Evaluation of antidiabetic activity of biogenic silver nanoparticles (AgNPs) using *Thymus serpyllum* on STZ-induced diabetic BALB/c mice

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Islamabad, Pakistan

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In

Healthcare Biotechnology

By

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This thesis is dedicated to my parents who have always stood by my side & supported every decision I've ever made.

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LIST OF ACRONYMS

AgNP	Silver Nanoparticles
AMPK	Adenosine Monophosphate Kinase
ANOVA	Analysis of Variance
AUC	Area under the Curve
DNS	Dinitro salicylic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDS	Energy Dispersive X-ray Spectroscopy
FBG	Fasting Blood Glucose
FTIR	Fourier Transform Infrared Spectroscopy
GLUT4	Glucose Transporter 4
H & E	Haematoxylin & Eosin
IPGTT	Intraperitoneal Glucose Tolerance Test
IR	Insulin Resistance
IRS1	Insulin Receptor Substrate 1
ITT	Insulin Tolerance Test
KBr	Potassium Bromide
SEM	Scanning Electron Micrograph
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus
XRD	X ray Dispersive Spectroscopy

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ABSTRACT

Type 2 Diabetes Mellitus is one of the common metabolic disorder which is characterized by abnormal blood sugar level due to impaired insulin secretion or impaired insulin action or both. Metformin is most commonly used drug for the treatment of Type 2 Diabetes Mellitus but due to its slow mode of action and various side effects it shows poor and slow therapeutic response in patients. Currently, scientists are trying to tackle these limitations by developing nanomedicines. These nanoparticles based medicines are usually synthesized using biological sources due to low cytotoxicity. Among these silver nanoparticles are gaining more importance in current era due to their strong antidiabetic properties along with antioxidant, antibacterial potential. The current study was conducted to examine the antidiabetic effect of silver nanoparticles using aqueous leaf extract of *Thymus serpyllum*. Furthermore, to characterize these nanoparticles FTIR, XRD and SEM were used. The alpha amylase inhibition and antioxidant activity were checked through α amylase and DPPH radical scavenging assay respectively. To check the effect of silver nanoparticles on blood glucose levels FBG, IPGTT, ITT tests were employed on STZ induced BALB/c mice. To assess the morphological changes in the anatomy of liver, pancreas and kidney of BALB/c mice due to silver nanoparticles, histological analysis was done through H&E staining system. Finally, AMPK and IRS1 genes expression analysis was carried out via real time PCR. Silver nanoparticles were found to be spherical in shape with an average size of 42nm. They showed an IC50 of 8 µg/ml and 10 µg/ml for α amylase and DPPH assay respectively. Our study suggests that silver nanoparticles specifically 10mg/kg causes significant increase in expression of AMPK & IRS1 which ultimately increase the glucose uptake in cells. Thus, *Thymus serpyllum* mediated silver nanoparticles possess the potential to cure Type 2 Diabetes Mellitus.

Chapter 1

INTRODUCTION

1.1 Type 2 Diabetes Mellitus

It is also known as Insulin Independent Diabetes Mellitus (IDDM) and is a multifactorial, chronic disorder responsible for high co-morbidity rates across the globe (Dixon, Zimmet et al. 2011).Type 2 Diabetes Mellitus (T2DM) involves failure of pancreatic β cells, thus causing insulinopenia and insulin resistance in liver, skeletal muscles and adipose tissues ultimately leading towards their metabolic derangements and failure (Brownlee 2005).Blood glucose levels are majorly regulated by pancreas which release the enzymes in accordance with the signals (Figure 1).

Mostly pregnant women when develops high blood glucose level without any kind of previous diagnosis suffers from gestational Diabetes which may lead to Type II diabetes. Diabetes in pregnancy may give rise to severe conditions, like stillbirth , macrosomia and respiratory distress (Alberti and Zimmet 1998).The risk can be reduced through strict metabolic control as the food we eat has the major impact on development of diabetes (Asif 2014).

1.2 Epidemiology

The rate of diabetes is increasing all over the world. Approximately 422 million people have diabetes, accounting 1.6 million causalities in year 2016, as per World Health Organization (WHO), hence making diabetes 7th driving reason of morbidity and mortality worldwide. While the number of diabetic patients worldwide is expected to rise to 640 million by 2040 (Marín-Peñalver, Martín-Timón et al. 2016).

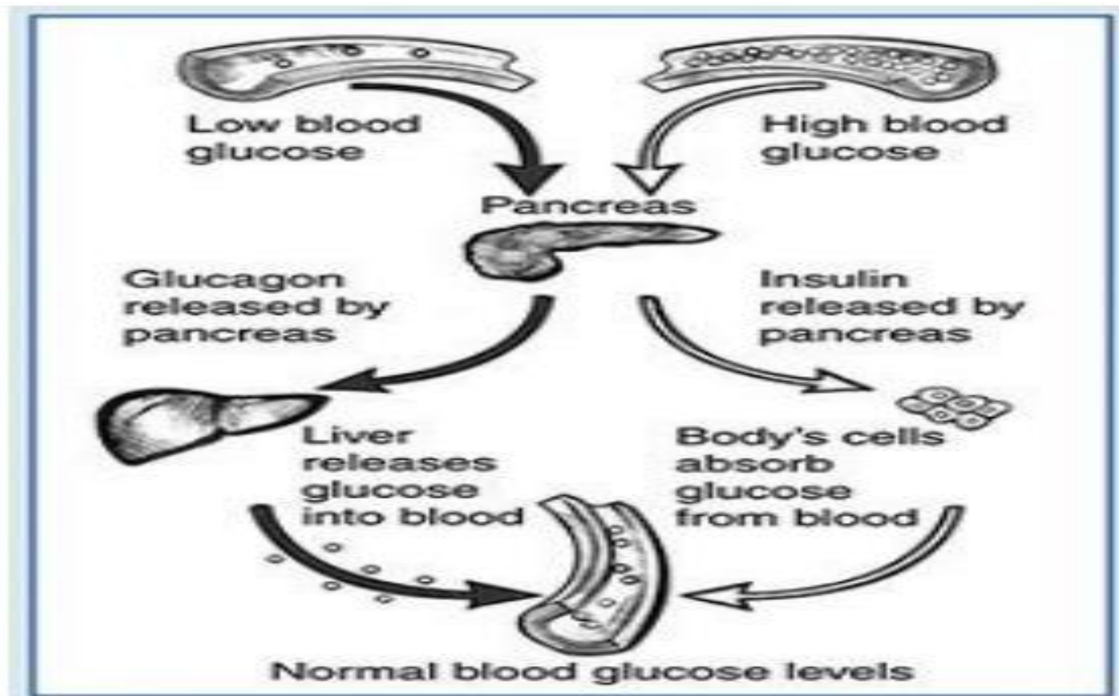

Figure 1 : Regulation of Blood Glucose (Control and Prevention 2011)

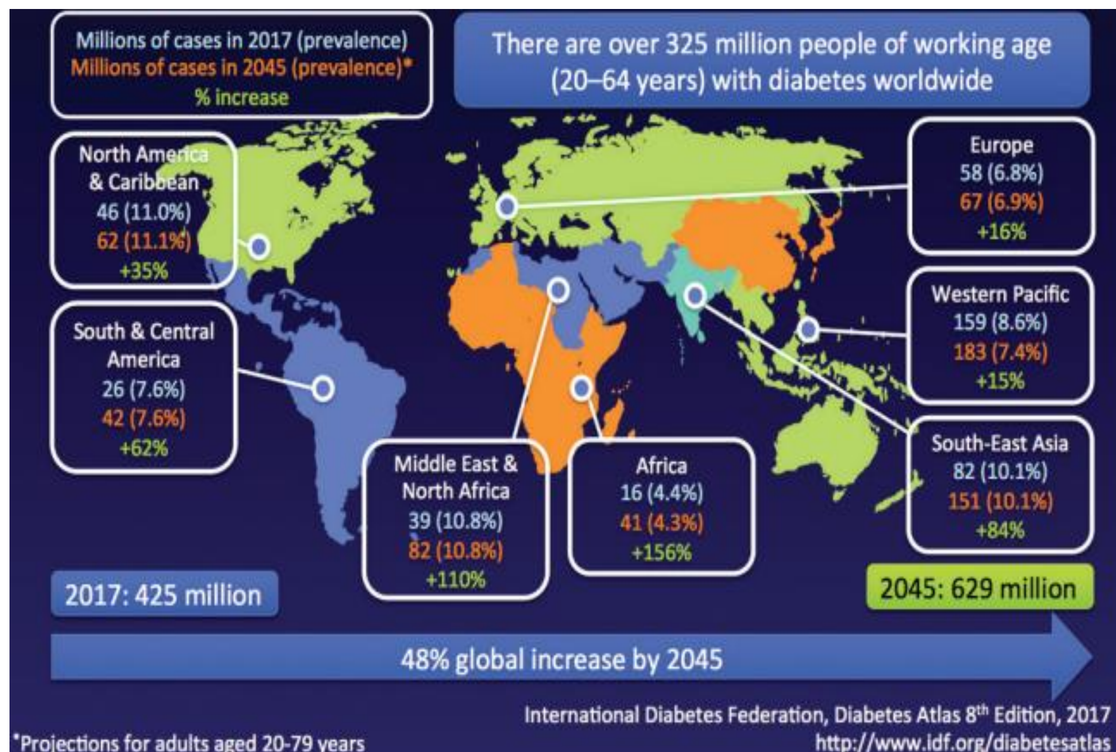

Figure 1.2 : Global prevalence of Diabetes [Mitchell, Annemans et al. (2012)]

Both the prevalence and number of cases have been steadily increasing over the past few decades, nominating it to be the major cause of stroke, cardiac arrest, lower limb amputation, kidney failure and blindness (Collaboration 2003, Murea, Ma et al. 2012).

Approximately 50% of the people are unaware that they are diabetic and 90% of such diabetic patients have T2DM while remaining 10% of people have T1DM (Chen, Magliano et al. 2012) .According to the atlas of International Diabetes Federation for 2017 , there are over 425 million people with diabetes worldwide and the prevalence is expected to increase to 629 million by 2045 i.e. an increment of 48% globally (International Diabetes federation , 2017).

The prevalence of diabetes is highest among the adults aged 20 or above .Diabetic prevalence has also increased in Pakistan which ranks it as the 10th highest number of diabetes cases with 7,474,000 adults affected at present compared to 5.2 million in 2012 (Sherin 2015).

1.3 Pathophysiology of Diabetes

By means of GLUT2 the glucose molecules after meal enter into the β-cells of pancreas. By the entry of intracellular glucose molecules into glycolysis and Krebs cycle in mitochondria there is production of ATP molecules. Potassium channel gets blocked when there is an increase in ATP: ADP ratio. As a result the membrane gets depolarized with potential being changed due to increased level of potassium ions which are positively charged (Lee and Halter 2017). There is an influx of calcium ions due to activation of voltage dependent calcium channel which in turn leads to expression of transcriptional activators e.g. hepatocyte nuclear factor 4 alpha (HNF4A) and hepatocyte nuclear factor 1 alpha (HNF1A). These transcriptional activators initiate synthesis and secretion of insulin. (Figure 1.3).

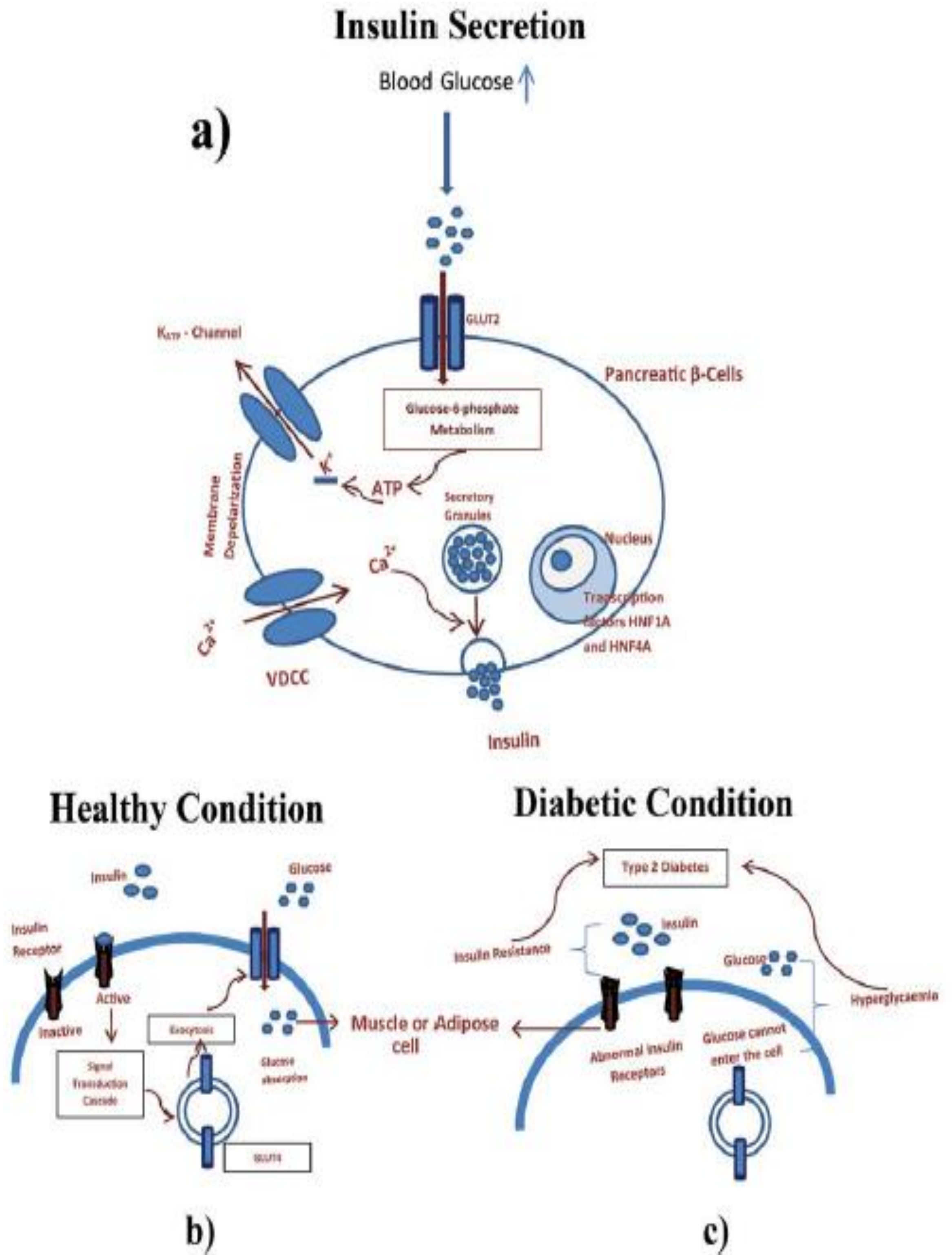

Figure 1.3 : Schematic diagram of T2DM pathophysiology (VSS, Adapa et al. 2018)

Secretion of insulin is reduced by β cells impairment due to which response of pancreas towards the glucose is decreased ultimately (VSS, Adapa et al. 2018). Hyperglycaemia is caused after meals due to impaired glucose tolerance (IGT), which is basically the decrease in glucose-responsive insulin secretion. There is further progression of impaired insulin secretion due to Lipotoxicity and glucose toxicity which are the associated risk factors. Overworking of the pancreatic β-cells can be caused if this condition is not treated. This overworking can cause progression of apoptosis and finally a decline in mass of the pancreatic β-cell (Cantley and Ashcroft 2015). The progressive decrease in the function and mass of pancreatic β-cell over time as well as increase level of glucose in blood (hyperglycaemia) result in the development of T2DM (Nolan, Ruderman et al. 2015). There is an increase in the blood glucose level when insulin resistance occurs i.e. when the cells of the body is not able to respond to physiological levels of insulin that is produced. In normal conditions GLUT4 translocate from cytoplasm to cell membrane. This happens due to the binding of insulin peptide to the insulin receptor that initiates signal cascade based on phosphorylation. GLUT4 helps in the uptake of glucose molecules into the cells and prevents the usage of stored fats for energy (VSS, Adapa et al. 2018). On the other hand in diabetes, insulin receptors remain inactive in the cells and there is no translocation of GLUT4 from cytoplasm to membrane. Therefore, there is no absorption of glucose molecules by the cells hence leads to the development of chronic hyperglycaemia. Combination of insulin resistance and inhibition of insulin secretion results in T2DM as influenced by genetic determinants, dietary pattern, lifestyle, level of physical activity and aging. Nutrient overload and imbalance which is caused by excessive intake of sugars, fats and oils can develop hyperlipidaemia and hyperglycaemia (Cnop, Vidal et al. 2007). There is glycation and lipid peroxidation due to persistent exposure

of carbohydrates and fats. These factors results in insulin resistance and are the key contributors to cause T2DM (Kahn, Hull et al. 2006).

1.4 Etiology of Diabetes

Multiple factors and mechanisms have been found to have hand in causing Diabetes Mellitus, yet the exact causing factors are uncertain (Pradhan, Manson et al. 2001).Being a disorder of multiple etiology, the genetic susceptibility superimposed by the environmental factors is involved undoubtedly in causing Type 2 Diabetes Mellitus (Group 1979) .

1.4.1 Genetic factors

More than 65 genetic variants have been revealed by Genome-wide association studies (GWAS) on T2DM, having potential of increasing risk of T2D by 10-30 %. Most of them are responsible for regulating insulin secretion (Lyssenko and Laakso 2013).In comparison to type 1 Diabetes Mellitus , genetic factors are evidenced to be stronger risk factors in Type 2 Diabetes Mellitus (Lyssenko and Laakso 2013). An offspring has 35-39% lifetime risk of developing T2D who has a single type 2 diabetic parent, while the risk rises to 60-70% for an individual with both parents having T2D where the risk for general population is 10% (Rich 1990). There are a number of complex interacting mechanisms involved in T2D pathogenesis, like insulin sensitivity, insulin secretion, beta cell functioning, replication of beta cells, obesity, role of adipocytes, hepatic glucose output etc. which may be controlled by various different genes themselves. The effect of genes may also vary in different populations. This complex aetiology explains the difficulty in unravelling the genetic basis of T2D and identifying the T2D causal genes (McCarthy 2003).

1.4.1.1 Role of AMPK and IRS1 in Diabetes

AMPK i.e. AMP activated protein kinase is the key sensor and regulator of energy status of cells in all eukaryotes. AMPK is regulated by AMP: ATP ratio so when this ratio increases, this causes activation of AMPK so its activated during energy depletion and regulates many different processes in the cell and thus it is known as master regulator of energy metabolism.

There are several factors activating AMPK like AMP in starvation and hunger and exercise which are the nucleotide dependent activation while two main upstream serine/threonine kinases LKB1 and CAMKKβ cause the nucleotide independent activation of AMPK by phosphorylating it at Thr172.AMPK deactivates all energy consuming pathways and activates the energy producing pathways.

The downstream effects of AMPK involves the negative regulation of mTOR (Mammalian Target of Rapamycin) signalling by phosphorylating it which actually is an activator of protein synthesis so AMPK blocks the protein synthesis (Figure 1.4.1.1) . Another function of AMPK is activation of ULK1 which further activates the macro autophagy i.e. degradation and recycling of cellular contents by vacuoles and lysosomes. The other downstream function of AMPK is the activation of fatty acid catabolism by activating ATGL (Adipose Triglyceride Lipase) which is the first enzyme responsible for the release of fatty acids from triglycerides .In another mechanism, AMPK inhibits the Acetyl CoA Carboxylase (ACC) which is absolutely necessary for fatty acid synthesis. AMPK also stops the cholesterol synthesis by blocking HMG-CoA reductase. The most important function of AMPK which makes it the molecule of interest in diabetes is its activation of glucose uptake by GLUT 4

transporters through the process involving TBC1D1 so that an insulin sensitive cell maybe able to uptake and utilize the glucose.

1.4.1.2 AMPK in T2DM

In T2DM , AMPK regulation is of immense interest due to growing evidences which suggest AMPK dysregulation has an immense role in the occurrence of T2DM and insulin resistance while the activation of AMPK will ameliorate the T2DM and IR pathologies .Several studies on animal models of metabolic diseases such as diabetes have shown decreased AMPK activity in muscle , liver and adipose tissues (Garcia and Shaw 2017).

Metformin which is the standard drug for diabetes , indirectly activates the AMPK by inhibition of mitochondrial function (Hardie 2013).Though Metformin , is the drug of first line for many T2DM patients but it cause abdominal discomfort, diarrhea, anorexia, flatulence and also decreases Vitamin B12 intestinal absorption (Hoffmann, Roa et al. 2003).

Silver nanoparticles can be a potential source of insulin sensitization as they increase the cytosolic Calcium ions concentration and activates the AMPK by phosphorylating it via CAMKKβ pathway in SH-SY5Y cells and also in rats (Li, Li et al. 2019).AMPK activation enhances the sensitivity towards the insulin and it could mediate the insulin by increasing its action (Tom, Garcia-Roves et al. 2014).

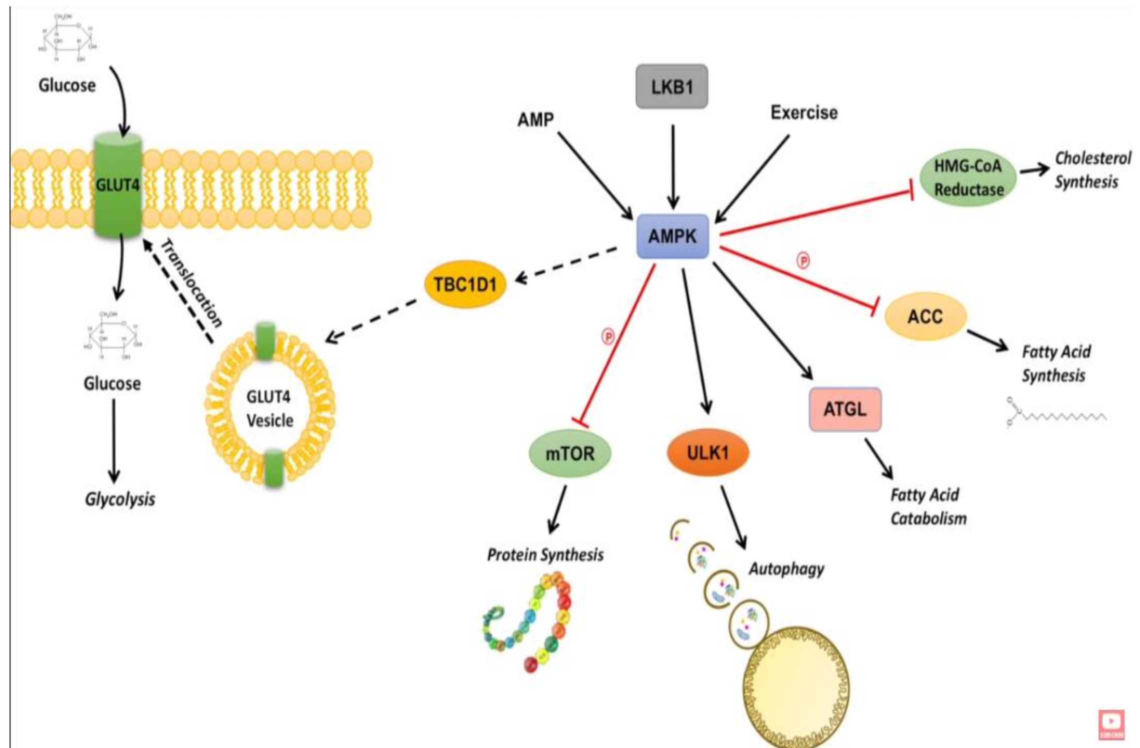

Figure 1.4.1.1: AMK Signalling Pathway Adapted from (Shackelford and Shaw 2009)

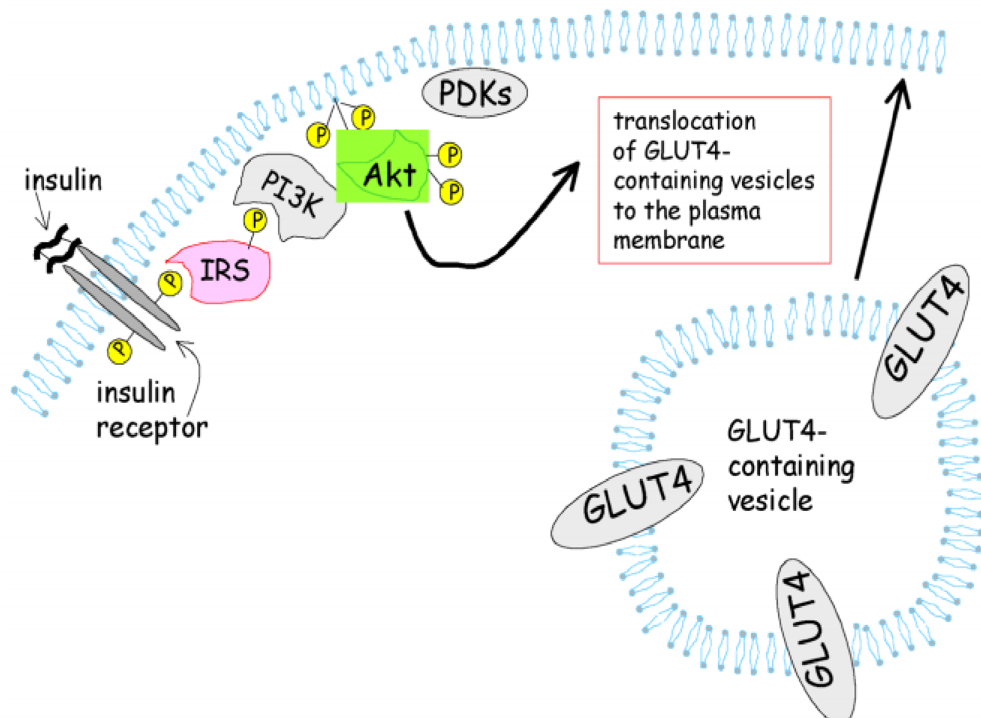

Figure 1.4.1.2: Insulin signalling elements leading to the transport of glucose (Garcia and Shaw 2017.

Insulin receptors (IRs) are expressed in different concentrations in almost all of the tissues that respond to insulin and for the insulin to perform its function it binds to the α and β subunits which induces the cytosolic substrates IRS1 and IRS2 phosphorylation (Figure 1.4.1.2) (White and Yenush 1998). Of the several AMPK targets which could most likely change the action of insulin after activation of AMPK , one of them is IRS1.Studies have shown that animal models lacking IRS1 developed hyperglycaemia or Type 2 Diabetes Mellitus hence increasing the protein levels of IRS1 will ultimately reduce the hyperglycaemia complications (Lavin, White et al. 2016).

Insulin binds to its receptor and activates the phosphorylation cascade from IRS1 which induces transport of glucose into the cells (Fisher 2006).

Silver nanoparticles lead towards the reduction in blood glucose levels by increasing the IRS1 and GLUT2 expression levels .Besides, silver nanoparticles elevate the expression levels of insulin and its secretion (Alkaladi, Abdelazim et al. 2014).

1.4.2 Epigenetic Factors

Epigenetics are the inheritable and reversible changes capable of altering the expression of genes while not disturbing the sequence of a DNA. Epigenetic methylation and histone acetylation have been seen to cause reduced expression of insulin, adiponectin and insulin-responsive glucose transporter-4 genes in diabetics (Pinney and Simmons 2010).

1.4.3 Inflammatory Factors

Growing evidence suggests that in T2DM pathophysiology, inflammation owes a significant role , thus linking T2DM to other metabolic disorders which partly or solely are caused due to inflammation like CVDs. Inflammatory markers and cytokines high

levels are responsible to develop glucose intolerance with time ,thus such cytokines can be used as early markers to predict the progression of diabetes (Pradhan, Manson et al. 2001). TNF- α also induces insulin resistance in obese rodent models (Xu, Barnes et al. 2003).

1.4.4 Environmental factors

The effect of T2D genes is greatly influenced by the environmental factors as some susceptible individuals would never develop T2D if they adopt a healthy lifestyle, while some non-susceptible individuals develop T2D due to their unhealthy practices (McCarthy 2003).

1.4.4.1 Diet

High intake levels of saturated fats and low levels of fibre intake is a very unhealthy diet and makes one prone to several metabolic disorders like cardiovascular problems and T2DM (Hu 2011).

1.4.4.2 Obesity

Weight gain and obesity are the crucial factors for the development of diabetes mellitus. Obesity increases the risk four folds for an individual with family history of T2D (Gong, Neuhouser et al. 2006) .In obesity the adipocytes enlarge in size, altering metabolism in different ways. Release of free fatty acid (Ghaffari-Moghaddam, Hadi-Dabanlou et al.) or non-esterified fatty acids and glycerol is increased due to increased lipolysis in obese individuals compared to lean, because IR is caused by these FFA in muscles (Davis, Christiansen et al. 2000).

Other Important Factors

Environmental factors include overeating, physical inactivity, psychological stress, certain dietary products, smoking and drinking alcohol. Increase in age also increases the risk of developing diabetes (Mokdad, Ford et al. 2003).

1.5 Symptoms of Diabetes

Symptoms of Type I Diabetes include excessive secretion of urine known as polyuria, thirst known as polydipsia, increased hunger , fatigue , sores that do not heal , blurred vision , numbness in hands or feet , weight loss and tiredness. In Type II Diabetes these symptoms are less noticeable. In this form, mostly symptoms appear years after its onset, when complications have already been increased (Drivsholm, de Fine Olivarius et al. 2005).

1.6 Diagnostic Criteria of Diabetes

To diagnose T2DM, blood tests are often used in which blood is drawn from an individual and sent to a laboratory for testing. Testing can enable health care providers to diagnose T2DM and prediabetes and help them to control and prevent complications to occur and delay onset of the disease, respectively. Various tests are used for the T2DM diagnosis including (a) A1C or HbA1c which is a glycohaemoglobin or haemoglobin test which measures the thickness of sugar bound to haemoglobin in red blood cells over the period of past three months (life span of a red blood cell).The thicker sugar coat on RBCs indicate the higher levels of glucose in blood , (b) The simplest blood test is the FPG i.e. Fasting Plasma Glucose which is done after several hours of fasting to help diagnose diabetes and prediabetes, (c) Another test is OGTT i.e. Oral Glucose Tolerance Test which measure the glucose utilizing ability of body in which the patients fast for about 8 hrs and then blood sample is taken at baseline ,

after which a glucose load of about 75grams is given and glucose level is measured after a period of 2 hrs. RPG which is the Random Plasma Glucose Test could also be used for diagnosis of T2DM.The individuals are diagnosed to be diabetic if the RPG level is equal to or higher than 200 µg/dL (Mellitus, Seino et al. 2010).

1.7 Treatment Strategies for Diabetes Type 2

T2DM can be managed by both drugs and by changes in lifestyle.

1.7.1 Management without Drugs

The first line of treatment for T2DM is diet control, weight management and physical activity. High caloric food intake and built up of excess adipose tissue induces insulin resistance and leads to decreased glucose uptake by cells and reduced glycogen synthesis. Increase in the levels of saturated fats has been associated with insulin resistance (Steyn, Mann et al. 2004). Moreover, various studies have shown that food items rich in carbohydrate while having least lipid content result in improved glycaemic control and reduction in low density lipids (LDL) as compared to diet with more fat and less carbohydrates. Also exercise and increased physical activity has also shown improvement in regulation of plasma glucose levels and improvement in insulin sensitivity (Mahan and Escott-Stump 2004).

1.7.2 Drug Therapies

There are various drugs available which are used for management of T2DM as listed below:

- Metformin: It is used to lower glucose levels in blood through suppression of glucose production in liver and increases the glucose uptake in cells by activating AMPK (Jackson, Hawa et al. 1987).

- Sulfonylureas: A class of drugs which stimulates glucose-dependent insulin secretion. However, it can cause hypoglycemia as a potential side effect.

- Thiazolidinedione: This medication works to increase the levels of receptor molecules, especially peroxisome proliferator-activated receptors which are nuclear receptor and acts as transcription factors for various genes involved in fat and glucose metabolism. However, thiazolidinedione's show some adverse effects including increased oedema and cognitive heart failure (Jackson, Hawa et al. 1987).

- α-Glucosidase Inhibitors: This medication is used to decrease glucose digestion by competitive inhibition of enzymes involved in catalysis of carbohydrate digestion in the intestine thus lowering postprandial blood glucose (Kavishankar, Lakshmidevi et al. 2011).

- Repaglinide: This drug is administered prior to intake of a meal in order to stimulate insulin secretion though with a shorter half-life as compared to sulfonylureas (Balfour and Faulds 1998).

1.8 Insulin Therapy

As progressive failure of β-cells occurs in T2DM, therefore over a period of time pancreas can no longer synthesize and secrete insulin via the use of oral medication. As a result, the patient is then recommended to be administered with insulin injections. One injection a day is administered at start along with oral medications but when with time being a single injection is unable to bring the glucose level closer to normal then multiple injections are administered a day .

1.9 Reactive oxygen species (ROS) and Diabetes

Our immune system face new challenges every day in the form of hazardous chemicals and pathogenic substances .ROS generation plays significant part in signalling of cell that's why are considered essential for various biological processes (Halliwell, Aeschbach et al. 1995). They also cause oxidative-stress cell damage by reacting with biomolecules such as proteins, lipids, enzymes and DNA/RNA. But to balance lethal oxidative stress, biological system have developed a defence mechanism known as antioxidative system. Recently, naturally plant-isolated phytochemicals have gained huge attention as modulators of ROS generations as well as a treatment strategy for inflammatory and aging diseases (Beatty, Koh et al. 2000).

T2DM is caused due to an imbalance among the antioxidants produced by body's natural mechanism and the cellular reactive oxygen species produced thus declaring the diabetes as an oxidative stress based disorder. Due to the excessive production of ROS , apoptosis and maturation of β cells increases while the synthesis and secretion of insulin decreases (Bai, Gao et al. 2018) .Several findings have reported that oxidative stress results in endothelial dysfunction, insufficient utilization of glucose in peripheral tissues and impaired secretion of insulin by β-cells of pancreas (Gothai, Ganesan et al. 2016).

Antioxidants are used to cure the oxidative stress and interest is diverting to the natural antioxidants rather than synthetic antioxidants (Scalbert, Johnson et al. 2005). Many plant mediated nanoparticles are potent source of antioxidants that act to reduce the oxidative stress produced as a metabolic response or induced exogenously (Garhwal 2010).

Silver nanoparticles are a rich source of antioxidants and they are readily available for its action into the tissues as they can easily penetrate deep down into the tissues (Johnson, Krishnan et al. 2018).It has been proved that free radicals especially oxygen based are effectively scavenged by silver nanoparticles (Seralathan, Stevenson et al. 2014).The synthesis of metallic nanoparticles from precursor salts occur via the oxidation reduction reactions. The reducing materials which are present in the plant extracts shifts the electrons to the ions of metal precursor, thus producing the nanoparticles (Goodarzi, Zamani et al. 2014).The synthesis and efficiency of nanoparticles depends on the amount of reducing compounds like phenols , flavonoids and terpenes etc. in the plant extracts .

1.10 *Thymus serpyllum* Description

One of such plants is *Thymus serpyllum* commonly known as Breckland thyme , which belongs to the family Lamiaceae and it possess several important compounds like minerals , phenols , flavonoids and many other reducing agents (Mushtaq, Bashir et al. 2016).Traditionally , its leaves and flowers in dried form are used as tea and infusions against fever , bronchitis , cold and cough (Jabeen, Ajaib et al. 2015) .Several studies have shown that it depicts anti-rheumatic, ant-inflammatory and hypoglycaemic activities (Malik, Ghumman et al. 2012, Mushtaq, Bashir et al. 2016).

In the present study , *Thymus serpyllum* will be analysed for its antidiabetic efficacy both in vitro and in vivo on streptozotocin induced mice models .For this, silver nanoparticles of *Thymus serpyllum* will be synthesized using the plant extract as the reducing and stabilizing agent of the metal precursor. As mentioned earlier, the source selected for the synthesis of silver nanoparticles i.e. Breckland thyme has antidiabetic properties, so it is expected that on nanoscale these biogenic nanoparticles may exhibit

additional medicinal properties and that additive effect may also have role in increasing the insulin sensitivity and glucose uptake in diabetic cells.

Current research is focused on biogenic synthesis of silver nanoparticles from *Thymus serpyllum* and their efficacy as antidiabetic agents in streptozotocin induced diabetic mice models.

Table 1.6: Diagnostic criteria of Diabetes

	Normal	Pre-Diabetic	Diabetic
Fasting Plasma Glucose	70-99 mg/dL	100 - 125 mg/dL	≥126 mg/dL
2-Hour Post Glucose Intake	<140 mg/dL	140 - 199 mg/dL	>200 mg/dL
HbA1c	4 - 5.9 %	5.7 - 6.4 %	≥ 6.5 %

Figure 1.10: *Thymus serpyllum (*Breckland Thyme)

1.11 Research Objectives

- Biosynthesis of silver nanoparticles from *Thymus serpyllum*
- Characterization of newly synthesized AgNP by FTIR , SEM & XRD
- Determination of antioxidant ability of AgNP by DPPH Assay
- Evaluation of α amylase inhibition activity of AgNPs
- Investigation of the antidiabetic potential of AgNP in an in house generated STZ induced mice model of diabetes.
- Histopathological examination of Liver, Kidney and Pancreas by Haematoxylin and Eosin (H & E) staining.
- To evaluate the differences in expression of AMPK & IRS1 between the T2DM mice models treated with standard drug metformin and various doses of AgNPs.

CHAPTER 2

LITERATURE REVIEW

The frontiers of research on diabetes are focused to update the best method of diabetes analysis, monitoring and cure. Defects in various molecular signaling pathways are shown to be associated with pathogenesis of type 2 diabetes. Insulin signaling, Adipocytokine signaling and glycation haxosamine signaling are some of the major pathways involved in regulating blood glucose homeostasis. Genes contributing in these pathways involved GLUT2, GLUT4, IRS, IRS1, PI3K, AKT, TNFα, mTOR, protein kinases and many more. Activation of upstream gene phosphorylate another target and hence lead to highly regulated signaling in the body to control glucose homeostasis. Any impairment in this signaling can lead to pathogenesis of type 2 diabetes via insulin resistance or β cell dysfunction. Current treatment employed for the diabetes include both oral and injectable medications (McGovern, Tippu et al. 2018). While these medications have been accepted and practiced for decades, they have their side effects and drawbacks.

2.1 Nanotechnology: Introduction to a New Era

In biotechnology one of the most emerging field is nanotechnology and nanoparticle research is very important nowadays because of the ways of synthesis and its applications.

Nanoscale is usually a size below 100nm. Nanoscience studies the responses, properties, and phenomena of materials at macromolecular, molecular and atomic scales usually at size ranges between 1-100nm (Logothetidis 2006)

Richard Feynman, a well-known Physicist who won Nobel Prize for Physics in 1965, gave a discussion at the conference of the American Physical Society at the California Institute of Technology on December 29, 1959. His talk was extraordinarily perceptive. He gave a speech which is entitled as "There is Plenty of Room at the Bottom, An Invitation to Enter a New Field of Physics," he suggested to employ "machine tools to make smaller machine tools, these in turn to be used in making still smaller machine tools and so on all the way down to the atomic level." So the earliest vision of nanotechnology started with his discussion (Asiyanbola and Soboyejo 2008). Japanese researcher Norio Taniguchi, in 1970 defined nanotechnology for the first time as "Nanotechnology majorly consist of processing of, separation of, deformation and consolidation of materials by atom or by molecules". In 1980, K. Eric Drexler worked on promotion of technological significance at Nano level(Corbett, McKeown et al. 2000).

2.2 Advancement of Nanotechnology

The application of nanotechnology for obtaining novel products has been going on since the ancient civilization. The ancient Romans used to colour glass with shades of mauve and yellow by using different concentrations of gold and silver (Daniel and Astruc 2004). Gold and silver nanoparticles were also used for aesthetic purpose in the famous Lycurgus cup from the 4th century, which is now placed in the British museum (Barber and Freestone 1990). Similarly, in Middle Ages, colloidal silver and gold nanoparticles were used to produce bright coloured stained windows, mostly red and purple in European cathedrals. For example, in Notre Dame, the red and purple colour of the rose window of cathedrals is due to presence of gold nanoparticles (Dreaden, Alkilany et al. 2012). The technique of nanoparticle synthesis was studied in the 9th century by Muslim scientists, who carried out the reduction of metal oxides upon

heating at high temperature which were already deposited on ceramic surfaces(Chandra, Chandra et al. 2015). This technique of glass colouring was further refined in 15th and 17th century by using precipitates of different colloids added to the glass (Daniel and Astruc 2004). The first ever documented chemical synthesis of metal nanoparticles was performed in 1857 by Michael Faraday (Klimov 2003), who reduced solution of chloroauric acid with carbon disulphide to obtain deep red coloured gold nanoparticle solution, and then by Zsigmondy in 1906 (Crookes 1913), who reduced chloroauric acid in the presence of formaldehyde to obtain monodisperse gold solutions. Zsigamody's method was then modified in 1951 through Turkuvish method that involves chloroauric acid reduction in the presence of sodium citrate to synthesize gold nanoparticles (Turkevich, Stevenson et al. 1951). This method has also been employed for the synthesis of silver nanoparticles.

2.3 Role of Nanotechnology in Clinical Therapeutics

The emergence of nanotechnology has made a significant impact on clinical therapeutics in the last two decades and enormous advancements have been done in developing the field of nanomedicine to detect, diagnose and effectively treat the diabetes (Babu, Templeton et al. 2013). Nanomedicine as per national institute of health is a formulation of drug whose end product's size is less than a micron (Babu, Templeton et al. 2013). Nanomedicine has gained much advantage due to its ability to overcome biological barriers, enhancing the bioavailability of drug (Lavan, McGuire et al. 2003), specifically target the disease sites and make the effective delivery of drug possible (Babu, Templeton et al. 2013).

2.4 Silver nanoparticles and its applications

Silver nanoparticles have a lot of applications in industries, biological sciences, biomedical sciences such as in targeted drug delivery systems (Dreaden, Alkilany et al. 2012)also in the production of many products such as in textile industries , deodorants, cosmetics and perfumes, food storage , as antimicrobial agents ,bandages, biosensors (Durán, Marcato et al. 2011), as orthopaedic and cardiovascular implants, bone biomaterials, surgical catheters, wounds and burns dressings as therapeutic agents, in various house hold products and cleaning solutions. Silver nanoparticles are also used in the burn and wound healing process due to its anti-inflammatory property (Jeyaraj, Varadan et al. 2013).

2.5 Different shapes of Silver Nanoparticles

Depending on the type of method, stabilizer and reducing agent various shapes of Silver nanoparticles can be synthesized. They can be rods, spherical (Wang, Wang et al. 2006) , prisms (Darmanin, Nativo et al. 2012), in the form of nanowires(Murphy and Jana 2002), Nano pyramids(Wiley, Im et al. 2006), nanobars (Wiley, Chen et al. 2007) and cubic (Wiley, Im et al. 2006).

2.6 Synthesis methods of Silver Nanoparticles

Silver nanoparticles have been synthesized by chemical, physical and biological methods. Physical methods used for the synthesis of silver nanoparticles are vapour condensation and arc discharge method (Sharma, Yngard et al. 2009). Chemical synthesis of silver nanoparticles is achieved by chemical reduction, photochemical reduction and electrochemical synthesis (Khan, Faisal et al. 2011).The physical and chemical methods require a lot of energy and time and are mostly toxic.

2.6.1 Green Synthesis of Silver Nanoparticles

In environmental and technological challenges nanomaterial can help including catalysis, conversion of solar energy, water treatment and medicine. Nanoparticles show improved properties on the basis of their morphology, size and distribution. For the preparation of nanoparticles various physical and chemical methods have been developed using metals i.e. copper, silver gold etc. These methods are very successful in the synthesis of well-defined and pure nanoparticles but these are hazardous and harmful to the nature as well as costly.

Recently an increase in awareness towards the green synthesis of nanoparticles have been observed. Different metal nanoparticles like copper, gold, zinc, titanium and silver have been prepared but the most effective are the silver nanoparticles as silver is nontoxic, harmless antimicrobial agent and has a very good antidiabetic and antioxidant activity.

For the production of silver nanoparticles the important thing is the presence of phytochemicals in plants. Terpenoids, flavonoids, Quinone's, carboxylic acids, ketones, amides and aldehydes are the phytochemicals that are responsible for the reduction of ions. There is a wide use of nanoparticles in electronics, medicine, diagnostic agents, and therapeutics. Medicines, medical devices, food storage cans and food wraps can be coated using nanoparticles. Toxicity can be caused by metal oxide nanoparticles which can have can have adverse effect on human health. Beside this metal oxide nanoparticles are also expensive. Green synthesis of nanoparticles does not require temperature, high energy and is cost effective and can be made at large scales. Most importantly, green synthesis of nanoparticles is not hazardous for human health (Wiley, Im et al. 2006).

2.6.1.1 Plant Mediated Silver Nanoparticles and their Antidiabetic Potential

Silver nanoparticles synthesis from plants is more beneficial as compared to microbes and algae especially because they do not require the tedious stages of growing the cultures on media ,hence less biohazardous and can be easily improved (Ghaffari-Moghaddam, Hadi-Dabanlou et al. 2014). Plants possess an industry of compounds like Phenols, flavonoids, terpenoids and a lot more which act as reducing, stabilizing as well as capping agents for the nanoparticles and enhance their biomedical properties (Iravani 2011). The current focus of anti-hyperglycemic drugs is turning towards inhibition of intestinal enzymes such as alpha amylase and alpha-glucosidase which would in turn decrease the elevation in the post prandial blood glucose level (Kazeem, Adamson et al. 2013). Biogenic silver nanoparticles thus serve as potent inhibitors of such digestive enzymes (Sorescu, Nuţă et al. 2016). Silver nanoparticles synthesized from different plants that are assessed for their anti-diabetic potential are summarized in Table 2.1

(Balan, Qing et al. 2016) reported silver nanoparticles synthesis using *Lonicera japonica* leaf extract, native to Eastern Asia and traditionally used as anti-viral and anti-inflammatory agent. UV visible spectra was recorded at 435nm due to SPR and they have spherical and hexagonal shape with an average size of 52 nm. Antioxidant efficacy was checked by DPPH assay where the radical scavenging response increased in a dose dependent manner and IC50 value was found to be 46.7µg/ml for AgNPs compared to the ascorbic acid with IC50 as 26.45 µg/ml. For diabetes care the most effective approach considered is the inhibition of alpha amylase and alpha glucosidase digestive enzymes (Rasouli, Hosseini-Ghazvini et al. 2017). Both enzymes were remarkably inhibited with increasing the concentration of *Lonicera* mediated AgNPs where IC50

value determined for alpha-amylase was 54.56 µg/ml while 37.86 µg/ml for alpha-glucosidase. LB and Dixon plot analysis identified AgNPs as reversible noncompetitive inhibitors (Balan, Qing et al. 2016).

Pterocarpus marsupium, a medicinal plant, bark and wood were collected from Kerala, India and 5ml of its aqueous extract was mixed with 50ml of 1mM silver nitrate solution and silver nanoparticles of 148.5nm size were formed within 90minutes (Sharifi-Rad, Hoseini-Alfatemi et al. 2015). The SPR Band was found at 279nm and FTIR identified the presence of phenols as capping agents while carboxyl and hydroxyl groups as stabilizing and reducing agents. The surface potential with zeta sizer was found to be -28mV. In vitro antidiabetic activity was accessed via alpha amylase inhibition assay showing concentration dependent increase in percent inhibition from 22 to 72 % varying dose from 100-1000 µg/ml.

Table 2.6: Medicinal plants exploited for silver nanoparticles synthesis against type 2 diabetes.

Plant's Name	Plant's Part	Size	Reference
Lonicera japonica	Leaves	52	(Balan, Qing et al. 2016)
Pterocarpus marsupium	Bark and wood	148.5	(Sharifi-Rad, Hoseini-Alfatemi et al. 2015)
Clausena anisata	Leaf and root	13-61	(Amoo, Aremu et al. 2012)
Calophyllum tomentosum	Leaves	24	(Govindappa, Hemashekhar et al. 2018)
Ocimum basilicum	Leaves	17	(Malapermal, Botha et al. 2017)
Ocimum sanctum	Leaves	15	(Malapermal, Botha et al. 2017)
Punica granatum	Leaves	35-60	(Saratale, Shin et al. 2018)
Azima tetracantha	Leaves	10-80	(B and S 2018)
Eysenhardtia polystachya	Bark	10-12	(Campoy, Gutierrez et al. 2018)
Withania somnifera	Root	123.23	(Uddin, Kavya et al.)
Alyssum homalocarpum	Seeds	30	(Ghasemian Lemraski and Valadbeigi 2018)
Andrographis echioides	Leaves	-	(Nirubama, Kanchana et al. 2015)
Musa paradisiaca	Stem	30-60	(Anbazhagan, Murugan et al. 2017)
Zingiber officinale	Rhizome	128	(Garg, Pandey et al. 2016)
Aloe vera	Leaves	30.5	(Ashraf, Ansari et al. 2016)

Silver nanoparticles were made via the green route with the aid of ethanolic leaf and root extract of *Clausena anisata* as both stabilizing and reducing agent. This shrub ,native to South Asia and Africa , dried leaves have multiple medicinal benefits against epilepsy , tuberculosis , migraine , rheumatoid and diabetes (Amoo, Aremu et al. 2012) (Yakoob, Tajuddin et al. 2016). Exposure of various on and off conditions like direct boiling and microwave and sunlight radiations synthesized the silver nanoparticles whose UV spectral peak was obtained at 488.9nm while XRD analysis confirmed its crystalline nature. FESEM analysis revealed that the biogenic nanoparticles are spherical in shape with 13-61 nm size range. Furthermore, they were found to exhibit great antioxidant activity and 500 µg/ml of silver nanoparticles exhibited 80.32% inhibition of alpha amylase in comparison to standard acarbose 85.24%. In addition, an increasing trend in glucose uptake by yeast cells was observed with an increase in concentration, i.e. 68.2 % at a concentration of 10mM. Meanwhile , the nanoparticles increased the glucose diffusion rate from 30 to 180 mints (Begum, MH et al. 2016) .

Calophyllum tomentosum, commonly grown in Sri Lanka and Western regions of India, leaves were used to make aqueous extract via microwave method and mixed with 5mM silver nitrate to get 24nm size silver nanoparticles at room temperature. Crystalline nature and the presence of phytochemicals was confirmed with XRD and FTIR respectively. The *Calophyllum tomentosum* mediated silver nanoparticles exhibited strong DPPH, H_2O_2 and nitric oxide scavenging activity. Bio-capping of functional groups on AgNPs made them potent inhibitors for α-amylase and α-glucosidase as well as β-glucosidase and DPPIV (Govindappa, Hemashekhar et al. 2018).

In another study ,antidiabetic properties of AgNPs (3-25nm) synthesized from combination of *Ocimum basilicum* and *Ocimum sanctum* were detected using *Bacillus*

stearothermophilus as enzymatic model of α-glucosidase, showing the great inhibitory potential compared to the standards (Malapermal, Botha et al. 2017).

(Saratale, Shin et al. 2018) contentedly used *Punica granatum* leaves as a mediator for synthesis of AgNPs and reported crystalline, spherical shape and stable nanoparticles with a surface charge of -26.6mV using XRD, FESEM-EDS and DLS respectively for characterization. The AgNPs caused a concentration dependent increase in the inhibition of digestive enzymes like alpha-glucosidase and alpha-amylase with an IC50 value of 53.8 and 65.2 µg/ml respectively. The results also illustrated that they have potent in vitro antioxidant activity determined by DPPH and ABTS with an IC50 value of 67.1 and 52.2 µg/ml respectively. In another study, Manimegalai *et al*. (B and S 2018) reported that synthesis of silver nanoparticles using *Azima tetracantha* leaf extract possess an efficient anti-hyperglycemic activity and potential antiglycating response in a dose dependent manner by inhibiting alpha amylase and alpha glucosidase with an IC5O value of 262.18 µg/ml and 271.78 µg/ml respectively.

According to another study *Eysenhardtia polystachya* rich in phenols and flavonoids have proved as an excellent anti-glycating agent for Type 2 Diabetes by reducing the oxidative stress in mice models (Perez-Gutierrez, Garcia-Campoy et al. 2016). Abraham et al.(Campoy, Gutierrez et al. 2018) synthesized *Eysenhardtia polystachya* loaded silver nanoparticles with an SPR band at 413 nm and surface charge of -32.25mV i.e. higher stability. TEM images revealed that the synthesized nanoparticles are spherical in shape with 10-12 nm diameter. Furthermore, in glucose-induced hyperglycemic zebrafish in-vivo model, these nanoparticles showed improved insulin secretion better survival of β cells of pancreas.

Silver nanoparticles were synthesized in aqueous medium using *Withania somnifera* root extracts as reducer and stabilizer. SEM images presented the nanoparticles to be spherical in shape with an average size of 123.23 nm. The root extract mediated nanoparticles were found to have the greatest potency for acting as inhibitors of metabolic enzymes in vitro. The percentage inhibition shown by *Withania somnifera* roots mediated nanoparticles was higher than that recorded by standard Acarbose for alpha amylase. Similar inhibition for alpha glucosidase prove their therapeutic role as anti-hyperglycemic agents.

The seeds of *Alyssum homalocarpum* were used by Lemraski et al. (Ghasemian Lemraski and Valadbeigi 2018) to get the methanolic extract which was used further to synthesize face centered cubic agglomerated nanoparticles functionalized with rich polyphenols hence , the molecules with high antioxidant activity. FTIR measurements revealed that the seed extract attaches to the nanoparticles surface by amide linkages. The nanoparticles showed strong α-glucosidase inhibitory activity but weak α-amylase inhibitory activity 221 µg/ml for 100mg/ml concentration.

Andrographis echioides is a medicinal herb used in various ailments cure. Nirubama *et al*. mixed its 80% ethanolic extract with 3mM silver nitrate to yield silver nanoparticles that were characterized using various techniques. *Andrographis echioides* mediated nanoparticles were found to strongly scavenge the radicals of DPPH, ABTS and nitric oxide assay, increasing in a dose dependent manner. Meanwhile , in vivo alpha glucosidase per cent inhibition in Wistar rats was found to be statistically significant (Nirubama, Kanchana et al. 2015).

(Anbazhagan, Murugan et al. 2017) noted that the process of synthesis was noticeably fast and spherical AgNPs (on average 30-60nm) got generated within few a minutes as

the stem extract of *Musa paradisiaca* came in contact with silver ions. Characterization for confirmation was done via SEM, UV Visible spectrophotometry, XRD and FTIR. These successfully synthesized nanoparticles from banana were tested in vivo for combined treatment of diabetes and malaria on streptozotocin induced diabetic rats and they were found to be effective in improving and moderating insulin as well as galactose and glucose.(Garg, Pandey et al. 2016) reported the utilization of ginger rhizome ethanolic extract for the first time as a reducer for silver nitrate to obtain biogenic crystalline nanoparticles of 128 nm, which were further analyzed via AFM, XRD and SEM. Silver nanoparticles dose of 200mg/kg on diabetic rats showed a gradual decrease in the glucose levels as compared to the standard metformin while the exact mode of action of these nanoparticles remained unknown. Ali Alkaladi et al. (Alkaladi, Abdelazim et al. 2014) concluded that male albino diabetic rats responded to 10mg/kg silver nanoparticles effectively and exhibited sufficient reduction in glucose levels. Furthermore, the nanoparticles elevated the serum insulin levels and the insulin receptors along with the higher expression of GLUT-2.

Advanced glycation end products (Drivsholm, de Fine Olivarius et al.) formation is the major cause of inflammation and is considered as oxidative derivative (Vlassara and Uribarri 2014). Furthermore, Advance Glycation End products have proved as potent risk factor for hyperglycemia and peripheral insulin resistance (Singh, Bali et al. 2014).In this context , Ashraf et al. (Ashraf, Ansari et al. 2016) synthesized silver nanoparticles on average 30.5nm spherical particles using Aloe vera leaf extract as a stabilizer and reducer. They concluded that these nanoparticles showed a favorable response on structure of protein and found to inhibit AGE in a dose dependent manner.

Approximately 320 tons of silver nanoparticles are synthesized yearly which are utilized in bio-sensing , food products and nano-imaging (Ahamed, AlSalhi et al. 2010).

Chapter 3

Materials and Methods

All the experimentation and work of this research was done in Atta Ur Rahman School of Applied Biosciences (ASAB) while for the characterization of nanoparticles, equipment in School of Chemical and Materials Engineering (SCME) and US Pakistan Centre for Advanced Studies in Energy (USPCASE) at National University of Sciences & Technology (NUST) were utilized. Green approach was adopted for the synthesis of biologically active silver nanoparticles from *Thymus serpyllum*. The details of the chemicals and the methodology used for this project is given as follows:

3.1 Plant Selection and Storage

Thymus serpyllum natural plant was collected from Rakaposhi Base Camp, Gilgit Baltistan for silver nanoparticles synthesis. The aerial parts of plant were dried and then ground into a fine powder by an automated electric grinder and stored at room temperature in a sterile sealed container.

3.2 Preparation of Plant Extract

Plant extract was prepared by modification of the protocol of Sun *et a l*(Sun, Cai et al. 2014). 10 gram of plant powder was soaked in 100 ml deionized water in Erlenmeyer flask at room temperature for few minutes and then heated at 60°C for 15mints on a hotplate. The extract was made to cool for half an hour and then supernatant was collected and twice filtered through 0.45 µm pore size filter paper using vacuum filtration assembly. The filtrate obtained was stored at 4°C as a stock solution for to be used within 1 week.

Figure 3.1: Dried aerial parts of *Thymus serpyllum*

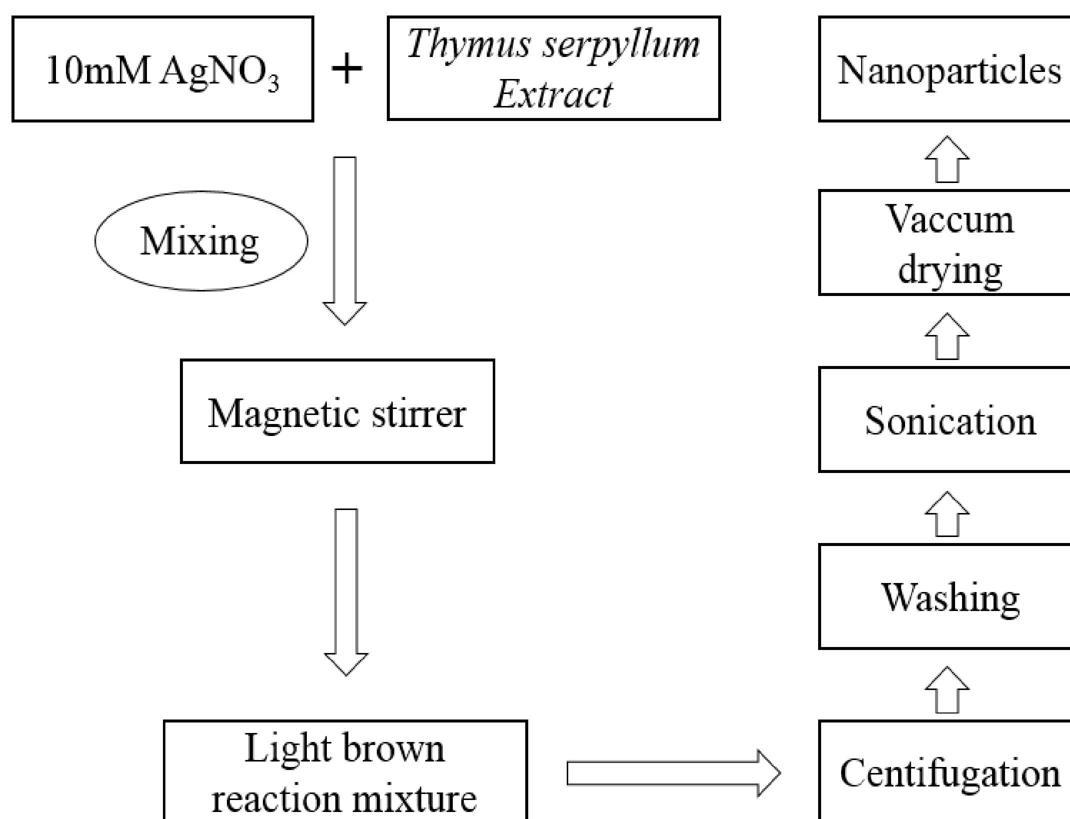

Figure 3.3: Schematic representation of AgNP synthesis

3.3 Synthesis of *Thymus serpyllum* Mediated Silver Nanoparticles

The stock solution of plant, acting as reducing and capping agent for the precursor, was diluted to 15% (v/v). 850µl of silver nitrate (10mM) was added drop by drop per second into the 14.25 ml solution taken from 15% diluted extract of *Thymus serpyllum* under magnetic stirring at 300 rpm and 25°C temperature. This working solution was then kept in dark in rotary orbital shaker for 5hrs at 700 rpm and 25°C temperature, followed by monitoring after every 1 hour using UV Spectrophotometer for SPR band.

3.4 Purification of Nanoparticles

The reaction mixture was centrifuged at 15000rpm for 30mints at 4°C in a refrigerated centrifuge. Supernatant was discarded and the pellet was re-suspended thrice in deionized water and centrifuged with above conditions to obtain a thicker pellet (Figure 3.4). The pellet was then air dried and collected and stored in Eppendorf tubes. This dried mass of particles was weighed and used for further activities.

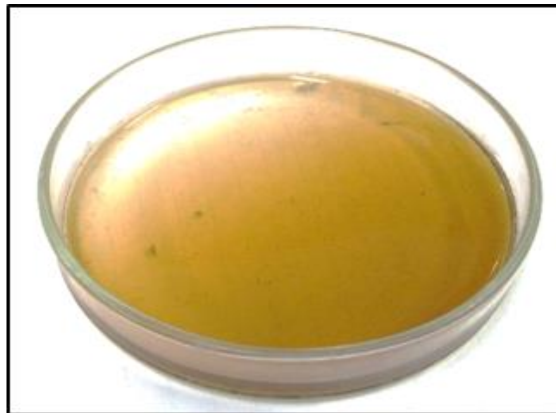

Figure 3.4: Thick pellet of purified AgNPs after centrifugation

3.5 Characterization of Silver Nanoparticles

3.5.1 UV Visible absorption Spectroscopy

This is one of the most widely used technique in both chemical and clinical laboratories. It actually measures the extent of absorption that occurs in a sample when the light beam passes through it. In this instrument , the beam of light is split , where one half of

the beam is directed towards the cuvette containing the reference solvent only and the other half is directed towards the cuvette containing the sample to be analyzed. It is a powerful technique to analyze the concentration, size and stability of nanoparticles.

Biogenic silver nanoparticles were initially characterized using Spectrophotometer (LABOMED, Inc. U.S.A, Model UVD-2950) to observe the specific peak of the silver nanoparticles on the UV visible spectra. The absorption spectra range was set from 300-600nm .Spectrophotometer was blanked first by filling both the cuvettes with deionized water .Then one quartz cuvette was filled with nanoparticle solution while the other with deionized water as reference and loaded in spectrophotometer chamber , where the silver nanoparticles absorbed the photons of specific wavelength depending upon the particle size distribution.

3.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

It was used to observe the functional groups attached to the surface of silver nanoparticles. The Perkin-Elmer Spectrum 100 FTIR by USA was used to analyse both the dried extract and dried silver nanoparticles powder for comparison of functional groups. KBr due to its hygroscopic nature was used to prepare the pellets of both the extract and AgNPs as water free samples are required for analysis. The KBr pellets were prepared using hydraulic press and the wavelength range was set from 450-4000 cm^{-1}.Both the extract and nanoparticles were loaded individually into the chambers where the infra-red waves pass in order to detect the functional groups attached at surface .The transmission spectra was saved for interpretation to identify the functional groups .

3.5.3 X-Ray Diffraction (XRD) Analysis

The atomic and molecular structure of silver nanoparticles crystals can be studied using X-Ray diffraction. XRD machine produce an intense beam of X-rays which is thrown on the test sample. These rays reflect at different angles and intensities by the sample. The data is calculated and a 3D structure is formed by the machine after reflection of X-rays. From this 3D structure the angle and the position of an atom in a crystal lattice can be revealed. The sample was highly concentrated using centrifuge for XRD analysis. 1ml of the sample was taken in the Eppendorf tube and was centrifuged for 15 minutes at 15,000 rpm. After 1st centrifugation the supernatant was removed and more sample solution was added to the pellet and centrifuged again. The same process was repeated at least 7-8 time until an enlarged pellet is formed that is the requirement for XRD. A glass slide was used for placing the pellet. The slide was placed under lamp for 20-25 minutes for drying. In this way a thick layer of AgNPs deposited on the glass slide which was ready for XRD analysis. The glass slide with sample was carefully placed in the XRD machine (STOE, model number theta, Germany). Copper K alpha was used as a radiation source, a 40 KV voltage, and 40 mA current was used. The angle used in the process was 20-80 theta.

3.5.4 Scanning Electron Microscopy

SEM model No 51-ADD0007 TESCAN VEGA3 was used for SEM analysis in order to analyse the morphology and average particle size of the AgNP. The sample which was used for SEM was first diluted and then sonicated. Under UV lamp a single drop of sample was dried for 20-30 minutes. In order to make the sample conductive and fixed for analysis it was sputter coated with gold.

3.5.5 Energy dispersive X-ray spectroscopy (EDS)

EDS is generally used for the chemical characterization or elemental analysis of a sample. In order to analyse the composition of sample EDS was done. The mode which was selected for determining the composition of the sample was PHA mode T3 with live time of 50 seconds and real time 56.03 seconds. The energy range was 0-20Kev while the counting rate was 2785 counts/second.

3.6 Free Radicals Scavenging Ability of AgNP

To evaluate the radical scavenging property of AgNP, DPPH (2, 2 Di Phenyl, 1 Picryl Hydrazyl) radical scavenging assay was carried out. The reagent which is used in this assay is DPPH which undergoes reduction by donating hydrogen when it reacts with anti-oxidant compounds. DPPH change its colour from violet to light yellow as it is a colorimetric reagent. Using UV/Vis spectrophotometer this visual change in colour can be observed at 517nm.

The protocol for this assay started by making fresh solution of DPPH (0.1mM) in methanol. Different concentrations of both ascorbic acid and AgNPs such as 10µg/ml, 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, and 100µg/ml were prepared. 200µl was taken from each of various concentrations of ascorbic acid and AgNPs and mixed thoroughly with 800µl of DPPH solution in separate tubes to make a final volume of 1ml. It was then followed by an incubation period of 30 minutes under dark condition at room temperature. After 30 minutes of incubation the absorbance was measured at 517nm with the help of UV/Vis spectrophotometer. The formula used for percent scavenging/inhibition was:

Percentage Scavenging/Inhibition = [(control sample absorbance value – test sample absorbance value)/ control absorbance value] x 100

This procedure was repeated thrice and the values obtained were used to plot a graph and relative comparison of the free radicals scavenging ability of different concentrations of silver nanoparticles with respect to ascorbic acid was done. Because of the high antioxidant activity ascorbic acid was used as a reference.

3.7 Alpha Amylase Inhibitory Assay

The effect of silver nanoparticles on alpha amylase activity was determined by making various concentrations of AgNPs and acarbose in separate tubes as 10, 20, 40, 60, 80 and 100 µg/ml in .02 M PBS with adjusted pH of 6.9, to which 500 µl of α-amylase was added and the reaction mixture was allowed to incubate for 10 minutes at 37°C .Afterwards, 500 µl of starch (1% solution) was poured into each of tubes containing the reaction mixture of varying concentrations and incubated for another 10 minutes. Then 1ml of DNS was added in all tubes in order to terminate the reaction, followed by placing the tubes in water bath at 60 °C for 15 minutes. Then cool the buffered mixture and add 10 ml of distilled water in each tube .Set the spectrophotometer at 540 nm and take absorption readings of each concentration and make comparisons with the standard amylase inhibitor i.e. acarbose. The spectrophotometer is blanked with phosphate buffer saline (PBS).

The formula used for % scavenging/inhibition was:

Percentage Scavenging/Inhibition = [(control sample absorbance value – test sample absorbance value)/ control absorbance value] x 100

3.8 Animals for in vivo study

The study was conducted on 4 weeks old male BALB/c mice (n=50), purchased from NIH Islamabad and bred and housed in animal house of Atta-ur-Rahman School of

Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). These mice were kept in cages (5 mice per cage) at constant temperature (25±2 ºC) and natural light-dark cycle (12-12 hours) and were given distilled water *ad libitum* and fed with basic chow diet.

3.8.1 Ethics Statement

The approval for all the protocols carried out during research was obtained from internal review board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST). All the tests and experiments performed were according to the guidelines provided by the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

3.8.2 Mice Model Construction for T2DM

T2DM mice model was constructed by combination of High Fat Diet (HFD) and low doses of Streptozotocin. The normal control mice (n=10) were fed with a diet consisting of following components in Table 3.8.2.The other mice models (n=40) for the purpose of diabetes induction, after weaning when they were 3 weeks of age, got switched to HFD and were given intraperitoneal STZ injections (100mg/kg) in 0.1 M citrate buffer (pH 4.5) at 6th and 9th week of age after overnight fasting. The control animals however, received citrate buffer injections only. After 2 days of 2nd STZ injection i.e. on 44th day, Fasting blood glucose levels of all mice were checked using On-Call EZ II Blood Glucose Monitoring System (Blood ACON International, USA).Mice with Blood Glucose levels greater than 126 mg/dL were considered as diabetic (Dong, Xu et al. 2013).

3.8.3 Treatment Design

A 50 days long protocol applied generated a T2DM BALB/c mice model by involving HFD and STZ 100mg/kg at 6th and 9th week shown in figure 3.8.3. Afterwards, in order to analyse the therapeutic potential of biogenic silver nanoparticles using *Thymus serpyllum* as antidiabetic agents, mice were divided into 5 groups. Each group had total of 10 animals of almost 10 weeks of age which were further taken through experiment. These groups were categorized as follows:

3.8.3.1 Normal control

Group 1 was assigned as normal group and included 10 BALB/ c mice receiving normal diet and water throughout the experimentation.

3.8.3.2 Diabetic Group

Group 2 was assigned as the negative control group for diabetes .The mice in this group were left untreated throughout the experiment and were used as comparison for the rest of treatment groups.

3.8.3.3 Metformin Treated Group

This group was used as the positive control group .This group mice received the standard drug Metformin (100mg/kg) orally in feed for consecutive 28 days. The effect on weight and blood glucose levels were noted throughout the experiment.

Table 3.8.2: Components of Basic Chow Diet

Component	**Quantity (%)**
Crude Fibre	4
Crude Fat	9
Crude Protein	30
Moisture	10.4

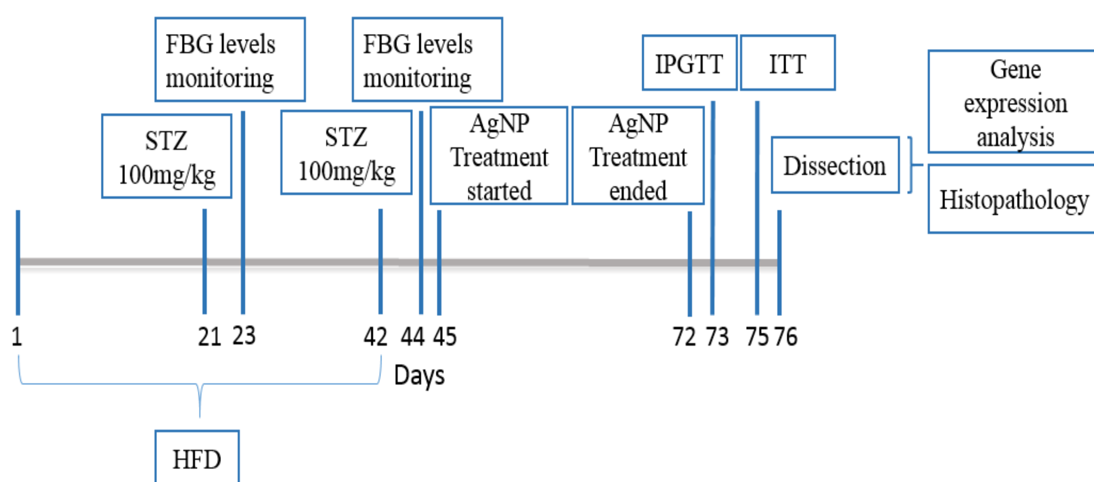

Figure 3.8.3: Timeline depicting Diabetic animal model construction and treatment period

3.8.3.4 Low Dose Treated Group

Group 4 was allocated as low dose silver nanoparticles treatment group. These mice were given silver nanoparticles 5mg/kg orally through feed for a period of 28 days. The reduction in blood glucose levels were noted after regular intervals for these mice.

3.8.3.5 High Dose Treated Group

The mice of this group were given silver nanoparticles 10 mg/kg in normal diet for 28 days and blood glucose levels and body weight was monitored weekly.

3.9 Glucose Levels Estimation tests

3.9.1 Fasting Blood Glucose Test

The glucose in the blood of normal and that of streptozotocin induced mouse models i.e. all groups was monitored on weekly basis. For fasting blood glucose test the animals were fasted for 8 hours. The blood was taken from the tail of mice and measured using ON Call glucometer.

3.9.2 Intraperitoneal Glucose Tolerance Test

On 29th day of treatment mice from each group were subjected to Intraperitoneal Glucose Tolerance Test (IPGTT). Mice were kept on fasting for 8-10 hours. 2.5g/kg of glucose was given to the animals. Blood samples of about 200 µl from the vein of each mouse was collected at different time intervals i.e. 0, 30, 60, 90 and 120 minutes in order to measure the level of glucose. Glucose-time curve was calculated using the values of Areas under the Curve (AUC).

3.9.3 Insulin tolerance test

On 31st day of model development insulin tolerance test was done. For this test mice were fasted for 4 hours and then injected with 0.5U/kg of human insulin subcutaneously. For the determination of blood glucose concentration the blood samples were collected after 0, 30, 60 and 120 minutes of insulin injection and the values of Area under the Curve (AUC) were calculated.

3.10 Real Time PCR (Gene Expression Studies)

3.10.1 PRIMER DESIGNING

Primer 3 software available at (http://frodo.wi.mit.edu) was used to design primers in order to quantify expression of AMPK, IRSI and GAPDH genes. After designing of primers BLAST was performed in NCBI to check the specificity of each primer. Primers sequences are shown in the table 3.10.1.

3.10.2 RNA EXTRACTION

RNA required for the RT-PCR was extracted using Tri-reagent. Extraction was done using the protocol provided by the manufacturer. Experimental animals were anesthetized and sacrificed on the 30th day of treatment. For anesthetisation, chloroform was used. Isolation of liver was done after dissection and then 1ml of Trizol was used for homogenization of isolated tissue. At room temperature samples were allowed to stand for 5 minutes to support the complete separation of nuclear protein. After 5 minutes, 0.2 ml of chloroform was added to the samples. Then samples were shaken for 15 sec until it turned milky and kept at room temperature for 10 minutes. For 15 minutes at 12,000 rpm at 4oC, samples were centrifuged which cause the formation of three 3 phases in the mixture. Proteins present at the bottom in the red organic phase, DNA in the white phase and RNA present at top of the tube which is a colourless phase.

Then aqueous phase was removed carefully to eliminate the DNA contamination by not allowing the mixing of other phases. The aqueous phase was transferred to another tube along with the addition of 0.5ml of isopropanol and allowed to set at room temperature for 10 minutes. Again samples were centrifuged at 4°C for 20 minutes at 12,000 rpm. After this step RNA was precipitated and pellet was clearly observed at the side of the tube. Washing of RNA pellet was done using 1ml of 75% ethanol and then samples were centrifuged for 5 minutes at 4°C at 7500rpm. Ethanol was removed and RNA pellet was allowed to dry which is then re-suspend in 30ul PCR water. RNA sample was stored at -80°C.

3.10.3 Quality of RNA

RNA isolated from all samples were run on gel (2%) in order to ensure the good quality of RNA and to ensure that the RNA is not degraded. 5ul of RNA and 2ul of loading dye was mixed and loaded in wells. 100 bp ruler was used. Voltage was set at 90 volts and current as 400mA and the time allowed for running of samples on gel was 35 minutes. Results were analysed using Dolphin Doc (Wealtech). The samples of RNA exhibiting sharp and clear bands are considered of good quality. Good quality RNA with sharp bands is very much important for obtaining accurate results.

3.10.4 RNA Quantification

Quantification of RNA was done using Thermo-scientific Nano-drop ND-2000 following by the cDNA synthesis.

3.10.5 cDNA Synthesis

Before cDNA synthesis concentration of RNA was determined using Nanodrop and the sample with good quality RNA was selected for further proceedings. 1ul RNA sample

along with 10mM oligodT and NF water were incubated for 5 minutes at 65°C and immediately placed on ice to give heat shock. Then 4µl of *Reverse Transcriptase* (RT) buffer (Thermo Scientific), 1 µl3. RNAse Inhibitor (Thermo Scientific), 2µl of 10mM dNTPs and 1µl of RT enzyme (Thermo Scientific), 2.8µl of RNA template and then 8.2µl of NF water was added to raise the volume to 20µl. The reaction mixture was incubated for I hour at 42°C and at 70°C for 10 minutes.

3.10.6 Conventional PCR

Conventional PCR was done for the confirmation of synthetic cDNA for the GAPDH (house-keeping gene). PCR determinants are listed in the table 3.10.6.

3.10.7 Expression Analysis of AMPK and IRS1 on Real Time PCR

Optimization of the primers for the genes AMPK and IRS1 and their Expression analysis was done on Real Time PCR. Passive dye ROX and Maxima® SYBER Green qPCR Master Mix (2X) (Catalog No: K0221, Fermentas) was used during the PCR run. The composition of the reaction mixture for the real time PCR is given below in the Table 3.10.7.

3.10.8 Real time PCR profile

Real time PCR profile used for the housekeeping gene i.e. GAPDH is shown in Figure 3.10.8.1 while PCR profile of AMPK and IRS1 is shown in Figure 3.10.8.2 and figure 3.10.8.3 respectively.

Table 3.10.1: Primer sequences used for the expression analysis of AMPK, IRS1 and GAPDH

Gene	**Primer Sequence 5' to 3'**	**Product Size**
GAPDH	F-ACCCAGAAGACTGTGGATGG R-CACATTGGGGGTAGGAACAC	175 bp
IRS1	F- ACATCACAGCAGAATGAAGACC R-CCGGTGTCACAGTGCTTTCT	232 bp
AMPK	F-GTCGACGTAGCTCCAAGACC R-ATCGTTTTCCAGTCCCTGTG	250bp

Table 3.10.6: Recipe for Conventional PCR

Constituent	**Quantity (µl)**
Nuclease free water	15.5
10mM Forward Primer	1
10mM Reverse Primer	1
10mM dNTPs mix	1
10X Taq Buffer	2.5
25mM MgCl2	2.5
Taq Polymerase	0.5
cDNA	1
Total	**25**

Table 3.10.7: Real Time PCR constituents

Constituent	**Quantity (µl)**
Nuclease free water	7.2
10mM Forward Primer	0.8
10mM Reverse Primer	0.8
2X SYBR Green/ROX Qrt PCR reaction mix	10
cDNA	1.2
Total	**20**

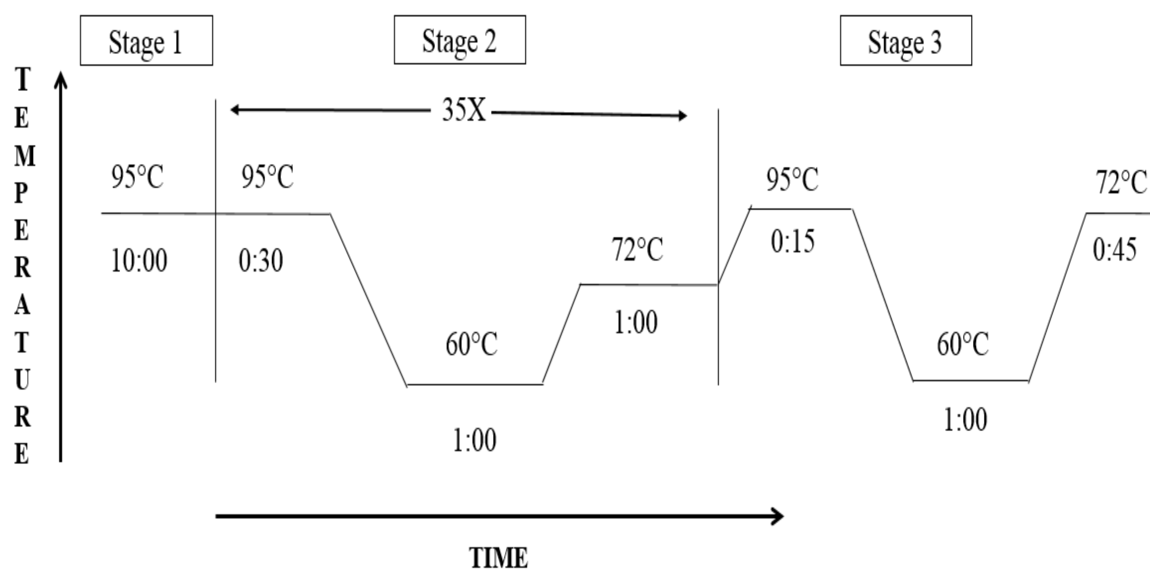

Figure 3.10.8.1: Real Time PCR profile for GAPDH Gene

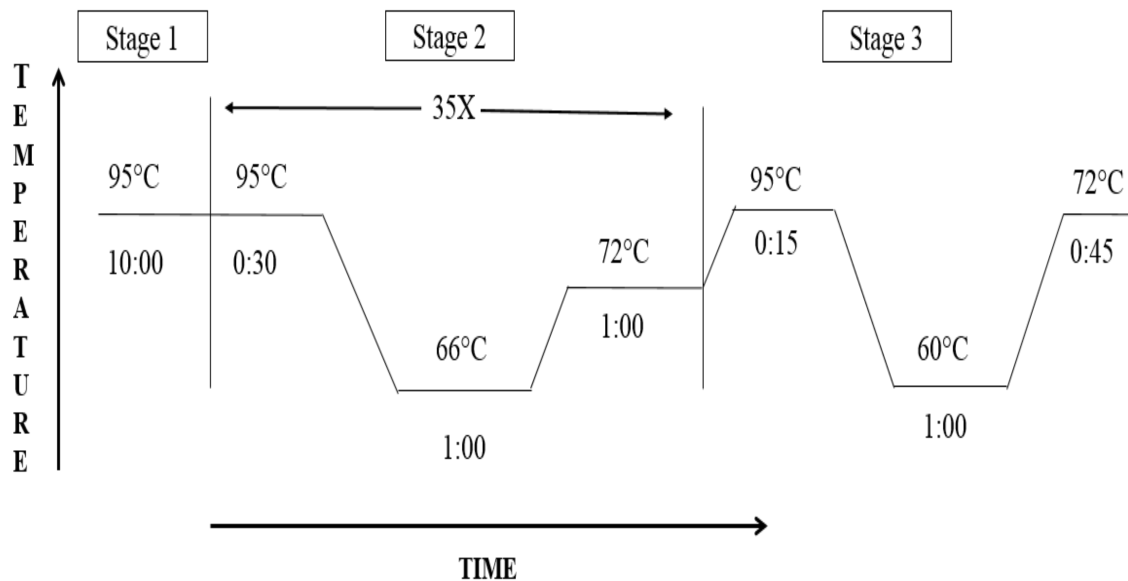

Figure 3.10.8.2: Optimized Real Time PCR for AMPK Gene

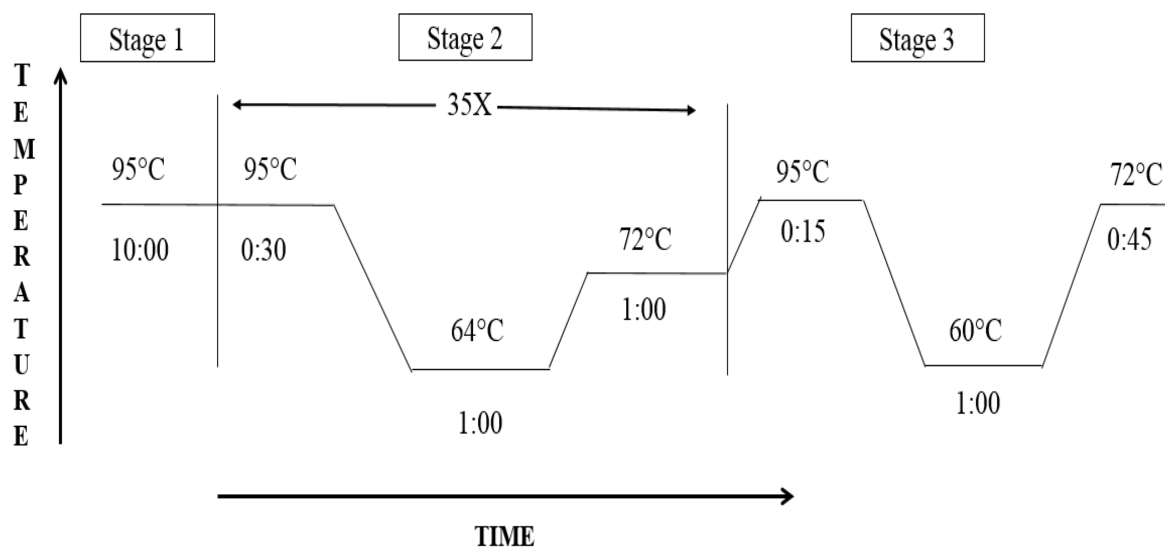

Figure 3.10.8.3: Real time PCR profile optimized for IRS1 gene at 64°C

3.11 Statistical Analysis

Statistical analysis of the expression of the AMPK, IRS1 and GAPDH genes in different diabetic mice and those treated with nanoparticles in comparison to normal control group (healthy mice) was done using One Way ANOVA and Post Hoc Bonferroni test on Graph Pad Prism version 5.01.

3.11.1 Real Time PCR Calculations:

Target genes average Ct values were compared with the average of Ct values of the reference gene i.e. GAPDH to get the ΔCt values .The fold change in the expression of the targeted markers was calculated as:

- Ct value of GAPDH in Control = X1
- Ct value of GAPDH in Experimental = X2
- Ct value of Target gene in Control = Y1
- Ct value of target gene in Experimental = Y2
- ΔCt Control = (Ct of Target in Control) – (Ct of GAPDH in Control) = Y1-X1 = ΔCt1
- ΔCt Exp. = (Ct of Target gene in Exp.) – (Ct of GAPDH in Exp.) = Y2-X2 = ΔCt2
- ΔΔCt = (ΔCt Exp.) – (ΔCt Control) = ΔCt2-ΔCt1
- Fold Change = $2^{(-\Delta\Delta Ct)}$

3.12 Histological Examination of Kidney, Liver & Pancreas

Histologically analysis was done to check the effects of biosynthesized silver nanoparticles on the anatomy of sample tissues. Right after anaesthesia liver, pancreas and kidney tissues of mice were isolated to avoid decomposition and post-mortem

autolysis. The tissues were preserved in 10% formalin solution. Formalin solution was prepared by adding 37% formaldehyde to distilled water in 1:9. To neutralize the effect of formalin, the solution was buffered with phosphate buffered saline (PBS) as formalin is acidic in nature and may react with haemoglobin present in the sample tissue which can affect the histological analysis.

3.12.1 Fixation of sample tissues and embedding in paraffin cassette

A typical process of dehydration was carried out through a series of alcohols (ethanol) i.e. 70%, 85%, and 100% for 1 hour in each before fixation in paraffin. After dehydration there should be not even a tiny residue of water in the sample. In order to displace ethanol completely the sample tissues were incubated for 2 hours in a clearing agent called Xylene.

For paraffin infiltration the sample tissues were incubated in molten paraffin for 4 hours and then solid blocks were formed using specific moulds. The blocks were kept at 4◦C for solidification.

3.12.2 Tissue sectioning and staining

Sample tissues were cut into thin sections of about 4 microns using microtome and placed in water maintained at 37°C. Sections were then transferred to the pre-coated adhesive glass slides. Deparaffinization was carried out in xylene after 20 minutes incubation at 60◦C. The slides were then placed for 3 minutes in different concentrations of ethanol i.e. 95%, 70% and 50% for rehydration. Followed by dehydration, H&E (Haematoxylin and Eosin) staining system was used for staining of sections. The slides were placed for 2 minutes in 70% acid alcohol to remove excess of stain. Acid alcohol is prepared by adding 2ml of glacial acetic acid to 200ml of 70% ethanol. Slides were dried for 2 hours in dark and followed by an incubation period of 2 minutes in xylene. With the help of micropipette a drop of DPX (used as mounting

medium) was placed on the slides. It was allowed to spread on the slide by gently placing the coverslips. The slides were then examined under light microscope.

Chapter 4

RESULTS

4.1 Visual Confirmation of *Thymus serpyllum* mediated AgNPs

When *Thymus serpyllum* extract was added to the 10mM solution of silver nitrate, a change in colour from transparent to light brown and then dark brown was observed gradually with time that preliminary confirms the formation of silver nanoparticles (Figure 4.1).After air drying the nanoparticles solution in a petri plate for 24 hrs, the nanoparticles were obtained in the form of solid brownish black powder.

4.2 Characterization of Biogenic Silver Nanoparticles

The characterization of thymus serpyllum mediated silver nanoparticles was done to evaluate and analyze their morphology, size, purity and many other factors.

4.2.1 UV Visible Absorption Spectroscopy

In the ultraviolet-visible spectroscopy (UV-Vis) the absorption of light by the molecules occurs in ultraviolet-visible region which corresponds to the colour of the solutions involved i.e., when the colour of solution changes from light yellow to dark brown. Absorption spectrum gets broadened and red shift occurs indicating that the hue of colour and its intensity depends on the stoichiometric ratios in which the extract and silver nitrate solution are mixed.

UV Visible absorption spectroscopy of silver nanoparticles showed the Surface Plasmon Resonance peak at 425nm (Figure 4.2) While $AgNO_3$ and extract do not have any peak in this region (250-600nm).

Figure 4.1: The color change represents the preliminary confirmation of silver nanoparticles. Purified AgNPs after drying are shown in the right panel

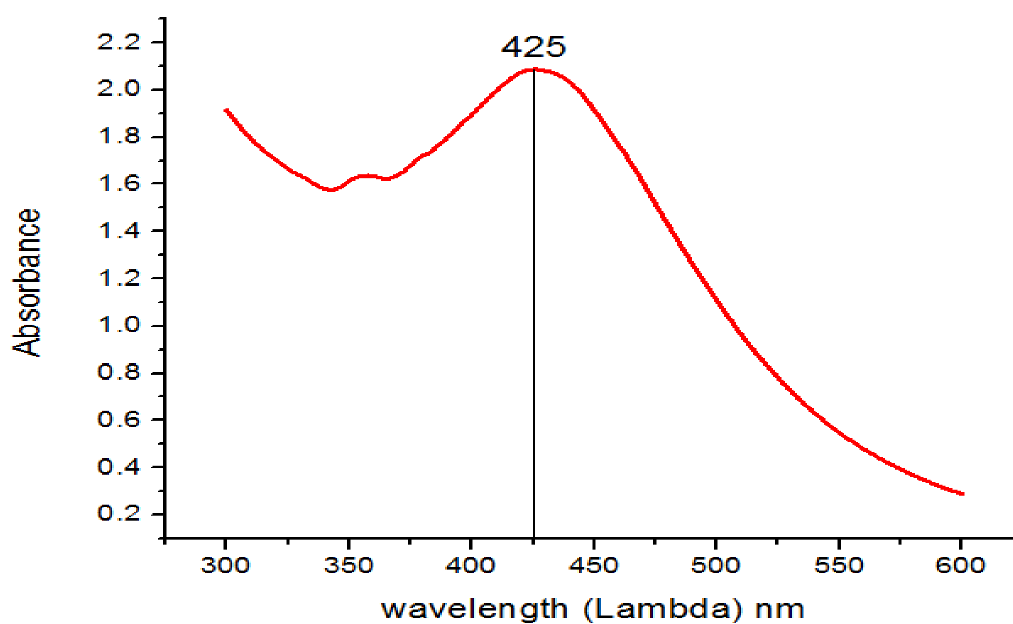

Figure 4.2.1: UV-Vis Spectroscopy of Reaction mixture indicating typical absorbance peak of AgNPs around 425nm.

4.2.2 X-Ray Powder Diffraction (XRD)

XRD is a non-destructive technique which identifies the crystalline nature at the atomic scale. This method has been used to identify the structure imperfections in sample and to measure the phase identification while its working principle is Bragg's Law.

The structure of synthesized nanoparticles has been investigated by X Ray Diffraction (XRD) analysis. The obtained XRD patterns of the sample were analyzed using Jade 8 software and the JCPDS Card Number 00-04-0783 was obtained accordingly confirming the formation of crystalline and stable silver nanoparticles (Lanje, Sharma et al. 2010). Structural information on the nanoparticles shows the XRD patterns of the nanoparticles are lying flat with their basal planes parallel to the substrate. (Figure 4.2.2) shows the XRD pattern of silver nanoparticles i.e. seven intensive peaks and these diffraction peaks obtained at 2θ were 28° , 32° , 39°,46°,55°,57 °and 68°which are indexed at (111), (200), (220), (311), (222), (400) and (331) facets of silver respectively. The sharp peaks indicate the crystallinity and purity of silver nanoparticles.

4.2.3 Fourier Transform Infrared Spectroscopy

The FTIR spectrum is a vibrational spectrum of the nanoparticles produced as a result of the oscillations of the atoms present on their surface. It is clear from the figure 4.2.3 that the silver nanoparticles (AgNPs) show significant variations in their vibrational spectra in comparison to the extract spectrum. In an IR spectrum the width and intensity of peaks depends upon the particle size, with the increase in particle size the width of the peak decreases and intensity increases. FTIR analysis was carried out for

nanoparticles and that of extract. Shift in the chemical groups was analysed as a function of reducing and capping agents for nanoparticles.

FTIR analysis depicts the similar functional groups among the extract and silver nanoparticles which enhances the stability and reduces cytotoxicity of nanoparticles.

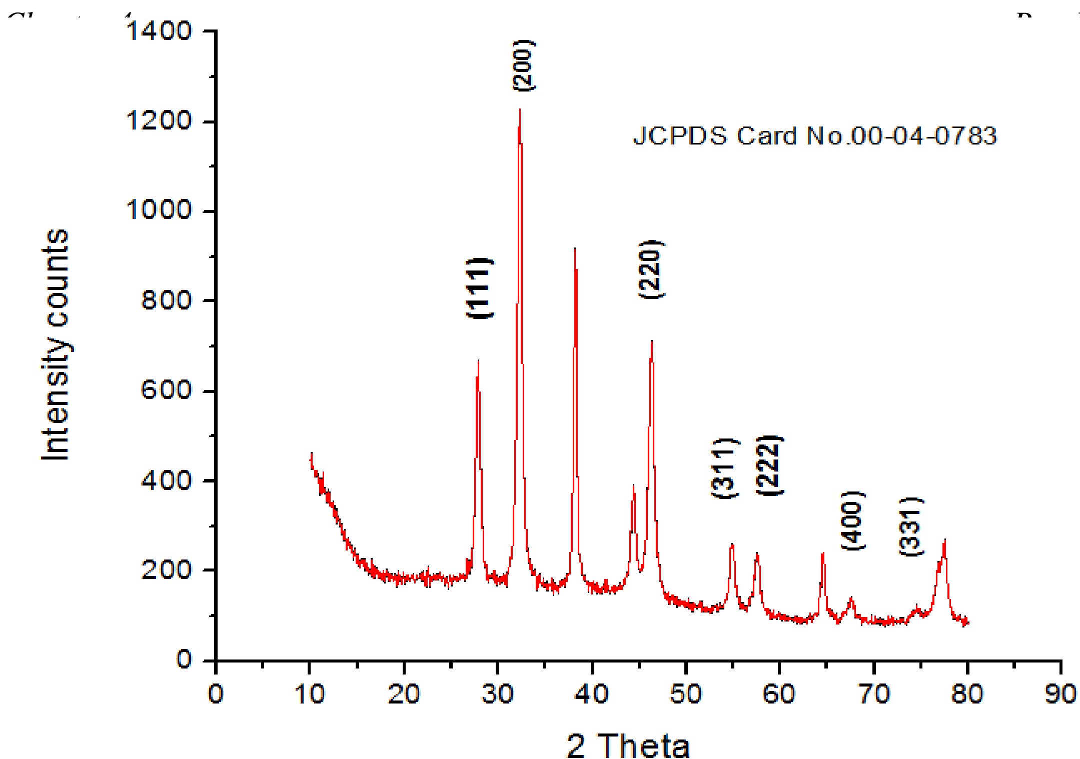

Figure 4.2.2: X-ray Diffraction pattern of Biogenic Silver Nanoparticles

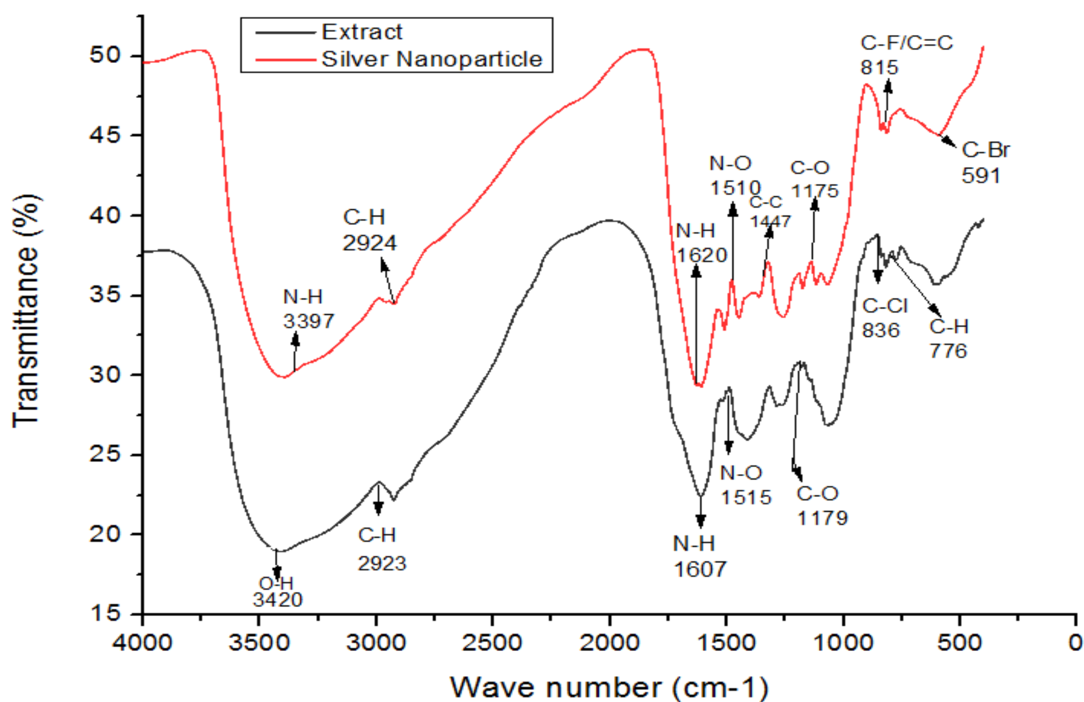

Figure 4.2.3: FTIR depicting functional groups of silver nanoparticles vs Thyme extract. FTIR peaks of AgNPs reveals few similar functional groups to that of thyme extract as 2924 cm^{-1} (C-H stretching), 1620 cm^{-1} (N-H bending) , 1510 cm^{-1} (N-O stretching and 1175 cm^{-1} (C-O bending) which potentially act as surface capping & stabilizing groups

4.2.4 Scanning Electron Micrograph

The SEM analysis reveals that smaller, spherical and monodispersed AgNPs are generated and the nanoparticles are uniformly distributed with an average size of 42nm.

4.2.5 Energy Dispersive X-Ray Spectroscopy (EDS)

The identification and quantification of elemental composition of AgNPs was done by EDS analysis at magnification of 90x with 512 x 384 pixel and probe current of 20Kv.EDS analysis of biogenic silver nanoparticles via SEM machine revealed the presence of silver in the colloidal solution of thymus serpyllum mediated silver nanoparticles. Silver exists in mass of 9.04%. The other elements present were carbon, oxygen and chlorine which attributes the presence of organic source i.e. plant extract.

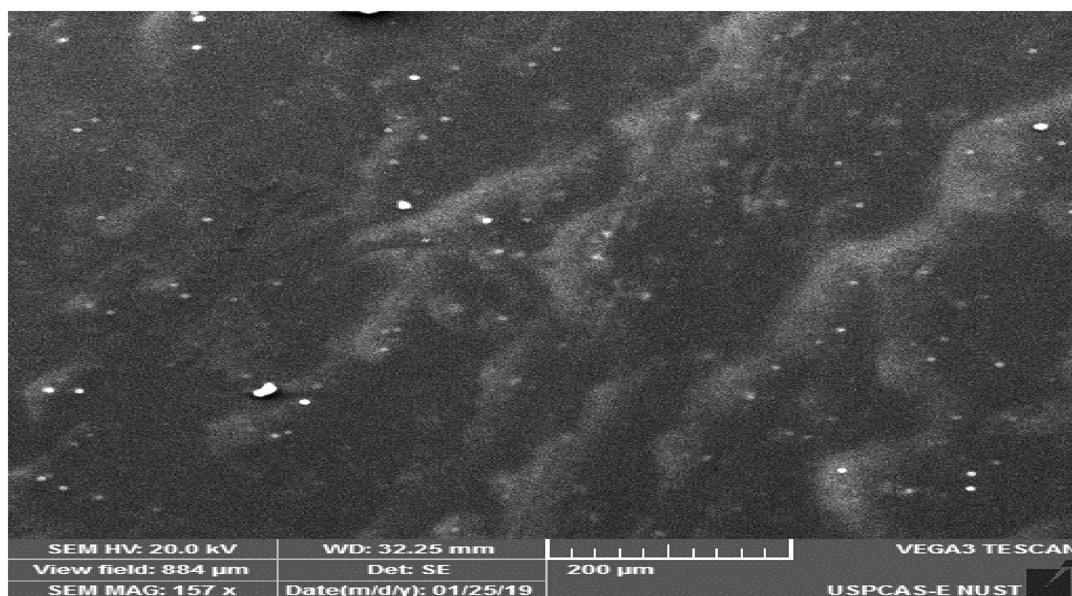

Figure 4.2.41: SEM image of biogenic AgNPs at 157kx and 200µm indicating formation of spherical nanoparticles

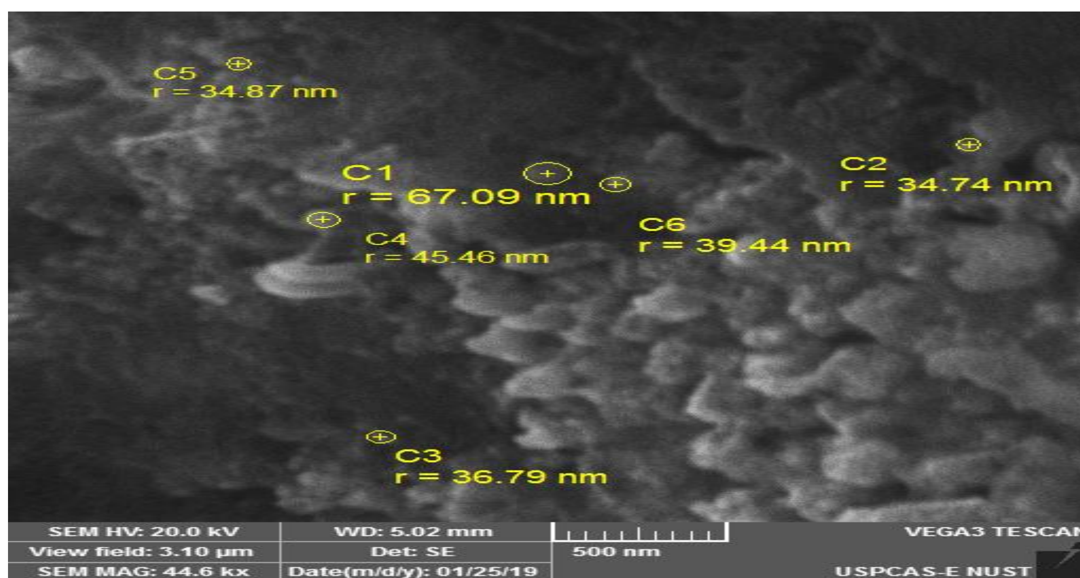

Figure 4.2.42: SEM image of biogenic AgNPs at 44.6 kx and 500nm indicating various sizes of nanoparticles

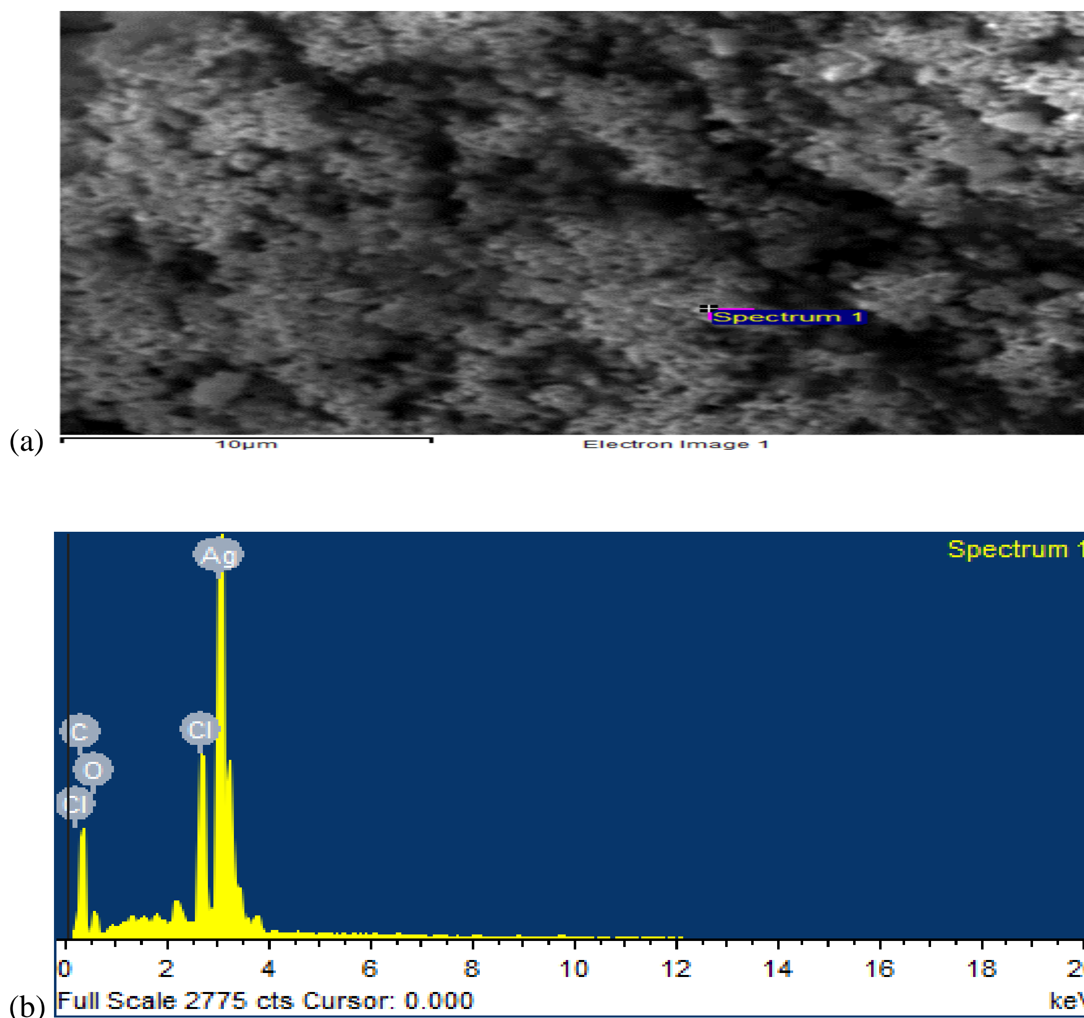

Figure 4.2.5: **Energy Dispersive X-ray Spectroscopy** (a) SEM region of sample from where EDS graph was taken (b) The EDS graph with energy on X-axis and counts on Y-axis , it shows the elemental composition of AgNPs in colloidal solution and confirms the presence of AgNPs at a highest spectrum peak of 3KeV

Table 4.2.5: Atomic percent and weight of the elements in AgNPs

Element	Weight %	Atomic %
Silver	43.82	9.04
oxygen	10.63	14.78
Chlorine	6.66	4.18
Carbon	38.89	72.02

4.3 Radical Scavenging Activity of *Thymus serpyllum* mediated AgNPs

DPPH assay was employed to evaluate the antioxidant ability of biosynthesised silver nanoparticles. The assay revealed that the scavenging ability of these nanoparticles increases as the concentration of nanoparticles increases Figure 4.7.

The standard ascorbic acid showed maximum free radicals scavenging ability of 80 % at a concentration of 100 µg/ml while the silver nanoparticles at the same concentration scavenged 78% of the free radicals .The IC50 of silver nanoparticles was found to be 8 µg/ml while that of standard ascorbic acid showed IC50 of 5.5 µg/ml. This suggests that the biologically synthesized AgNPs can be used as antioxidants.

4.4 Enzyme Inhibition Ability of AgNPs by α Amylase Inhibition Assay

The enzyme, alpha amylase catalyzes the hydrolysis of α 1-4 glycosidic linkages in polysaccharides so its inhibition possess a significant role in controlling hyperglycemia. The assay was performed to assess the enzyme inhibition potential of the synthesized silver nanoparticles. Acarbose served as a standard while the sample without AgNPs and containing enzyme was used as standard. The inhibition potential was expressed as percent inhibition. The synthesized silver nanoparticles inhibited the alpha amylase in a dose dependent manner comparable to that of standard acarbose Figure 4.4. The standard acarbose showed maximum enzyme inhibition of 90% at a concentration of 80 µg/ml while *Thymus serpyllum* mediated AgNPs inhibited the enzyme upto 83% at a concentration of 80µg/ml. The IC50 of standard acarbose is 7.5µg/ml and that of silver nanoparticles has an IC50 of 10 µg/ml .Thus the synthesized silver nanoparticles could potentially be use as antidiabetic agents.

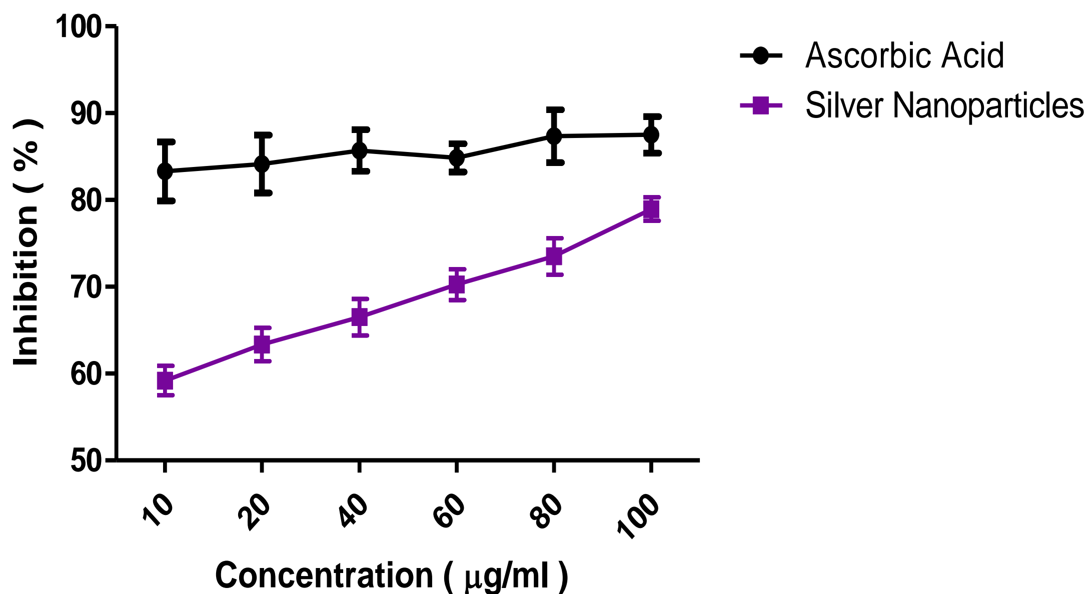

Figure 4.3: Comparisons of absorbance values of different concentrations of ascorbic acid and AgNP in DPPH assay

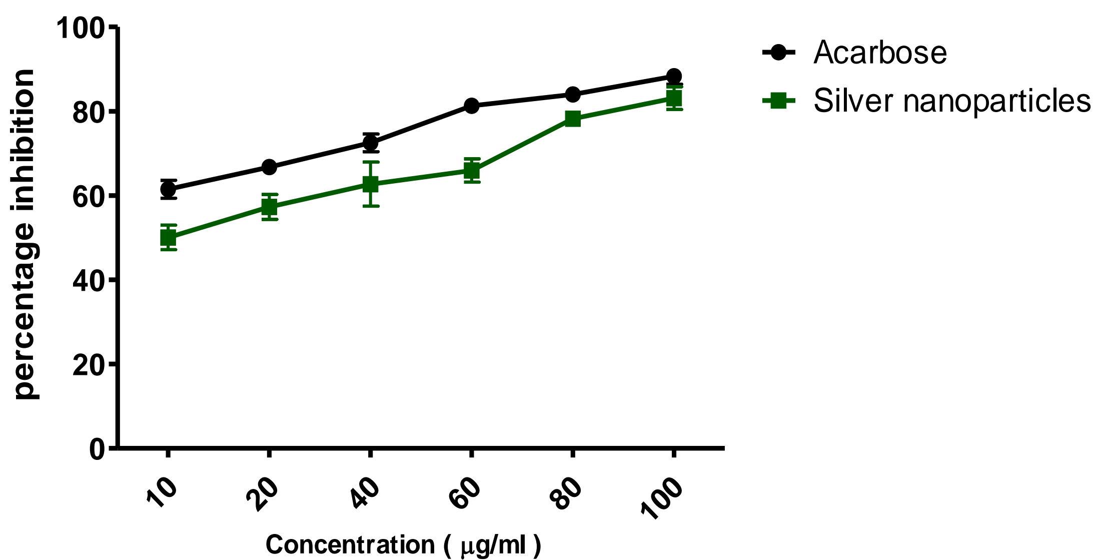

Figure 4.4: Comparison of % α-amylase inhibition of various concentrations of AgNPs and acarbose

4.5 Evaluation of therapeutic potential of AgNP (in vivo)

4.5.1 Estimation of Blood Glucose Levels during Diabetic Mouse Model Construction

The fasting blood glucose levels of all mice (n=50) i.e. control and Diabetic were measured after the first and second streptozotocin injection in diabetic mice at 6th at 9th week respectively in order to assure that diabetes is induced successfully after following a 42 days long protocol.

4.5.2 Estimation of AgNP treatment on Body weight

Effect on body weight of mice due to exposure to AgNPs was studied over the span of 4 weeks. The average weight of normal mice was compared to the diabetic group and the rest of the treated groups as metformin, AgNPs low and high dose treated respectively. The body weight of the diabetic group significantly increased since it did not received any treatment after HFD. While in those of treated groups i.e. the standard metformin, 5mg/kg AgNPs and 10mg/kg AgNPs in comparison to control showed no significant effect on body weight.

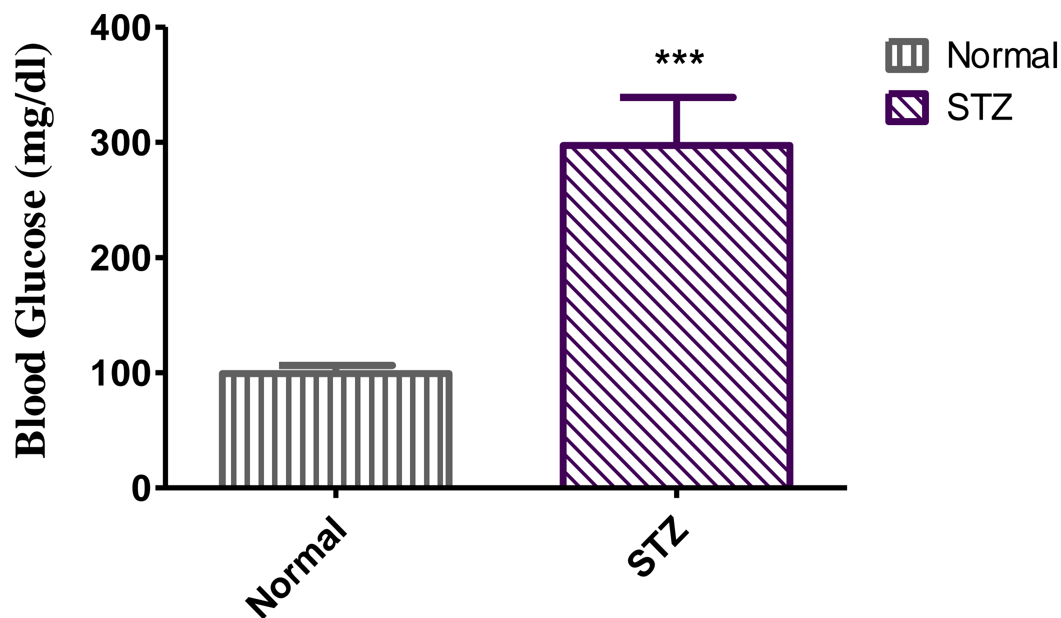

Figure 4.5.1: Average FBG levels (mmol/L) in control mice and mice fed with HFD from age of 3 to 9 weeks of age and rendered two doses of 100mg/kg STZ.

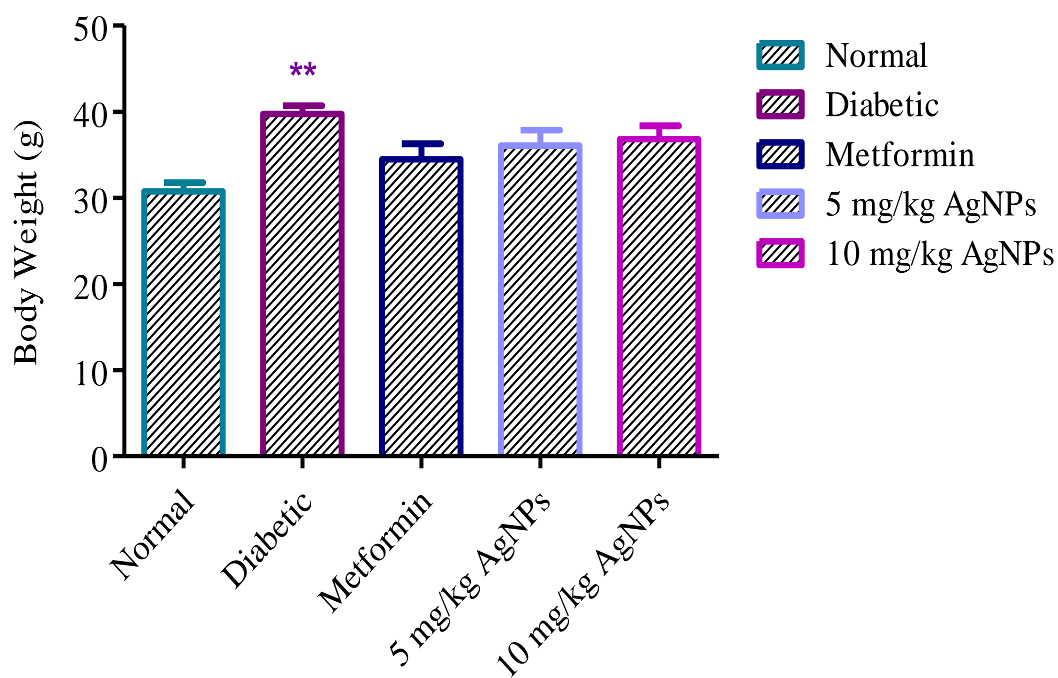

Figure 4.5.2: Body weights of treated and non-treated mice after 28 days treatment (** $p < 0.01$)

4.5.3 Comparative Analysis of Fasting Blood Glucose Levels in Treated Mice Groups

During 28 days long treatment, the blood glucose levels of mice of all groups were checked every week after overnight fasting. Due to HFD and STZ administration, the blood glucose levels of untreated group noticeably raised to a significant level from the normal group. During the initial two weeks of treatment, no significant reduction in the blood glucose levels of treated groups was observed.

Comparatively, the 10mg/kg AgNPs treated group after 21 days of treatment significantly reduced the blood glucose levels. At the end of treatment (after 28 days), the FBG levels of AgNPs treated groups were slightly higher than the normal, but in comparison with the untreated diabetic mice models, there was a significant reduction in their glucose levels. Moreover, the metformin and 5mg/kg AgNP treatment had almost similar effects, however, the 10mg/kg AgNP treated group showed a better reduction in blood glucose levels comparable to the control group.

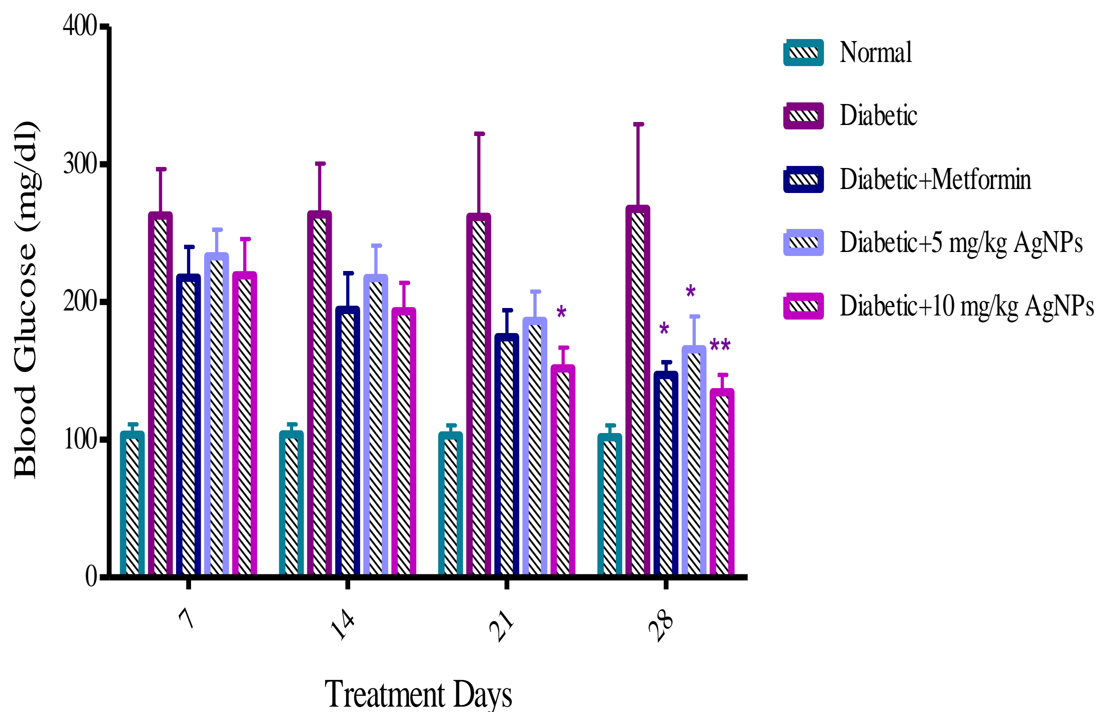

Figure 4.5.3: Histograms represent the effect of AgNPS Treatment on Fasting Blood Glucose (FBG levels (mmol/L) measured from Day 1 to Day 28 in comparison to Diabetic group. The data was analyzed using Two-way ANOVA following Bonferroni Post Hoc test and is shown as mean ± SEM (* $p<0.05$ and ** $p<0.01$).

4.5.4 Assessment of Glucose Tolerance in mice by Intra-peritoneal Glucose Tolerance Test

One of the main feature of IR is inability to tolerate the glucose. The glucose tolerance ability of diabetic mouse models was analyzed after 28 days of treatment with AgNPs. The untreated diabetic mice shows glucose intolerance while the metformin treated, 5mg/kg and 10mg/kg AgNP treated group improved the glucose tolerance to a noticeable level.

Area under the Curve was plotted to show the glycemic index of each group. 10mg/kg AgNPs treated group showed similar effect to that of metformin treated diabetic mice on enhancement of glucose tolerance, as shown by the expression of glycemic response in AUC form.

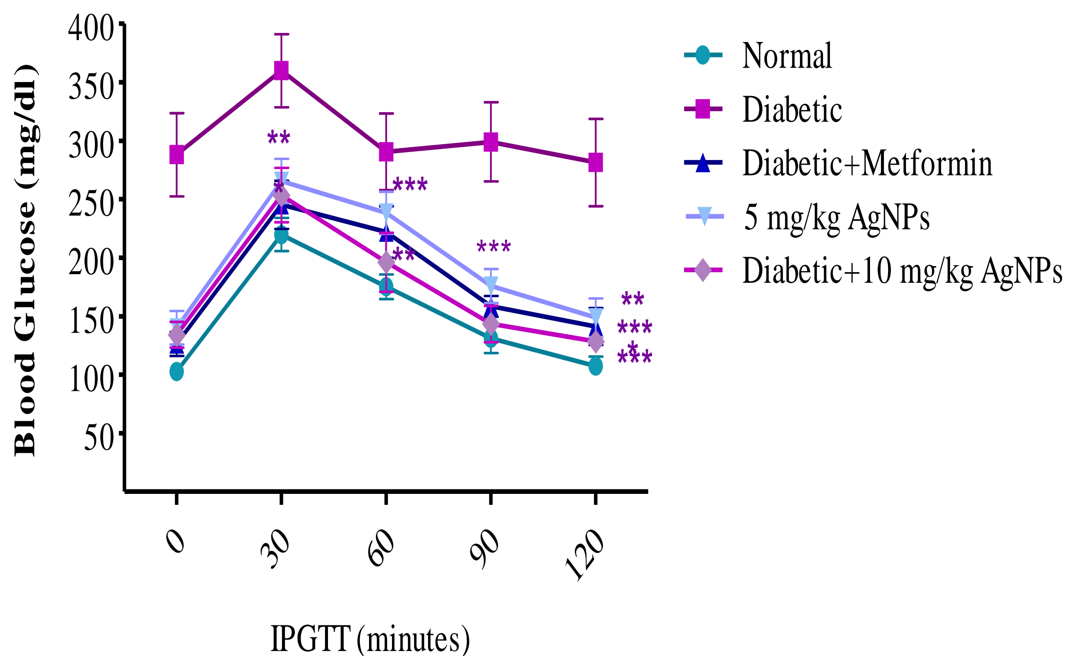

Figure 4.5.4.1: IPGTT results of the healthy, diabetic, metformin treated and AgNPs low (5mg/kg) and high dose (10mg/kg) mice post treatment. The statistical significance of differences among different groups was analyzed using Two Way ANOVA Bonferroni test as implemented in Graph pad prism 5.0 software (* $p<0.05$ ** $p<0.01$, *** $p<0.001$).

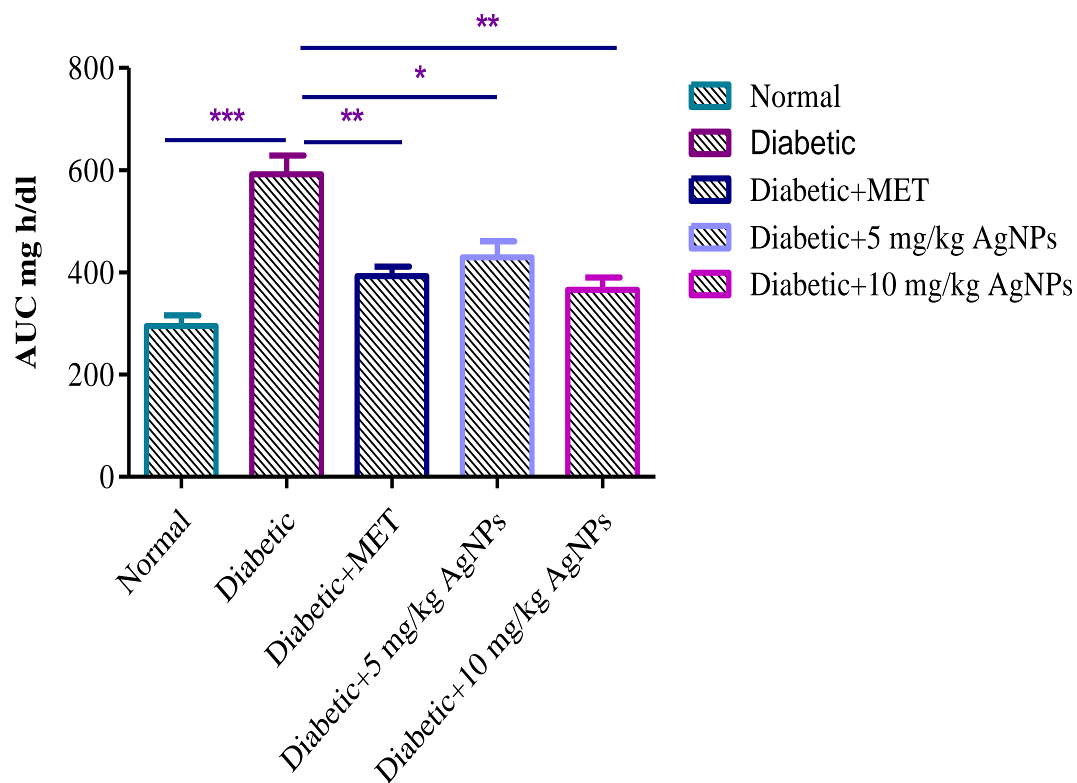

Figure 4.5.4.2: Area under the Curves (AUC) for IPGTT .The statistical significance of differences among different groups was analyzed using Two Way ANOVA Bonferroni test as implemented in Graph pad prism 5.0 software (* $p<0.05$ ** $p<0.01$, *** $p<0.001$).

4.5.5 Estimation of Insulin Tolerance in Treated Mice by Insulin Tolerance Test

Insulin tolerance test was carried out to determine the effect of AgNP treatment on action of insulin. After the completion of treatment duration, Insulin tolerance test was performed after 36 hrs. After injecting insulin to all groups, the glucose levels in blood was measured using glucometer at intervals of 0, 30, 60, 90 and 120 minutes. The untreated diabetic mice showed a greater extent of insulin intolerance so the glucose levels remained significantly higher from the normal range, while, the metformin treated, 5mg/kg and 10mg/kg AgNP treated group showed sensitivity towards the insulin and utilized the insulin to lower the blood glucose levels (Figure 4.5.5.1).

Area under the Curve in untreated diabetic group was much higher after insulin administration as compared to the normal (Figure 4.5.5.2). In comparison with diabetic group, all treated groups i.e. diabetic standard drug treated, 5mg/kg and 10mg/kg treated mice groups significantly lowered the AUC levels in almost similar manner.

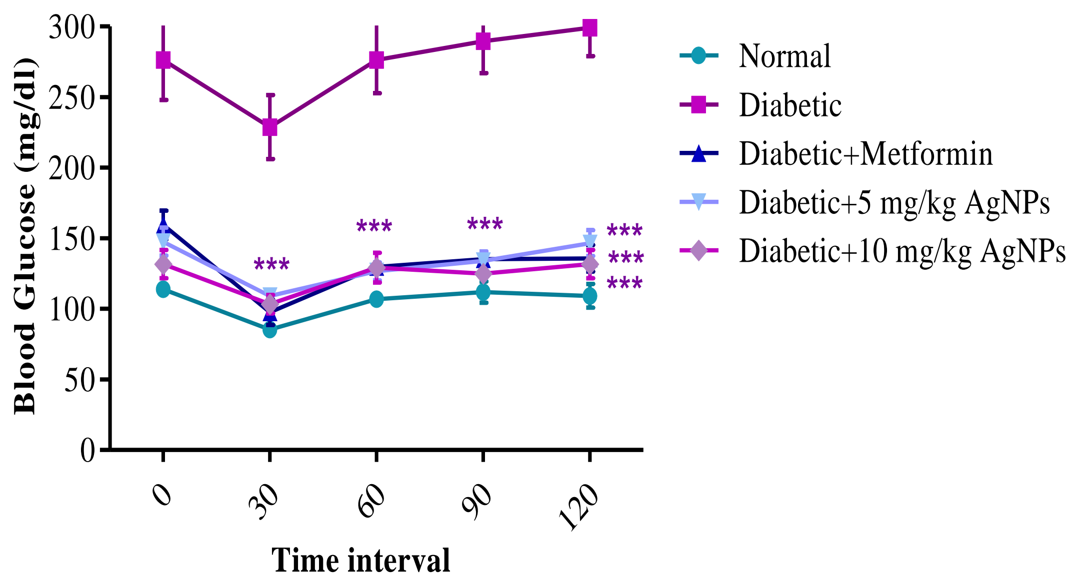

Figure 4.5.5.1: Effect of AgNPs on Insulin tolerance (day 31).Results of the healthy, diabetic, metformin treated and AgNPs low (5mg/kg) and high dose (10mg/kg) mice post treatment. The statistical significance of differences among different groups was analyzed using Two Way ANOVA Bonferroni test in Graph pad prism 5.0 software (*** $p < 0.001$).

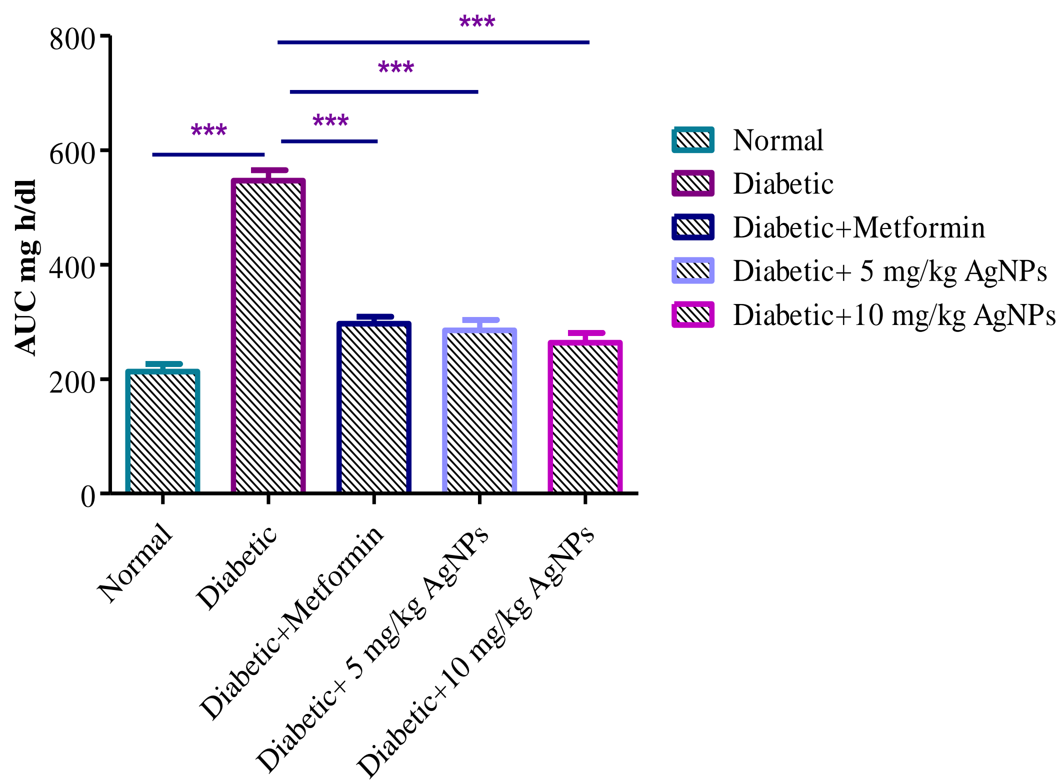

Figure 4.5.5.2: The Area under the Curve (AUC) for Insulin Tolerance Test (ITT).Analyzed Two Way ANOVA followed by Bonferroni Post Hoc test (*** $p < 0.001$)

4.6 AMPK and IRS1 Gene Expression Analysis through Real Time PCR

AMPK and IRS1 genes expression level in liver tissues of control, diabetic, diabetic mice treated with standard metformin and diabetic mice treated with low (5mg/kg) and high dose (10mg/kg) AgNPs were evaluated by semi-quantitative PCR and quantitative real time PCR.

4.6.1 Confirmation of cDNA synthesis

The real time primers of AMPK and IRS1 were optimized at 66°C and 64°C respectively and cDNA synthesis of all groups was confirmed through amplification with GAPDH Figure 4.6.1.

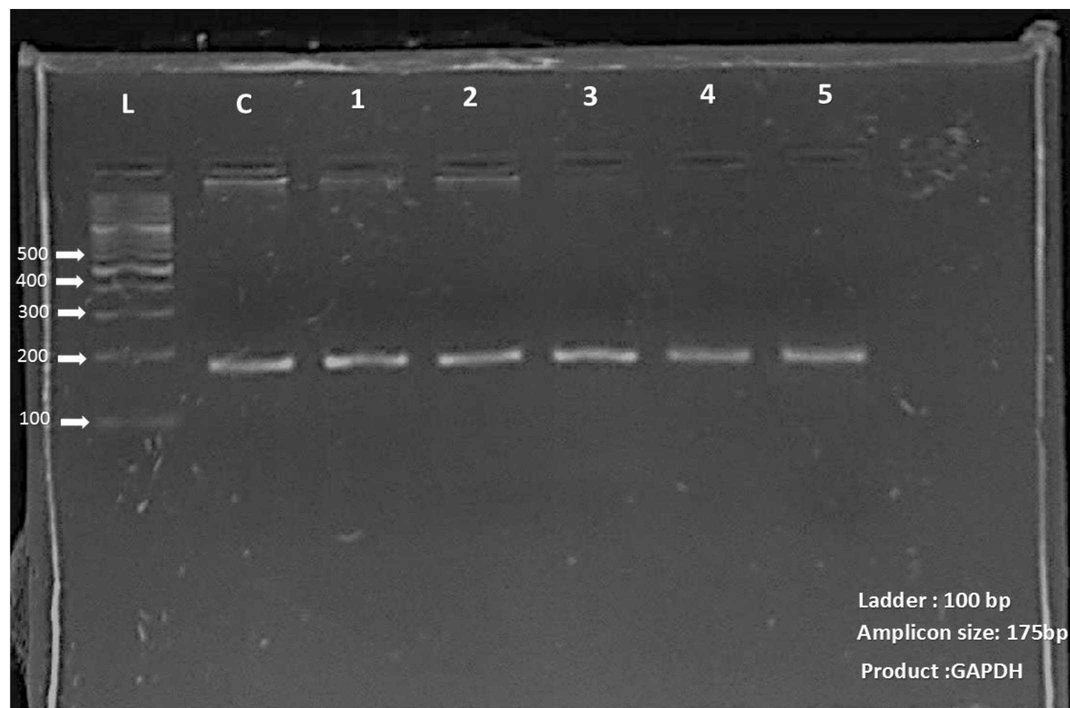

Figure 4.6.1: cDNA synthesis confirmation by amplification of GAPDH in Normal, Diabetic, metformin and AgNPs treated liver of BALB/c mice. L: Ladder, C: Positive Control, 1-5 cDNA amplification with GAPDH

4.6.2 Expression Analysis of IRS1 Gene

Quantitative real time PCR (qRT-PCR) was carried out to observe the expression of IRS1 gene in liver of normal, diabetic , metformin treated and AgNP (low and high dose) treated groups. It was observed that the expression level of IRS1 in diabetic group was significantly decreased (* $p<0.05$) as compared to the control (Figure 4.6.2).Upon comparison between Diabetic and Metformin treated group, a significant increase was observed (* $p<0.05$) in IRS1 expression in liver of metformin treated group.IRS1 expression in 5mg/kg AgNP treated group was upregulated to almost 2.5 folds in comparison to the untreated Diabetic group. 10mg/kg AgNP treated mice depicted approximately 4.5 fold increase in expression of IRS1 in comparison to the untreated diabetic mice ($p<0.001$).

4.6.3 Expression Analysis of AMPK Gene

The following graph shows the mean fold change in the expression of AMPK in Diabetic untreated , Metformin treated and Silver Nanoparticles treated mice groups (Figure 4.6.3).The expression of AMPK is significantly reduced in the diabetic untreated group (* $p<0.05$) in comparison to the control.

AMPK is the main target of the standard drug metformin so metformin treatment in diabetic mice increased its expression to almost 3 folds. But AMPK expression in low dose treated silver nanoparticles group increased to almost 1.5 fold while in the 10mg/kg AgNPs treated diabetic mice the expression fold increased almost 3 times comparable to the metformin treated group with a significance of (*** $p<0.001$).

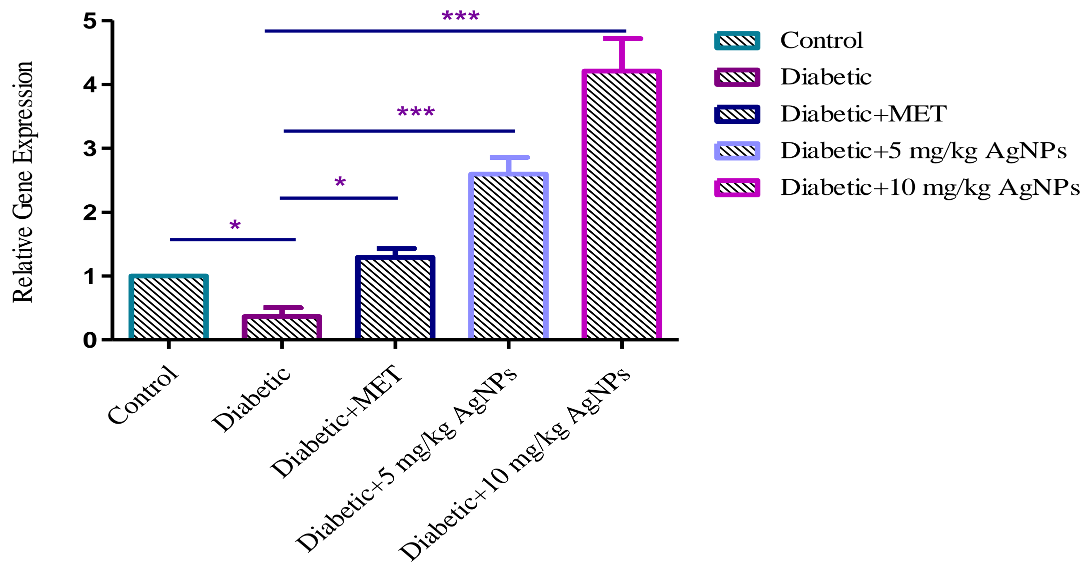

Figure 4.6.2: Relative expression of IRS1 in control, diabetic, metformin and AgNPs treated (28 days) groups. Significance was determined by One Way ANOVA following Bonferroni's Multiple comparison Post Hoc Test. Error bars represent the SEM (standard error of the mean) (* p<0.05 *** p<0.001)

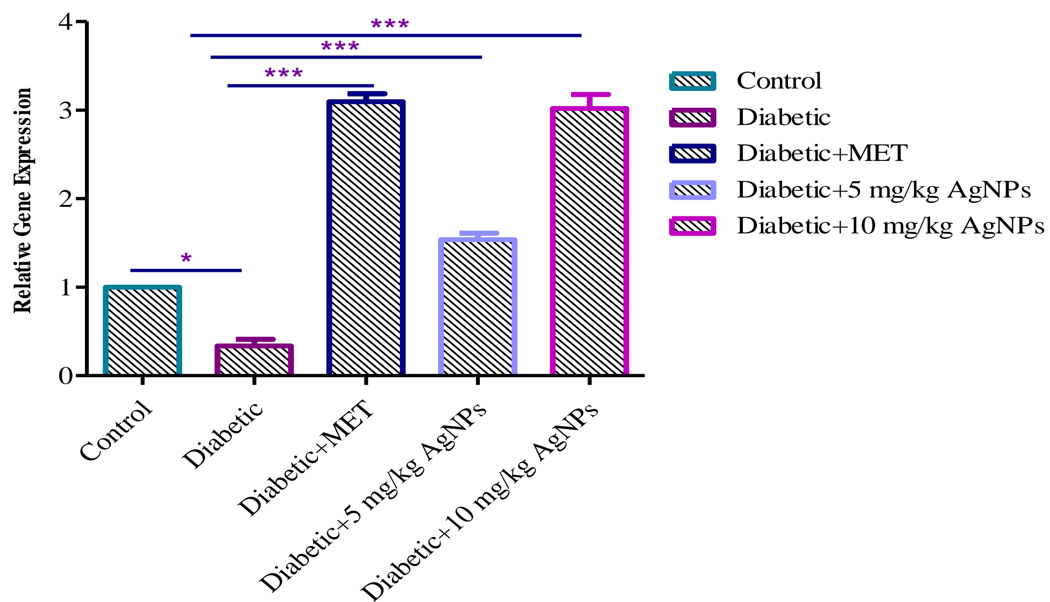

Figure 4.6.3: Relative expression of AMPK in control, diabetic untreated, diabetic metformin and AgNPs treated (28 days) groups. Significance was determined by One Way ANOVA following Bonferroni's Multiple comparison Post Hoc Test. Error bars represent the SEM (standard error of the mean) (* p<0.05 *** p<0.001)

4.7 Histopathological Analysis

The Liver, Kidney and Pancreas were from all groups were obtained for histological analysis after the required treatment was carried out. The histological examination overall showed that high dose silver nanoparticles restored the damaged and necrotic tissues to a greater extent.

4.7.1 Histopathological Examination of Control, diabetic and NP treated Liver

Haematoxylin and Eosin stained sections of Liver at 40X depicts the portal vein and the portal triad in normal, diabetic, metformin treated and silver nanoparticles treated groups of BALB/c mice Figure 4.7.1.1.

4.7.2 Histopathological Examination of Control, diabetic and NP treated Kidney

H & E stained sections of kidney from all groups were analyzed focusing on glomerulus and renal tubules arrangement and boundary (Figure 4.7.2).The cellular morphology and density is better restored by high dose silver nanoparticles i.e. 10mg/kg.

4.7.3 Histopathological Examination of Control, diabetic and NP treated Pancreas

Pancreas from all the groups was H&E stained and histological examination was carried out on various magnifications to compare the morphology of all treated mice versus diabetic groups. Islets of Langerhans and pancreatic acini were focused on 40X in all groups.

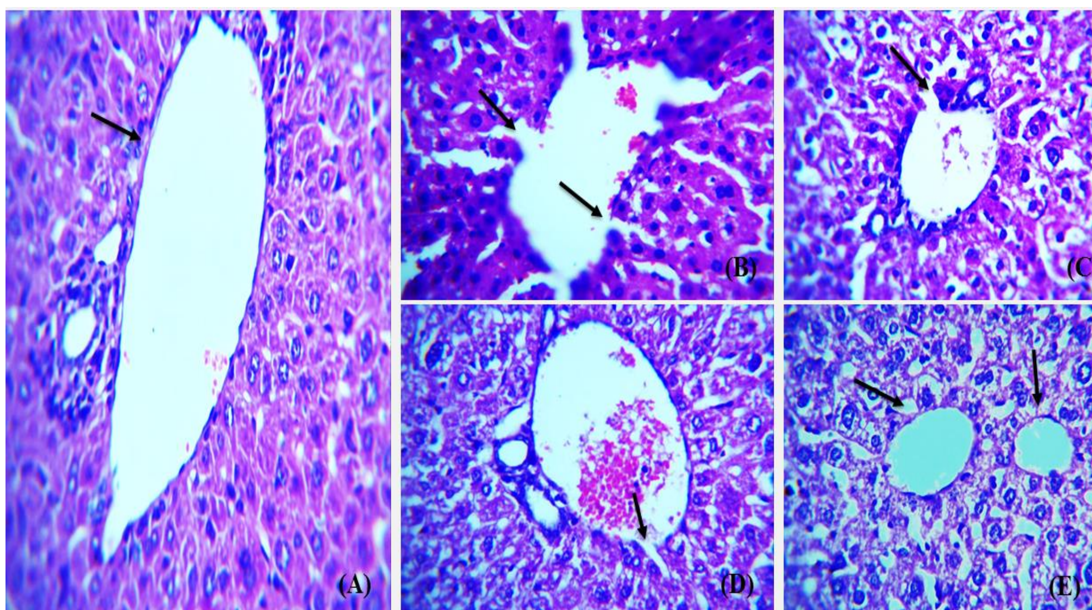

Figure 4.7.1: Representative images showing Hematoxylin and Eosin stained (H&E) (×40) sections of Liver **(A)** Control, Normal liver histology with portal triad and portal vein (arrow) with hepatocytes radiating from the portal vein **(B)** Diabetic group showing complete distortion of portal vein architecture and congestion of portal triad **(C)**Diabetic Metformin treated group showing somewhat restoration of hepatic architecture but dilated vascular channels**(D)**Diabetic mice liver treated with 5mg/kg AgNPs showing greater restoration of portal vein boundary with little dilation (arrow) and normal portal triad **(E)** Diabetic AgNP (10mg/kg) treated mice liver showing restored architecture comparable to that of normal with intact portal triad and normal physiology of kupffer cells (arrow).

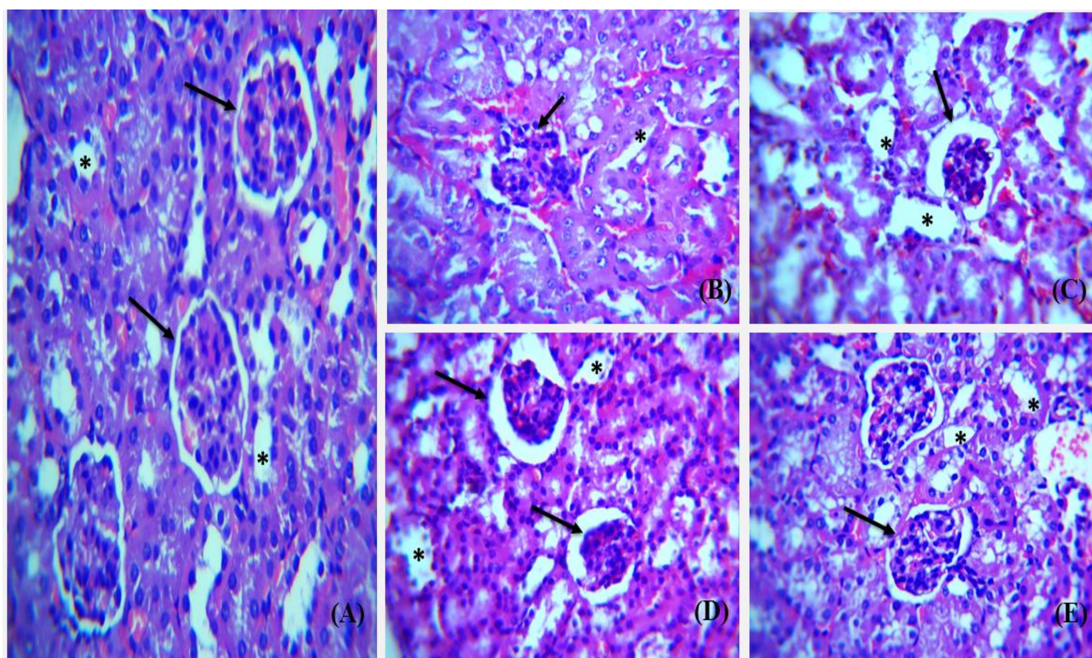

Figure 4.7.2: Photomicrograph showing Hematoxylin and Eosin stained (H&E) (×40) sections of kidney with asterisk showing kidney tubules & arrow showing glomerulus **(A)** Control group with regular shape of glomerulus and distinguishable collecting duct and renal tubule **(B)** Untreated Diabetic group showing irregular renal cells distribution ,tubules dilation and glomerulosclerosis **(C)** Diabetic Metformin treated group shows somewhat normal appearance of tubules and intact glomerular boundary when compared to the diabetic group **(D)** Diabetic AgNPs treated (5mg/kg) group showing restoration of glomerular boundary and intact kidney tubules **(E)**Diabetic AgNP (10mg/kg) treated group show more reno-protective activity as compared to the standard metformin with complete restoration of glomerulus and renal tubules

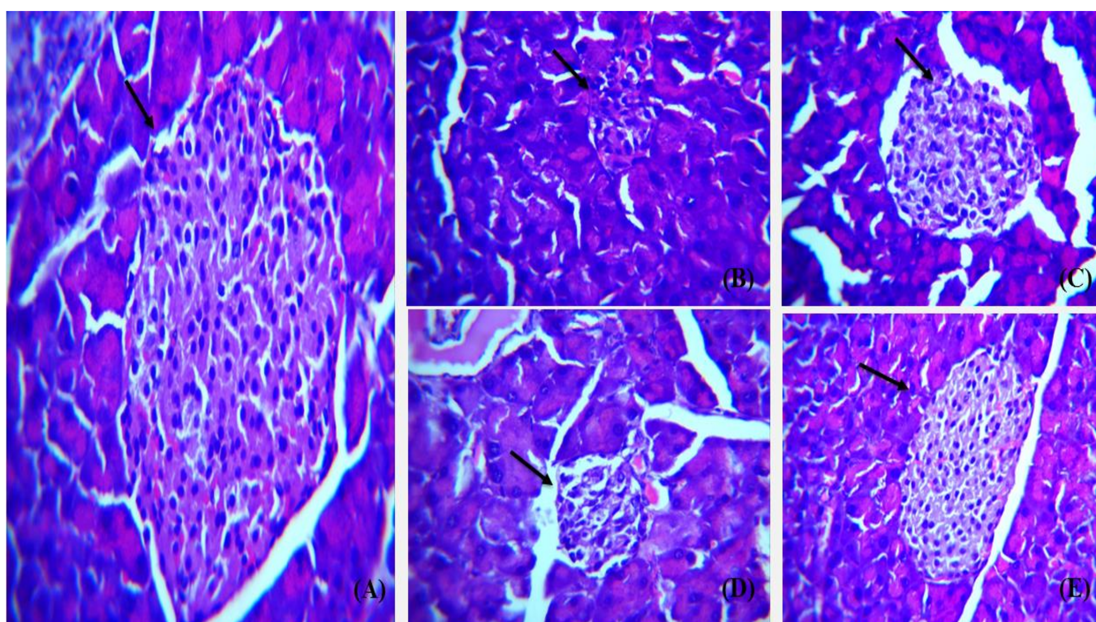

Figure 4.7.3: Representative images showing Hematoxylin and Eosin stained (H&E) (×40) sections of pancreas **(A)** Control, normal pancreas showing normal islets of Langerhans in between normal pancreatic β cells (arrow) **(B)** Diabetic pancreas showing ruptured and destructed islets of Langerhans with damage in B-cells **(C)** Diabetic and Metformin group pancreas showing some normal islets of Langerhans **(D)** Diabetic and AgNP (5mg/kg) mice pancreas showing some restoration in islets of Langerhans with damage in β-cells (arrow); and **(E)** Diabetic and AgNP (10mg/kg) pancreas showing complete restoration of islets of Langerhans .

Thus the cellular morphology and density of liver, kidney and pancreas is better restored by 10mg/kg dose of silver nanoparticles and also it does not cause any noticeable harm so the silver nanoparticles are safe to use as antidiabetic agents with respect to our study.

Chapter 5

DISCUSSION

Diabetes Mellitus is a multifactorial disorder encountering insulin resistance or failure of insulin action. The current available therapeutic approaches fail in the management of diabetes either due to the several side effects caused by them or due to their inefficacy in targeting the right pathway or mechanism. Scientists are now leading towards the Nanobiotechnology with great interest for digging the solution for diabetes management due to their untiring benefits at smaller scale. One of the aspect of nanotechnology is nanomedicine which will overcome the side effects of conventional drugs by better pharmacokinetic control and sustained release of therapeutic substance. Currently nanoparticles are employed generally in diabetes for monitoring of blood glucose levels, in bio sensing and imaging and as delivery vehicles for the insulin to eradicate the use of injectable insulin in Type 1 Diabetes Mellitus (T1DM). But there is a need to focus on insulin sensitization and Beta cell mass regeneration in T2DM (Mukhopadhyay and Prosenjit 2018). However, recent research on plant extract mediated nanoparticles has gained more attention because of excessive presence of various chemicals compounds that act as a reducing as well as capping agents to stabilize the nanoparticles (Parveen, Banse et al. 2016). Silver nanoparticles from biogenic sources have been elucidated to reduce the oxidative stress in diabetes and act as alpha amylase inhibiting agents (Muniyappan and Nagarajan 2014).

In view of this knowledge, this study aimed to develop such nanoparticles which have therapeutic efficacy as antidiabetic agents. Biogenic silver nanoparticles using *Thymus serpyllum* extract were successfully synthesized for the first time in this study. *Thymus serpyllum* extract either methanolic or aqueous have been reported in many studies to possess different biological effects as anti-rheumatic, anti-cancer, anti-inflammatory

and anti-diabetic (Nikolić, Glamočlija et al. 2014). So, the current study aimed on analyzing the antidiabetic effect of *Thymus serpyllum* mediated silver nanoparticles by estimating the blood glucose levels in mice by FBG, ITT & OGTT tests and determining their radicals scavenging potential by DPPH assay. The effect of silver nanoparticles on genes of AMPK pathway and Insulin signaling pathway was assessed by conducting the expression profile of IRS1 and AMPK gene and associated toxicity with silver nanoparticles was determined by histological examination of tissue samples from liver, kidney and pancreas of mice.

In this study, protocol for the synthesis of silver nanoparticles from *Thymus serpyllum* was optimized by varying the concentrations of extracts and the silver nitrate which was used as a precursor. This plant is rich in the bioactive compounds like amine, alkynes, flavonoids, alkenes and phenols (Jovanović, Đorđević et al. 2017) .These compounds aid in reduction of the silver ions and act as stabilizing agents for silver nanoparticles.

The color change of reaction mixture from light yellow to brown during a five hour reaction indicated the preliminary confirmation of silver nanoparticles synthesis, likewise, the SPR band for AgNP was obtained at 425nm. The optimal conditions for AgNP synthesis were 25°C for 5 hours for a 10mM silver nitrate and 14.25ml diluted plant extract.

The XRD pattern of silver nanoparticles in our study showed seven intense peaks at 28°, 32°, 39°, 46°, 55°, 57° and 68° which were indexed at (111), (200), (220), (311), (222), (400) and (331) facets of silver respectively which correspond to the JCPDS Card No. 00-04-0783 in Jade software. Similar peaks for silver nanoparticles are obtained and cited in various literature (Hu, Wang et al. 2008) (Theivasanthi and Alagar

2011, Bindhu and Umadevi 2015). The SEM images revealed the mean particle size of 42nm and showed that the AgNPs are uniformly distributed and are spherical in shape , this shape is in accordance with the various nanoparticles synthesized by other researchers (Bar, Bhui et al. 2009, Iravani 2011).

EDS was done to figure the elemental composition and their % weight in nanoparticles .In our results the percentage of silver occurred to be 9.04% which is appropriate , as drug and phytochemicals were also the part of synthesis .The presence of other elements like carbon , oxygen and chlorine were due to biological source i.e. plant as reported in literature (Imelouane, Tahri et al. 2011).FTIR results revealed that silver nanoparticles and the plant extract have some shared peaks. The peaks at 2924 cm^{-1} (C-H stretching), 1620 cm^{-1} (N-H bending), 1510 cm^{-1} (N-O stretching and 1175 cm^{-1} (C-O bending) were present in both plant extract and the silver nanoparticles. Similar results were reported in other studies where silver nanoparticles were synthesized from plant extract (Kumar, Pammi et al. 2014, Ahmed, Saifullah et al. 2016).

The second objective of our study was the evaluation of antidiabetic potential of these *Thymus serpyllum* mediated silver nanoparticles. The bioassays such as DPPH showed that the synthesized nanoparticles scavenge the free radicals in a dose dependent manner and similarly inhibit the amylase activity with respect to the dose of AgNPs in alpha amylase inhibition assay. Ascorbic acid which is standard showed 80% scavenging of free radicals at 100µg/ml concentration while our nanoparticles scavenged 78% of the radicals at the same concentration. Likewise , the silver nanoparticles showed alpha amylase inhibition activity of upto 83% while the standard acarbose had 90% enzyme inhibition activity at a concentration of 80µg/ml. Biogenic silver nanoparticles have potent alpha amylase inhibition and DPPH radicals scavenging ability reported in various findings (Balan, Qing et al. 2016).

To further assess the antidiabetic potential of our synthesized nanoparticles, in vivo studies on BALB/c mice models were conducted. Silver nanoparticles treated diabetic rats have previously been reported as effective insulin sensitizing agents and in improvement of expression level of GLUT2 and IRS1 genes (Alkaladi, Abdelazim et al. 2014). Results shown by efficacy assessment of biosynthesized nanoparticles in vivo were synchronous to the results obtained in vitro against DPPH and alpha amylase assays. The 5mg/kg AgNP treated mice group enhanced the expression levels of AMPK & IRS1 genes somehow similar to the results obtained for metformin. But the 10mg/kg dose of silver nanoparticles significantly improved the said genes levels and didn't show any contradictory toxicity.

In support of recent findings, our histopathological assessment presented more promising observations for tissue sections treated with silver nanoparticles. The silver nanoparticles treatment did not disturbed the normal morphology of liver, kidney and pancreas, in contrast, they improved the beta cell mass and restored the degenerated boundaries of the defected organs.

In short , the 10mg/kg dose of silver nanoparticles give better results for expression studies of IRS1 & AMPK and restored the morphology of pancreas , liver and kidney in diabetic mice to a greater extent in comparison to the 5mg/kg dose of silver nanoparticles and the standard metformin drug.

Chapter 6

CONCLUSION & FUTURE PERSPECTIVE

The main objective was the formulation of a nanodrug for diabetes using green route which could be safer and biocompatible than the synthetic drugs with fewer side effects. The medicinal plant "*Thymus serpyllum*" aqueous extract rich in bioactive compounds served as a reducing agent to synthesize and then as capping agent to stabilize the nanoparticles .The newly biosynthesized silver nanoparticles were confirmed via characterization techniques such as SEM, XRD, EDS and FTIR depicting spherical 42nm size nanoparticles with JCPDS Card No. 00-04-0783.Smaller size nanoparticles show better antioxidant and alpha amylase inhibition activity and increasing the dose of AgNPs increased the scavenging ability as well as amylase inhibition. The synthesized nanoparticles improved the insulin sensitivity and glucose tolerance in treated mice and reduced fasting glucose levels significantly. We conclude that the synthesized nanoparticles are more powerful in reducing the hyperglycaemia and enhance the IRS1 and AMPK expression for treatment of diabetes which aid in glucose uptake and inulin sensitization. Our results suggest that Silver Nanoparticles potentially restore the cellular morphology of liver, kidney and pancreas specifically. However, further research is needed to confirm these findings.

There is a need of further *in vivo* pharmacological investigations to elucidate the mechanism of action of AgNPs by targeting other genes involved in diabetes. Furthermore, functionalization of these AgNPs with various drugs could be done to provide sustained release of the drug at site of action in body.

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