of antihyperglycemic, antihyperlipidemic and antioxidant activity can be beneficial in the prevention of DM and its complications (Patel et al., 2009).

**Disease profile**

**a) Definition**

Diabetes is defined as a state in which homeostasis of carbohydrate, protein and lipid metabolism is improperly regulated by insulin. This results primarily in elevated fasting and postprandial blood glucose levels. In diabetic condition, dyslipidemia, lipid abnormalities are the unbalanced metabolic states of diabetes (Bhandari et al., 2002). DM may present with characteristic symptoms such as polyphagia, polydypsia, polyuria, blurring of vision and weight loss.

**b) Prevalence**

There are two types of diabetes- Type-1 diabetes mellitus formerly known as insulin dependent diabetes mellitus (IDDM) and Type-2 diabetes mellitus formerly known as non-insulin dependent diabetes (NIDDM). The vast majority of diabetic patients are Type-2 diabetes mellitus. Diabetes patients are 25 times more prone to blindness, 2 times more prone to heart attacks, 2-6 times more prone to stroke and 17 times more prone to kidney damage as compared to non diabetics (Kohli, 2006).
Type 1 Diabetes

It is characterized by severe lack of insulin due to the destruction of most or all of the beta cells in the islets of Langerhans by an autoimmune process, usually leading to absolute insulin deficiency (Robertson, 2006). Over 95% of persons with type 1 diabetes mellitus develop the disease before the age of 25 and most often between the ages of 10 and 16, with an equal incidence in both sexes and an increased prevalence in the white population Fig. 5.

![Fig. 5. Type 1 diabetes](image)

Type 2 Diabetes

Insulin resistance in peripheral tissue and an insulin secretory defect of the beta cell of pancreas, so that less glucose is produced and to an impairment of insulin's ability to stimulate the uptake of glucose in muscles and other tissues Fig. 6. The cause of this insulin resistance has not yet been fully established, but may involve defects in the action of insulin after it has bound to the insulin receptor on the surface of cells. This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise (Mohan et al., 2007).

![Fig. 6. Type 2 diabetes](image)
**Gestational diabetes:** Occur during pregnancy, sensitivity to insulin decreases (placental hormones affect glucose tolerance). Beta cells may not be able to meet this increased need for insulin gestational diabetes. They are occurs in up to 14% of pregnancy. This increases subsequent risk of developing Type 2 diabetes. Increased risk for perinatal mortality and neonatal morbidity.

**Other types of diabetes mellitus:** Specific genetic/molecular defects have been identified in a minority of what were considered Type 2 diabetes:

1) Genetic defects of function of beta cell. e.g. Hepatic nuclear factor 4 alpha - autosomal dominant condition of impaired insulin secretion; early onset and slowly progressive; type 1 (mature onset diabetes of the young).e.g. Mutation of mitochondrial DNA

2) Genetic defects in the action of insulin: e.g. insulin receptor - (severe insulin resistance) Lipoatrophic diabetes

3) Endocrine disorders
   - Diseases of the pancreas, e.g. pancreatitis, neoplasia, cystic fibrosis, haemochromatosis
   - Endocrinopathies, e.g. acromegaly, Cushing's syndrome, hyperthyroidism, pheochromocytoma

4) Drug/chemical induced, e.g. vacor, pentamidine, glucocorticoids, thiazides, dilantin

5) Infection, e.g. congenital rubella, cytomegalo virus

6) Immune mediated (uncommon), e.g. Stiff man syndrome, anti-insulin receptor antibodies.

c) **Epidemiology**

DM in humans is undergoing a remarkable upsurge in prevalence in the India. Historically, the usual ratio for Type -1 to Type- 2 diabetes has been 1:20. Classically, Type- 1 diabetes is described as an autoimmune disease in which a foreign protein is incorporated into islet β cells, perhaps via viral infection. In
response, the patient's lymphocytes attack the foreign protein and inadvertently destroy the patient's β cells as collateral damage. This leads to a state of absolute insulin deficiency. The pathogenesis of Type-2 diabetes is less well defined, however, it is invariably associated with defective sensing of glucose signals by the β cell. It is often associated with a state of insulin resistance, which means insulin that is secreted by the β cell and bound to liver, muscle and fat cells is subnormally efficacious in carrying out its metabolic actions. The WHO has predicted that the global prevalence of Type-2 diabetes will be more than from 135 million in 1995 to 300 million in 2025 and that this increase will affect both industrialized and developing countries expecting the greatest increase in India, from 19.4 to 57.2 million (Chatterjee, 1997).

d) Classification of diabetes mellitus

<table>
<thead>
<tr>
<th>Classes</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type-1 diabetes</td>
<td>Islet β-cell destruction, autoimmune, idiopathic</td>
</tr>
<tr>
<td>Type-2 diabetes</td>
<td>Insulin resistance, insulin deficiency</td>
</tr>
<tr>
<td>Genetic defects of β-cell function</td>
<td>Chromosome 20, HNF 4α, chromosome 7, Glucokinase</td>
</tr>
<tr>
<td>Genetic defects in insulin action</td>
<td>Type A insulin resistance, lipoatrophic diabetes</td>
</tr>
<tr>
<td>Disease of the exocrine pancreas</td>
<td>Pancreatitis, neoplasia, cystic fibrosis, pancreatectomy</td>
</tr>
<tr>
<td>Endocrinopathies</td>
<td>Cushing’s syndrome, hyperthyroidism</td>
</tr>
<tr>
<td>Drug- or chemical-induced</td>
<td>Nicotinic acid, thiazides, glucosteroids</td>
</tr>
<tr>
<td>Infections</td>
<td>Congenital rubella, cytomegalovirus</td>
</tr>
<tr>
<td>Uncommon forms of immune-mediated</td>
<td>Insulin auto immune syndrome, Anti-insulin receptor antibodies</td>
</tr>
<tr>
<td>Other genetic syndromes</td>
<td>Down’s syndromes, Huntington’s chorea</td>
</tr>
</tbody>
</table>
e) Different forms of Diabetes mellitus

General: Type- 1 Diabetes mellitus (formerly called insulin dependent diabetes mellitus or IDDM)

- Autoimmune Type -1 diabètes mellitus (Type- 1A).
- Non-autoimmune or idiopathic Type- 1 diabètes mellitus (Type- 1B)

Other Specific forms of Diabetes:

1) Diseases of the exocrine pancreas

Fibrocalkaneous pancreatopathy, Pancreatitis, Trauma, Pancreatectomy, Neoplasia, Cystic fibrosis, Haemochromatosis and others

2) Endocrinopathies

Cushing's syndrome, Acromegaly, Pheochromocytoma, Glucagonoma, Hyperthyroidism, Somatostatinoma etc.

3) Infections

Congenital rubella, Coxsackie B, Cytomegalovirus, Mumps, Adenoviruse etc.

4) Drug or chemical induced diabetes mellitus

Nicotinic acid, Glucocorticoids, Thyroid hormone, Thiazides, Dilantin, Vacor, Interferon therapy etc.

5) Other genetic syndromes sometimes associated with diabetes

Down's syndrome, Friedreich's ataxia, Huntington's chorea, Klinefelter's syndrome, Laurence – Moon – Biedel syndrome, Porphyria, Prader willi syndrome, Turner's syndrome, Wolfram's syndrome etc.
6) **Associated with Pregnancy**

Gestational Impaired Glucose Tolerance (GIGT)

Gestational Diabetes Mellitus (GDM)

**Insulin**

The DM has been well known as a wasting disease due to insulin deficiency in human beings. The pancreas secretes insulin. Carbohydrate metabolism is primarily under the control of insulin. Insulin deficiency occurs in a person due to the functional disorder of the pancreas.

**a) The Endocrine part of the Islets of Langerhans**

The normal human adult pancreas contains on an average some 500,000 islets of langerhans, distributed in scattered manner within the gland, comprising 1 to 3% of the total tissue. Each group of cells of the endocrine part is surrounded by the acini of the exocrine part, they look like islands and are hence termed as islets. The distribution of islets is maximum in the tail and minimum in the head of the gland. Three types of cells are found in the islets. These are called the $\alpha$ (alpha), $\beta$ (beta) and (delta) types. The $\beta$ cells are fewer in number about 20% and they exist peripherally in the islets, while the most numerous $\beta$ cells (about 75% to 80%) are situated centrally in the form of lumps. The synthesis of two hormones insulin and glucagon takes place in the $\beta$ cells, respectively in the islets of Langerhans. Both hormones play an important role in carbohydrate metabolism (Rama Rao, 2001). The function of the $\beta$ cells (about 5% in number) is not clearly known. It is assumed that they may secrete serotonin but some others believe that gastrin is secreted by these cells.

**b) Chemistry**

Insulin from different sources (e.g. pig, cattle, sheep and horses) shows minor differences in amino acid composition and immunological activity. The nearest to human insulin in structure is insulin from pig. Insulin is destroyed by action of digestive enzymes and is hence inactive when given (administered) by mouth (Pilkis, 1988). The biological action of the hormones can be prolonged by
combining it with protamine or globin (protamine zinc insulin and globin insulin) or by altering the size of the crystals (ultralente insulin; large crystals and slow acting).

c) Metabolism of Insulin

Insulin is believed to be transported in the plasma bound to a specific insulin transporting protein. Insulin is degraded primarily in the liver and kidney by the enzyme, "Glutathione insulin transhydrogenase". The half life of plasma insulin is only 7-15 minutes (Koolman, 1999).

d) Mode of action of Insulin

1. Muscle, adipose tissue and liver are the major sites of its action
2. It is active on the lens and leukocytes
3. It has minor action on the metabolism of renal tissue, erythrocytes

e) Extrahepatic tissues

- It facilitates the transport of glucose across the cell membrane.
- Insulin promotes metabolic pathways like glycogenesis, glycolysis and HMP pathways.
- Insulin stimulates intracellular transport of all sugars e.g. arabinose, xylose and galactose.
- Insulin stimulates uptake of amino acids by the cell.
- Insulin stimulates the activity of enzymes hexokinase and glycogen synthetase.
- Insulin stimulates oxidative phosphorylation in mitochondria of muscle.
- Insulin stimulates the entry of Na⁺, K⁺ & PO₄⁻ into adipose tissue.
- Liver: Insulin is an anabolic hormone causing increased carbohydrate metabolism, glycogen formation, lipid synthesis, amino acid uptake and protein synthesis.
Glucose Homeostasis

A carbohydrate, particularly glucose, is an important source of fuel for living organisms. It has been found that glucose homeostasis contributes to two kinds of hormones, including insulin and anti-insulin or counter-regulatory hormones (glucagon, growth hormones, cortisol and catecholamines). Maintenance of serum glucose concentrations within a normal physiological range is primarily accomplished by two pancreatic hormones, insulin and glucagon. Derangements of glucagon or insulin regulation can result in hyperglycemia or hypoglycemia. Glucose penetrates most tissues slowly unless, insulin is present to facilitate its uptake; however, central nervous system (CNS) cells, capillary endothelial cells, gastrointestinal epithelial cells, pancreatic cells and renal medullary cells are freely permeable to glucose Fig. 7.

![Glucose Metabolism Diagram](image)

**Fig. 7. Glucose Homeostasis**

The endocrine portion of the pancreas, called the islets of Langerhans, consists of cordlike groups of cells arranged along pancreatic capillary channels. These pancreatic cells monitor changes in the availability of small calorigenic molecules, namely glucose and to a lesser extent amino acids, ketone bodies and fatty acids. Pancreatic β-cells appropriately alter their rates of insulin secretion in response to fluctuations in the levels of these calorigenic molecules, with glucose
playing the dominant role in regulation of insulin secretion. Pancreatic β-cells secrete glucagon in response to increases in amino acid and fatty acid levels; however, glucose inhibits glucagon secretion. If blood glucose levels fall (e.g., during hypoglycemia or fasting), glucagon secretion is augmented, providing a counter regulatory hormonal response that stimulates gluconeogenesis in the liver and other tissues to avoid hypoglycemia. Circulating glucose levels are determined by the balance among absorption, storage, production and use (metabolic rate). Glucagon and insulin are the two most important hormones that maintain glucose homeostasis when blood concentrations are disturbed.

**Insulin deficiency and its effects**

In simplified terms, they can be described as stimulation of glucose utilization and inhibition of gluconeogenesis (Pilkis, 1988). In addition, the transport of glucose from the blood into most tissues is also insulin-dependent (exceptions to this include the liver, CNS and erythrocytes).

**a) Fat metabolism**

The presence of insulin favors the production of triglycerides from free fatty acids (FFAs). When insulin deficiency causes an energy deficit, FFAs are oxidized to β-hydroxybutyric acid, acetoacetic acid and acetone. β-Hydroxybutyric acid can be used as an energy source, but in the absence of insulin the production of the keto acids eventually is greater than their metabolism and excretion. If insulin is not given to the patient, metabolic ketoacidosis ensues. The keto acids cause the blood pH to decline. The body’s neutralizing factors eventually are depleted and the patient continues to deteriorate to the point of coma and possibly death.

**b) Protein metabolism**

The presence of insulin favors the production of structural proteins from constituent amino acids. When glucose is present intracellularly in sufficient quantities for needed energy production, most structural proteins retain their integrity. In the absence of insulin, structural protein production is not favored and intracellular glucose levels are insufficient to match energy demands. In attempt to produce energy, skeletal muscle converts its structural proteins to constituent amino
acids. The liberated amino acids are transported to the liver, where they are converted to glucose via gluconeogenesis (Setter et al., 2000). In patients with diabetes, glucose enters the blood but is not taken up by tissues because of a true or relative lack of insulin. Thus, hyperglycemia is escalated and structural proteins are wasted.

A) Pathogenesis of Type-1 Diabetes mellitus

Three interlocking mechanisms are responsible for the islet cell destruction showed in Fig. 8.

1. Genetic Susceptibility

2. Auto-Immunity

3. Environmental

1. Genetic Susceptibility

At least one of the susceptibility gene for Type-1 diabetes resides in the region that encodes the class II antigens of the Major Histocompatibility Complex (MHC) on chromosome GP21 (HLA-D). The HLA-D region contains three classes of genes (DP, DQ and DR). The class II molecules are highly polymorphic and each has numerous alleles. About 95% of white patient with Type-1 diabetes have either HLA-DR3 or HLA-DR4 alleles or both where as in the general population the prevalence of these antigens is only 45%. It is thought that genetic variations in the HLA class II molecules may alter recognition by the T-cell receptor, or may modify the presentation of the antigen because of variations in the antigen-binding cleft, thus, class II HLA gene may effect the degree of immune responsiveness to a pancreatic β-cell autoantigen or a β-cell autoantigen may be presented in a manner that promotes an abnormal immunologic reaction (Michael, 2000).

2. Auto-Immunity

Clinical onset of Type-1 diabetes is abrupt; this disease in fact results from a chronic auto-immune attack of β-cells that usually exists for many years before the disease becomes evident. A lymphocyte with rich inflammatory infiltrate (Insulitis) is observed in the islets of patients in early diabetes. The infiltration
consists mostly of CD8 T-lymphocytes. CD4 T cell from animals with autoimmune diabetes can transfer diabetes to normal animals, thus establishing the primary of T-cell auto-immunity in Type-1 diabetes.

The Insulitis is associated with increase expression of class I MHC molecules and aberrant expression of class II MHC molecules on the β-cells. This aberrant expression is mediated in part by locally produced cytokines [eg. Interferon-gamma (IFN-γ) derived from activated T-cells]. Genetic dysregulation of a cytokine that induce IFN-γ production promotes the development of diabetes in a mouse model. About 70%-80% of patients with Type-1 diabetes have islet cell auto antibodies against intracellular islet cell antigens, such as Glutamic Acid Decarboxylose (GAD) “islet auto antigen 2” (1a-2a tyrosine phosphatases), insulin and gangliosides.

![Fig. 8. Overview of the pathogenesis of Type-1 diabetes mellitus](image)

3. Environmental factors

Viruses

A viral infection has long been noted in the diagnosis of new cases and has the association between coxsackie viruses of group B and pancreatic diseases including diabetes. Other implicated viral infections include mumps, measles, cytomegalovirus, rubella and infections mononucleosis. It has been postulated that
one of these viruses causes mild β-cells injury, which is followed by an auto-immune reaction against previously sequestered antigens in virally altered β-cells in persons with HLA-linked susceptibility. Another is that an immune response develops against a viral protein that shares amino acid sequences with a β-cell protein (molecular mimicry).

Others

Antigenic exposure may also come from other sources. Children who ingest cow’s milk products early in life (before age of 4 months) have a 1.5 fold increase risk for Type-1 diabetes relative to those who do not, raising the spectrum of a cross-reacting antigen in cow’s milk.

B) Pathogenesis of Type-2 diabetes mellitus

The two metabolic defects that are characterizing Type-2 diabetes mellitus are showed in Fig. 9.

1. A derangement in β-cell secretion of insulin

2. A decrease response of peripheral tissue to respond to insulin (Insulin resistance)

1. Deranged β-cell Secretion of Insulin

A modest hyperinsulinemia may be observed, attributed to β-cell hyper responsiveness to physiological elevations in blood glucose, with the development of overt disease. The pattern of insulin secretion exhibits a subtle change. Early in the course of Type-2 diabetes, insulin secretion appears to be normal and plasma insulin levels are not reduced.

However, the normal pulsatile oscillating pattern of insulin secretion is lost and the rapid first phase of insulin secretion triggered by glucose is obtunded. Collectively, these and other observations suggest derangements in β-cell response to hyperglycemia early in Type-2 diabetes, rather than deficiencies in insulin synthesis per se. Later in the cause of Type-2 diabetes a mild to moderate deficiency of insulin develops which is less severe than that of Type-1 (Willium, 1989).
Evaluation of Antioxidant, Antidiabetic and Wound Healing Potential of Chromolaena Odorata (L.) King And Robinson Using In Vitro and In Vivo Models

2. Insulin Resistance

Insulin resistance (IR) is a common pathological state in which target cells fail to respond to ordinary levels of circulating insulin showed in Fig. 10. It results in inability of insulin to provide normal glucose and lipid homeostasis (Barbara, 2007). Insulin resistance is also a feature of a number of other health disorders, including obesity, glucose intolerance, dyslipidemia and hypertension clustering in the so-called metabolic syndrome (also commonly referred to as syndrome X).

a) Symptoms of insulin resistance

- Feeling agitated, jittery, moody, nauseated, or having a headache is common in insulin resistance, with almost immediate relief once food is eaten.
- Intestinal bloating.
- Sleepiness.
- Weight gain.
- Fatigue.
- Increased triglycerides.
- Increased blood pressure.
b) Causes and associated conditions of insulin resistance

A number of factors increase the risk for insulin resistance, including genetic predisposition, obesity and inactivity, aging, medications, polycystic ovary syndrome and rare disorders such as partial lipodystrophy. Concomitant conditions that are associated with insulin resistance include Type 2 diabetes, hypertension, dyslipidemia, atherosclerosis and polycystic ovarian syndrome (Giorgio, 2006).

Fig.10. Insulin resistance and associated conditions.

Pharmacological therapy

A) For Type-1 Diabetes mellitus: Principal types of insulin preparations include-

1) Rapid-acting insulins – Insulin lispro and insulin aspart.

2) Short-acting insulin – Regular humulin, velosulin BR.

3) Intermediate-acting and long-acting insulins – Lente humulin, NPH (neutral protamine hagedorn) humulin, ultralente insulin and insulin glargine-lantus (Daniel, 2007).

B) For Type-2 Diabetes mellitus: Oral Hypoglycemic agents

1. α-glucosidase inhibitors (AGIs): Acarbose and miglitol.

An enzyme in the brush border of proximal small intestinal epithelium α-glucosidase serves to breakdown disaccharides and more complex carbohydrates.
By competitive inhibition of this enzyme, the AGIs delay intestinal carbohydrate absorption. Their greatest effect is on post-prandial glucose levels and effect on fasting blood glucose level is small.

**Adverse effects:** Flatulence, abdominal discomfort, diarrhea.

**2. Sulfonylureas (SUs)**

They have been available in United States since 1954. First generation SUs: Chloropropamide, tolbutamide, acetohexamide and tolazamide. Second generation SUs: Glyburide, glipizide, glimepiride, glibenclamide. SUs bind to the SU receptor found on the surface of pancreatic β-cells. This interaction leads to a closure of voltage-dependent KATP channels, facilitating cell membrane depolarization, calcium entry into the cell and insulin secretion. The possibility that such agents may also directly enhance peripheral glucose disposal (i.e. decrease insulin resistance) has also been raised.

**Adverse effects:** Weight gain, hypoglycemia. They must be used cautiously in hepatic or renal impairment.

**3. Biguanides**

Over 30 years ago, biguanides like metformin, phenformin, buformin were used for treatment of diabetes. Metformin’s major action is to decrease hepatic glucose output primarily by decreasing gluconeogenesis, but it may also increase glucose uptake by skeletal muscles. Metformin activates hepatic and muscle AMPK, a cellular signal for increased energy requirements. Activation of hepatic AMPK results in phosphorylation and inhibition of acetyl-coenzyme A carboxylase, which catalyzes the rate-limiting step of lipogenesis. This block in fatty acid synthesis promotes fatty acid oxidation.

**Adverse effects:** Gastrointestinal, lactic acidosis (rare) and renal dysfunction.

**4. Non-sulfonylureas:** Nateglinide, repaglinide.

The mechanism of action of these drugs is similar to that of SUs (closure of KATP channel leading to calcium-dependent insulin secretion). However they bind to the SU receptor at a different site and with different kinetics than SUs. Their onset of action is faster and half-life is shorter, which results in brief stimulation of insulin release (Silvio, 2002).
Adverse effects: Hypoglycemia, weight gain, contraindicated in liver, kidney dysfunction and concomitant use of repaglinide with gemfibrozil is avoided.

5. Insulin sensitizers (Thiazolidinediones)

The currently available thiazolidinedione is pioglitazone. Troglitazone an earlier introduced thiazolidinedione was removed from market because of risk of hepatic failure. Thiazolidinediones function as ligands for the PPARγ, which is most highly expressed in adipocytes. These nuclear receptors, which are ligand-activated transcription factors, play an integral part in the regulation of the expression of a variety of genes involved in carbohydrate and lipid metabolism. Thiazolidinediones improve insulin sensitivity, particularly in the peripheral tissues. In adipocyte differentiation is enhanced, lipolysis is reduced, adipokines are altered, namely a decrease in TNF and free fatty acid levels and increased adiponectin levels. These effects enhance insulin sensitivity.

Adverse effects: Weight gain, edema, anemia, pulmonary edema, congestive heart failure, contraindicated in liver dysfunction.

6. Intestinal lipase inhibitor: Orlistat

It is an antiobesity agent that acts as a selective inhibitor of gastric and pancreatic lipases and thereby inhibits the hydrolysis of dietary fat into absorbable free fatty acids and monoglycerides.

Adverse effects: Flatulence, oily spotting, fecal urgency, increased frequency of defecation and fecal incontinence. Absorption of fat-soluble vitamins can be adversely affected. Contraindications are chronic malabsorption syndrome, cholestasis and known hypersensitivity.

7. Herbal Drug

Diabetes mellitus is a common chronic endocrine disorder. Since ancient time a number of herbal medicines were used in the treatment of DM. Many studies have been carried out in search of a suitable plant drug that would be effective in DM. Herbal medicines for diabetes can be classified into four categories according to their mode of action:
i) Drugs acting like insulin
ii) Drugs acting on insulin secreting beta cells
iii) Drugs acting by modifying glucose utilization

Animal models for experimental diabetes mellitus

There are many advantages of using animals models in research work on diabetes as various aspects of the disease like the etiology, its multifactorial genetics, pathogenesis of the disease and its complication can be explicitly understood. Secondly, it also helps in the development and evaluation of newer agents for the treatment of diabetes. However, there are some limitations in the use of animal model for studies on diabetes. Induction of diabetes in animals can be carried out by various ways—by using different chemical diabetogenic agents, surgically by partial Pancreatectomy, by viral induction and genetic manipulation by selective in breeding Various diabetic chemicals.

Induction of diabetes by various chemical diabetogenic agents is also dependent on the species, the strain, sex and the diet of the animals. Variations in susceptibility have also been observed amongst male and female mice of same strain, males being more susceptible to insulin dependent diabetes mellitus (IDDM) than females.

1. Alloxan

Diabetogenic action of alloxan is mediated by reactive oxygen species. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic concentrations causes rapid destruction of β-cells. The action of alloxan in the pancreas is preceded by its rapid uptake by the β-cells. Since alloxan exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein bound sulphhydryl groups (including SH-containing enzymes) are very susceptible to its action. The reaction between alloxan and dialuric acid is a process in which intermediate alloxan radicals (HA•) and an unidentified “compound 305” (maximum absorption at 305nm) is formed.
Alloxan is converted into unstable dialuric acid which is then reoxidised back to
alloxan. Increased production of OFR in the islets, together with inadequate defense
makes the β-islet cells susceptible to alloxan. In normal non fasted animals, the
blood glucose level after alloxan injection fluctuates in a triphasic pattern.

Triphasic response of alloxan

1. Early hyperglycemia of short duration (about 1-4 h) due to a sudden short
lasting decrease or cessation of insulin release and a direct glycogenolytic
effect on the liver.

2. Hypoglycemia phase lasting up to 48 h and often resulting in convulsion
and death (which may be prevented by treatment by glucose) due to
uncontrolled leakage of insulin from the damaged cells.

3. Chronic diabetes phase, consequence of insulin lack histologically only a
few β-cells if any, are detectable in animals with fully developed alloxan
diabetes.

2. Streptozotocin

Streptozotocin [2-deoxy-2-{3-(methyl-3-nitrosoureido)-D-glucopyranose}] is
synthesized by streptomycetes achromogenes and is used to induce both Type-1 and
Type-2. It is freely soluble in water, unstable at room temperature and has to be
stored below -200C.

Streptozotocin induces diabetes in almost all the species. Diabetes dose varies
with the species and the optimal dose required to produce diabetes in rat was found to
be (50 – 60 mg/kg i.p. or i.v.), in mice (175-200 mg/kg i.p. or i.v.) and in dogs (15
mg/kg, for 3 days). Due to its low stability the rapid i.v. injection appears to be the
best route of administration. STZ induces diabetes in hamster, monkey and guinea
pigs. STZ diabetes can be induced by two ways either by single injection of STZ or
by multiple low dose injection of STZ. Like alloxan, it shows triphasic fluctuation
pattern in diabetes. Initial hyperglycemia is observed by 1 h after the injection
followed by hyperglycemia and again a hyperglycemia state at 48 h, the elevated
blood glucose level is observed by 48- 72 h (peak effect) and is maintained
thereafter. Different mechanism of action on the β- cells destruction by STZ has been
proposed. It mainly acts through free radical generation.
Other report proposed that STZ exerts lethal damage by alkylating DNA or its phosphate backbone as well as glycolytic or mitochondria enzyme. STZ also influence the immune system by suppressing the T-cell function associated with atrophy of the thymus and peripheral lymphoid tissue. Like alloxan, STZ also induces OFR induced lipid peroxidation and DNA strand breaking in pancreatic islet cell Streptozotocin enters the β-cell via a glucose transporter (GLUT 2) and cause alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation leads to depletion of cellular NAD+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage.

3. Other diabetogenic agents

- Dehydroascorbic acid 650 mg/kg for three days in rat
- Dehydroisoascorbic acid 1.5 mg/kg in rat
- Dehydroglucoascorbic acid 3.5-3.9 gm/kg in rat
- Methyl Alloxan 53 mg/kg in rat
- Ethyl Alloxan 53-130 mg/kg in rat
- Oxime & Dithizone 53 mg/kg in rabbit
- Sodium Diethyldithiocarbonate 0.5-1 g/kg in rabbit
- Potassium Xanthate 200-350 mg/kg in rabbit

4. Non-insulin dependent diabetes mellitus (NIDDM) resembling animal models

By altering the dose and the day of the STZ injection, the n-STZ models exhibit various stages of Type-2 diabetes mellitus, such as impaired glucose tolerance, mild, moderate and severe hyperglycemia. Neonatal STZ-induced rat (n-STZ) model of Type 2 diabetes mellitus model is generated by injecting Wistar rats
on the day of their birth (n0=birth) intravenously (saphenous vein) or intraperitoneally with 100 mg/kg of STZ. Also, the n-STZ rat model is developed by varying the day of the STZ injection after the birth, such as 2nd day or 5th day of the birth and these are alternatively called n2-STZ and n5-STZ models respectively. The rats treated with STZ on the day of birth, exhibit insulin deficient acute diabetes mellitus 3-5 days after birth. They showed high plasma glucose and about 93% decrease in plasma insulin and high plasma glucagon content. It was found that only by 8 weeks of age and thereafter n0-STZ rats showed mild hyperglycemia.

Sprague-Dawley pups were injected intraperitoneally on the 2nd day after birth with 90 mg/kg STZ and on 1.5 days after birth with 120 mg/kg STZ. By 6 weeks of age these animals showed basal hyperglycemia and abnormal glucose tolerance. The above two animal models are based mainly on β-cell deficiency and these models are useful for evaluating the effect of β-cell deficiency in the development of NIDDM. NIDDM animal models can also be prepared by neonatal alloxan induced diabetes by injecting alloxan 200 mg/kg body weight i.p. to neonates of 6 days old.

5. Hormone induced diabetes

*Growth hormone induced diabetes*: In intact adult dogs and cats repeated administration of growth hormone induces an intensively diabetic condition with all symptoms of diabetes including severe ketonemia and ketonuria.

*Corticosteroid induced diabetes*: Hyperglycemia, glucosuria are observed in forced fed rats treated with cortisone. In guinea pig and rabbit, experimental corticoid diabetes could be obtained without forced feeding.

6. Insulin deficiency due to insulin antibodies

Bovine insulin (1mg) is injected subcutaneously to guinea pigs at monthly intervals and is bleed by cardiac puncture two weeks after the second and subsequent doses of antigen. Intravenous injection (0.25 – 1.0 ml) of guinea pig anti-insulin serum to rats induces a dose dependent increase of blood glucose. This effect is due to neutralization by insulin antibodies secreted by the injected animal.
7. Virus induced diabetes

Type 1 diabetes mellitus may be due to virus infection and β-cell specific autoimmunity. The D-variant of the encephalomyocarditis virus (EMC-D) selectively infects and destroys the β-cells in the male Swiss mice similar to the human insulin-dependent diabetes.

8. Models of diabetes accelerated atherosclerosis

Accelerated cardiovascular disease is a leading cause of both morbidity and mortality in diabetic patients. Aggressive therapy of dyslipidemia is necessary, since the risk of myocardial infarction is the same as in nondiabetic patients with previous myocardial infarction. Currently, rats and mice are the most widely used models to study diabetes.

9. Pancreatectomy

The technique of complete Pancreatectomy in the dog has been used by many scientists as a relevant animal model for diabetes mellitus in man. Polyuria, polydipsia, polyphagia and severe glucosuria were noted following removal of the pancreas in dogs.

Precise evaluation of consequences of reduced β cell mass in rats can be achieved by partial Pancreatectomy. After 90% of the pancreas is removed, animals maintain moderate hyperglycemia in fed state but show no differences in body weight and plasma insulin concentrations as compared with sham-operated control animals. Loss of glucose-stimulated insulin secretion was documented in the animal after oral or intravenous glucose challenge. No glucose stimulated insulin release can be seen in perfused pancreases in these animals. In contrast the reaction to other secretagogues is retained.

Causes of Diabetes

a. Heredity i.e. family history of late onset diabetes

b. Obesity i.e. over weight

c. Lack of physical activity i.e. sedentary life style
d. Women with prior gestational diabetes

e. Stress

**Diabetes Symptom**

**A. Symptoms of type I diabetes may include**

- Increased thirst and urination
- Blurred vision
- Feeling very hungry
- Weight loss in spite of increased eating

**B. Symptoms of Type 2 diabetes may include**

- Feeling tired or ill
- Frequent urination (especially at night)
- Unusual thirst
- Weight loss
- Blurred vision
- Frequent infections
- Slow healing of sores.
- Having dry, itchy skin

**Complications occur in Diabetes**

**Acute Complications**

**Type – I Diabetes (IDDM)**

- Due to illness or fever, insulin requirement increases if additional requirement of insulin is not met with, a diabetic coma can develop.
• Diabetic Ketoacidosis – Appearance of large amount of glucose along with Ketone bodies in urine.

Type – II Diabetes (NIDDM)

• Dehydration coma – Loss of excessive amounts of water and salt.
• Skin problems.

Long term complications

**Eyes:** Progressive loss of vision, leading to blindness, diabetes is among the three common causes of blindness today.

**Heart:** Diabetics are very prone to developing high blood pressure.

**Blood vessels and circulation:** The arteries may develop fat deposits hindering flow of blood, affecting the blood supply to extreme parts of limbs.

**Kidneys:** More susceptible to infections of the urinary bladder and Kidneys. It may also lead to failure of kidney functions.

**Nervous System:** Diabetes affects the nerves leading to loss of sensation. In contrast certain diabetics may suffer from tingling or burning sensation in extreme parts of limbs

**Plant as a Diabetic Agent**

**Invitro Enzymatic Activity**

The intestinal digestive enzymes alpha-glucosidase and alpha-amylase are plays a vital role in the carbohydrate digestion. One antidiabetic therapeutic approach reduces the post prandial glucose level in blood by the inhibition of α- amylase and α-glucosidase enzymes. These can be an important strategy in management of blood glucose. The study was to investigate the phytochemical bioactive compounds of the methanolic extract of *Psidium guajava* leaves, its in vitro anti-diabetic activity. The assay results suggests that the presence of bioactive compounds, could be responsible for the versatile medicinal properties of this plant including diabetes, the extract exhibit the dose-dependent increase in inhibitory effect on α-glucosidase enzyme
(upto 89.4%) and α- amylase enzyme (upto 96.3%). The study proves that the antidiabetic activity of methanolic extract of *Psidium guajava* leaves by in vitro studies (Manikandan *et al.*, 2013).

Diabetes is a clinical syndrome characterized by hyperglycemia due to absolute or relative deficiency of insulin. Recent decades have experienced a sharp increase in the incidence and prevalence of diabetes mellitus. One antidiabetic therapeutic approach is to reduce gastrointestinal glucose production and absorption through the inhibition of carbohydrate digesting enzymes such as α- amylase and α-glucosidase. Inhibition of amylase and glucosidase enzymes involved in digestion of carbohydrates can significantly decrease the post prandial increase of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in management of blood glucose. The study was to screen the methanol extract of root of *Caesalpinia digyna* for its in vitro antidiabetic activity. The result suggests that methanol extract of *Caesalpinia digyna* exhibit dose-dependent increase in percentage inhibitory activity on α-glucosidase enzymes IC₅₀ 402.23±10.14 µg/ml and α- amylase IC₅₀ of 686.94 ± 3.98 µg/ml (Narkhede *et al.*, 2011).

Medicinal plants have been reported to play an important role in modulating glycemic responses and have preventive and therapeutic implications. The intestinal digestive enzymes play a vital role in the carbohydrate digestion. The possible action of isolated compounds through which *Pithecellobium dulce* fruit peel exerts its hypoglycemic effect using suitable in vitro techniques (Praylin Singh *et al.*, 2015). The isolated compounds were subjected to inhibitory effect of non-enzymatic glycosylation of hemoglobin assay and enzymatic alpha-amylase inhibition assay using specific standard in vitro procedure. Non-enzymatic glycosylation of hemoglobin assay showed inhibitory activity of 73.7 % and 53.9 % at 1mg/ml. The IC₅₀ values of amylase inhibitory activity of compounds from *Pithecellobium dulce* fruit peel was found to be 80.9 % and 56.5 % at 1mg/ml. Results in two different compounds revealed that the compound 1was found to be more potent than compound 2 at the concentrations 0.2 mg/dl to 1.0 mg/dl. The findings indicate *Pithecellobium dulce* fruit peel possess hypoglycemic effect and hence can be utilized as an adjunct in the management of *diabetes mellitus*. 
The Polyherbal formulation was screened for its phytochemical constituents, total phenols, flavonoids, and vitamin C content. It was also analyzed for its inhibitory effect against the digestive enzymes α amylase and α glucosidase, compared with the standard drug acarbose. The formulation showed the presence of major constituents such as steroids, cardiac glycosides, phenols, flavonoids, and saponins. It also had a high level of phenols (340 ± 2.5 mg/g), flavonoids (235.4 ± 8.3 mg/g), and vitamin C (470.8 ± 16.6 mg/g), and showed a half-maximal inhibitory concentration (IC50) value of 0.41 ± 0.03 mg/mL and 0.51 ± 0.01 mg/mL for amylase and glucosidase, respectively. The results showed that ADJ6 had a significant inhibitory activity on α-amylase and α-glucosidase; however, its inhibitory activity was less than that of acarbose. The plants that are formulated in ADJ6 possess potent antidiabetic activity (Anand Duraiswamy et al., 2015).

The inhibitory effects of ethanolic extract of the leaves of *Senna surattensis* (EESS) on α amylase and α glucosidase. The *in vitro* antidiabetic activity of *S. surattensis* using the glucose uptake by isolated rat hemidiaphragm model. In *vitro* studies using mammalian alpha-glucosidase extracted from the small intestine homogenate of mouse showed that the extract was found to be more effective in inhibiting the activities of maltase half maximal inhibitory concentration (IC50 209.15 mg/mL) and sucrase (IC50 366.44 mg/mL), when compared with the control group (acarbose). The extract of *S. surattensis* were further quantified with respect to porcine pancreatic α amylase inhibition using the chromogenic 3, 5-dinitrosalicylic acid method. Interestingly, *S. surattensis* was also found to exhibit α amylase (IC50: 123.95 mg/mL) inhibitory activity. The glucose uptake in the rat hemidiaphragm was significantly (p < 0.01) increased by EESS (220.95 ±5.4 mg/g/30 minute) when compared with the control group. The total polyphenolic content of EESS was found to be 98 mg pyrocatechol/ mg of the extract. These results suggest that EESS inhibited carbohydrate digestive enzymes and increased the peripheral uptake of glucose. This study endorses the use of this plant for further studies to determine their potential for managing Type 2 diabetes (Ellappan et al., 2013).

The amino acid and fatty acid composition of polypeptide k and oil isolated from the seeds of *Momordica charantia* was analysed. The analysis revealed polypeptide k contained 9 out of 11 essential amino acids, among a total of 18 types
of amino acids. Glutamic acid, aspartic acid, arginine and glycine were the most abundant (17.08%, 9.71%, 9.50% and 8.90% of total amino acids, respectively). Fatty acid analysis showed unusually high amounts of C18-0 (stearic acid, 62.31% of total fatty acid). C18-1 (oleic acid) and C18-2 (linoleic acid) were the other major fatty acid detected (12.53% and 10.40%, respectively). The oil was devoid of the short fatty acids (C4-0 to C8-0). Polypeptide k and oil were also subjected to in vitro α-glucosidase and α-amylase inhibition assays. Both polypeptide k and seed oil showed potent inhibition of α-glucosidase enzyme (79.18% and 53.55% inhibition, respectively). α amylase was inhibited by 35.58% and 38.02%, respectively. Collectively, the in vitro assay strongly suggests that both polypeptide k and seed oil from Momordica charantia are potent potential hypoglycemic agents (Zuraini Ahmad et al., 2012).

**In vivo antidiabetic activity**

Oxidative stress is the root cause of diabetic macro and microvascular complications. Biochemical and epidemiological studies indicate that current treatments for diabetes do not reduce risks of developing complications, suggesting their inability to alleviate the levels of oxidative stress. This study in streptozotocin (STZ)-induced diabetic rats was carried out to investigate the effect of combining the antidiabetic drug, metformin, with an ethanolic extract of Scutellaria baicalensis, a plant whose root is known for its radical scavenging activity.

Plasma and hepatic lipid peroxide concentrations in the herb-treated and herb + metformin-treated groups were also significantly reduced (p < 0.05). In addition, the combined treatment caused significant elevations of plasma and pancreatic insulin levels and reductions of plasma and hepatic triglycerides (TG) and cholesterol levels. The study thus showed that S. baicalensis enhanced the antidiabetic effect of metformin in STZ-induced diabetic rats by improving the antioxidant status (Waisundara et al., 2008). It also increased pancreatic insulin content as well as improved the lipid profile in these rats.

Diabetes mellitus is one of the major causes of morbidity and mortality worldwide. Despite lack of scientific evidences to support its therapeutic efficacy, the use of herbal supplements has significantly increased. Guar gum has a wide variety
of non-food and food uses as a stiffener in ice cream, yogurt, bakery, and soups; as a stabilizer for cheeses, puddings, and cream; and as a meat binder. Therefore being safe, it was tried in model Type 2 diabetes induced by streptozotocin (STZ) in rats, compared to gliclazide. Healthy Sprague Dawley male adult rats were randomly divided into 5 equal groups namely A, B, C, D, and E (group A and B) were kept as normal control and diabetic control. The other diabetic groups from group C to group E were treated as follow Gliclazide 4.5 mg/kg, Guar gum 200 mg/kg and Guar gum 200 mg/kg plus gliclazide 4.5 mg/kg respectively for 14 consecutive days. Blood glucose, insulin, glycosylated hemoglobin (HbA1c), superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), lactate dehydrogenase (LDH), urea, insulin resistance, insulin sensitivity and β-cell function were measured. Guar gum exerted antidiabetic activity nearly similar to that of gliclazide as indicated by significant reduction of glucose, HBA1c, and insulin resistance. Elevation of insulin and β-cell function was also observed. Guar gum and gliclazide showed antioxidant activity as seen by significant reduction of MDA and LDH (Nasry et al., 2013).

*Areca catechu* L. (Palmaceae), commonly known as Areca nut in English, is a perennial tree occurring throughout the Indian subcontinent and used traditionally for several medicinal purposes (Suvankar Mondal *et al.*, 2012). The present study was expected to evaluate anti diabetic activity of petroleum ether, chloroform and methanol extracts of *A. catechu* leaf in Wister rats. Diabetes mellitus was induced in rats by single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg body weight). After STZ induction, the hyperglycemic rats were treated with all three extracts orally at the dose 200 mg/kg body weight daily for 15 days. Glibenclamide (0.5 mg/kg b. w., p.o.) was used as reference drug. The fasting blood glucose levels were measured on every 5th day during the 15 day treatment. All the extracts at 200 mg/kg orally significantly (p < 0.001) exhibited anti diabetic activity in STZ-induced diabetic rats by reducing and normalizing the elevated fasting blood glucose levels as compared to those of STZ control group. The methanol extract was most active. The present study concludes that *A. catechu* leaf confirmed promising anti diabetic activity in STZ-induced diabetic Wistar rats.
Triumfetta pilosa for the treatment of diabetes. Antidiabetic activity of ethanolic extract of Triumfetta Pilosa Roth was evaluated for in-vivo hypoglycemic activity using Streptozotocin induced diabetic rats. Different biochemical parameters were used to determine the blood glucose levels using streptozotocin induced diabetes and analyzed it effect in kidneys after 21 days treatment (Ramakrishna et al., 2011). Ethanol extract had shown significant protection and lowered the blood glucose levels when compared to normal in glucose tolerance test. In kidney the changes caused after induction of diabetes showed degeneration’s in proximal tubular epithelial cells in the cortex of kidneys, hemorrhage in the interstitial area and periglomerular lympholytic infiltration and hyalinization of the arterioles which was reduced after feeding with Triumfetta Pilosa. Ethanolic extract of Triumfetta Pilosa prevented alteration in kidney pathology.

The anti-diabetic and nephroprotective activity of the Ethanolic extract of the whole plant of Canthium dicoccum (family: rubiaceae) was investigated on alloxan induced diabetic albino rats. A comparison was made between both plant extract and a known antidiabetic drug glibenclamide (5mg/kg-1). The dried bark of Canthium dicoccum was subjected to extraction by continuous hot extraction method using ethanol as a solvent. Phytochemical estimation was done for the presence of phytoconstituents. Dose selection was made on the basis of acute oral toxicity study (200mg/kg-1, 400mg/kg-1 bodyweight) as per OECD guidelines. Oral administration of extract of Canthium dicoccum for 21 days resulted in significant reduction in blood glucose level. Alloxan induced diabetic rat model and oral glucose tolerance test (OGTT) model was used for evaluation of antidiabetic activity. The biochemical parameters were analysed. All rats in the diabetic groups had FBG levels well within the diabetic range (>150 mg dL-1) at the initial stage of the experiment but after 21 days of treatment with extracts or glibenclamide the FBG significantly dropped in dose-dependent manner. The results suggest that the ethanol extracts of the Canthium dicoccum restored the metabolic changes in alloxan-induced diabetic rats.

The anti-hyperglycemic potential of the aqueous fruit extract of amla (E. officinalis, for eleven weeks in streptozotocin-induced diabetic obese rats. The study utilized forty eight rats divided into four groups as follows. Untreated diabetic control (group 1) received 2% gum acacia as vehicle; groups 2 and 3 were diabetic
rats administered the fruit extract in 400 and 200 mg/kg doses, respectively; while group 4 (diabetic rats) received metformin (600 mg/kg) as reference drug (Mai et al., 2015). The parameters assessed weekly were body weight, as well as fasting blood glucose, cholesterol and triglyceride levels in venous blood. Results: Both plant extract-treated groups showed significant (p ≤ 0.001) reduction in blood glucose levels in the fifth and sixth weeks compared to the metformin-treated group. Body weight significantly increased during the fourth, fifth and sixth weeks, being more pronounced in the extract-treated groups (272 ± 15.0 g and 227 ± 7.23 g for 200 and 400 mg/kg doses, respectively; the corresponding body weight for untreated diabetic control was 197 ± 9.83 g. Furthermore, both extract doses (200 and 400 mg/kg) produced significant decrease (p ≥ 0.05) in serum glucose (186 ± 15.5 mg/L and 146 ± 15.1 mg/L), cholesterol (143.6 ± 0.86 mg/L and 151.0 ± 0.77 mg/L) and triglyceride (82.6 ± 0.51 mg/dl and 84.8 ± 0.84 m/dl) levels, respectively, similar to the metformin treated group.

Based on ethnopharmacological information, Barleria montana has been used to treat diabetes by the tribals in and around tropical and subtropical areas. But there are no more scientific reports available about the antidiabetic activity of this plant (Shyam Ganapaty, 2013). Hence the study was carried out to ascertain the activity. The plant was extracted with methanol in soxhlet apparatus and the extracts thus obtained were examined for acute toxicity studies in wistar albino rats at different doses upto 2000 mg/kg body weight. The plant extracts were also evaluated for antidiabetic activity at a dose levels of 100, 200 and 400 mg/kg in streptozotocin induced diabetic rats. The plant extracts have not produced any toxic symptoms within the treated animals. The maximum reduction in blood glucose level was observed at 8 and 12th hour after the oral administration of the 400 and 200 mg/kg b. w of methanolic extract of Barleria montana aerial parts respectively. From the observations, it was concluded that the reduction of blood glucose levels in diabetic rats was found to be dose dependent and also dependent on duration of action. So it might be useful in the treatment of diabetes without toxicity.

Orthosiphon stamineus is a popular folk medicine widely used to treat many diseases including diabetes. Previous studies have shown that the sub-fraction of chloroform extract was able to inhibit the rise of blood glucose levels in a glucose
tolerance test. This study was carried out to evaluate the chronic effect and possible mechanism of action of the bioactive chloroform sub-fraction of *O. stamineus* using streptozotocin-induced diabetic rats and *in vitro* methods. Administration of the chloroform extract sub-fraction 2 (Cf 2-b) at a dose of 1 g/kg twice daily on diabetic rats for 14 days showed a significant lowering (p < 0.05) of the final blood glucose level compared to the pretreatment level. However, there were no significant differences in the plasma insulin levels post-treatment compared to the pretreatment levels for all doses of Cf 2-b. Conversely, Cf 2-b at a concentration of 2 mg/mL significantly increased (p < 0.001) the glucose uptake by the rat diaphragm muscle. The increase in glucose uptake was also shown when the muscle was incubated in a solution containing 1 IU/mL of insulin or 1 mg/mL of metformin. Furthermore, the effect of this sub-fraction on glucose absorption in the everted rat jejunum showed that Cf 2-b at concentrations of 0.5 mg/mL, 1 mg/mL and, 2 mg/ml significantly reduced the glucose absorption of the jejunum (p < 0.05-0.001). Similarly, the absorption of glucose was also inhibited by 1 mg/mL and 2 mg/mL of metformin (p < 0.001). These results suggest that the effect of Cf 2-b may be due to extrapancreatic mechanisms. There was no evidence that the plant extract stimulated the release of insulin in order to lower the blood glucose level (El snoussi *et al.*, 2013).

The antihyperglycaemic activity of the hydroalcoholic extract of the leaves of *C. procera* of occurrence in coast of Pernambuco, Brazil. The hydroalcoholic extract of the leaves of *C. procera* (300 and 600 mg/kg/day), vehicle, insulin or metformin (500 mg/ kg/day) were administered orally to streptozotocin induced diabetic rats (n = 7/group) for four weeks. Changes in body weight, food and water intake, biochemical markers, fasting glucose levels and oral glucose tolerance test were evaluated. The results showed that the *C. procera* dried extract (300 and 600 mg/kg) reduced significantly the level of blood glucose throughout the evaluation period and improved metabolic status of the animals and ameliorate the oral tolerance glucose test. The phytochemical screening revealed and quantified the presence of phenolic compounds and flavonoids in a percentage of 29.1 and 2.9%, respectively. Thus, the extract of the leaves of *C. procera* has antihyperglycemic activity (Mário *et al.*, 2013).
Antidiabetic effect of aqueous extract of *C. pareira* leaves (CPAE) was evaluated at 250 mg/kg and 500 mg/kg body weight, p.o. doses in male albino mice over the period of 14 days. Random blood glucose level and body weight were observed periodically. Liver glycogen level, organ coefficient and other biochemical parameters were also determined after completion of the study. Significant (p < 0.001) changes were observed in random blood glucose levels of mice after 14 days of treatment period at 500 mg/kg CPAE treatment. Aspartate transaminase, alanine transaminase, alkaline phosphatase, total bilirubin, triglycerides and creatinine levels were significantly (p < 0.05) decreased while liver tissue glycogen and serum protein levels were significantly (p < 0.05) increased after CPAE administration. No significant changes were observed in body weight and organ coefficient (Kuldeep et al., 2013).

The antidiabetic effect of various extracts of *Desmodium gangeticum* was evaluated on normal and streptozotocin (STZ)-nicotinamide induced type-2 diabetic animals. Type-2 diabetes was induced in Wistar albino rats of either sex by the administration of STZ-nicotinamide (40, 110 mg/kg b.w., respectively) intraperitoneally. *D. gangeticum* (100 mg/kg b.w.) extracts in different solvents (viz. pet. ether, benzene, chloroform, acetone, ethanol and water) were administered to diabetic rats for 21 days. The effect of extracts on blood glucose, lipid profile (TC, TG, LDL-C and HDL-C) and body weight was studied in diabetic rats. *D. gangeticum* extracts significantly reduced the elevated blood glucose, TC, TG, LDL-C level. Reduced HDL-C level and body weight in diabetic animals were found to be elevated significantly by the *D. gangeticum* extracts. But among all the extracts aqueous extract exhibited the best antidiabetic, antihyperlipidemic activity and positive effect on weight of diabetic rats. The results of our study suggest that the aqueous extract of *D. gangeticum* possesses a promising effect on STZ-nicotinamide induced type-2 diabetes (Rekha Bisht, 2013).

*In vitro* evidences for antioxidant and anti diabetic potential of *Trichodesma indicum* to generate a stronger biochemical rationale for further *in vivo* studies. The hydroalchohol extract of *Trichodesma indicum* whole plant was screened for its in vitro antioxidant activity using 2, 2-Diphenly 1-picryl hydrazyl assay, Scavenging of Nitric oxide and Superoxide radical methods, evaluation of Total antioxidant
capacity of the extract and Reducing power assay. Cytotoxic effect of the extract was evaluated by MTT assay and in-vitro antidiabetic effect was studied using the glucose uptake model in rodent skeletal muscle cells (L-6 cells) involved in glucose utilization. The extract of Trichodesma indicum showed good antioxidant properties against 2, 2-Diphenly 1-picryl hydrazyl radical with low IC$_{50}$ values 135µg/ml and against nitric oxide and superoxide radical exhibited poor scavenging properties with IC$_{50}$ value of > 1000µg/ml. The total antioxidant activity which expressed equivalent to Ascorbic acid has 225.28 mg per gram of dried extract. The increased absorbance at 700 nm indicates an increase in reducing power of samples. The drug extract showed percentage growth inhibition value of 500 µg/ml and showed average glucose uptake 91.03±10.12 over the control. The extract of Trichodesma indicum exhibited significant antioxidant activity and moderate antidiabetic activity and merits further investigation in animal models and support traditional claim (Sudharshan et al., 2012).

Terminalia bellirica is a native plant of India belonging to the family Combrataceae (Mary Shoba Das, 2015). The results showed that the extracts did not confer any cytotoxicity and the ethanolic extract showed better glucose uptake potential. The results were compared with insulin and metformin, which were used as the standard antidiabetic drugs. Insulin (1IU/ml) and metformin (100 µg/ml) enhance the glucose uptake over control.

The effect of antidiabetic activity of andrographis paniculata, salacia reticulata and Ocimum sanctum by in vitro screening. Cells were cultured on 6 well plates and incubated for 48 hrs at 37°C in CO$_2$ incubator. When semi confluent monolayer was formed, the culture were renewed with serum free DMEM containing 0.2 % BSA and incubated for 18 hrs at 37°C in CO$_2$ incubator. The cells are treated with Insulin, Standard drug and plant extract and added glucose (1M) and incubated for half an hour. The supernatant was collected for glucose estimation and glucose uptake was terminated by washing the cells three times with 1 ml ice-cold KRP buffer. Cells were subsequently lysed by freezing and thawing three times. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium by GOD-POD method. Results: In vitro study on glucose utilization in L-6 cells showed that the effects of both the extract were found to be
mild over control. *Andrographis paniculata* enhanced the glucose uptake by 16.11 ± 2.76% over control *salacia reticulata* stimulated the uptake of glucose only by 12.44 ± 2.35% over control *Ocimum sanctum* enhanced the glucose uptake by 11.75 ± 2.06 over control. Conclusion The drugs discussed in these studies have exhibited hypoglycemic activity and stimulates glucose uptake in L-6 skeletal muscle cells. Antidiabetic activity of *Andrographis paniculata* was found to be prominent over salacia reticulate, which has shown better activity than *Ocimum sanctum*. This study can bring a promising role for these plants in the management of Diabetes mellitus (Prem Kumar *et al*., 2012).

*Invitro* glucose uptake assay of hydromethanolic leaves extract of *Syzygium jambos* (L) Alston (SJM) on L6 Cell lines. *Syzygium jambos* (L) Alston Commonly known as rose apple belongs to family Myrtaceae, It is one of the most important medicinal plant in India, Brazil. In Brazil decoction of leaves used for diabetes. Hydromethanolic leaves extract of SJM shows cytotoxic concentration (320μg/ml) in normal cells. In the present study we demonstrated that SJM enhance glucose transport in L6 myotubes, the extract possesses significant glucose uptake (% of glucose uptake 113±12.26) compared with standard Insulin (% of glucose uptake 131±17.57) if these effects can be confirmed clinically, SJM Extract may used for prevention of DM. The presented data about hydromethanolic leaf extract of *Syzygium jambos* (L) Alston having activity such as enhancing glucose uptake in skeletal muscle (L6) cell lines. To conclude, this study supports by using natural plant sources (SJM leaves) we can prevent diabetic complications such as diabetic nephropathy, diabetic retinopathy, and diabetic cancer (Yarasu Nagamuni *et al*., 2013).

**ANTIOXIDANT**

Antioxidants have possibly been used to preserve fats long before recorded history. In prehistoric times, it is conceivable that aromatic plants were used to give fats, to be used for medicinal purposes and foods, pleasant odors and flavors. Retardation of oxidative reactions by certain compounds was first recorded by (Berthollet, 1926). Early workers explain the phenomenon as “catalysts poisoning”. In the mid-nineteenth century, Chevreul observed that linseed oil dried much more slowly in oak wood than on glass or natural surfacing; other woods had the same
effect but not to the same extent and observed that oak wood was an anti-drying agent for linseed oil. The course of rancidification of fats remained unknown until Duclaux demonstrated that atmospheric oxygen was the major causative agent of free fatty acid oxidation. Several years later, perhaps, the first report of antioxidants used in fat was by Deschamps observed that gum benzoin and poplar tree extracts could retard rancidity of ointments made with lard. Wright observed that American Indians in the Ohio Valley preserved bear fat using bark of the slippery elm and found that elm was effective to preserve lard and butter fat. Elm bark was patented as an antioxidant 30 years later. Present knowledge of the properties of various chemicals to prevent oxidative breakdown of fats and fatty foods began with the classic studies by Moureu and Dufraise.

During World War I and shortly thereafter these workers tested more than 500 compounds for AA (Hui, 1996). This basic research, combined with the vast importance of oxidation in practically all manufacturing operations, triggered a search for chemical additives to regulate oxidation. According to The Mitochondrial Free Radical Theory of Aging (Cook, 1996), free radicals are a class of molecule with a very simple definition. The nature of atomic structure and of the covalent chemical bond (the features that give an atom its valency) are fixed by the rule that electrons occupy orbitals of atoms, such that an orbital can contain zero, one or two electrons, and that electrons carry less energy when they are one of a pair in an orbital than when they are unpaired. A molecule is only a free radical if it possesses one or more unpaired electrons Fig. 11.

AA is a fundamental property important for life. Many of the biological functions, such as antimutagencity, anticarcinogenicity, and antiaging, among others, originate from this property (Huang, 1992). Many of the natural antioxidants, especially flavonoids, exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory actions (Zhou et al., 2004). The AA of several plant materials has recently been reported however, information on the relationship between AA and phenolic content and composition of many food plants is not available (Cao, 1996).
Antioxidants and Free Radicals

Numerous antioxidants are plant-based and play a fundamental role protecting plants that are open to the elements such as sunlight and severe oxygen stress (Wallace, 1997). It has been suggested that antioxidants may amend cellular oxidative status and prevent biologically significant molecules such as DNA, proteins, and membrane lipids from oxidative damage and as a result lessen the risk of several chronic diseases including cancer and cardiovascular disease (Amarowicz et al., 1996). A sufficient ingestion of natural antioxidants in food is, therefore, of great consequence for the defense of macromolecules against oxidative damage. According to (Stratil, 2006), the cells most frequently damaged by oxidative stress are unsaturated fatty acids in lipids, cholesterol, different functional polypeptides, proteins and nucleic acids. Some antioxidants provide increased protection with increasing concentration, while others have optimal levels after which higher levels exert prooxidant effects. (Moure et al., 2001) reported that there is a general trend for AA to increase with the concentration of the antioxidant up to a certain limit, depending on the antioxidant and the test used, most natural extracts and tests have shown a maximum AA at a 0.05% concentration.

Various phenolic extracts from plant sources have been shown to increase in AA with increasing concentration of the extract. (Gordon, 1992) showed that IP of lard samples increased as concentration of hexane extracts of powdered tanshen (Salvia miltiorrhiza Bunge) increases. (Onyeneho, 1992) also found that increasing the concentration of freeze dried extracts from the bran of durum wheat led to increase AA showed by a decrease in PVs of soy oil. Similarly, ethanolic extracts...
from red grape marc, peels and seed showed increased AA with increase in extract concentration. (Gamez-Meza, 1999) showed that AA of extracts of Thompson grape Bagasse increased as the concentration increased which was shown by the increased stability of soybean oil. The increase in AA of phenolic compounds due to increase in their concentration could be due to an increase in the concentration of potential sites for radical scavenging (Gordon, 1990). On the other hand, at high concentrations, prooxidant effects could arise due to the involvement of the phenolic compounds in initiation reactions (i.e., formation of radicals).

Fig. 12. Complications of Free Radical Oxidative Stress

Extraction Methods

Plant-derived antioxidants, especially, the phenolics have gained considerable importance due to their potential health benefits. Epidemiological studies have shown that consumption of plant foods containing antioxidants is beneficial to health because it down-regulates many degenerative processes and can effectively lower the incidence of cancer and cardio-vascular diseases (Johnsen et al., 2003). Recovery of antioxidant compounds from plant materials is typically accomplished through different extraction techniques taking into account their chemistry and uneven distribution in the plant matrix. For example, soluble phenolics are present in higher concentrations in the outer tissues (epidermal and sub-epidermal layers) of fruits and grains than in the inner tissues (mesocarp and pulp).
Solvent extraction is more frequently used for isolation of antioxidants; both extraction yield and AA of extracts are strongly dependent on the solvent due to different antioxidant potential of compounds with different polarity. (Julkunen-Tiitto, 1985) found a different behaviour in the extraction of different compounds and total extractable polyphenols (TEP). Maximum total phenolic extraction yields were attained with methanol, where as 50% acetone extracted more selectively leucoanthocyanins and no significant effects were observed in the extraction of glycosides (Pin- Der Duh, 1998). Also for extracts from burdock roots, water yielded the greatest amount of extract and exhibited the strongest AA. (Aziza, 1999) reported maximum AA from cocoa by products in the methanol followed by mixtures of chloroform, ether and dichloroethane or chloroform, methanol and dichloroethane. Also (Przybylski, 1998) reported that the AA of buckwheat extracts varied with the polarity of the solvent, those extracted with methanol being the most active.

Literature suggests limitations on reporting antioxidant capacity in cereals based on the various extraction methods. First, the procedure used to extract antioxidants may be incomplete. However, the extract yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent (Yu et al., 2002).

Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable of these solvents are (hot or cold) aqueous mixtures containing methanol, ethanol, acetone, and ethyl acetate. Methanol and ethanol have been extensively used to extract antioxidant compounds from various plants and plant-based foods (fruits, vegetables etc.) such as plum, strawberry, pomegranate, broccoli, rosemary, sage, sumac, rice bran, wheat grain and bran, mango Kernel, mango, citrus and many other fruit peels (Adom et al., 2003). In most of these experiments, the most commonly employed solvent is absolute ethanol or ethanol: water in different proportions although the extraction of phenolic compounds could be improved by using more polar solvents such as methanol (Liyana et al., 2006).
Some authors use methanol: water as an extraction solvent, but the solvent dilution is not regularly acidified, which has shown to improve the extraction. The solid-liquid extraction is a heterogeneous, multi component operation involving the non-steady transfer of solutes from a solid to a fluid. Vegetable materials include different solutes that can be extracted simultaneously at different rates depending on the location (outer surface, pores, vacuoles, etc.) and their partition coefficients (Diaz-Reinoso et al., 2006).

Stability of different extracts from same material was dependent on extracting solvent used for solubilization and removal of polyphenolic compounds. Methanol extracts from cocoa by-products were stable up to 50°C where as other extracts were less stable. (Bonoli, 2004) reported that maximum phenolic compounds were obtained from barley flour with mixtures of ethanol and acetone. Similarly, aqueous methanol was found to be more effective in recovering highest amounts of phenolic compounds from rice bran and Moringa oleifera leaves. (Anwar, 2003) extracted antioxidant compounds from various plant materials including rice bran, wheat bran, oat grouts and hull, coffee beans, citrus peel and guava leaves using aqueous 80% methanol (methanol: water, 80:20 v/v). A study conducted by (Peschel, 2006) on some fruits and vegetable by-products showed that apple, strawberry, cucumber, chicory showed highest phenolic content in methanol extract than water, ethanol, acetone and hexane. It was found that methanol extract was also having high AA when tested by superoxide anion scavenging activity, FTC, DPPH and Rancimat methods.

The AA also depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, as well as the test system and substrate to be protected by the antioxidant (Meyer et al., 1998). It has been suggested that the determining factor for the AA is the lipophilic nature of the molecules and the affinity of the antioxidant for the lipid (Brand-Williams, 1995) A close dependency on the AA of phenolic compounds, and even the recommended concentration of synthetic antioxidants has been indicated for some tests (Von Gadow, 1997). The antioxidant potential of a compound is different according to different antioxidant assays or, for the same assay when the polarity of the medium differs, since the interaction of the antioxidant with
other compounds play an important role in the activity, dramatic difference in the relative antioxidant potential of model compounds were observed when one model compound is strongly antioxidant with one method and prooxidant with another. A phenomenon known as “polar paradox” has been repeatedly reported; hydrophilic antioxidant are more effective than lipophilic antioxidants in bulk oil, whereas lipophilic antioxidants present greater activity in emulsions.

**Total Phenolic Content (TPC)**

It is highly impractical to quantify all of the compounds in plants that exhibit antioxidant activities; a variety of methods have been developed to quantify total antioxidant capacity of plant extracts (Pekkarinen et al., 1999).

Most natural antioxidants are multifunctional in complex heterogeneous foods; their activity cannot be assessed by any one method (Karamac, 1997). Measurement of TPC quantifies the total concentration of phenolic hydroxyl groups present in an extract being assayed, regardless of the specific molecules in which the hydroxyl groups occur (Kuti, 2004). The assays used to measure total phenols do not, therefore, provide information on the particular phenolic compounds present in a sample. Some common methods for the measurement of total phenols included the colorimetric Prussian blue, Folin-Denis ferric ammonium citrate or FC methods. The FC method, however, appears to be the popular method widely used for the determination of total phenols. The method is based on the reducing power of hydroxyl groups, which react with the FC phenol reagent (an oxidizing agent comprised of heteropolyphosphotungstate-molybdate) under basic conditions to form chromogens that can be detected spectrophotometrically at 760 nm.

The FC method has been used for many years as a means to determine total phenolics in natural products (Frankel, 2000). Many studies show the recommended reference standard (gallic acid) being replaced with tannic acid equivalents, caffeic equivalents, vanillic acid equivalents and catechin equivalents, among others. Phenolic compounds can be found in flavonoids, phenolic acids, hydroxycinnamic acid derivatives and lignans.
Antioxidant Capacity Assays

It is of great interest to the general public, medical and nutritional experts, and health and food science researchers to know the antioxidant capacity and constituents in the foods we consume. Due to the complexity of the composition of foods, separating each antioxidant compound and studying it individually is costly and inefficient, notwithstanding the possible synergistic interactions among the antioxidant compounds in a food mixture. A total antioxidant capacity assay using only one chemical reaction seems to be rather un-realistic and not easy to rely, yet, there are numerous published methods claiming to measure total antioxidant capacity in vitro. In fact, phenols are presumed to be responsible for the beneficial effects derived from the consumption of whole grains, fruits, and vegetables. Phenolic compounds have strong in vitro and in vivo AAs associated with their ability to scavenge free radicals, break radical chain reactions, and chelate metals. Moreover, high phenol consumption has been correlated with a reduced risk of cardiovascular diseases and certain cancers (Sanchez- Maren, 2002).

Several methods have been described to assess AA of compounds both in vitro and in vivo, such as the measurement of prevention of oxidative damage to biomolecules such as lipids or DNA and methods assessing radical scavenging ability (Waterman, 1994). The following in vitro methods have been described: Ferricthiocyanate (FTC) method, β-carotene-linoleate model system and free radical scavenging using 2, 2-diphenyl-1 picrylhydrazyl (DPPH).

No single assay will accurately reflect all of the radical foundations or all antioxidants in a mixed or complex system and it must be appreciated at the outset that there are no simple universal methods by which antioxidant capacity can be measured accurately and quantitatively; also too many analytical methods result in inconsistent results, inappropriate application and interpretation of assays and improper specifications of antioxidant capacities (Prior, 2005). There are two reaction mechanisms in which antioxidants can deactivate radicals. The first of these methods is the single electron transfer (SET) assay which detects the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals.
According to (Prior, 2005), SET reactions are usually slow and can require a lengthy time to reach completion, so the antioxidant capacity calculations are based on per cent decrease in product rather than kinetics. The second method is the hydrogen atom transfer (HAT), which measures the antioxidant’s ability to quench free radicals by hydrogen donation. HAT reactions are solvent and pH independent and are usually quite rapid. The presence of reducing agents, including metals, is a complication in HAT assays and can lead to erroneously high apparent reactivity (Halliwell, 1995). There are methods utilizing both HAT and SET mechanisms. The TEAC and DPPH assays are usually classified as SET reactions, however, these two indicator radicals in fact may be neutralized either by direct reduction via electron transfers or by radical quenching via HAT (Kim, 2010).

2, 2-Diphenyl-1-picrylhydrazyl Activity

DPPH is one of a few stable and commercially available organic nitrogen radicals bearing no similarity to the highly reactive and transient peroxyl radicals involved in various oxidative reactions in vivo (Wanasundara, 1996). This assay is based on the measurement of the reducing ability of antioxidants towards DPPH. The ability can be evaluated by electron spin resonance or by measuring the decrease of its absorbance. The measurement of the loss of DPPH color at 515 nm following the reaction with test compounds is what the antioxidant assays are based on (Prior, 2005). DPPH radical, with a deep violet color, receives a hydrogen atom from the antioxidant and is converted to a colorless molecule. Using this reagent, the free radical scavenging ability of the antioxidant can be determined by spectrophotometric methods. Yen and Duh 1994, Chen and Ho 1995 have reported that inhibition of free radical formation by different antioxidants can be measured using very stable free radicals such as 2, 2-diphenyl-1-picrylhydrazyl. Using the DPPH assay has its advantages. It is simple, rapid and needs only a UV vis spectrophotometer to carry out the experiment.

Reducing Power (RP)

RP increases according to the increase in absorbance. As more Fe$^{3+}$ are reduced to the ferrous form or when more electrons are donated by antioxidant components; the colour of Perl’s Prussian blue will be darker, resulting in an
increased absorbance reading. Reductants also react with certain precursors of peroxides, thus preventing the formation of peroxide (Jayaprakasha et al., 2001). The RP is found to be dependent on extract concentration. Yen (1995) also reported that RP of tea extracts increased markedly with increasing extract concentration.

**Ferric Thiocyanate (FTC) Capacity**

Real food Systems generally consists of multiple phases in which lipid and water co exists with some emulsifier. Hence an antioxidant assay using heterogenous systems such as oil-in water emulsion is one of the model systems for such evaluation, satisfying above conditions. The linoleic acid emulsion system/thiocyanate method has been used here for evaluation under above conditions (Osawa Toshihiko, 1981). During peroxidation in an incubator the absorbance values increased owing to oxidation products, which react to form ferric thiocyanate the colour of red blood. Antioxidants can hinder the oxidation and consequently, the increase in absorbance will be less.

**β-carotene bleaching assay**

Mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from hydroperoxides formed from linoleic acid. B-carotene, in this model system undergoes rapid discolouration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecule and looses its chromophore and characteristic orange colour (Ramakrishna et al., 2008).

**Lipid Oxidation**

Lipid oxidation is a general term used to describe a complex sequence of chemical interactions between unsaturated fatty acyl groups in lipids with active oxygen species (Coupland, 1996). The unsaturated fatty acids on triacylglycerols and phospholipids have low volatility and do not directly contribute to the aroma of foods. However, these fatty acids will decompose during lipid oxidation to form small, volatile molecules that produce the off-aromas associated with oxidative rancidity. These volatile compounds are detrimental to food quality except in the case of food products such as fried foods, dried cereal, and cheeses, where small amounts of these volatile compounds are important in their flavour profile.
The oxidative deterioration of fats and oils in foods is responsible for rancid odours and flavours, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic, compounds. The addition of antioxidants is required to preserve flavor and colour and to avoid vitamin destruction (Abdalla, 1999). The mechanisms of lipid oxidation in a particular food depend on the nature of the reactive species present and their physico-chemical environment.

Lipid oxidation reactions are dependent on the chemical reactivity of numerous components including reactive oxygen species, prooxidants, and antioxidants. However, research over the past few decades has shown that the physical properties of food systems are extremely important to the chemistry of lipid oxidation (Frankel, 1994 and Nawar, 1996).

Understanding how the physical nature of bulk oils impacts lipid oxidation reactions could lead to the development of new antioxidant technologies and to the more efficient use of existing antioxidant ingredients. For example, if association colloids are found to be the major site of oxidation reactions in bulk oils, then altering these structures or more efficiently delivering antioxidants to the site of oxidation could significantly improve the oxidative stability of oils. Thus, increasing the understanding of oxidative mechanisms in oils could increase the use of nutritionally beneficial polyunsaturated lipids in foods.

**a) Mechanism of Lipid Oxidation**

Theoretically, lipid oxidation is a free radical chain reaction between unsaturated fats and oxygen that can occur in an autocatalytic manner. However, lipid oxidation in many food systems is accelerated by prooxidants such as transition metal ions, photosensitizers, UV light, and certain enzymes. The overall mechanism of lipid oxidation involves three stages:

- initiation - the formation of free radicals
- propagation - the free-radical chain reactions and
- termination - the formation of nonradical products
In the initiation step, a fatty acid radical known as the alkyl radical (L•) is formed by abstraction of a hydrogen from a fatty acid in the presence of an initiator (In•). Once the alkyl radical forms, the free radical can delocalize over the double bond system resulting in double bond shifting and in the case of polyunsaturated fatty acids, formation of conjugated double bonds. In bulk oils, the ease of formation of alkyl radicals in fatty acids increases with increasing unsaturation. The first step of propagation involves the addition of oxygen to the alkyl radical resulting in the formation of peroxyl radical (LOO•).

b) Lipid Oxidation Decomposition Products

Rancidity in food occurs when unsaturated fatty acids decompose into volatile compounds. These volatile oxidation products are produced from the decomposition of fatty acid hydroperoxides. The homolytic cleavage of hydroperoxides (LOOH) between these two oxygen molecules is the most likely hydroperoxide decomposition pathway. This reaction yields an alkoxy (LO•) and a hydroxyl radical (•OH). The alkoxy radical (LO•), which is more energetic than either the alkyl (L•) or peroxyl radical (LOO•), can enter into a number of different reaction pathways. Alkoxy radicals can attack another unsaturated fatty acid, a pentadiene group within the same fatty acid or the covalent bonds adjacent to the alkoxy radical. This last reaction is known as β-scission reaction and is important to food quality as it can cause fatty acids to decompose into low molecular weight, volatile compounds that cause rancidity.

c) Antioxidants (oxidation inhibitors)

Incorporation of antioxidants into foods is one of the most effective methods of retarding lipid oxidation. However, many factors can impact the activity of antioxidants with some antioxidants retarding lipid oxidation under certain conditions but promoting lipid oxidation under other conditions. Several degradation reactions, which may occur on heating or during long term storage, deteriorate fats and oils and the lipid constituents of foods.

Oxidation reactions and the decomposition of oxidation products are the main processes which result in decreased nutritional value and sensory quality (Huang et al., 1994). Research has implicated oxidative and free-radical-mediated reactions in
degenerative processes related to ageing and diseases such as cancer, coronary heart disease and neurodegenerative disorders such as Alzheimer's disease (Gordon, 2001). The prevention or retardation of these oxidation processes is essential for the food producer and almost everyone involved in the entire food chain from “farm to fork”. Various methodologies may be employed to inhibit oxidization, including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure and the use of suitable packaging (Lachance et al., 2001). Another common method of protection against oxidation is to use specific additives which inhibit or retard oxidation. These oxidation inhibitors are generally known as antioxidants.

Antioxidants can be classified according to their mechanisms of action as either primary or secondary antioxidants. However, some substances have more than one mechanism of antioxidant activity and are referred to as multiple-function antioxidants.

**Primary Antioxidants**

Primary or chain-breaking antioxidants retard lipid oxidation by interfering with chain propagation, initiation, reactions by accepting free radicals and forming stable free radicals that do not further promote initiation or propagation reactions (Yanishlieva et al., 2001). For a primary antioxidant to be effective it must inactivate free radicals before they can attack unsaturated fatty acids. Although, there are many synthetic free radical scavenging antioxidants (BHA, BHT, PG and TBHQ), that are efficient and relatively cheap these synthetic compounds are “label unfriendly” additives. Special attention has been given to the use of natural free radical scavengers due to the worldwide trend to avoid or minimize the use of synthetic food additives. Many natural free radical scavengers such as catechins (catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate) also have health benefits as inhibitors of biologically harmful oxidation reactions in the body (Li et al., 2000). Therefore, a better understanding of the properties of natural free radical scavengers could lead to the development of food additives that could both prevent oxidative deterioration of foods while also providing health benefits. Taking advantage of rancidity prevention and health promoting properties of natural free radical scavengers could help overcome their limitation in effectiveness, price, associated flavours and colours.
Secondary Antioxidants

Secondary antioxidants prevent the formation of free radicals in the initiation step by scavenging oxygen, e.g. ascorbic acid or chelating metal ions, e.g. citric acid and flavonoids. Some phenolic antioxidants have synergistic effects with ascorbic and citric acids. Ascorbic acid is primarily an effective oxygen scavenger, but when used in combination with tocopherol gives rise to a stronger synergistic antioxidant effect. The synergistic effect of ascorbic acid with tocopherol is believed to be due to the ability of ascorbic acid to regenerate tocopherol by reducing the tocopheroxyl radical resulting from the primary antioxidant effect of the tocopherol (Vinson et al., 2001).

Chelators

Transition metals are prooxidants capable of accelerating lipid oxidation reactions. Chelators are a group of secondary antioxidants that can bind and thus inactivate or reduce the activity of prooxidant metals. The most common food chelators are citric acid (and its lipophilic, mono glyceride ester), phosphoric acid (and its polyphosphate derivatives), and ethylene diamine tetra acetic acid (EDTA). It should be noted that some chelators can be ineffective when used alone but can greatly enhance the action of phenolic free radical scavengers when used in combination. In addition, some chelators may increase oxidative reaction under certain conditions by increasing metal solubility.

Oxygen Scavengers and Reducing Agents

Oxygen is a substrate in lipid oxidation reactions. Ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate, and sulfites are able to scavenge oxygen and act as reductants. Ascorbic acid and sulfites retard lipid oxidation by reacting directly with oxygen to eliminate it from food. In recent years, oxygen scavengers have been directly incorporated into packaging materials, however, these technologies are still too expensive for most food packaging applications (Whysner et al., 1994).
Singlet Oxygen Quenchers

Singlet oxygen (O_2) is an excited state of oxygen that can be formed by enzymatic reactions in biological systems or in the presence of photosensitizer, light and triplet oxygen. Singlet oxygen can be inactivated through both chemical and physical quenching pathways by compounds such as tocopherols, carotenoids (including β-carotene, lycopene, and lutein), polyphenols (including catechins, and flavonoids), amino acids, peptides, proteins, urate, and ascorbate (Khokhar, 2003).

Interactions between Antioxidants and Multiple Antioxidant Functions

It has been reported that when two antioxidants are used together, a synergistic or additive effect can be observed. Vitamin E and vitamin C are a well-known example of a synergistic system. Vitamin E acts as a lipid soluble chain-breaking antioxidant and vitamin C reduces the tocopherol radical back to its original state so that it can continue to inactivate free radicals in the lipid phase. If a chain-breaking and a secondary antioxidant are mixed, both initiation and propagation are suppressed. Thus, a combination of chelators and free radical scavenging antioxidants are often very effective. For instance, chelating antioxidants such as citric or other acids can enhance the activity of phenolic antioxidants (Kikugawa, 1990). A synergistic relationship between ascorbic acid and phosphates in retarding lipid oxidation was also observed in meat.

Antioxidants can also have multiple functions such as free radical scavenging and metal chelation. Examples include propyl gallate (PG), proteins and proanthocyanidins. Ascorbic acid is also a multifunctional antioxidant in food systems. It can quench singlet oxygen, reduce free radicals and antioxidant radicals, and remove molecular oxygen in the presence of metal ions (Decker, 2002). The antioxidant principles were characterized as phenolic compounds and phospholipids (Mahoney, 1986). The phenolics were assumed to be mainly gallic acids and gallates. In another study, gallotannins and condensed tannin-related polyphenols were reported in mango kernels (Tong et al., 2000). Ethanolic extracts of mango kernels displayed a broad antimicrobial spectrum and were more effective against Gram-positive than against Gram-negative bacteria. Their active component was shown to be a polyphenolic-type structure; however, its exact nature still remains to be elucidated.
NATURAL ANTIOXIDANTS

Natural versus synthetic antioxidants

Plants contain a variety of substances called “Phytochemicals” that owe to naturally occurring components present in plants. The phytochemical preparations in preventing lipid oxidation have tremendous potential for extending shelf life of food products. Several research groups around the world have succeeded in finding and identifying natural antioxidants from herbs and spices using different model systems (Yamaguchi et al., 1999).

Increased intake of dietary antioxidants may help to maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organisms (Schuler, 1990). Antioxidants are widely used in the manufacture, packaging and storage of lipid-containing foods. Much interest has developed during the last few decades in naturally occurring antioxidants because of the adverse attention received by synthetic antioxidants (Purvankara et al., 2000) and also the worldwide trend to avoid or minimize the use of artificial food additives. Halliwell, 1989 defined an antioxidant as any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate.

The primary purpose of adding antioxidants to lipids is to delay the onset of oxidation and accumulation of oxidative products (Wanasundara, 1998). Antioxidants may delay or inhibit oxidation, but cannot improve the quality of an already oxidized product (Kochhar, 1990). Antioxidants may act either as primary (chain breaking) or as secondary (preventive) antioxidants. Some phenolic antioxidants are synergists due to the presence of other non-phenolic substances such as citric acid.

Hundreds of materials, both synthetic and of natural origin, have been developed as antioxidants for food preservation, but only BHA and BHT as synthetic antioxidants and tocopherols as natural ones are practically used. Synthetic antioxidants such as BHT, BHA, TBHQ and PG are currently widely used in the food industry to increase the oxidative stability of fats and oils (Chu, 1999). However, the most commonly used synthetic antioxidants, BHA, BHT, PG and TBHQ are faced
resistance in recent times due to accumulating evidence that they could be toxic, carcinogenic or mutagenic (Frankel, 1996) and also suspected of causing liver damage. There has been a growing interest in the exploration of natural antioxidants as food additives due to these safety concerns (Malecka, 2002).

On the other hand, tocopherols are widely used as safe natural antioxidants, but they are not as effective as synthetic antioxidants and the manufacturing cost is high. These circumstances stimulated the isolation of new antioxidants from natural sources, especially from plant materials. They are added to an extensive variety of foods in order to prevent or retard oxidation. Since continued use of chemical antioxidants such as BHA and BHT has been reported to cause teratogenic and carcinogenic effects in small animals and primates, commonly used vegetable leaves which are rich in antioxidants would probably prove safe to the health of the consumers.

(Shi, 2001) stated that natural antioxidants are safer, more potent and more efficient than synthetic antioxidants. For example, α-tocopherol (the most active primarily because α-tocopherol transfer protein selectively recognizes natural α-tocopherol. In addition, the possible activity of synthetic antioxidants as promoters of carcinogenesis has become a concern. Therefore, replacing synthetic antioxidants with natural alternatives, or simply replacing all synthetic food additives with natural choices, has attracted great interest over the past two decades.

**Plants as sources of antioxidants**

Antioxidant properties of common spices and herbs were systematically investigated in the 1950s. Since then, numerous studies have been devoted to vegetable sources, not only for their possible utilization as antioxidative extracts but also to look for new, naturally efficient antioxidants. Several antioxidants have been characterized from plant leaves and some products or extracts proved to be as good as the synthetic BHA and BHT (Sherwin, 1990).

Natural antioxidants may be found in any plant part. Fruits, vegetables, spices, nuts, seeds, leaves, roots and barks have been considered as potential sources of natural antioxidants. Antioxidants in flaxseed, sunflower, soybean, cottonseed and
canola typify those found in oilseeds. The majority of natural antioxidants are phenolic compounds, and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids that are common to all plant sources (Vekiari et al., 1993).

Naturally occurring antioxidants are presumed safe since they are found in food that has been used by man for centuries. The natural antioxidants in food may also be beneficial because there is evidence that they prevent cancer development and that they inhibit oxidative reactions in the body system. Phenolic compounds have both primary and secondary antioxidant activities by acting both as chain breaking antioxidants by donating hydrogen atoms to lipid radicals (radical scavenging) or by preventing initiation reactions by such means as chelating metals (Pratt, 1990). Both the chain breaking (radical scavenging) and metal chelating properties are known to be possible due to the structural features of the phenolic compounds.

Phenolic extracts from several plant sources have been shown to have antioxidant activities. These include potato peels (Rodriguez, 1994), tomatoes (Abushita et al., 1997), garlic and ginger (Aruoma et al., 1997), sunflower seed, wheat germ, fruits, vegetables and medicinal plants (Velioglu et al., 1998), grape seed, tea, sage and thyme, plums, grape marc, red clove and tree spinach. Thompson grape Bagasse is byproduct with high phenolic compounds and is a source of natural antioxidant. Phenolic compounds from sorghum such as condensed tannin (Naczk et al., 1998), flavones (such as naringen) and anthocyanins (Chun et al., 2003) have also been reported to possess AA.

An increasing demand for natural additives has shifted the attention from synthetic to natural antioxidants. As leafy vegetables are found to be good source of antioxidants and the present study is to examine the antioxidant potential and antimicrobial activity of leaf extracts of Pimpinella tirupatiensis. Antioxidant potential of leaves of P. tirupatiensis was studied using different methods like DPPH, nitric oxide, hydrogen peroxide scavenging activity. Reducing power and antimicrobial activity was estimated by using both gram positive and gram negative microorganisms by using DMF as solvent. The aqueous extracts showed maximum scavenging activity of DPPH followed by nitric oxide, hydrogen peroxide and reducing power respectively. Benzene and alcoholic extract showed maximum antimicrobial activity (Tharun et al., 2013).
In vitro anti-oxidant activities of n-hexane, chloroform and ethanol extract of Citrullus lanatus seeds to find out the extract having highest anti-oxidant activity. The In-vitro anti-oxidant activity of the extracts were studied using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, ferric reducing power activity, hydrogen peroxide (H2O2) scavenging activity and nitric Oxide (NO) scavenging activity. The total Phenolic contents and flavanoid contents were estimated taking gallic acid and quercetin calibration curve respectively. In in-vitro anti-oxidant studies it was found that all the extracts posses In-vitro anti-oxidant activities. But the order of possessing activities were n-hexane>ethanol>chloroform extracts of Citrullus lanatus seeds. The total Phenolic content was highest in n-hexane extract and total flavanoid content was highest in ethanol extract. It can be concluded that Citrullus lanatus seed extracts possess anti-oxidant activities and the potency of anti-oxidant activities depends on the type of extract. The n-hexane extract of Citrullus lanatus seeds possess highest anti-oxidant activity in vitro (Habibur et al., 2013).

Herbal drugs are frequently considered to be less toxic and also free from side effects, than synthetic ones. Hence, the present study was designed to investigate one such combination of herbal drugs, Asystasia gangetica and Morus indica for their antidiabetic and antioxidant potential against alloxan-induced diabetes in albino rats. The effect of both individual and a combination of Asystasia gangetica and Morus indica on blood glucose and liver glycogen were studied in the diabetic rats. The study also assessed for the effect of selected plant extracts for their effect on superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and lipid peroxidation (LPO) in the homogenates of the pancreas. The results of the present study attests significant antidiabetic and antioxidant potential for the selected plants individually and also in combination as a prominent decrease in blood glucose and liver glycogen was observed in the rats treated with the extracts of the selected plants. Similarly, the levels of the protective antioxidant enzymes like SOD, CAT and GSH were increased along with decrease in the LPO levels. The present study provides a scientific evidence for antidiabetic and antioxidant potential of Asystasia gangetica and Morus indica. Further studies to isolate bioactive compounds will pave the way to identify potential lead compounds for developing safe and efficacious antidiabetic agents (Pradeep Kumar, 2010).
The methanolic crude extracts of some commonly used medicinal plants were screened for their free radical scavenging properties using ascorbic acid as standard antioxidant. Free radical scavenging activity was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The overall antioxidant activity of green tea (Camellia sinensis Linn.) was the strongest, followed in descending order by black tea (Camellia sinensis Linn.), Eugenia caryophyllus (Spreng.) Bullock and Harrison, Piper cubeba Linn, Zingiber officinale Roscoe and Piper nigrum Linn. Trigonella foenum graecum Linn. and Elettaria cardamomum (Linn.) Maton showed weak free radical scavenging activity with the DPPH method. All the methanolic extracts exhibited antioxidant activity significantly. The IC$_{50}$ of the methanolic extracts ranged between 6.7 $\pm$ 0.1 and 681.5 $\pm$ 8.4 $\mu$g/ml and that of ascorbic acid was 8.9 $\pm$ 0.1 $\mu$g/ml. The study reveals that the consumption of these spices would exert several beneficial effects by virtue of their antioxidant activity (Nooman et al., 2008).

The antioxidant and free radical scavenging activity, five different extracts of Terminalia arjuna bark were examined. In the present study, the free radical scavenging potential of five extracts of the bark of Terminalia arjuna was assessed by measuring its capability for scavenging 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical, hydrogen peroxide radical, nitric oxide radicals (NO), as well as its ability in reducing power capacity assessment, cupric reducing antioxidant capacity, using appropriate assay systems compared to natural and synthetic antioxidants. Total antioxidant capacity, phenolic and flavonoid contents were determined spectrophotometrically. In DPPH free radical scavenging activity, the highest IC$_{50}$ value was showed by methanol extract with a value of 6.34 $\mu$g/ml followed by ethanol and petroleum ether having value of 7.76 and 25.63, respectively, as opposed to that of the scavenging effects of ascorbic acid and butylated hydroxytoluene (BHT) of 5.698 and 8.816, respectively. Methanol extract showed highest activity having IC$_{50}$ value of 14.436 and 25.184 $\mu$g/ml in hydrogen peroxide and nitric oxide scavenging assay, respectively. All the five fractions showed good reducing power and cupric reducing capacity with increasing concentration again taking methanol extract to the top position. The methanol extract yielded 817.488 $\pm$ 8.108 mg/g gallic acid equivalent phenolic content and 199.122 $\pm$ 8.282 mg/g quercetin equivalent flavonoid content that was highest among five extracts. Methanol extract of T. arjuna was found to possess the highest total antioxidant capacity (415.925 $\pm$ 2.291)
followed by ethanol (377.675 ± 1.889) mg/g Ascorbic Acid Equivalent, respectively. A linear correlation appeared between the total antioxidant capacity and the total phenolic contents of the extracts with good correlation coefficient (R2 = 0.891). n-Hexane and chloroform extract showed least activity in all the measures. The results obtained beacon that T. arjuna is a potential source of antioxidants and thus could prevent many radical related diseases (Mohammad Shahriar et al., 2012).

2.4 TLC AND HPTLC

Science and technology have never been so promising nor have delivered so many opportunities to improve health and extend lives, but continued investments are being invested in both the public and private sector, in spite of the current economic climate. Increasing pharmaceutical industry success rates and delivering more medicines are very challenging, but very few predictive scientific and analytical tools are available. Research on drugs involves production control of bulk drug and final product, toxicological analysis of side effects of the drug or its possible impurities, and determination of the fate of a drug and its metabolites in an organism by the monitoring of body fluids. Common criteria for drug evaluation include the quality and therapeutic value of the bulk drug and pharmaceutical product, identification studies, purity, content, uniformity, chemical and physical stability, and biological availability.

Analysis of pharmaceutical compounds and newer drugs is commonly used in all the stages of drug discovery and development process. These analytical techniques provide more accurate and precise data, not only supporting drug discovery and development but also postmarket surveillance. Pharmaceutical analysts work regularly to improve the reliability of existing techniques to cope up the demands for better chemical measurements. Modern pharmaceutical analysis is mainly dominated by costlier instrumental analysis. Hence, many analysts’ focus is on developing newer applications, discoveries, and new methods of analysis to increase the specificity and sensitivity of a method. Analytical methods used in drug analysis are diversified and are still being improved to find better solutions to satisfy manufacturers and institutions that test drug quality. Official documents dealing with the problem of QC of pharmaceutical products recommend diversified analytical techniques, with chromatographic methods playing a significant role in pharmaceutical analysis.
Thin layer chromatography studies are among the key identity tests in most pharmacopoeial monographs. Pharmacopoeial standards are typically used by industry as a basis for meeting QC requirements and current good manufacturing practices (cGMPs). An extension of TLC is high-performance thin layer chromatography (HPTLC) is robust, simplest, rapid, and efficient tool in quantitative analysis of compounds. HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution. The separation can be further improved by repeated development of the plate, using a multiple development device. As a consequence, HPTLC offers better resolution and lower Limit of Detection (LODs).

Visual detection is suitable for qualitative analysis, but a more specific detection method is needed for quantitative analysis and for obtaining structural information on separated compounds. UV, diode-array and fluorescence spectroscopy, mass spectrometry (MS), Fourier-transform infrared (FTIR), and Raman spectroscopy have all been applied for the in situ detection of analyte zones on a TLC plate. Van Berkel and coworkers have recently described couplings of TLC to atmospheric pressure chemical ionization and electrospray ionization. In both couplings, a special surface sampling probe is used for extracting the analyte on-line from the TLC plate to MS analysis. The usage of HPTLC is well appreciated and accepted all over the world. Many methods are being established to standardize the assay methods. HPTLC remains one step ahead when compared with other tools of chromatography.

One of the available chromatographic techniques is HPTLC, which is used for the identification of constituents, identification and determination of impurities, and quantitative determination of active substances. The use of modern apparatus such as video scanners, densitometers, and new chromatographic chambers, and more effective elution techniques, high-resolution sorbents with selected particle size or chemically modified surface, the possibility of combining with other instrumental methods, and development of computer programs for method optimization all make HPTLC an important alternative method to HPLC or gas chromatography.
Specifically, HPTLC is one of the ideal TLC technique for the analytical purposes because of its increased accuracy, reproducibility, and ability to document the results, compared with standard TLC. Because of this, HPTLC technologies are also the most appropriate TLC technique for conformity with GMPs. Today the comprehensive use of TLC in pharmaceutical analysis is demonstrated by the great number of articles published in this field.

HPTLC remains one of the most flexible, reliable, and cost-efficient separation technique ideally suited for the analysis of botanicals and herbal drugs. Used with standardized procedures, it guarantees reproducible results, a vital element in the routine identification of complex fingerprints of plant extracts and pharmaceutical products. It has established itself as the method of choice for handling complex analytical tasks involving herbal drugs and botanicals. The unique combination of state-of-art instrumentation, standardized procedures, and solid theoretical foundations enables it to deliver reliable, cGMP-compliant results time after time.

High-throughput analysis using HPLTC is being aimed at the rapid analysis of large numbers of compounds. This field has been expedited by the requirement to provide analytical support for multiple drug targets emerging from the field of molecular biology, human genetics, and functional genomics. Further, drivers for development have been in the support for the analysis of large compound libraries arising from parallel and combinatorial chemistry, and economic pressure to reduce time-to-market for new drug candidates.

2.4.1 Applications

HPTLC is one of the most widely applied methods for the analysis in pharmaceutical industries, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food and drug analysis, environmental analysis, and other areas. It is due to its numerous advantages, for example, it is the only chromatographic method offering the option of presenting the results as an image. Other advantages include simplicity, low costs, parallel analysis of samples, high sample capacity, rapidly obtained results, and possibility of multiple detection. Le Roux et al evaluated a HPTLC technique for the determination of salbutamol serum levels in clinical trials.
and established as a suitable method for analyzing samples from the serum. Many lipids have also been analyzed and studied using HPTLC; 20 different lipid subclasses were separated using HPTLC with the reproducible and promising results. Many reports on studies related to clinical medicine have already been published in many journals. HPTLC is now strongly recommended in the analysis of drugs in serum and other tissues. HPTLC is also used in analyzing the purity and efficacy of many pharmaceutical preparations and dosage forms. Puranik et al developed and validated a simple, rapid, and accurate chromatographic methods (HPLC and HPTLC) for simultaneous determination of ofloxacin and ornidazole in solid dosage form. The amount of ofloxacin and ornidazole estimated as percentage of label claimed was found to be 100.23 and 99.61% with mean percent recoveries 100.47 and 99.32%, respectively. Both these methods were found to be simple, precise, accurate, selective, and rapid and could be successfully applied for the determination of pure laboratory prepared mixtures and tablets.

TLC has been known as the fast tool for the detection of compounds. Another advantage of TLC is the capability to detect more compounds than HPLC, although the resolution is poorer. In this regard, the compounds which cannot be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g., sugar, still can be detected by reagent spraying. The TLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. Thus, it can be used as a tool in the quality control in order to warranty that the active compounds are extracted. HPTLC is commonly applied for the identification, assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal & animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics and nutrients (Biringanine et al., 2006).

The main advantages of HPTLC over other conventional analytical methods, is that many samples can be run simultaneously using a little volume of mobile phase, thus reducing the time and cost per analysis (Faisal et al., 2009). These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post chromatographic derivatization reagents. HPTLC has become a
routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness. It has proved a very useful technique because of its low operating cost, high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis (Alam et al., 2011).

The fruits of *Averrhoa bilimbi* L. are used to treat skin disorders, fever, for scurvy and beneficial in diarrhoea, hepatitis and in inflammatory condition. It is also used to treat hyperlipidaemia and possess potential antibacterial and antioxidant activity. Pharmacognostical standards on bilimbi fruits are not yet available for correct identification of plant material and to ascertain its quality and purity. The present investigation was therefore undertaken to determine the requisite pharmacognostical standards according to the Pharmacopoeial guidelines for evaluating the fruit. Pharmacognostical evaluation included examination of morphological and microscopical characters, physicochemical properties, phytochemical analysis, fluorescence study and HPTLC fingerprint. The powder microscopy showed the presence of simple and glandular trichomes and spiral thickening of vessels. Phytochemical screening reported the presence of carbohydrates, proteins, amino acids, flavonoids, tannins and hydrolysable tannins. The HPTLC fingerprint developed for the separation of phytoconstituents is unique to *A. bilimbi* L. fruit powder. HPTLC fingerprint has been developed; as the chemical fingerprint obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines. This unique band pattern obtained from HPTLC fingerprint can be used for the identification of *A. bilimbi* L. fruits. These studies provide referential information for correct identification and standardization of *A. bilimbi* L. fruit (Avinash et al., 2013).

Most of the traditional medicinal plants in India are not scientifically validated. Scientific evaluation along with traditional knowledge is essential to obtain effective drugs for commercial purpose. *Solena amplexicaulis* belongs to the family, Cucurbitaceae, a traditional medicinal plant species of TamilNadu, India is being prescribed to cure various diseases. The present study is to establish the chemical fingerprint through TLC and HPTLC analyses for various secondary metabolites in this species. The profiles of various individual secondary metabolites were made and
developed for authentication. The methanolic tuber extract showed the presence of 5 alkaloids, 6 flavonoids, 2 glycosides, 10 saponins and 7 terpenoids. The development of such fingerprint can be used in differentiation of the species from the adulterant in terms of phytochemical constituents and hence act as biochemical markers in the pharma industry and plant systematic studies (Karthika et al., 2014).

The aerial parts (fresh leaves, pods, flowers) of the plant *Sesbania grandiflora* were collected powdered and the powder was initially defatted with petroleum ether (60-80°C). The marc was further macerated with ethanol for 72 hrs. The extract was preserved at room temperature for further studies. Preliminary Phytochemical screening was done which revealed the presence of sugars, tannins, polyphenols, flavonoids, aminoacids, triterpenoids, saponins, flavonoids. The TLC techniques were used for qualitative determination of possible number of components from the ethanolic extract. A solvent system was optimized in order to get maximum separation on plate and presence of various phytochemicals present was confirmed by the use of different spraying reagents (Avalaskar et al., 2011).

There are a numbers of bioactive compounds in plants, such as alkaloids, tannins, flavonoids, sterols, triterpenes, etc., noted to have the major role in nutrition, physiology and control of diseases. Flavonoids constitute one of the most characteristic classes of compounds in higher plants. The foremost important task in this paradigm is the screening of these compounds in the plants. The chromatographic study of the compounds serves to be a very useful and reliable source in the process of bioactive compounds screening in plants. According to the ethnobotanical information, it has been reported that the plant *Launaea procumbens* possesses the anticancer potential. Hence in the present study, an attempt has been made to identify the flavonoid constituent of *Launaea* by using TLC and chemical derivatization method. Further, the isolation of the same compound is carried out by preparative HPTLC using the standardized solvent system viz., ethyl acetate: formic acid: glacial acetic acid: water (12.1: 1.3: 1.1: 2.8). The confirmation of the isolation was done by IR Spectroscopy (Gaurav et al., 2012).

Plants of medicinal importance though are quite well known among the Ayurvedic practitioners since years, yet many of them have not been standardized, validated and documented completely. Among such medicinally valuable plants,
Salacia holds a place as an effective antidiabetic herb and many species of this plant are in use as anti-diabetics. The present study reports the phytochemical analysis of one of its species Salacia oblonga (SO) (roots and stems), belonging to the family Celastraceae (bittersweet), through various biochemical and chromatographic methods. TLC and HPTLC analysis of six different extracts of SO revealed numerous bands, indicating the presence of diverse groups of phytocompounds, many of them are assumed to contribute significantly to its antioxidant activity as well as, other biological activities. This type of analysis can help in fingerprint profiling of the plant and its various species. The identification and characterization of the phytocompounds can further help in finding out molecular targets/mechanism of action of the constituents of this herb that are responsible for its biological activities (Sujata Basu et al., 2013).

The methanol extracts of Abrus precatorius in five different accessions were screened and their phytochemical compounds were separated using the advanced chromatographic techniques TLC and HPTLC. For TLC analysis the chromatogram was observed under UV (365nm), UV (265nm), visible light and Iodine chamber to observe the variously colored bands. Maximum numbers of bands (twelve) were observed in leaves extracts of Ap1 accession. In HPTLC analysis 13 peaks were eluted in Ap1 accession, of these the 1st peak had the maximum height (160.7 AU) with Rf value 0.01 and percentage of area (13.05%), the 2nd peak had the minimum height (15.5 AU) with Rf value 0.12 and percentage of area (2.42 %). Hence the present study clearly shows that A. precatorius in Hosur accession can be used as potential medicinal plant for having more phytochemicals among the five populations (John et al., 2014).

Preliminary phytochemical screening of the extract showed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, terpenoids, phenolic compounds, proteins, reducing sugars, fats and oils. HPTLC finger printing of petroleum ether extract of Moringa oleifera leaf powder under white light reveal presence of eight components 0.24, 0.29, 0.34, 0.54, 0.76, 0.83, 0.93, 0.99. Component number 8 at Rf 0.99 showed maximum concentration. Petroleum ether extracts of Phyllanthus emblica leaf powder showed seven peaks with Rf values in the range with their Rf value - 0.26 0.34 0.55, 0.77 0.80, 0.95. Component number 6 at 0.95 Rf value
showed maximum concentration. It can be concluded that HPTLC fingerprint analysis of Petroleum ether extract of leaf powder extracts of *Phyllanthus emblica* (PEPE) and *Moringa oleifera* (PEMO) can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also a good estimator of genetic variability in plant populations (Malliga *et al.*, 2013).

The high performance thin layer chromatography (HPTLC) fingerprint profile of methanol and ethyl acetate extracts of leaves of *Cassia fistula*. Chromatographic technique was used for separation of components from different extracts of leaves. This study was planned to develop a HPTLC fingerprint profile of extracts in different solvents such as petroleum ether, toluene, ethyl acetate, chloroform, acetone and formic acid. A HPTLC method for the separation of the active constituents in extracts has been developed and TLC of these extracts on silica gel pre-coated aluminum plates of Merck by automatic TLC applicator and using solvent system toluene: ethyl acetate: formic acid: 5:4:1 was performed. HPTLC profiling of the extract confirm about the presence of various phytochemicals. HPTLC fingerprint was scanned at 400 nm for methanol and ethyl acetate leaf extracts revealed 15 and 16 peaks with Rf values in the range of 0.06 to 0.99 and 0.02 to 0.98 respectively. The bands revealed presence of greenish, purple, pink and light yellowish orange bands showing the presence of steroids, terpenoids and saponins after spraying with anisaldehyde sulphuric acid reagent. The HPTLC method for routine quality control of present species can be carried out using this method for extracts of plant and serve in qualitative, quantitative and was appropriate for standardization of the extract (Leena Seasotiya *et al.*, 2014).

*Wrightia tinctoria* using high performance thin layer chromatography (HPTLC). Different solvent extracts such as petroleum ether, chloroform, ethanol and methanol were prepared and phytochemical screening was made using standard procedures. All the solvent extracts were also subjected to HPTLC analysis. The preliminary phytochemical analysis confirmed the presence of alkaloids, triterpenoids, proteins, amino acids, flavanoids and steroids. Comparatively, chloroform and ethyl acetate extracts resolved to show more number of peaks than other solvents used for the preparation of leaf extracts of *Wrightia tinctoria*. It was apparent that the Rf values were mostly different irrespective of solvents used for
extraction. The similarity in few Rf values of 0.21, 0.24 and 0.27 at 254nm and 0.21 and 0.44 at 366nm evidences the presence of specific components among solvent extracts of this plant. The difference in Rf values in most of the appeared peaks reflected qualitative variation in the phytocompounds. It can be concluded that HPTLC fingerprinting of *Wrightia tinctoria* may be useful in differentiating the species from the adulterant and act as a biochemical marker for authentication of this medicinally important plant in the pharmaceutical industry and plant systematic studies (Eswaran *et al.*, 2011).

Seeds of *Thevetia nerifolia* were reported as rich sources of cardiac glycosides, and peruvoside is one among the valuable cardiotonic drug used for the treatment of congestive heart failure. Samples were extracted with different solvent systems. Occurrence of peruvoside was analyzed by TLC technique; its presence was detected in chloroform and ethyl acetate fractions, both petroleum ether and methanol fractions gave negative results. Quantification of peruvoside in positive fractions was done using HPTLC fingerprinting. Densitogram showed scattered Rf values, and a comparison of Rf values of samples with standard peruvoside established the existence of different isomeric forms of the drug within same fraction. Extraction of peruvoside was partial with chloroform but complete with ethyl acetate. A total of 20% peruvoside can be extracted successively using chloroform (3-7%) and ethyl acetate (12-16%) from all three studied morpho variant plants. Considering the valuable therapeutic applications of cardiac glycosides, it is anticipated that isolation of this powerful drug economically from the natural resources will help to compensate the high cost alternatives for treating congestive heart failures (Nesy *et al.*, 2014).

**ACUTE TOXICITY**

Acute toxicity tests can provide preliminary information on the toxic nature of a material for which no other toxicology information is available. In most acute toxicity tests, each test animal is administered a single (relatively high) dose of the test substance, observed for 1 or 2 weeks for signs of treatment-related effects, then necropsied. Some acute toxicity tests (such as the "classical" LD$_{so}$ test) are designed to determine the mean lethal dose of the test substance. The median lethal dose (or LD$_{so}$) is defined as the dose of a test substance that is lethal for 50% of the animals in
a dose group. LD₅₀ values have been used to compare relative acute hazards of industrial chemicals, especially when no other toxicology data are available for the chemicals. However, many important observations of toxicity are not represented by LD₅₀ values or by slopes of dose-response curves for lethality. For example, information about morbidity and pathogenesis may have more toxicological significance than mortality, and these endpoints also should be evaluated in short term toxicity tests.

The Agency does not recommend that petitioners determine the median lethal dose (LD₅₀) for direct food additives or color additives used in food. However, if a petitioner decides to conduct an acute oral toxicity test, alternative test protocols can provide useful information about the acute toxicity of a substance. These protocols generally use fewer animals, and are thus more cost efficient, than tests designed to determine LD₅₀. The following guidelines should help the petitioner design acute oral toxicity tests when the petitioner has decided that such information is useful.

The main focus of the acute toxicity test should be on observing the symptoms and recovery of the test animals, rather than on determining the median lethal dose (LD₅₀) of the substance. The rat often is used as the animal model in acute toxicity tests, but other species also may be used. Often only one sex is studied in an acute toxicity test; generally, the female is assumed to be more sensitive to the acute toxic effects of chemicals than the male.

Before deciding on the dose of a test compound that will be used in studying its acute toxicity, the compound's chemical and physical characteristics (including molecular weight, partition coefficient, and the toxicity of related chemicals) should be considered; otherwise, oral toxicity including lethality caused by relatively large doses of a chemical may have no biological relevance to the chemical's effects at lower doses.

The following brief descriptions of oral toxicity tests may help the petitioner choose a test that meets his needs; detailed information about each type of test is available in the referenced material.

**a. Limit Tests** To determine the acute toxicity of a new food additive that is not expected to be particularly toxic, 5 gm (or ml) of the compound/kg body weight
of the test animal should be administered orally by gavage to several (perhaps 5) animals that have been fasted (overnight for rats, 4 hours for mice). Test animals should be observed closely for up to 14 days; symptoms of toxicity and recovery should be noted. Gross and histopathological examination of the test animals at the end of the study may help identify toxic effects on target organs. If no animals die as a result of this dose, there is no need to test higher dosages. The acute toxicity of the compound can then be expressed as being greater than 5 gm (or ml)/kg body weight of the test animal. This method is called the "limit test." In general, 5 gm or 5 ml of the test substance/kg body weight is the practical upper limit for the amount of test material that can be administered in one oral gavage dose to a rodent. If there are deaths following administration of an acute dose of 5 gm/kg body weight, then a lower dose should be administered to several (perhaps 5) animals and the results evaluated as discussed above. For compounds expected to be acutely toxic at 5 gm/kg body weight, it would be wise to select a lower initial "limit" dose.

b. Dose-Probing Tests Dose-probing acute toxicity protocols may have value when the petitioner has no preliminary information about the test substance that would help him select appropriate doses for toxicity studies. In a dose-probing acute toxicity test, one animal per each of 3 widely spaced dosages should be used and a sufficient observation period should follow administration of the doses. Subsequent toxicity studies may be based on the results of the doseprobing study. Variations of dose-probing acute toxicity studies are described in the literature. Other methods of determining appropriate doses for longer-term toxicity studies include a simple test wherein 3 or 4 doses are each administered to 1 or 2 test animals and the animals are observed for up to 14 days. If some of the animals die, one can estimate an approximate median lethal dose, termed ALD.

c. Up-and-Down Tests The "up-and-down" procedure involves dosing animals one at a time: First one animal at one dose, then another animal one or two days later at a higher dose (if the first animal survives) or a lower dose (if the first animal dies). This process continues until the approximate LD50 has been determined. One disadvantage to this test is the length of the study. Each animal should be observed for at least seven days after dosing so that delayed deaths can be recorded. However, this method usually requires only six or eight test animals as compared with the 40 to 50 test animals that may be used in the "classical" LD50 test.
d. Pyramiding Tests Pyramiding studies involve a minimum number of animals: Two animals are given successively increasing doses of the test substance on alternate days until an acutely toxic dose or some practical upward limit is reached. This test does not yield a lethality curve and often is used to assess acute toxicity in non-rodents. This test, although more like a short-term, repeated dose toxicity study than a true acute toxicity study, can provide useful preliminary information on the toxic nature of a new material for which no other toxicology information is available.

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys. Hence, evaluation of toxic properties of a substance is crucial when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being. In practice, the evaluation typically includes acute, sub-chronic, chronic, carcinogenic and reproductive effects (Asante-Duah, 2002).

Toxicology may be defined as the study of harmful, poisonous and adverse effects of drugs and other chemicals constituents found in plants, which may increase the chances of mortality or weakness in the general health, physically as well as mentally. The present study has been under taken to study the adverse or hazardous effects of methanolic extract from Tridex procumbens, dissolved in Dimethyl sulphoxide (DMSO) and accordingly to determine LD$_{50}$, to establish the safety of methanolic extract of *Tridex procumbens* in SD Rats as per OECD guidelines 423. All the Rats were sequentially administered orally the methanolic extract first in a single dosage of 2000 mg/kg body weight. All the animals were observed for mortality, wellness parameters and body weight for 14 days and due to some morbidity and mortality the experiment was again performed at same dosage and same results were observed. Then decrease in the dosage to 300 mg/kg body weight was performed and accordingly observed as per OECD Guidelines 423. No mortality or any significant change was observed at 300 mg/kg body weight, however at 2000
mg/kg body weight dose the mortality rate was 2/3. Conclusively indicates the LD₅₀ value of *Tridex procumbens* methanolic extract to be less than 2000 mg/kg body weight and more than 300 mg/kg body weight (LD₅₀ ≥ 300 mg/kg body weight, but < 2000 mg/kg body weight (Abrar Hussain *et al.*, 2012).

The oral acute toxicity of *Brucia javanica* Merril extract on both Male and Female DDY-Mice. Brucia javanica leaves function as a cytotoxic, anti-diarrhoea, etc. Brucia javanica leaves extract was administered orally for the first 24 hours at various dose levels (562.5 mg/kg bw (body weight), 1125 mg/kg bw, 2250 mg/kg bw, and 4500 mg/kg bw) to determine the toxicity effects. The treatment groups were compared to the normal control. Vital organs (liver, heart, lymph, lungs, etc) and body weight were analyzed to study the toxicity. LD₅₀ was determined using Reed and Munich formula. Vital organs average weight showed no difference between the control group and the treatment groups from the lowest dose until the highest dose. In addition, the body weight data showed no difference between the control group and the treatment groups. LD₅₀ for *Brucia javanica* extract using Reed and Munich formula was 1003.65 mg/kg b.w (Marissa Angelina *et al.*, 2012).

The effects of ethanol extract of *Trigonella foenum-graecum* (Fenugreek) seeds on the blood glucose levels in alloxan-induced diabetic rats at different doses (2g/kg, 1g/kg, 0.5g/kg and 0.1g/kg) were studied. The hypoglycemic effect of extract was compared with that of the standard antidiabetic drug (glimepiride, 4mg/kg) single dose. The extract showed significant activity against the diabetic state induced by alloxan but the intensity of hypoglycemic effect varied from dose to dose. The most effective dose recognized was 1g/kg but that is still lower than the standard antidiabetic drug. No acute toxicity was observed for ethanol extract of *T. foenum-graecum* seed when it was administered orally at high dose level (3 g/kg body weight), which is higher than effective antihyperglycemic dose, and closely observed for 24 hrs for any mortality and next 10 days for any delayed toxic effects on gross behavioral activities. Phytochemical group tests were also accomplished and presence of alkaloids, steroids and carbohydrates were recognized in the extract.
WOUND HEALING ACTIVITY

In this modern jet age, the incidence of accidents has steeply risen, which is responsible for the different types of wounds. A wound is disruption of the anatomic structure and its functional continuity of living tissue. Healing is the process of repair that follows injury to the skin and other soft tissues. Wound healing is essentially, a survival mechanism and represents an attempt to maintain the normal structure and function. The capacity of a wound to heal depends partly on its depth, as well as on the overall health and nutritional status of the individual. Clinically wound may be categorized as acute or chronic based on the timeliness of healing. The acute wound is a breakdown of the integrity of the soft tissue envelope surrounding any portion of the body. Acute wound is defined by its size, depth and involved anatomic structures. However, the exact duration of healing and the distinction between acute and chronic is arbitrary and often based on variables including the site and cause of the wound, age and physical condition of the patient. The time course between an acute versus chronic wound is a continuum between 4 and 6 weeks. It is during this time that if an acute wound has not healed spontaneously, it is likely to become a chronic, “problem wound” that requires further intervention.

The acute wound can present as simple or complex, depending on its location, size, involved anatomic structures and bio-burden. The foundation for closure of an acute wound lies in an adequate surgical debridement and a systematic approach to options for closure. Etiology of acute wound is usually violation of the skin and subcutaneous tissue integrity through multiple mechanisms. These mechanisms include penetrating or blunt trauma as well as various environmental exposures, such as chemical substances, extremes of temperature, prolonged or excessive pressure and radiation. Disruption of the continuity of the skin from any of these mechanisms allows entry of organisms that can lead to local or systemic infection. Irrespective of the nature of the cutaneous injury, acute wounds are expected to heal within a predictable timeframe, although the treatment required to facilitate healing will vary according to the type, site and depth of a wound.

Chronic wound is defined as those wounds that fail to progress through a normal, orderly and timely sequence of repair or wounds that pass through the repair process without restoring anatomic and functional results. Orderly refers to the
progression of the wound through the biologic sequences that comprise the phase of repair of acute wounds. Timeliness relates to the progression of phases of repair in a manner that will heal the wound expeditiously. Timeliness is determined by the nature of the wound pathology, medical status of the patient, and environmental factors. Most chronic wounds are associated with a small number of clinical entities, particularly chronic venous stasis, diabetes mellitus and pressure necrosis. Although some components of the healing process have regenerative aspects, skin is an example of a tissue in which the response to injury is predominantly one of repair.

Phases of wound healing are hemostasis, inflammation, proliferation, epithelization and maturation-remodeling. All the phases may occur in orderly and overlapping manner. The primary processes that contribute to the closure of skin wounds are epithelization, wound contraction and collagenous scar formation. Although the relative contribution of each process is different depending on the type of wound, all of these processes are stimulated in response to injury. A partial thickness burn will heal primarily by epithelization, whereas collagenous scar formation is much more important in the healing of an incisional wound. Wound contraction is the primary process involved in the secondary healing of large open wounds such as pressure sores. Epithelization, wound contraction and collagenous scar formation represent terminal aspects of the wound healing response. There are multiple necessary precursors to these terminal events. Wound healing consists of the combined effect of all these preliminary and terminal processes occurring in a carefully regulated manner.

2.6.1 Phases of healing

When human skin tissue is injured, the body supports a complex variety of cellular and molecular reactions in order to return the tissue to homeostasis. These are categorized into 4 phases.

2.6.1. a Hemostasis/ coagulation

This phase involves a series of complex reactions leading to hemostasis and clot formation. The clot consists of a fibrin mesh with aggregated platelets embedded in it. The mesh traps red cells that become another component of the clot plug. Fibrin is the end product of the coagulation cascades that are stimulated by vascular injuries. There is an intrinsic and an extrinsic coagulation cascade triggered by separate events.
Activation of factor XII initiates the intrinsic coagulation pathway and occurs when blood is exposed to foreign surfaces. An exposure to tissue factor that binds factor VII initiates the extrinsic coagulation pathway. Tissue factor is not found on vascular endothelial cells, but is found in abundance on extravascular cellular surfaces, especially on adventitial fibroblasts. On injuring to the cells the factor is released. Both coagulation pathways result in the production of thrombin catalyzing the conversion of fibrinogen to fibrin. In addition to contributing to hemostasis, fibrin is also the primary component of the provisional matrix that forms in the wound during the early healing period. Fibronectins are a class of glycoproteins that facilitate attachment of migrating cells onto the fibrin latticework and they are an extremely important component of the early matrix as well as mature dermis. Fibronectin is produced by fibroblasts and epithelial cells. Stimulation of the haemostatic mechanisms is limited to the site of injury in that normal endothelial cells produce prostacyclin that inhibits platelet aggregation. In addition, in uninjured areas, antithrombin III binds thrombin and limits its activity and protein C degrades factors V and VII.

The processes of blood coagulation and platelet aggregation terminate when the stimuli for clot initiation dissipate. Clot breakdown begins as soon as the clots form. Plasminogen activator mediates the clot lysis and converts plasminogen to plasmin, an extremely potent enzyme that can degrade a wide variety of extracellular matrix proteins.

**Fig. 13. Four Phases of Wound Healing**
b Inflammation

Following an injury, inflammatory response is an essential part of the wound-healing process. A reaction of vascularized living tissues after injury is inflammation. The physiologic process underlying this inflammation begins immediately upon tissue injury, but reaches completion usually after neutralization of the injurious influence.

The inflammatory response triggers events that have implications for the entire healing process. The physical signs of inflammation include erythema, edema, pain and heat. These signs are largely a result of changes that occur in the microcirculation and particularly in microvessels of 15 to 20 μm in diameter. Areas of injury cause vasodilatation that generate erythema and heat. Vasodilatation is mediated by histamine, kinins, prostaglandins and possibly additional factors such as leukotrienes and endothelial cell product. In addition to vasodilatation, the capillaries develop gaps between the endothelial cells lining them. Gap formation and increased permeability are also partially mediated by histamine and prostaglandins, although neutrophil factors contribute as well. These gaps allow plasma to leak from the intravascular space to the extravascular compartment. The prostaglandins PGE1 and PGE2 stimulate vasodilatation as well as capillary permeability. Prostaglandins affect vasodilatation through activation of adenyl cyclase and production of cAMP. Prostaglandins accumulate in injured tissue, probably from activation of phospholipases on injured cell membranes. The migration of cells and fluid into the injured area generates edema.

Role of white blood cells in inflammation

Neutrophils are the first of the leukocytes found in wounded tissue in large numbers. Neutrophils function as defensive units that engulf foreign material and digest it through the action of hydrolytic enzymes and oxygen radicals. After phagocytosing damaged tissue or bacteria, neutrophils are themselves phagocytosed by macrophages and destroyed. Alterations in pH resulting from breakdown products of tissue and bacteria, along with swelling and decreased tissue oxygenation resulting from damage to the blood supply, produce the pain noted in areas of injury. As monocytes migrate from the capillaries into the extra vascular space, they transform
into macrophages in a process mediated by serum factors and fibronectin. Specific factors that stimulate macrophage migration include collagen fragments, fibronectin fragments and elastin derived from damaged matrix, as well as complement components. Macrophages are tremendously important in normal wound healing.

Macrophages phagocytose bacteria and dead tissue and also secrete collagenase and elastases that break down damaged matrix. Lymphocytes produce factors essential for normal healing. In addition to functioning as immunoreactants, it is involved in cellular immunity and antibody production. IL-2 and other factors have been demonstrated to be chemotactic for lymphocytes. Eosinophils are only present in limited quantities in the peripheral circulation under normal circumstances. They can also migrate into the extravascular tissues in response to injury. In normal healing, changes that occur in tissue over time are extremely reproducible. After hemostasis is accomplished, inflammatory cells migrate into the wound with neutrophils initially predominating. At 48 to 72 hours, macrophages begin to outnumber neutrophils and large number of macrophages remains in the wound for several days. This is critical in that macrophages unlike neutrophils are essential for normal healing. After 5 to 7 days, few inflammatory cells remain in normally healing wounds and fibroblasts become the predominant cell type.

2.6.1. c Proliferative Phase

This phase begins approximately 2-3 days after formation of wound. During the proliferative phase, the repair processes are angiogenesis, fibroplasia and epithelisation. This stage is characterized by the formation of granulation tissue, consisting of a capillary bed, fibroblasts, macrophages and a loose arrangement of collagen, fibronectin, hyaluronic acid and bacteria. The desired outcome of proliferative phase is to fill the wound defect with connective tissue and cover it with epithelium. These sub phases do not happen in discrete timeframe but constitute an overall and ongoing process.

i. Epithelization

Occurs early in wound repair. Following injury, renewal of the epithelial barrier is essential to re-establish the barrier functions of skin. Epithelial cells cover all surfaces of the body including internal surfaces such as the gastrointestinal,
respiratory and genito-urinary tract. Injury results in discontinuity of the epithelium. The epithelial cells migrate and initiate the process of epithelization. This process is important to complete healing quickly, as it reforms the body’s barrier with the outside by minimizing chance of infection and water loss at the wound site. If the basement membrane remains intact, the epithelial cells migrate upwards in the normal pattern. This is equivalent to a first-degree burn of the skin. The epithelial progenitor cells remain intact below the wound and the normal layers of epidermis are restored in two to three days. If the basement membrane is destroyed, similar to a second degree or third degree burn, then the wound is reepithelialized from the normal cells in the periphery and from the skin appendages (hair follicles and sweat glands). Epithelization in an incisional wound involves cellular migration over a distance of less than a millimetre from one wound edge to the adjacent one and is a minor contributor to the healing process. Incisional wounds are generally completely reepithelialized in 24 to 48 hours. Epidermal growth factor and platelet-derived growth factor are postulated to stimulate epithelial migration. Transforming growth factor (TGF) is also a potent stimulant of epithelial cell migration and proliferation.

ii. Fibroplasia

This process predominates in 2 to 4 days after formation of wound and mediated by cytokines. Fibroblasts are the primary mesenchymal cells in dermis and they are the most important mesenchymal cells involved in wound healing. Injury damages smooth muscle cells and other cell types and are involved in the healing response. Fibroblasts from the surrounding undamaged tissue migrate into wound matrix under the influence of chemotactic cytokines derived from inflammatory cells and other factors, some of which may be bound to the matrix. The fibroblasts themselves may contribute chemotactic cytokines that further stimulate their migration. Undifferentiated mesenchymal cells in the neighbourhood of the wound may differentiate into fibroblasts when stimulated by macrophage products and migrate into the wound as well. Platelet derived growth factor (PDGF) has been demonstrated to be chemotactic for both fibroblasts and smooth muscle cells and has been demonstrated to be present at the site of wound.
iii. Angiogenesis

Reconstructs the vasculature in areas damaged by wounding. The capillary sprouts grows through the proliferation of endothelial cells at the sprout bases cells within the sprout develop a curvature, which results in a lumen. The capillary sprouts continue to grow until they contact other sprouts growing from other directions. The sprouts then interconnect forming a vascular loop, and the sprouting process begins anew. After a portion of the wound becomes revascularized with new capillaries, the vascular system subsequently matures possibly through the aggregation of capillaries, resulting in fewer larger vessels in the healed wound. Cytokines, many of which are macrophage derived, directly and indirectly stimulate the endothelial cell migration and proliferation required for angiogenesis. The lactic acid, biogenic amines and low oxygen tension in the injured tissue stimulated the release of cytokines.

iv. Granulation tissue formation:

This new tissue fills the wound space and revascularizes the injured site. The concurrent infiltration into the wound site of macrophages, fibroblasts and blood vessels allows granulation tissue formation to occur. Granulation tissue consists of a combination of cellular elements including fibroblasts and inflammatory cells along with new capillaries embedded in a loose extracellular matrix of collagen, fibronectin and hyaluronic acid. As early as 24 hours after injury, fibroblasts and vascular endothelial cells begin proliferating to form granulation tissue. It is specialized and its formation is the hallmark of healing.

v. Collagenation

Collagen synthesized primarily by fibroblasts, is a major component of normal skin, granulation tissue and mature scar. Collagen makes up 25% of all body proteins and more than 50% of the protein found in scar tissue. Fibroblasts produce large quantities of collagen, a family of triple chain glycoproteins, which form the main constituent of the extracellular wound matrix, which are ultimately responsible for imparting tensile strength to the scar. Detection of collagen in the wound occurs around the third day of post-injury, thereafter the levels increase rapidly for approximately 3 weeks. It then continues to accumulate at a more gradual pace for up to 3 months. Initial deposition of collagen is in a haphazard fashion. Subsequently,
these individual collagen fibrils are reorganized by cross-linking into regularly aligned bundles. Fibroblasts have migrated into the wound, laying down new collagen of the subtypes III and I. Early in normal wound healing type III collagen predominates but is later replaced by type I collagen. Tropocollagen is the precursor of all collagen types that transforms within the cells rough endoplasmic reticulum, where proline and lysine are hydroxylated. Disulfide bonds are established allowing 3 tropocollagen strands to form a left-handed triple helix, termed procollagen. As the procollagen is secreted into the extracellular space peptidases in the cell wall cleave terminal peptide chains, creating true collagen fibrils. The critical component of collagen synthesis is the hydroxylation of lysine and proline moieties within the polypeptide chains, and this occurs in the endoplasmic reticulum. Hydroxylysine is required for covalent cross-link formation. Hydroxyproline is found almost exclusively in collagen and serves as a marker of the quantity of collagen in tissue. This hydroxylation process requires specific enzymes for lysine and proline in addition to oxygen, vitamin C, alpha-ketoglutarate, and ferrous iron functioning as cofactors. Deficiencies in vitamin C, oxygen, or suppression of enzymatic activity by corticosteroids may lead to under hydroxylated collagen, which is incapable of generating strong cross-links and which is early broken down early. Age56, mechanical pressure and tension affect the rate of collagen synthesis. Transforming growth factor (TGF) is the most potent stimulant of collagen synthesis. The TGF derives from both inflammatory cells and the fibroblasts themselves. Specific antibodies to TGF can limit collagen accumulation in wounds. Fibroblast growth factor and epidermal growth factor have also been shown to stimulate collagen synthesis.

vi. Wound Contraction

The process by which the wound edges pull together to reduce the defectis wound contraction. It plays a considerable role in reducing the volume of tissue for repair. It is the result of the contraction of myofibroblasts in the granulation tissue. These are attached to each other and to the adjacent matrix components so that granulation tissue as a whole contracts and draws together the surrounding tissues.
Wound contraction, like collagen synthesis, begins approximately 4 to 5 days after wounding. Wound contraction represents the centripetal movement of the wound edge towards the center of the wound. Maximal wound contraction continues for 2 to 15 days, though it can continue for longer periods if the wound remains open. The wound edges move towards each other at an average rate of 0.6 to 0.75 mm/day. The rate of contraction is dependent on tissue laxity, and there is significant variability among different tissues. A wound in the buttock where the tissue is loose will contract much more than a wound on the scalp or pretibial area where the skin is tighter. Wound shape can also affect contraction. Wounds with square edges contract more rapidly than circular wounds. Forces of contraction in a circular wound cancel each other to some degree, preventing effective centripetal movement of the wound edge. Radiation and cytolytic drugs delay contraction adding further evidence that cellular activity is required. TGF-β can stimulate collagen lattice contraction and appears to be a mediator of wound contraction. Splints can temporarily slow wound contraction, though wound contraction will proceed at an accelerated rate after splint removal. Topical dressings may also delay wound contraction.

2.6.1. d Scar formation:

Scar formation is the hallmark of the final product of healing process. This relatively avascular and cellular mass of collagen serves to restore tissue continuity, strength and function. At approximately 21 days following injury, net accumulation of wound collagen becomes stable. During this period the wound becomes less cellular as apoptosis occurs. Endothelial cells appear to be the first cell type to undergo apoptosis followed by myofibroblasts, which disappear on completion of wound contraction approximately 21 days after wounding. The process of scar remodeling dramatically increases wound bursting strength. The greatest rate of increase occurs between 3 and 6 weeks after wounding. By 6 weeks after wounding, the wound has reached 80% to 90% of its eventual strength.

Wound healing types

a. Primary healing or healing by first intention

This occurs on closing a wound within a few hours of its creation. Approximation of a wound edges is done surgically or mechanically and collagen metabolism provides long-term strength. This surgical incision results in the mortality of a minimal number of cellular constituents.
b. Secondary healing or healing by second intention

This involves no formal wound closure. It occurs on allowing an open full thickness wound to close by wound contraction and epithelization. Secondary healing results in an inflammatory response that is more intense than seen in primary wound healing. Fibroblastic differentiation into myofibroblasts, which resemble contractile smooth muscle, contributes to wound contraction. These myofibroblasts are maximally present in the wound from 10-20 days.

c. Delayed primary closure or healing by third intention

If the wound edges are not re-approximated immediately, delayed primary wound healing transpires. Contaminated wounds despite this type of healing. Macrophages, metamorphose into epitheloid cells, encircled by mononuclear leukocytes forming granulomas, wall off the foreign materials. Now surgical closure of the wound is usually done and if the ‘cleaning’ of the wound is incomplete, chronic inflammation can ensue resulting in prominent scarring.

Complications of wound healing

Abnormalities, in any of the basic healing process can result in complications.

a. Deficient scar formation: Inadequate formation of granulation tissue or inability to form a suitable extracellular matrix, can lead to deficient scar formation and thereby wound dehiscence.

b. Ulceration: Ulceration of a wound may be due to inadequate blood supply and vascularization.

c. Excessive scar formation: This may be due to excessive deposition of extracellular matrix at the wound site. It results in a hypertrophic scar or keloid. The rate of collagen synthesis and the number of reducible cross-links remain high leading to a situation that indicates ‘maturation arrest’ or block in the healing process.

Factors affecting wound healing

Healing does not always occur in a straightforward, undisturbed fashion. Classification of several factors interfering with healing is as follows
a. **General** i) Local factors ii) Systemic factors

b. **Specific** i) Growth factors and Cytokines ii) Endocrine hormones

c. **Drugs** i) Corticosteroids ii) NSAIDs iii) Anticancer drugs iv. Drug resistance

a) **General**

i. **Local factors**

Most commonly used terms in delayed wound healing are wound contamination, wound colonization, critical colonization and wound infection. Wound contamination is the presence of bacteria within a wound without any host reaction. Wound colonization refers to the presence of bacteria within the wound that multiply or initiate a host reaction. Critical colonization means multiplication of bacteria causing a delay in wound healing, usually associated with an exacerbation of pain not previously reported but still with no overt host reaction. Wound infection is the deposition and multiplication of bacteria in tissue with an associated host reaction. This is the most common reason for delay in poor wound healing. Wound infection is determined by host immune competence and the size of the bacterial inoculum. With normal host defenses and adequate debridement, a wound may bear a level of 1, 00,000 microorganisms per gram of tissue and still heal successfully. Beyond this number, a wound may become infected.

**Wound Colonization**

Microbial colonization is usually described as a process that occurs when exposed subcutaneous tissue provides a favorable substratum for a wide variety of microorganisms to contaminate and colonize. If the involved tissue is devitalized (e.g., ischemic, hypoxic, or necrotic) and the host immune response is compromised, the conditions become optimal for microbial growth. Wound contaminants are likely to originate from either exogenous or endogenous sources. Exogenous contamination is either from environment or from the surrounding skin (involving members of the normal skin microflora such as Staphylococcus epidermidis, micrococci, skin diphtheroids, and propionibacteria). Endogenous contamination may occur from mucous membranes (primarily the gastrointestinal, oropharyngeal, and genitourinary
mucosa). The normal microfloras of the gut, the oral cavity, and the vagina are both diverse and abundant, and these sources (particularly the oral and gastrointestinal mucosa) supply the vast majority of microorganisms that colonize wounds. A minor healing wound may allow sufficient time for only relatively small number of skin contaminants to take residence, but the continued exposure of devitalized tissue associated with a slowly healing chronic wound is likely to facilitate the colonization and establishment of a wide variety of endogenous microorganisms. Many researchers opine that aerobic or facultative pathogens such as Staphylococcus aureus, Pseudomonas aeruginosa, and beta-hemolytic streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds. Such opinion has been formed on the basis of referenced comments and studies performed largely during the last two decades that have investigated the role of microorganisms in wound healing. The role of anaerobic organisms in causing wound infection has been underestimated. The failure to recognize the prevalence of anaerobic bacteria in wounds may be due to several reasons. Firstly, anaerobes, were not regarded as being detrimental to normal wound healing, secondly compared with aerobic and facultative microorganisms, the culture, isolation, and identification of anaerobic bacteria is more time-consuming, labor-intensive and expensive, it is deemed to be too demanding for many diagnostic microbiology laboratories. Lastly, anaerobes perceived to die rapidly in air, the method of specimen collection and transportation to the laboratory is critical for maintaining viability and for effective culture.

**Pathogenic effects of virulent micro-organisms:**

Some species of micro-organisms such as Staphylococcus and Streptococcus produce superantigens. Superantigen released into the blood stream, initiates an uncontrolled proliferation of T lymphocytes allowing the release of cytokines that initiates cell and tissue damage. Biofilm, usually attached to a wound surface is a microbial colony encased in an adhesive polysaccharide matrix. Cells in biofilms exhibit a decreased sensitivity to host immunological defense mechanisms, decreased susceptibility to antimicrobial agents and increased virulence. They have also been implicated in persistent infections.
Diabetes mellitus is a classic example where wound healing is slow because of interruption of the inflammatory phase as well as the proliferative phase. Neutrophils and macrophages cannot adequately keep the bacterial load of the wound in check, as their glycosylation is inhibitory to phagocytic function. Infection thus prolongs inflammatory phase. Tissue Perfusion: A good blood supply is a basic factor in the success of wound repair. It is essential for the supply of oxygen, other nutrients required in the cellular, and biochemical process of repair and it is necessary for the removal of wound metabolites. Arterial diseases that limit blood flow and venous abnormalities retarding drainage are, well documented to cause impairment to the healing of wounds.

**ii. Systemic factors**

**Age:** Proteolysis is an essential component of wound healing, but if uncontrolled, it may lead to degradation of the neo-matrix and a delay in wound repair. Despite numerous reports of impaired wound healing associated with increasing age, the control of proteolysis is completely unknown. Ashcroft GS et al have reported that tissue inhibitor of matrix metalloproteinases (TIMP)-1 and -2 inhibit the activity of matrix metalloproteinases and the pattern of regulation of these molecules determines in part the spatial and temporal regulation of proteolytic activity. Antioxidant defense system: Free radicals and other oxygen-derived species are constantly generated in the body during metabolism. Oxidant induced protein denaturation with subsequent breakdown is a well-recognized process after wound injury. A marked increase in oxidant release and a decrease in antioxidant defense is well defined after post wound. The reactivity of some free radicals can cause severe damage to biological molecules especially to DNA, lipids and proteins. Antioxidant defense systems like reduced glutathione, glutathione peroxidase and superoxide dismutases are produced by the body to protect the cellular constituents from the damages caused by reactive oxygen species. The leaves of plant Adhatoda vesica Nees (Acanthaceae) has shown to exert antioxidant activity by scavenging the lipid peroxidation. The increased lipid peroxide formation in the tissues of carbon tetrachloride (CCL4) treated rats have shown significant inhibition by *Glycyrrhiza glabra* Linn.
**Tissue oxygen:** This is the most important factor-regulating wound healing. Oxygen influences angiogenesis, epithelization and resistance to infection. Reduced wound oxygen tension can delay wound healing by slowing the production of collagen. Collagen fibril cross-linking begins to fail as tissue oxygen pressure falls below 40 mm Hg because oxygen is required for the hydroxylation of proline and lysine to synthesize mature collagen. Wound hypoxia also predisposes to bacterial infection as the leukocyte’s oxidative phosphorylation, bactericidal activities are severely impeded without normal tissue oxygen levels. Oxygen enhanced environments have been shown to be bactericidal for most clostridial species and inhibit alpha toxin release. Thus, cell death and tissue necrosis caused by tissue hypoxia or anoxia creates ideal growth conditions for members of the wound microflora, including fastidious anaerobe that proliferate as residual oxygen is consumed by facultative bacteria. Oxygen is a critical component of the respiratory burst activity in polymorphonuclear leukocytes (PMNs), resulting in the intracellular production of highly potent antimicrobial metabolites. A significant reduction in the killing capacity of PMNs at a pO₂ of 30 mm Hg has been reported. This signifies, poorly perfused wound tissue is more susceptible to infection than are wounds involving well-perfused tissue. Although many endogenous anaerobes survive prolonged periods of exposure to air and tolerate oxygen tensions up to 60 mm Hg (8% oxygen), the redox (oxidation-reduction) potential (Eh) of tissue is also important for their survival. Generally, a low Eh (measured in millivolts) favors the growth of anaerobic bacteria. Hypoxic or anoxic wound environment that has a low oxygen tension and a low redox potential will facilitate the development of poly microbial aerobic-anaerobic populations.

**Nutrition:** Nutrition is a crucial aspect of a holistic approach to the healing of wounds. Poor nutritional status can delay the wound healing process or cause inadequate healing when nutritional deficiencies are not corrected. Synthesis of collagen appears gets inhibited in protein deficient animals. A light protein diet hastens the acquisition rate of tensile strength. Malnutrition causes a decreased rate of fibroblastic proliferation and neovascularization and impairs both cellular and humoral immunity.
**Vitamin deficiency**

**Vitamin C:** Participates in the process of wound healing by promoting the synthesis of collagen. Hence, its deficiency contributes to fragile granulation tissue. It affects collagenation and causes impaired wound healing. Supplementation with Vitamin C twice a day accelerates ulcer healing.

**Vitamin A:** Rats fed with vitamin A supplemented diet showed enhanced wound healing compared to those fed a standard diet. The beneficial effect of vitamin A on wound healing may be due to an increase in collagen synthesis. Vitamin A has a stabilizing effect on lysosomal membrane. Thus, excessive doses of vitamin A being reported to increase inflammatory reactions. It is known to reverse the inhibitory effect of cyclophosphamide and corticosteroids on wound healing.

**Trace elements:** *Copper* is a cofactor required for the enzyme lysyl oxidase, which plays a role in cross-linking and strengthening of connective tissue. A copper supplement as a part of a comprehensive nutritional program has been recommended to promote wound healing. Copper complexes have antineoplastic activity. Copper complexes of Tolmetin (Tol-Cu) found to have prohealing action. *Zinc* is a component of many enzymes that is necessary for repair wounds. Even a mild deficiency of zinc can interfere with optimal recovery from everyday tissue damage as well as from more serious trauma. It is found that zinc reverses the healing suppressant effect of corticosteroids. Zinc salts also reverse the healing suppressant effects of non-steroidal anti-inflammatory agents.

**b. Specific**

**b. i Growth Factors and Cytokines**

Growth factors and cytokines are two distinct categories of signaling proteins that modulate wound healing at a molecular and cellular level. Growth factors are constitutively present, released by a few selected subsets of cells and have primarily tropic effects on cells. However, they may have an indirect inflammatory influence. Cytokines are small molecular weight mediators, which primarily have variable effect on inflammatory process by their influence on the cells of the immune system. All nucleated cells release this expressing transient, local and elicit varying responses in different cells.
b. ii Endocrine hormones

Several hormones may enhance or alter healing. Growth hormone has shown to increase angiogenesis and myofibroblast differentiation in wounded mice. It also stimulates the production of collagen. Its deficiency severely limits bone growth and hence the accumulation of bone mass. Treatment with oxytocin activated the process of vasculogenesis, proliferation of endotheliocytes that turn resulted in the effective clearance of the wound and optimal granulation tissue formation. Leptin accelerates the healing of colonic anastomoses. Insulin induces accelerated wound healing associated with diminished inflammation and increased collagen deposition in rats. Injury evokes the secretion of hormones. The adrenocorticotrophic hormone released from pituitary or exogenously administered cortisone exert a deleterious effect on the wound healing process, which has been suggested to result from the anti-inflammatory action of these steroids. The corticosteroids lower transforming growth factor – beta (TGF-beta) and insulin like growth factor –1(IGF – 1) levels and tissue deposition in wounds.

c. Drugs

Corticosteroids

Steroids are known to inhibit all aspects of wound healing like tensile strength of closed wound, rate of epithelization and appearance of new blood vessels in healing tissue. The impaired healing, results from derangements in cellular function induced by steroids. A primary feature of wounds in steroid treated individuals is a deficiency in inflammatory cell function. Inflammatory cells, particularly macrophages mediate essentially all aspects of healing through cytokines. By diminishing supply of cytokines, steroids and other immunosuppressive agents profoundly impair all aspects of healing. Macrophage migration, fibroblast proliferation, collagen accumulation and angiogenesis are among the processes diminished by steroid administration.

All anti-inflammatory steroids have an inhibitory effect on wound healing. The effect of cortisone on healing is that it prevents the inflammatory phase, which is essential to the healing process. Cortisone and other anti-inflammatory steroids increase the integrity of the lysosome, the subcellular particle that contains an
assortment of acid hydrolases. It is known that lysosomal enzymes take a prominent part in the inflammatory process. Glucocorticoids represent the most important and frequently used class of anti-inflammatory drugs. These are widely used for the treatment of various diseases despite known side effects like skin atrophy and immunosuppressive effects. These are known to adversely affect wound healing in experimental skin models.

**Nonsteroidal anti-inflammatory drugs (NSAIDS)**

Nonsteroidal anti-inflammatory drugs, are being used to suppress postoperative inflammatory edema and pain. The usefulness of nonsteroidal anti-inflammatory drugs is limited due to adverse gastrointestinal tract events. Adverse effects like gastric mucosal injury are known to be caused by aspirin and to retard gastric wound healing. NSAIDS such as aspirin, indomethacin and ibuprofen are the most widely used drugs for pain, arthritis, cardiovascular diseases and more recently for the prevention of colon cancer and Alzheimer’s disease.

c. iii. Anticancer Agents

Anticancer agents are antiproliferative and adversely affect the healing process. Hence they can delay wound healing when used perisurgically in cancer. Cyclophosphamide interferes with wound contraction, epithelization, tensile strength and granulation tissue formation. Supplementation of vitamin A reverses the anti-healing effect. The 5-fluorouracil (5-FU) and Colchicines (COL) also suppress wound healing. The drug probably decreases the collagen content by affecting the fibroblast proliferation and hence decreases the tensile strength. Cyclosporine is an immunosuppressant drug which causes a significant reduction in tensile strength of healing incision wound and granulation tissue weight of dead space wound and does not affect the wound contraction and epithelization period of excision wound. Prevention of impaired wound healing and reduction the adverse effects of radiation can be done using vitamin A supplementation.

c. iv. Drug resistance:

Antimicrobial resistance is the ability of microbes, such as bacteria, viruses, parasites or fungi to grow in the presence of a chemical (drug) that would normally
kill it or limit its growth. In 1928 while working with *Staphylococcus* bacteria, Scottish scientist Alexander Fleming noticed that a type of mold growing by accident on a laboratory plate was protected from, and even repelled, the bacteria. The active substance, which Fleming called penicillin, was literally an antibiotic - it killed living organisms. Thus began the age of using natural and, later, synthetic drugs to treat people with bacterial infections. Though not widely popular until the 1940s, antibiotics and other antimicrobials (medicines that kill or slow growth of a microbe) have saved countless lives and blunted serious complications of many feared diseases and infections. The success of antimicrobials against disease-causing microbes is among modern medicine's great achievements. Soon after its use however, some strains of Staphylococcus Aureus began to produce the enzyme penicillinase inactivating the antimicrobials like ampicillin, other penicillins and cepha laosporins. Methicillin, used to treat S. aureus, was the first penicillinase–resistant semisynthetic penicillin. Late 1960 and 1970’s saw the emergence of MRSA, (Methicillin resistant *Staphylococcus aureus*) with the first report out breaks in both acute and long-term care facilities. Emergence of MRSA in wound was of concern because of resistance to other antimicrobials. A gene on the bacterial chromosome that codes for abnormal penicillin binding protein (PBP) carries resistance to Methicillin. This abnormal PBP has minimal affinity for all penicillins, so very little Methicillin binds to it. Hence, all penicillin is ineffective against MRSA. Some strains of MRSA are becoming resistant to other antimicrobial agents like vancomycin. Treatment of MRSA wound infection involves antimicrobial therapy and prevention of cross contamination. An alternative method to control MRSA in wound is the use of ultraviolet rays. The emergence of antibiotic resistance is a major problem in antimicrobial therapy.

**Mechanisms causing antibiotic resistance:**

1. Permeability: Some microorganisms change their cell permeability to the drug, possibly by alteration in the chemical nature of outer membrane; example is tertracycline resistant to *Pseudomoans Aeruginosa*.

2. Production of enzymes: Penicillin resistant *Staphylococcus Aureus* produces an enzyme β lactamase that destroys the penicillin.
3. Aminoglycoside attaches with 30 S subunit of ribosome but resistant bacteria develop an altered receptor.

4. Altered metabolic pathway by altering the metabolic pathway, bacteria bypasses the reaction inhibited by the drug. Sulphonamide resistant bacteria utilize preformed folic acid and do not require the conversion of PABA (Para-Aminobenzoic acid) to folic acid (a reaction inhibited by sulphonamides).

**Genetic basis of resistance:**

1. **Chromosomal resistance:** This occurs, because of spontaneous mutation. In clinical practice, mutational resistance is of great importance in tuberculosis. The antimicrobial drugs selectively suppress susceptible organisms but resistant mutants will multiply unchecked. On using two or more anti-tubercular drugs for treatment, resistant mutant does not occur, as, a mutant resistant to one drug, will be destroyed by another drug.

2. **Extra chromosomal resistance:** This occurs by transfer of plasmids and genetic material. Transferable drug resistance mediated by the R factor is the most important. R factors are plasmids that contain genes that code for drug resistance against one and often several antimicrobial drugs. Inactivation of drugs occur by plasmid coded enzymes example β lactamases destroy the β –lactam ring which is responsible for the antibacterial action of β lactam antibiotics like penicillins and ceaphalosporins.

   One group of β lactamases that is occasionally found in certain species of gramnegative bacilli usually *Klebsiella pneumoniae* and *Escherichia coli*. These enzymes are termed as extended spectrum β lactamases (ESBL) because they confer upon the bacteria the additional ability to hydrolyze the β-lactam rings of cefotaxime, ceftazidime, aztreonam and these enzymes have developed resistance to antibiotics like penicillin. ESBL enzymes can also be found in bacteria such as *Salmonella, Proteus, Morganella, Enterobacter, Citrobacter, Serratia*, and *Pseudomonas*. Enzymes are proteins produced by living organisms possessing the ability to speed up biochemical reactions. In most cases, the body successfully fights off ESBL-producing bacteria. However, because of the enzymes' ability to fight off antibiotics, people with weak immune systems are at risk like children, the elderly and people with other illnesses.
The drug resistance in India is particularly grim due to various factors. Generally, there is little control on the use of antibiotics. Community awareness of the issues involved in antibiotic therapy is poor and this is compounded by over-the-counter availability. Coupled with primitive infection control in hospitals and weak or deficient sanitation, the conditions are suited for transmission and acquisition of antibiotic resistance. The facility with which enteric pathogens spread widely in India illustrates this point. On the other hand countries with a good sanitary infrastructure are hardly bothered by the import of cholera cases. Large parts of the country do not have the technical infrastructure to generate useable data on the ground. Thus, the contribution of infectious diseases is greater in impoverished societies. In the absence of a Central Monitoring Agency, the national scene in India with regard to antimicrobial resistance is unknown. The two probable exceptions are *M. tuberculosis* and *Leishmania donovani*. *Staphylococcus aureus* has developed resistance to newer antibiotics over the years. Methicillin resistance is quite frequent approaching and at time exceeding 50% in tertiary care centers. Vancomycin resistance has been very low. However, the reckless use of the antibiotic may alter the scenario. This coupled with the emergence of Community Acquired MRSA would pose serious clinical problems with global ramifications. Likewise, coagulase negative staphylococci have acquired multiple resistance and become important nosocomial pathogens. *Vancomycin Resistant Enterococci* (VRE) are being isolated in Indian hospital laboratories and tertiary care centers, one centre in north India reported to isolate 38% of blood culture was positive for VRE in Intensive Care Unit. UVR is very effective against resistant microbial in very less duration, only in seconds. Research showing the effectiveness UVR will be discussed in detail in the next section.

**Monitoring of wound repair through different wound models**

To study the rate and extent of several processes that occur during wound healing a variety of wound models are employed. Wound repair is a complex phenomenon having various phases like granulation, collagenation, collagen maturation; scar remodeling, scar maturation, wound contraction and epithelization. Some of these phases are independent while many of them are sequential. Quite often, these phases progress concurrently
a. Resutured incision wound

This is the much routinely used wound model. Skin is split through its full thickness and after securing haemostasis; the wound edges are approximated by interrupted sutures and wound allowed to heal by first intention. One of the important aspects of incision wound healing is the rate at which the wound gains it strength (tensile strength or breaking strength). the ability to resist rupture can be assessed in various ways and has been studied for centuries. Paget166 introduced the measurement of tensile strength to assess the progress of incisional wounds. The breaking strength required to disrupt the healed wound at various time intervals or at a given age of the wound, can be converted into tensile strength if cross sectional area of a wound edge is taken into account. The breaking strength is measured by applying tractional force on wound edge. Instead of traction face, if a pressure is applied to break the wound of a wall of viscus or skin it is called bursting strength. This wound model can also be employed to study histological and biochemical parameters and is not suitable for study on wound contraction and epithelization.

b. Dead space wounds

In this wound model, granulation tissue is harvested over a subcutaneously implanted foreign body. To produce dead space wounds workers have used various foreign body implants like cotton pellets. Propylene130 and silicon implants169 of uniform thickness and weight can be placed in dead space made in the axilla and groin regions. After a definite period, these pellets or tubes can be taken out, granulation tissue harvested on it, carefully dissected and tensile strength of the tissue measured. The dry granulation tissue weight can be estimated. This granulation tissue can be used for histological studies and estimation of hydroxyproline content of collagen.

c. Excision wound

Conventionally a wide-open dermal wound (of known dimensions) is created by cutting away a piece of skin in its full thickness. To study physical aspects of wound healing, excision wounds, of different shapes and sizes in different locations has been employed. Rat, rabbit and guinea pig are used for this purpose.
(Morton and Malone, 1972) have used circular wound of 2.5 cm diameter made on depilated dorsal thoracic region in rats. This wound permits monitoring of wound contraction and epithelization; and it is possible to differentiate the process of contraction and epithelization in this wound model study by physical attribution. Repeated wound tracings are used to study the rate of contraction. Results are expressed as percentage of closure of their original wound size for the purpose of comparison.

**Wound and Diabetes**

Diabetes mellitus can be virtually harmless if controlled, but the state of abnormally high blood glucose levels associated with the condition can lead to some serious complications. If left uncontrolled for a long time, or if diabetic patients fail to adapt their lifestyles in order to manage the disease, they will have more difficulty preventing complications from occurring. A serious complication that diabetics may encounter are diabetic wounds.

A metabolic disorder caused primarily by a defect in the production of insulin by the islet cells of the pancreas resulting in an inability to use carbohydrates are characterized by hyperglycemia, glycosuria, polyuria, hyperlipemia (caused by imperfect catabolism of fats), acidosis, ketonuria, and a lowered resistance to infection. Periodontal manifestations may include recurrent and multiple periodontal abscesses, osteoporotic changes in alveolar bone, fungating masses of granulation tissue protruding from periodontal pockets, a lowered resistance to infection, and delay in healing of the wounds the most common being Type-1 diabetes and Type-2 diabetes (Greenhalgh, 2003). These are diseases of the metabolic system and involve the body's ability in metabolizing sugar using the hormone insulin. Diabetes mellitus is one of the major contributors to chronic wound healing problems. When diabetic patients develop an ulcer, they become at high risk for major complications, including infection and amputation. The pathophysiological relationship between diabetes and impaired healing is complex. Vascular, neuropathic, immune function and biochemical abnormalities each contribute to the altered tissue repair. Despite treatment of these chronic wounds, which involves tight glucose control and meticulous wound care, the prognosis for their healing is quite poor. Wound healing is impaired in diabetic patients with infection or hyperglycemia. The heat shock proteins (HSPs), originally identified as heat-inducible gene products, are a highly conserved family of proteins that respond to a wide variety of stress.
Wounding induces HSPs, particularly in the epidermis. In the initial phase of wound healing there is an inflammatory response, followed by organization of the fibrin-rich exudates and subsequent re-epithelialisation and formation of granulation tissue. The wound bed contains abundant inducible HSP70 which contributes to protein homeostasis and cell survival within the healing wound. HSP functions are compromised under conditions of diabetes (Mustafa Atala, 2009). Both type 1 and Type 2 diabetes are characterized by an increased risk for the development of microvascular and macrovascular complications. In diabetes, endogenous defence systems are overwhelmed, causing various types of stress. Uncontrolled oxidative stress represents a characteristic feature of diabetes. Among the other important conditions related to diabetes are dyslipidemia, modification of proteins and lipids, and perturbations in the tissue antioxidant defence network. In traditional medicine plants are generally used for treatment of various diseases and abnormalities in the body.

Fig. 14. Diabetic Wound
Fig. 15 Pathway of Diabetic Wound

i. Causes of Diabetic Wounds

The main concern with diabetic wounds is poor or delayed healing. Healing problems are caused by the peripheral arterial diseases and peripheral neuropathy that can occur with diabetes, wherein the small blood vessels in different parts of the body, especially in the extremities (hands and feet), grow narrower and reduce the blood circulation to those areas. A lack of circulation in the extremities can result in a reduced supply of oxygen and nutrients to the body tissue and nerves, which is necessary for healing. Over time, nerves in these areas may become damaged, decreasing the sensation of pain, temperature and touch, making patients vulnerable to injury.

ii. Types of Diabetic Wounds

For a diabetic patient, every wound is a health concern and requires immediate attention. The most common two types are wounds of external origin and wounds of internal origin. Due to peripheral neuropathy, wounds of external origin, such as skin cuts, burns, bumps and bruises, may often go unnoticed by the diabetic patient. If external wounds go unnoticed for some time, delayed treatment can put the patient at risk for further complications. Wounds of internal origin, such as skin ulcers, ingrown toenails or calluses, can lead to the breakdown of skin and surrounding tissue, increasing the risk of bacterial infections.
iii. Signs and Symptoms of Diabetic Wounds

Diabetic wounds may present with the following signs and symptoms:

- Chronic pain or completely painless
- Signs of inflammation (swelling, redness, heat, pain and loss of function)
- Signs of infection (pus drainage, discharge, bad odor and dead tissue)
- New numbness and dullness (signs of nerve damage)
- Fever and/or chills (signs of progressively worsening infection that can be limb-threatening or even life-threatening)

Having high levels of blood glucose for extended periods makes the immune system function improperly. Due to reduced blood flow and damaged nerves, patients with uncontrolled diabetes are at high risk of developing non-healing or infected wounds. The risk is higher for diabetic patients who also have other chronic diseases, such as atherosclerosis, high cholesterol levels, obesity or AIDS. If a patient has an unhealthy diet, smokes and does not exercise, they are at greater risk for diabetic wounds. Any patient who has a hazardous occupation, such as work that involves operating heavy machinery, using sharp tools for building and construction, or violent sports, is at greater risk.

iv. Treatment of Diabetic Wounds

The best treatment is prevention, since medical treatment for diabetic wounds provides limited help. If a wound occurs, treatment can include:

- Keeping all wounds clean and properly dressed
- Antibiotics (for infected wounds or as a preventive measure for wounds at risk of getting infected)
- Surgical debridement (the dead or infected tissue is removed to allow the healthy tissue to heal and regenerate)
• Referral to a podiatrist or a wound care center (for patients with calluses, corns, hammertoes, bunions, toenail problems or chronic non-healing ulcers)

• Limb amputation (to save as much of a limb as possible when there is a serious infection)

v. Prevention

Prevention of diabetic wounds is critical for diabetic patients to ensure a normal and active healthy life. It is important to remember that diabetic wounds can be disabling and life threatening in some cases. Prevention should begin with:

• Controlling diabetes by following your doctor's recommendatrions for treatment and lifestyle modifications that include a healthy diet, regular exercise, cessation of smoking and regular monitoring of blood glucose levels

• Daily inspection and cleaning of your extremities as they are more prone to ulcers and injuries

• Carefully trimming the nails with a safe nail trimmer (refer to an expert if the patient requires extra care or if there are skin lesions

• Always wear dry, clean socks to help protect your feet, and never walk barefoot (avoid tight socks that may reduce the blood circulation to the feet

Medicinal Plants Having Wound Healing Activity

The plant Centella asiatica is used in traditional medicine to treat a variety of disorders. The objective of this study presented in this report was to evaluate the wound healing potential of ethanolic extract of the plant in streptozotocin-induced diabetic rats. The study was carried out by using Wistar albino rats by creating excision and dead space wounds. The measured wound area of the Centella asiatica treated group was reduced significantly when compared to diabetic control animals. Significant increase in the weight of the granulation tissue and the hydroxyproline content were observed. The histological study of the healing tissue obtained from the experimental diabetic animals showed the fast lay-down of collagen when compared to the normal and diabetic control group. The fasting blood glucose values of the diabetic (Somashekar Shetty et al., 2013) experimental group animals were
significantly reduced when compared to the diabetic control animals. We noticed the correlation between the wound contraction rate and the blood glucose values. The results of the present study clearly demonstrate that the ethanolic extract of *Centella asiatica* possesses a definite prohealing action in normal healing as well as in the diabetes induced wound healing.

The antidiabetic activity is evaluated by estimating the blood glucose level, total protein, total cholesterol, creatinine and blood urea and nitrogen in alloxan induced diabetic rats. The wound healing activity property was studied by excision and incision methods. There is a significant decrease in the blood glucose levels from 1 week to 3 week in n-hexane extract, ethyl acetate and methanolic extract treated groups when compared to the diabetic control group. The n-hexane extract treated group has shown significant increase in the total cholesterol, creatinine and urea levels when compared to the other treated groups and is almost similar to the standard group. In contrast the methanolic extract has brought has brought the total protein level to the normal in diabetic induced rats (Aparna lakshmi *et al.*, 2011).

Wounds generally termed as physical injuries that result in an opening or breaking of the skin. There are different types of wounds which range from mild to potentially fatal. Wound healing is impaired in diabetic patients with infection or hyperglycaemia. Diabetes mellitus is one of the major contributors to chronic wound healing problems. The diabetic patients with ulcer become at high risk for major complications which include infection and amputation. In traditional medicine plants are generally used for treatment of various acute and chronic diseases and abnormalities in the body. Due to the present fast life of the humans a drastic increase in chronic disease conditions mainly diabetes has been determined. Most of these patients tend to face a tremendous problem when they get an infected wound.

*Lantana camara* (Verbanacea) is a commonly available medicinal plant throughout India. Wound healing property of the plant in various wound models has been studied. Thorough literature survey revealed that the wound healing property of Lantana camara in diabetic wound was not studied. This study was aimed to evaluate the wound healing property of Lantana camara in diabetic rats. Methods: Group-1 rats served as normal control in which excision wound was created in normal, non-diabetic rats and wound was topically applied with vehicle. To induce diabetes
mellitus in group 2-5, a single injecting of streptozotocin (45 mg/kg, i.p.) prepared by dissolving in 0.9% ice cold citrate buffer was given. Excision wound was inflicted in the back of the rats. Group-2 was the diabetic control in which diabetic rats received vehicle ointment topically. Group 3, 4 and 5 were the test drug groups in which diabetic rats were topically applied ethanolic extract of Lantana camara in three doses 10%, 15% and 20% respectively. Wound healing parameters such as percentage of wound contraction rate and epithelialization period were observed. Data was analyzed using SPSS software by one way ANOVA and the statistical significance was fixed as p < 0.005. Results: There was a delay in wound healing in diabetic rats compared to non-diabetic rats. The extract showed dose dependent increase in wound contraction rate and hastened the epithelialization period. Extracts enhanced contraction rate only during later phase of wound healing process. High dose (20%) extract showed maximum healing effect (Sivanageswararao et al., 2014).

The anti-diabetic activity of the Methanolic extract of the bark of Ficus mollis (family: moraceae) was investigated on alloxan induced diabetic wistar rats. A comparison was made between both plant extract and a known antidiabetic drug glibenclamide (5mg/kg). The dried bark of Ficus mollis was subjected to extraction by continuous hot extraction method using methanol as a solvent. Phytochemical estimation was done for the presence of phytoconstituents. Dose selection was made on the basis of acute oral toxicity study (200mg/kg, 400mg/kg bodyweight) as per OECD guidelines. Oral administration of extract of ficus mollis for 21days resulted in significant reduction in blood glucose level. Alloxan induced diabetic rat model and oral glucose tolerance test (OGTT) model was used for evaluation of antidiabetic activity. The biochemical parameters were analysed. All rats in the diabetic groups had FBG levels well within the diabetic range (>200 mg dL-1) at the initial stage of the experiment but after three weeks of treatment with extracts or glibenclamide the FBGL significantly dropped in dose-dependent manner, and also correct the lipid profile and liver enzymes. The results suggest that the methanol extracts of the bark of Ficus mollis restored the metabolic changes in alloxan-induced diabetic rats. The wound healing activity of the methanolic extract of the bark of Ficus mollis (family: moraceae) was investigated on alloxan induced diabetic wistar rats. A comparison was made between both plant extract and a known wound healind drug povidone iodine. For wound healing activity the bark of ficus mollis linn was powdered and
then makes simple ointment base preparation. The ointment preparation was made in two concentrations 5% and 10% the wound healing effect was evaluated in control and alloxan induced diabetic rats, respectively. Povidone iodine is used as reference drug (Jameeluddin et al., 2014).

*Alternanthera brasiliana* Kuntze is a neotropical native species used against inflammation, cough and diarrhea etc. Traditionally it is used as hemostatic in north east India, but its excellent wound healing activity in excision, incision, aged, burn, immunocompromised wound were reported by us. Keeping in mind the delayed healing of wound in diabetic patients and its complications, the present study was undertaken to evaluate the healing efficacy of topical application of *Alternanthera brasiliana* in experimentally induced diabetic wounds in Sprague Dawley rats. The animals were divided into three groups of six animals each. Group I is control, group II is treated with topical application of the extract and group III is used as standard. Prohealing activity was assessed by wound contraction, histopathological study, modulation of enzymatic and non enzymatic parameters. There was significant (P<0.01) increase in wound contraction, augmented levels of superoxide dismutase, catalase, reduced glutathione, hydroxyproline, protein and ascorbic acid level in the treated group as compared to the control group. It can be hypothesized that *A. brasiliana* favours wound healing in diabetic animals, due to the presence of various phytoconstituents which are known for augmenting healing and its antioxidant activity as well (Chandana Choudhury Barua et al., 2013).

The wound healing activity of *Phyllanthus niruri* leaves extract in rats. Five groups of adult male Sprague Dawley rats were experimentally wounded in the posterior neck area. Group 1 animals were treated with sterile deionized water (negative control). Thin layer of blank placebo was applied topically to Group 2 rats. Groups 3 and 4 rats were dressed topically with thin layer of placebo containing 5% and 10% *P. niruri* extract, respectively. Intrastie gel was used as a positive control. Grossly, wounds treated with placebo containing 5%, 10% *P. niruri* extract or Intrastie gel significantly accelerated the rate of wound healing compared to wounds treated with sterile deionized water or dressed with blank placebo. Histological analysis of healed wounds confirmed the gross observations. Wounds dressed with placebo containing 5%, 10% *P. niruri* extracts or intrastie gel
showed markedly less scar width at the wound enclosure with large amounts of fibroblasts proliferation, more mature and densely packed collagen and angiogenesis compared to wounds dressed with sterile deionized water (Khaled Abdul et al., 2012).

**HERBAL MEDICINE**

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Neube et al., 2008). Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician. Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans (Handa et al., 2008). The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, countercurrent extraction, microwave-assisted extraction, ultrasound extraction
(sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression may be employed. Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase microextraction, protoplast extraction, microdistillation, thermomicrodistillation and molecular distillation. The basic parameters influencing the quality of an extract are:

1. Plant part used as starting material
2. Solvent used for extraction
3. Extraction procedure Effect of extracted plant phytochemicals depends on
4. The nature of the plant material
5. Its origin
6. Degree of processing
7. Moisture content
8. Particle size

The variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract depends upon

1. Type of extraction
2. Time of extraction
3. Temperature
4. Nature of solvent
5. Solvent concentration
6. Polarity

Plants are potent biochemists and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. Scientific analysis of plant components follows a logical
pathway (Das et al., 2010). Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found. Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues. The logic behind this came from the ethno medicinal use of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues, plants are usually air dried to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 h (Parekh et al., 2006).

In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial properties. Choice of solvents successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants. The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be nontoxic and should not interfere with the bioassay.

1. Water: Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound.
2. **Acetone:** Acetone dissolves many hydrophilic and lipophilic components from the two plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol. Both acetone and methanol were found to extract saponins which have antimicrobial activity (Eloff, 1998).

3. **Alcohol:** The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unpolar character and cause polyphenols to be released from cells. More useful explanation for the decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive. Moreover, water is a better medium for the occurrence of the micro-organisms as compared to ethanol (Lapornik *et al.*, 2005). The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased. Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

4. **Chloroform:** Terpenoid lactones have been obtained by successive extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Bimakr, 2010).
5. **Ether**: Ether is commonly used selectively for the extraction of coumarins and fatty acids. Dichloromethanol: It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoids.

Methods of extraction Variation in extraction methods usually depends upon:

1. Length of the extraction period,
2. Solvent used,
3. pH of the solvent,
4. Temperature,
5. Particle size of the plant tissues
6. The solvent-to-sample ratio

The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal.

**Extraction procedures**

a. **Plant tissue homogenization**: Plant tissue homogenization in solvent has been widely used by researchers. Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5 - 10 min or left for 24 h after which the extract is filtered. The filtrate then may be dried under reduced pressure and redissolved in the solvent to determine the concentration. Some researchers however centrifuged the filtrate for clarification of the extract (Wang, 2010).

b. **Serial exhaustive extraction**: It is another common method of extraction which involves involves successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. Some researchers employ soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.
c. Soxhlet extraction: Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent.

If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.

d. Maceration: In maceration (for fluid extract), whole or coarsely powdered plant drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile drugs.

e. Decoction: this method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume.

f. Infusion: It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water.

g. Digestion: This is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstrum is increased thereby.

h. Percolation: This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to
form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

i. **Sonication**: The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

**Standardization**

Standardization is an essential factor for polyherbal formulation in order to assess the quality of the drugs based on the concentration of their active principle. It is very important to establish a system of standardization for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous. Plant material when used in bulk quantity may vary in its chemical content and therefore, in its therapeutic effect according to different batches of collection e.g. collection in different seasons and/or from sites with different environmental surroundings or geographical location. WHO has appreciated the importance of medicinal plants for public health care in developing nations and has evolved guidelines to support the member states in their efforts to formulate national policies on traditional medicine and to study their potential usefulness including evaluation of its quality, safety and efficacy (Cowan, 1999).

The process of evaluating the quality and purity of crude drugs by means of various parameters like morphological, microscopically, physical, chemical and biological observation is called standardization. Standardization involves adjusting
the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity by adding excipients or by mixing herbal drugs or herbal drug preparations. Botanical extracts made directly from crude plant material show substantial variation in composition, quality, and therapeutic effects. The presence of the word “Standardized” on a supplement label does not necessarily indicate product quality. When the active principles are unknown, marker substances should be established for analytical purposes and standardization.

**Importance of herbal plants in the treatment of diseases**

Nature always stands as a golden mark to exemplify the outstanding phenomena of one race depending on other for food. Natural products from plant, animal and minerals have been the basis of the treatment of human disease from the times immemorial. Today it is estimated that about 80% of people in developing countries are still depending on traditional medicine based largely on species of plants and animals. Herbal medicines are currently in demand and their necessity is increasing eventually. About 500 plants with medicinal use are mentioned in ancient literature by Theophrastus and 800 plants have been used in indigenous systems of medicinal system. India is a rich depository of medicinal plants are used in traditional medical treatments. The various indigenous systems of medicine such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat different diseases. The use of plant medicines is becoming popular due to toxic and side effects of allopathic drugs. This led to sudden increase in the number of herbal drug industries. Herbal medicines are the major remedy in traditional system of medicine have been used in medical practices since ages. The practices are continuing till today because of its biomedical benefits as well as its cultural beliefs in many parts of world. It have made a great contribution towards maintaining human health care system. In India around 20,000 medicinal plant species have been recorded but more than 500 traditional plant communities use about 800 plant species for curing different diseases.

Currently 80% of the world population rely on plant-derived medicine for the first line of primary health care because it has no side effects. Plants are important sources of medicines. Presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. In the 20th century,
roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various pharmacopeias. There are many evidences that many herbal plants are used for the treatment of diabetes milletus. Plant families which are confirmed to show hypoglycemic activity include: Leguminoseae, lamiaceae, lilliaceae, cucurbitaceae, asteraceae, moraceae, rosaceae, euphorbiaceae, araliaceae, polygalaceae, asclepidaceae, meliaceae etc. Many clinical studies have conformed the therapeutic importance of medicinal plants in the treatment of diabetes milletus disease. The effect of the medicinal plants may delay the diabetic complications and rectify the metabolic abnormalities. However during the past few decades new bioactive compounds are being isolated from the hypoglycemic plants. They showed hypoglycemic activity with more efficacy and are used in effective treatment of diabetes milletus.

Fig. 16. Parameters for standardization and quality evaluation of herbal drugs
WHO guidelines for standardization of herbal formulations

The standardization of crude drug materials includes the following steps

i. Authentication: Herbal drugs should be authenticated by considering parameters like; stage of collection, parts of the plant collected, regional status, botanical identity like phytomorphology, microscopy and histological analysis, taxonomical identity, etc.

ii. Foreign matter: Herbs collected should be free from insect parts or animal excreta and other contaminants like dust, soil, stones and extraneous matter etc. Foreign matters also include parts or the organ of the plant other than require for drug by definition or beyond limits set by the WHO guidelines.

iii. Organoleptic evaluation: Sensory characters, taste, appearance, odour, texture etc of the drug, are the various Organoleptic parameters should be evaluated for identification and purity before undertaking further tests.

iv. Presence of important diagnostic tissues: Microscopically identity of medicinal plant material is indispensable for the identification of broken or powdered material. An examination by microscopy alone cannot always provide complete identification, though when used in association with other analytical method.

v. Ash values: The amount of material remaining after ignitionis called total ash. It is also further determined by acid insoluble ash and water soluble ash.

vi. Extractive values: This determines the amount of active constituents extracted with different solvents like water, alcohol, ether, chloroform etc. from a given amount of medicinal plant material.

vii. Moisture content and volatile matter: Determination of moisture content or loss on drying helps in the estimation of the amount of volatile matter. It is more appropriate for substances contain water as the only volatile constituent. An excess of water in medicinal plant material will encourage microbial growth so that limits for water content should be set for every plants.
viii. **Chromatographic and spectroscopic evaluation:** TLC, HPTLC, HPLC methods will provide qualitative and semi quantitative information about the main active constituents present in the crude drug as chemical markers in the TLC fingerprint evaluation of herbals. The quality of the drug can also be assessed on the basis of the chromatographic fingerprint.

ix. **Microbial contamination and aflatoxins:** Usually medicinal plants containing bacteria and molds are coming from soil and atmosphere. Analysis of the limits of *E. coli* and molds clearly throws light towards the harvesting and production practices. The substance known as aflatoxins will produce serious side-effects if consumed along with the crude drugs. Aflatoxins should be completely removed or should not be present.

x. **Radioactive contamination:** Microbial growth in herbals is usually avoided by irradiation. This process may sterilize the plant material but the radioactivity hazard should be taken into account. The radioactivity of the plant samples should be checked accordingly to the guidelines of International Atomic Energy (IAE) in Vienna and that of the WHO.

In order to obtain quality oriented herbal products care should be taken right from the proper identification of plants; season and area of collection, extraction, isolation and verification process.

**Quality control parameters for herbal formulations**

i. **Physical parameters:** It include colour, appearance, odour, clarity, viscosity, moisture content, ash values, pH, disintegration time, friability, hardness, flow property, flocculation, sedimentation and settling rate.

ii. **Chemical parameters:** It includes limit tests for heavy metal, extractive values, chemical assays for active constituents, etc.

iii. **Chromatographic analysis of herbals:** Chromatographic analysis can be carried out using TLC, HPLC, HPTLC and GC, UV, Fluorimetry, GC-MS.

iv. **Microbiological parameters:** It includes total viable content, total mold count, total entero-bacteriaceae and their count.
REVIEW OF CHROMOLAENA ODORATA

Botanical name: Chromolaena odorata

Family: Asteraceae

Common names include Siam weed, Christmas bush, devil weed, camphur grass, common floss flower.

Description

C. odorata is a herbaceous to woody perennial with a bushy habit which forms a very dense thicket about 2 m high, in almost pure stands. This many-branched plant becomes lianescenent when it has the opportunity to climb on a support. Isolated individuals start to branch when they reach a height of about 120 cm. After the first year of growth, the plant develops a strong, woody underground storage organ, which can reach a diameter of 20 cm. Stems are terete and become woody. Twigs are slightly striolate longitudinally, pubescent, opposite-decussate. Leaves are simple, opposite-decussate and without stipules. They are rhomboid-ovate to ovate with an acute apex and a cuneate base. The blades are trinerved a few millimetres after the base, roughly crenate-serrate beyond their maximum breadth, slightly pubescent above and pubescent with numerous small yellow dots below (a lens is needed to see this). The petiole is 1-3 cm long, and the blade 5-14 cm long and 2.5-8 cm broad. Leaves and twigs produce a characteristic smell when crushed. Capitula are grouped in one, three or five convex trichotomic corymb 5-10 cm in diameter, at the end of the twigs. The involucre is cylindrical, 8-10 mm long by 3-4 mm broad. It is made of a series of four or five oblong bracts, the external being the shorter. These bracts are obtuse, chartaceous, pale in colour with three or five nerves. The receptacle is convex, without scales. There are 15-35 florets per capitulum. The corolla is 5 mm long and has five lobes. Its colour ranges from pale-lilac to white. Styles are of the same colour, exserted and flexuous. Cypsela are composed of a 3- to 4-mm-long fusiform blackish achene, with five beige barbelate ribs, overtopped by a pappus of about 30 barbelate beige capillary bristles which are 4-5 mm long (Gautier, 1992).
Leaves

The leaves of Siam weed are soft, green, hairy and triangular in shape, with a distinctive three-vein ‘pitchfork’ pattern (see Figure 1). New growth exhibits a purple colouration. The stems are smooth, round and fairly brittle, becoming woody at the base when old.

Flowers

Siam weed flowers from May to July and again in September to October, producing masses of pale lilac flowers that appear white from a distance. They turn a darker lilac when mature. Flowering is triggered by the shorter day lengths in winter and, due to the prolific flowering, this can be a good time to identify new infestations. Any new infestations should be treated immediately to reduce the production of viable seed.

Seeds

Siam weed produces huge numbers of windborne seeds within 8–10 weeks after flowering (more than 80 000 seeds per plant per season). Each seed has a tuft of white hairs that allow it to be transported by wind and water. Seeds will also attach to vehicles, machinery, clothing, footwear and animals. Most seeds germinate immediately after rain, though some appear to remain dormant for several years. Seed longevity research is continuing.
Life cycle

Siam weed is a perennial that can out-compete and smother crops and native vegetation because of its phenomenal growth rate (20 mm per day or 5 m per year) and ability to scramble up taller plants to a height of 20 m.

Habitat

Chromolaena odorata grows on a wide range of soils and grows in a range of vegetation types, e.g. forests (annual rainfall 1500mm), grassland and arid bushveld (annual rainfall less than 500mm). In arid areas, it is restricted to riverbanks and it will only become invasive in the frost-free areas of medium to arid woodland which are not water-stressed in the growing season. For good growth of Siam weed seedlings, the relative humidity should be in the range of 60 – 70%; at values higher than 80% the growth performance was poor. Experiments show that Siam weed seedlings grew well at 30°C and even better on mulched soils at 25°C. In heavy shade, Siam weed will not seed. It has a negative relationship with tree canopy cover and appears to be most abundant on the edge of forested areas reports that in northeastern India, Siam weed is regarded as a nutrient-demanding early successional species (Vanderwoude et al., 2005). It takes advantage of the flush of soil that becomes available after a disturbance, such as fire or land clearing for agriculture, and exhibits relatively high foliar N, P and K contents.

The medicinal values of plants lie in their component phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body (Hill, 1952). A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceautical and drug research. Therefore, the present work has been designed to evaluate the antioxidant potential of C. odorata with a view to contributing to the search for beneficial uses of this invasive plant which is a menace to farmers.

Control

It is important that Siam weed be contained to currently infested areas. This can be achieved by:
- Restricting entry to Siam weed infestations through fencing or other means
- Cleaning down vehicles, machinery and equipment (if restricting entry is not possible)
- Cleaning all clothing, shoes and camping gear before leaving an area known to have been infested with Siam weed
- Quarantine of livestock for at least one week before they leave a Siam-infested property
- Requesting a weed hygiene declaration when buying anything that may be contaminated with Siam weed seed.

**Mechanical control**

Physical removal of the basal/root ball is very effective and recommended for smaller infestations. However, it is extremely important to make sure the removed plant does not remain in contact with soil, as any contact will result in the plant re-shooting.

**Medicinal Uses**

A very important though facultative use of *C. odorata* is as a green manure or fallow component. Being invasive, it has replaced native secondary successional species in much of the tropical Old World, and farmers have learnt to live with as a component of the farming system, especially in shifting cultivation and rotational practices. It is often preferred over native species as it easier to cut and clear, and is observed to suppress another invasive species, *Imperata cylinrdrica*, which has much worse impacts on agriculture. Reports that *C. odorata* improves soil fertility require confirmation, though clearly the fallow period will have a positive effect. *C. odorata* can also be cut and cleared prior to seed set, composted and used as a valuable organic soil amendment.

Traditional Uses: In traditional medicine, a decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. Other traditional medicinal uses include anti-diarrheal, astringent, antispasmodic, antihypertensive, anti-inflammatory, diuretic, tonic,
antipyretic and heart tonic (Vital, 2009). The fresh leaves and extract of *C. odorata* are a traditional herbal treatment in some developing countries for burns, soft tissue wounds and skin infections (Suksamrarn et al., 2004).

**Newer Findings**

*Chromolaena odorata* is found to be a highly efficacious medicinal herb according to the traditional and folk medicinal systems. The same is proved by its pharmacological evaluation performed by scientific community across the world. The most established and discussed aspect of Chromolaena is its role in wound healing. Extracts from the leaves of *Chromolaena odorata* have been shown to be beneficial for treatment of wounds. In traditional usage, the leaf is ground into a paste and is applied topically on affected places to heal wounds (Ling et al., 2004). The aqueous extract and the decoction from leaves of this plant have been used throughout Vietnam for the treatment of soft tissue wounds and burns for decades. A product made from *Chromolaena* named eupolin have already been licensed for use in Vietnam for soft tissue burns and wounds. Studies *in vitro* of these extracts demonstrated enhanced proliferation of fibroblasts, endothelial cells and keratinocytes, stimulation of keratinocyte migration in an *in vitro* wound assay, up-regulation of production by keratinocytes of extracellular matrix proteins and basement membrane components, and inhibition of collagen lattice contraction by fibroblasts (Ayyanar, 2009).

A study on chromolaena has demonstrated that the extract increased expression of several components of the adhesion complex and fibronectin by human keratinocytes. The process of wound healing is also enhanced by the antimicrobial activity of the *Chromolaena* (Raina, 2008). The ability of wound healing is attributed to the antioxidant property of the drug which helps in conserving the fibroblast and keratinocyte proliferation on the site (Phan, 1998). The basis for the external applications of *Chromolaena* was found to be its profound antioxidant action. The antioxidant effects of purified fractions on cultured fibroblasts and keratinocytes were investigated using colorimetric and lactate hydrogenase release assay. The results showed that the phenolic acids present (protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids) and complex mixtures of lipophilic flavonoid aglycones (flavanones, flavonols, flavones and chalcones) were major and powerful
antioxidants (Panda et al., 2010). The nitric oxide scavenging activity of the Chromolaena extract was demonstrated. Quantitative determination of the total phenolic content shows that the extract contains an appreciable amount of phenolic compounds and may be responsible for the observed antioxidant potential (Phan et al., 2001). The anti-inflammatory, analgesic and antipyretic activities of the Chromolaena are evident from its traditional usage in rheumatic fever and similar conditions. In one of the recent studies, the pharmacological evaluation of the drug extract was performed by using standard experimental models which includes; hot plate and formalin paw licking tests for analgesic activities, carrageenan paw oedema and cotton pellet granuloma for anti-inflammatory activities and Brewer’s yeast induced pyrexia for antipyretic tests. The result shows that the extract produced consistent analgesic, anti-inflammatory and antipyretic activities (Mahmood et al., 2005). Other studies have shown that the anti-inflammatory activity is accounted by the presence of flavonoids in the extract. The antimicrobial activity of Chromolaena was evaluated and proved in a number of experiments.