EFFECTS OF GINKGO BILOBA AND PANAX GINSENG ON METABOLISM OF CARBOHYDRATE, LIPIDS AND INSULIN RECEPTOR GENES IN DIABETIC RATS

MAHRUKH NASEEM 2011-VA-531

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To

The Controller of Examinations,
University of Veterinary and Animal Sciences, Lahore.

We, the Supervisory Committee, certify that the contents and form of the thesis, submitted by MAHRUKH NASEEM, have been found satisfactory and
recommend that it be processed for the evaluation by the External Examination (s) for the award of the degree.

Dr. Muhammad Quaid Zaman
(Supervisor)

Dr. Imtiaz Rabbani
(Member)

Dr. Hafsa Zaneb
(Member)

Dedicated
To
My PARENTS
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**CHAPTER 1**

**INTRODUCTION**

Overwhelming evidence shows that diabetes mellitus (DM), a cluster of metabolic alterations known as ‘chronic metabolic syndrome’ mainly associated with substantial mortality and morbidity, has become one of the major health issue worldwide in the last few decades. In 2010 diabetes affected 285 million and is expected to affect 438 million people by the year 2030 globally (Diabetes in the UK, 2010). After cancer and cardio-vascular disease, DM is the third recognized major pathology, causing deterioration in human health with frequently lethal outcome. Its prevalence has been gradually increasing over the years (Li-Xia et al. 2011). Depending on the form, diabetes is characterized either by lack of insulin or insulin resistance, both of which lead to hyperglycemia, oxidative stress and subsequently to neuropathy, retinopathy, nephropathy, stroke, cardiovascular disease, gum
infections, necrosis of extremities, polydipsia, polyphagia, polyuria, muscular weakness, weight loss, and glucosuria (Marles and Farnworth 1995; Mohanty et al. 2000; Sowers et al. 2001; Wilson 2002; Lteif et al. 2003; StittCavanagh et al. 2009; Hui et al. 2009; Krishnamurthy et al. 2011). The cause of diabetes type I, also known as “juvenile onset diabetes” is insufficient secretion of insulin from pancreatic β cells. In diabetes type II, or “late onset diabetes”, the sensitivity of cells to insulin is reduced. Both forms of diabetes can, in principle, affect individuals of any age group and lead to disorders in the metabolism of carbohydrates, proteins or fat (Nayak and Roberts 2006).

Worldwide, diabetes type II is the most common form of diabetes. Although increasingly, diabetes type II is also affecting teenagers or even children, people who are at high risk are typically those over 48 years of age with a modern life style, excessive body fat, abnormal cholesterol level, high blood pressure, depression, improper diet, lack of physical activities and other environmental conditions. Beside these factors, inheritance also plays a vital role in diabetic development (Cooke and Plotnick 2008; Riserus et al. 2009; Ripsin et al. 2009; Babish et al. 2010).

There is strong evidence supporting the fact that hyperglycemia is linked to enhanced generation of reactive oxygen species (ROS), the free radicals that are the metabolic products of the body. Excess ROS in the body causes oxidative stress in the diabetic state (Ihara et al. 1999; Mohanty et al. 2000; Lin et al. 2008). Oxidative stress during diabetic condition reflects an imbalance between the increased generations of free radicals and decreased anti-oxidant potential, which may contribute to the development and progression of diabetes-associated complications by depleting the activity of the anti-oxidative defense system of the body (Baynes and Thorpe 1999; Pazdro and Burgess 2010; Savu et al. 2012; Maritim et al. 2003; Kumar 2012). ROS are highly toxic for cells, including the β cells of the pancreas, since they interact with nucleic acids, cellular proteins and lipid metabolism. Accordingly, ROS are associated with many complications and ultimately cause cellular death (Vaya and Aviram 2001; Wada and Ou 2002; Kajikawa et al. 2002; Sakai et al. 2003; Rahimi et al. 2005; Maltas and Yildiz 2012). However, the body has different anti-oxidant endogenous defenses like catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) or exogenous defenses originating from diets to scavenge ROS (Andersen and Markham 2006). This anti-oxidant defense system of the body fails to meet the protection demand if the level of ROS increases as in case of many several diseases, such as in DM.

Chronic hyperglycemia causes β cell dysfunctioning and/ or may also induce β-cell apoptosis thence, the insulin concentration begins to fall below normal levels in the blood in patients suffering from type II diabetes (Ibrahim et al. 2008; Chen et al.
Hepatic insulin resistance is one of the major contributing factors in diabetic development. Reduction in hepatic insulin sensitivity causes post-prandial hyper-glycemia and also causes an increase in hepatic glucose production, hence causes further elevation in the hyperglycemic state and chronic hyperinsulinemia. Besides enhancing the glucose level in the blood, hepatic insulin resistance could also cause dysregulation of lipid synthesis which may also lead to hepatic steatosis (Taniguchi et al. 2005), which, in turn, contributes to the inability of hepatic cells to respond normally to insulin.

Oral hypo-glycemic agents, such as biguanides (e.g. metformin), non-sulfonylurea secretagogues, thiazolidinediones (e.g. TZD or glitazone) and sulfonylurea, are available along with insulin for the treatment of diabetes type II but these products have prominent side effects, such as weight gain, impaired liver function, peripheral edema and gastro-intestinal disorders (Maghrani et al. 2004; Yeo et al. 2011). Since pharmacologically, these agents stimulate insulin secretion by the β-cells, they are ineffective in conditions where β-cell function is impaired (e.g. diabetes type I and chronical diabetes type II) (Cheng et al. 2013a). Instead of these pharmaceutically derived treatments, natural remedies can be safer (Murphy 2000). In the early stages of diabetes type II, lifestyle changes with less intake of sugars and fats, more exercise and loss of weight should be a primary goal that can frequently restore normal insulin sensitivity before β-cell necrosis becomes the leading symptom. Furthermore, medicinal plants and their products contain therapeutically and health beneficial effects and have been used since ancient time (Joseph and Raj 2010; Joseph et al. 2012; Singh 2011). Interest in these natural remedies, especially herbs, which show potent antihyperglycemic activities, are greatly increasing.

*Ginkgo biloba* has been considered one of the popular functional medical plants; it is oldest still existing tree species on the earth, with no closely living relative, being the last residual member of the *Ginkgoaceae* family commonly known as, ginkgo, maidenhair tree, gymnosperm tree and dates back 250 million years in China. Individual trees may live as long as one thousand years and grow to a height of about 38–40 m. The word *Ginkgo* is derived from the Chinese “Yin-kuo” for golden apricot and *biloba* refers to the bilobular structure for the leaves (DeFeudis 1998). *G. biloba*, due to its wide beneficial effects, is one of the top-selling herbal products worldwide and grows throughout China, Korea, Japan, Europe and United States (Jacobs and Browner 2000). Because of the applied beneficial properties for the prevention of human diseases, all parts of the *G. biloba*, such as leaves, roots and bark are the most frequently used natural remedies that have been used as traditional Chinese herbal medicines for several thousand years to treat diabetes, aging, asthma, bronchitis, cancer, impair sexual dysfunction, chilblains, peripheral arterial diseases, hypertension, cerebral insufficiency
associated with aging, preventive to drunkenness, stress, Alzheimer’s dementia, neuronal and cerebral diseases for thousand years (Marwick 1995; Oken et al. 1998; Kudolo 2001; Maclellan et al. 2002; Rai et al. 2003; Nakanishi 2005; Saw et al. 2006; Boveris et al. 2007; Chan et al. 2007; Dekosky et al. 2008; Janssen et al. 2010; Bachinskaya et al. 2011; Unger 2013). This defensive effect is attributed to anti-oxidant effect of \textit{G. biloba} leaves (Pietta et al. 2000; Maltas et al. 2011; Maltas and Yaldiz 2012).

Ginseng refers to the roots of numerous plant species belonging to the genus Panax of the family \textit{Araliaceae}, is a slow mounting perennial herb with fleshy root. Ginseng was first cultured around 11 BC, has a therapeutic history of more than five thousand years. Ginseng is widespread herbal medicine, has been used for several thousands of years in Asian countries (Wen et al. 1996; Rhim et al. 2002; Radad et al. 2006; Wang et al. 2007; Kim et al. 2013). Ginsenosides is an active ingredient of ginseng (Murphy and Lee 2002). Ginseng has traditionally been used mainly to “strengthen the weak body” and helps in the restitution of homeostasis, CNS disorders, cardiovascular syndromes, cancer, immune deficiency, sexual dysfunction, and liver disease due to pharmacological properties that are anti-oxidant, anti-inflammatory, anti-apoptotic and antimitotic (Wen et al. 1996; Attele et al. 1999; Kaneko and Nakanishi 2004; Rai et al. 2003; Radad et al. 2006; Amin at al. 2011).

According to our best knowledge, no data is available on the anti-diabetic effects for the combination of these two natural remedies. The motive of this study was to contribute to the understanding of the molecular mechanisms involved in diabetes by evaluating the anti-diabetic effects of \textit{G. biloba} leaf extract (GBE) and/ or \textit{P. ginseng} root extract (PGE) on several genes involved in the metabolism of carbohydrates and fats and biochemical parameters and to study the synergistic or energetic effects of these two herbs in combination.
2.1 Diabetes Mellitus

The two common types of diabetes are: type-I (juvenile diabetes) and type-II (late onset diabetes). Both types are manifested by elevated blood glucose concentration. Type-I DM, also known as insulin dependent diabetes (IDDM), is an autoimmune disorder with destruction or apoptosis of pancreatic β-cells, resulting in insufficient insulin secretion. Accordingly, individuals suffering from type-I DM are entirely dependent on exogenous sources of insulin. Type II DM is also known as non insulin dependent diabetes (NIDDM) and is a metabolic disorder, in which the patient is unable to respond to insulin, characterized by progressively rising insulin resistance and declining pancreatic β cell function. Dysregulation of glucose and lipid metabolism are the major factors determining the course of type-II DM. Type II diabetes commonly occurs in elder people, and treatment should begin with dietary changes and more exercise, although in most cases, additional medication will be needed (Aroson 2008; Babish et al. 2010; Singh 2011; Nagy et al. 2012, Chen et al. 2012). Although the two types of diabetes have different life-threatening complications, long-standing hyperglycemia is common in both (Gaster and Hirsch 1998; DeFronzo 1999; Xie et al. 2005a).

According to a recent WHO report, around 171 million population in 2000 were suffering from diabetes worldwide and this number is expected to double by 2030. Heart disease and stroke are among the primary causes of death among diabetic patients in developed countries (Tewari and Venkatesh 2004; Wild et al. 2004; Cho et al. 2006).

Risk of fatal and nonfatal cardiovascular disorders is greater in postprandial diabetic condition rather basal hyperglycemia and this risk further enhances when postprandial hyperglycemia is associated with postprandial hyperlipemia; i.e. increase in lipid peroxidation, increasing lipoprotein atherogenic capacity and
decreasing plasma anti-oxidants. Presumably higher the glycemic concentration in blood higher will be the degree for the reduction of antioxidant status of the body (Erlinger and Brancati 2001; Gagliardino 2005).

The World Health Organization (WHO) has listed 21,000 plants, used for medicinal purposes worldwide. Among these, 150 species are used commercially on large scale (Zohary and Hopf 2000). Martes and Farnsworth (1995) worked on anti-diabetic plants and listed about 1200 species of plants having anti-diabetic activities. They also investigated the hypoglycemic activities, potential toxicity problems and mechanism of actions of these anti-diabetic plants.

The blood glucose concentration is precisely controlled by the liver. During fasting, the liver splits more glycogen to glucose and thus increases the glucose production to maintain the normal circulatory glucose status. In comparison, an increase in blood glucose level as happens immediately after meal is reduced by an increased glucose uptake into hepatic and adipose tissue and into skeletal muscles (Klover and Mooney 2004). Several complex mechanisms are involved in regulating systemic glucose homeostasis. In diabetes, the gluconeogenic pathway is aberrantly activated, thus supplying relatively large quantities of glucose into the circulation (Klover Mooney 2004; Hanhineva et al. 2010), in addition to hepatic insulin resistance (Hanhineva et al. 2010). As has been stated, long term increases or decreases in the blood glucose concentration impairs bodily function in multiple ways. For instance, hypoglycemia may lead to coma, seizures and eventually death. In contrast, long lasting hyperglycemia, causes many complications, such as blindness, renal failure, cardio-vascular disorders and neuropathy (Watson et al. 2004).

Free fatty acids (FFA) play a vital role in the development of insulin resistance and thence, in the pathogenesis of diabetes. When insulin activity is inhibited, the rate of lipolysis increases so that large quantities of FFAs are released from adipose tissue. (Prato et al. 1990; Campbell et al. 1994; Wang et al. 2006). The subsequent elevation of plasma FFA may also reduce the utilization of glucose by skeletal muscle and increase hepatic glucose production by stimulating glycogenolysis and gluconeogenesis by liver cells (Bergman and Ander 2000). Moreover, intracellular lipid accumulation in muscle follows elevated FFA (Perseghin et al.1999). This elevated FFA, increased deposits of fat in the liver, pancreatic tissues and skeletal muscle dysregulate glucose disposal as a manifestation of diabetes and other associated metabolic syndromes (Guilherme et al. 2008). In pancreatic β cells, lipid accumulation may also impair insulin secretion and lead to apoptosis (Shimabukuro et al. 1998).

Regulation of body fat and energy balance are long standing pharmacological issues in obesity and diabetes. Adipose tissue serves as the largest energy reservoir of the
body; plays an important role in the regulation of cell function, capable of influencing multiple physiological and pathological processes through complex mechanisms of paracrine, endocrine and autocrine signals. The primary and unique role of adipose tissue is to store triacylglycerol and also to mobilize this reserve. Energy is assimilated by fat cells and stored as triglycerides in lipid droplets in adipocytes (Fruhbeck et al. 2001). To carry out the regulation of both lipolysis and lipogenesis, enzymes and hormones are essential.

For a long time, it was believed that adipose tissues functioned primarily as a storage site for excess body energy, but this concept has changed with the discovery of specific adipocyte derived hormones, i.e. adiponectin and leptin. Now, it is a well known fact that adipocytes not only play a passive role for the storage of excess energy but also function as important endocrine secretory cells that secrete dozens of factors that normalize food intake and regulate many metabolic processes. In adipose tissue, insulin resistance is a major cause of DM that leads to the reduction of glucose uptake and increases in FFA release into the blood due to an increase in lipolysis. Dysregulation of adipokine, an important bioactive substance secreted from adipocytes, plays a vital role in insulin resistance development and thence diabetes. An alteration of adipocytes has been implicated in cardiovascular and metabolic complications of obesity and type II DM (Hauner 2004; Babish et al. 2010; Lee et al. 2010).

Sagara et al. (1996) reported that oxidative stress caused neurological, cardiovascular, retinal and renal diabetic complications. Some important defensive mechanisms against oxidative stress are: (a) reduced generation of ROS, (b) enhancement in the production of antioxidant enzymes, like CAT, SOD, glutathione reductase (GSH) and GPX. They further reported that in hyperglycemic state, diminished glutathione levels causes reduction in the defense against oxidative stress. N-acetyl cysteine, a precursor of glutathione, inhibited the functional and structural abnormalities within the peripheral nerves in the diabetic state. Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules, like ROS, e.g. Superoxide, peroxy, hydroxyl, hydroperoxyl, nitric oxide, nitrogen dioxide and reactive nitrogen species (Ebuchi et al. 2010). Glucose oxidation increases the generation of free radicals in the blood and produces superoxide anion radicals. Hyperglycemia causes lipid peroxidation, Advanced Glycated End products and finally activation of the sorbitol pathway, which leads to NADPH and glutathione depletion. Decreases in the activities of antioxidant enzymes, like SOD, CAT and GPX were found in the kidney of diabetic rats (Kedziora-Kornatowska et al. 2000; Jialal et al. 2002).
In oxidative stress, there is an imbalance between the production of reactive oxygen species, reactive chlorine species and reactive nitrogen species and defensive mechanisms. In both the two types of DM, hyperglycemia results in the extensive production of reactive species during glucose oxidation, subsequent oxidative degradation of glycated proteins and nonenzymatic glycation of proteins (Rosen et al. 2001). Moreover, these increased free radicals destroy the β-cells and thus reduce the insulin secretion (Kim et al. 2011). Anti-oxidant enzymes, such as CAT, GXP, MDA and SOD help to decrease the oxidative stress in diabetes (Mexwell 1995). Reduction in anti-oxidant enzymes has been suggested to be an important co-factor in diabetes (West 2000).

SOD is an important anti-oxidative enzyme that contributes to cellular defense and plays a primary role for the conversion of superoxide anions into hydrogen peroxide (Kim et al. 2011). Glutathione reductase indirectly contributes in maintaining homeostasis and cellular protection by reducing the glutathione pool in cells via oxidation-reduction reaction (Kitamura et al. 1983; Kim et al. 2011). According to Son (2012) the diabetic condition is characterized by an increase in lipid peroxidation, increased in the activity of free radicals and enhancement in cellular protein oxidation as the important cofactors of cellular injury and cardiovascular disease. According to Jacob (1995), GPX plays a crucial role in reducing H$_2$O$_2$ in the presence of reduced glutathione. These detoxifying strokes of GPX against H$_2$O$_2$ protect the cell membrane against oxidative damage.

The liver serves as an important site for energy homeostasis and glucose metabolism. Hepatic insulin resistance enhances the process of gluconeogenesis and plays a key role in the development of diabetes type II (Friedman et al. 1997; Law 2002). Peroxisome proliferator-activated receptors (PPAR), Glucose transporters (GLUT), insulin receptor substrate (IRS) and sterol regulatory element-binding protein (SREBP) play crucial roles in this process. GLUT-2 is the main glucose transporter isoform in hepatic tissue and is crucial in glucose regulation by mediating bidirectional glucose transport. PPAR and SREBP are important transcription factors. In adipocytes PPARs were deemed the main isoform, but has also been found to mediate lipid and energy homeostasis by changing their expression in the liver. SREBP-1c is essential for the regulation of lipogenic genes (Memon et al. 2000; Zhou et al. 2011).

Watson et al. (2004) reported that insulin induced GLUT-4 translocation from intracellular membrane compartments into the plasma membrane surface, where it catalyzes the uptake of the glucose into adipose tissues and muscle. In mammals, glucose clearance from the blood is mainly by facilitative transporters that transport glucose down its concentration gradient in an energy independent manner. These are comprised of a family of highly related 12 transmembrane proteins. Usually all
mammalian cells are net consumers of glucose and maintain low intracellular glucose concentration and thus favor glucose entering into the cell. However, hepatocytes function as a net producer of glucose during periods of reduced insulin as occur in the fasting/basal state. During fasting, hepatic glycogenolysis and gluconeogenesis enhances intracellular glucose concentration. The resulting net efflux of glucose from the liver provides the brain and other tissues with a stable glucose supply.

GLUT-4 is the main glucose carrier expressed in the rats skeletal muscles; however, GLUT-1 is also expressed to some extent in rats muscles. Whereas, GLUT-1, GLUT4 and GLUT5 usually occur in human skeletal muscle. Under fasting state, the majority of the GLUT-4 is found in the intracellular membrane, but when activated by exercise or insulin, GLUT-4 is translocated from the endosomal compartment on the surface membrane which in turn enhances muscle glucose uptake (Zorzano et al. 2005). In rats GLUT-4 is differentially expressed in oxidative and glycolytic muscle fibers (Neufer et al. 1993; Zorzano et al. 2005).

Insulin receptor (IR) is transmembrane receptors that is regulated by the insulin and have important physiological effects in glucose homeostasis (Lee and White 2004). Any pathophysiological conditions that fail to synthesis IR may cause many clinical manifestations like diabetes and insulin resistance (He et al. 2011). The main bio-function of the IR is to enhance intracellular glucose uptake by the targeted tissue, so in case of suppression of IR signaling or insulin insensitivity, the body will suffer with hyperglycemia and many diabetic related complications (Krishnapuram et al. 2013).

Taniguchi et al. (2005) and Kim et al. (2009) reported that liver insulin resistance may compromise signalling via IRS protein, a family of docking molecules that connect insulin receptor activation to crucial downstream kinase cascades, such as activation of PI3 kinase (PI3K) or MAPK pathways. The two major IRS isoforms that are strongly expressed in the hepatic tissue are IRS-1 and IRS-2. Both these isoforms of IRS are downregulated in the liver in case of diabetes mellitus. They have further reported that IRS-1 is closely associated with glucose homeostasis gene regulation, whereas IRS-2 mediates the insulin signal in hepatic cells and has a regulatory role in the liver lipid metabolism. The dysfunctioning of IRS-1 and/or IRS2 in the liver causes basal hyperinsulinemia, hyperglycemia, dyslipidemia, glucose intolerance, insulin resistance and various metabolic disorders. IRS-2 is the key isoform in liver. It compensates for the lack of IRS-1 in the IRS-1−/− mouse model. Hepatic insulin signaling is mediated chiefly through IRS-2 (Bell et al. 1990; Rother et al. 1998; Memon et al. 2000; Zhou et al. 2011).
Saad et al. (1992) studied the effect of the streptozotocin-induced diabetes on IRS-1 phosphorylation and IR in the rats muscle and liver. In their study, they reported that insulin binding to the plasma membrane enhanced both in diabetic rats muscle and liver. This increase in insulin binding with muscle and liver in the diabetic condition has been attributed to increased number of binding receptors rather than to change in binding affinity of insulin for the receptors. They further reported that this enhancement in insulin binding in diabetic rats was due to dysfunctions in receptor binding activity which causes reduction in receptor phosphorylation in the diabetic condition. Phosphorylation of IRS-1 showed non-significant changes in liver and muscle of diabetic rats after insulin stimulation. Hepatic insulin signaling is usually mediated by IRS-2, the liver isoform (Rother et al. 1998).

The liver has a major role in metabolism; having two key roles in metabolizing glucose i.e. it plays a key role in balancing and regulating energy; also in the production and utilization of glucose. Down-regulation of IRS-1 is related to a reduction in glucokinase expression and enhanced blood glucose, whereas, decrease in IRS-2 up-regulates lipogenic enzymes SREBP-1c and fatty acid synthesis as well as enhancing hepatic lipid accumulation (Taniguchi et al. 2005).

Phosphoenolpyruvate carboxykinase (PEPCK) is the liver enzyme that plays a rate controlling role in gluconeogenesis cycle from lactate, pyruvate and alanine (Hanson and Garber, 1972). PEPCK is a liver enzyme that catalyzes the conversion of oxaloacetate into phosphoenolpyruvate.

In a normal condition of the body, insulin rapidly declines the PEPCK gene transcription in the liver, however in the pathophysiological conditions, such as diabetes and obesity, gluconeogenesis and PEPCK expression both markedly increase despite to the high level of insulin in blood circulation (Hofmann et al., 1992). It is believed that insulin may decrease PEPCK gene transcription by activating putative insulin receptor factor present in IRS (Lucaset al. 1991; O’Brein et al. 1994). Thus, the defect in binding, expression or phosphorylation of nuclear proteins activities via IRS might impair the insulin ability to reduce transcription of PEPCK gene which in turn leads to enhanced gluconeogenesis in diabetes (Friedman, 1997).

Horton et al. (2002) reported that SREBPs involved in lipid metabolism. Three isoforms of SREBPs are: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a is one of the most important isoforms and is a powerful activator for all SREBP genes associated with the synthesis of triglycerides, cholesterol and fatty acids. Role of SREBP-1c and SREBP-2 are more limited than SREBP-1a. SREBP-1c has a role in enhancing gene transcription essential for fatty acids synthesis rather than cholesterol
synthesis. However, SREBP-2 has a role to activate cholesterol synthesis. SREBP-1c and SREBP-2 both are predominant in hepatic and other intact tissues. Since, on normal expression level, SREBP-1c favors fatty acid biosynthetic pathway, whereas, SREBP-2 favors the cholesterologenesis. Over-expression of SREBP-1c in hepatic tissue of the transgenic mice produces a triglyceride enriched fatty liver without increasing cholesterol level; however, mRNA for the fatty acid synthetic enzymes and the rate of fatty acid synthesis are enhances four folds in this tissue. Moreover, they found no enhancement in the mRNA expression of the genes of cholesterol synthetic enzymes and also in the rate of cholesterol synthesis. Conversely, the over-expression of SREBP-2 in the liver enhances the mRNAs encoded all cholesterol biosynthetic enzymes and thus increases rate of cholesterol synthesis. This enhancement of cholesterol synthesis is even more prominent when the extent of cholesterol overload in the tissue is high; might be normally reduces SREBP processing and basically eliminate cholesterol synthesis.

In mice, the plasma lipoprotein concentration tends to decrease due to dysfunctioning of SREBPs (either up-regulation or down-regulation). If the SREBPs in the liver of the mice are over-expressed, it tends to lower the plasma triglyceride and cholesterol. Hepatocytes of SREBP1a in the transgenic mice overproduced VLDL-C, but these particles are quickly removed by the action of LDL receptors and do not accumulate in plasma. In fact some nascent VLDL particles are degraded before secretion by the process that is medicated by LDL receptors. The high level of SREBP-1a support sustained expression of LDL receptor, even in the cells having increased cholesterol level. In LDL receptor-deficient transgenic mice carrying the SREBP-1a, plasma cholesterol and triglyceride level increased about ten times (Horton et al. 1999; Gillian-Daniel et al. 2002).

Fatty acid synthase (FAS) is regulated by insulin (Real et al. 2010). The biosynthesis of FFAs is catalyzed by these FAS, which control the synthetic pathway of long chain fatty acid by using malonyl-coenzyme A (CoA) as a carbon donor, acetyl-CoA as a primer and NADPH as a reducing equivalent (Menendez et al. 2009). Although FAS is also present in adipose tissues however, their contribution to whole body lipogenesis is considered to be lower as compare to liver (Marin et al. 1992; Letexier et al. 2003), but adipose tissues remain an important site of endogenous FA synthesis (Swierczynski et al. 2000).

Claycombe et al. (1998) reported that insulin increased the gene expression of FAS and FAS activity in the cultured human adipocytes, suggesting the fact that insulin sensitivity in their regulation. Various studies showed that insulin resistance increased the expression of FAS and FAS protein production in adipose tissue (Bluher et al. 2002; 2004).
FAS have a strong influence both peripherally to enhance fatty acid oxidation and centrally to decrease food intake (Mobbs and Makimura 2002; Kumar et al. 2002), leading to suppressed fat mass and resolution of fatty liver in mice (Loftus et al. 2000). The mRNA expressions of FAS are frequently used marker of de novo lipogenesis and any alteration in FAS activity are considered due to changes in the transcription rate of FAS gene (Berndt et al. 2007).

PPARs are well characterized transcription factors, are the members of the nuclear hormone receptor family and have distinct tissue distribution. There are three isoforms of PPARs i.e. α, δ and γ that have distinct tissue distribution pattern. PPAR-α is primarily present in heart, hepatic and kidney tissues. PPAR-δ is ubiquitously expressed and plays a crucial role in gene regulation involved in lipoprotein metabolism, lipid utilization and storage, insulin action and glucose metabolism (Memon et al. 2000). PPAR-γ is mainly present in the adipose tissue, macrophages, and cells of the vasculature, play an essential role in adipogenesis, glucose and lipid homeostasis (Lehrke and Lazar 2005; Puhl et al. 2012), exhibits anti-inflammatory actions (Ricote and Glass 2007) and regulates acetyl-CoA synthetase and fatty acid transporters (Memon et al. 2000). Memon et al. (2000) reported that PPAR-γ down-regulates the mRNA expression of TNF-α and leptin in adipose tissues. They reported markedly increase in both PPAR-α and PPAR-γ mRNA expression level in hepatic region of the diabetic mice model. They found more pronounced effects of PPAR-γ mRNA expression of genes of liver in diabetic mice, compared with PPAR-α mRNA level. Moreover, up-regulation of PPAR-γ appears to be more sensitive to develop obesity

PPAR-α ligands have been found to improve lipid profile and are also involved indirectly to improve the insulin sensitivity by enhancing the β-oxidation of fatty acids which in turn decreases the lipid accumulation and toxicity of the liver as well as in the skeletal muscle (Li and Glass 2004; Banz et al. 2007). Since PPAR-γ plays a pivotal role in the adipogenesis, it is widely distributed in the adipocytes and also stimulates the production of insulin signaling pathway. Yet it is not clear that what mechanism involved by the PPAR-γ to improves insulin sensitivity in the adipocytes, however, it is a well known fact that PPAR-γ induces the genes expression involved in the insulin signaling pathway (Kintscher and Law 2005; Benz et al. 2007). Studies had shown that PPAR-γ activities enhance the number of small adipocytes having more sensitivity to insulin and reduces the number of large adipocytes in white adipose tissue (Okuno et al. 1998). Further; PPAR-γ serve as essential regulator for the metabolic activities of glucose and insulin leads to enhancement of
insulin sensitivity in type II diabetic patients and rodent models (Hallakou et al. 1997). Thus, PPAR-γ in white adipose tissue may play a key role in insulin sensitivity.

TNF-α is a pleiotropic cytokine having complex physiological functions, interacts with the array of other cytokines to initiate a cascade of cellular activities downstream. It also stimulates macrophages and monocytes phagocytosis to clear pathogens, induces expression of vascular adhesion molecules on endothelial cells, chemokines on macrophages and neutrophils to stimulate cell trafficking. Thence, in acute infections, TNF-α is mostly beneficial to host (Barbara et al. 1996; Lee and Lau 2011). TNF-α plays a key role to initiate and to amplify the inflammatory response. In the signaling pathway, TNF-α is triggered by binding one of the two cell surface receptors either to TNF-R1, which helps to associated pro-inflammatory and apoptotic pathways activated by TNF-α or TNF-R2, which mediated via TNF tissue repair and angiogenesis (Lee and Lau, 2011).

TNF-α enhances the insulin resistance in the muscle, hepatocytes and adipocytes. In obesity, TNF-α is up-regulated in the adipocytes and promotes the chances of diabetes by increasing apoptosis of the β cells or impacting IRS-1 (Badawi et al. 2010). However, over production of TNF-α may increases the induction of cytokines e. g interlukine-1 (IL-1), IL-6 and IL-8. Additionally, the glucocorticoids might develop local inflammation, tissue damage (Elenkov and Chrousos 2002; Bradley 2008). In addition, TNF-α is capable to induce cachexia in animals (Tracey et al. 1987) and also inhibit lipoprotein lipase (LPL) activity in the cell culture of adipocytes (Kawakami and Cerami 1981) and thence enhances triglycerides level in the blood.

Up-regulation of TNF-α in the adipose tissues occurs in the insulin resistance. The downregulation of TNF-α impaired insulin signal by suppresses the expression of IRS-2 and GLUT-4 (Hotamisligil et al. 1993; Lagathu et al. 2003; Shoelson et al. 2006).

2.2 Effects of Ginkgo biloba on diabetes mellitus

Cheng et al. (2013b) reported that the most important areas of G. biloba cultivation are Europe and China. The European applicants started earliest along and are developing smoothly, moreover their patents were of best quality. The Chinese applicants started later but are now growing fast with certain research capabilities, however, the patents quality needs to be improved.
The two major fractions in the standardized leaf extract (EGb 761) of *G. biloba* are: terpenoids and flavonoids. These two major components have different properties, responsible for divert and unique therapeutic action of this natural remedy. Ginkgo contains 6-7% terpenoids, 24-26% flavonoids and minor quantities of organic acids. Flavonoids present in *G. biloba* are: biflavones, (ciadopitysin, ginkgetin, bilobentol, amentoflavones, isoquingetin and 5metoxibilobetol), kaempferol, flavones, tannins, flavonols and quercetin glucosides. All these components act as free radical scavengers/anti-oxidants, cation chelating agents and enzyme inhibitors (Maclennan et al. 2002; Smith and Luo 2003; Hochmann et al. 2006).

The flavonoids present in ginkgo are responsible for the free radical scavenging and antioxidant activities and terpenes inhibit platelet activation factor (Huang et al. 2004; Chen et al. 2005). Goh and Barlow (2004) reported that flavonoids shows low bioavailability because they are poorly absorbable by the intestine in their glycosylated form and are rapidly eliminated. These flavonoids are absorbed only when they are presented as aglycones.

According to DeFeudis and Drieu (2000), non-absorbable flavonoids that reach the colon are subject to the bacterial enzyme degradation and then the metabolites may be absorbed. Once absorbed, they are metabolized in the liver into their conjugated derivatives. They further reported that the anti-oxidant activities of the plant is mainly attributed to the flavonoids fraction which has the ability to neutralize the free radicals, which may be achieved by direct attenuation of ROS, chelation of pro-oxidant transitional metal ions, expression of anti-oxidant metabolites, such as glutathione. Therefore, the flavonoids react preferentially with hydroxyl radicals and directly scavenge them.

Structurally flavonoids consist of an aromatic ring that has a double bond to react preferentially with hydroxyl radicals (Zimmerman et al. 2002). Extract of *G. biloba* also contains terpenoids, which are non-saponifiable lipids of the cyclic ester type (lactones). The two major types of terpenoids present in the *G. biloba* extract are: ginkgolides (major terpene molecules of ginkgolides A, B, C. J and M), present in 3.1% of the total extract and bilobalide collectively called terpene trilactones present in 2.9% of total extract. Other constituents contain proanthocyanadins, glucose, sterols, inositols. rhamnose, organic acids, D-glucaric acids, vanillic acids and ginkgolic acid (Goh and Barlow 2002; Smith and luo 2004; Nakanishi, 2005).

Vaya and Aviram (2001) studied the anti-oxidants properties of ginkgo and reported that phenolic compounds, like phenolic acids and flavonoids, found in fruits, vegetables and plants, have the ability to scavenge active oxygen, superoxide and
hydroxyl radicals by single-electron transfer. Flavonoids showed anti-oxidant property and to act as metal chelator.

Kose and Dogan (1995) compared the ability to prevent lipid peroxidation of ginkgo with a variety of other water-soluble (vitamin C, glutathione and uric acid) as well as fat soluble (vitamin E and vitamin A) anti-oxidants. Their results showed that ginkgo was more effective than water soluble anti-oxidants and as effective as the fat soluble anti-oxidants.

Shankar et al. (2005) studied the anti-oxidant effects in STZ induced diabetic rats and found that anti-diabetic activities of ginkgo showed tremendous anti-oxidant effects without showing any metal ion mediated lipid peroxidation. They reported that rats treated with ginkgo showed significant reduction in fasting blood sugar level; however, enhanced blood glutathione activities. In diabetes, oxidative stress is because of increased plasma free radicals concentration and a sharp reduction of anti-oxidant defenses.

Zhou et al. (2011) studied the effects of *G. biloba* on hepatic cells and reported that extract of ginkgo stimulated expressions of PPARs and IRS-2 in the normal status. Whereas, during insulin resistance, ginkgo extract stimulate the mRNA expressions of IRS-2 and GLUT-2; down-regulates the mRNA expression of SREBP1c expression. According to Zhou et al. (2011), GLUT-2 is a key glucose transporter in hepatic cell, up-regulation in its expression might be helpful for glucose absorption. SREBP1c have an important role in lipid metabolism; thus, its down-regulation may reduce lipid production, on the other hand, SREBP directly reduces mRNA expression of IRS-2 at transcriptional level, this reduction may be due to enhancement in the mRNA expression of IRS-2.

2.3 Effects of *Panax ginseng* on diabetes mellitus

The genus name of *Panax ginseng*, “Panax” was given by the Russian botanist C.A. Meyer, derived from the Greek words: “pan” meaning all and “axos” meaning cure. The specie name “ginseng” derived from the Chinese word “rensheng” meaning human; as ginseng roots look like the human body. Thus, *P. ginseng* means the “all-healing man”. It is a shade-loving perennial herb, cultivated mainly in China, Japan, Eastern temperate forest regions of the North America from Minnesota, Korea, Southern Quebec, Wisconsin to Georgia, Ozark Plateau and Oklahoma in South (Assineure et al. 2003; Qi et al. 2011). In China, ginseng roots are harvested when the plant is 3 – 6 years old, roots may be submitted to air drying (white ginseng) or are steamed (red ginseng) (Nocerino et al. 2000; Radad et al. 2006).

The most effective anti-diabetic species of ginseng are: Chinese ginseng (Ohnishi et al. 1996), Korean Red ginseng, Sanchi, Siberian (Ohnishi et al. 1996; Lee et al. 2009a), American ginseng (Amin et al. 2011) and Asian ginseng (Lim et al. 2009).
Ginseng has been highly cultivated and used in several Chinese herbal medicines since ancient time. Today, ginseng occupied a well-known position, in the list of top selling natural remedies. More than six million Americans are regularly using ginseng products (Radad et al. 2006). It shows highly pharmacological effects, including cardiovascular disorders, neurodegenerative diseases, central nervous system, immunomodulatory, anticancer, anti-stressor, reproductive and anti-diabetic properties. (Assineure et al. 2003; Yun et al. 2004; Qi et al. 2011; Kim et al. 2012).

*P. ginseng* is categorized as either cultivated or wild depending upon different nurturing methods. Cultivated ginseng farmed systematically on open land and harvested after a 5–6 years of cultivation period. Whereas, wild ginseng is slow growth, sensitive to atmospheric changes, planted at altitude between 800 and 1500 m and in a deep mountain. Due to these differences in their cultivation method and habitat, there may be some difference of active ingredients between cultivated and wild ginseng. In China and Korea, it is generally accepted that wild ginseng is more reactive than cultivated one (Jung et al. 2005).

Recently, seven types of beneficial health claims for the red ginseng have been approved by Korean food and drug administration for products available in the market, including five health claims, i.e. fatigue recovery, blood flow improvement by inhibiting platelet aggregation, immunity boasting, memory improvement and anti-oxidant efficacy for red ginseng and only two health associated claims namely fatigue recovery and immunity boosting for the white ginseng (Kim et al. 2012). Medical effects of the ginseng are attributed to its active compound, known as ginsenosides, present in the leaf, berry and root of ginseng. However, different parts of the plant contain distinct ginsenoside ratio and these parts contain different therapeutic activities. The basic structure of all ginsenosides is similar. It consists of a group of steroidal sapogenins nucleus with 17 carbon atoms arranged in four rings. (Huang 1999; Attele et al. 1999 Attele et al. 2002). The ginsenosides have powerful anti-oxidant activities; stimulates the expression of gene involved in anti-oxidant enzymatic action (Kim et al. 1996; Surh et al. 2001). The radical scavenging, chelation and oxidant activity of ginsenosides depends upon its linkage positions and sugar moieties, the types of aglycone, and total number of hydroxyl groups. In contrast to flavonoids, ginsenosides are more powerful role in oxidative stress than glycosides, and sugar moieties are positively correlated to their activities (Qi et al. 2011).

*P. ginseng* root has been shown to enhance fructose-rich chow-induced, insulin resistance in rats. Furthermore, hypoglycemic activity of *P. ginseng* root extract is due to enhancement in insulin secretion induced by activation of muscarinic M3 receptors in pancreatic β-cells through acetylcholine released from cholinergic nerve
terminals. All these results propose that *P. ginseng* root can be used potentially to treat disorders of glucose homeostasis. (Jung and Kang 2013).

Sotaniemi et al. (1995) conducted a clinical trial to see the anti-diabetic properties of ginseng and found non-significant change in TC, TG, HDL-C and LDL-C. Jung et al. (2005) conducted a study to see the anti-diabetic activities of wild *P. ginseng* leaf extract on rats. Their study protocol was of 4 weeks and they grouped rats as: normal group, normal rats fed with 200 mg/Kg ginseng leaf extract, diabetic control rats, diabetic rats treated with 200 mg/Kg ginseng leaf extract and diabetic rats group fed on 40 mg/Kg leaf extract. At the end of their study period of 4 weeks they killed the rats and collected kidneys, spleen and liver. They reported significant reduction in blood glucose concentration in both diabetic treated with ginseng leaf extract i.e. 40 mg/kg and 200 mg/Kg, they also studied the anti-oxidant status for CAT, GSH-Px and SOD in the collected organs, and found that ginseng leaf extract protect against CAT and GSH-Px to certain degree in all the three organs i.e kidneys, liver and spleen. However the supplement enhances the activity of SOD in the spleen and liver, whereas, its activity is significantly decreased in kidney. Thus, they concluded that ginseng leaf extracts causes both reactivation of the scavenging of free radicals and anti-oxidant enzymes. Ibrahim et al. (2008) compared the anti-oxidant properties of vitamin E, A, C and ginseng in diabetic rats. They found 57.5% increase in glutathione with vitamin E treatment, 63.7% with vitamin A, 64.4% with vitamin C, 74.7% with ginseng.

American ginseng extract showed anti-oxidant activities both in lipid soluble and water soluble medium by chelating metal ions and directly removing 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals. In diabetes, the supposed mechanism of ginsenosides could be to protect anti-oxidant protein or enzyme responsible for the removal of free radicals and thus neutralizing the oxidative stress. Ginsenosides protected the cells from lethal oxidant damage (Xie et al. 2009).

Ginsenosides are amphiphilic and have the ability to intercalate into the plasma membrane, leads to change fluidity of membrane and affects membrane function, eliciting a cellular response. Evidences showed that ginsenosides interact directly with specific membrane proteins. Moreover, like steroid hormones, ginsenoside are also lipid soluble, that could traverse the plasma membrane and initiate genomic effects. As plasma membrane have both lipid and protein portions. The physio-chemical properties of the membrane are sensitive to changes in membrane components and lipophilic agents that might modulate the curvature stress. The scattered protein ion channels, transporters and receptors have a role in ionic transportation between intracellular and extracellular environments of the cell. Since the ginsenosides are amphiphilic in nature, so ginsenosides interacts with polar heads
of membrane phospholipids and the β-OH of cholesterol through their OH groups. Furthermore, their hydrophobic steroid backbone could intercalate into the hydrophobic interior of the bilayer. Both of these effects might contribute to altering the lipid environment around membrane protein. Cholesterol shares ginsenosides amphipathic nature and steroid backbone, is an intrinsic membrane lipid, Ginsenoside Rb1 have ability to increase Na\(^+\)-K\(^+\) ATPase and Ca\(^{2+}\)-Mg\(^{2+}\) ATPase neuronal activities. It is possible that some ginsenosides may interact with membrane cholesterol and displace it from the immediate environment of ATPase. Ginsenosides may also alter membranous protein structure by altering membrane modulating and dynamics actions of ions channels, membrane enzymes and binding receptors (Attele et al. 1999).

More than 40 ginsenosides, which have various biological properties, have been identified and isolated from white and red ginseng. The variation in properties of ginsenosides could be based upon structural variations. More than 80% of identified ginsenosides belong either to propanaxatriol (PPT), such as ginsenosides Re, RF and Rg1 and propanaxadial (PPD) i.e. ginsenosides Rb1, Rb2, Rc and Rd (Murphy and Lee 2002; Lim et al. 2009; Kim et al. 2014). Many studies have aimed to convert major ginsenosides to the more active minor ginsenosides using heating, acid treatment, enzymatic and microbial conversion (Kim et al. 1999; Kim et al. 2005a). Beside ginsenosides, ginseng also contains glycosides mixture, trace minerals, carbohydrates, proteins, peptides and amino acid (Radad et al. 2006; Amin et al. 2011). The ginsenosides an active ingredient of ginseng is depend on its species, harvesting season, plant age, part of the plant, preservation and extraction methods (Kaku et al. 1975; Kim et al. 1998; Huang 1999; Attele et al. 1999; Yoon et al. 2003). The amount of ginsenosides is highest in flower buds (8.4%-26.4%), then berry (8.25%-21.8%), crown (4.29%-17.4%), rootlet (9.2%-12.3%), side root (6.5%-12%), leaf (7.6%-12.6%), seed bud (3.19%), stem (2.1%), and seed (0.7%). After several hours of steaming, the quantity of ginseng saponins (GS) would decrease by more than 30%. Some GS are increased (Rg2, Rg3, Rh1 and Rh2), whereas some are decreased (Rb1, Rb2, Rb3, Rd, Re, Rc and Rg1) (Cheng et al. 2008).

From the ginsenoside, PPT such as ginsenosides Rh2, Rh1 and compound k have the ability to enhance proliferation and differentiation of brown marrow and have anti-cancerous properties. Among PPD ginsenosides Rg1, Re and Rh are the main constituents. However, ginsenoside Rg1 and Re are metabolized to ginsenoside Rh1 by human intestinal microflora. The Rh1 contains anti-obesity, anti-inflammatory, anti-allergic, and estrogenic properties (Gu et al. 2013).
According to Shang et al. (2007; 2008), Rb1, one of the ginsenosides, is the most abundant in all the ginsenosides, which facilitates the process of adipogenesis by enhancing the PPAR-γ genes expression. Rb1 binds to the PPAR-γ ligand binding domain as measured by the surface Plasmon resonance, which shows that Rb1 activates PPAR-γ. Rb1 leads to increased basal and insulin-mediated glucose uptake. They also reported that Rb1 have a role in possessing insulin like effects as Rb1 inhibits lipolysis in adipocytes. Thus, it stimulates glucose transport and inhibits lipolysis in adipocytes via enhancing insulin signaling.

Ginseng radix can ameliorate hyperglycemia possibly by blocking intestinal glucose absorption and inhibiting hepatic glucose-6-phosphatase, ginseng roote can do it through the upregulation of adipocytic PPAR-γ expression as well as inhibiting intestinal glucose absorption in KKAY mice (Chung et al. 2001).

Some other reports revealed that Rh2, one of the ginsenosides, lowered plasma glucose concentration by enhancing beta-endorphin secretion in diabetic rats, which activates opioid mureceptors, which in turn regulates the expression of GLUT-4. This GLUT-4 enhances glucose sensitivity in the muscle and adipose tissue, thereby promotes tissue glucose absorption and reduces blood glucose concentration (Lai et al. 2006; Han et al. 2006).

Kim and Kim (2012) reported that *P. ginseng* might mediate its anti-hyperglycemic effects through different mechanisms, including insulin secretory mechanism by pancreatic β-cells and glucose take up by the targeted tissues. *In vivo* study has demonstrated that *P. ginseng* treatment increases insulin secretion from the rat islets and inhibit β-cell apoptosis. They studied the mechanism on the antidiabetic effects of *P. ginseng* on the expression of signaling molecular associated with insulin action, insulin secretion and β-cell mass action in Goto-Kakizaki rats. They have found that *P. ginseng* significantly has reduced protein tyrosine phosphatases (PTP)1B expression of adipose tissue and muscles, they further studied the expression of protein involved in insulin secretion namely insulin and uncoupling protein (UCP) 2 from the pancreatic cells and the expression of protein involved in β-cells mass, i.e. Bax and PPARs and found 25% up-regulation in insulin and 82.1% down-regulation in UCP-2 expression in the pancreatic cells and 93.8% and 76.5% down-regulation for Bax and PPARs respectively. The exact mechanism that how ginseng regulates the glucose concentration is yet not cleared; however there are several hypotheses, which may explain the working mechanism of ginseng. As the inhibition of the neuronal discharge frequently from the gastric secretion has been observed in the rats treated with ginseng, so one hypothesis is that modulating effects of ginseng on the digestive system may be involved (Yuan et al. 1998).
The other mechanism is that, ginseng may exert its effect directly through modulation of insulin secretion from the pancreatic β-cells. As it is observed by several researchers that ginseng effectively increase the blood insulin and glucose concentrations in alloxan-induced diabetic rats. This unique effect of ginseng might be mediated by nitric oxide (NO). It is now clear that NO stimulates glucose-dependent insulin secretion in the islet cells of pancreas in rats (Kimura et al. 1981; Spinas et al. 1998).

And the last hypothesis which may also explain the mechanism of the ginseng is that it improved the glucose transport in the liver of rats by increasing the activity of GLUT-2. This effect may also be mediated by NO. Evidences show that the insulin-stimulated glucose uptake in the muscle and adipose tissues are NO-dependent in rats and this increase in NO to elicit the insulin stimulation might be due to ginseng. Enhancement in the NO synthesis by ginseng in kidney, corpus cavernosum and in the endothelium of lungs has been reported (Ohnishi et al. 1996; Gillis, 1997; Roy et al. 1998; Vukan et al. 2000).

**Statement of Problem:**

Diabetes is a metabolic disorder, becoming one of the burning health issues worldwide and is the third largest known disease of the world. Many synthetic drugs are used now-a-days to treat diabetes, sometimes with health threatening side effects. Due to these side effects, interest in developing natural remedies has been increasing in the last few years.

In the present study, we try to evaluate the anti-diabetic properties of *G. biloba* leaf extract and *P. ginseng* root extracts individually and in combination on the metabolic genes involved in carbohydrate, lipid, insulin metabolism and biochemical parameters, to see the synergistic or energetic effects of these two herbs in combination.
CHAPTER 3
MATERIALS AND METHODS

3.1 Experimental animals
A total of fifty-six normo-glycemic male adult Wistar rats, weighing 150-200 g, 120 days old, were selected for the present study. Rats were acclimated for a period of 12 days in the animal shed of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan, prior to the experiment. All the animals were housed in stainless steel cages, 2 animals per cage, with wood litter bedding that was changed on a weekly basis to maintain hygienic conditions. They were kept in an environmentally controlled room with a temperature of 24±5°C; and were maintained under a 12-h light: 12-h dark cycle. All animals were given free access to water and feed (standard rats chow) (Amin et al. 2011; El-Mesallamy et al. 2011). Body weight (BW) was measured weekly; food intake was measured twice a week for each rat by determining the pre and post-weights of the food jars. Duration of the experiment was 14 weeks. Animal experiments were carried out according to the instructional ethical committee guidelines for the care of laboratory animals of UVAS, Lahore, Pakistan.

3.2 Experimental Design
Rats were allowed to feed on high-fat diet (HFD: 12.7% maize starch, 6.5% dextrose, 3.9% sunflower oil, 31.3% beef tallow and 28.6% casein by weight) for two weeks, and then divided randomly into following seven groups (8 rats in each group):
**Non-diabetic group:** Rats of this group were served as non-diabetic or negative control and given standard diet without any supplementation of *Ginkgo biloba* leaf extract (GBE) or *Panax ginseng* root extract (PGE).

**Diabetic group:** This group included diabetic control or positive control rats and given the standard diet without any supplementation of GBE or PGE.

**GBE group:** This group was comprised of diabetic rats with standard diet supplemented with GBE at the dose of 100 mg/kg/day.

**PGE group:** This group was comprised of diabetic rats with standard diet supplemented with PGE at the dose of 300 mg/kg/day.

**Mixed 1 group:** The diabetic rats received standard diet with the combination of both GBE and PGE at dose of 200 mg/kg/day (50mg/kg/day of GBE and 150mg/kg/day of PGE).

**Mixed 2 group:** The diabetic rats received standard diet with the combination of both dose of GBE and PGE at dose of 400mg/kg/day (100mg/kg/day of GBE and 300mg/kg/day of PGE).

**Mixed 3 group:** The diabetic rats received standard diet with the combination of both GBE and PGE at dose of 600mg/kg/day (150mg/kg/day of GBE and 450mg/kg/day of PGE).

At the end of 14th day, the rats were kept in fasting condition overnight and then received a single intraperitoneal injected of alloxan monohydrate (Sigma, USA) dissolved in 0.5 ml of saline solution at the dose of 120-130 mg/kg BW. For the non-diabetic group, 0.5 ml normal saline solution was injected intraperitoneally. 20%
glucose solution was given to the rats to drink instead of water after 6h of alloxan induction. For the next 24h, rats were kept on 5% glucose solution to prevent hypoglycemia (Brownlee 2001; Ebuehi et al. 2010).

After 3 days of alloxan induction, overnight fasting blood samples were collected from tip of tail and glycemia was measured (ACCU check, Germany). Only rats having fasting blood glucose levels higher than 250 mg/dl were considered diabetic and included in the study (Shankar et al. 2005; Cheng et al. 2013a). Supplementation of the standardized \textit{G. biloba} leaf extract (terpenoids 6%/flavonoids 24%) (GBE) and \textit{P. ginseng} root extract (4%HPLC) (PGE) was purchased from Hunan Nutramax Inc (COCO CHEN, China) and treatment was started by mixing GBE and PGE into the standard diet (39.7% maize starch, 20% dextrose, 5.8% sunflower oil and 20.5% casein by weight) for 14 weeks.

\section*{3.3 Blood sampling}

At the end of the experimental period of 14 weeks, blood samples in fasting/ basal state were collected directly from the heart in a dry glass centrifuge tubes, coagulated at room temperature and then centrifuged at 3000 rpm for 15 minutes at room temperature for the separation of serum. The clear non-hemolysed supernatants sera were separated using micropipettes and stored at -20°C for further biological analysis (Kumar 2012).

\section*{3.4 Tissue sampling and mRNA expression of genes in the liver, skeletal muscle and visceral adipose tissue:}

Rats were sacrificed; liver, skeletal muscle and adipose tissues were collected and cleaned with normal saline solution and were stored immediately at -80 °C.

RNA was extracted (Laboratoire de Biochimie, MMS, Nantes, France) using trizol reagent (ambion, USA), according to the manufacturer’s instructions. The total RNA concentration was quantified by measuring the absorbance at 260 nm. Total RNA (1 µg) was then reverse-transcribed in to cDNA using Super-ScriptIII Reverse Transcriptase (Invitrogen, France) in a 20 µl reaction volume. An initial denaturation step for 5 min at 70°C was followed by an elongation phase of 45 min at 55°C.

Quantitative PCR was performed on a MyiQ2 Real-Time PCR Detecting system (Bio-Rad, Marnes-la-coquette, France) using SYBR Green (Bio Rad) supermix. The PCR was carried out for 45 cycles of 95°C for 30 s and 60°C for 30 s (Wang et al. 2006). The
fluorescence was read during the reaction, allowing continuous monitoring of amount of PCR product. The sequence of primers was determined by the primer3 website. The mRNA levels were normalized using GADPH as a housekeeping gene. Relative quantification was performed using the \( \Delta\Delta CT \) method. Primers used for qRT-PCR are listed in Table 1.

Table 1: Genes and the primer sequences used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TCCCATTCTTCCACCTTTGAT GCT</td>
<td>ACCCTTTGCTGTAGCCATATT CAT</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>GCTTCTGTTGCCCTTCTGTC</td>
<td>TGGACGCTCTCTTTCCAACCT</td>
</tr>
<tr>
<td>IR</td>
<td>GTGCTGCTCATGTCTTAAGA</td>
<td>AATGGTCTGTGCTCTTCGTG</td>
</tr>
<tr>
<td>IRS-1</td>
<td>GCCAATCTTCTCAGTTGC</td>
<td>CATCGTGAAAGAAGGCATAGG</td>
</tr>
<tr>
<td>PEPC K</td>
<td>AGTCACCATCACTTCTGGA AGA</td>
<td>GGTGCAGAATCAGCGGTTT</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GGCATGAAACCTGAAGTGTT</td>
<td>TGGGCTTTTACCTGGTTATC</td>
</tr>
<tr>
<td>FAS</td>
<td>CGCCGTTGGCTGGAGATTG</td>
<td>CGGGCCGAGGTGGTGGGAA G</td>
</tr>
<tr>
<td>PPAR-( \alpha )</td>
<td>GAGACCTCGGGGATCTTGCAGGCTTGTGAGCGGGAAG</td>
<td></td>
</tr>
<tr>
<td>PPAR-( \gamma )</td>
<td>CTGACCAATGGTACGTGCAGGCTTCCTGAGCTTGG</td>
<td></td>
</tr>
<tr>
<td>TNF( \alpha )</td>
<td>GCAGAGCCTTCCAAGGCCCTACC</td>
<td>GTTACCAGGCCCACCCTCTCTTTGG</td>
</tr>
</tbody>
</table>

**Abbreviations:** GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), GLUT-4 (Glucose transporter-4), IR (Insulin receptor), IRS-1 (Insulin receptor substrate-1), PEPCK (Phosphoenolpyruvate carboxykinase), SREBP-1c (Sterol regulatory element binding protein-1c),
FAS (Fatty acid synthase), PPAR-α (Peroxisome proliferator-activated receptor- α), PPAR-γ (Peroxisome proliferator-activated receptor- α), TNF-α (tumor necrosis factor-α)

3.5 Determination of biochemical parameters: The biochemical analyses of FSG (fasting serum glucose), CAT (catalase), MDA (malondialdehyde), ALT (alanine aminotransferase), AST (Aspartate aminotransferase), creatinine were done using commercially available kits (Randox, UK).

Serum TC (total cholesterol) and TG (triglycerides) were measured using enzymatic kits (Bio-Merieux, Marcy-l'Etoile, France). VLDL-C (Very low density lipoprotein-cholesterol), LDL-C (low density lipoprotein-cholesterol) and HDL-C (high density lipoprotein-cholesterol) profiles were performed using fast protein liquid chromatography (FPLC) (AKTA FPLC SYSTEM, GE Healthcare, USA) (Chadli et al. 2014).

3.6 Statistical analysis:

Results were expressed as Mean ± S.E.M. Statistical analyses were performed using Stat view software (SAS Institute Inc., SAS Campus Drive, Cary, NC, USA). Two-way repeated measure analysis of variance (ANOVA) was performed for body weight and weekly glucose. For the rest of the parameters one-way repeated measure analysis of variance (ANOVA) followed by PLSD Fisher's test was performed to assess the effect of herbal drugs. Differences were considered significant at P<0.05.

CHAPTER 4
RESULTS

4.1 Body weight

Table 2 shows the values of BW of non-diabetic, diabetic and all the five treated groups
(GBE, PGE, mixed 1, mixed 2 and mixed 3) at the first week of the experiment and at the end of 14th week of the experiment. A significant reduction in the BW of diabetic group (P < 0.0001) was recorded compared to non-diabetic rats on week 14. A significant reduction in the BW of diabetic group (P < 0.0001) was recorded on week 14 compared to week 1. Furthermore, GBE (P < 0.0001), PGE (P < 0.0001), mixed 1 (P < 0.0001), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) showed significant reduction in the BW compared to the non-diabetic group at the end of week 14. For the GBE and PGE treatment groups, the reduction in BW was significant as (P < 0.05), for all the three mixed groups it was highly significant (P < 0.0001) compared to the diabetic group at the end of week 14.

4.2 Plasma glucose concentration

We investigated the anti-hyperglycemic activities of GBE and/or PGE in diabetic rats. Blood glucose level was monitored on the week 1 and week 14. We found a significant increase in the blood glucose concentration in the diabetic control and in the treated groups compared to the non-
diabetic group on the 1st week of treatment. Significant enhancement (P < 0.0001) in blood glucose level was recorded on week 1 and week 14 in diabetic group compared to the nondiabetic group and a significant (P < 0.0001) reduction in blood glucose level was recorded for all treated groups compared to diabetic group (Table 2).

**Results**

We also investigated the FSG concentration in alloxan-induced diabetic rats at the end of our experimental trial and found significantly higher (P < 0.0001) FSG concentration in the diabetic group than the non-diabetic group. A significant decrease (P < 0.0001) was found in all the five treated groups compared to the diabetic group (Fig 1; Table 3).

Fig 1: Fasting serum glucose concentration of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced diabetic rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic group and non-diabetic group, b presents comparison between treatment groups and diabetic group. *** presents P < 0.0001.
### Results

Table 2: Effect of *Ginkgo biloba* leaf extract (GBE) and/or *Panax ginseng* root extract (PGE) on body weight and blood glucose concentration at week 1 and week 14 in alloxan-induced diabetic rats (n=08).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>GBE</th>
<th>PGE</th>
<th>Mixed 1</th>
<th>Mixed 2</th>
<th>Mixed 3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 14</td>
<td>Week 1</td>
<td>Week 14</td>
<td>Week 1</td>
<td>Week 14</td>
<td>Week 1</td>
<td>Week 14</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>165.36 ±1.51&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>206.20 ±2.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165.93 ±1.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>143.75 ±2.20&lt;sup&gt;f&lt;/sup&gt;</td>
<td>166.87 ±0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>166.87 ±0.99&lt;sup&gt;f&lt;/sup&gt;</td>
<td>166.91 ±1.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>159.08 ±2.53&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>83.43 ±1.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.68 ±1.35&lt;sup&gt;f&lt;/sup&gt;</td>
<td>417.07±25.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>485.58 ±25.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>339.63 ±14.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>227.38 ±14.65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>422.16 ±17.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>412.81 ±15.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± S.E.M. Different superscripts<sup>a-f</sup> represents significant difference between the groups in a row at P < 0.05.
**4.3 Serum lipid Profile**

A significant increase in serum TC (P < 0.0001), VLDL-C (P < 0.0001), LDL-C (P < 0.0001) and significant decrease in HDL-C (P < 0.0001) levels in the diabetic group were observed compared to non-diabetic rats. In case of TC, a significant reduction was measured only for mixed group 3 (P < 0.001). A significant reduction in VLDL-C was recorded for mixed 1 (P < 0.05), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) compared to diabetic group. Like VLDL-C, LDL-C also showed a significant reduction (P < 0.0001) for all the three mixed groups compared to the diabetic group (Table 3). Whereas, our data showed a significant increase for HDL-C in all the five treated groups, i.e. GBE (P < 0.001), PGE (P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) compared to diabetic rats. We measured a significant reduction in TG in all the five treated groups and it was significant increased (P < 0.0001) in diabetic rats (Table 3).

**4.4 Determination of oxidative stress**

We found a significant increase in plasma CAT level after treatment with GBE (P < 0.05) PGE (P < 0.05), mixed 1 (P < 0.05), mixed 2 (P < 0.05) and mixed 3 (P < 0.05) which had been found to be significantly decreased (P < 0.0001) in diabetic rats (Table 3). We observed significant increase (P < 0.001) in plasma MDA level in diabetic rats compared to non-diabetic rats and it was found to be significantly decreased after treatment of 14 weeks with GBE (P < 0.05), PGE (P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.001) and mixed 3 (P < 0.0001) in comparison to diabetic group (Table 3).
4.5 Serum Biochemical parameters

We measured the serum concentrations of creatinine, AST and ALT in non-diabetic, diabetic and all the five treated groups (Table 3). Significant increase (P < 0.001) in serum creatinine concentration was recorded in diabetic rats as compared to non-diabetic rats. While only three treated groups, i.e. PGE group (P < 0.05), mixed 1 group (P < 0.05) and mixed 3 group (P < 0.001) showed a significant reduction for creatinine concentration compared to diabetic group.

A significant increase in both AST (P < 0.0001) and ALT (P < 0.001) levels was recorded in diabetic group as compared to non-diabetic group; however, a significant reduction for AST level was noticed in PGE (P < 0.05), mixed 2 (P < 0.05) and mixed 3 (P < 0.001) treated groups. Whereas, in case of serum ALT level, a significant reduction was recorded in GBE (P <
0.001), PGE (P < 0.05) and mixed group 3 (P < 0.05) only.
Table 3: Effect of *Ginkgo biloba* leaf extract (GBE) and/or *Panax ginseng* root extract (PGE) on fasting serum glucose, Creatinine, AST, ALT, Anti-oxidative Stress (CAT and MDA) and serum lipid concentration (TC, VLDL-C, LDL-C, HDL-C, TG and VLDL-TG) in alloxan-induced diabetic rats (n=08).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>GBE</th>
<th>PGE</th>
<th>Mixed 1</th>
<th>Mixed 2</th>
<th>Mixed 3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSG (mg/dl)</td>
<td>83.68±1.35d</td>
<td>485.58±25.52a</td>
<td>227.38±10.89b</td>
<td>187.97±3.22c</td>
<td>178.05±1.63c</td>
<td>178.87±1.22c</td>
<td>162.55±0.91c</td>
<td>0.0001</td>
</tr>
<tr>
<td>TC (g/L)</td>
<td>0.82±0.01c</td>
<td>1.34±0.01a</td>
<td>1.29±0.02ab</td>
<td>1.34±0.02a</td>
<td>1.29±0.01ab</td>
<td>1.32±0.01a</td>
<td>1.25±0.02b</td>
<td>0.0001</td>
</tr>
<tr>
<td>VLDL-C (g/L)</td>
<td>0.07±0.005c</td>
<td>0.28±0.005a</td>
<td>0.29±0.005a</td>
<td>0.29±0.005a</td>
<td>0.26±0.01b</td>
<td>0.22±0.002c</td>
<td>0.18±0.003d</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL-C (g/L)</td>
<td>0.06±0.001d</td>
<td>0.55±0.01a</td>
<td>0.54±0.01a</td>
<td>0.53±0.01a</td>
<td>0.43±0.004b</td>
<td>0.43±0.005b</td>
<td>0.30±0.005c</td>
<td>0.0001</td>
</tr>
<tr>
<td>HDL-C (g/L)</td>
<td>0.68±0.01b</td>
<td>0.49±0.009c</td>
<td>0.54±0.008d</td>
<td>0.54±0.01d</td>
<td>0.54±0.01d</td>
<td>0.647±0.007c</td>
<td>0.76±0.01a</td>
<td>0.0001</td>
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<tr>
<td>TG (g/L)</td>
<td>1.25±0.007d</td>
<td>2.12±0.02a</td>
<td>1.56±0.01b</td>
<td>1.42±0.02c</td>
<td>1.38±0.03c</td>
<td>1.28±0.01d</td>
<td>1.23±0.01d</td>
<td>0.0001</td>
</tr>
<tr>
<td>CAT (KU/L)</td>
<td>20.83±0.26a</td>
<td>18.60±0.65c</td>
<td>19.99±0.16ab</td>
<td>19.56±0.18b</td>
<td>19.55±0.13b</td>
<td>19.50±0.12b</td>
<td>19.49±0.16b</td>
<td>0.0005</td>
</tr>
<tr>
<td>MDA (mmol/L)</td>
<td>6.54±0.23b</td>
<td>7.55±0.28a</td>
<td>6.95±0.22b</td>
<td>6.59±0.22b</td>
<td>6.35±0.21b</td>
<td>6.34±0.11b</td>
<td>6.32±0.11b</td>
<td>0.001</td>
</tr>
<tr>
<td>AST (µ/L)</td>
<td>76.88±1.43c</td>
<td>210.06±3.08a</td>
<td>209.43±1.56a</td>
<td>195.95±1.13ab</td>
<td>199.66±0.36ab</td>
<td>195.28±1.15ab</td>
<td>194.3±12.23b</td>
<td>0.0001</td>
</tr>
<tr>
<td>ALT (µ/L)</td>
<td>36.23±0.72d</td>
<td>40.9±1.68a</td>
<td>36.88±0.19d</td>
<td>37.37±0.35cd</td>
<td>39.48±0.64abc</td>
<td>39.96±0.47ab</td>
<td>37.58±0.61bcd</td>
<td>0.0007</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.63±0.12c</td>
<td>2.01±0.03a</td>
<td>1.95±0.04ab</td>
<td>1.8±0.04bc</td>
<td>1.75±0.06bc</td>
<td>1.82±0.04abc</td>
<td>1.7±0.03c</td>
<td>0.002</td>
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</table>

Data is presented as Mean ± S.E.M. Different superscripts a-d represents significant difference between the groups in a row at P < 0.05.
FSG (Fasting Serum Glucose), TC (Total Cholesterol), VLDL-C (Very Low Density Lipoprotein-Cholesterol), LDL-C (Low Density Lipoprotein-Cholesterol), HDL (High Density Lipoprotein-Cholesterol), TG (Total triglyceride), MDA (Malondialdehyde), CAT (Catalase), AST (Asparatate aminotransferase), ALT (Alanine aminotransferase).
4.6 mRNA expressions of genes

To further understand the underlying mechanisms that how GBE and/or PGE improve the glucose tolerance and dyslipidemia in diabetic state, the mRNA levels of key genes involved in the metabolism of carbohydrates and lipids were studied in liver, skeletal muscles and adipose tissues.

4.6.1 mRNA expressions of genes in liver

The mRNA expressions of eight genes, i.e. GLUT-4, IR, IRS-1, PEPCK, SREBP-1c, FAS, PPAR-α and TNF-α were studied in liver (Table 4). For both mRNA expressions of GLUT-4 and IR, we observed non-significant down-regulation in diabetic rats in comparison to non-diabetic rats and in case of GLUT-4, only mixed 3 showed significant up-regulation (P < 0.05) compared to diabetic group (Fig 2). However, no significant change for mRNA expressions of IR was found in any of the treated group (Fig 3). In case of IRS-1, a significant downregulation (P < 0.001) was found in diabetic rats in comparison with non-diabetic rats; and the mRNA gene expression of IRS-1 was significantly up-regulated in GBE (P < 0.05) and mixed 3 group (P < 0.05) compared to diabetic group; however, other three groups, i.e. PGE, mixed 1 and mixed 2 groups showed no change after treatment (Fig 4). For the mRNA expressions of PEPCK, a significant down-regulation (P < 0.05) was recorded in diabetic group compared to non-diabetic rats; however, a significant up-regulation was found for GBE (P < 0.05), mixed 1 (P < 0.05), mixed 2 (P < 0.05) and mixed 3 (P < 0.05) treated groups compared to the diabetic group (Fig 5).

SREBP-1c showed significant up-regulation (P < 0.0001) in diabetic rats in comparison with non-diabetic rats. A significant down-regulation in mRNA expression of SREBP-1c was
found for mixed 2 (P < 0.05) and mixed 3 (P < 0.001) groups in comparison with diabetic rats (Fig 6). For mRNA expressions of FAS, we observed significant down-regulation (P < 0.001) in diabetic group as compared to non-diabetic group and found significant up-regulation in mixed 2 (P < 0.05) and mixed 3 (P < 0.05) treated groups compared to diabetic rats (Fig 7). A nonsignificant change for the expression of PPAR-α in liver of all groups including diabetic rat group were reported (Fig 9). However, TNF-α showed significant up-regulation (P < 0.0001) in diabetic rats compared to non-diabetic group and found a significant down-regulation for GBE (P < 0.001), PGE (P < 0.0001) and mixed 3 (P < 0.0001) treated groups compared to diabetic group (Fig 8).
Table 4: Effect of *Ginkgo biloba* leaf extract (GBE) and/or *Panax ginseng* root extract (PGE) on the mRNA expressions in liver, skeletal muscle and adipose tissue in alloxan-induced diabetic rats (n=08).

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Genes</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>GBE</th>
<th>PGE</th>
<th>Mixed 1</th>
<th>Mixed 2</th>
<th>Mixed 3</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td>GLUT-4</td>
<td>1.10±0.18</td>
<td>0.83±0.06</td>
<td>1.07±0.09</td>
<td>1.19±0.13</td>
<td>1.14±0.13</td>
<td>1.08±0.13</td>
<td>1.24±0.17</td>
<td>0.4864</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>1.11±0.20</td>
<td>1.01±0.10</td>
<td>1.06±0.10</td>
<td>1.14±0.12</td>
<td>1.14±0.12</td>
<td>1.03±0.19</td>
<td>1.11±0.12</td>
<td>0.9749</td>
</tr>
<tr>
<td></td>
<td>IRS-1</td>
<td>1.05±0.10</td>
<td>0.50±0.06</td>
<td>0.83±0.05</td>
<td>0.73±0.17</td>
<td>0.71±0.09</td>
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<tr>
<td></td>
<td>PEPCK</td>
<td>1.10±0.18</td>
<td>0.52±0.06</td>
<td>0.98±0.11</td>
<td>0.83±0.11</td>
<td>0.97±0.17</td>
<td>1.02±0.12</td>
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<tr>
<td></td>
<td>SREBP1-c</td>
<td>1.20±0.28</td>
<td>2.54±0.21</td>
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<td>2.36±0.16</td>
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<td>1.85±0.17</td>
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<td>0.72±0.05</td>
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<td>0.64±0.06</td>
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Data is presented as Mean ± S.E.M. Different superscripts represent significant differences among the groups in a row at P<0.05.

GBE (*G. biloba* extract), PGE (*P. ginseng* extract), Glucose transporter-4 (GLUT-4), Insulin receptor (IR), insulin receptor substrate-1 (IRS-1), Phosphoenolpyrovate carboxykinase (PEPCK), Sterol regulatory element binding protein-1c (SREBP1-c), Fatty acid synthase (FAS), Peroxisome proliferator-activated receptor-α (PPAR-α), Peroxisome proliferator-activated receptor-γ (PPAR-γ), tumor necrosis factor-α (TNF-α).

Results
Fig 2: mRNA expression of GLUT-4 in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced diabetic rats (n=8). All values are presented as Mean ± S.E.M. b presents comparison between treatment groups and diabetic group. * presents P < 0.05.

Fig 3: mRNA expression of IR in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced diabetic rats (n=8). All values are presented as Mean ± S.E.M.
Results

Fig 4: mRNA expression of IRS-1 in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced diabetic rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic group and non-diabetic group, *b* present comparison between treatment groups and diabetic group. * presents P < 0.05, ** presents P < 0.001.

Fig 5: mRNA expression of PEPCK in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic groups and non-diabetic group, *b* presents comparison between treated groups and diabetic group. * presents P < 0.05.
Fig 6: mRNA expression of SREPB-1c in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic group and non-diabetic group, b presents comparison between treated groups and diabetic group. * presents P < 0.05, ** presents P < 0.001, *** presents P < 0.0001.

Fig 7: mRNA expression of FAS in the liver of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic groups
and non-diabetic group, b presents comparison between treated groups and diabetic group. * presents P < 0.05, ** presents P < 0.001.

Fig 8: mRNA expression of PPAR-α in the liver of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M.

Fig 9: mRNA expression of TNF-α in the liver of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced
rats (n=8). All values are presented as Mean ± S.E.M. \(^a\) presents comparison between diabetic groups and non-diabetic group, \(^b\) presents comparison between treated groups and diabetic group. ** presents \(P < 0.001\), *** presents \(P < 0.0001\).

4.6.2 mRNA expressions of genes in skeletal muscles

The mRNA expressions of GLUT-4, IR, IRS-1, PPAR-\(\alpha\) and TNF-\(\alpha\) were studied in skeletal muscle (Table 4). A significant down-regulation (\(P < 0.05\)) in the mRNA expression of GLUT-4 was found in the muscles in diabetic rats compared to non-diabetic and significant upregulation was recorded in all the treated groups, i.e. GBE (\(P < 0.001\)), PGE (\(P < 0.001\)), mixed 1 (\(P < 0.0001\)), mixed 2 (\(P < 0.0001\)) and mixed 3 (\(P < 0.001\)), after treatment compared to diabetic rats (Fig 10). A significant down-regulation in the mRNA expressions of IR (\(P < 0.0001\)) and IRS-1 (\(P < 0.0001\)) was found in diabetic group compared to non-diabetic rats. We, recorded a significant up-regulation in the mRNA expression of IR in skeletal muscle in mixed 3 group (\(P < 0.001\)) compared to diabetic group (Fig 11), but we measured no significant change in the mRNA expression of IRS-1 in any of the treated groups (Fig 12). A significant downregulation (\(P < 0.05\)) was measured for the gene expression of PPAR-\(\alpha\) in diabetic rats compared to non-diabetic rats and significant up-regulation was found in GBE (\(P < 0.001\)), PGE (\(P < 0.05\)), mixed 1 (\(P < 0.001\)), mixed 2 (\(P < 0.001\)) and mixed 3 (\(P < 0.001\)) treated groups compared to diabetic group (Fig 14). A significant up-regulation (\(P < 0.0001\)) in the mRNA expression of TNF-\(\alpha\) was measured in diabetic rats compared to the non-diabetic group and a significant downregulation was found in mixed 2 (\(P < 0.001\)) and mixed 3 (\(P < 0.0001\)) treated groups compared to diabetic group (Fig 13).
Fig 10: mRNA expression of GLUT-4 in the muscle of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic groups and non-diabetic group, b presents comparison between treated groups and diabetic group. * presents P < 0.05, ** presents P < 0.001, *** presents P < 0.0001.

Fig 11: mRNA expression of IR in the muscle of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic groups and non-diabetic group, b presents comparison between treated groups and diabetic group. *** presents P < 0.0001.
Diabetic

Fig 12: mRNA expression of IRS-1 in the muscle of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic groups and non-diabetic group, *b* presents comparison between treated groups and diabetic group. *** presents P < 0.0001.

Diabetic

Fig 13: mRNA expression of PPAR-α in the muscle of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic groups and non-diabetic group, *b* presents comparison between treated groups and diabetic group. * presents P < 0.05, ** presents P < 0.001.
Fig 14: mRNA expression of TNF-α in the muscle of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. "a" presents comparison between diabetic groups and non-diabetic group, "b" presents comparison between treated groups and diabetic group. ** presents P < 0.001, *** present P < 0.0001.
4.6.3 mRNA expressions of genes in adipose tissues

The mRNA expressions of GLUT-4, IR, IRS-1, FAS, PPAR-γ and TNF-α were done in the adipose tissues (Table 4). No significant change was observed in any of the groups in the mRNA expressions of GLUT-4 (Fig 15) and FAS (Fig 18). For the mRNA expression of IR, our data showed a significant down-regulation (P < 0.05) in diabetic rats compared to non-diabetic rats and a significant up-regulation was recorded in mixed 3 group (P < 0.05) compared to diabetic rats (Fig 16). We measured a significant down-regulation in the mRNA expression of IRS-1 (P < 0.0001) in diabetic rats compared to non-diabetic rats and found a significant upregulation in its expression in PGE (P < 0.001) and mixed 3 (P < 0.0001) treated groups compared to diabetic group (Fig 17).

A significant down-regulation (P < 0.001) was recorded in the mRNA expression of PPAR-γ in diabetic group in comparison with non-diabetic group; however, significant upregulation was recorded for PGE (P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.001) and mixed 3 (P < 0.0001) treated groups (Fig 19). In case of mRNA expression of TNF-α, our results showed significant up-regulation (P < 0.0001) in diabetic rats compared to non-diabetic rats; however a significant down-regulation was recorded after treatment with GBE (P < 0.0001), PGE (P < 0.0001), mixed 1 (P < 0.0001), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) compared to diabetic rats (Fig 20).
Fig 15: mRNA expression of GLUT-4 in the adipose tissue of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic groups and non-diabetic group, b presents comparison between treated groups and diabetic group.

Fig 16: mRNA expression of IR in the adipose tissue of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between
diabetic groups and non-diabetic group, \( b \) presents comparison between treated groups and diabetic group. * presents P < 0.05.

Fig 17: mRNA expression of IRS-1 in the adipose tissue of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxaninduced rats (n=8). All values are presented as Mean ± S.E.M. \( a \) presents comparison between diabetic groups and non-diabetic group, \( b \) presents comparison between treated groups and diabetic group. ** presents P < 0.001, *** present P < 0.0001.
Fig 18: mRNA expression of FAS in the adipose tissue of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic groups and non-diabetic group, *b* presents comparison between treated groups and diabetic group.

![Graph of mRNA expression of FAS](image)

Diabetic

Fig 19: mRNA expression of PPAR-γ in the adipose tissue of non-diabetic, diabetic, *Ginkgo biloba* leaf extract (GBE), *Panax ginseng* root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. *a* presents comparison between diabetic groups and non-diabetic group, *b* presents comparison between treated groups and diabetic group. * presents P < 0.05, ** presents P < 0.001, *** presents P < 0.0001.

![Graph of mRNA expression of PPAR-γ](image)

Diabetic
Fig 20: mRNA expression of TNF-α in the adipose tissue of non-diabetic, diabetic, Ginkgo biloba leaf extract (GBE), Panax ginseng root extract (PGE), mixed 1, mixed 2 and mixed 3 groups of alloxan-induced rats (n=8). All values are presented as Mean ± S.E.M. a presents comparison between diabetic groups and non-diabetic group, b presents comparison between treated groups and diabetic group. *** presents P < 0.0001.
Diabetes is a chronic metabolic syndrome with disturbance of fat, carbohydrate and protein metabolisms (Amin et al. 2011), affecting approximately 3% of the total population globally. Type II diabetes is a complex disease characterized by insulin resistance, pancreatic islet β-cell dysfunction, hyperglycemia, dyslipidemia and inflammation. DM is a global health problem, becoming more prevalent in the coming few decades and it is estimated that six people die every minute due to diabetes worldwide (Wild et al. 2004). According to Xie et al. (2002) currently used pharmacological drugs for the treatment of diabetes have certain limitations and are also involved high risk of secondary failure. Thus due to these risks, natural remedies, as they contain less side effects, are more beneficial and more popular worldwide for diabetic treatment. Herbal medication has been used as therapeutic agents in East-Asian courtiers and account for approximately 80% of the medical treatment in the developing countries. Moreover the use of herbal medicines is also significantly increased in the last few decades in the Western part of the world (Park et al. 2005). Clinical studies suggest that high risk of type II diabetes in human beings is due to inherited insulin resistance. In victims of diabetes the insulin stimulated glucose disposal is markedly reduced (Attele et al. 2002).

Current studies provided the facts that HFD develops insulin resistance (Schrauwen
2007; Zheng et al. 2011) however; alloxan has been well known to cause β-cells necrosis (Brownlee 2001; Ebuehi et al. 2010). Thus, HFD followed by alloxan in rat model was used in the present study to mimic natural metabolic characteristics of type II DM in human. It is well known that alloxan induces diabetes through the reduction of alloxan to dialuric acid by oxidation reduction cycle with the formation of superoxide anion. These negative charged ions undergo dismutation to H$_2$O$_2$. Thereafter, highly reactive hydroxyl radicals can be formed if a divalent metal, like Cu (II) is present by a Fenton like reaction. These ROS may cause selective damage of the pancreatic islet β cells (Etuk 2010).

Recently, there has been increased interest in investigating the pharmacological uses of _G. biloba_ and _P. ginseng_ via biochemical and molecular biological techniques. Both these natural remedies are widely used for the treatment of cancer, neural-treatment, liver functional protective agent, atherosclerosis, allergy, sexual dysfunctioning and diabetes (Jeong et al. 2001; Attele et al. 2002; Choo et al. 2003; Yun et al. 2004; Kim et al. 2005b; Chen et al. 2005; Saw et al. 2006; Lopez et al. 2007; Kang et al. 2007; Boveris et al. 2007; Chan et al. 2007; Varjas et al. 2009; Ni et al. 2010; Amin et al. 2011; Bang et al. 2014). The demand of _G. biloba_ in the international markets is increasing due to its beneficial health effects (Yildiz and Maltas 2012).

_P. ginseng_ root occupies a well-known position in the herbal drugs and is one of the world’s best-selling medicinal plants (Yun 2001).

**5.1 Effect of _G. biloba_ and _P. ginseng_ on Body weight**

A significant reduction in the BW of alloxan-induced diabetic rats was recorded from week 1 to week 14, but suppression was observed in the decrease of BW after treatment with PGE and/or
GBE compared to diabetic rats at the end of the study. Our results for the reduction of BW in diabetic rats are in compliance with previous data (Ebuehi et al. 2010; Cheng et al. 2013a), who reported a significant decrease in diabetic rats and significant reduction in weight loss after treatment with GB leaf extract. The data for the treatment of ginseng are in agreement with Xie et al. (2002), Xiong et al. (2010) and Lee et al. (2012), who reported significant reduction in the BW after treatment with ginsenosides (an active ingredient of ginseng).

Reduction in the BW in diabetic condition might be attributed due to improper glucose/lipid metabolic actions which lead to tissue breakdown and muscle loss (Baynes 1991), degeneration of adipocytes and muscle tissue to retain the energy lost from the body due to bulk conversion of glycogen to glucose and frequent urination (Ene et al. 2007; Ramadan et al. 2009) or to loss of tissue proteins (Swanston-Flatt et al. 1990).

5.2 Effects of G. biloba and/or P. ginseng on carbohydrate metabolism in diabetic rats

Impaired glucose metabolism is the hallmark in diabetic state (Aroson 2008). Diabetic rats have impaired glucose tolerance. Prolonged untreated hyperglycemia may leads to β cells glucotoxicity, increased advanced glycation end products and increased influx through the polyol pathway (Rolo and Palmeira 2005; Ahmad 2005; Alam et al. 2014). Since we measured a blood glucose concentration in the 1st and last week of our study and found a sustain reduction in all the treated groups from week 1 to week 14. We further measured a FSG level at the end of the study and found a significant increase in FSG level in diabetic group and a significant reduction for all five treated groups. Other researchers also showed reduction in glycemia with GBE treatment (Shankar et al. 2005; Zhou et al. 2011; Cheng et al. 2013a; Ren et al. 2013). This blood glucose
reduction may be either an effect of improvement in the plasma insulin concentration due to positive influence of flavonoids on the pancreatic β-cells or it may be due to enhancement in the blood glucose transport to the peripheral tissues (Cheng et al. 2013b). For PGE group our results are compatible with the findings of other researchers (Attele et al. 2002; Xie et al. 2005b; Banz et al. 2007; Lim et al. 2009; Xiong et al. 2010; El-Khayat et al. 2011; Lee et al. 2012; Park et al. 2012; Shang et al. 2013; Jeon et al. 2013; Bang et al. 2014). Since, the β-cells are responsible for the secretion of insulin and thus directly regulate the blood glucose concentration. Insulin resistance usually occurs during diabetes. As the name implies, that in insulin resistance state, there is higher insulin concentration is required for the regulatory control of the blood glucose concentration (Attele et al. 2002; Lim et al. 2009; Jung and Kang 2013). Chronic hyperglycemia reduces the insulin concentration in the blood (Ibrahim et al. 2008); because hyperglycemia causes β-cell dysfunctioning and might also induce β-cell apoptosis in type II diabetic state (Chen et al. 2012). Abnormal elevation of blood glucose level is one of the predominant effects due to dysfunctioning in the action of insulin. Sustained, decreased in hyperglycemia in diabetic state helps to reduce the risk for the development of macro-vascular and micro-vascular complications (Wild et al. 2004).

To further understand the mechanisms that how GBE and PGE act as hypoglycemic agents, we investigated the mRNA expressions of genes involved in the carbohydrate/glucose metabolic activities. In the present study, no change was recorded in mRNA expression of GLUT-4 in the liver and adipose tissues in any of the treated groups, but a significant upregulation was observed in skeletal muscles after treatment in all the groups. This regulates glucose utilization by skeletal
muscle in response to increased levels of insulin in blood (Huang and Czech 2007; Leto and Saltiel 2012). In response to insulin, intracellular vesicles containing glucose transporter proteins translocated on the cell surface and thus increased glucose uptake (Brannmark et al. 2013). The skeletal muscles constitute the main body mass, serve as a main site of cellular mechanism to dispose of an exogenous glucose uptake, stimulated by insulin for the glucose transport. Furthermore, the skeletal muscles serve as storage site for both glucose and glycogen and oxidize to produce energy by various transport mechanisms, like GLUT-4. Glucose transport is a rate limiting step in glucose metabolism and transporter mediates glucose removal from circulation and maintains the proper glucose metabolism (Huang and Czech 2007).

According to our best knowledge, no data is available for mRNA expressions of GLUT4, IR and IRS-1 in rats treated with *G. biloba*, so we will discuss it with the studies of flavonoids. Flavonoids, natural occurring phenolic compound, are widely distributed in many plants and evidences support the fact that it contains various biochemical having therapeutically actions that may affect many functions of body cells (Middleton et al. 2000; Brahmachari 2011). Lee et al. (2010a) found increase in GLUT-4 protein expression in adipose tissues and muscles in mice after treatment with nobiletin flavonoids. Our results are also in agreement with the work of other researchers who worked on the flavonoids (Alam et al. 2014; Dong et al. 2014; Kandasamy and Ashokkumar 2014). Our findings for ginseng in rats are in agreement with (Kim et al. 2009; Lee et al. 2009a; Gao et al. 2013; Jeon et al. 2013). Since the expression of GLUT-4 in the skeletal muscles is regulated by insulin metabolism, thus any dysfunctioning of pancreatic β cells, as in case of diabetes, also impair the expression of GLUT-4 (Zorzano et al. 2005). It is possible that diabetic complication cause impairment of insulin action as reflected from reduction in the mRNA
expression level of GLUT-4 transporter in liver, muscles and adipose tissues without disturbing IR and IRS-1 genes in the body. Evidences support the fact that decrease in glucose uptake in insulin resistance was not necessarily linked with expression of GLUT-4 (Pedersen et al. 1990) and in fact the intrinsic activities of GLUT-4 are involved in this uptake (Konrad et al. 2002). Kim and Kim (2012) found a significant reduction in the hyperglycemic stage after treatment with P. ginseng and reported significant increase in the mRNA expression of GLUT-4 in adipose tissue, whereas they found non-significant change in the expression of GLUT-4 in skeletal muscles. Jung and Kang (2013) studied the efficacy of Korean red ginseng on the muscle glucose uptake in high-fat fed rats and found non-significant difference for the glucose transport rate in the skeletal muscle and GLUT-4 contents in the skeletal muscles. Gao et al. (2013) found that ginsenoside Re shows no significant increase in the expression of GLUT-4, however, they found enhancement in the glucose uptake in a dose dependent fashion and ginsenoside Re facilitated the translocation of GLUT-4 or it may directly enhanced the synthesis of GLUT-4 protein in 3T3-L1 adipocytes cells.

Insulin resistance is a key factor underlying type II diabetes (Wang et al. 2011). In skeletal muscles the insulin resistance is as associated by decreased insulin-stimulated glucose uptake, mainly results from impaired insulin signaling and multiple post-receptor intracellular defects including glucose phosphorylation, reduced glucose oxidation, impaired glucose transport and glycogen synthesis (Abdul-Ghani and Defronzo 2010; Yuan et al. 2011a).

Insulin regulates the glucose uptake by adipose tissue and skeletal muscle via complex cascade signaling pathway. Insulin causes the phosphorylation of IR by activating phosphatidylinositol 3-kinase (PI3K) which stimulates the protein kinase B and in turn enhances
the translocation of GLUT-4 on plasma membrane (Schenk et al. 2008; Lee et al. 2010a). IR is a protein to which insulin must bind to carry out its various biological actions in the cells (Calle et al. 2008). Evidences show an increase in IR number and mRNA level of the IR in the liver, muscles and fats in the diabetic rats (Tozzo and Desbuquois 1992; Secchi et al. 1992; Amessou et al. 1999) that could be compensatory mechanism of the body to cope with the diabetic condition. Impairment in the cascade of insulin signaling specially at IR level reduces the efficacy of insulin at its normal or high level and develops the insulin resistance (Calle et al. 2008). In our study a significant down-regulation in IR gene expression was observed in the skeletal muscles and adipose tissues in diabetic rats.

IRS is one of the vital molecules for the transduction of intracellular insulin signal. Hence, it is able to up-regulate its expression by enhancing the intracellular insulin sensitivity (Zhou et al. 2011). The IRS is a member of the ligand-activated receptor and tyrosine kinase family of transmembrane signaling proteins (Huang and Czech 2007). Although insulin signaling is intracellularly transducted via a complex signaling pathways, IRS-1 is believed to be the first basic cytosolic mediator (Cheatham and Kahn 1995). IRS-1 has a key role for insulin-mediated signal transduction pathway and links the IR to its final biological actions via a series of intermediate effectors. Saad et al. (1992) said that variable responses for IRS-1 regulation in liver and muscle were found in diabetic animals, which might create various modifications in insulin signaling and contribute to insulin resistance for liver and muscle. According to Friedman et al. (1999) and Carvalho et al. (2001), expression of IRS-1 decreased in diabetes, furthermore the reduction in the expression of IRS-1 may also be linked with age and impaired insulin sensitivity. Hepatic insulin resistance is the chief component responsible for the development of type II DM. In liver insulin
resistance is associated with down-regulation in the mRNA expressions of IRS-1 and IRS-2 (Taniguchi et al. 2005). As in our study, we also noticed a significant down-regulation for IRS-1 in diabetic state for all the studied tissues (i.e., liver, skeletal muscle and adipose tissue). Interested results after treatment for both IR and IRS-1 were reported in our study. We found no significant change in the mRNA expression of IR for hepatic tissues in any of the treated group; however in case of skeletal muscles and adipose tissues, a significant up-regulation was observed in mixed 3 group and no other treated group showed any significant change. A significant up-regulation in gene expression of IRS-1 in hepatic tissue was observed in GBE and mixed 3 group, and a significant up-regulation in IRS-1 in adipose tissue was found for PGE group and mixed 3 group, whereas in case of skeletal muscles no significant was recorded in any of the treated groups. Zhou et al. (2011) found a significant up-regulation in the expression of IRS-2 in hepatic cells after treatment with ginkgo but they did not measure the expression for IRS-1. Brahmachari (2011) reported that the flavonoids may mimic the insulin action, insulin secretary effect, stimulating peripheral tissues glucose uptake and regulates the expressions and activities of enzymes involved in carbohydrate metabolism. The results for the GBE in case of IR are in line with previous results of other demonstrating the effects of flavonoids (Wu et al. 2013; Herrera et al. 2013). The results for IRS-1 are in agreement with (Zheng et al. 2011; Herrera et al. 2013; Vareda et al. 2014; Kandasamy and Ashokkumar 2014). Lim et al. (2009) reported that ginsenoside Rg3, one of the active ingredients of P. ginseng, enhances phosphorylated IRS-1 level and also increases the total IRS-1 protein level. They further reported in the same study that ginsam, component of P. ginseng produced by vinegar extract,
significantly reduces the blood glucose level and increases the expression of IRS-1 in the muscles of Otsuka Long-Evans Tokushima fatty rats.

Lee et al. (2012) conducted a study to determine the anti-diabetic properties of Korean red ginseng and determined that red ginseng up-regulates the translocation of GLUT-4 and expression of IRS-1 in Sprague-Dawley (SD) rats. Gao et al. (2013) found up-regulation in the expression of IRS-1 after treatment with ginsenoside Re in 3T3-L1 adipocytes cells.

There are evidenced that suggest the fact that in diabetes the basic cause of enhancement in gluconeogenesis is due to incapability of insulin to inhibit hepatic insulin glucose synthesis and the ability of insulin to regulate transcription of rate controlling gluconeogenic enzymes. Dysfunctioning of PEPCK also plays a vital role in this problem (Law et al. 2002). Despite of increase in blood insulin concentration, many researchers found increased in PEPCK expression in diabetic animal models (Friedman et al. 1997; Valera et al. 1999; Law et al. 2002). However we found a significant down-regulation in PEPCK level in diabetic state and significant upregulation in all treated groups except PGE group. Flavonoids are present in many vegetables, fruits, berries, tea, coffee and red grape wine (Hanhineva et al. 2010; Alam et al. 2014). Law et al. (2002) found a decrease in the expression of PEPCK after treatment with flavonoids present in green tea. Other researchers also found down-regulation for PEPCK after treatment with different flavonoids (Jung et al. 2006; Collins et al. 2007; Cederroth et al. 2008; Herrera et al. 2013). Li et al. (2012) found a significant down-regulation in PEPCK expression after treatment with ginsenosides. We are unable to understand reason for down-regulation of mRNA expressions of PEPCK.

Thus regulation in mRNA expressions of GLUT-4 (in mixed group 3 in liver and in all treated groups in muscle), IR (in mixed group 3 in muscle and adipose tissue) and IRS-1 (in GBE
and mixed 3 in liver; PGE and mixed 3 in adipose tissue) in our study indicated that *G. biloba* and/or *P. ginseng* have the ability to enhance insulin sensitivity and also enhance the ability of the cell to increases glucose uptake.

### 5.3 Effect of *G. biloba* and/or *P. ginseng* on lipid metabolism in diabetic rats

Dyslipidemia and hypertriglyceridemia are the important primary factors for cardiovascular disorders in the diabetic state. Diabetic patients have dysfunctioning in packaging cholesterol and have higher serum TG level (Cho et al. 2006). It is well documented fact that dyslipidemia (high TG and LDL-C and Low HDL-C) is the main cause of coronary arterial disorder and also cause the development of atherosclerosis lesions which contain lipid foam cells and also scavenger receptors which can recognize different modified LDL species, like malondialdehyde-LDL (Witztum and Steinberg 1991; Witzum 1994; Benzi and Morretti 1995). Metabolic disorders due to diabetes are characterized by non-alcoholic fatty liver, insulin resistance and dislipidemia (Semenkovich 2006).

Lipase activities in insulin resistance results to enhance lipolysis and free fatty acid concentration in the plasma that is one of the leading causes of hypercholesterolemia and hypertriglyceridemia (Bland 1995; Grammer 2000). Excess of fatty acids are metabolized to acetyl CoA, which is used by liver for the cholesterol synthesis and thus elevated the level of cholesterol in diabetes (Nakazawa et al. 1996; Hallliwell and Gutteridge 1999; Grammer 2000).

DM is associated with prominent alteration of plasma lipid and lipoprotein profile (Zheng et al. 2011). As in our study, we also reported a significant increase in TC, VLDL-C, LDL-C and a significant decrease in HDL-C in diabetic rats. Ebuehi et al. (2010) found a significant increase in TC and TG concentration in alloxan induced diabetic rats. The data in our study demonstrated
that both theses natural remedies improved the dyslipidemia and hypertriglyceridemia especially for all the three mixed treated groups. Hypertriglyceridemia is one of the important markers of insulin resistance (Schwartz 2006). The reduction in the TG level is the indication to enhance energy expenditure of the whole body, thus it is important to reduce hypertriglyceridemia in diabetic state (Defronzo and Farrannini 1991; Liu et al. 2013). The ability of *G. biloba* to reduce the concentration of MDA would, therefore, be helpful to reduce the dyslipidemia condition and thence these diseases (Kudolo et al. 2005). Kudolo (2001) found non-significant reduction in TC, TG and LDL-cholesterol; however they reported no change in the values of HDL-cholesterol after and before treatment with ginkgo in the NIDDM patients.

Attele et al (2002), Yoon et al (2003), Cho et al (2006), Banz et al. (2007), Lee et al. (2009a), Xiong et al. (2010) and Shang et al. (2013) found significant reduction for TC; Yoon et al (2003), Banz et al (2007), Lee et al. (2009a), Gu et al. (2013), Chan et al (2013), Jung and Kang (2013) and Shang et al. (2013) reported significant reduction for TG after treatment with ginseng. Lim et al. (2009) reported that ginsam, component of *P. ginseng* produced by vinegar extract, significantly reduces TC, LDL-C and HDL-C concentrations. Lim et al. (2009), Lee et al (2009a) and Shang et al. (2013) reported significant reduction for LDL-C; Yoon et al (2003), Lim et al (2009) and Shang et al. (2013) found significant increase for HDL-C after treatment with ginseng. Bang et al. (2014) found no significant changes for TC, TG, HDL-C and LDL-C levels after treatment with Korean red ginseng in type II diabetic patients. Reduction in plasma total cholesterol levels by the extracts may have an important clinical significance, since hyperlipidemia is often associated with type II diabetic patients.
We studied the genes involved in the lipid metabolism to better understand how GBE and PGE play a role to correct the dyslipidemia and hypertriglyceridemia in diabetic state. Liver plays a major role in the metabolic activities of the body; serves as a main storage releasing site for carbohydrate and fatty acid synthesis (Quan et al. 2012). SREBP-1c plays a pivotal role lipogenic transcriptional factor and lipolytic genes that is regulated by insulin and glucose (Kersten 2001; Horton et al. 2002; Goldstain and brown 2008; Yuan et al. 2011b). Lipid homeostasis is regulated by SREBPs and directly regulates the expression of more than 30 genes involved in the synthesis and uptake of the TC, TG, phospholipids and FAs (Horton et al. 2002). We reported significant change in the expression of SREBP-1c only in mixed 2 and mixed 3 treated group and the results for both ginkgo (Zhou et al. 2011) and for ginseng contradictory results were reported by different researchers Lee et al. (2010b) found significant reduction in the expression of SREBP-1c, however, Yuan et al (2008); Quan et al. (2012) found up-regulation in its expression after treatment with ginseng. SREBP-1c enhances the transcription of gene required for fatty acids and cholesterol synthesis (Horton et al. 2002).

It is believed that FAS catalyzes the biosynthesis of FA particularly in the liver (Menendez et al. 2009). The gene expression of FAS is predominantly regulated by nutritional and hormonal signals (Sul and Wang 1998; Menendez et al. 2009). Diabetes suppressed the expression of glycolytic and lipogenic gene (e.g. FAS and L-PK) however, enhances the expression of gluconeogenic enzyme PEPCK (Wang et al. 2006). Beside up-regulating the expression of FAS transcription in the adipocytes, insulin also enhances human FAS gene expression and FAS enzymatic activities. However, FAS is markedly down-regulated under metabolic complications, like insulin resistance and diabetes in liver (Real et al. 2010), as also reflected by our data. A
significant up-regulation was reported only for mixed 2 and mixed 3 treated groups in hepatic tissue. Flavonoids down-regulates the mRNA expression of FAS (Goldwasser et al. 2010; Wu et al. 2011). These findings for ginseng are in line with other researchers (Bluher et al. 2002; Bluher et al. 2004; Banz et al. 2007; Real et al. 2010). However, Yuan et al. (2008) and Gu et al. (2013) found down regulation in the expression of FAS after treatment with ginseng.

According to Berndt et al. (2007), excess energy intake induce elevated TG, free fatty acid and hyper-insulinemia may have increased or decreased circulatory adipokines, leading to up-regulation in the expression of FAS and FAS activity, resulting in increased lipogenesis.

PPAR-α promotes the targeted genes involved in metabolic pathways of lipids and lipoproteins, through binding with PPAR response elements (PPREs). PPAR-α is involved in the lowering effect of TG via transcriptional activation of apolipoprotein (apo) CIII, lipoprotein lipase and fatty acid β-oxidizing enzymes in human. PPAR-α is also well characterized to increase the circulating amount of HDL level through inducing the gene expression of apo AI and AII. Thus dysfunctioning of the PPAR-α results in the abnormalities in TC and TG metabolism because of impaired FA and lipoprotein metabolisms (Vu-Dac et al. 1995; Yoon et al. 2003). However, in rats and mice PPAR-α induces a pronounced reduction in the expression of apo A-1, one of the major HDL apolipoproteins and thence, also decreased in apo A-1 (Peters et al. 1997; Lee et al. 2006).

According to Asayama et al. (1999), enhancement in the FA supply to the liver in diabetes, stimulates the mRNA expression level of PPAR-α and thus also enhances the function of targeted genes involved in the metabolic activities of fatty acids. PPAR-α improves the lipid profile and also insulin sensitivity by enhancing the β-oxidation of FA (Li and Glass 2004; Banz et al. 2007),
thus dysfunctioning of PPAR-α results the metabolic abnormalities in TC and TG by causing impairment in fatty acid and lipoprotein metabolism (Yoon et al. 2003).

PPAR-γ are widely distributed in the adipocytes; regulate the expression of key genes involved in the glucose and lipids metabolism, also play an important role in the induction of adipocyte differentiation (Spiegelman 1998; Desvergne and Wahli 1999; Lee et al. 2010a), improve insulin sensitivity, reduce the circulatory level of fatty acids (Lalloger and Staels 2010; Kahn et al. 2010) and stimulate the production of small insulin signaling pathway by inducing the expression of genes involved with the insulin signaling cascade (Benz et al. 2007). Two major isoform of PPAR-γ are: PPAR-γ1 expressed in several tissues and PPAR-γ2 expressed in adipose tissues (Kota et al. 2005).

The present study proved that *G. biloba* and *P. ginseng* traditionally used anti-diabetic herbs; have an effective role to improve lipid metabolism through up-regulation of PPAR-α in skeletal muscle and PPAR-γ in adipose tissues. In case of PPAR-α, our data also showed agreement with other researchers (Cederroth et al. 2008; Wu et al. 2013). However, Fan et al. (2014) found down-regulation both for the expression of PPAR-α and PPAR-γ after treatment with flavonoids present in okra.

According to Zhou et al. (2011), PPAR-γ may regulate insulin sensitivity and may also be involved in insulin signaling pathway. They found significant up-regulation in the expression of PPAR-γ after treatment with ginkgo leaf extract. Flavonoids, present in many plants, including ginkgo have beneficial health effects and reduce the risk of metabolic syndrome (Sabu et al. 2002; Jung et al. 2004). Numerous flavonoids act as ligands of PPAR-γ and activate its expression (Liang
et al. 2001; Zheng et al 2011; Yoshida et al. 2013). Lee et al. (2010a) reported significant reduction in the mRNA gene expression of PPAR-γ in adipose tissues after treatment with nobiletin, a flavonoids present in many citrus fruits.

Park et al (2005) and Jeon et al. (2013) reported non-significant change for PPAR-α expression in the liver after treatment with ginseng. Yoon et al (2003) and Banz et al. (2007) found significant decrease for the expression of PPAR-α in adipose tissue and muscle after treatment with ginseng.

Banz et al. (2007) and Chan et al. (2013) after treatment with ginseng reported significant enhancement in the expression of PPAR-γ in adipose tissue and liver; whereas, reduction in the expression of PPAR-γ in muscle. Park et al (2005) found significant up regulation in mRNA expression of PPAR-γ in adipose tissues. PPAR-γ has a role in insulin signaling pathway and helps to regulate the insulin sensitivity, thus increased in expression of PPAR-γ also enhances the sensitivity of the cells to insulin (Zhou et al. 2011).

According to Edvardsson et al. (1999), the activation of PPAR-α causes Peroxisome proliferation and leading to the enhancement of β-oxidation of the fatty acids. However the activation of PPAR-γ leads to adipocytes differentiation and also improves insulin signaling to the mature adipocytes. It is believed that in the hyperlipidemic state of diabetes both PPARs are the functional target for the treatment. In these lines, our data suggests that GBE and/or PGE could more efficiently contribute to regulate the lipid homeostasis.
5.4 Effect of *G. biloba* and/or *P. ginseng* on oxidative stress and TNF-α gene expression in diabetic rats

Hyperglycemia, one of the major contributing factors to increased ROS production in diabetes, plays an important role in the lipid peroxidation, protein oxidation; causes severe tissue damage and consequently pathogenesis of vascular diseases, the main cause of morbidity and mortality in diabetic state (Ceriello 2006; Giacco and Brownlee 2010; Son 2012; Cheng et al. 2013a). Previously, it was studied in various experimentally induced diabetic animal models that the oxidative stress in diabetes caused persistent and chronic hyperglycemia, therefore, depleting activities of antioxidant defense system and, if remain untreated, could promote free radicals generation, leading to severe complications (Bohar et al. 2004; Jung et al. 2005).

Reduction in the anti-oxidative enzymatic activities is due to depletion of oxygen free radicals, glycosylation of enzymes or it may also be due to hyperglycaemic condition which produced structural and functional changes in anti-oxidant enzymes and thus reduces its activation level. Hyperglycemia also causes inefficiency of glycosylation for anti-oxidant enzymes (Lalla et al. 2000).

Our data showed a significant reduction in CAT and a significant increase in MDA were recorded in diabetic state. Evidences support the fact that alloxan generates the free radicals and destroyed the pancreatic β-cells. Although CAT and MDA have an important role to neutralize the toxicity of active oxygen, however, reduction in anti-oxidant enzymes has been observed in diabetes (West 2000). We found significant enhancement in CAT and a significant reduction in the level of MDA activities in all the five treated groups. The anti-oxidant properties of *G. biloba*
may be attributed to the flavonoids, important active ingredient heavily present in ginkgo (Pincemail et al. 1989; Pietta et al. 2000; Maclennan et al. 2002; Smith and Luo 2003; Maltas et al. 2011; Yildiz and Maltas 2012). Okezie et al. (2007) reported that flavonoid, considered among the most powerful anti-oxidants existing in nature, may effectively ameliorate diabetes and its complications. Ginsenoside, an active ingredient of ginseng, is a strong anti-oxidant activity; having radical scavenging activities by stimulating mRNA expression of anti-oxidative enzymes and also enhancing activities of anti-oxidant enzymes which are necessary to maintain the cell viability by reducing the generation of oxygen radicals from intracellular metabolism (Kim et al. 1996; Sur et al. 2001; Jung et al. 2005; Xie et al. 2009; Lee et al. 2009b; El-Khayat et al. 2011; Amin et al. 2011; Liu et al. 2013). In addition, saponins, another active ingredient, also contain powerful anti-oxidant activities (Kim et al. 2003; Salih 2012). According to Xie et al (2004), the anti-oxidant activities of ginseng contain a defensive role to protect the pancreatic β cells and also protect other tissues from oxidative stress due to hyperglycemia.

CAT plays an important role to protect the body against damage of active oxygen stress by converting the endogenous hydrogen peroxide (H$_2$O$_2$) into water (Kim et al. 2011; Kodydkova et al. 2014). In mammals, CAT belongs to a large mono-fuctional, haem-containing group, almost all aerobically respiated organisms contain the member of this sub-group (Chelikani et al. 2004; Kodydkova et al. 2014). CAT is an intracellular, tetrameric proteinous enzyme (Kodydkova et al. 2014), commonly found in mammalian erythrocytes, liver and occasionally in kidney (Deisseroh and Dounce 1970). In the liver, CAT is predominantly found in peroxisomes (Quan et al. 1986).

Ren et al. (2013) and Cheng et al. (2013a) also reported a significant reduction in serum MDA, however they found significant enhancement in other anti-oxidant enzymes after treatment.
with ginkgo leave extract. Flavonoids in ginkgo known to be efficient scavenging properties thus react very strongly with superoxide anion and hydroxyl radicals and also suppress the lipid peroxidation (Shankar et al. 2005). DeFeudis and Drieu (2000) reported that flavonoids fraction have the ability to neutralize the free radicals, which may be achieved by direct attenuation of ROS, chelation of pro-oxidant transitional metal ions, expression of anti-oxidant metabolites, such as glutathione. Therefore, the flavonoids react preferentially with hydroxyl radicals and directly scavenge them.

Amin et al. (2011) also reported a significant increase in CAT and significant decrease in MDA level after treatment with ginseng. El-Khayat et al. (2011) investigated that ginseng resulted a significant reduction of the elevated blood glucose concentration and serum creatinine concentrations; ginseng also decreased MDA but increased GSH, glutathione S-transferase GST and SOD in diabetic rats. Kim et al. (2011) found a significant enhancement for SOD, GPX, GSH and CAT in diabetic rats and after treatment with fermented ginseng extract. They found significant reduction in the activities of all these anti-oxidant enzymes.

Evidence suggests that, in diabetes, a complex alteration in the activities of anti-oxidant enzymes as well as the oxidative stress marker MDA occur (Scott and King 2004). The MDA, formed during arachidonic acid oxygenation, is a very strong reactive metabolic product formed from free radicals induced lipid peroxides and concentration of MDA is usually used to measure the index of the lipid peroxides in the body (Kudolo et al. 2005). MDA, the final product of lipid break down, is an important marker of oxidative stress in the body (Ren et al. 2013) and is one of the important indicators used for the free radicals induced lipid peroxidation, plays an important role in pancreatic damage during diabetes (Okur et al. 1995; Ilhan et al. 2001; Bicakci et al. 2005).
Cho et al. (2006) showed non-significant variations both for MDA and glutathione after treatment with Re ginsenosides. The significant increase in MDA in our study indicates the generation of free radicals, which enhances the cellular damage (El-Khayat et al. 2011).

TNF-α has been proposed a link between adiposity and the development of insulin resistance. Further studies revealed that the concentration of plasma TNF-α could be linked with abdominal adiposity. TNF-α is an important contributor to systemic insulin resistance by impeding insulin’s actions in liver and skeletal muscle (Hotamisligil and Erbay 2008). Other studies demonstrated the contribution of TNF-α for inhibiting insulin for the glucose uptake in the cell culture of adipocytes (Hotamisligil et al. 1994) or induction of insulin resistance in the rodents (Lang et al. 1992).

Recent data showed that oxidative stress and inflammation play major roles for the onset and development of chronic diseases (Camps and Garcia-Heredia 2014). Excess adiposity stimulates recruitment of macrophages and secretes large amounts of TNF-α. The resulting chronic inflammatory state causes adipocyte lipolysis, release of free fatty acids, exacerbating insulin resistance, metabolic risk factors and finally diabetes (Slawik and Vidal-Puig 2006; Babish et al. 2010). Down-regulation in the expression of TNF-α was found in all the three tissues after treatment with GBE, PGE and all the three mixed groups, which shows effective therapeutic effects of these two herbs on diabetes. According to our best knowledge, there is no data for mRNA expression of TNF-α treated with G. biloba, so we compared the oxidative stress with flavonoids and the results are compatible with other researchers (Lee et al. 2010a; Okada et al. 2010; Yoshida et al. 2013). Flavonoids are the common component, usually present in different plants and fruits including ginkgo, and has been reported to have a pivotal role to improve hyperglycemia (Hsu et
al. 2003; Jung et al. 2004), IR function (Shisheva and Sherchter 1992), insulin like properties (Choi et al. 1991), anti-oxidant and lipid lowering properties (Choi et al. 2001). In case of ginseng, our findings are in line with previous results demonstrating the effects of ginseng on mRNA expression of TNF-α (Shang et al. 2013; Gao et al. 2013; Gu et al. 2013; Jeon et al. 2013; Chan et al. 2013). According to our results, we propose that GBE and/or PGE interact with alloxan and blunt its oxidative potential. This significantly decreases the pancreatic β-cell damage and hence is able to slow down the process of development of diabetes when given in combination with alloxan.

5.5 Effect of G. biloba and/or P. ginseng on LFT and serum creatinine in diabetic rats

There is strong evidence to support the facts that liver enzymes, AST and ALT, are linked with increased risk of diabetes and considered as an important liver marker to identify liver diseases (Kunutsor et al. 2013). Both these liver enzymes are found in serum, liver and in various tissues (Kim et al. 2005c) and become elevated in any liver disease (Ruhl and Everhart 2009). In the present study, PGE showed significant reduction both for AST and ALT, but ginkgo reduced ALT and had no effect on AST. In case of mixed groups, only mixed 2 and mixed 3 showed significant reductions in the dose dependent manner in the level of AST, however mixed 3 group showed a significant reduction in ALT. Kudolo (2001) conducted the clinical trial on NIDDM patients. They gave the treatment of 120mg/kg supplementation of ginkgo for 3 consecutive months and found no change in AST and ALT levels after and before treatment. Our results for ginseng are not in agreement with other researchers (Xiong et al. 2010; Kim et al. 2011; Jeon et al. 2013). However, Lim et al. (2009) studied the liver enzyme activities, and reported lowering level of ALT and AST in ginseng treated rats. Vuksan et al. (2008) conducted a clinical trial to evaluate
the effects of *P. ginseng* on diabetic patients and reported significant reduction in AST and non-
significant change for ALT, creatinine and body weight were observed by them.

An elevated ALT has strong correlation with accumulated liver fat (Ander et al. 2005; Westerbacka et al. 2009) and also an important liver marker of nonalcoholic fatty liver disease
(Marchesini et al. 2001) and is an important pathophysiological mechanism for development of
diabetic complications (Sattar et al. 2004). Elevated liver fat has strong correlation with hepatic
insulin resistance, causes increase in liver glucose output and finally development of diabetes
(Kunutsor et al. 2013). It is difficult to explain the mechanism of AST associated with diabetes;
however AST also has a strong association with liver fat accumulation (Kunutsor et al. 2013).

Creatinine is a waste product of the body which must be excreted via kidneys through
glomerular filtration. Increased in blood creatinine level indicates impaired renal function (Salih
2012). In case of diabetes, the serum creatinine level increases showing the nephropathy as we
also observed. In the present study, a significant decrease in serum creatinine level was found only
in the PGE, mixed 1 and mixed 3 treated groups. These findings are in agreement with other
researchers (Yokozawa et al. 1999; Sabu et al. 2002; Renno et al. 2008; Kang et al. 2008; Elkhayat
et al. 2011; Amin et al. 2011). The creatinine reducing effect of ginkgo and ginseng might be due
to the flavonoids and ginsenosides respectively. The stability of creatinine level after treatment
indicates the protective effects against alloxan toxicity of this natural remedy. Lee et al. (2009a)
found that ginseng also has effect on liver functions. They found increased ALT but significantly
decreased AST and creatinine.
Handful of data is available to reveal that in traditional medical systems, diabetes in rats that is similar to type II diabetes of humans was successfully treated with plant extracts (Ackerknecht 1982). For instance the leaf extract of *G. biloba* (Kudolo 2000, 2001; Zhou et al. 2011) and root extract of *P. ginseng* (Sotaniemi et al. 1995; Vuksan et al. 2000; Attele et al. 2002; Vuksan et al, 2008; Reeds et al. 2011) have also been used as a tonic without any evidences of toxicity (Attele et al. 1999). In vitro and in vivo studies, conducted on human and animals, support the claim that the leaf extract of ginkgo and root extract of ginseng possess antihyperglycemic, anti-hypertriglyceridemia and anti-hyperlipidemia activities. Detailed human studies will be helpful to evaluate the anti-diabetic activities of leaf extract of *G. biloba* and root extract of *P. ginseng* both at biochemical and molecular level.

**Conclusion:**

• GBE contains anti-hyperglycemic (up-regulating the gene expressions of IRS-1 in liver and GLUT-4 in muscle), anti-hypertriglyceridemic (up-regulating the gene expression of PPAR-α in muscle) and anti-oxidative (down-regulation the gene expression of TNF-α in liver and adipose tissue) properties in alloxan-induced diabetic rats after 14 weeks of treatment.

• PGE has strong anti-hyperglycemic (up-regulating the gene expressions of GLUT-4 in muscle and IRS-1 in adipose tissue), anti-hypertriglyceridemic (up-regulating the gene expressions of PPAR-α in muscle and PPAR-γ in adipose tissue) and anti-oxidative (downregulating the gene expression of TNF-α in liver and adipose tissue) effects in alloxan-induced diabetic rats after 14 weeks of treatment.

• When both these natural remedies were given in combination, synergistic effects were recorded in a dose dependent manner. The results revealed that mixed group with
150:450mg/kg/day of GBE and PGE showed more effective anti-hyperglycemic (up-regulating the gene expressions of GLUT-4 and IRS-1 in liver; GLUT-4 and IR in muscle; and IR and IRS1 in adipose tissue), anti-hypercholesterolemia (up-regulating the gene expression of FAS in liver), anti-hypertriglyceridemic (up-regulating the gene expressions of SREBP-1c in liver; PPAR-α in muscle and PPAR-γ in adipose tissue) and anti-oxidative (down-regulating the gene expression of TNF-α in liver, muscle and adipose tissue) effects in alloxan-induced diabetic rats after 14 weeks treatment.

• Thus GBE and/or PGE showed anti-diabetic activities by improving the metabolic activities of carbohydrate (glucose uptake by cells) and lipids (hypo-cholesterolemic, hypotriglyceridemic) and also helped the body to recover from the oxidative stress. The anti-diabetic properties of these two natural remedies were found to be greatly enhanced in the mixed group 3 (150mg/kg/day of GBE and 450mg/kg/day of PGE).
CHAPTER 6
SUMMARY

Diabetes is a major public health issue. As conventional pharmaceutical agents have greater incidences of adverse effects so the interest in the natural remedies has increased greatly in the last few decades. *Ginkgo biloba* leaf extract (GBE) and *Panax ginseng* root extract (PGE) are ancient Chinese herbal drugs that have prominent position in the list of the best-selling natural remedies and are increasingly being used for the treatment of diabetes. The anti-diabetic effect of GBE is attributed to flavonoides while that of PGE is attributed to ginsenosides. In this study, GBE and PGE in combination showed significantly higher anti-diabetic effects than individual extracts in diabetic rats.

Adult Wistar rats were allowed to feed on a high fat diet (HFD: 12.7% maize starch, 6.5% dextrose, 3.9% sunflower oil, 31.3% beef tallow and 28.6% casein by weight) for two weeks. The rats were divided into seven groups (08 rats in each group): Non-diabetic control group, Diabetic group, Diabetic + 100 mg/kg *G. biloba* leaf extract treated group (GBE), Diabetic + 300 mg/kg, *P. ginseng* root extract treated group (PGE), mixed 1 group : Diabetic + combination of both GBE and PGE at dose of 200 mg/kg/day (50mg/kg/day of GBE and 150mg/kg/day of PGE), mixed 2 group : Diabetic + combination of both GBE and PGE at dose of 400mg/kg/day (100mg/kg/day of GBE and 300mg/kg/day of PGE), mixed 3 group : Diabetic + combination of both GBE and PGE at dose of 600mg/kg/day (150mg/kg/day of GBE and 450mg/kg/day of PGE). At the end of
the 14th day, the rats were kept in fasting condition overnight and then a single intra-peritoneal injection of alloxan monohydrate (Sigma, USA) dissolved in 0.5 ml of saline solution at a dose of 120-130 mg/Kg body weight was injected in all rats except for the non-diabetic group which were injected with an equal volume of normal saline. Body weight (BW) and blood glucose were measured at week 1 and week 14. At the end of the experimental period, blood samples in fasting/basal state were collected from heart puncture for the biochemical parameters. Liver, muscles and adipose tissue were also collected for mRNA expression of genes involved in carbohydrate and fat metabolism.

Results were expressed as Means ± S.E.M. Statistical analyses was performed using Statview software (SAS Institute Inc., SAS Campus Drive, Cary, NC, USA). Two-ways repeated measure ANOVA followed by PLSD Fisher's test was performed for BW and blood glucose to assess the effects of time and herbal drugs. For the rest of the parameters, one-way ANOVA followed by PLSD Fisher's test was performed to assess the effect of herbal drugs. Differences were considered significant at P < 0.05.

A significant (P < 0.0001) reduction in the BW of the diabetic group was recorded compared to non-diabetic rats and a significant reduction in BW was observed after treatment in all the five treated groups compared to diabetic group. Glycemia was significantly higher in the diabetic rats (P < 0.0001) compared to non-diabetic rats and a significant reduction in the blood glucose level was recorded in all the five treated groups (P < 0.0001) group in comparison to the diabetic group. A significant reduction for fasting serum glucose (FSG) (P < 0.0001) was recorded for all the five treated groups compared to the non-treated diabetic rats. We linked the reduction in
hyperglycemia to the mRNA expression of genes involved in glucose metabolism. In particular, we studied the gene expressions of GLUT-4, insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and phosphoenolpyruvate carboxykinase (PEPCK) in liver, muscle and adipose tissue. A significant up-regulation for the mRNA expression of GLUT-4 was observed only in muscle in all the five treated groups, i.e. GBE (P < 0.001), PGE (P < 0.001), mixed 1 (P < 0.0001), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001). We found a significant downregulation in the mRNA expression of IR in muscle (P < 0.0001) and adipose tissue (P < 0.05) in the diabetic group compared to non-diabetic rats, however, a significant up-regulation was found in mixed 3 group in muscle (P < 0.001) and adipose tissue (P < 0.05). We found a significant down-regulation (P < 0.001) for IRS-1 in liver in diabetic state and a significant up-regulation was recorded in GBE (P < 0.05) and mixed 3 (P < 0.05) groups only. We found a significant down-regulation of IRS-1 in muscle (P < 0.0001) and adipose tissues (P < 0.0001) in the diabetic group. None of the treated group showed significant results in muscles however, a significant upregulation was found only in PGE (P < 0.001) and in the mixed 3 group (P < 0.0001) in adipose tissue. A significant up-regulation was recorded for PEPCK in GBE (P < 0.05), mixed 1 (P < 0.05), mixed 2 (P < 0.05) and mixed 3 (P < 0.05) groups in liver.

A significant increase of blood cholesterol was found in rats in the diabetic state (P < 0.0001) and a significant reduction was found only in the mixed 3 (P < 0.001) treated group. A significant decrease was found for VLDL-C in mixed 1 (P < 0.05), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) groups. A significant decreased was observed for LDL-C in mixed 1, mixed 2 and mixed 3 (P < 0.0001) groups which previously found to be enhanced in diabetic condition. In case of
HDL-c a significant decreased was found for GBE (P < 0.001), PGE (P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.0001) and mixed 3 (P < 0.0001) which was previously found to be increased in the diabetic group (P < 0.0001). Conversely, a significant increase was seen for TG (P < 0.0001) in the diabetic state and a significant reduction was found in all the five treated groups (P < 0.0001).

We further studied genes involved in lipid metabolism. A significant up-regulation was found for SREBP-1c in diabetic group (P < 0.0001) and a significant down-regulation was found to occur in mixed 2 (P < 0.05) and mixed 3 (P < 0.001) treated groups compared to untreated diabetic rats. In the liver, a significant up-regulation in the mRNA expression of FAS was found only in mixed 2 (P < 0.05) and mixed 3 (P < 0.05) treated groups which found to be down regulated in the untreated diabetic group (P < 0.001). A significant down-regulation in the mRNA expression of PPAR-α was found in diabetic rats skeletal muscle (P < 0.05), however, a significant up-regulation was found in GBE (P < 0.001), PGE (P < 0.05) mixed 1 (P < 0.001), mixed 2 (P < 0.001) and mixed 3 (P < 0.001) treatment groups in comparison to diabetic rats. We studied PPAR-γ in adipose tissue and found a significant up-regulation in PGE (P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.001) and mixed 3 (P < 0.0001) groups which had previously been found to be down regulated (P < 0.001) in diabetic rats compared to non-diabetic rats.

We found that the body of the diabetic rats suffer with oxidative stress and measured a significant decrease for CAT (P < 0.0001) in diabetic group and significant increase was found in GBE (P < 0.05), PGE (P < 0.05), mixed 1 (P < 0.05), mixed 2 (P < 0.05), mixed 3 (P < 0.05) groups compared to diabetic rats. Whereas, a significant decreased was recorded for MDA in GBE (P < 0.05), PGE
(P < 0.05), mixed 1 (P < 0.001), mixed 2 (P < 0.001) and mixed 3 (P < 0.0001) groups, which previously showed a significant increased (P < 0.001) in diabetic group compared to non-diabetic. We linked oxidative stress with TNF-α and found a significant upregulation (P < 0.0001) for all the three studied organs in diabetic groups compared to the nondiabetic group. In case of liver a significant down-regulation was found for GBE (P < 0.0001), PGE (P < 0.0001) and mixed 3 (P < 0.0001) groups compared to untreated diabetic rats. A significant down-regulation in the expression of TNF-α in muscle was recorded only in the mixed 2 (P < 0.001) and mixed 3 (P < 0.0001) groups compared to diabetic rats. However, a significant down-regulation in the expression of TNF-α in adipose tissue was observed for all the treated groups (P < 0.0001 for all groups) in comparison to the diabetic group.

For serum creatinine a significant enhancement was observed for PGE (P < 0.05), mixed 1 (P < 0.05) and mixed 3 (P < 0.05) groups which were previously found to be reduced in diabetic rats. A significant increase for AST was found in diabetes (P < 0.0001) compared to non-diabetic rats, while a significant reduction was found to occur only for PGE (P < 0.05), mixed 2 (P < 0.05) and mixed 3 (P < 0.001) treated groups in comparison to the untreated diabetic group. Like AST a significant reduction was recorded for ALT in the diabetic group (P < 0.001) and only GBE (P < 0.001), PGE (P < 0.05) and mixed 3 (P < 0.05) showed a significant decreased in ALT level compared to untreated diabetic rats.

In conclusion, we found that both GBE and PGE have strong individual antihyperglycemic, anti-hyper-triglyceridemic and anti-oxidative effects in an alloxan monohydrate induced rat model of diabetes. Both also showed strong influence on the activation on the expression of genes
involved in the metabolic pathways of glucose and lipid which previously became dysfunctional in diabetic rats. When both these natural remedies were given in combination, synergistic effects were recorded in a dose dependent manner. Further work is needed to evaluate the way by which human beings suffering from diabetes are safely treated with these herbal remedies.


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