CHAPTER-2: LITERATURE REVIEW

2.1. Herbal Medicines Used in the Treatment of Diabetes

Considering the multifactorial aetiology of diabetic complications, an ideal drug or drug combination should target several key pathogenetic mechanisms (Dewanjee et al., 2011). Medicinal plants that possess substantial quantity of antioxidant components have been found to be useful against diabetes and its related complications (Bhat et al., 2008; Choi and Son 2011)

2.2. Criteria for Selection of Herbs for Developing a Herbal Formulation

- Relevant ethnobotanical information
- Scientific proof studies for their anti-hyperglycaemic effect
- Knowledge about the toxicity and margin of safety
- Availability of the plant
- Legal access to plant from other countries
- Globally accepted ingredients, not banned by regulatory agencies of some countries (Paul A Devasagayam 2007)

2.3. Rationale of Selection of Herbs

Diabetes is a multifactorial disorder and ayurveda recognizes oxidative stress as the major cause of diabetes and its complications. It also recommends using formulation containing multiple herbs or active ingredients to normalize the altered pathophysiology. Each ingredient serves a specific function in the formulation and is used to achieve synergetic therapeutic action (Tiwari 2005). Addition of multiple ingredients makes the formulation too complex and makes the process of formulation and standardization difficult. Use of herbs with multiple therapeutic actions could eliminate the use of multiple ingredients making the formulation simple and facilitates standardization (Ofner III 1996).
2.4. Monograph of *Allium sativum* (color plate- 5a)

**Family** : Liliaceae, Alliaceae.

**Habitat** : Native to Central Asia. Cultivated all over India.

**English** : Garlic.

**Ayurvedic** : Lashuna, Rasona, Yavaneshta, Uragandha, Mahaushadh, Arishta.

**Siddha/Tamil** : Ullippoondu, Vellaippondu.

**Action** : Antibiotic, bacteriostatic, fungicide, anthelmintic, antithrombic, hypotensive, hypoglycemic, hypocholesterolaemic. Also used for upper respiratory tract infections and catarrhal conditions (Khare 2007).

**Scientific Literatures on Allium Sativum**

Garlic (*Allium sativum* L. fam. Alliaceae) is a commonly used spice and the best-selling herbal drug (Aviello et al., 2009). Diabetes is a life style disorder and could be managed with use of functional foods (Ballali and Lanciai 2011). A combination of garlic with standard antidiabetic therapeutic regime could improve the glucose and lipid homeostasis by virtue of their anti-diabetic and anti-hyperlipidemic property (Ashraf et al., 2011; Madkor et al., 2011; Hfaiedh et al., 2011). Clinically, garlic has been evaluated for a number of conditions, including hypertension, hypercholesterolemia, diabetes, rheumatoid arthritis and for the prevention of arteriosclerosis and cancer. Garlic appears to be clinically safe although allergic reactions may occur (Aviello et al., 2009). The multiple therapeutic effects of garlic, such as insulin secretagogue, insulin sensitizer, antioxidant, antiglycative properties could be beneficial in preventing diabetes progression and the development of diabetes-related complications (Liu et al., 2007). S-allyl cysteine, present in garlic, an organosulphur compound inhibits the formation of advanced glycation products *in vitro* (Ahmad et al., 2007, Ahmad and Ahmed 2006). The organosulfur compounds are potent antioxidants that prevent oxidation...
Fig 5a: Color plate of bulbs of *Allium sativum*.
of LDL. They also have anti-glycation property which could prevent complications in patients with diabetes mellitus or cardiovascular diseases (Ou et al., 2003; Huang et al., 2004).

### 2.5. Monograph of *Lagerstroemia speciosa* (color plate-5b)

**Synonym**: *L. reginae* Roxb.

**Family**: Lythraceae.

**Habitat**: Tropical Himalaya, and Assam, Western and Eastern Ghats, up to 1000 meter.

**English**: Pride of India, Queen’s Flowers, Queen Crape Myrtle.

**Tamil**: Kadalai, Pumaruttu.

**Action**: Seed - narcotic. Root - astringent, stimulant, febrifuge. Fruit - used for aphthae of the mouth. Leaves—purgative, diuretic, deobstruent. Bark—an infusion is given in diarrhoea and abdominal pain. A decoction of the leaves, also of dried fruits, is used like tea for diabetes mellitus in Philippines. Mature leaves and fruits, in fresh condition, exhibit hypoglycaemic activity experimentally. The potency decreases on storing the material. The leaf extract, when administered as powder and as tannin-free extract, showed hypoglycaemic activity in mice. Amino acids constitute the insulin-like principle. The plant contains triterpenoids, colocolic acid and maslinic acid. Colocolic acid is known to possess hypoglycaemic activity. Leaves contain lageracetal and sitosterol. Ellagitannins have been isolated from fruits and leaves (Khare 2007).

**Scientific Literatures on *Lagerstroemia speciosa***

*Lagerstroemia speciosa* (Lythraceae) extracts, a folk medicine used since 1940 by Philippinos for treatment of diabetes. Corosolic acid (CA), a pentacyclic triterpene and Lagerstroemin, a ellagitannin were reported for their hypoglycemic effects. CA has also been reported for its antihyperlipidemic, antioxidant, anti-inflammatory activities. CA has also claimed to have multiple beneficial effects on glucose and lipid metabolism, which includes enhanced cellular uptake of glucose,
Fig 5b: Color plate of aerial part of *Lagerstroemia speciosa*. 
impaired hydrolysis of sucrose and starches, decreased gluconeogenesis and the regulation of lipid metabolism. (Stohs et al., 2011). A herbal formulation (DLBS3233) was developed using a combination of standardized extract of Lagerstroemia speciosa and Cinnamomum burmannii, which is indented for its anti-diabetic effect (Tandrasasmita 2011). It has the potential to inhibit lipid peroxidation and scavenges reactive oxygen species (super oxide) and NO based free radicals (Saumya and Basha 2011). CA shows better alpha-glucosidase inhibitory activity than alpha-amylase inhibitory activity (Hou et al., 2009) and inhibits the hydrolysis of sucrose by sucrase (Takagi et al., 2008). CA ameliorates the altered hepatic glucose metabolism by decreasing the rate of gluconeogenesis and promotes the glycolysis of sugar in the liver which may be one of the possible mechanisms for its anti-diabetic effect (Yamada et al., 2008). Ellagitannins (lagerstroemin, flosin B and reginin A) have been reported to increase glucose uptake in adipocytes and could be responsible for the antidiabetic effect of Lagerstroemia speciosa (Hayashi et al., 2002). Except a few, the majority of antidiabetic drugs cause hypoglycaemia, promote weight gain; worsen the risk of diabetes mellitus and its associated complications. Adipogenesis is the process of differentiation and proliferation of adipocytes and is a major mechanism leading to weight gain and obesity. Drugs that could reduce blood glucose levels without inducing adipogenesis are highly desirable for the treatment of diabetes mellitus. The Penta-O-galloyl-glucopyranose (Klein et al., 2007) and tannic acid (Liu et al., 2005) available with Lagerstroemia speciosa has both glucose uptake and anti-adipogenesis activity, which may indicate the therapeutic potential of Lagerstroemia speciosa in the management of obesity associated diabetes.

2.6. Standardization of Herbal Medicines

India has been recognized for its wide variety of spices and medicinal plants (Krishnaswamy 2008). Herbal medicines are generally prepared as a complex mixture using medicinal plants (Patra et al., 2010). Herbal medicines have drawn
increased attention, since they have easy access and do not need laborious pharmaceutical synthesis like modern medicines (Girish et al., 2008). Due to their complex nature, herbal formulations pose enormous analytical challenges in quality control (Li et al., 2010). They lack effective quality control methods right from the selection of raw materials till the production of finished products (Patra et al., 2010). In the modern era, advancement of scientific knowledge has taken forward the development of analytical techniques for quality evaluation and standardization of herbal based formulations (Aravind et al., 2008). High Performance Thin Layer Chromatographic (HPTLC) technique may be used as a rapid analytical method to control the quality of herbal extracts and their formulations (Gallo et al., 2008). HPTLC fingerprint may also be used as a chemical fingerprint of the herb(s)/formulations for quality control (Srivastava et al., 2004). It may also serve to assess the uniformity of the active ingredients and to identify batch-to-batch variation during formulation (Patra et al., 2009). HPTLC fingerprint developed with or without marker facilitates the authentication and assessment of quality of herbal drugs (Rajani and Kanaki, 2008) and provides the concept of phytoequivalence (Sudberg et al., 2010). HPTLC chromatogram can also be used as an ideal tool for conducting stability studies, to predict the shelf life, monitor adulterations and extraction processes of herbal medicines (Meier and Spriano, 2010). Compared to pharmaceuticals, stability studies of herbal medicines is difficult to conduct due to the presence of mixture of various types of phytoconstituents. Preliminary phytochemical analysis of herbal drugs also provides necessary information pertaining to the nature of phytoconstituents present in the herbal drug and helps in devising the mobile phase of different polarity to establish a HPTLC fingerprint profile of good resolution (Rajani and Kanaki, 2008). The complexity of herbal formulation, due to inclusion of multiple interactive ingredients hinders the standardization of these drugs. Standardization of herbal preparations will be much
easier when formulated with minimum number of standardized ingredients than inclusion of crude extracts or multiple ingredients.

Herbal medicines are usually made up of simple or complex ingredients. The ingredients are mainly of herbal origin since they are largely available. Herbs may contain varying concentration of chemical compounds and this variation could be due to many factors like the geographical conditions, time of harvesting and processing of the herb. Consistency in the chemical composition is a requirement for the safety and efficacy of an herbal medicine. Herbal medicines are considered to be dietary or food supplements in many countries and therefore are subjected to a very limited form of regulation. They are often considered to be safe since they are of natural origin; however many toxic effects have been reported due to their direct toxic effects, allergic reactions, effects from contaminants, and interactions with drugs and other herbs. Herbal manufacturers attempt to provide products with consistent levels of suspected active ingredients through a process known as standardization; this technique has uncertain effects on the safety and efficacy of the final product (Bent and Ko 2004). Standardized herbal medicines could be the starting point for approval of regulatory agencies of various countries (Rajani and Kanaki 2008). The major pharmaceuticals standardize their herbal medicines using modern analytical techniques such as HPTLC and HPLC fingerprinting. Fingerprint analysis could be a tool to control the quality of herbal medicines and is used to determine their identity and stability, detect adulteration and batch-to-batch consistency in raw material and in finished products (Xie et al., 2006). The role of fingerprinting in quality control and assessment of herbal medicines has been demonstrated in earlier studies (Qian et al., 2007; Chaudhary et al., 2007 and Ip et al., 2010). Fingerprint profiles form a benchmark for the herbal drug when the identity of the active principles is not known or when chemical markers are not available. Ideally, a marker compound is the one chemical compound specific to the plant material. These marker compounds are not easily available in the market.
and they are very expensive because of their low availability in the plant. For those plants for which active principles are known, quantification of the active principles is carried out. Fingerprint profiles with marker compounds, and quantification of marker compound(s) with single herbal drugs are generally performed. In the case of polyherbal preparations it is practically impossible to have marker compounds specific to each of the ingredients of the formulation. HPTLC fingerprint profiles generated with or without marker compounds could be well appreciated in quality control herbal medicines (Rajani and Kanaki 2008).

Another approach to herbal medicine standardization is establishment of phytochemical profile, since it has a direct bearing on the therapeutic activity. It covers all the possible information generated with regard to the chemical constituents present in a herbal drug. Quantification of specific chemical groups of interest (e.g., total alkaloids, total phenolics, and total tannins) gives more precise information pertaining to the nature of chemical compounds available in a drug. The preliminary phytochemical analysis of a plant extract provides the nature of phytochemicals available in them and also enables to devise the mobile phase based on the polarity of these phytochemicals during the establishment of HPTLC fingerprint profiles to achieve good resolution of the components of different polarity (Rajani and Kanaki 2008). Botanical extracts standardized with marker compound provides a certain level of quality control, but not complete quality assurance. Apart from estimation of the concentration of the marker compounds, industries are looking for other satisfactory systems to improve standardization and measurement of antioxidant capacity (Ninfali et al., 2009).

Some of the reported standardization techniques on *Allium sativum* and *Lagerstroemia speciosa* are reviewed. A HPTLC method has been proposed for the analysis of garlic and its formulations for their alliin content, which involves densitometric evaluation of alliin after resolving it by HPTLC on silica gel plates with n-butanol:acetic acid: water (6:2:2, v/v) as the mobile phase. The peak areas were
recorded at 540 nm after derivatizing the resolved bands with ninhydrin reagent. (Kanaki and Rajani 2005) S-allylcysteine sulfoxide (alliin) is a far more stable and commercially prevalent compound than allicin and is thus more amenable for use as a standard for routine analysis (Rybak et al., 2004) The quality of most of the preparations made from garlic powder or garlic dry extract is determined by their content of alliin. (Keusgen 1998). A novel HPLC method for determination of a wide variety of S-substituted cysteine derivatives in Allium species has been developed and validated (Kubec and Dadáková 2009).

Chromatographic methods have been developed for the determination of corosolic acid in Lagerstroemia speciosa leaf extracts using high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) (Vijaykumar et al., 2006). The maximum corosolic acid content was found in the leaves of Lagerstroemia speciosa and a HPTLC densitometric quantification of corosolic acid in leaf extracts was performed in absorbance mode at 366 nm after derivatization with chloroform-methanol 8.5:1.5 (v/v) as mobile phase (Mallavadhani 2008).

2.7. Methods for Assessing Antioxidant Activity of Herbal Medicines

Free radicals are considered to be an important cause in triggering the development of type 2 diabetes and its complications. Drug that control hyperglycemia and free radical induced oxidative tissue damage could benefit the diabetics to control their elevated blood sugar level and hyperglycemia induced oxidative stress as well. A wide variety of in vitro assay methods have been developed to assess the ability of compounds to prevent oxidative damage. Amongst the tests that measure radical scavenging capacity, the DPPH assay is the widely used method (Locatelli et al., 2009). Nitric oxide is a free radical whose half life is very short. Nitrite and nitrate are the final products of nitric oxide (NO) oxidation pathways whose half life is greater when compared to nitric oxide. The concentrations of nitrite and nitrate are frequently assessed as an index of systemic
NO production. Different methods have been used for the estimation of nitrite/nitrate, with the most commonly used being the spectrophotometric assay, based on the Griess reagent (Giustarini et al., 2008). Lipid peroxidation end products such as malondialdehyde (MDA) are used as indicators for determining the cellular oxidant status and such endproducts could be measured via a thiobarbituric acid reactive substances (TBARS) assay (Potter et al., 2011). Super oxide radical could be generated in vitro using the PMS-NADH-nitroblue tetrazolium system and the ability of compounds to scavenge these radicals is measured spectrophotometrically. 2, 7-dichlorofluorescein diacetate (DCFH-DA) staining is also widely used to directly measure the redox state of a cell. The nonfluorescent dye of 2′,7′-dichlorofluorescin diacetae (DCF-DA) is converted to fluorescent 2′,7′-dichlorofluorescein (DCF) by peroxidase (H₂O₂) and the ability of test compounds to inhibit the fluorescence is assessed (Carter et al., 1994; Eruslanov and Kusmartsev 2010)

2.8. Methods for Assessing Antidiabetic Activity of Herbal Medicines

2.8.1. In Vitro Models for Antidiabetic Activity

Antidiabetic effects of herbal medicines could be assessed using a wide range of in vitro methods. These methods could play an important role in the evaluation of herbal medicines, as preliminary screening tools or as follow-up to animal studies. In vitro assays are performed based on a specific biological process relevant to the disease and its treatment. These assays are of considerable value in identifying the possible mechanism of action. A wide range of tests are available to evaluate the potential antidiabetic activity based on the primary need to control hyperglycemia and its associated complications.

2.8.1.1. In Vitro Assays to Study Inhibition of Carbohydrate-Digesting Enzymes

Alpha-glucosidase is a group of membrane-bound enzymes of the small intestinal villi, involved in the breakdown of α-linkages of complex sugars like
oligosaccharides and disaccharides into simple sugars like glucose, fructose, and galactose. Inhibition of intestinal carbohydrate metabolising enzymes with alpha-glucosidase inhibitors reduces the postprandial increase of blood glucose level after a mixed carbohydrate diet and is considered as one of the therapeutic approaches for developing novel antidiabetic and antiobesity agents (Soumyanath and Srijayanta 2006). Presently, there is renewed interest in plant-based medicines in the prevention and management of diabetes and obesity. Therefore, natural alpha-glucosidase and alpha-amylase inhibitors from plant sources offer an attractive strategy for the control of hyperglycaemia (Tundis et al., 2010). The in \textit{vitro} inhibitory effect of herbal extracts on \(\alpha\)-glucosidases could be assessed by determining the degree of decrease in the amount of glucose liberated from molecules of the substrate after incubation with the enzyme. Glucose can be determined colorimetrically using the glucose oxidase method. The choice of substrate used depends on the enzyme of interest; if the activity of sucrase is to be determined, sucrose will be used as the substrate, whereas maltose and isomaltose would be used to determine maltase and isomaltase respectively. (Soumyanath and Srijayanta 2006).

\textbf{2.8.1.2. \textit{In Vitro} Assays to Study Inhibition of Tissue Sorbitol accumulation}

Aldose reductase is the key enzyme of the polyol pathway which catalyzes the reduction of glucose to sorbitol. During diabetes mellitus, the increased availability of glucose in insulin-insensitive tissues (eye lens, nerves, and retina) leads to the increased formation of sorbitol. Sorbitol does not readily diffuse across cell membranes and the intracellular accumulation of sorbitol has been implicated in the chronic complications of diabetes such as cataract, neuropathy, and retinopathy. Therefore, the AR inhibitor can prevent the conversion of glucose to sorbitol and may have the capacity to prevent and/or treat several diabetic complications (Yoshikawa and Matsuda 2006). Human erythrocyte was used as an insulin insensitive tissue to access the effect of ascorbic acid on erythrocyte sorbitol
content. Ascorbic acid supplementation could effectively reduce sorbitol accumulation in the erythrocytes of diabetics (Vinson et al., 1989; Cunningham 1994). Few herbal medicines were also evaluated for their inhibitory effect on erythrocyte sorbitol accumulation (Kato et al., 2006; Zhou1989).

2.8.1.3. In Vitro Assays to Study Inhibition of Protein Glycation

Glycation is a non-enzymatic reaction (Maillard reaction) between the free amino groups of the protein and the keto group of the reducing sugars to form a Schiff base that further rearranges to form a ketoamine adduct, which eventually undergoes irreversible chemical modifications to form advanced glycation end products. Bovine serum albumin was used as a model of protein, which reacts with sugars (glucose, fructose, ribose) allowing the formation of AGE products and could be used to evaluate compounds having protein glycation inhibitory activity in vitro (Sharma et al., 2002). The end products of polyol pathway namely sorbitol and fructose plays a major role in the formation of advanced glycation end products (Oimomi et al., 1989). The rate of glycation is greater with fructose than glucose (Suárez et al., 1989). An equal molar concentration of glucose and fructose was used as glycating agent by Nandhini et al., 2004. Aminoguanidine, a known antiglycation agent was used as a positive control by Rajasekar and Anuradha 2007. Few reports have revealed the in vitro protein glycation inhibitory potential of certain herbal medicines indicating their use in mitigating diabetic complications (Dong et al.,2010; Adisakwattana et al.,2010; Sultana et al.,2009).

2.8.1.4. In Vitro Assays to Study Adipocyte Function

Adipocytes are insulin-sensitive cells that play a major role in energy homeostasis and also in the pathogenesis of metabolic disorders like insulin resistance, obesity, and type-2 diabetes. At the clinical level, adipose tissue insulin resistance contributes to type 2 diabetes and an increase in the adipose deposits causes obesity, resulting from an imbalance between food intake and energy expenditure. When expansion of the adipose tissue reaches its maximum limit, as
in obesity, fat accumulates in non-adipose tissues such as liver, heart, muscle and pancreas, developing a toxic response known as lipotoxicity, a condition that promotes the development of insulin resistance and other metabolic complications (Medina-Gomez et al., 2007). Insulin resistance results in decreased insulin-stimulated glucose transport into peripheral tissues like skeletal muscle and adipocyte tissue. The stimulation of glucose uptake into peripheral tissues is an important mechanism for the removal of glucose in blood and for the management of type-2 diabetes mellitus (Muthusamy et al., 2008). The glucose uptake process is achieved by activation of a complex signal transduction cascade that stimulates the signalling of the insulin responsive glucose transporter protein, GLUT-4 from specific intra-cytoplasmic pool (Morgan et al., 2011). Adipogenesis is the process of cell differentiation by which preadipocytes become adipocytes. The process of adipogenesis is controlled by several transcriptional factors (Laudes, 2011). Dysregulation of the mechanisms that control adipocyte leads to metabolic disorders like obesity. Dysregulation in the signalling cascade mechanism of transcriptional factors during adipocyte differentiation results in metabolic diseases. This highlights the importance of understanding adipocyte differentiation mechanisms. Significant advances towards an understanding of the regulatory processes involved in adipocyte differentiation have largely been made by the identification of transcription factors that contribute to the adipogenic process (White and Stephens, 2010). PPARγ, a nuclear receptor is a transcription factor that is abundantly expressed in adipocytes and plays a key role in the differentiation of preadipocytes to adipocytes and in the regulation of insulin resistance. PPARγ is an important target of anti-diabetic class of compounds and is known as the master regulator of adipocyte cell biology. Although PPARγ regulates hundreds of adipocyte genes, several other members like SREBP-1c, leptin have been shown to have significant roles in controlling adipogenesis (Han et al., 2006). Sterol regulatory element binding proteins (SREBPs) are essential transcriptional factors
for lipid synthesis and adipocyte differentiation (Yang et al., 2007). Leptin, a key polypeptide hormone in regulating energy homeostasis, is mainly produced by adipocytes (Ikeda et al., 2011; Pan 2012).

In spite of numerous preclinical and clinical interventions, the prevalence of obesity and obesity associated diabetes mellitus are on the rise demanding an urgent need for exploring novel therapeutic agents that can regulate adipogenesis and glucose homeostasis as well. Agents that stimulate glucose uptake and regulate adipogenesis may be useful in the management of type 2 diabetes mellitus (Thyagarajan-Sahu et al., 2011).

The 3T3-L1 model is well established model to study the effect of drugs in vitro on adipogenesis and glucose uptake (Thyagarajan-Sahu et al., 2011; Elefanty and Stanley 2012) and measuring the oxidative stress. It has provided key insights to gain a better understanding of the molecular mechanism(s) underlying the GLUT-4 protein expression and transcriptional factors involved in lipid metabolism. Adipose tissue oxidant stress is emerging as an important mediator of adipocyte dysfunction (Espiritu and Mazzone 2008). Intracellular production of reactive oxygen species (ROS) was determined spectrofluorimetrically using 2', 7'-dichlorofluorescin diacetate (Xi et al., 2007). 3T3-L1 pre-adipocytes are ideal cells to study the adipocyte differentiation process and the expression of transcriptional genes and proteins involved during adipogenesis and glucose uptake. These cells could be easily grown and differentiated into mature adipocytes using appropriate differentiation media. Adipocyte differentiation could be evaluated by oil red O staining (Haridas Nidhina et al., 2011). Gene expression and protein expression could be evaluated by RT-PCR and Western blot analysis techniques respectively. Glucose uptake could be determined with radio-labelled deoxy glucose.

2.8.2. Experimentally induced animal models of NIDDM

In vitro assays have their own limitations since they do not assess the bioavailability and metabolism of the herb does not occur during these assays. The
activity of a herb may be due to metabolites formed in vivo rather than compounds originally present in the herbal extract. The herbal extract may be effective at a particular concentration range in vitro but its relevance in vivo has to be considered. Animal models have been used to investigate the mode of action and side effects of medicinal plants and to assess their antidiabetic activity in vivo. The most widely used animal models are small rodents, since they are less expensive, easy to maintain and generally exhibit a more rapid onset of diabetic condition consistent with their short lifespan. In humans, due to the heterogeneity of diabetic conditions, no single animal model mimics entirely the pathophysiology of diabetes. Thus, many different animal models have been used, each displaying unique features of human diabetic states. Insulin resistance and beta-cell function are major predictors that contribute to the pathophysiology of type 2 diabetes and it is advantageous if an animal model designed to test efficacy exhibits both of these pathogenic features. There are several chemicals that induce diabetes, of which streptozotocin (STZ) produces a more specific and controllable destruction of islet β-cells compared to other chemicals like alloxan and is now the most widely used model of insulin-dependent diabetes for testing new therapies. A rat model of type 2 diabetes could be established using high fat diet (HFD) combined with low dose of streptozotocin (STZ) or multiple low-dose STZ injections, which exhibits both the pathogenic features of type 2 diabetes mellitus. Chronic administration of HFD to rats could initiate insulin resistance and administration of STZ could induce type-2 diabetes mellitus by inducing β cell death through alkylation of DNA. Administration of high-dose STZ to HFD fed rats severely impairs insulin secretion mimicking type 1 diabetes, whereas low-dose STZ is known to induce a mild impairment of insulin secretion mimicking type 2 diabetes (Srinivasan et al., 2005, Zhang et al., 2008). To test the anti-diabetic effect of medicinal plants using such animal models, it is convenient to commence the therapy within a few days of STZ administration before hyperglycemia becomes severe.
2.9. Biomarkers of Oxidative Stress

High concentration of glucose during diabetes mellitus exhibits a more specific triggering effect on reactive oxygen species (ROS) production. The overproduction of ROS is accompanied by an increased nitric oxide (NO) generation, a phenomenon favoring the formation of the stronger oxidant, peroxynitrite that initiates various events like lipid peroxidation, protein oxidation, and DNA damage. (Kocić and Cirić 2009). Type 2 diabetes mellitus is associated with increased lipid peroxidation (LPO) process (Marjani 2010). Chronic hyperglycemic state promotes disease progression by increasing oxidative damage to proteins, lipids and DNA as a result of a combination of increased free radical production and an impaired ability of cells to detoxify the radicals and repair damaged molecules (Mattson 2009). Lipid peroxidation is an important process in the development of diabetic complications (Nakhjavani et al., 2010). The measure of end products of this process such as reactive carbonyl compounds, malonic dialdehyde and methylglyoxal could be a biomarker in the prediction of the levels of tissue damage in the target organ during diabetes mellitus (Marjani 2010). The reactive carbonyl compounds formed during lipid peroxidation and sugar glycoxidation, namely advanced lipid peroxidation end products and advanced glycation end products accumulate in tissues causing stress to tissues that lead to diabetic complications. The development of drugs having both antioxidant and carbonyl scavenger activity forms a new therapeutic challenge in the treatment of oxidative stress associated diseases like diabetes mellitus (Negre-Salvayre et al., 2008).

Glutathione (GSH) is an endogenous antioxidant which serves as a biomarker of the antioxidative capacity of the cell. Several clinical conditions are associated with reduced GSH levels which as a consequence can result in a lowered cellular redox potential. High glucose concentrations during diabetes mellitus metabolises the excess of glucose through aldose reductase pathway,
where reduced nucleotides are used as the cofactors during the conversion glucose to fructose, leading to decreased reduced glutathione (GSH) content (Kocić and Cirić 2009). Therefore, restoration of the intracellular glutathione levels could protect the cells from damage caused by reactive oxygen species. The modulation of GSH metabolism might provide a useful adjuvant therapy in diabetes and cardiovascular disease (Exner et al., 2000).

The non-enzymic reaction between a free amino group in proteins and a reducing sugar, called the glycation process also inactivates glutathione and in addition, glutathione could also be glycated. Alterations in the concentration of GSH in tissues have also been demonstrated to be a common feature of many pathological conditions like diabetes and other disease like cancer, AIDS, neurodegenerative and liver diseases (Ganea and Harding 2006). Glutathione peroxidase, is also a biomarker of oxidative stress from the glutathione family, whose activity is decreased during chronic hyperglycemic state (Goyal et al., 2011). Oxidative damage to proteins could be measured as protein carbonyl content in tissues. Protein carbonyl groups result from free radical-induced protein oxidation and their level in tissues serves as a biomarker of oxidative damage (Chevion et al., 2000).

Chronic hyperglycemia could cause tissue damage leading to micro and macro vascular complications. Deregulation in the glucose metabolic pathway plays major role in the progression of diabetic complications. Increased extra cellular glucose concentration causes dysregulation in the reactive oxygen and nitrogen signalling pathways that lead to diversion of glycolytic intermediates into pathological pathways, the causative factor in the development of diabetic complications (Bonnefont-Rousselot 2002). It is widely recognized that the activity of hexokinase and the content of glycogen are decreased whereas the activities of glucose 6-phosphatase and fructose 1, 6-bisphosphatase are increased in the tissues during diabetes. The mechanism underlying the effect of oxidative stress on
key enzymes of the metabolic pathway is still under research and novel pharmacological targets designed to stimulate glycolytic pathway and inhibit gluconeogenic pathway and restore the glycogen through regulating key enzymes in the glucose metabolism could potentially enhance protection against hyperglycemia induced oxidative tissue damage and prevent or reverse complications of diabetes mellitus (Folli et al., 2011).

2.10. Safety Studies on Herbal Medicines

The uncertain composition of many herbal medicines raises questions about their safety and their adverse effects are probably not reported. Most of the herbal preparations are not prepared using standardized ingredients, are consumed as dietary supplements, and are not subjected to extensive pre-clinical or clinical trials, like allopathic drugs by the drug regulatory authorities (Goldman 2001).

2.10.1. Acute Oral Toxicity

Acute oral toxicity testing is still required for the classification and labelling of chemicals or mixture of chemicals. There have been increasing efforts over the last two decades to reduce the number of animals needed for this testing, according to the three 3Rs concept. An in vitro cytotoxicity test is used as an alternate for acute oral toxicity testing (Schrage et al., 2011). The oral acute toxic class method (ATC method) was developed as an alternative to replace the oral LD$_{50}$ test. The ATC method is a sequential testing procedure using only three animals of one sex per step at any of the defined dose levels. Depending on the mortality rate three but never more than six animals are used per dose level. This approach results in the reduction of numbers of animals used in comparison to the LD$_{50}$ test by 40-70% (Diener and Schlede 1999; Holzhütter et al., 2003; Schlede et al., 2005).

2.10.2. Repeated Oral Toxicity

Reverse pharmacology is defined as the science of integrating documented clinical experiences and experimental observations into leads by transdisciplinary
exploratory studies and further developing these into drugs candidates or formulations through robust preclinical and clinical research. Reverse pharmacology concept is very popular in Indian herbal industry. It is proposed that all non-clinical development may be done after initial clinical trials are successful. A salient feature of this approach is the combination of knowledge learned from traditional or folk medicine and the modern technology to provide better and safer leads. In this process safety remains the most important starting point and the efficacy becomes a matter of validation (Patwardhan and Mashelkar, 2009). Reverse pharmacology can be perceived to comprise of three phases. First, the experiential phase that includes robust documentation of clinical observations of the biodynamic effects of standardized ayurvedic drugs by meticulous record keeping. Second, the exploratory studies for tolerability, drug-interactions, dose-range finding in ambulant patients of defined subsets of the disease and paraclinical studies in relevant in vitro and in vivo models to evaluate the target-activity. Third phase includes experimental studies, basic and clinical, at several levels of biological organization, to identify and validate the reverse pharmacological correlates of ayurvedic drug safety and efficacy. The scope of reverse pharmacology is to understand the mechanisms of action at multiple levels of biology and to optimize safety, efficacy and acceptability of the leads in natural products based on relevant science. In this approach the candidate travels a reverse path from ‘clinics to laboratory’ rather than classical ‘laboratory to clinics’

Toxicity studies need to be done even if the herbs have a long history of safety and usage, and do not have any documented toxicity, as they can lead to adverse effects especially during long term administration for chronic diseases or when used as food supplements and nutraceuticals. In case of multi-herb preparation, the toxicity or therapeutic efficacy may be due to the synergism between the different active constituents present in the preparation. Toxicological studies should include tests such as acute, subchronic and chronic toxicology that could detect clinically
signs of immunotoxicity, genotoxicity, carcinogenicity and reproductive toxicity (Remirez 2006). These tests could help in the identification of possible target organs involved and the toxic symptoms. Studies of special toxicology such as carcinogenesis are very important if the plant contains compounds with known mutagenic or carcinogenic activities. Moreover, human ethics committees suggest a subchronic oral toxicity study before they approve a longer treatment trial for an unproven nutraceutical (Chanabra et al., 2003).

Subchronic oral toxicity study is screened using the OECD test guideline-407 (Repeated Dose 28-day Oral Toxicity Study in Rodents) to access the toxic effect of a test material on repeated oral exposure. Groups of five male and five female rats were treated by oral gavage with various doses of the test material and or vehicle for at least 28 days. The toxicity of test material is evaluated based on clinical observations, ophthalmic examination, body weight, food and water consumption, urinalysis, haematology, clinical biochemistry and pathology. The results obtained from the study allow characterization of the test substance for toxicity, access the dose response relationship and determine the No-Observed Adverse Effect Level (NOAEL) (Gelbke et al., 2007). Several studies have been performed to access the NOAEL of herbal mixtures using repeated oral toxicity studies (Yoshioka et al., 2010; Park et al., 2010; Li et al., 2011).