CHAPTER – 5: DISCUSSION

Hyperglycemia is a metabolic abnormality in diabetes mellitus and is responsible for the development of diabetic complications. During chronic diabetic conditions, high concentration of glucose cause irreversible tissue damage thorough various mechanisms which includes increased polyol pathway flux, activation of protein kinase C isoforms, and formation of advanced glycation end products. All the above mechanisms seem to be due to overproduction of reactive oxygen species in the mitochondria observed with hyperglycemia (Brownlee 2001). Most of the antidiabetic class of drugs have better glycemic control by acting on individual molecular targets but they fail to control oxidative stress, the major cause involved in the onset and progression of diabetic complications. Multitarget therapeutics act on multiple targets exhibiting synergistic effect but its use still on long term use aggravates the incidence to side effects (Tiwari 2005). A therapeutic regime that could normalize both hyperglycemia and the oxidant/antioxidant status with minimal side effects could be beneficial in the management of diabetes mellitus and diabetic complications.

Herbal drugs are used worldwide for prophylactic and therapeutic purposes. Most of the herbal drugs contain number of ingredients which are not standardized but instead employ the use of crude extracts or crude powders as active ingredients. These preparations are marketed to the public. They either lack efficacy or safety data to support their use clinically. The quality control of herbal formulation is difficult since it uses a combination of several herbs to achieve synergistic or antagonist activity. The use of minimum number of standardized ingredients in a herbal formulation could enable the production of reproducible safety and efficacy data. Standardization of herbal medicines helps to assure the minimum quality so that it is compliant with national and international regulations.
Setting standards for quality control of a single plant drug is comparatively easier. It is more difficult to deal with multiherbal preparations (Ankli et al., 2008).

DIA-2 is an herbal mixture containing fixed combination (1:1 w/w) of *Allium sativum* (ASE) and *Lagerstroemia speciosa* (LSE), formulated with an intention to manage metabolic disorders such as diabetes with well experimented, documented and clinically proven herbs. The distinct features of DIA-2 compared to that of other herbal formulations were that they contain minimum number of ingredients and contain standardized extracts of ASE and LSE instead of crude herbal extracts.

Phytochemicals are secondary metabolites of plants, which play a crucial role in regulating and modulating human health and disease (Murakami and Ohnishi 2012). Phytochemical analysis is one of the tools to assess the quality of herbal drugs, which include preliminary phytochemical screening and chemoprofiling using modern analytical techniques. The qualitative and quantitative phytochemical tests establish the chemical composition of ASE and LSE, demonstrates that both ASE and LSE possess some bioactive constituents that could contribute towards the treatment of diabetes and in the management of hyperglycemia induced oxidative stress.

High-performance thin-layer chromatography (HPTLC) has emerged as an important analytical tool for qualitative and quantitative analysis of phytochemicals (Raman 2006) and characterization of the phytoconstituents in herbal drugs. HPTLC fingerprints are accepted analytical tools for identification and quality control of herbal drugs (Alaerts et al., 2007). The concept of phytoequivalence was developed to ensure consistency of herbal products, where a chromatographic fingerprint of an herbal drug was constructed and compared with the profile of a reference product (Mauji Ram et al., 2011). The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs (Liang et al., 2004). The phytocompounds used in standardization of ASE and LSE were alliin and corosolic acid respectively. There are some reports on the application of
chromatographic techniques for the analysis of ASE (Blania and Spangenberg 1991; Kanaki and Rajani 2005) and LSE (Gao et al., 2010 Liu et al., 2011), but attempts to apply these techniques for developing chromatographic fingerprints as a herbal formulation are not available. Phytochemical markers based standardization was employed as an analytical tool in quality control of herbal medicines. This approach would only serve as an additional parameter in assessing the quality of the drug since phytochemical markers are difficult to obtain and are often quite expensive. The chromatographic fingerprints are also useful in monitoring the entire process of formulation. Regulatory agencies also recommend chromatographic fingerprint as the basis for proper identification and quality control of herbal medicinal products (Eike Reich and Anne Schibli 2007). Herbal drugs with similar chromatographic fingerprint have similar properties and a similarity in chromatographic fingerprint pattern has been a suitable analytical approach in quality control of multi-herbal drug products (Xiao-Hui Fana et al., 2006).

In the present study a densitometric method was developed to construct HPTLC chromatographic fingerprint of ASE, LSE and DIA-2. Derivatization is mainly used in the post chromatographic mode for localization of the separated component zones on the layer (Monika Waksmandzka-Hajnos et al., 2008). In our study, we attempted to develop chromatograms without derivatization which can compromise the selectivity of the method by reacting with compounds that may not be resolved from the compound of interest (Biringanine et al., 2006). However, in our study; we were able construct the chromatogram for ASE only after derivatization with 0.2% ninhydrin reagent, a reagent used for identification of compounds containing an amino group in their structure (Monika Waksmandzka-Hajnos et al., 2008).

In vitro tests can play an important role in the evaluation of antioxidant or antidiabetic activity of medicinal plants and are considered as initial screening tool before performing an in vivo study. Both the component herbs of DIA-2 namely
ASE (Eidi *et al.*, 2006; Chung 2006) and LSE (Priya *et al.*, 2008, Saumya and Basha 2011) were known for their antioxidant effect and anti-diabetic effect. In the present study, we attempted to test our hypothesis, whether DIA-2 or its component herbs show a synergistic effect when tested on various *in vitro* antioxidant and anti-diabetic screening systems.

Hyperglycemia is the main causative factor for oxidative stress during diabetes mellitus. Chronic hyperglycemic conditions enhance the production of free radicals, resulting in end organ tissue damage leading to diabetic complications (Brownlee 2001; Chan *et al.*, 2008). Recent research studies indicate that current treatment available for diabetes though have a good control over hyperglycemia do not control the progression of diabetic complications, suggesting their inability to improve the altered antioxidative defense mechanisms (Waisundara *et al.*, 2008). Consequently, the current research and development of drugs has targeted on oxidative stress pathways for the prevention of diabetic complications (Omar *et al.*, 2010). Use of antioxidants has been considered till date as one of the therapeutic remedies for the prevention of diabetic complications (Xie *et al.*, 2009). Use of synthetic antioxidants is not helpful and has adverse effects (Radulovic *et al.*, 2007). In recent years, plant based medicines have been found to possess excellent antioxidant activities and demonstrate significant therapeutic effects when used in combination than when used alone (Yang *et al.*, 2009). Plant drug combinations are widely used in the adjuvant therapy of type 2 diabetes mellitus for the prevention of complications (Szentmihályi *et al.*, 2010).

Determination of the reducing capacity of a plant extract may reveal its potential antioxidant activity (Samarakoon *et al.*, 2011). The reducing ability is generally associated with the presence of reductones which break the free radical chain by donating a hydrogen atom. (Harbaum *et al.*, 2008). The reducing power exhibited by ASE and LSE could be attributed due to the presence of organosulphur compounds (Yin *et al.*, 2002) and phenolic compounds (Odabasoglu
et al., 2005) respectively. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

The phosphomolybddate method is a quantitative method to assess the total antioxidant capacity (Prieto et al., 1999) and the antioxidant capacity is expressed as grams equivalent of vitamin E per gram of extract. In the presence of ASE or LSE, Mo (VI) is reduced to Mo (V) and forms a green colour phosphomolybdenum V complex, which shows a maximum absorbance at 695 nm. LSE was found to possess higher antioxidant activity when compared to ASE.

The effect of ASE and LSE on nitric oxide radical scavenging was determined by the decrease in intensity of pink coloured chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethlenediamme measured at 540 nm. It was found that IC_{50} of both ASE and LSE showed an inhibitory activity on NO free radical compared to Vitamin E. Nitric oxide plays a dual beneficial/deleterious role, depending on its concentration (Bencini et al., 2010). Low concentrations of NO are essential for normal cellular homeostasis and higher concentrations have been identified as a key pathogenic factor leading to cellular damage in many chronic human diseases (Amiri et al., 2011). During chronic hyperglycemic conditions, there is an over production of superoxide free radical, which is accompanied by an increased NO generation, resulting in the formation of the stronger oxidant peroxynitrite (Brownlee 2001). The nitric oxide scavenging effect might be associated with oganosulfur compounds (Ippoushi et al., 2002) and phenolic compounds (Conforti and Menichini 2011) found in ASE and LSE respectively. Our previous finding also reveals that ASE and LSE could inhibit the reactive oxygen species and super oxide radicals in vitro (Kesavanarayanan et al., 2012); suggesting its beneficial use in preventing superoxide radical induced tissue damage and superoxide radical induced nitric oxide production.
The approach of scavenging the stable DPPH radicals is a widely used method to evaluate the hydrogen donating capacity of antioxidants, since it involves a relatively short time compared to other methods. After addition of alcoholic solution of DPPH to various concentrations of test compounds (ASE, LSE and Vitamin E), we found that there was gradual decrease in absorbance in all the treatment groups. The decrease in absorbance may be due to the proton donating ability (Mathiesen et al., 1997) of phenolic constituents present in ASE, LSE (Kesavanarayanan et al., 2012) and Vitamin E (Mukai et al., 2005).

Accumulation of end products of lipid peroxidation in tissues results in cellular dysfunction which plays a major role in the development of oxidative stress-related diseases such as diabetes (Negre-Salvayre et al., 2008 and 2010). Several medicinal plants have been reported to inhibit the lipid peroxidation (Steinrut et al., 2011; Jadhav and Bhutani 2002) and are found to be beneficial in the management of diabetic complications. Recent studies on LSE reveal its ability to lower lipid peroxidation activity (Saumya and Basha 2011; Priya et al., 2008). ASE has also been reported to reduce the lipid peroxidation activity (Borek 2006) due its rich organosulfur compound content (Augusti and Sheela 1996).

Hyperglycemia induced metabolic disturbance generates increased production of ROS leading to the development of diabetic complications. In an effort to elucidate the ROS production, super oxide scavenging assay, ABTS radical scavenging assay and \( \text{H}_2\text{O}_2 \) induced intracellular ROS production in 3T3-L1 adipocytes was performed. DIA-2 synergistically inhibited the ABTS free radical, super oxide generation and ROS generation. The activity of DIA -2 may be attributed to the individual polyphenolic compounds that are reported to scavenge the free radicals by virtue of their antioxidant property. The antioxidative potential of ASE varies according to the nature of its chemical moieties and standardization process. However, chemical moieties other than organosulphur compounds may contribute to the biological activities. The inhibitory activity of DIA-2 on \( \text{H}_2\text{O}_2 \)
induced ROS production and super oxide radical production may be due to the presence of polyphenols and nonsulfur compounds present in LSE and ASE.

Inhibition of α-glucosidase is one of the therapeutic approaches for delaying carbohydrate digestion, resulting in reduced postprandial glucose. The in vitro antihyperglycemic effect of DIA-2 and its individual herbs were assessed by their ability to inhibit carbohydrate hydrolyzing enzyme like, α-glucosidase. Polyphenols have been reported to have α-glucosidase enzyme inhibitory property (Ranilla et al., 2010). There are some earlier reports to suggest that polyphenolic compounds have both antioxidant and α-glucosidase enzyme inhibitory potential (Gamberucci et al., 2006; Ranilla et al., 2010). Similarly, a recent study has shown the antioxidant and inhibitory potential of ASE against α-glucosidase enzyme (Cazzola et al., 2011). The synergistic effect of DIA-2 to inhibit α-glucosidase could be due to ubiquitous polyphenolic compounds and non sulphur compounds.

In the diabetic state, to maintain glucose homeostasis the excess glucose enters the polyol pathway, which includes enzymatic reduction of glucose to sorbitol by NADPH dependent aldose reductase (AR) followed by oxidation of sorbitol to fructose by NAD dependent sorbitol dehydrogenase (SDH). The impermeability of sorbitol through the cell membrane causes osmosis in the cell, one of the main causative factors for the long term complications. In non-diabetics, sorbitol is converted to fructose and is easily excreted from the cell. AR inhibitors like hydantoin derivatives (Sorbinil) and carboxylic acid derivatives (Epalrestat) are useful, but limited in use because of undesirable side effects. We have demonstrated the decreasing order of potency in terms of IC$_{50}$ value: DIA-2 < Ascorbic acid < ASE < LSE. It was found that tannoid principles of Emblica officinalis were potent inhibitors of rat lens AR and human recombinant AR (Suryanarayana et al., 2004). The tannins present in LSE might have a role in inhibition of AR. In diabetes, AR levels are expressed intensely in insulin-independent tissues, over-expression of AR enhances production of reactive
oxygen species (ROS), which cause membrane damage and cellular leakage. On the other hand the organosulfur compound (DAS) present in garlic was reported to reduce the expression of AR and subsequently lower the production of ROS (Ibrahim et al., 2008). DAS present in ASE may be responsible for reduced expression of AR. AA inhibits the enzymatic conversion of glucose to sorbitol by virtue of it structural similarity with glucose. Due to this structural similarity, AA competes with glucose for transport into cells. In the diabetic state, the uptake of AA into cells appears to be impaired. When ascorbic acid (AA) is supplemented orally to diabetic patients it may help in preventing many of the complications of diabetes (Wang et al., 1995). DIA-2 showed enhanced inhibitory activity on sorbitol accumulation in erythrocytes at 0.16 μg/mL compared to its individual ingredients which might be due to multiple effects of tannins and organosulphur compound (DAS) present in LSE and ASE respectively.

Glycation is a complex chemical reaction and occurs between reactive aldose or ketose sugars and protein bound free amino groups. An initial labile Schiff’s base formed during the reaction undergoes Amadori rearrangement and yields a stable ketoamine derivative. Following dehydration and subsequent chemical modification, some Amadori products accumulate with time as advanced glycation end products (AGEs). The formation of AGEs has been proposed to play an important role in the pathogenesis of the long term complications of diabetes. AGE inhibitors inhibit the glycation cascade and offer a promising therapeutic approach for the prevention of diabetic complications. Unfortunately, clinical trials of these inhibitors in diabetic patients have been suspended due to their adverse effects. In the present study, the effect of ASE, LSE, DIA-2 and aminoguanidine (AG) on inhibition of AGEs formation was demonstrated by in vitro method using bovine serum albumin (BSA) as a model of protein because it is a key protein found abundantly in human plasma. The nature of the reducing sugars influences the rate and extent of the glycation. A mixture of fructose and glucose was included in our
study, because fructose is present in tissues at a concentration comparable to that of glucose and reacts with protein approximately 10 times more rapidly than glucose. DIA-2 showed maximum % at 10 μg/mL, while ASE, LSE and amino guanidine showed maximum % of inhibition at 30 μg/mL. S-allyl cysteine, present in garlic is responsible for inhibition of AGE formation (Ahmad and Ahmed 2006; Ahmad et al., 2007). Non-enzymatic glycation plays a major role in the development of diabetic complications (Szwergold 2006). Transglycation is a deglycation process operating on the very first product of nonenzymatic glycation i.e. Schiff’s bases. Glucose-cysteine is one of the transglycation products found at increased concentration in diabetes (Szwergold et al., 2005). ASE is rich in S-allyl cysteine, S-ethylcysteine, N-acetylcysteine (Ahmad and Ahmed 2006; Ahmad et al., 2007; Huang et al., 2004; Ou et al., 2003); we assume that the presence of these cysteine residues may influence the formation of sugar cysteine adduct rather than sugar-protein adduct, thereby preventing the formation of AGE end products. No studies on LSE pertaining to its role on glycation have been carried out so far, but it has been reported that polyphenols, condensed tannins and flavonoids can inhibit the glycation process. LSE is reported to be rich in these bioactive principles which may have a potential role in inhibiting the glycation process (Adisakwattana et al., 2010). Administration of organosulphur compound, diallyl tetrasulfide isolated from ASE was found to normalize the elevated liver protein carbonyl content in cadmium induced oxidative damage in rats (Murugavel and Pari 2007). Tannins have shown to be beneficial in protecting protein oxidation and glycation. LSE may have probable action on protein oxidation as well due to rich availability of tannins (Nakagawa et al., 2002).

Assays like MTT reduction and LDH release have been used to determine cell survival or death (Maioli et al., 2009; Chen and Li 2006; Khanavi et al., 2010; Kumar et al., 2011). Cytotoxicity of ASE and LSE were evaluated primarily to identify their cytotoxic concentrations. Among the extracts, ASE was ~ 6.6 fold cytotoxic
than LSE. Based on these results, the herbal mixture (ASE:LSE) was prepared in the ratio of 1:1 and 1:2 and tested for cytotoxicity. Herbal mixture in the ratio 1:2 revealed ~ 4.4 fold cytotoxicity compared to 1:1 herbal mixture. The organosulfur compounds in ASE has been reported to trigger the production of ROS (Das et al., 2007) and the organosulphur compound, allicin is reported to be responsible for the cytotoxic effect of ASE directly affecting the cell microtubules (Prager-Khoutorsky et al., 2007). The organosulphur compounds have also been reported to activate cell apoptosis by virtue of their oxidative inactivation of essential cellular thiol-containing enzymes like glutathione [GSH] (Gruhlke et al., 2010). Sulphydryl reducing reagents (i.e antioxidant) reduce the cytotoxic effects of ASE which have been reported earlier (Prager-Khoutorsky et al., 2007). Antioxidant rich polyphenolic compounds (phenols, tannins, flavonoids) are comparatively higher in LSE than that of ASE and may protect the cells from sulphydryl oxidation (Pandey and Rizvi 2010). The cytotoxic effect of 1:1 mixture was found to be less when compared to 1:2 mixture. The high cytotoxic effect of 1:2 mixtures may possibly be due to the higher concentration of corosolic acid, a naturally occurring triterpenoid present in LSE (Ali et al., 2007). Similar findings on cytotoxic effect of Perilla frutescens var. japonica leaf extract (Banno et al., 2004; Akihisa et al., 2006) and Crataegus pinnatifida var. psilosa extract (Ahn et al., 1998) was observed due to high concentration of triterpenoids. Based on these findings, 1:1 herbal combination [DIA-2] was considered for further investigations. Cytotoxicity assay also formed the basis to identify the non-cytotoxic dose of DIA-2 and its individual ingredients in the 3T3-L1 preadipocytes and further investigations were made at non-cytotoxic dose range levels.

In the insulin resistance state, the glucose transporter-4 (GLUT-4) translocation is decreased. In the present investigation, ASE, LSE and DIA-2 were tested for their glucose uptake enhancing effect. 3T3-L1 differentiated adipocytes are the most preferred cell line to study insulin-stimulated glucose uptake assay by
in vitro method. 3T3-L1 preadipocytes express non-insulin-sensitive glucose transporter GLUT-1, but on differentiation it expresses the insulin-responsive glucose transporter GLUT-4. The maximum percentage of 2-Deoxyglucose (2-DOG) uptake into the differentiated 3T3-L1 adipocytes was found to be 145.16±1.32% on addition of 0.01 μg/mL of LSE compared to vehicle control. Corosolic acid, a triterpenoid compound (Shi et al., 2008) and Lagerstroemin, a potent ellagitannins present in the leaves of LSE are responsible for glucose transport stimulatory activity (Bai et al., 2008; Hayashi et al., 2002). Earlier reports also support our finding that LSE possesses glucose uptake stimulatory activity (Liu et al., 2001) and the activity is due to the tannic acid present in LSE (Liu et al., 2005). Similarly at 0.01 μg/mL ASE showed 122.32±0.58% of glucose uptake.

The pharmacologic properties of garlic are extremely complex and are mainly derived from the organo-sulfur compounds. Administration of fresh garlic juice and processed garlic has been reported to modulate GLUT-4 and peroxisome proliferator-activated receptors (PPARs) expression in heart tissues, through the up regulation of GLUT-4, PPARα and PPARδ. Both freshly crushed garlic and processed garlic provide cardioprotection, the former has additional cardioprotective properties presumably due to the presence of H₂S (Mukherjee et al., 2009). In the present status, it is assumed that the glucose uptake activity of ASE may be due to the presence of these organosulfur compounds. DIA-2 showed maximum of 151.51±0.9% glucose uptake at a 10 fold lower concentration (0.001 μg/mL), than that of ASE and LSE. PPARγ agonist, RG (50 μM), was used as a positive control which showed 163.9±0.42% of glucose uptake compared to vehicle control.

3T3-L1 cells are convenient cell culture model for the investigation of adipose differentiation. They differentiate into adipocytes and accumulate lipid droplets in the cytoplasm. When fat cells are stained with oil red O, the degree of staining seems to be proportional to the extent of cell differentiation (Zou and
Most of the antidiabetic drugs such as insulin or thiazolidinediones (TZD), except few, up regulate both glucose transport and lipid biosynthesis in adipocytes and cause gain in body weight. Thus, these drugs treat one of the key symptoms of type 2 diabetes, namely hyperglycemia, but predispose to obesity. Therefore, while these drugs are beneficial over the short term, they may not serve the need for long term health. The most desirable situation would be the development of new types of antidiabetic drugs that are either hypoglycemic or anti-hyperglycemic without promoting weight gain. The ellagitannins present in banaba (Hou et al., 2009) and diallysulphide (DAS) present in garlic (Lee et al., 2008; Ambati et al., 2009) were also responsible for anti-adipogenic effect. Both LSE and ASE have better glucose uptake activity and anti-adipogenic effect. DIA-2 is a unique combination having both these properties, and may be useful for the management of hyperglycemia and obesity in type II diabetes.

The differentiation of preadipocytes to adipocytes is governed by a cascade of transcription factors. Insulin, an adipogenic hormone triggers a series of transcriptional factors that are involved in the adipocyte differentiation process. Most notable adipogenic transcription factors are peroxisome proliferator-activated receptor gamma, sterol regulatory element binding proteins, and adipocyte differentiation dependent factors leptin and GLUT-4 which plays an important role in the complex transcriptional cascade during adipocyte differentiation. Peroxisome proliferator-activated receptor (PPAR) gamma and Sterol regulatory element binding proteins (SREBPs) are transcriptional factors that control expression of lipogenic genes that mediate fatty acid and cholesterol metabolism. Among the various SREBP isoforms (SREBP-1a, SREBP-1c and SREBP-2) SREBP-1c is involved in FA synthesis and insulin induced glucose metabolism. In addition to transcriptional factors, adipocyte characteristic proteins such as glucose transporter 4 (Glut 4) and leptin are also involved in the adipocyte differentiation cascade. Leptin is an adipocyte-derived hormone involved in the regulation of lipid
metabolism, adipocyte differentiation, body weight and food intake. (Schupp et al., 2009). GLUT4 is the main insulin-responsive glucose transporter and is expressed predominantly in muscle and adipose tissues (Im et al., 2006). The growing epidemic of obesity and diabetes mellitus needs a better understanding of adipocyte biology. Therefore targeting the key factors involved in the adipocyte differentiation process may be an effective approach to regulate adipose mass (Lo et al., 2011). DIA-2 synergistically inhibited the lipid droplet accumulation and down-regulated the levels of mRNA expressions of PPARγ, leptin and SREBP-1c comparatively to its individual treatments. Our data demonstrate that component herbs of DIA-2 could synergistically inhibit the adipocyte differentiation via modulation of these three pathways. DIA-2 may also have anti-diabetic effects at least in part through up regulation of GLUT-4 protein in 3T3-L1 adipocytes.

The rat model of T2DM was developed by combination of insulin resistance induced by feeding HFD with intraperitoneal injection of low dose streptozotocin, which has been known to induce a mild impairment of insulin secretion. Body's resistance to insulin and the falling production of insulin by the β cells of the pancreas are the two important factors associated with type 2 diabetes. The experimental animal model aimed at mimicking these clinical features of human type 2 diabetes mellitus. Several studies have reported that HFD feeding to rats leads to insulin resistance followed by administration of low-dose STZ which induces a mild impairment of insulin secretion resembling the clinical feature of type 2 diabetes (Zhang et al., 2008).

Baseline biochemical markers (plasma glucose, insulin, triglyceride, cholesterol) and body weight changes were used to evaluate the effect of DIA-2 on sugar and lipid metabolism while developing the animal model (Sugano et al., 2006). One of the classical symptoms of diabetes mellitus is weight loss. Induction of diabetes with STZ showed significant loss in body weight which may be due to increased muscle wasting and proteolysis of muscle proteins. Treatment with RG
increased the body weight in animals however DIA-2 treated animals showed non-significant change in body weight at all dose levels. Newer classes of antidiabetic drugs are beneficial only over a short term period; they are not optimal for long term use, since they promote weight gain and obesity, one of the leading causes of type 2 diabetes.

Administration of HFD and low dose of STZ was considered as an ideal model for pharmacological screening of drugs for type 2 diabetes mellitus (T2DM) since it resembles the clinical manifestation of type 2 diabetes in humans. Administration of low dose of STZ to rats fed with HFD for 4 weeks elicited signs of T2DM such as hyperglycemia and light impaired insulin secretion. The low dose of STZ partially destroys the pancreatic beta cells with deficiency in insulin secretion rather than destroying it completely and its administration along with high fat diet induces insulin resistance in rats. The DIA-2 restored 18.52% and 39.26% of insulin levels respectively in a dose dependent manner at 62.5 and 125 mg/kg when compared to the hyperglycemic group. Capacity of DIA-2 to increase insulin and decrease plasma glucose level demonstrates the insulin secretagogue and sensitizer effect. Hypercholesterolemia and hypertriglyceridemia are the common lipid metabolic abnormalities in diabetes resulting in insulin resistance, thereby, reducing the capability of glucose utilization stimulated by insulin. This may be the reason for perpetuation of elevated glucose level (> 250 mg/dl) in hyperglycemic group throughout the study. The DIA-2 significantly decreased the plasma TC and TG levels in diabetic rats, thereby, improving glucose metabolism and utilization. The presence of organosulphur compounds and nonsulfur compounds such as saponins in ASE may work synergistically to produce various biological effects. Corosolic acid, a triterpenoid compound present in LSE has been widely claimed for its antidiabetic, anti-adipogenic, anti-oxidant, anti-obesity and insulin-like properties. It has also been reported to have possible role on carbohydrate and lipid metabolism. DIA-2 treatment has the ability to normalize the altered lipid and
glucose metabolism and may hence have the potential to manage diabetes mellitus. However, the research on botanical based preparations is not easy to pursue. We have attempted to develop this unique herbal preparation with minimum number of standardized ingredients and investigated its ability to manage diabetes without gain in body weight with grounded scientific evidence.

In an attempt to understand the underlying biochemical mechanism of the antidiabetic effect of DIA-2, we investigated the key glycolytic (hexokinase) and gluconeogenic enzymes (glucose-6-phosphatase and fructose 1, 6 bisphosphatase) along with glycogen content in the liver of diabetic rats. The key plasma biomarkers of hepatocellular injury (ALT, ALP, AST, total protein and albumin) and renal injury (BUN and creatinine) were evaluated to access the hepatoprotective and nephroprotective role of DIA-2 during diabetes. The tissue (Brain, heart, liver, kidney and pancreas) antioxidant status, protein carbonyl content in liver and Na⁺K⁺ATPase activity were respectively assessed to ascertain the role of DIA-2 in ameliorating the oxidative stress and providing membrane stabilisation activity. In addition, the histopathological changes were also observed in the target organs (pancreas, liver, kidney and brain).

The liver is an important insulin dependent tissue that plays a major role in the glucose homeostasis (i.e. glucose production and utilization) by strictly controlling the activities of the key enzymes involved in the pathways of carbohydrate metabolism (glycolysis and gluconeogenesis). Liver also controls the synthesis (glycogenesis) and degradation of glycogen (glycogenolysis) as a part of its glucose homeostasis mechanism. During diabetes mellitus due to lack of insulin, increase in hepatic gluconeogenesis, glycogenolysis and decrease in glycolytic, glycogenic activity was observed (Postic et al., 2004).

Hexokinase is the first key enzyme involved in the glycolytic pathway which phosphorylates glucose to glucose-6-phosphate (G-6-P). Glucose-6 phosphatase (G-6-Pase), a key enzyme involved in the gluconeogenesis and glycogenolysis...
catalyzes the last common step of both the processes i.e., the dephosphorylation G-6-P to glucose (Chatelain et al., 1998). Fructose 1, 6-bisphosphatase (F-1, 6-BPase), a gluconeogenic enzyme controls the production of hepatic glucose through gluconeogenesis process.

High fat diet (HFD)/low-dose streptozotocin (STZ) induced diabetes causes insulin resistance and subsequent insulin secretory defect in rats that closely resembles the metabolic disturbances that are seen in T2DM in humans. Compared to normoglycemic animals, the diabetic group of animals showed a significant reduction in insulin levels, decrease in the activities of hexokinase, significant elevation in the activities of glucose-6-phosphatase and fructose 1, 6-bisphosphatase in the liver. Similarly, the glycogen content was decreased in diabetic rats. Activation of hexokinase activity, inhibition of activities of enzymes involved in gluconeogenesis and restoration of glycogen content have been proposed as potential targets for suppressing hepatic glucose levels during T2DM. The use of herbal medicines to modulate the altered carbohydrate metabolism during diabetes mellitus is quite high and therefore finds its own importance in the management of this metabolic disorder. Unfortunately there is an insufficient evidence to actively recommend the use of herbal medicines in improving the altered carbohydrate metabolism (Prabakar et al., 2008).

The individual ingredients of DIA-2 were reported earlier for their possible role on carbohydrate metabolism (Chang et al., 1980; Yamada et al., 2008). The combined effect of ASE and LSE (i.e. DIA-2) on hepatic carbohydrate metabolising enzymes, viz. hexokinase, glucose-6-phosphatase and fructose 1, 6 diphosphatase and hepatic glycogen in diabetic rat were investigated in the present study. Administration of DIA-2 has enhanced the hepatic activity of hexokinase and decreased the G-6-Pase and F-1, 6-BPase activity. DIA-2 treatment has also restored the levels of glycogen when compared to diabetic animals. Recent reports reveal that polyphenols may influence carbohydrate metabolism mainly by affecting
glucose transport and insulin-receptor function and insulin release from β cells of
the pancreas (Hanhineva et al., 2010). S-allyl cysteine sulfoxide (SACS), a
sulphur containing amino acid of ASE could modulate the carbohydrate metabolism
through activation of glycolysis, inactivation of gluconeogenetic enzymes and
stimulation of insulin secretion from β cells (Sheela et al., 1992; Augusti et al.,
1996) The pentacyclic triterpenoid and corosolic acid available with LSE have been
reported to inhibit the activity against glycogen phosphorylase, a rate limiting
enzyme during glycogenolysis that breaks glycogen into glucose (Zhang et al.,
2009; Wen et al., 2007) and the presence of such a compound may be responsible
for the restoration of hepatic glycogen after DIA-2 treatment.

Oxidative stress is one of the causative factors in the development of
consequences of diabetes. The endogenous antioxidant levels such as reduced
glutathione (GSH) and glutathione peroxidase [GPx] were decreased in HFD/STZ
treated animals as compared to normoglycemic animals. These changes were
restored on DIA-2 treatment. Conversely, the increased levels of TBARS in
HFD/STZ group were attenuated significantly in DIA-2 pre-treated group when
compared with STZ lesioned group. The antioxidant activity of DIA-2 could be
attributed to the presence of biologically active compounds such as allicin, S-allyl-
cysteine (SAC), diallyl-di-sulphide (DADS), and diallyl-sulphide (DAS) available with
ASE (Augusti et al., 1996), and ellagitannins, corosolic acid available with LSE
(Priya et al., 2008).

Insulin and oral hypoglycemics agents are reported to cause liver damage
(Chitturi et al., 2002). Regular monitoring of liver enzymes is still recommended
during therapy with few anti-diabetic classes of drugs. Liver enzymes such as ALT,
AST and ALP are marker enzymes for liver function. Hyperglycemia is usually
accompanied by an increase in the activities of the enzymes of the liver (Mori et al.,
2003). Administration of DIA-2 has led to a significant decrease in ALT, AST and
ALP levels. The improvement in the hepatic maker enzymes level by DIA-2
treatment indicates that it does not cause hepatotoxicity which is likely to be caused by few classes of antidiabetic drugs. DIA-2 seems to possess hepatoprotective potential along with antidiabetic activity. The active constituent in the ingredients of DIA-2 could be responsible for antidiabetic and hepatoprotective activity. ASE was reported to decrease AST and ALT levels during hyperglycaemic conditions (Eidi et al., 2006). Improvement in the activities of liver marker enzymes level by DIA-2 treatment indicates that DIA-2 has hepatoprotective action along with antidiabetic activity.

The protein status is usually determined by measuring the levels of total proteins and albumins. Albumin is the most abundant protein synthesised by the liver. During diabetes mellitus, the protein metabolism is altered and remains unaltered in diabetes associated obesity. Insulin plays a major role on protein metabolism in the liver (Abu-Lebdeh et al., 1996). Administration of STZ to high fat diet treated rats did not affect the protein metabolism in both hyperglycemic animals and DIA-2 treated animals.

Experimental animal model of type-2 diabetes mellitus has provided considerable insight into the histological alterations during diabetic state. DIA-2 treatment preserved the normal hepatocyte architecture and suppressed the diabetes induced morphological alterations in the liver. Biochemical analysis of liver tissues and histopathological evaluation of PAS-stained liver sections revealed hepatocellular glycogen depletion in HFD/STZ induced diabetic animals when compared to normoglycemic rats. After DIA-2 treatment the hepatic glycogen content was restored when compared to the diabetic rat as confirmed by biochemical and histopathological analysis. From these observations, it is evident that the DIA-2 treatment restored the glycogen levels in the liver of diabetic animals.

Herbal medicines are consumed by humans either as food or medicine (Perera and Li 2012). *Allium sativum* and *Lagerstroemia speciosa* are one such
herbal medicine consumed either as food or medicine by humans for treating various disorders. Though the therapeutic effect of these medicinal plants in metabolic disorder management are established (Stohs et al., 2012; Madkor et al., 2011) there is limited or no scientific data regarding their safety aspects when given as a herbal mixture.

High dose (500 mg/kg body weight/day) of *Allium sativum* has been reported for its hepatotoxic potential and low doses of garlic (100 or 250 mg/kg body weight/day) are considered to be safe (Rana et al., 2006 Nakagawa et al., 1984 Sumiyoshi et al., 1984). High concentrations of triterpene acids, like corosolic acid have also been known for their toxic effects (Akihisa et al., 2006). The acute and repeated oral toxicity study was undertaken to access the possible effects of DIA-2 after single and repeated oral administration for 28 days to SD rats.

The determination of repeated oral toxicity of DIA-2 could be evaluated only after obtaining initial toxic information on its acute oral exposure. The acute oral toxicity study demonstrated the oral safety in female SD rats. To ascertain the toxicity related information, single administration of DIA-2 at a dose (2000 mg/kg) as recommended by OECD-423 test guideline was done. The absence of clinical signs of toxicity, lack of changes in body weight and gross pathology of vital organs on necropsy suggested that DIA-2 is orally safe up to 2000 mg/kg body weight (Kesavanarayanan et al., 2012).

The selection of dose for the study is based on the therapeutic dose of DIA-2. The low dose (62.5 mg/kg), high dose (250 mg/kg) is selected based on its anti-hyperglycemic effect as investigated in our earlier study (Kesavanarayanan et al., 2012) and the intermediate dose (125 mg/kg) is a geometric mean between the high and the low dose. Administration of DIA-2 at these selected dose levels for 28 days to either sex of SD rats showed no treatment related changes regarding clinical signs, mortality, body weights, feed and water consumption, hematology, clinical chemistry, organ weight, gross and histopathology in rats of either sex.
Clinical signs are used as a measure of humane endpoints in safety evaluation studies; administration of DIA-2 did not produce any toxic clinical signs or mortality during the experimental period. Weekly changes in body weights revealed that administration of DIA-2 had no significant effect on the body weight in either sex when compared to vehicle control group. No significant difference was also observed on weekly feed and water consumption between the DIA-2 and vehicle treated groups.

Few studies on garlic use have reported to cause allergic reactions, alteration of platelet functions (Borrelli et al., 2007), however the platelet function is not impaired by single and repeated oral consumption at dietary dose (Scharbert et al., 2007). The effect of DIA-2 on haematological tests performed at the end of the study did not differ between groups. DIA-2 treatment also showed no significant changes in plasma biochemistry compared to the control group.

Organ weight data obtained from toxicology studies is an integral component in the safety assessment of pharmaceuticals (Sellers et al., 2007) and food/nutritional products (Michael et al., 2007). The organ weight is considered to be an important endpoint for identification of toxic effects of chemicals on target organs (Bailey et al., 2004). The relative organ weight (organ weight expressed as a percentage of body weight) was calculated from the terminal body weight. There was no significant difference in relative organ weight in either sexes of rats in both DIA-2 and vehicle treated animal groups.

Histopathological examinations of the major organs [liver, kidney, brain, heart] were performed. On histological examination of all the organs in high dose of DIA-2 (250 mg/kg body weight) treated animals, exhibited no apparent pathological alterations when compared to vehicle treated animals. In the safety assessment of chemicals to humans, it is essential to determine no observed adverse effect levels (NOAEL) or lowest observed adverse effect levels (LOAEL) from animal experiments (Aida et al., 1992). The lowest observed adverse effect level (LOAEL)
of DIA-2 was ≥ 250 mg/kg and the no-observed adverse effect level (NOAEL) was
≤ 125 mg/kg.