CHAPTER – 1: INTRODUCTION

1. Diabetes Mellitus

Diabetes mellitus is defined as a group of metabolic diseases of multiple aetiologies characterized by chronic hyperglycemia with disturbances in carbohydrate, lipid and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO 1999). Chronic hyperglycemia is implicated in the development of microvascular complications such as diabetic retinopathy, diabetic nephropathy, diabetic peripheral neuropathy and macrovascular complications such as coronary heart disease, peripheral vascular disease, cerebrovascular disease. Many metabolic pathways have been suggested to be involved in the pathogenesis of these vascular complications (Brownlee 2001). Some of the important pathways are,

I. Aldose reductase or Polyol pathway
II. Advanced glycation end product (AGE) pathway
III. Protein Kinase C (PKC) activation pathway
IV. Hexosamine pathway
V. Oxidative stress pathway

1.1. Pathogenesis of Diabetic Complications

High levels of glucose in diabetes mellitus cause overproduction of superoxide in the mitochondrial electron transport chain, which inhibits the activity of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Decreased activity of GAPDH was found to be a consequence of poly (ADP-ribosyl) ation of GAPDH by a nuclear enzyme, poly (ADP-ribose) polymerase (PARP), which is activated by DNA strand breaks caused by superoxide overproduction. Inhibition of GAPDH by superoxide leads to an increase in the level of upstream metabolites from glycolysis into five major glucose-driven signalling pathways. This includes the following (Fig-1)
1. the aldose reductase or polyol pathway
2. the advanced glycation end products (AGEs) pathway
3. the protein kinase C (PKC) pathway
4. the hexosamine pathway
5. the oxidative stress pathway

1.1.1. Polyol pathway

Polyol pathway refers to the enzymatic reduction of glucose to sorbitol followed by enzymatic oxidation of sorbitol to fructose (Fig-2). It is also called as sorbitol-aldose reductase pathway. Aldose reductase (AR) is the rate-limiting enzyme which reduces glucose to sorbitol using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Sorbitol is further oxidized to fructose by sorbitol dehydrogenase (SDH) using nicotinamide adenine dinucleotide (NAD+) as a cofactor. During normoglycemic state, AR has a low affinity for glucose and the amount of glucose that is metabolized by the AR is virtually negligible. However, during hyperglycemic state, high concentration of glucose activates AR enzyme activity resulting in an intracellular accumulation of sorbitol with concomitant decrease in NADPH levels. Further, conversion of sorbitol to fructose leads to the cytosolic accumulation of NADH. SDH has a low enzymatic capacity for sorbitol, so the rate of sorbitol oxidation to fructose is relatively slow. Therefore, sorbitol accumulates in the cell and causes osmotic cell damage.

Another proposed mechanism that explains how activation of polyol pathway causes cell damage is as follows, increased AR activity depletes its co-factor NADPH; which also acts as a co-factor for glutathione reductase (GR) during the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), a major intracellular antioxidant. The competition between AR and GR for NADPH during hyperglycemic state causes depletion of GSH. On the other hand, an increased intracellular level of NADH is the substrate for NADH oxidase to generate reactive oxygen species (ROS). Fructose and its metabolites (fructose-3-phosphate
Figure-1: Biochemical pathways involved in hyperglycemia induced mitochondrial superoxide overproduction and damage (Brownlee 2001).

Figure-2: The polyol (sorbitol) pathway. First glucose is reduced to sorbitol by the enzyme, aldose reductase (AR) and it further oxidise to fructose by sorbitol dehydrogenase (SDH) [Brownlee 2001]
and 3-deoxyglucosone) are more potent nonenzymatic glycation agents that would increase the formation of advanced glycation end products (AGE) and are known to cause oxidative stress. Thus, increased AR activity during hyperglycemia diminishes the cellular antioxidant capacity resulting in tissue damage due to cellular oxidative stress (Brownlee 2001).

1.1.2. Advanced glycation end products

During hyperglycemic conditions, the excess of glucose undergoes an irreversible non-enzymatic amino-carbonyl reaction called the Maillard reaction (Fig-3). In this reaction, the aldehyde group of sugar reacts with free amino group of a wide range of proteins to form Schiff bases, which further undergo rearrangements to form amadori products, which further undergoes oxidative degradation to form advanced glycation end products (AGE). Increased production and accumulation of AGE activates NADPH oxidase, an enzyme that effectively reduces $O_2$ to superoxide ($O_2^-$) resulting in over production of super oxide radicals. Increased accumulation of AGE activates the receptor for AGE (RAGE), resulting in the production of ROS, the major causative factor in the development of diabetic complications (Brownlee 2001).

1.1.3. Protein kinase C

Accumulation of glyceraldehyde-3-phosphate (G-3-P) stimulates synthesis of diacylglycerol (DAG), which in turn activates one or more isoforms of protein kinase C (PKC) [Brownlee 2001]. One of the main mechanisms by which the body regulates the activity of tissue proteins is by adding and removing phosphate groups. PKC adds phosphate groups to host protein substrates in tissues throughout the body at their serine and threonine residues and modifies the activity of key signalling proteins. PKC is over expressed during hyperglycemic state and is known to trigger various diabetic complications. Over expression of PKC is accompanied by increased activation of the pro-oxidant enzyme NADPH oxidase, which may exacerbate oxidative stress (Liu et al., 2012). Selective inhibition of this
enzyme will be one of the favorable approaches to treat diabetes-mellitus-related complications (Sobhia et al., 2012).

1.1.4. Hexosamine pathway

During hyperglycaemia, super oxide anion activates the hexosamine pathway as a consequence of decreased activity of the enzyme GAPDH in the glycolytic pathway and diverts the upstream metabolite, fructose-6-phosphate into the hexosamine pathway (Brownlee 2001). The rate-limiting enzyme in this pathway is glutamine: fructose-6-phosphate amidotransferase (GFAT), which catalyses the conversion of fructose-6-phosphate to glucosamine-6-phosphate. The latter gets metabolized to UDP-N-acetyl-glucosamine (UDP-GlucNAc), which acts as a substrate for O- and N-glycosylation of protein, with pathophysiological outcomes that lead to several complications (Rajapakse et al., 2009; Rajamani 2011).

1.1.5. Oxidative stress pathway

Oxidative stress is defined as excessive production of reactive oxygen species (ROS) in the presence of diminished antioxidant substances. It is evident that a single hyperglycemia-induced free radical generation and/or impaired endogenous antioxidant defence system forms the unifying theme in the development of diabetic complications. Oxidative stress arises from several sources during diabetes and hyperglycemia, the major being hyperglycemia induced over production of ROS, especially superoxide anions. Superoxide overproduction caused due to inhibition of GAPDH activity is accompanied by increased nitric oxide generation, due to an endothelial NOS and inducible NOS uncoupled state, a phenomenon favouring the formation of the strong oxidant peroxynitrite, which in turn damages DNA. The peroxynitrite anion is cytotoxic because it oxidizes sulfydryl groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine, which affects many signal transduction pathways (Ceriello 2003).
Hyperglycemia can increase oxidative stress through several pathways and major mechanism appears to be over production of the superoxide anion by the mitochondrial electron transport chain. The primary mechanism of diabetic complications is centred around oxidative stress. It has been suggested that oxidative stress triggers sorbitol pathway, AGE pathway, hexosamine pathway and PKC activation.

During chronic hyperglycemic state, the conversion of glucose to polyols via polyol pathway occurs; AR reduction of glucose to sorbitol probably contributes to oxidative stress by depleting its cofactor NADPH, which is also required for the regeneration of GSH (Fig-4). Sorbitol dehydrogenase, the second enzyme in the polyol pathway that converts sorbitol to fructose, also contributes to oxidative stress; this is possibly because of depletion of its cofactor NAD+ that leads to more glucose being channelled through the polyol pathway. Free radicals are also formed disproportionately in diabetes by nonenzymatic glycation of proteins resulting in the formation of advanced glycation end products (AGE) and interaction of AGE with their receptor RAGE.

1.2. Target Genes Involved in Glucose and Lipid Metabolism

Over production of ROS not only causes cellular damage but also activates the signal transduction cascade that activates specific target genes (Kakehi and Yabe-Nishimura 2008). During oxidative stress, a variety of transcription factors like Peroxisome proliferator-activated receptors- gamma (PPAR-γ), ligand-activated transcription factors, and a nuclear hormone receptor involved in regulating expression of genes involved in different aspects of lipid and glucose metabolism are activated. These are implicated in the pathogenesis of obesity and obesity associated diabetic complications (Arck et al., 2010). Another transcription factor is Sterol response element binding protein [(SREBP) 1c] that plays an important role in the regulation of fatty acid, triglyceride, and cholesterol synthesis in the liver and over expression of this gene is found in obesity and diabetes mellitus (Shimomura
Figure-3: The formation of advanced glycation end products (AGE) after several modifications (Brownlee 2001).

Figure-4: Oxidative stress is involved in the pathogenesis of diabetes and hyperglycemia-associated vascular complications (Giacco and Brownlee 2010)
et al., 1999). In obesity, the adipose tissue secretes a variety of metabolically important substances including adipokines which affect insulin sensitivity and form a link between obesity and type 2 diabetes. Obesity is associated with elevated leptin levels and is considered to be the major risk factor in the development of cardiovascular disease. Therefore, the investigation of the specific genes involved in glucose and lipid metabolism and their drug treatment related alterations might be a novel strategy in the management of metabolic disorders (Baranova et al., 2006).

1.3. Therapeutic Approaches in the Management of Diabetes Mellitus

The major classes of oral anti-hyperglycemic drugs used in the management of type-2 diabetes mellitus could be classified as follows (Tielmans et al., 2007; Phillips and Twigg 2010)

1.3.1. Insulin secretagogues

Meglitinides: Repaglinide and Nateglinide

Sulfonylureas: Glyburide, Glipizide, and Glimepiride

1.3.2. Insulin Sensitizer

Biguanides: Metformin

Thiazolidinediones: Rosiglitazone, Pioglitazone

1.3.3. Agents that Modify Carbohydrate Absorption

Alpha-glucosidase inhibitors: Acarbose, Voglibose and Miglitol

1.3.4. Glucagon-Like Peptide-1 (GLP-1) Agonists

Incretin Mimetics: Exenatide

DPP-IV Inhibitors: Vildagliptin, Sitagliptin and Saxagliptin

Amylin Analogs: Pramlintide

1.4. Mechanism of Action

1.4.1. Insulin secretagogues

Insulin secretagogues are medications that stimulate the beta cells in the pancreas to secrete insulin. Sulfonylureas (Glyburide, Glipizide, and Glimepiride)
are one of the most commonly used oral hypoglycaemic agents. Sulfonylureas act by binding to the sulfonylurea receptor, which results in a calcium influx and stimulation of insulin secretion. Meglitinides (Repaglinide and Nateglinide) are short-acting oral hypoglycemic agents which increase the insulin secretion in the pancreatic beta-cells in a similar fashion to that of sulfonylureas, through activation of ATP dependent K channels.

1.4.2. Insulin Sensitizer

Biguanides (Metformin) improve insulin resistance by increasing peripheral utilization of glucose and reducing hepatic glucose production, mainly by inhibiting gluconeogenesis. Thiazolidinediones (Rosiglitazone, Pioglitazone) improve insulin action in peripheral tissues and enhance glucose uptake in the cells via binding to an intranuclear agent, peroxisomal proliferator-activated receptor (PPARγ) that activates genes involved in glucose and lipid metabolism (Joshi 2005).

1.4.3. Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors (Acarbose, Miglitol) inhibit alpha-glucosidase activity in the intestinal brush border, where complex carbohydrates get converted into monosaccharides (Fig-5). By slowing this transition, these drugs delay glucose absorption and slow the postprandial rise of blood glucose (Van de Laar 2005; Brewer 2006).

1.4.4. Glucagon-Like Peptide-1 (GLP-1) Agonists

Glucagon-like peptide (GLP-1) and gastric inhibitory peptide (GIP) are two of the gut hormones in the incretion family that increase glucose induced insulin release. Dipeptidyl peptidase-4 (DPP-4) is the enzyme responsible for degrading GLP-1 and GIP. GLP-1 mimetics (Exenatide) are GLP-1 receptor agonists resistant to degradation by DPP-4. These agents bind to the GLP-1 receptor and stimulate insulin synthesis and secretion. In contrast, DPP-4 inhibitors (Vildagliptin Sitagliptin, Saxagliptin) prevent DPP-4 from degrading endogenously produced GLP-1 (Scheen 2008).
Figure-5: Multiple therapeutic approaches involved in the treatment of type 2 diabetes mellitus.
1.4.5. Amylin Analogs

Amylin is a pancreatic islet amyloid polypeptide that is secreted by the beta cells of the pancreas along with insulin. Insulin and amylin complement each other’s action on the regulation of glucose homeostasis. Amylin lowers circulating glucose by delaying gastric emptying, suppressing glucagon secretion, and decreasing appetite. Amylin analogues (Pramlintide) control glycemia by slowing gastric emptying time and suppress postprandial secretion of glucagons (Kesty et al., 2008).

1.5. Therapeutic Approaches in the Management of Diabetic Complications

The incidence of diabetic complications is directly related to glycemic control. Glycemic control is considered to be the mainstay for the prevention and progression of these complications; however, such control is not easily achieved. Currently, therapeutic approaches to manage the progression of diabetic complications are being undertaken by targeting the molecular pathways involved in the pathogenesis (the polyol pathway, advanced glycation end products, protein kinase c, and the superoxide pathway) of diabetic complications. Therapeutic targets include agents that could inhibit the activation of polyol pathway, inhibit the formation of advanced glycated end product, inhibit activation of protein kinase C and reduce the overproduction of superoxide and its associated oxidative tissue damage. This leads to beneficial effect in the management of hyperglycemia induced complications. However, clinical trials targeting these biochemical alterations or their combination with oral hyperglycemic drugs have failed to show significant beneficial effects (Cumbie and Hermayer 2007).

1.5.1. Aldose reductase inhibitors

Aldose reductase inhibitors (Epalrestat, Ranirestat, and Fidarestat) prevent the breakdown of glucose by a specific metabolic pathway called the polyol pathway. Their mechanism of action involves competitive binding to aldose reductase and slowing down of sorbitol production. Dysregulation of the polyol
pathway has been implicated as a major cause of diabetic complications (retinopathy, neuropathy, and nephropathy). The rate of conversion of glucose to sorbitol by aldose reductase in tissues that are not insulin sensitive (lenses, peripheral nerves and glomerulus) is high during hyperglycemic conditions like diabetes mellitus. Sorbitol does not diffuse through cell membranes easily and therefore accumulates, causing osmotic stress and target organ tissue damage.

1.5.2. Advanced Glycation Inhibitors

Accumulation of advanced glycation end products have been implicated in the pathogenesis of diabetic complications (neuropathy, retinopathy and nephropathy). Inhibition of AGE formation shall be beneficial as a promising target for therapeutic intervention of diabetic complications (Rahbar 2007). AGE-inhibitors or AGE-breakers are compounds that inhibit the formation of AGE or disrupt the cross-link of formed AGE. Pyridoxamine (Metz et al., 2003), benfotiamine (Köpcke et al., 2001), aminoguanidine (Kobayashi et al., 1993) and α-lipoic acid (Thirunavukkarasu et al., 2005) are some of the AGE inhibitors that have been tested for the prevention of AGE formation.

1.5.3. Protein Kinase-C Inhibitors

Increased formation of diacylglycerol (DAG) and the subsequent activation of protein kinase C (PKC) isoforms may contribute in the development of vascular abnormalities during diabetes mellitus. PKC inhibitors could delay the onset and reduce the burden of diabetic complications. Ruboxistaurin mesylate (selective inhibitor of PKC-β) is used therapeutically in the management of diabetic retinopathy [Danis and Sheetz 2009]

1.5.4. Antioxidants

Antioxidants are substances which protect biological tissues from free radical damage. They can be recycled or regenerated by biological reductants. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes,
increased lipid peroxidation, and development of insulin resistance. The free radicals are naturally eliminated by the presence of enzymatic and nonenzymatic antioxidant mechanisms in the body. Classical antioxidants, such as Vitamin C and Vitamin E have failed to demonstrate their beneficial effect to combat the free radical induced damage during diabetes mellitus. New low-molecular mass compounds that act as SOD or catalase mimetics or L-propionyl-carnitine and lipoic acid may be good candidates for reducing oxidative stress.

1.6. Side Effects of Drugs used in the Management of Diabetic Complications

The clinical potential of drug used in the management of diabetic complications is still controversial due to the lack of conclusive evidence (Table-1). The safety of this category of drugs is also uncertain. The main adverse effects with aldose reductase inhibitors include hypersensitivity reactions (Sorbinil) and liver toxicity (Tolrestat). PKC-β selective inhibitor (Ruboxistaurin) is an investigational drug intended for diabetic peripheral retinopathy. Chronic studies are required to evaluate its efficacy and side effects. Aminoguanidine, an AGE inhibitor has limited clinical use due to its side effects like nausea and headache. The use of antioxidants to combat diabetic complications has failed to demonstrate its benefit, since large clinical trials do not include oxidative stress biomarkers as a criterion for inclusion in these clinical trials. The overwhelming failure of antioxidant therapy to prevent disease can be explained by inadequacy of the doses of antioxidants used, short duration of therapy, or poor timing of initiation of the supplementation. A more likely reason for failure of antioxidants to reduce diabetes-related complications is the multiplicity of mechanisms of glucotoxicity (Hill 2008; Mooradian and Haas 2011).

1.7. Comorbid Disorders and Related Therapeutic Approach during Diabetes Mellitus

Diabetes is associated with multiple comorbidities (cardiovascular diseases, obesity) that may affect life expectancy. Obesity associated diabetes mellitus is one
### Table-1: Side Effects of oral hypoglycaemic agents (Asche et al., 2008; Hamnvik and McMahon 2009; Wirth 2011)

<table>
<thead>
<tr>
<th>Class of Drug</th>
<th>Side Effect</th>
<th>Effect on Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonylurea</td>
<td>Hypoglycemia</td>
<td>Weight gain</td>
</tr>
<tr>
<td>Meglitinide</td>
<td>Hypoglycemia</td>
<td>Weight gain</td>
</tr>
<tr>
<td>Biguanide</td>
<td>Diarrhea, Lactic acidosis, Metallic taste in mouth</td>
<td>Weight neutral</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Increased risk of hypoglycemia when taken with insulin, Hepatotoxic</td>
<td>Weight gain</td>
</tr>
<tr>
<td>α-glucosidase inhibitors</td>
<td>Abdominal pain, diarrhea, flatulence</td>
<td>Weight neutral</td>
</tr>
<tr>
<td>Incretin therapy</td>
<td>Hypoglycemia possible if used in combination with sulfonylureas</td>
<td>Weight neutral or Weight loss</td>
</tr>
<tr>
<td>DPP-4 Inhibitors</td>
<td>Nasopharyngitis, hypersensitivity and skin reactions</td>
<td>Weight neutral</td>
</tr>
<tr>
<td>Amylin</td>
<td>Nausea, Vomiting, Hypoglycemia when used in combination with insulin</td>
<td>Weight loss</td>
</tr>
</tbody>
</table>
such comorbidity that leads to the development of cardiovascular disease (CVD) and its management is of prime importance. Dietary modifications and physical activity have failed to manage obesity and only three medications (orlistat, sibutramine, and rimonabant) are currently available for long-term therapy of obesity. The common adverse effect is gain in body weight when they are discontinued after long term use (Dybicz et al., 2011; Davis et al., 2011; Woodard et al., 2011).

1.8. Traditional Medicine

Traditional medicines are unscientific knowledge based medicine systems that are developed and practiced by different cultures over generations in the maintenance of health before the era of modern medicine. Herbal medicines are the most beneficial form of traditional medicine (Said et al., 2008; Hamza et al., 2011).

1.8.1. Traditional Medicines: Constraints

There are numerous plant based therapeutic approaches all over the world. Most of the herbal preparations are mixtures of plants and minerals, prepared on the basis of their synergistic or additive therapeutic value to achieve an appropriate clinical response. The major problems posed with these medicines are the lack of standardization and consistency, lack of studies for toxicity, safety and quality which are unacceptable by the regulatory authorities. Heavy metal content above permissible limits and presence of inorganic minerals are also important causes for nonacceptance of these medicines by the regulatory authorities. Standardization is an important process where the active constituents are known in a preparation. The complex composition of these medicines makes the standardization, assessment of safety and therapeutic potential difficult. Herbal medicines also lack reproducibility of activity due to differences in the biochemical profiles of plants harvested at different times and locations, differences in variety, and variation in the methods used for extraction. Good Agricultural Practices (GAP), qualitative and quantitative
analysis of bioactive compound of herbs could overcome this variation in biological activity. Good Manufacturing Practices (GMP) could improve the acceptability and quality of these medicines. Preclinical and clinical testing of these medicines conducted as per Good laboratory practices (GLP) and Good Clinical Practices (GCP) respectively supports the safety and efficacy of these medicines. Modern analytical tools like chromatographic techniques are useful in marker based standardization and promote consistency from batch-to-batch preparation of these medicines. By this integrated approach, herbal medicines could be accepted globally and integrated with modern medicines for effective health care (Ahmad et al., 2006; Rosenbloom et al., 2011).

1.8.2. Herbal Drug Research: Bottlenecks and Steps to be taken

To give credibility to herbal medicine in health care industry, rigorous procedures need to be followed for the standardization and biological evaluation of safety and efficacy.

In the present study, an attempt has been made to develop a herbal mixture containing minimum number of standardized ingredients. The individual ingredients and herbal mixture were standardized using modern analytical tools. The safety of this herbal mixture on acute and repeated oral exposure was investigated. The anti-diabetic activity and their possible mechanism of action were investigated using \textit{in vivo} and \textit{in vitro} models.

1.9. Objectives of the Study

The objectives of the present study are to assess the

1. \textit{In vitro} antioxidant and antidiabetic effect of the individual herbs of DIA-2.
2. HPTLC fingerprint of DIA-2 and its component herbs.
4. Effect of DIA-2 and component herbs on ROS production in H$_2$O$_2$-treated
differentiated 3T3-L1 adipocytes.

5. Effect of DIA-2 on glucose, lipid profile, body weight and tissue biochemical/antioxidant status in high fat diet/low dose streptozotocin (STZ)-induced type II diabetes in rats.