Therapeutic Efficacy of *Thymus serpyllum* Derived Silver Nanoparticles in Streptozotocin Induced Diabetic BALB/c Mice

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A thesis submitted in partial fulfilment of the requirement for the degree of

Master of Science (MS)

In

Healthcare Biotechnology

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DECLARATION

I, **Ayesha Imran,** declare that this research work titled **"Therapeutic Efficacy of *Thymus serpyllum* Derived Silver Nanoparticles in Streptozotocin Induced Diabetic BALB/c Mice"** is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of Dr. Peter John. The material that has been used from other sources has been properly acknowledged/ referred.

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Ayesha Imran

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CERTIFICATE FOR PLAGIARISM

It is certified that MS Thesis entitled **"Therapeutic Efficacy of *Thymus serpyllum* Derived Silver Nanoparticles in Streptozotocin Induced Diabetic BALB/c Mice" of Ms. Ayesha Imran,** Regn. No. 00000277617 has been examined by me. I undertake that:

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Ayesha Imran

I dedicate the outcome of my efforts to my beloved parents Mr. and Mrs. Imran and my sister (Laiba Noureen) who have always stood by my side and supported every decision I've ever made.

TABLE OF CONTENTS

LIST OF ACRONYMS

AgNPs	Silver Nanoparticles
AgNO3	Silver nitrate
µg	Microgram
2D	2 Dimensional
3D	3 dimensional
ANOVA	Analysis of Variance
GLUT4	Glucose Transporter 4
GLUT2	Glucose Transporter 2
IRS2	Insulin Receptor Substrate 2
INS2	Insulin 2
AMPK	Adenosine Monophosphate Kinase
T2DM	Tpe2 Diabetes Mellitus
T1DM	Tpe1 Diabetes Mellitus
STZ	Streptozotocin
UV Vis	Ultraviolet Visible
XRD	X-ray Dispersive Spectroscopy
FBG	Fasting Blood Glucose level
PDB	Protein Data Bank

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Type 2 diabetes mellitus (DM) is a chronic metabolic disorder and is characterized by means of consistent hyperglycemia as a consequence of either entire or incomplete deficiency of insulin secretion and action of insulin. The most commonly used therapeutic agent used for type 2 diabetes mellitus is metformin. However, it has various side effects which include its slow mode of action and gastrointestinal infections. Therefore scientists are trying to overcome these limitations by promoting nanomedicine that can be synthesized from biological sources. In nanomedicine, silver nanoparticles have gained more importance due to their strong anti-diabetic, anti-oxidant and antimicrobial properties.

The present study reports a green, economical and biological means for the efficient production of the silver nanoparticles (AgNPs) from the extract of *Thymus serpyllum*. This synthesis method was also optimized under different physiochemical parameters such as temperature and molar concentration ratios to obtain narrow size distribution of AgNPs. XRD was used to characterize these silver nanoparticles. To induce diabetes in BALB/c mice, streptozotocin (STZ) injections were given along with high fat diet. Primers for Glut4, INS2, IRS2 were designed by Primer 3 Plus and then validated by UCSC In-Silico PCR.

The results were initially assessed by UV-VIS spectroscopy, showing maximum absorbance at the characteristic wavelength of 400nm. The shape of these silver nanoparticles was found spherical. XRD pattern of silver nanoparticles showed six intensive peaks. The difference between Fasting Blood Glucose (FBG) level and body weight of control and experimental mice showed the successful induction of diabetes in BALB/c mice. UCSC In-Silico PCR gives the primers that showed binding to the target region and showed no off-target binding. Thus, silver nanoparticles having anti-diabetic potential can be synthesized from *Thymus serpyllum* extract under optimized conditions.

CHAPTER 1

INTRODUCTION

1. Diabetes mellitus

Diabetes mellitus is delineated through means of persistent hyperglycemia as well as deterioration in the metabolism of carbohydrates, lipids, proteins that is imputable to either absolute or incomplete deficiency of insulin secretion or insulin action. The persistent hyperglycemia of the diabetes is interconnected with protracted damage, pathology, anomaly, and malfunction of various organs, substantially the eyes, kidneys, nerves, heart, and blood vessels. There are two crucial forms of diabetes, Type1 Diabetes Mellitus, T1DM and Type2 Diabetes Mellitus, T2DM (Galtier, 2010; Diabetes Care 2014).

1.1 Type2 Diabetes Mellitus

Type2 Diabetes Mellitus is additionally known as **Insulin Independent Diabetes Mellitus** (IDDM). It is an inimitable category of DM that corresponds about 90% to 95% of whole diabetic inmates (Tripathi, 2006). It is inevitable that the cases will be increase to 439 million by 2030 (Chen et al., 2011). T2DM is a **polygenic disorder** delineated by various inadequacies in insulin activity within internal organs and the scarcities in pancreatic secretion of insulin, a fact which ultimately stimulates failure insulin secreting cells in pancreas (Israili, 2011). Type2 DM occurs as a result of interactions between genetic, behavioral as well as environmental risk factors (Chen, 2011).

T2DM inpatients can further vulnerable to diverse sorts of complications i.e. transitory and protracted time period complications. The complicacies (figure 1.1) consist of **macrovascular**

problems (hypertension, heart attacks, hyperlipidemia, strokes, coronary disorders, cerebral and peripheral vascular abnormalities), **microvascular** problems (retinopathy, neuropathy, and nephropathy) and cancers (Yanling et al., 2014).

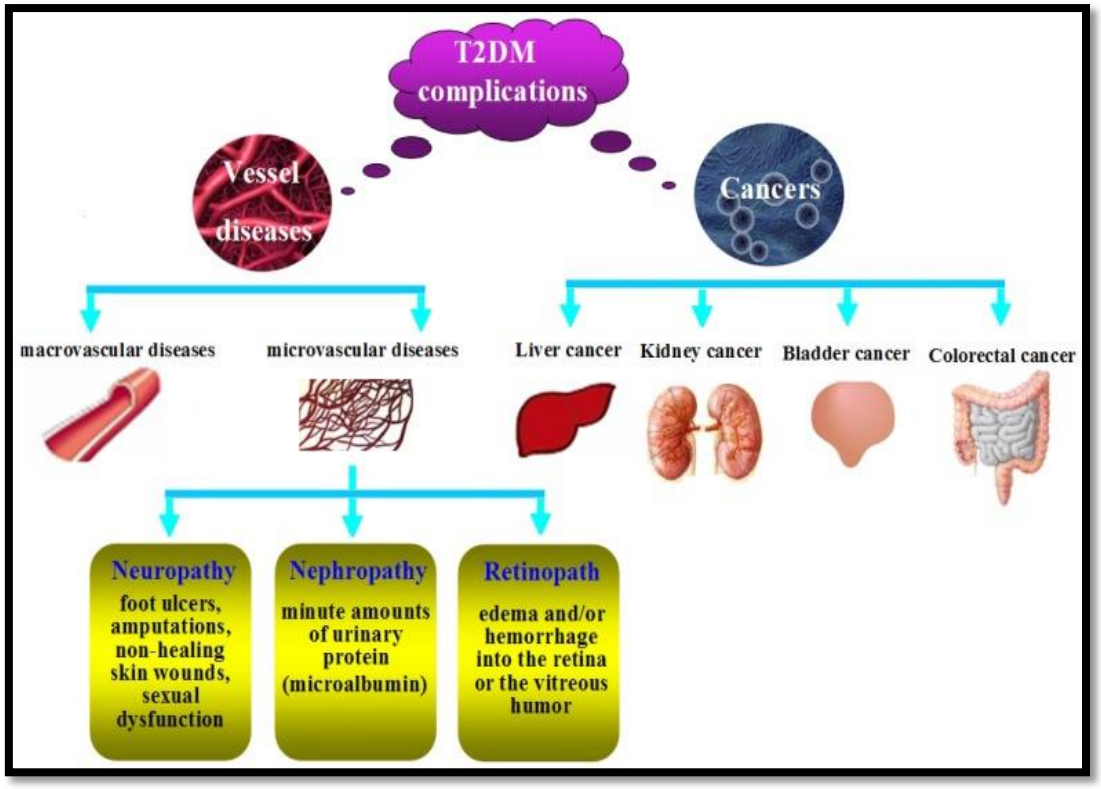


Figure 1.1: Complication of Type2 Diabetes Mellitus (Yanling et al., 2014)

Cardiovascular disorder is the crucial incentive of fatality and morbidness in prediabetes as well as T2DM, for which oxidative stress is effectual and influential mechanism (Chaturved, 2007). Diabetic neuropathy can additionally established a correspondence among foot ulcers, amputations, noxious skin injuries and sexual abnormalities. The neuropathy will result into failure of defensive sensibility in the foot that utmost terminates in callous formulation, contusion as well as auxiliary impairment and could additionally bring about contamination in the epidermis (Sanghera DK, Blackett PR, *2012)*. Epidemiologic data has established that diabetes can enhance the contingence of cancer for instance colorectal cancer, , bladder

cancer, liver cancer, breast cancer , kidney cancer , that deteriorates relying on sub sites of distinctive cancers (Donadon et al., 2008; Larsson and Wolk, 2011).

1.2 Epidemiology/Prevalence of Diabetes Mellitus

Diabetes is a most important health issue as it has progressed to the petrifying levels. In 2019, there are almost semi quadrillion people (9.3% of adult's 20–79 years) that have diabetes across the board. The predictable numeral of personages (20–79 years) that are suffering from diabetes has been increased by 62% during the period of last 10 years; from 285 million in 2009 to 463 million, at present and will intensified to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045. The prevalence of diabetes by age and gender in is given in figure 1.2 (Saeedi et al., 2019).

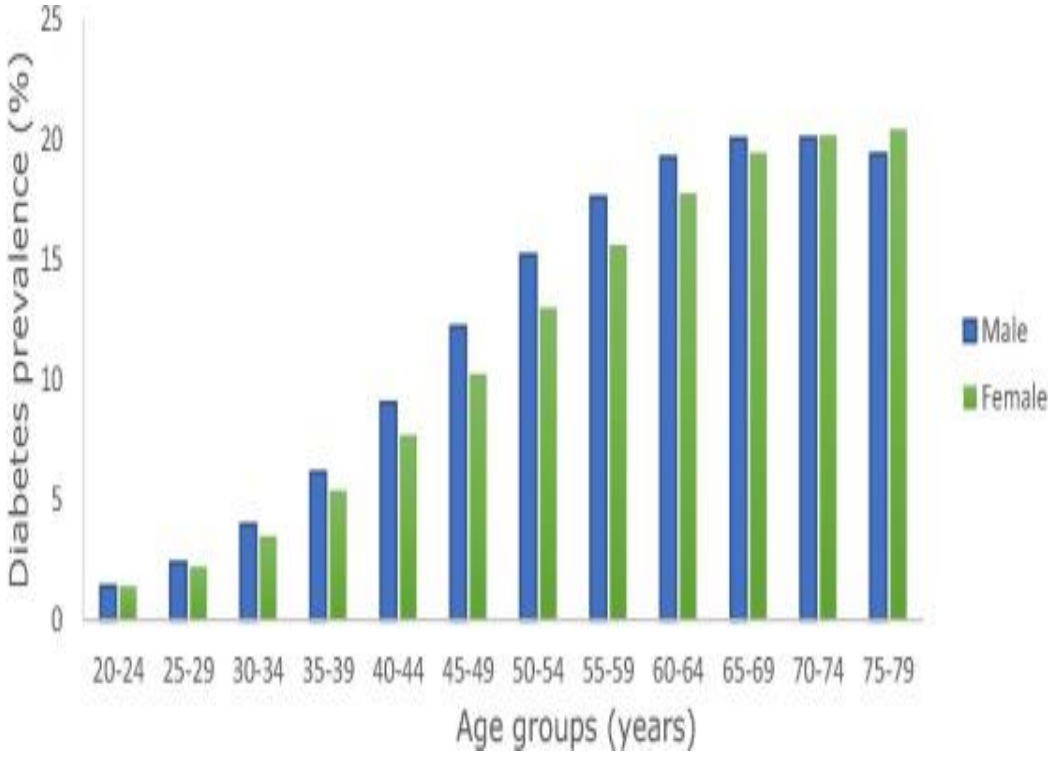


Figure 1.2: Prevalence of diabetes (Saeedi et al., 2019)

The incidence of diabetes has been expanding rapidly and this is the foremost incentive of presbyopia, retrogression of kidneys, stroke, acute myocardial infarction as well as inferior forelimb disjuncture. In 2016, there was a probability that almost 1.6 million deaths were directly caused by diabetes. According to World Health Organization (WHO), diabetes is the seventh leading cause of death in 2016 (International Diabetes Federation, 2017).

Approximately, semi percent (50.1%) of the population/inhabitants accompanied by diabetes do not comprehend that they are suffering from diabetes. Type 2 diabetes depicts the most prevalent category of diabetes. It is responsible for about 90% of entire diabetic inpatients as compare to type 1 diabetes that accounts for approximately 10% (Sarwar et al., 2010). The pervasiveness of type 2 diabetes and prediabetes has increased in Pakistan. Pakistan has approximately 74 lac adults with diabetes and therefore ranked as 10^{th} because of highest number of diabetes cases (Sherin, 2015).

1.3 Pathophysiology/Pathogenesis of Diabetes Mellitus

1.3.1 Normal Glucose Homeostasis

Glucose is transported in the blood to certain tissues to be used as energy. **Insulin** that helps the absorption of glucose originating at blood circulation with the aid of fat tissue and skeletal muscles, is itself symphonized as well as originated by Beta cells that are present inside the pancreas. Insulin serves as the hormone (peptidal hormone) which has phenomenal ability to regulate the assimilation of fat as well as carbohydrate within an individual's body (AlSaraj, 2015).

There are four transporters of glucose (GLUT1, GLUT2, GLUT3 and GLUT4). After intake of meal, blood glucose enters amongst the cell which is facilitated by Glucose transporter type 2 (GLUT2) membrane transporters as shown in the figure 1.3

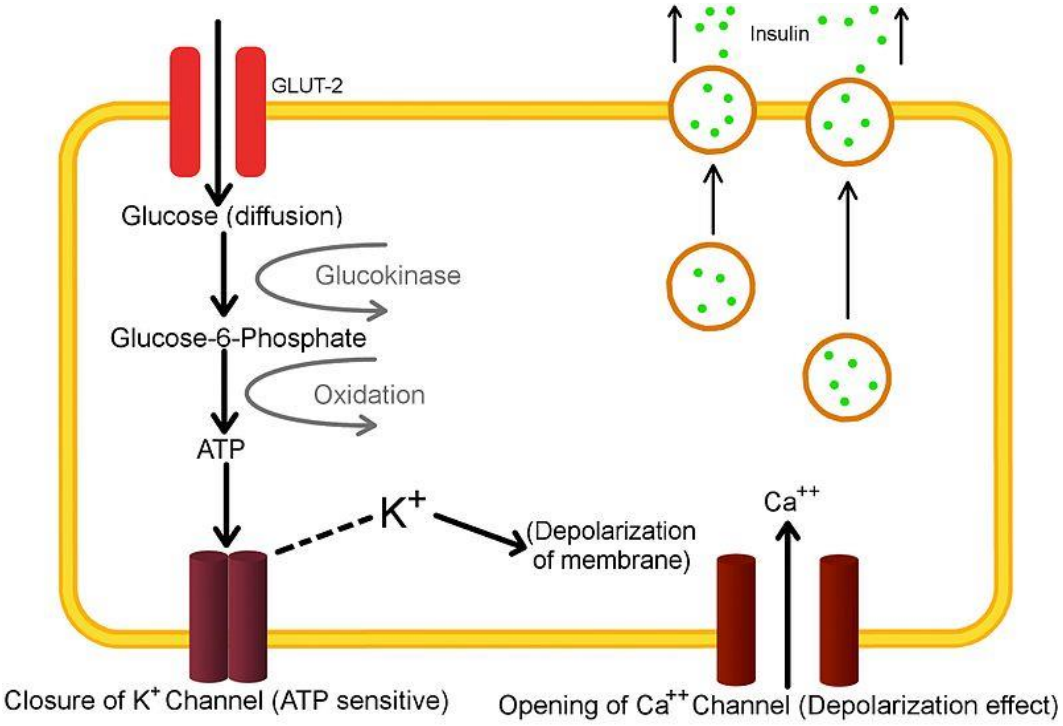


Figure 1.3.1: Normal Glucose Homeostasis (Arthur, 2006)

The subsequent glucose within the cells is then phosphorylated via means of glucose-6phosphatase toward glucose-6-phosphate which will ultimately enter into glycolysis series, thus triggering pyruvate in addition to adenosine triphosphate (ATP). Pyruvate then intrudes into next phase i.e. tricarboxylic acid (TCA) cycle, as a result of which further ATP will generate. KATP channel will block due to increase in ATP: ADP ratio, which leads to the depolarization of membrane and hence permitting Ca 2+ ions towards the cell facilitated by voltage dependent calcium channel. These ions will then eventually exhilarate the release of insulin (peptidal hormone); Insulin will then bind to the receptors of insulin which will ultimately initiates the phosphorylation based signaling of cascade. For uptake of glucose molecules, the translocation of intracellular GLUT4 to membrane will occur (Lee and Halter, 2017; Arthur, 2006).

1.3.2 Pathophysiology of Type2 Diabetes Mellitus

Impaired secretion of insulin and insulin resistance make a contribution towards the development of pathophysiological conditions. Various organs participate during the physiological progression of type2 diabetes (figure 1.3.1). Interruption in the connection among various organs i.e. pancreas, liver, adipose tissue, gut and central nervous system direct towards modification in normal glucose metabolism and type2 diabetes (DeFronzo, 1988; Reaven,1995)

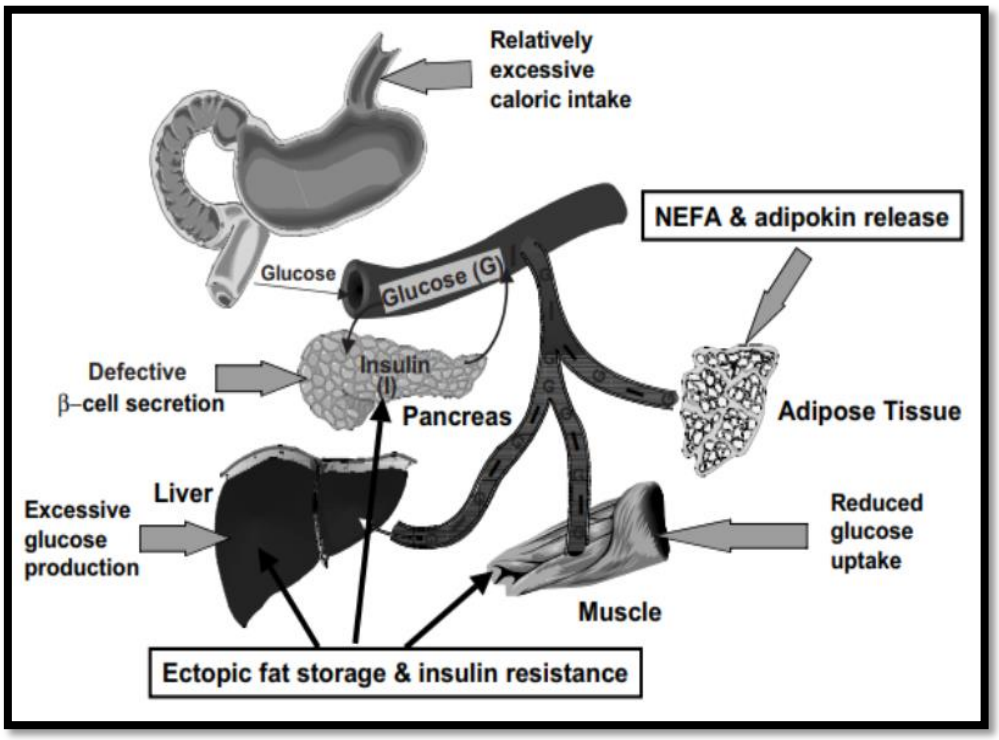


Figure 1.3.2: Role of organs in pathogenesis of type2 diabetes (DeFronzo, 1988; Reaven, 1995)

1.3.2.1 Impaired insulin secretion

Impaired secretion of insulin will results in decrease in responsiveness of glucose, as it has been observed earlier than pathological progression carried out by complication. Further distinctively, impaired glucose tolerance (IGT) is driven via reduction within initial-phase insulin secretion, which is glucose-responsive. And therefore, a reduction in supplementary

secretion of insulin occurs after meals. This will ultimately results postprandial hyperglycemia. Impaired secretion of insulin is generally progressive, and its development includes toxicity of glucose as well as lipo-toxicity. When it will remain untreated, this leads to the reduction of pancreatic cell (Ghani et al., 2008). The procession regarding effective decline relating to pancreatic cell utility consequently causes the permanent blood glucose elevation (figure 1.3.2.1).

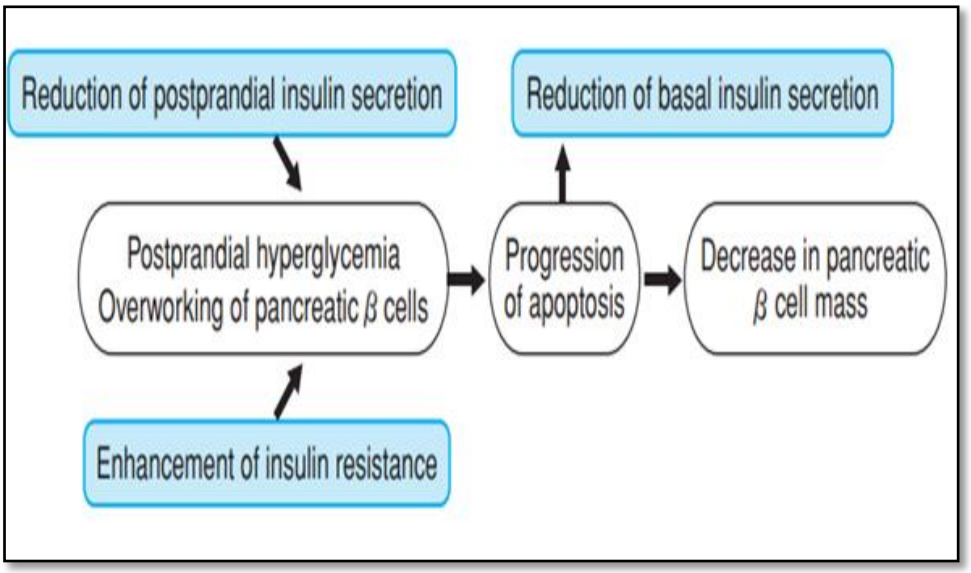


Figure 1.3.2.1: Development of type2 diabetes from pancreatic cells (Kaku, 2010)

1.3.2.2 Insulin resistance

This is the critical situation of the type that insulin is not able to perform its function into the body. This deprivation in the efficiency of insulin within predominant organs i.e. liver and muscles is one of the frequent procession characteristic of type 2 diabetes. The subatomic process for the activity of insulin has shown that this complication of resistance in insulin is associated in conformity with inherited factors along with environmental elements (hyperglycemia, free fatty acids, inflammatory mechanism, etc.) (Kaku, 2010).

There are three potent mechanisms for the control of metabolism of glucose. These mechanisms are present in skeletal muscles. This includes the foremost insulin stimulated

glucose transporter GLUT4, hexokinase and glycogen synthase. Consequently, malfunction in skeletal muscles glycogen production plays a chief role in the origination and development of processes involved in Insulin resistance. Similarly, the reduced capability of typical functioning of the above mentioned mechanisms that helps in circulation of insulin is major fundamental for the progression in insulin resistance (Petersen, 2002).

Genetic factor involves not solely polymorphisms in insulin receptor and insulin receptor substrate (IRS)-1 gene that has the ability to influence the signals of insulin. The polymorphisms of thrifty genes such i.e. 3 adrenergic receptor genes as well as uncoupling protein (UCP) gene are related to the genetic factors. These polymorphisms are related with visceral obesity and develop insulin resistance (Matsuda and DeFronzo, 1999).

1.4 Etiology of Type2 Diabetes Mellitus

The origin of T1DM is ultimate. However, T2DM do not have an ultimate and legitimate cause i.e., type2 diabetes mellitus is multifactorial (figure 1.4). The frequencies of development of abnormalities along with its related complexities are dissimilar in the inmates suffering from type2 diabetes. Furthermore, numerous determinants are present which somehow implicated in insulin resistance and other complications (Ismail et al., 2010; Thomas and Philipson, 2015; Zoungs et al., 2009; DeFronzo, 2015).

Type2 diabetes mellitus which is complicated multifactorial ailment, also involves multitudinous genes that are geographically dispersed amongst specific chromosomal regions. It is also related by means of distinctive pathways i.e. metabolic along with cellular signaling mechanisms. The possibility of developing Type2 diabetes can also be associated with dietary as well as environmental constituents. On the other hand, hereditary factors can also participate as a considerable factor for the elucidation of Type2 diabetes (Kaku, 2010)

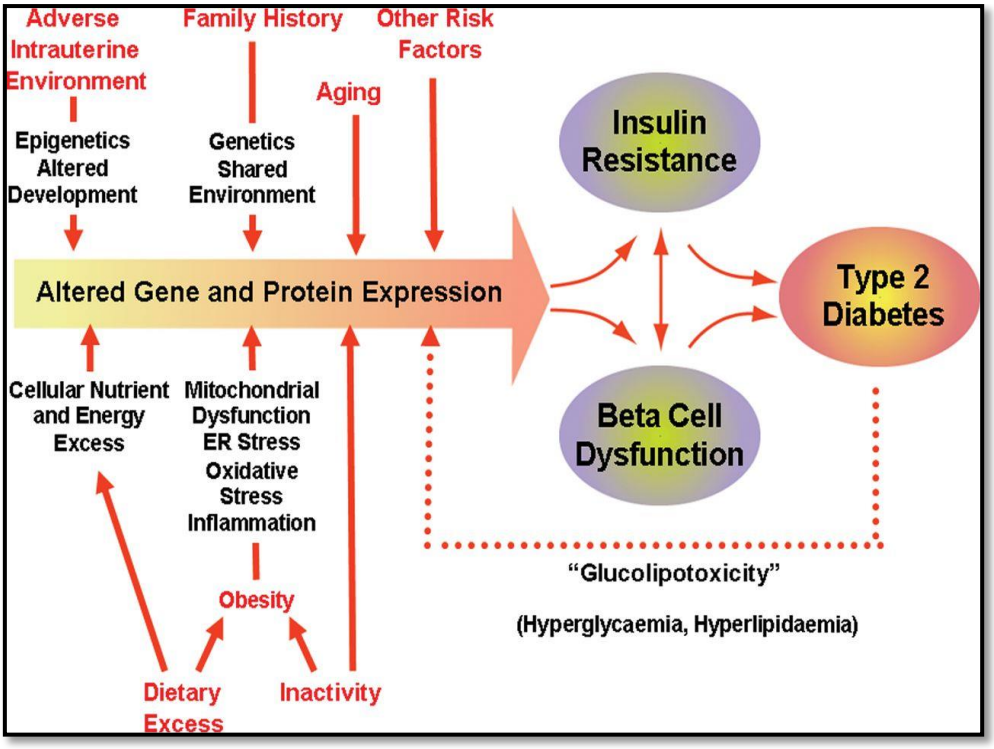


Figure 1.4: Etiology and pathophysiology of type2 diabetes (DeFronzo, 2015)

1.4.1 Genetic factors

Genome wide association studies (GWAS) has pinpointed 70 loci that are associated among type2 diabetes in range of populations. Stumvoll et al in 2015 had given the substantiality about chances to develop type2 diabetes if the family has positive history of diabetes. This depiction imparts about 2.5 times foremost possibility of developing Type2 diabetes. The foremost pedigree of Type2 diabetes inpatients has almost 15-25% chance to initiate malfunctioned glucose tolerance. This will ultimately lead the procession of Type2 diabetes. In 1995, Pierce et al., illustrated that possibility of the procession and progression of Type2 diabetes is approximately 38-40%, if only a mother or only a father are suffering from Type2 diabetes (Pierce et al., 1995). Tattersal and Fajans in 1975 demonstrated that approximately above 50% chances that the children will develop diabetes, if together mother and father both are suffering from diabetes (Tattersal and Fajans, 1975).

IRS2 gene encodes a protein that has the capability to act as an adaptor to insulin receptor. Here it activates cascade of kinase in pathways i.e. insulin signaling. However in case of type2 diabetes, the resistance in insulin secretion will occur due to suppression of *IRS2* gene. Thus IRS2 gene plays a major role in normal insulin signaling pathway (Zhang and Sun, 2009). In the previous years, it was observed that hyperglycaemia causes some major changes especially in the nervous system by modifying the function of IRS proteins. This ultimately leads to the impairment in diabetic marginal neuropathy as well as insulin signaling mechanism and cascade (Manu, Rachana and Advirao, 2017). In the latest findings, it is determined that polymorphism in Gly1057Asp of IRS-2 gene is mainly associated with T2DM (Ay et al., 2018).

1.4.2 Epigenetic Factors

Viral infections and toxins have the ability to modify the regular metabolism that influence for the epigenetic changes (figure 1.4.2). These modifications have the ability to manipulate the genes. This occurs as a result of two main processes i.e. by developing resistance and by the increasing the susceptibility to disease complications through allele polymorphism (Desiderio et al., 2016). Dietary factors have the ability to modulate epigenetics when an individual has sedentary lifestyle, has large intake of fatty foods and less intake of healthy food (Etchegaray and Mostoslavsky, 2016).

Methylation of DNA is determined as one of the main factor for the initiation of Type2 diabetes that affects secretion as well as sensitivity of insulin. Zhou and his co workers had illustrated almost 17 diverse genes. All these genes are found to be associated with development of type2 diabetes caused by epigenetic factors (Zhou, Sun, Li, & Zhu, 2018). Among all these genes, two genes i.e. insulin present in pancreas and metformin transporter genes that are present in liver are determined to be susceptible for demethylation.

The microvascular complexities of Type2 diabetes is related with the mitochondrial DNA. Methylation of this DNA takes place which ultimately cause various complications (Rorbach-Dolata, Kubis and Piwowar, 2017). Arginine and lysine amino acids present in the histone proteins are acetylated which is one of the factors for procession of type2 diabetes. This Acetylation is considered as an important modification in epigenetics (Rorbach-Dolata, Kubis and Piwowar, 2017).

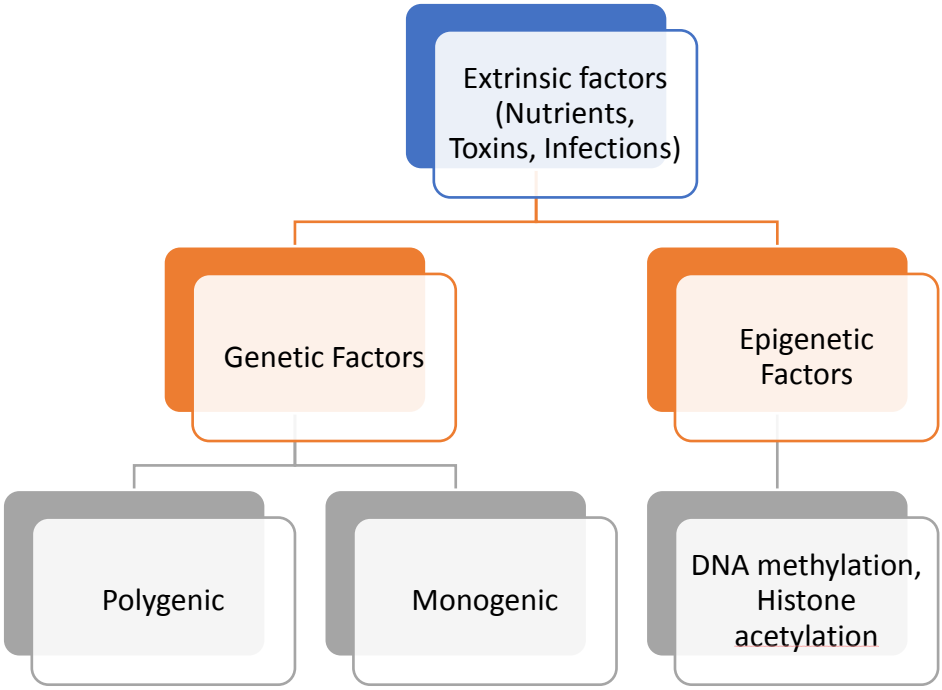


Figure 1.4.2: Classification of Epigenetic Factors

1.4.3 Ecological Factors

Increase in age along with weight, inadequate consumption of energy, alcohol intake, smoking, etc. are equitable threat elements that leads to the pathogenesis of type2 diabetes mellitus (figure 1.4.3) (Hu et al., 2001) (Belkina and Denis, 2010). **Obesity** (mainly visceral fat obesity) because of deficiency of exercise followed via the reduction in muscle mass, also culminates in insulin resistance, and also closely related to the immediate increase in the magnitude of patients with ages 30 and above (Zimmet et al., 2001). Even mild obesity (weight problems) increases the risk of developing diabetes, approximately 4- to 5-fold.

World Health Organization (WHO) in 2011 illustrated that around 90% of inpatients had developed Type2 diabetes as a result of excess body weight (WHO, 2011).

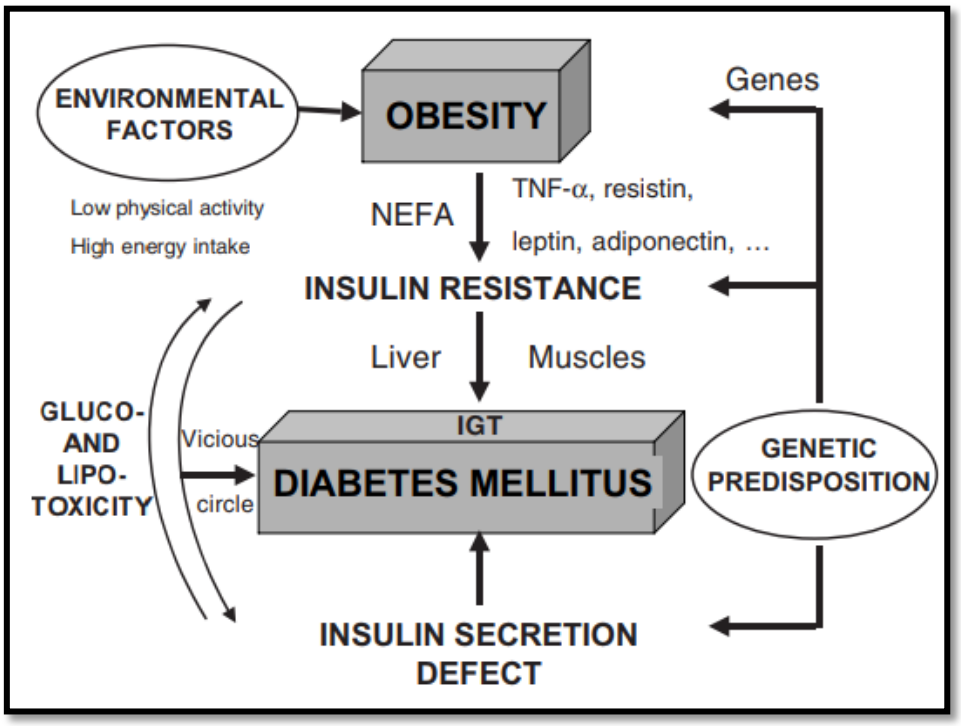


Figure 1.4.3: Contribution of environmental factors in type2 diabetes (Scheen, 2001)

The modifications in the **nutritional energy resources**, especially the proliferation in intake of fat, reduce intake of starch along with increment in the consumption of plain sugars and reduction in intake of dietary fiber, make a contribution toward obesity as well as initiate deterioration of glucose tolerance (Schulze et al., 2004; Dhingra et al. 2007).

1.4.4 Inflammatory factors

Oxidative stress culminates in the assembly of **reactive oxygen species (ROS)** together with other **pro-inflammatory cytokines** and **chemokines** in the beta cells. This will interrupt the blood circulation within the pancreatic cells thus destroying their utility (Ehses et al., 2007; Dedon and Tannenbaum, 2004).

According to different studies, **IL-6** as well as various other inflammatory cytokines has the capability to induce apoptosis in pancreatic islets. They can also act as a predictor and pathogenic marker for the development of T2DM (Pradhan, 2001; Akash et al., 2012). **TNF-alpha** creates an interrelation amongst complications in insulin secretion, obesity and islet hypersensitivity. Therefore, it is considered to play an essential part in the procession of Type2 diabetes (Tilg and Moschen, 2008). Its overproduction among adipose tissue appears to cause the hypersensitivity and beta cell loss present in pancreatic islets. It also produces extra complications in insulin secretion in the marginal tissues (Rosenvinge et al., 2007; Ruan, 2002).

1.5 Symptoms of Diabetes

If type2 diabetes proceeds without treatment, glucose levels in the circulation continue to remain elevated every time. However, this isn't always obvious at first. Type2 diabetes may extend progressively above several years devoid of any evident symptoms. Glucose levels in the blood which remains excessive all the time can initiate the subsequent symptoms: **Extreme thirst, frequent urination, tiredness, nausea along with dizziness**. If an individual has extremely high blood sugar levels, he may experience burdens' and drowsiness and ultimately he will lose consciousness (diabetic coma) (American Diabetes Association, 2017; Yan et al., 2016).

1.6 Diagnostic tests

Diabetes can be diagnosed by the hemoglobin A1C criteria or plasma glucose absorption (fasting or 2-hour plasma glucose) as shown in table 1.6.

1.6.1 Two-Hour Oral Glucose Tolerance Test (OGTT)

In this diagnosis, the plasma glucose level is computed before 2 hours of intake of glucose and 2 hours after the intake of approximately 75-80 gm of glucose. The individual is considered diabetic if glucose level is more than 200 mg/dL in sample taken after 2 hours of glucose intake

1.6.2 Fasting Plasma Glucose (FPG)

A blood sample is withdrawn from an individual after overnight fasting for about 8 hours. As per ADA, if the fasting glucose level is more than 126 mg/dL than the individual is considered diabetic.

1.6.3 Glycated Hemoglobin (Hb) A1C

This test provides an estimate of average blood glucose level in the previous 2 to 3 months of an individual. Patients in which Hb A1C is more than 6.5% are considered as diabetic. it measures the thickness of sugar bound to haemoglobin in red blood cells. The thicker sugar coat on red blood cells indicates the higher level of glucose in blood (Martinez et al., 2019; Lai et al., 2019).

Diagnostic Test	**Normal**	**Diabetic**
2-Hour Post Glucose Intake	≤140 mg/dL	≥200 mg/dL
Fating Plasma Glucose	70-99mg/dL	≥126 mg/dL
Glycated Hemoglobin	4-6%	≥6.5%

Table 1.6: Diagnostic criteria of Diabetes

1.7 Treatment of type 2 diabetes

1.7.1 Diet Therapy

Almost 50% cases of type2 diabetes are due to elevated blood sugar levels accompanied by overweight and sedentary lifestyle. Therefore, the aim to decrease the weight of diabetic individual can come under this diet treatment (Wing, 1995; Henry and Gumbiner, 1991). Food items rich in carbohydrate while having least lipid content result in improved glycaemic control and reduction in low density lipids (Franz et al., 1994)

1.7.2 Exercise

In the previous studies (Helmrich et al., 1994; Martin and Wahren, 1993) it is illustrated that exercise has numerous advantages in the anticipation of progression of type 2 diabetes mellitus. It also has the ability to increase sensitivity of glucose and thus to improve levels. Reduced intraabdominal fat, increased activity of glucose transporters (GLUT-4) that are sensitive to insulin present in muscle, improved blood circulation in tissues that are sensitive to insulin, are all the mechanisms through which sensitivity of insulin is restored by exercise (Erisonn et al., 1997)

1.7.3 Pharmacotherapy

The curative agents accessible for treatment of type2 diabetes consist of sulfonylureas and related compounds, biguanides, thiazolidenediones, α-glucosidase inhibitors. Pharmaceutical agents that can act through diverse mechanisms of action must be selected in order to improve glucose values and at the same time minimizing unfavorable effects. Some of the therapeutic agents are enlisted in the table 1.7.3 (Wu et al., 2014).

Class	Drug	Target	Disadvantage
Biguanides	Metformin	Amp-Kinase	• Vitamin B12 deficiency • Gastrointestinal problems
α-glucosidase inhibitors	Acarbose/ Miglitol/ Voglibose	α-glucosidase	• Gastrointestinal problems
Thiazolidinediones	Troglitazone/ Pioglitazone	PPAR-ϒ	• Blood cancer risk • Weight gain

Table 1.7.3: Therapeutic Agents (Wu et al., 2014)

1.8 Insulin Therapy

Insulin therapy can be used for treatment of type2 diabetes as foremost remedy of extreme hyperglycemia along with additional critical hyperglycemic circumstances. Insulin can be used along with other treatments and combinations for curing type2 diabetes. Burge and Schade illustrated large numbers of latest analogs of insulin that are in pharmaceutical trials (Burge, Schade, 1997). Lispro insulin is the foremost accessible analog of insulin. It represents a two amino acid alteration as compared to typical insulin present in humans (Holleman F, Hoekstra, 1997).

1.9 Antioxidant therapy

The hindrance of intracellular free radical development would present a therapeutic approach in order to avoid oxidative stress and the interrelated diabetic vascular problems. Antioxidants may additionally proceed at diverse levels, hindering the development of ROS or scavenge free radicals, and enhancing the antioxidants defense enzyme performances (Jakus, 2000).

Medicinal floras having antioxidant properties have the ability to be defensive in diabetic rats. This ability is due to scavenging free radicals especially oxygen radicals along with reduction in expressions of molecule-1 protein intercellularlly (Ghamarian et al., 2012; Rafieian and Baradaran, 2013; Nasri and Rafieian, 2013). Medicinal floras bear an extensive history for curing the complications. In conventional medicinal treatment it involves almost 800 plants that have the ability to cure diabetes (Rafieian and Baradaran, 2013; Hung et al., 2013; Nasri et al., 2014).

A probable assistance about vegetables and fruit is because of antioxidant components that are present inside them. Therefore, they made their contribution for the suppression of oxidative stress (Hulbert et al., 2005). Vegetables as well as fruits exhibited elevated accumulation of antioxidants, that has the ability to decrease the possibility of developing diabetes especially type2 diabetes (Tamadon et al., 2013).

Plants encompass to a great extent of enchantment due to their defensive along with curative characteristics for the diseases that are very difficult to cure which involves memory deterioration, diabetes, cancer along with heart diseases and many more, all these properties are due to their antioxidant potentials (Taghikhani et al., 2014; Beladi et al., 2014).

1.9.1 Nanoantioxidants

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). Metallic nanoparticles (NPs) such as magnetic, silver, and gold NPs, nanosized entities that have dimension between 1–100 nm, has the potential to treat type2 diabetes because of their physical along with chemical characteristics and their capability to transform the level of oxidative stress (Lushchak et al., 2018).

Silver nanoparticles have the ability to restore the normal **glucose**, serum **insulin** levels and **glucokinase** activity. It has also the potential to reduce lipid profile by suppressing the oxidative stress and elevating the antioxidant defense system (Shaheen et al., 2016).

1.10 *Thymus serpyllum*

Thymus serpyllum (wild thyme) associated with the family of Lamiaceae and as mentioned by the World Checklist, constitutes 7534 species, which includes the genus *Thymus* L. having 220 kind of species (Harley et al., 2004).

It is a necessary herbal plant with ethno-botanical importance in diverse regions of the World, peculiarly in Gilgit Baltistan region of Pakistan. Wild thyme for the most part repeatedly intended for treatment of sickness and complications interrelated with gastrointestinal along with respiratory systems (Menkovic et al., 2011; Benitez at al., 2016). *Thymus serpyllum* is considered an imperative supply of remedial agents having **antioxidant, antimicrobial, antitumor properties**. Wild thyme has various important properties due to which it can be exploited in various industries i.e. medicinal, food, cosmetics and many more (figure 1.10) (Jaric et al., 2015).

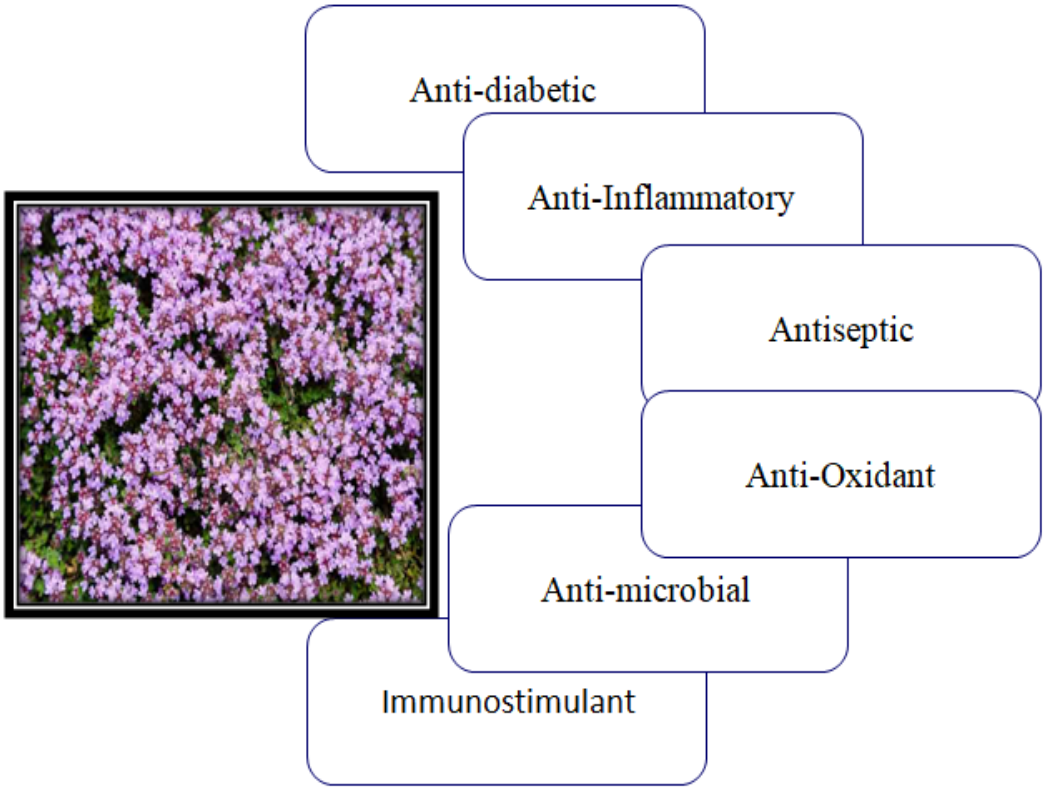


Figure 1.10: *Thymus serpyllum* and its properties (Jarić et al., 2015)

1.11 Objectives

- Docking of Silver with Glut 4
- Synthesis of cost effective, nontoxic environment friendly *Thymus serpyllum* mediated silver nanoparticles.
- Characterization of the synthesized biogenic silver nanoparticles by UV-Visible Absorption Spectroscopy and X-Ray Diffraction
- Induction of diabetes mellitus in BALB/C mice.
- Primer designing of INS2, IRS2, Glut4 and β-actin by primer 3 plus and validation by UCSC In-Silico PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Nanotechnology

Nanotechnology is the art about manipulating matter considered as the billionths of meters and nanometer, approximately the mass of 2 or 3 atoms (Kaira and Singh, 2012). Nanotechnology has provided absolutely innovative concepts and innovative approaches/processes in medicinal drug as well as in dentistry which will facilitate us in making superior, former and sure prognosis and to treat patients with least feasible interventions and without any unfavorable outcomes (Tevatia et al., 2016).

On 29th December 1959, the deceased Nobel Prize–winning physicist Richard P. Feynman published a discussion at Caltech on a title "There's Plenty of Room at the Bottom" (Feynman, 1960) at the yearly conference, organized by American Physical Society. He recommended that certain nanomachines, nano-robots as well as nano-devices eventually might be exploit to build up the comprehensive array of atomically defined microscopic equipments, technical as well as assembling materials. Thus the revelation of nanotechnology was evolved (Asiyanbola and Soboyejo, 2008) (Tevatia, 2016). The word "nanotechnology" was described by Tokyo Science University Professor Norio Taniguchi in 1974 paper. Norio defined this term as follows: "'Nano-technology' primarily comprises of the processing, dispensation, separation, consolidation, as well as deformation of materials and substances by solitary atom or by solitary molecule" (Taniguchi, 1974). "Engines of Creation: The Coming Era of Nanotechnology" by Dr. K. Eric Drexler is considered as the first book on the subject of nanotechnology (Corbertt, McKeown et al., 2016). In 2000, the United States National Nanotechnology Initiative was inaugurated in order to synchronize with the Federal nanotechnology research, progression and development (Ratner, 2002).

2.2 Nanotechnology in medicine (Nanomedicine)

The appliance of nanotechnology in favor of therapeutics, prognosis, examination, as well as inhibition of various complications and diseases is called "nanomedicine". In 1965, the first illustration regrading lipid vesicles by Bangham and his co-workers was presented, that was later on become acknowledged by the term liposomes (Bangham et al., 1965); In 1976 the primary managed dispensed polymer structures related to macromolecules were discussed and demonstrated by Langer and Folkman (Langer and Folkman, 1976); In, 1994, the initial elongated diffusing sheath of nanoparticles made up of polymers was once illustrated by Gref and his co-workers (Gref et al., 1994); In 1998 the foremost quantum dot produced as result of bioconjugation was discussed and illustrated by Bruchez and his co-workers (Bruchez et al., 1998) (Chen and Nie, 1998); and the earliest nanowire nanosenser was first demonstrated IN 2001 (Cui et al., 2001).

With an enhanced perceptive and understanding of brain functioning, improved prognosis as well as therapeutics intended for neurodegenerative disorders for example, Alzheimer's was presented through nanotechnology (Singh et al., 2008). Nanomedicine has the capability to conquer and overcome biological hurdles, to enhance and develop the bioavalability of drugs (Lavan, McGuire et al., 2003), to target disease and infectious sites only as well as for effectual and targeted drug delivery (Babu, et al., 2013)

The current and novel aged medications includes the use of nanoparticles that are composed of biopolymers, substances, metals and ceramic objects as they have the potential to combat against conditions and prerequisites similar to cancer (Farokhzad et al., 2006) as well as to combat against bacteria that are pathogenic for humans and many more characteristics (Morones et al., 2005) (Panacek at al., 2006).

The role of nanomedicine in biomedical research is illustrated in figure 2.2.

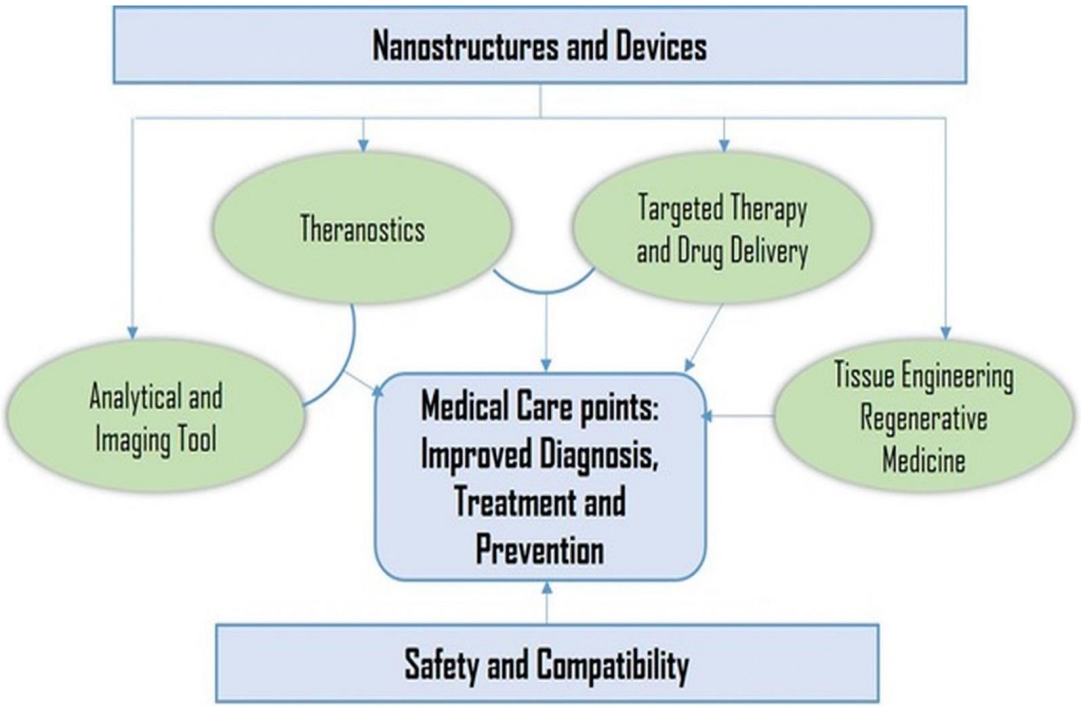


Figure 2.2: Nanomedicine Applications in biomedical research (Patra et al., 2018)

2.3 Nanoparticles in Therapeutics

Polymer-based nanoparticles, synthetic as well as natural, present another different approach for therapeutic and beneficial applications due to certain and definite characteristics such as biocompatibility, non-immunogenicity, non-toxicity as well as biodegradability (Crucho and Barros, 2017). **Natural polymer-based** nanoparticles have the potential to overwhelm toxicity hurdles and issues. They also have a potential to bring consequential progression and development in the effectiveness of therapeutical and remedial agents than already available well known treatments (Letchford et al., 2009).

Metallic nanoparticles that are used in medicinal applications are 1–100 nm in size. It is generally made up of cobalt, nickel, iron, gold, silver as well as their respective oxides such as magnetite, maghemite, cobalt ferrite, and chromium dioxide. **Magnetic nanoparticles** have the potential to target a precise location in the body through the help of an exterior magnetic field (Cuenca et al., 2006). The property of super-paramagnetic assist the secure

and stable release of curative agents to the body/cell in addition to appropriate accumulation at the site of target tissue, thus provides a reproducible and secure therapeutic technique (Fan et al., 2011) (Reilly, 2007). Anti-inflammatory action of AgNPs was identified formerly. This property was due to its potential to inhibit interferon-γ, tumor necrosis factor alpha (TNF-α) and to reduce matrix metallo proteinase (MMP) in addition to proinflammatory cytokines (Kirsner et al., 2001) (Tian et al., 2007). Biosynthesized magnetic nanoparticles extensively suppress the intensity of many enzymes present in liver such as alanine transaminase, alkaline phophatase, serum creatinine as well as uric acid in diabetic mice model (Swarnalatha et al., 2012).

Gold nanoparticles (AuNP) are comprehensively utilized metallic nanoparticles, particularly in cancer prognosis and its treatment. AuNPs can be used for targeted delivery of drugs, where the light irradiation has the ability to activate the release of drug (Mura et al., 2016) (Tian et al., 2016). **Silver nanoparticles** account for approximately more than 23% of all nano based products. It has been extensively used for prognostic as well as in therapeutics (e.g. in healing of wounds, arthritic disease and many more). These nanoparticles have been extensively well-known for their antibacterial, antiviral effects and antifungal effects (Kanav et al., 2016).

2.4 Silver Nanoparticles

AgNPs of diverse shapes and sizes hold distinct catalytic distinctiveness, together with surface plasmon resonance (SPR) as well as strong and vivid toxicity to extensive array of microorganisms. Silver ions along with its various salts have comprehensively been utilized due to their ability of prohibitory consequences against various microorganisms for a long period (Kim et al., 2007). Silver can be used as an antibacterial agent. In wound dressings, topical creams, antiseptic sprays, as well as fabrics, silver can be added (Ahmed et al., 2016). AgNPs has the ability to apply their activity of antibacterial, by performing as antibacterial

complements to antibiotics (Beyth et al., 2015). AgNPs reduce the actions of proinflammatory cytokines, interferon gamma and tumor necrosis factor alpha (Shin et al., 2007).

2.5 Characterization of Silver Nanoparticles

UV-spectrometry is utilized in order to validate the configuration of nanoparticles by observing their SPR. The solution of Silver nitrate is used to make silver nanoparticles (AgNPs). This indicates a highest absorbance of approximately 436 nm (Nithya and Ragunathan, 2009). The morphology (shape) along with particle mass of AgNPs can be identified using transmission electron microscopy, scanning (TEM and SEM) as well as atomic force microscopy (AFM). In addition, dynamic light scattering and X-ray diffraction can be utilized intended for examination of size distribution of particle along with its crystallinity (Nour et al., 2010). Silver nanoparticles can be spherical, rod shaped (Wang et al., 2006), nanowires (Murphy and Jana 2002) and many more shapes.

2.6 Synthesis of Silver Nanoparticles

Different synthetic Silver Nanoparticles methods yield different as well as inconsistent sizes, shapes as well as morphology. They also differ in their stability. Generally, there are three main methods: physical, chemical, and biological/green synthesis given in the figure 2.6:

2.6.1 Physical synthesis

Evaporation/condensation, laser ablation are considered as major substantial approaches for synthesizing and originating nanosilver from the samples like metal. The **evaporation/condensation** approach uses a tube of furnance which is kept under atmospheric pressure to manufacture AgNPs (Jung et al., 2006). **Laser synthesis** approach does not use chemical reagents. It only uses the laser ablation of metals in solution that ultimately leads to pure colloids of nanosilver (Tsuji et al., 2002).

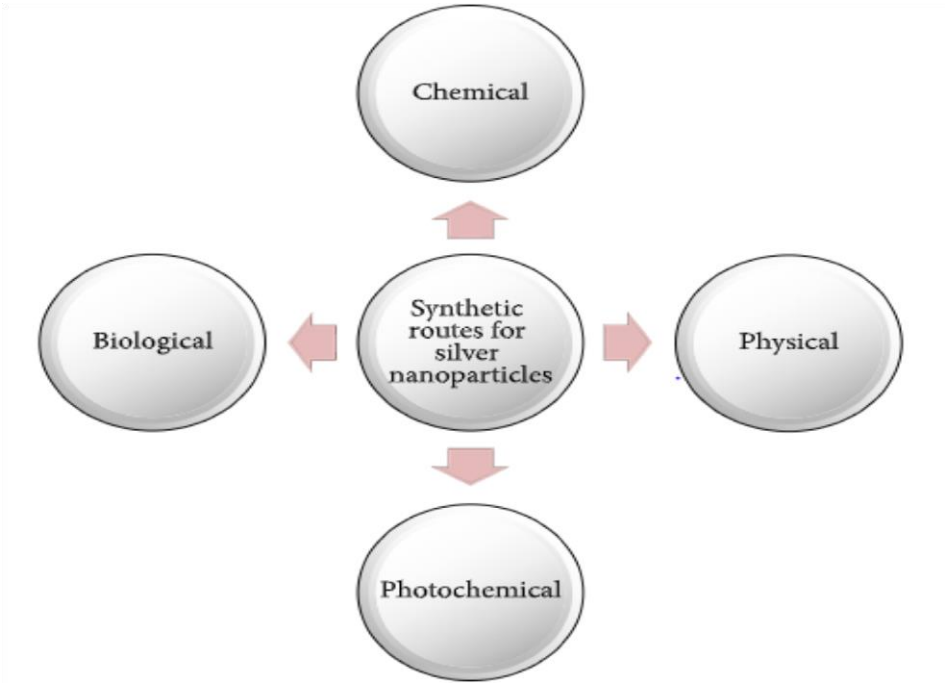


Figure 5.6: Methods for the synthesis of AgNPs (Adnan and Kang 2015)

2.6.2 Chemical synthesis

Chemical reduction is considered as a common process of production of nanosilver. This technique utilizes silver salt, reductants, along with a stabilizer or capping agents. Among all of them, silver nitrate (silver salt) is frequently used for silver nanoparticles synthesis. The reason is that silver nitrate is cost effective as well as it has chemical stability in comparison to various accessible salts of silver (Tien et al., 2008). The reductants contain borohydride (Evanoff and Chumanov, 2005), citrate (Pvatenko et al., 2007), ascorbate (Blanco et al., 2010), and hydrogen gas (Moore, 2006).

2.6.3 Biological synthesis of Silver nanoparticles

In biological strategies, Ag-NPs are synthesized by the use of plants, algae, yeast, fungi, and bacteria as reducing and stabilizing agents in the synthesis (Sintubin et al., 2012).

Shewanella oneidensis which is a metal reducing agent was utilized in order to biologically synthesize Ag-NPs utilizing silver nitrate [Ag (NO_3)] solution as precursor. The resultant

obtained Ag-NPs size was approximately lesser than 15 nm, having consistent dispersion, spherical shape, improved stability, and bulk surface area (Suresh et al., 2012). Stable Ag-NPs which has the size lesser than 20 nm can be made by means of airborne bacteria (***Bacillus* sp**.) in which silver nitrate $Ag(NO_3)$ will be used as the precursor (Thirumalai et al., 2010).

In a different study that was illustrated by Venkata Subbaiah and Savithramma, they discovered that Ag-NPs can also be produced from ***Cadaba Fruticosa*** leaves, in which silver nitrate $Ag(NO_3)$ will be utilized as precursor (Subbaiah and Savithramma, 2013). Some of the plant synthesized silver nanoparticles having antidiabetic potential have been enlisted in the table 2.6.3

Plant	Shape/Size (nm)	Plant's Part	Reference
Cymbopogon citrates	75-138	leaves	Agarwal et al., 2018
Alyssum homalocarpum	30	Seeds	Ghasemian Lemraski and Valadbeigi 2018
Argyreia nervosa	5-35	leaves	Saratale et al., 2017
Punica granatum	35-60	leaves	Saratale, Shin et al., 2018
Ananas comosus	Spherical	Outer peel of fruit	Gitishree et al., 2019
Musa paradisiaca	30-60	Stem	ANbazhagan, Muurugan et al., 2017
Sonneratia apetala	20-70	Leaf extract	Thatoi et al., 2016
Aloe vera	30	Leaves	Ashraf, Ansari et al., 2016
Heritiera fomes	400	Bark extract	Thatoi et al., 2016
Solanum nigrum	4–25	Leaves	Sengottaiyan et al., 2016
Holoptelea integrifolia	32-38	Leaves	Kumar et al., 2019
Withania coagulans	14	Leaves	Tripathi et al., 2019

Table 2.6.3: Green synthesized silver nanoparticles to treat diabetes

2.6.4 Comparison of chemical and biological method

Most common method is chemical method for industrial scale synthesis of Silver nanoparticles. Chemical methods however require stabilizing agent to prevent agglomeration of silver nanoparticles. Although the production rate is fast but chemical method is an intensive and energy consuming method. Contamination of reducing agent during the preparation of silver nanoparticles limits their biomedical application (Krutyakov et al 2008).

Therefore, eco-friendly nanoparticles are needed to be synthesized. Biological synthesis on the other hand is eco-friendly, green process not consuming energy. Therefore, use of organisms and plants for preparation of silver nanoparticles is eco-friendly approach. (Ghorbani et al 2011).

2.7 Role of Silver Nanoparticles for treatment of Diabetes:

Antidiabetic potential of silver nanoparticles (AgNPs) is possibly because of **inhibition of Reactive Oxygen Species (ROS), inflammation** and **enzymes involved in diabetes.** Reactive Oxygen Species (ROS) that includes superoxide radical (.O2), hydrogen peroxide (H2O2) and hydroxyl radical (OH) amplified levels have potential to originate damage in DNA along with prohibition of proteins directly (Chopra at al., 1998). The foremost target substrates to initiate free oxygen radical activity includes polyunsaturated fatty acids, after modification the end product is malondialdehyde (MDA) which is excreted in urine, blood, as well as other body fluids. Consequently it can also serves as a marker of lipid peroxidation along with oxidative stress respectively (Patterson et al., 1998) (Marks et al., 1996).

Abdelazim and Afifi silver (SNPs) tried to find whether silver nanoparticles ameliorate the oxidative stress resulted from diabetes in diabetic rats. A significant increase in the activity and mRNA expression level of superoxide dismutase enzymes (SOD), Catalase (CAT), Glutathione peroxidase (GPx) as well as reductase (*GRd*) was observed. Prabhu et al. in 2017

found the antioxidant potential of AgNPs biologically synthesized silver nanoparticles using Pouteriasapota. The activity of superoxide dismutase enzymes and Catalase was normal in liver tissue homogenates from AgNPs- treated groups (Afifi, M. and A.M. Abdelazim,2015).

Principally two carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase) are responsible for postprandial hyperglycemia. These two enzymes convert the carbohydrates into monosaccharides, as c consequent of which postprandial hyperglycemia occurs. Therefore inhibitors of α-amylase and α-glucosidase can play an important part in the control of hyperglycemia, because they have the potential to delay digestion of carbohydrate and thus reducing postprandial plasma glucose level(Prabhu et al., 2018) (Hara and Honda 1990). Balan et al. synthesized AgNPs using the aqueous leaf extract of *Lonicera japonica.* These silver nanoparticles inhibit carbohydrate digestive enzymes, thus showing strong anti-diabetic potential **(**Balan et al., 2016).

AgNPs have been reported in reduction of inflammation that is believed to be the cause of insulin resistance in diabetes Mellitus[58]. Sengottaiyan et al. in 2016 showed that in addition to control blood glucose level and body weight of mice, AgNPs significantly improves the dyslipidemic condition which is clearly observed in the diabetic control (Sengottaiyan et al., 2016).

2.8 Toxicity of Silver Nanoparticles

The toxicity regarding silver nanoparticles (AgNPs) is interrelated with their alteration under biological circumstances as well as environmental media. It also includes their interactions with organic macromolecules, surface oxidation, as well as the release of silver ions (McShan et al., 2014). AgNPs are usually offered as extremely efficient antimicrobial agents which has non-toxic effects to healthy cells of mammals (Stensberg et al., 2011). On the other hand, different in vitro research established the nanosilver-related toxic consequences in

hepatocytes of rat as well as neuronal cells (Mahdy et al., 2015), murine stem cells, human lung epithelial cells (Pinzaru et al., 2018).

In a previous oral toxicity investigation of rats, Kim and his co-workers additionally observed that silver nanoparticles can accumulate in blood, liver, lungs, kidneys, stomach, testes, and brain. However AgNPs confirmed no considerable genotoxicity after the silver nanoparticles of approximately 60 nm were orally administered for about 28 days at various concentrations (Kim et al., 2008).

2.9 Anti-oxidant and Pharmacological Properties of *Thymus serpyllum*

Hussain and his co-workers suggested that *T. serpyllum* essential oils have enhanced radical scavenging activity than *Thymus linearis* essential oil (Hussain et al., 2013). Petrović and his co-workers estimated the antioxidant capability of wild thyme, in which T. serpyllum converts DPPH (1,1-diphenyl-2-picryl-hydrazyl) into its reduced form DPPH-H. In this study, Thymus serpyllum showed even better antioxidant activity than synthetic antioxidants (Petrovic et al., 2014). Mihailović-Stanojević and his co-workers demonstrated the antioxidant potential of wild thyme aqueous extracts in rats' model. In this aqueous extract, phenols and flavonoids were abundantly present, (Mihailovic et al., 2013).

Table 2.9: Pharmacological properties of different extracts of *T. Serpyllum*

T. Serpyllum Extract	Pharmacological Activity	References
Aqueous	• Anti-hypertensive • Anti-diabetic	(Mihailovic-Stanojevic et al., 2013) (Mushtaq et al., 2016)
Ethanol	• Anti-oxidant • Anti-diabetic	(Joshi and Joyal, 2018) (Mushtaq et al., 2016)
Methanol	• Anti Cancer • Epigenetic modifications	(Bozkurt et al., 2012)
Ether	Anti-diabetic	(Mushtaq et al., 2016)
Aqueous-Ethanol	• Anti tumor • Lipid peroxidation	(Aralbaeva et al., 2017)

Wild thyme in addition to flavonoids and phenol, it also has carboxylic acids along with their derivatives, triterpenes as well as tannins (Wichtl, 2001). Pharmacological properties of different extracts of *Thymus serpyllum* is given in the table 2.7.According to the PDR for Herbal Medicines, the principal constituent of *T. serpyllum* is carvacrol, although it also has borneol, isobutyl acetate, caryophyllene, 1,8-cineole, citral, citronsellal, citronellol, *p*-cymene, geraniol, linalool, α-pinene, γ-terpinene, α-terpineol, terpinyl acetate, and thymol in comparatively elevated concentrations (Thomson, 2004).

CHAPTER 3

Materials and Methods

All the experimentation of the research was done in Atta-ur-Rahman School of Applied Biosciences (ASAB). For the characterization of silver nanoparticles, equipment in School of Chemical and Material Engineering (SCME) at National University of Sciences and Technology (NUST). Details of the methodology are given as follows:

3.1 Docking Analysis

3.1.1 Prediction of tertiary structure:

3D structure of GLUT4 was unavailable in Protein Data Bank (PDB) (https://www.rcsb.org/), therefore, it was estimated via online bioinformatics tools. Amino acid sequence of GLUT4 protein was attained from NCBI Database (ncbi.nlm.nih.gov/) (Jenuth, 2000) and GOR4 server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) (Garnier et al., 1996) was run to build up the secondary structure of this sequence. Software which is TMHMM was adopted to find out the trans membrane domains in GLUT4 protein (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001).

All three tactics of 3D modeling were utilized in order to develop the tertiary structure of GLUT4 i.e. Ab initio, Threading and Homology modeling. FALCON (http://protein.ict.ac.cn/TreeThreader/) and i-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) softwares were operated for ab-initio 3D modeling (Wang et al., 2015; Zhang, 2008), whilst MUSTER (https://zhanglab.ccmb.med.umich.edu/MUSTER/) (Wu and Zhang, 2008) and Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/help.cgi?id=help/faq) were recruited for threading based development of tertiary structure. IntFOLD (https://www.reading.ac.uk/bioinf/IntFOLD/) (Kelly et al., 2015) and Swiss Model

(swissmodel.expasy.org/) (Benkert et al., 2010) were employed for homology modeling of GLUT-4 protein in order to construct its 3D tertiary structure.

3.1.2. Validation of Tertiary Structure

Best developed structures by above used software were then compared by exploiting Rampage and SAVES analysis. Rampage examined them on the basis of Ramachandran plot (Lovell et al., 2003) whereas SAVES analyze them on the basis of ERRAT (Colovs and Yeates, 1993), WHATCHECK (Hooft, 1996) and PROCHECK (Laskowski et al., 1996). The best structure was then manipulated for docking scrutiny.

3.1.3 Preparation of 3D structure of Ag

The .SDF file of silver (Ag) was downloaded from Pubchem database (Kim et al., 2015). This file was converted into PDB format by adopting Discovery Studio 2017 (Studio, 2008).

3.1.4. Molecular docking

Molecular docking analysis was executed to observe the binding capability of Ag with GLUT4 protein. PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/) was utilized to perform the docking (Schneidman et al., 2005). PatchDock is a geometry-based flexible docking method that is accessed to study the biomolecular complexes (Dominguez et al., 2003). RMSD value was set on 4 Å and PDB files of Ag and GLUT4 were uploaded in the designated areas. On the basis of docking scores, docked file were chosen and then uploaded on PDBsum generate to find out the interactions. The result was then visualized through the PyMOL molecular graphic system v.1.3 (Delano, 2002).

After docking of silver with Glut4, *thymus serpyllum* plant mediated silver nanoparticles were then synthesized and characterized.

3.2 Plant Selection and Storage

Thymus serpyllum plant was collected from Rakaposhi Base Camp, Gilgit Baltistan for the synthesis of silver nanoparticles. The aerial parts of the plant were then dried (figure 3.2).

Figure 3.2: Dried *Thymus serpyllum*

These parts were then ground into a fine powder, using an automated electric grinder. This powder was then stored at room temperature in a sterile sealed container.

3.3 Preparation of Plant Extract

By the modification of the protocol of Sun et al (Sun, Cai et al., 2014), plant extract was prepared. 10 gram of the plant *Thymus serpyllum* plant powder was soaked in 100 ml deionized water in Erlenmeyer flask at room temperature for few minutes.

This mixture was then heated at 60°C for 15 minutes on a hotplate. The extract was then cooled for about half an hour and then the supernatant was collected. The supernatant was then twice filtered with 0.45 µm pore size filter paper using vacuum filtration assembly. The filtrate obtained was then stored at 4°C as a stock solution. This stock solution can be used within 1 week.

3.4 Synthesis of Silver Nanoparticles using *Thymus serpyllum*

The stock solution of the plant acts as reducing and capping agent for the precursor. This solution was diluted to 15% (v/v). 850µl of (10mM) silver nitrate was then added drop wise per second into the 14.25 ml solution taken from 15% diluted extract of *Thymus serpyllum*

under 25°C temperature and continuous magnetic stirring at 300 rpm. This working solution was then kept in rotary orbital shaker in dark for overnight at 700 rpm and 35°C temperature, followed by monitoring using spectrophotometer for SPR band after every 1 hour.

3.5 Purification of Nanoparticles

The reaction mixture was then centrifuged in a refrigerated centrifuge at 15000 rpm for 30 min at 4°C. Supernatant was then discarded. The obtained pellet was re-suspended thrice in deionized water. And it was centrifuged with the same conditions (15000 rpm for 30 min at 4°C) in order to obtain a thicker pellet. This pellet was then air dried, collected and stored in Eppendorf tubes. This dried mass of silver nanoparticles was weighed. The dried mass (silver nanoparticles) was now ready to use for further activities.

3.6 Characterization of Silver Nanoparticles

3.6.1 UV Visible Absorption Spectroscopy

In chemical and clinical laboratories, this technique is the most widely used. This technique helps to measure the amount of absorption that occurs in a sample when the beam of light passes through it. In this instrument, the beam of light is split in two parts. One half of the beam is directed towards the cuvette containing the reference solvent (deionized water) and the other half is directed towards the cuvette containing the sample (*thymus serpyllum* mediated silver nanoparticles) to be analyzed. This is a powerful technique in order to analyze the size, stability and the concentration of silver nanoparticles.

Thymus serpyllum mediated silver nanoparticles were first characterized using Spectrophotometer (LABOMED, Inc. U.S.A, Model uvd-2950) in order to observe the specific peak of the silver nanoparticles in the UV visible absorption spectroscopy. The spectra range for absorption was set from 300-600 nm. Spectrophotometer was first blanked by filling both cuvettes with deionized water. After this one cuvette was filled with silver nanoparticle solution and the other with deionized water as reference solvent. Both cuvettes

were then loaded in the spectrophotometer chamber, where the silver nanoparticles, depending upon the particle size distribution, absorbed the photons of specific wavelength.

3.6.2 X-Ray Diffraction (XRD) Analysis

X-Ray diffraction can be used to study the atomic and molecular structure of silver nanoparticles crystals. XRD produce an intense beam of X-rays which is thrown on the test sample. These X-rays reflect with different intensities and at different angles by the sample. After reflection of X-rays, the data is calculated and a 3D structure is formed by XRD. The angle and the position of an atom in a crystal lattice can be revealed from this 3D structure.

For XRD analysis, the sample was made highly concentrated using the centrifuge. For this, 1ml of the sample was taken in Eppendorf tube and was then centrifuged for about 15 min at 15,000 rpm. Subsequent to 1st centrifugation the supernatant was removed. More sample solution was added in the pellet and then centrifuged again. This process was repeated for about 7-8 time until enlarge thick pellet is formed, which is the important requirement for XRD. Pellet was placed in a glass slide. This slide was then placed under the lamp for 20-25 minutes for drying of the pellet. As a result, a thick layer of silver nanoparticles was deposited on the glass slide for XRD analysis. The glass slide containing the sample was then carefully placed in the XRD machine (STOE, model number theta, Germany). The radiation source used was Copper K alpha, with 40 KV voltage, and 40mA current. The angle used in the process was 20-80 theta (θ).

3.7 Animals for *In vivo* Study

4 weeks old male BALB/c mice (n=20) were taken from animal house of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) in which the study was conducted. These male BALB/c mice were kept in cages (5

mice per cage) at constant temperature (25±2°C) and natural light-dark cycle (12-12 hours) and were initially fed with basic chow diet and were given glucose water.

3.7.1 Ethics Statement

The approval for all the protocols that were carried out during the research was obtained from internal review board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). All the 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).

3.7.1 Antiglycation Assays

3.7.1 BSA-glucose

With the modification of protocol of Zheng et al., 2008, this assay was performed. Briefly, BSA (1200mg) and glucose (1.5mM) were dissolved in PBS (pH 7.4) with 0.1% sodium azide. Test samples containing silver nanoparticles (15, 7.5, 3 and 0.6mg/mL) and aminoguanidine (15, 7.5, 3 and 0.6mg /mL) were added in the above solution. Aminoguanidine was used as positive control. All these samples were then incubated at 37°C for 7 days. After this incubation period, fluorescence intensity was measured at 340nm (excitation wavelength) and 420nm (emission wavelength).

3.7.2 BSA-methyglyoxal

With the modification of protocol of Zheng et al., 2008, this assay was performed. Briefly, BSA (1200mg) and methylglyoxal (4.32mg) were dissolved in PBS (pH 7.4) with 0.1% sodium azide. Test samples containing silver nanoparticles (15, 7.5, 3 and 0.6mg/mL) and aminoguanidine (15, 7.5, 3 and 0.6mg /mL) were added in the above solution. Aminoguanidine was used as positive control. All these samples were then

incubated at 37°C for 7 days. After this incubation period, fluorescence intensity was measured at 340nm (excitation wavelength) and 420nm (emission wavelength). To calculate %age inhibition, following formula was used:

$$\textbf{Inhibition\%} = [1-(A_i / A_o)] \times 100$$

A_i = Fluorescence of BSA and glucose/ methylglyoxal with nanoparticles/aminoguanidine

A_o = Fluorescence of BSA and glucose/ methylglyoxal with phosphate buffer

3.8 Animals for *In vivo* Study

3-4 weeks old male BALB/c mice (n=20) were taken from animal house of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) in which the study was conducted. These male BALB/c mice were kept in cages (5 mice per cage) at constant temperature (25±2°C) and natural light-dark cycle (12-12 hours) and were initially fed with basic chow diet and were given glucose water.

3.8.1 Ethics Statement

The approval for all the protocols that were carried out during the research was obtained from internal review board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). All the 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 to induce T2DM

T2DM mice model was constructed by combination of High Fat Diet (HFD) along with low doses of Streptozotocin. The normal control mice (n=10) were fed with basic chow diet consisting of the following components given in table 3.7.2

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

Table 3.8.2: Components of Basic Chow Diet

The mice models (n=10) for the purpose of diabetes induction were switched to high fat diet when they were 3 weeks of age. These mice were given intraperitoneal Streptozotocin (100mg/kg) injections prepared in 01.1M citrate buffer (pH 4.5) at 6^{th} and 9^{th} week of age (Day 21 and 42) after overnight fasting. The control animals received citrate buffer injections only. After 2 days of 2^{nd} STZ injection i.e. on 44^{th} day, Fasting blood glucose levels of all mice were checked using EasyGluco Blood Glucose Monitoring System. Mice with Blood Glucose levels greater than 126 mg/dL were considered as diabetic (Dong, Xu et al., 2013).

Group Number	**Type**	**Administration**	**No. of mice**
1	Control Healthy Mice	Normal Saline	10
2	Diabetic mice	None	10

Table 3.8.3: Mice Grouping

3.8.3.1 Normal Control

Group 1 was assigned as normal group and included 10 BALB/c mice, receiving normal diet and saline water.

3.8.3.2 Experimental Group

Group 2 was assigned as the experimental group for diabetes. The mice in this group were given Streptozotocin injections on day 21 and 42 and fed with high fat diet during the experiment. This group will be used for the comparison with the control to confirm whether diabetes is induced or not.

3.9 Confirmation of type2 diabetes in mice model

3.9.1 Body Weight measurements

Effect on body weight of mice due to high fat diet and Streptozotocin injections was studied for 6 weeks. The average weight of the normal mice was compared to the experimental group.

3.9.2 Fasting Blood Glucose Test

The glucose in the blood of the normal mice as well as Streptozotocin induced diabetic BALB/c mice was monitored on weekly basis. For fasting blood glucose test, the animals were fasted for 8 hours. Then the blood was taken from the tail of mice and measured the glucose level using the glucometer.

3.10 Primer Designing

For the designing of Primers, four different genes were selected namely; GLUT4, INS2, IRS2 and beta actin, where beta actin acts as house-keeping gene. Primers were designed through primer 3 plus software.

3.11 UCSC In-Silico PCR

Validity of selected primers was checked through In-silico PCR of UCSC browser (Rhead et al., 2010). Sequence of Forward and Reverse primers were pasted in designated boxes on the official website of In-silico PCR of UCSC browser (https://genome.ucsc.edu/cgi-bin/hgPcr). Target organism i.e. *mus musculus* (mice), was selected in nominated box, and PCR was run. Primers that showed binding to the target region and showed no off-target binding were chosen for further study. Primer sequences are shown in the table 3.10.

3.12 Primer BLAST

Primer BLAST is a tool to design target-specific primers and to confirm the validity of the primers and in this tool a combination of BLAST and a global alignment algorithm occurs to get a full primer-target alignment. Sequence of Forward as well as Reverse primers were pasted in designated boxes on the official website of Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). Target organism i.e. *mus musculus* (mice), was selected in nominated box, and Primer BLAST was run.

Primer Type	**Gene**	**Primer sequence**	**GC content**	**Melting Temp.**	**Self-complementarity**	**Product size (bp)**
Forward Primer	GLUT4	TTTGCCCCTCAGTCATTCTC	50%	60.2	3.00	243
Reverse Primer	GLUT4	TTCTATTTGCCGTCCTCCTG	50%	60.2	1.00	
Forward Primer	IRS2	CCTTGCTCCTCCACTTCTTC	55%	59.0	2.00	207
Reverse Primer	IRS2	GCCCGAACCTCAATAACAAC	50%	59.4	2.00	
Forward Primer	INS2	GACTCCCAGAGGAAGAGCAG	60%	59.1	3.00	243
Reverse Primer	INS2	CCAGTAACCACCAGCCCTAA	55%	60.0	3.00	
Forward Primer	Beta Actin	TGTCCACCTTCCAGCAGATGT	61.39	52.38	4.00	101
Reverse Primer	Beta Actin	AGCTCAGTAACAGTCCGCCTAG	61.53	54.55	4.00	

Table 3.11: List of Primers

CHAPTER 4

RESULTS

4.1 Primary Structure of Glut4

Tertiary structure of GLUT-4 was not found in PDB; therefore, it was predicted using bioinformatics tools. Primary sequence of a protein is simply the sequence of its constituent amino acids. Primary structure of Glut-4 protein was downloaded from NCBI database in FASTA format with an accession no of P14142.3 (Figure 4.1)

```
>sp|P14142.3
MPSGFQQIGSEDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETWLGR
QGPEGPSSIPPGTLTTLWALSVAIFSVGGMISSFLIGIISQWLGRKRAMLVNNVLAVLGGSLMG
LANAAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILIAQVLG
LESLLGTASLWPLLLGLTVLPALLQLVLLPFCPESPRYLYIIQNLEGPARKSLKRLTGWADVSG
VLAELKDEKRKLERERPLSLLQLLGSRTHRQPLIIAVVLQLSQQLSGINAVFYYSTSIFETAGV
GQPAYATIGAGVVNTVFTLVSVLLVERAGRRTLHLLGLAGMCGCAILMTVALLLLERVPAM
SYVSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRPAAMAVAGFSNWTSNFIIGMGFQYVAEA
MGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQISAAFHRTPSLLEQEVKPSTELEYLGPDEN
D
```

Figure 4.1: Primary sequence of GLUT4 in FASTA format, downloaded from NCBI

4.2 Secondary Structure

Primary sequence retrieved from NCBI was used for the prediction of the secondary structure of GLUT4 by implementing GOR4 online tool. Results of GOR4 are shown in Figure 4.2 from where it is clear that this protein has 206 amino acids (40.47%) forming alpha helix, 96(18.86%) and 207 (40.67%) amino acids involved in extended strands and random coil formation respectively. Trans membrane domains were established by TMHMM (Krogh et

al., 2001) software and it showed that there are 12 trans membrane domains with GLUT4 (Figure 4.3).

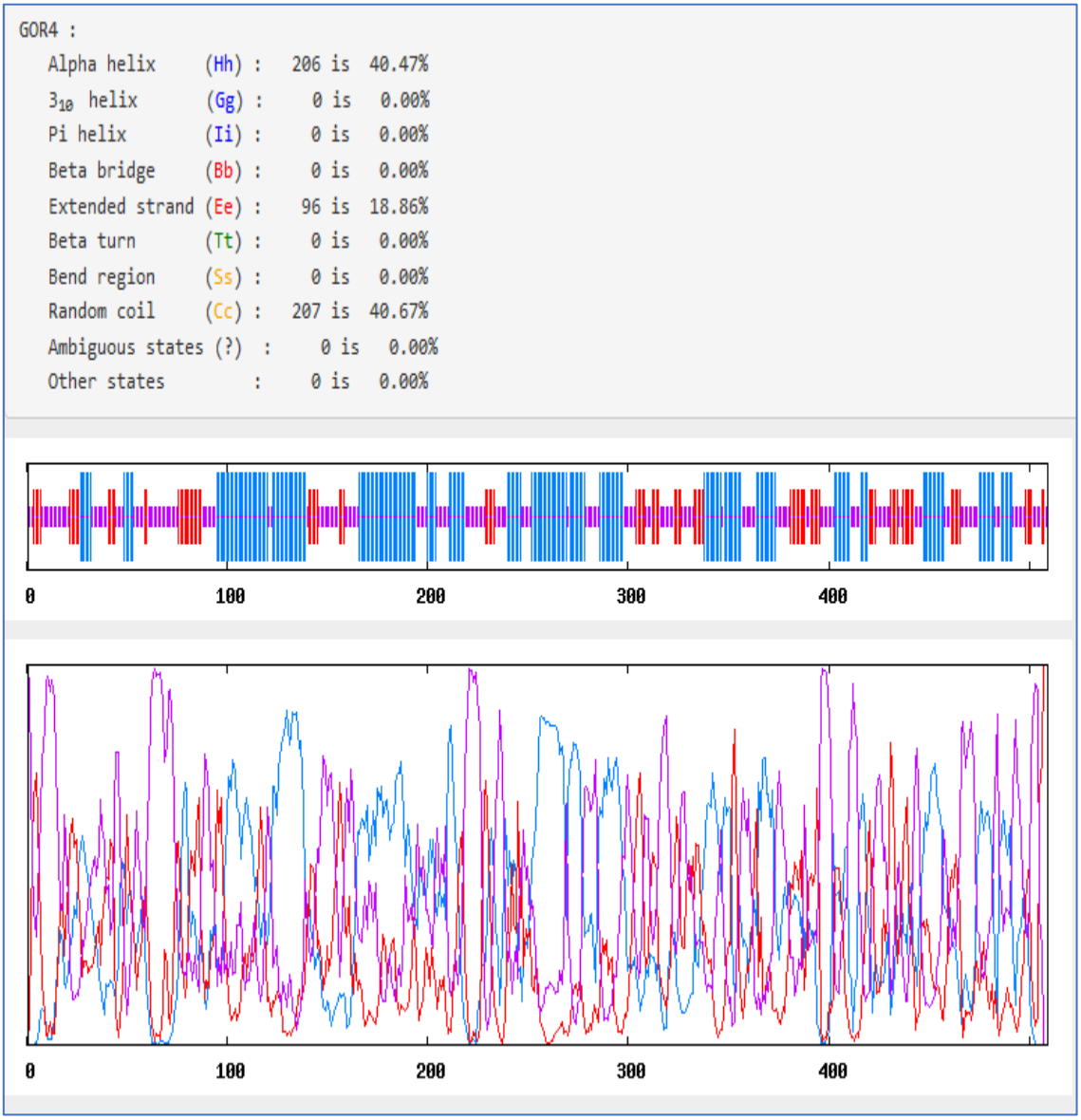


Figure 4.2: Secondary structure of GLUT4 protein, anticipated by GOR4 software

4.3 Transmembrane Domains (TMHMM)

TMHMM result

HELP with output formats

```
# sp|P14672|1-509 Length: 509
# sp|P14672|1-509 Number of predicted TMHs:  12
# sp|P14672|1-509 Exp number of AAs in TMHs: 262.31291
# sp|P14672|1-509 Exp number, first 60 AAs:  19.07592
# sp|P14672|1-509 Total prob of N-in:        0.83293
# sp|P14672|1-509 POSSIBLE N-term signal sequence
sp|P14672|1-509 TMHMM2.0        inside       1    19
sp|P14672|1-509 TMHMM2.0        TMhelix     20    42
sp|P14672|1-509 TMHMM2.0        outside     43    78
sp|P14672|1-509 TMHMM2.0        TMhelix     79   101
sp|P14672|1-509 TMHMM2.0        inside     102   112
sp|P14672|1-509 TMHMM2.0        TMhelix    113   135
sp|P14672|1-509 TMHMM2.0        outside    136   138
sp|P14672|1-509 TMHMM2.0        TMhelix    139   161
sp|P14672|1-509 TMHMM2.0        inside     162   167
sp|P14672|1-509 TMHMM2.0        TMhelix    168   190
sp|P14672|1-509 TMHMM2.0        outside    191   199
sp|P14672|1-509 TMHMM2.0        TMhelix    200   222
sp|P14672|1-509 TMHMM2.0        inside     223   287
sp|P14672|1-509 TMHMM2.0        TMhelix    288   307
sp|P14672|1-509 TMHMM2.0        outside    308   321
sp|P14672|1-509 TMHMM2.0        TMhelix    322   344
sp|P14672|1-509 TMHMM2.0        inside     345   350
sp|P14672|1-509 TMHMM2.0        TMhelix    351   373
sp|P14672|1-509 TMHMM2.0        outside    374   382
sp|P14672|1-509 TMHMM2.0        TMhelix    383   405
sp|P14672|1-509 TMHMM2.0        inside     406   417
sp|P14672|1-509 TMHMM2.0        TMhelix    418   440
sp|P14672|1-509 TMHMM2.0        outside    441   443
sp|P14672|1-509 TMHMM2.0        TMhelix    444   466
sp|P14672|1-509 TMHMM2.0        inside     467   509
```

Figure 4.3: Tran membrane domains in GLUT; established by TMHMM

4.4 Tertiary Structure

Tertiary structure was constructed using homology modeling, Threading and ab-initio approaches. SAVES and RAMPAGE online tools were utilized to choose the best one. Structures that had number of amino acids lower than 509 were excluded at once, no matter how much good they were. Figure 4.4.1 and 4.5.2 shows the 3D model of Ramachandran plot is also determined by using RAMPAGE. Ramachandran Plot of I-TASSER developed model of GLUT4 shows 85.3% amino acids in favored region, 8.5% in allowed and 6.2% in disallowed region.

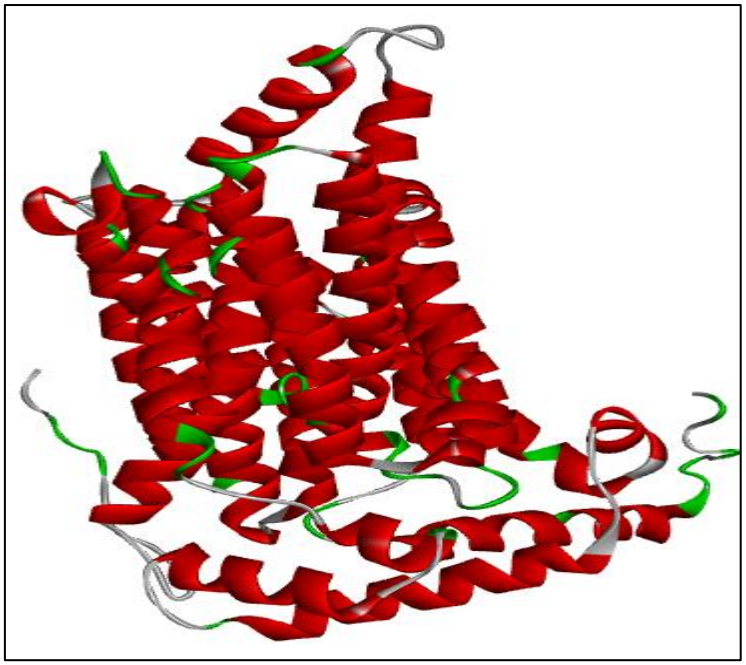


Figure 4.4.1: 3D structure of GLUT-4, predicted by I-TASSER

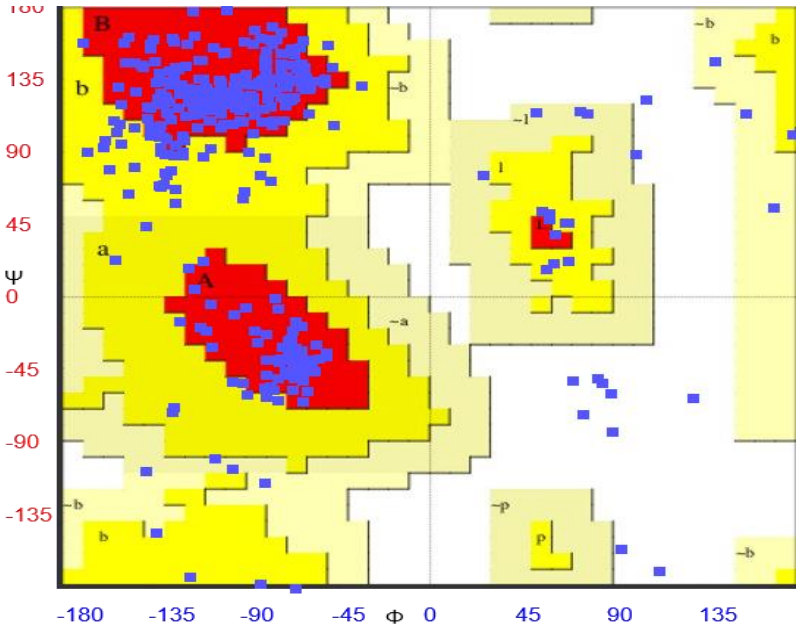


Figure 4.4.2: Ramachandran Plot of I-TASSER developed model of GLUT4

In the **Table 4.4** the results of SAVES analysis are given. In this analysis, comparison of different developed structures by using various softwares of 3D modeling was done, the I-TASSER developed model was the best one among all others and it is highlighted**.**

Table 4.4: Results of SAVES analysis; comparison of different developed structures by using various softwares of 3D modeling, the best one is highlighted.

Model	(Software)	Amino acid residues in final structure	Ramachandran Plot Residues in fav region: In allowed region: In outer region	ERRAT (Quality Factor A)	WhatCheck Error: Warning: Pass	Procheck Error: Warning: Pass
Model 1	Swiss-Model	451	97.3: 2.4: 0.2			
Model 1a	Swiss-Model	392	90.5: 7.9: 1.5			
Model 2	I-TASSER	509	87.4: 11.6: 1	**87.885**	6: 11: 30	3: 2: 3
Model 2a	I-TASSER	509	85.3: 8.5: 6.2	92.3868	6: 10: 31	2: 2: 4
Model 3	IntFOLD	509	80.7: 16.9: 2.4	**96.4072**	8: 16: 23	6: 1: 2
Model 3a	IntFOLD	509	79.7: 17.1: 3.2	**97.6048**	8: 17: 23	6: 1: 2
Model 4	FALCON	509	79.2: 20: 0.8	**92.0408**	6: 11: 30	3: 2: 3
Model 4a	FALCON	509	77.6: 22.2: 0.2	**88.3576**	6: 11: 30	3: 2: 3
Model 5	PHYRE2	468	95.9: 2.8: 1.3			
Model 5a	PHYRE2	451	88.9: 8.9: 2.2			
Model 6	MUSTER	509	78: 21: 1	**86.2705**	6: 10: 31	3: 2: 3
Model 6a	MUSTER	509	77: 22.4: 0.6	**89.749**	6: 11: 30	3: 2: 3

4.5 Docking Analysis

Molecular Docking was conducted to identify the binding affinity of Ag with the GLUT4 active sites. From docking results, it was speculated that silver is embedded in the active sites of Glut4 (Figure 4.5.1) thus indicating that silver has interactions with Glut4. PDBsum generate showed that asparginine residue of Glut4 has interactions with silver (Figure 4.5.2)

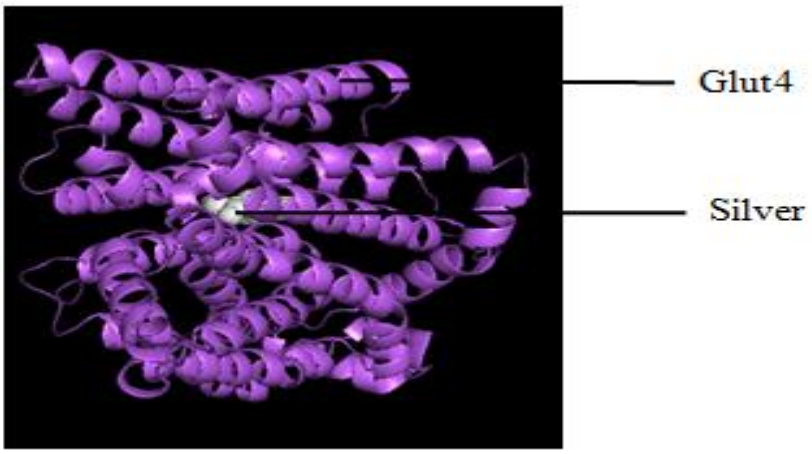


Figure 4.5 Interaction of with the binding pockets of Glut-4 protein

List of protein-ligand interactions

Hydrogen bonds

<----- A T O M 1 ----->					<----- A T O M 2 ----->				
Atom no.	Atom name	Res name	Res no.	Chain	Atom no.	Atom name	Res name	Res no.	Chain	Distance
2138	ND2	ASN	304	A -->	3429	Ag	LIG	1	_	2.87

Figure 4.5.2 List of protein-ligand interactions by PDBsum generate

4.6 Preliminary Confirmation of *Thymus serpyllum* mediated silver NPs

After the addition of 10mM solution of silver nitrate to the aqueous extract of *Thymus serpyllum*, light brown color changes to dark brown gradually with time that visually confirms the formation of silver nanoparticles. After 24 hours of air drying of silver nanoparticles in a petri plate, solid blackish brown powder of silver nanoparticles was obtained as shown in fig 4.6.

Figure 4.6: Purified Silver Naonoparticles

4.7 Characterization of Biogenic Silver Nanoparticles

To analyze the morphology, size and purity of the nanoparticles, the characterization of *Thymus serpyllum* mediated silver nanoparticles was performed.

4.7.1 UV Visible Absorption Spectroscopy

UV-Vis spectroscopy shows peak with maximum absorbance at 400nm which indicates the uniform shape of silver nanoparticles as shown in figure 4.7.1. However AgNO3 and extract do not have any peak in this region (250-500nm).

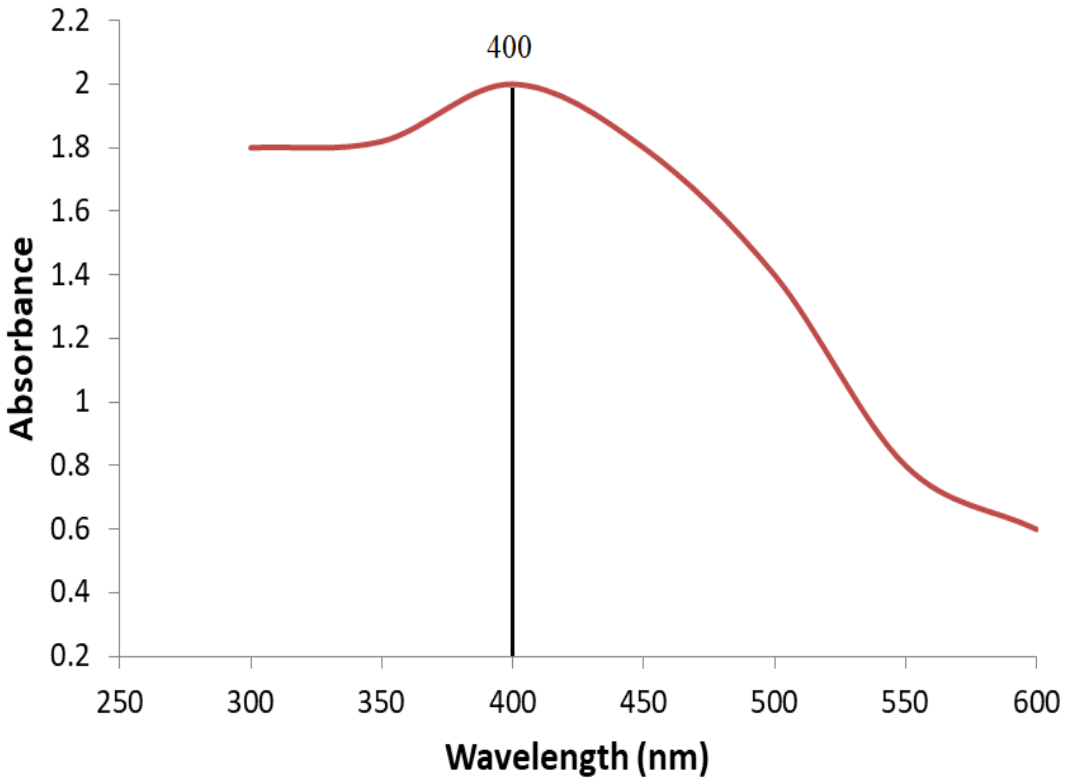


Figure 4.7.1: UV-Vis Spectroscopy of Reaction mixture indicating typical absorbance peak of AgNPs around 400nm.

4.7.2 X-Ray Diffraction (XRD)

X-Ray Diffraction is a technique that identifies the structure imperfections and crystallinity in the sample. The patterns that were obtained by X-Ray Diffraction were analyzed by Jade 8 Software and the JCPDS card number (00-004-0783) was obtained that confirms the crystalline and stale formation of biogenic silver nanoparticles.

The XRD pattern shows that they are present in flat form with their basal planes that are parallel to the substrate. The XRD pattern of the silver nanoparticles is shown in the figure 4.7.2. This pattern indicates six intensive peaks i.e. **28°, 32°, 46°, 55°, 57° and 77°** obtained at 2θ. These are indexed at (111), (200), (220), (311), (222), and (400).

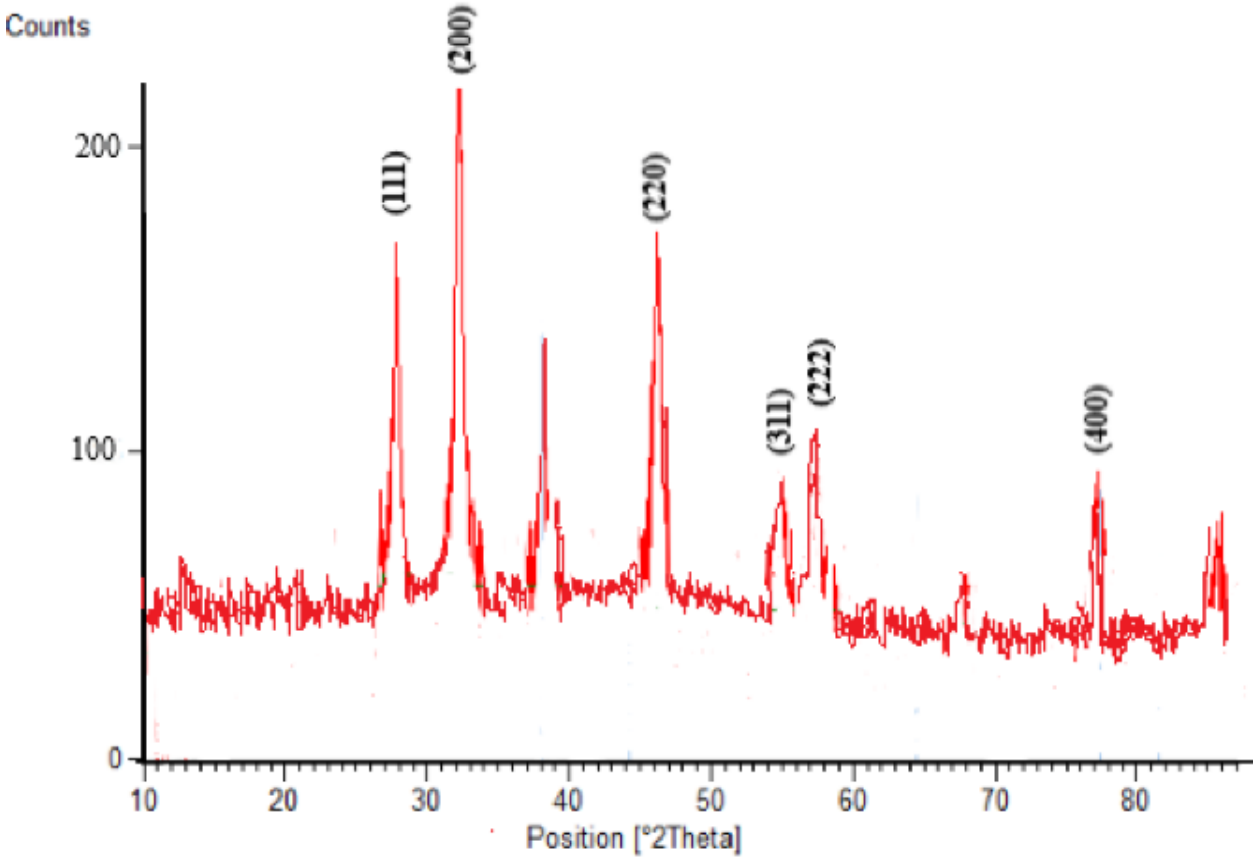


Figure 4.7.2: X-ray Diffraction of Thymus serpyllum mediated silver nanoparticles

4.8 Antiglycation Assays

4.8.1 BSA-glucose

Aminoguanidine gives %age inhibition (80%, 64%, 40% and 12%) at 15mg/mL, 7.5mg/mL, 3mg/mL and 0.6mg/mL. However biogenic silver nanoparticles give 78%, 55%, 28% and 6% at 15mg/mL, 7.5mg/mL, 3mg/mL and 0.6mg/mL as shown in the figure 4.8.1:

4.8.2 BSA-methylglyoxal

Aminoguanidine gives %age inhibition (72%, 62%, 44% and 24%) at 15mg/mL, 7.5mg/mL, 3mg/mL and 0.6mg/mL. However biogenic silver nanoparticles give (68%, 52%, 30% and 16%) at 15mg/mL, 7.5mg/mL, 3mg/mL and 0.6mg/mL as shown in the figure 4.8.2:

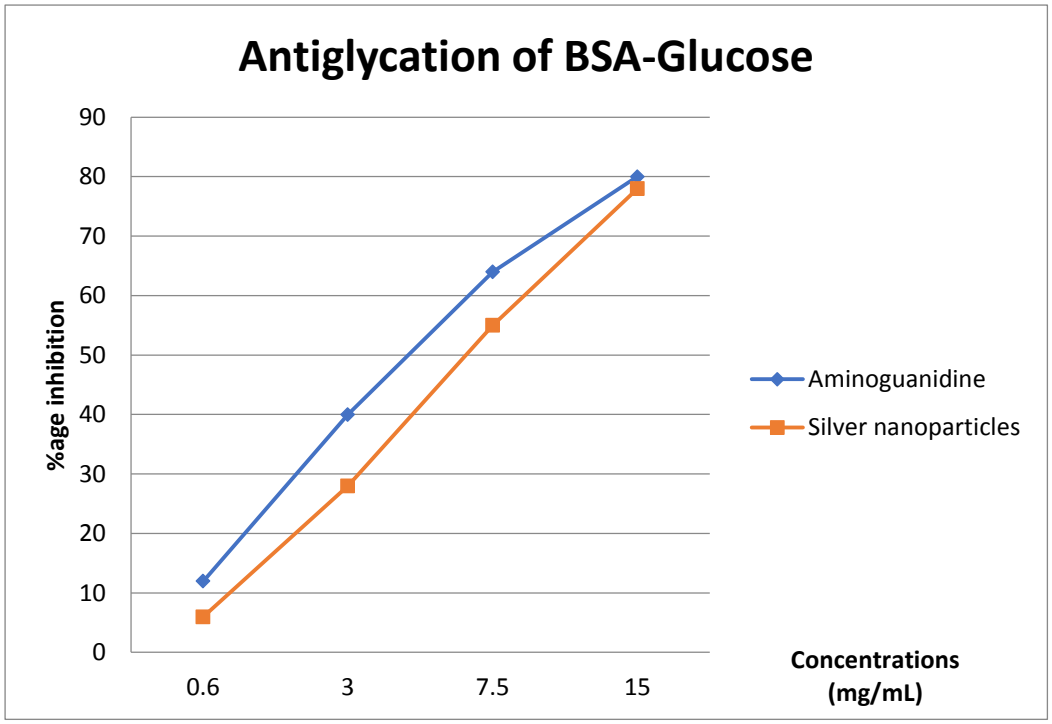


Figure 4.8.1: Antiglycation of BSA-Glucose

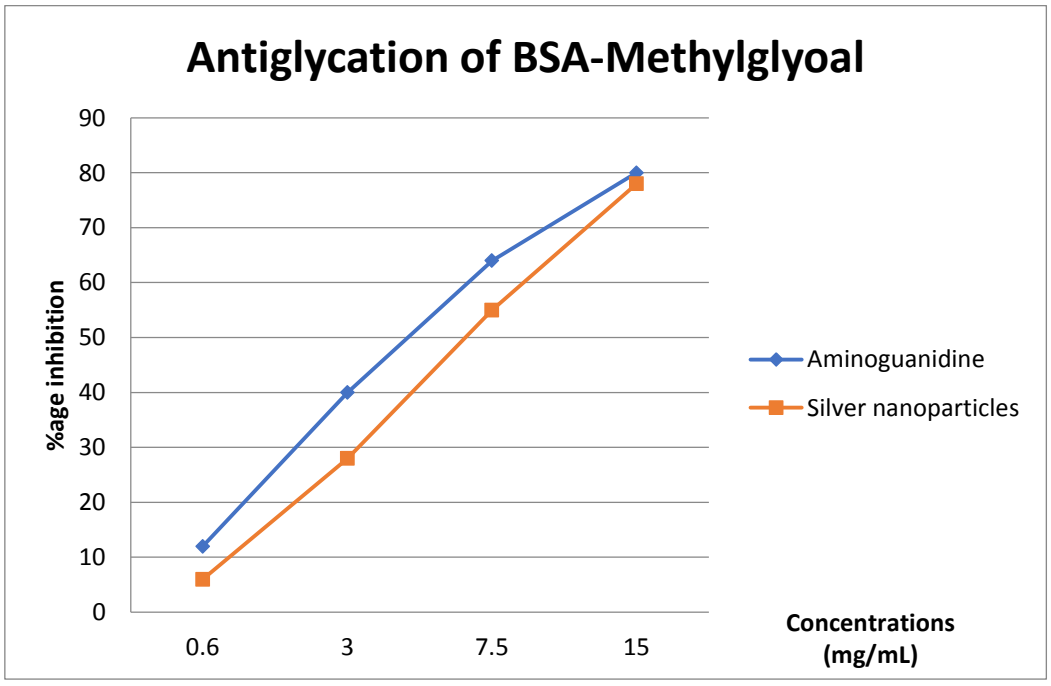


Figure 4.8.2: Antiglycation of BSA-Methylglyoxal

4.9 Evaluation of Induction of Diabetes Mellitus in BALB/c mice

4.9.1 Estimation of Body weight during Diabetic mice model construction

Effect on body weight of mice due to high fat diet and Streptozotocin injections was studied for 6 weeks. The average body weight of normal mice was compared to the diabetic group. The body weight of the diabetic BALB/c mice significantly increased due to High fat diet and Streptozotocin injections. Figure 4.8.1 demonstrates that there is a significant ($p<0.05$) difference between the body weights of control and diabetic mice groups.

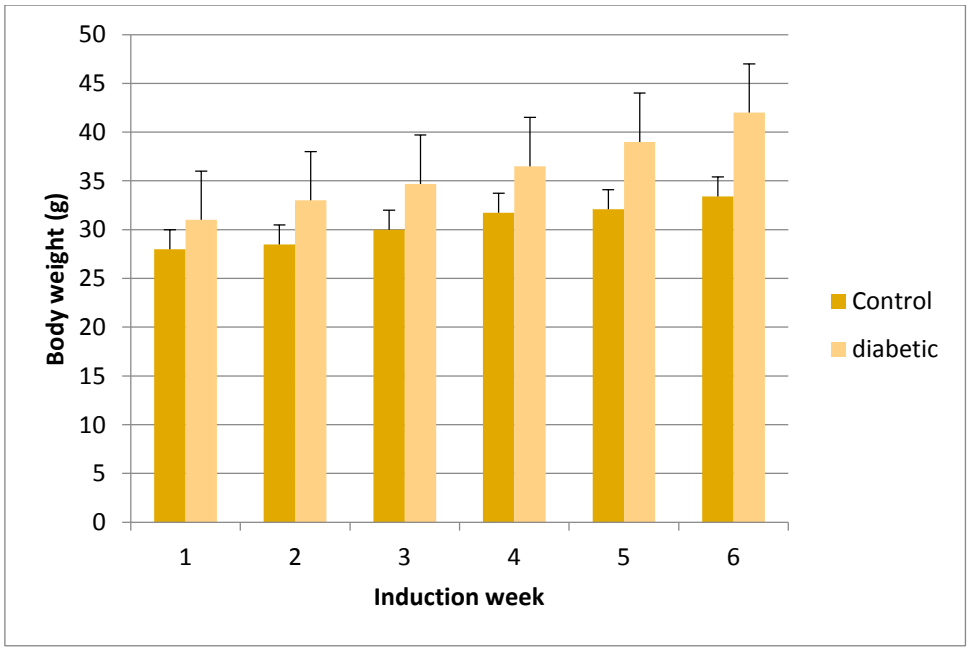


Figure 4.9.1 Measurement of body weight after every 1 week of Induction (*P<0.05)*

4.9.2 Estimation of Fasting Blood Glucose during Diabetic mice model construction

Fasting blood glucose levels of the diabetic and control mice were measured every week, in order to confirm that diabetes is successfully induced after 42 days protocol. Figure 4.8.2 depicts that there is a significant ($p<0.01$) difference in FBG levels of diabetic and control mice groups.

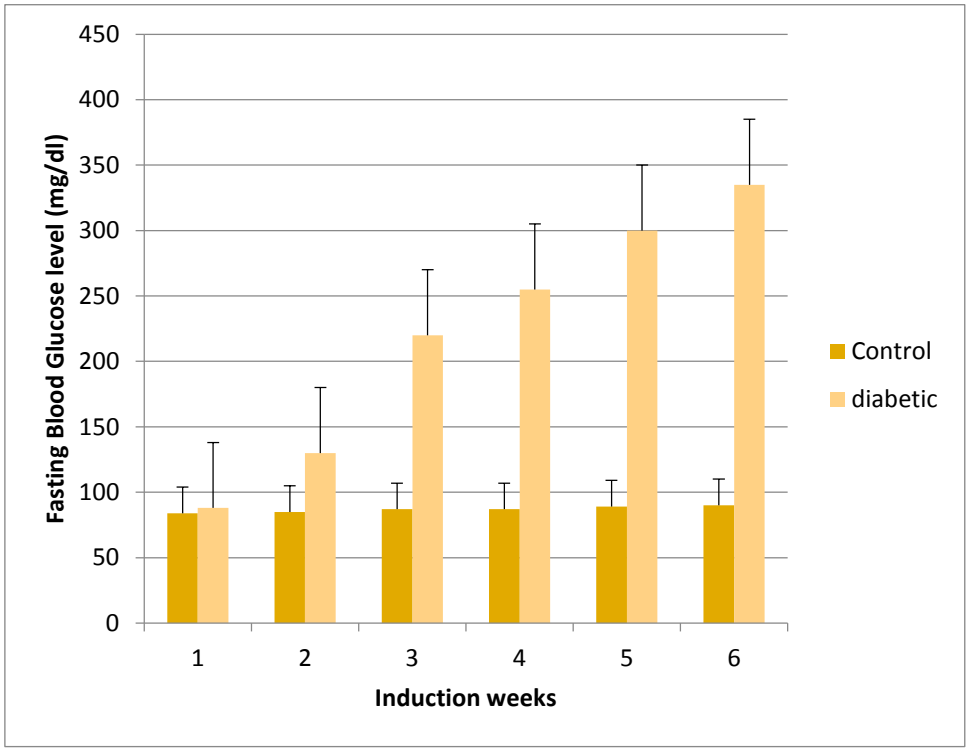


Figure 4.9.2 Measurement of fasting blood glucose after every 1 week of Induction *(P<0.01)*

4.10 Primer Validation

4.10.1 UCSC In-Silico PCR and Primer BLAST

Primers designed through Primer 3 Plus software were validated by Insilico PCR tool of UCSC Browser and Primer BLAST to design target-specific primers for polymerase chain reaction. Results depicted that the chosen primers for β-Actin (House Keeping Gene) Glut4, INS2 and IRS2 binds to the target region well and shows no off-target effects. Results of In-silico PCR are shown in figures 4.9.1.1 - 4.9.1.7.

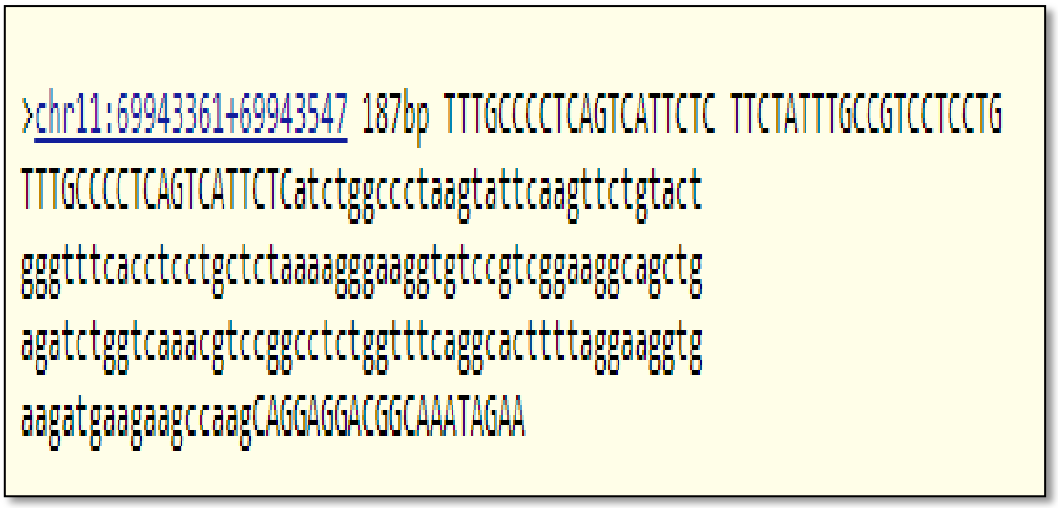


Figure 4.10.1.1: Insilico PCR results of GLUT4

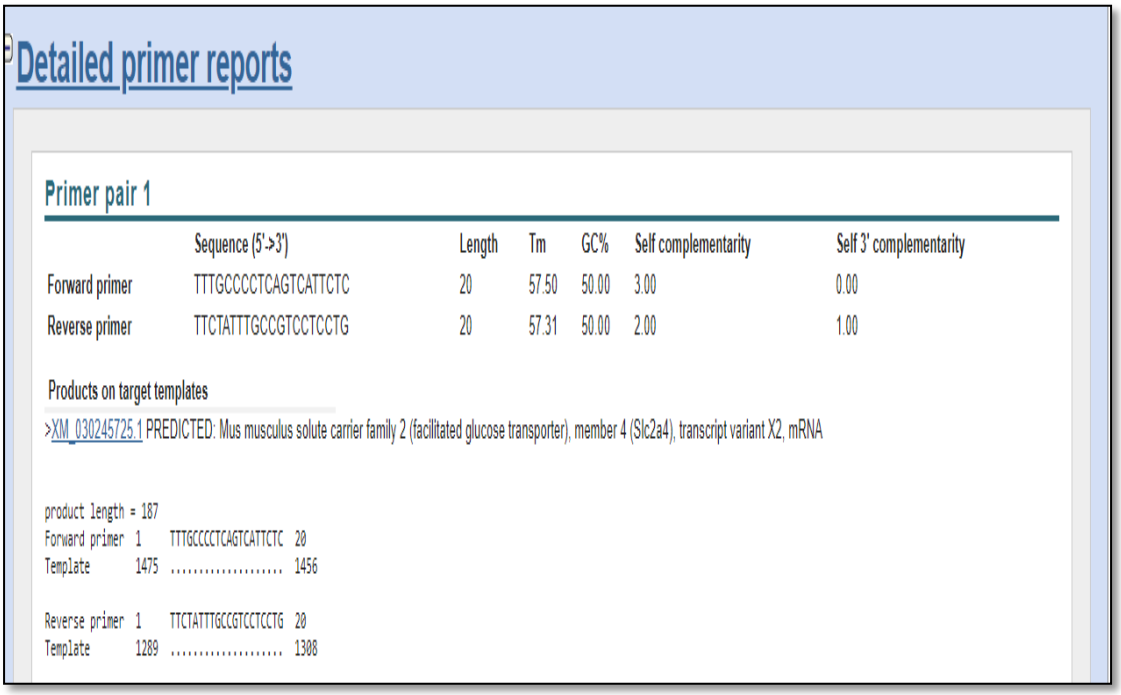


Figure 4.10.1.2: Primer-BLAST results of GLUT4

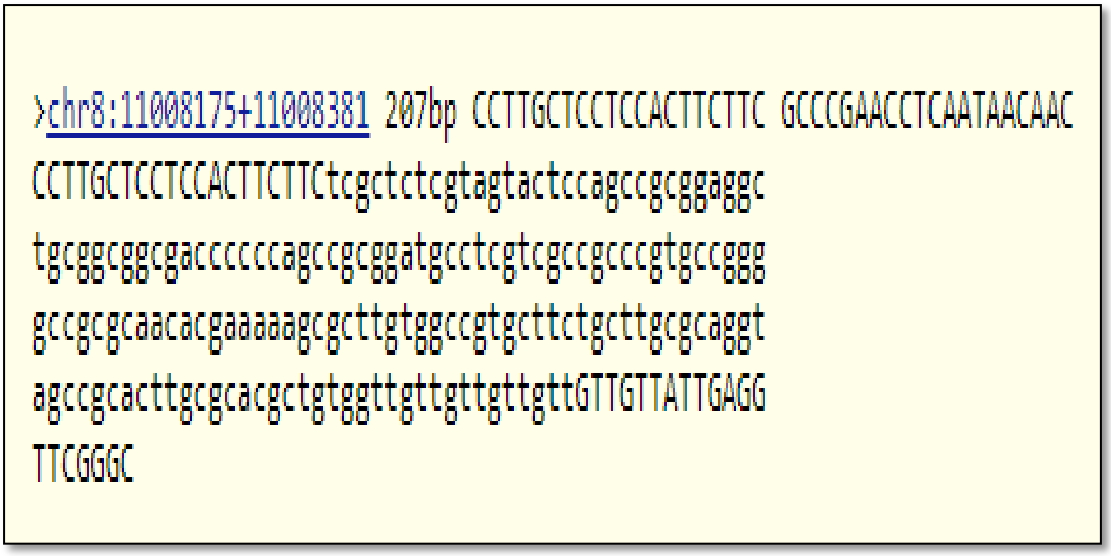


Figure 4.10.1.3: Insilico PCR results of IRS2

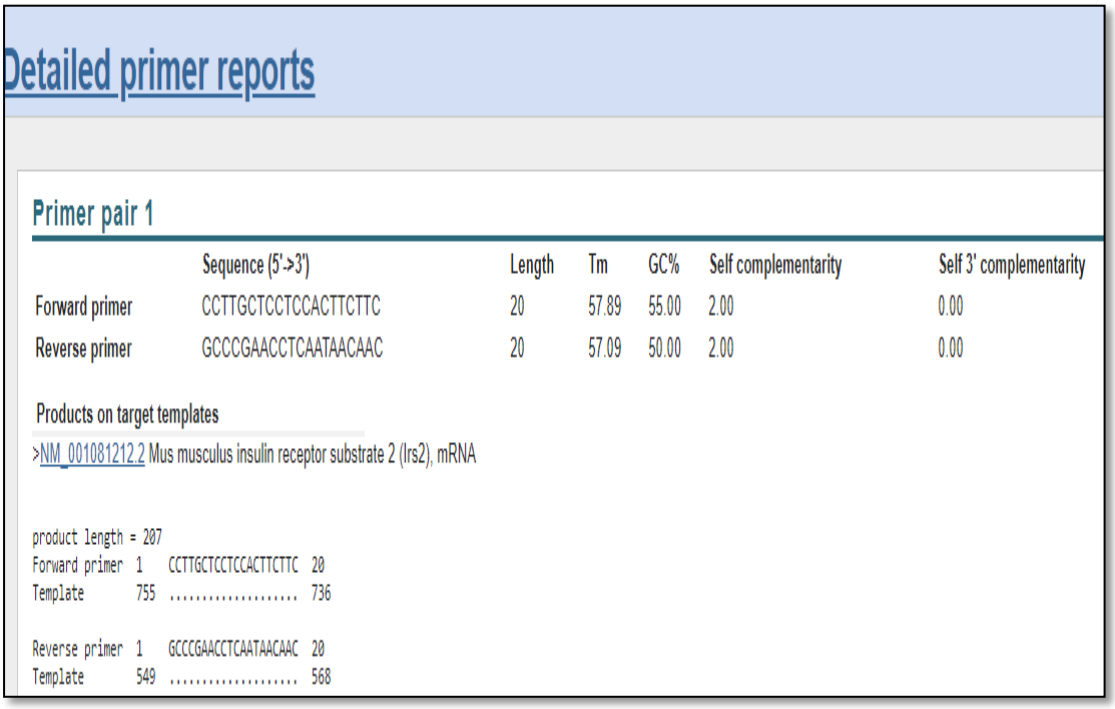
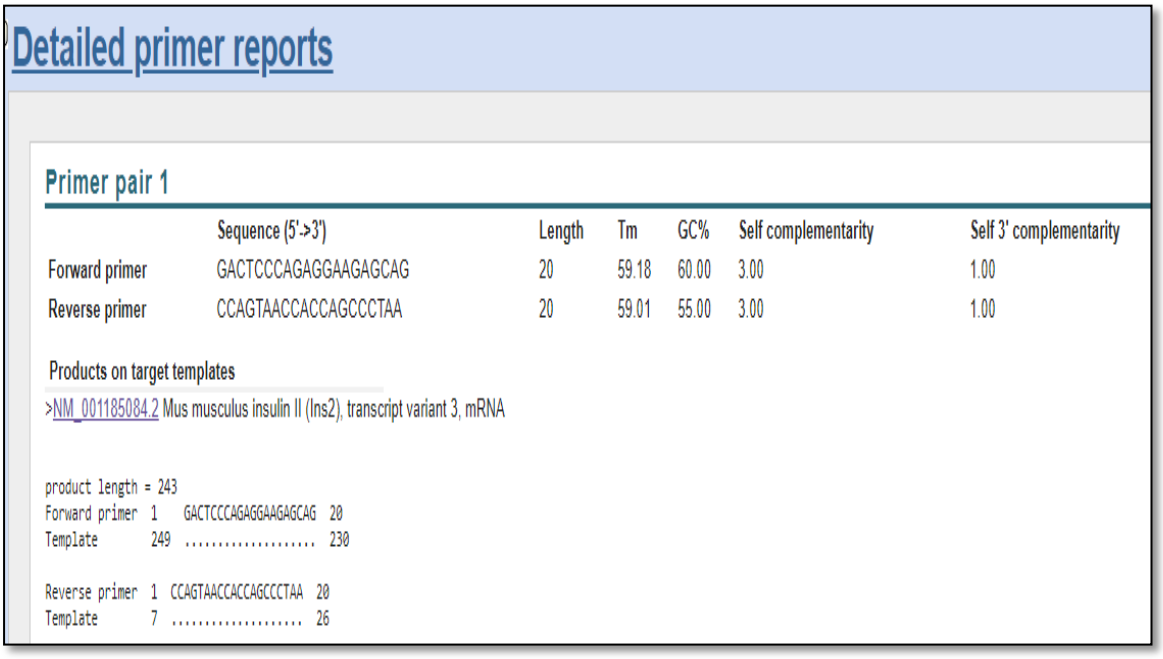


Figure 4.10.1.4: Primer-BLAST results of IRS2

>chr7:142679478+142679720 243bp GACTCCCAGAGGAAGAGCAG CCAGTAACCACCAGCCCTAA
GACTCCCAGAGGAAGAGCAGggccagcaggggcaggaagcgcatccacag
ggccatgttgaaacaataacctggaagataggctgggttgaggatagcaa
aagtttcacgtaagagaggaggctatatcctacctcaagtccctgaggtc
ttagctggggagccaggcccactgagaagagtaccttcctgcttgctgat
ggtttttgattgtagcggatcacTTAGGGCTGGTGGTTACTGG

Figure 4.10.1.5: Insilico PCR results of INS2



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACTCCCAGAGGAAGAGCAG	20	59.18	60.00	3.00	1.00
Reverse primer	CCAGTAACCACCAGCCCTAA	20	59.01	55.00	3.00	1.00

Products on target templates

>NM_001185084.2 Mus musculus insulin II (Ins2), transcript variant 3, mRNA

product length = 243
Forward primer 1 GACTCCCAGAGGAAGAGCAG 20
Template 249 230

Reverse primer 1 CCAGTAACCACCAGCCCTAA 20
Template 7 26

Figure 4.10.1.6: Primer-BLAST results of INS2

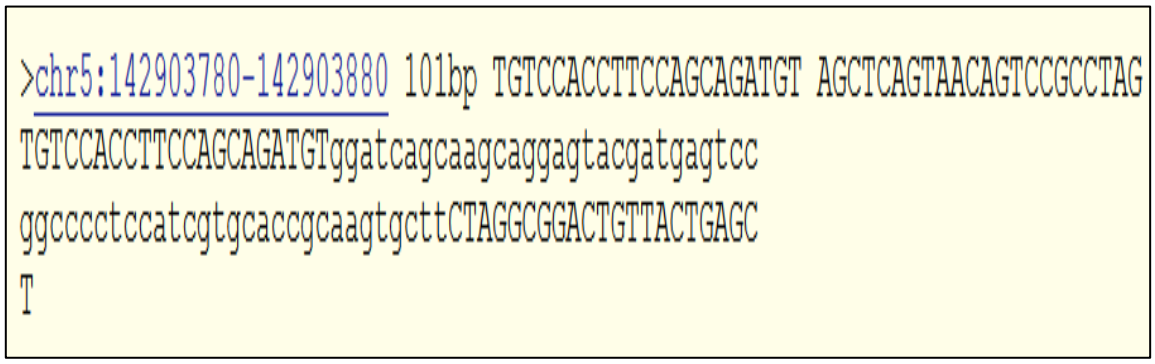


Figure 4.10.1.7: Insilico PCR results of Beta Actin

The primers of INS2, IRS2 and Glut 4 that are validated by UCSC In-Silico PCR can further used for in vivo studies to determine the mechanism of action of the biogenic silver nanoparticles to treat diabetes mellitus.

CHAPTER 5

DISCUSSION

Type 2 diabetes mellitus (DM) is a persisting metabolic complication and is distinguished by means of persistent hyperglycemia that is the consequence of either entire or incomplete deficiency of insulin secretion or insulin action. The most commonly used therapeutic agent used for type 2 diabetes mellitus is metformin. However, it has various side effects which include its slow mode of action and gastrointestinal infections. Scientists are therefore trying to overcome these limitations by leading towards the nanobiotechnology. Nanotechnology has provided absolutely innovative concepts and innovative approaches/processes in medicinal drug as well as in dentistry which will facilitate us in making superior, former and sure prognosis and to treat patients with least feasible interventions and without any unfavorable outcomes (Tevatia et al., 2016). The nanomedicine is the appliance of nanotechnology for treatment, prognosis, monitoring, as well as control of disease. Nanomedicine has the potential to overwhelm the consequences of conventional therapies.

In diabetes, nanoparticles are used to monitor blood glucose levels, in bio-imaging and bio-imaging as well as for targeted insulin delivery vehicles (Mukhopadhyay and Prosenjit, 2018). Silver nanoparticles have large potential for biomedical applications in diagnostics, imaging and treatment. Silver can be used as an antibacterial agent. In wound dressings, topical creams, antiseptic sprays, as well as fabrics, silver can be added (Ahmed et al., 2016). AgNPs reduce the actions of proinflammatory cytokines, interferon gamma and tumor necrosis factor alpha (Shin et al., 2007). However, toxic effects of silver nanoparticles should not be ignored.

It is important to evaluate the dimension of nanoparticles so that they can be used in therapeutics and biomedical research as in an in-vivo studies by Zang et al, have confirmed that nanoparticles with size greater than 80 nm gets entrapped in liver and can exhibit long

term cytotoxicity. However, small nanoparticles with size less than 10 nm can excreted via urine. (Zang et al 2014).

In the recent research, the synthesis of nanoparticles by plants have gained much importance because of the presence of various phytochemicals in plants that act as capping and reducing agents (Taghikhani et al., 2014). *Thymus serpyllum* is a significant origin of therapeutic substances that have antioxidant, anti-diabetic, antimicrobial, antitumor potential, as well as it has utility in pharmaceutical, food, and cosmetic industries (Jaric et al., 2015). Mihailović-Stanojević and his co-workers illustrated the antioxidant potential of wild thyme aqueous extracts in rats' model. Phenols and flavonoids were abundantly present in the extract (Mihailovic et al., 2013).

The aims of this study were to dock silver with GLUT4, to synthesize green, cost effective silver nanoparticles (AgNPs) from the extract of *Thymus serpyllum*. This synthesis method was also optimized under different physiochemical parameters such as temperature and molar concentration ratios to obtain narrow size distribution of AgNPs. XRD was used to characterize these silver nanoparticles. To induce diabetes in BALB/c mice, streptozotocin (STZ) injections were given along with high fat diet. Primers for Glut4, INS2, and IRS2 were designed by Primer 3 Plus and then validated by UCSC In-Silico PCR.

Molecular Docking was conducted to identify the binding affinity of Ag with the GLUT4 active sites. Depending upon the docking pose, it was speculated that the Ag demonstrated strong interactions with the active site of GLUT4 and their nearby residues.

By the modification of the protocol of Sun et al (Sun, Cai et al., 2014), silver nanoparticles were synthesized from *Thymus serpyllum* plant extract. The protocol was optimized by varying the concentration of plant extract, silver nitrate and temperature. The bioactive

compounds such as flavonoids, phenols etc present in *Thymus serpyllum* (Mihailovic et al., 2013) act as stabilizing agent during the synthesis of silver nanoparticles.

After overnight incubation, the light yellow shade of the reaction mixture was altered to blackish brown which indicated the preliminary confirmation of synthesis of *Thymus serpyllum* mediated silver nanoparticles. The optimal conditions for *Thymus serpyllum* mediated silver nanoparticles were 35°C with overnight incubation for 14.25ml diluted *Thymus serpyllum* extract and 10mM silver nitrate.

These biogenic silver nanoparticles were initially characterized by UV-VIS spectroscopy, showing maximum absorbance at the characteristic wavelength of 400nm. The shape of these silver nanoparticles was found spherical. X-ray Diffraction pattern of these biogenic silver nanoparticles showed six intensive peaks. Similar peaks are also cited in the literature for the silver nanoparticles (Hu, Wang et al., 2008).

The second objective of the study was the induction of diabetes mellitus in BALB/c male mice. In the study by Magalhães et al, they have also successfully induced diabetes mellitus type 2 with streptozotocin injections combined with High fat diet (Magalhães et al., 2019). Male BALB/c mice were divided into a control group (normal feed with saline water), and experimental group (High fat diet and Streptozotocin injections). Intraperitoneal streptozotocin injections were given to experimental male BALB/c mice on day 21 and 42. Fasting blood glucose and weight of the control and experimental mice were monitored every week. As a result of Streptozotocin injections and high fat diet, the fasting blood glucose level and weight of male BALB/c mice was greater than the control, which indicated the induction of type 2 diabetes mellitus in BALB/c mice.

Next objective was primer designing and their validation. The genes related to obesity and reactive oxygen species i.e. Glut4, INS2 and IRS2 were selected for primer designing. β-actin was used as house-keeping gene. Primer 3 plus was used to design primers. The primers with

good GC content and melting point were selected. These were further validated by UCSC In-Silico PCR. UCSC In-Silico PCR gives the primers that showed binding to the target region and showed no off-target binding and hence selected for further in vivo analysis.

Thus, silver nanoparticles having anti-diabetic potential can be synthesized from *Thymus serpyllum* extract under optimized conditions and type 2 diabetes mellitus can be induced by using streptozotocin injections and high fat diet.

CHAPTER 6

CONCLUSION AND FUTURE PROSPECTS

This study addresses the biosynthesis of anti-diabetic silver nanoparticles using *Thymus serpyllum* plant extract in order to minimize the side effects of synthetic drugs used for the treatment of diabetes. The aqueous extract of *Thymus serpyllum* is rich in phenols, flavonoids and many other bioactive compounds that act as capping and reducing agents. These *Thymus serpyllum* mediated silver nanoparticles were characterized by UV-Visible Absorption Spectroscopy showing maximum absorbance at the characteristic wavelength of 400nm and X-ray Diffraction.

Intraperitoneal streptozotocin injections and continuous feeding of high fat diet to experimental male BALB/c mice induces type 2 diabetes mellitus in male mice. Our results of UCSC In-Silico PCR of Glut4, INS2 and IRS2 genes suggest that these primers show binding to the target region in mice genome and showed no off-target binding and therefore can be used for further in-vivo study.

There is a need of investigation of INS2, Glut4, IRS2 and other genes involved in diabetes to explain the exact mechanism of action of biogenic silver nanoparticles. In future, the testing of anti-diabetic potential of these biogenic silver nanoparticles in diabetic cell line is also needed. Blood Brain Barrier permeability of *Thymus serpyllum* mediated silver nanoparticles for their toxicological assessment.

CHAPTER 7

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