TO STUDY THE DIABETES MEDIATED CNS COMPLICATIONS IN MOUSE MODEL OF DIABETES MELLITUS II

ENROLLMENT NO. – 142554

STUDENT NAME - Poonam Kumari

SUPERVISOR NAME - Dr. Udayabhanu M

MAY - 2016

Submitted in partial fulfillment of the requirement for the award of the Degree of

Master of Technology

in

Biotechnology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION AND TECHNOLOGY

WAKNAGHAT, SOLAN – 173234, HIMACHAL PRADESH

TABLE OF CONTENTS

CERTIFICATE

This is to certify that the work titled "**TO STUDY THE DIABETES MEDIATED CNS COMPLICATIONS IN MOUSE MODEL OF DIABETES MELLITUS II**" submitted by "**Poonam Kumari**" in partial fulfillment for the award of M. Tech (Biotechnology) of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor ……..

Name of Supervisor ……..

Designation ……..

Date ……..

ACKNOWLEGMENT

Feelings of gratitude are perhaps the most difficult one to express literally. During my research work, I have been accompanied and supported by so many people. I consider this an opportunity to express my gratitude to all those who have been involved directly or indirectly with the successful completion of this dissertation.

I express my deep sense of gratitude from the inmost recess of my heart to project supervisor Dr. Udayabanu M. for his valuable guidance, suggestions, encouragement and interest throughout the completion of my project work. His presence has always been as source of inspiration for me, which has gained tune through this work.

It is my pleasure to express my thanks to Dr. R.S Chauhan for providing me this opportunity facilities and encouragement for doing this project work.

I sincerely thank Mr Vineet Mehta for his cooperation, coordination, valuable suggestions and keen care bestowed on me during my work.

I would also thanks to biotechnology department for their support and encouragement.

Signature of the student ……..

Name of Student ……..

Date ……..

SUMMERY

Diabetes Mellitus is a chronic metabolic disorder which is becoming epidemic globally. India has second highest number of diabetic patients thus it needs immediate attention. CNS complications of diabetes are major cause of morbidity in diabetics and the restrict person's normal day to day life. Several medications are available for the management of diabetes but none is capable of preventing, halting or reversing neurological complications. Further, all these medications provide symptomatic relief and has no effect on the cause of disease due to which diabetes as well as associated complications keeps on intensifying. Therefore, aim of the present study was to identify and evaluate certain natural molecules which might be beneficial in the management of diabetes as well as its neurological complications. We evaluated several small molecules of natural origin through *in-vitro* assays to evaluate their potential to alleviate oxidative stress, genotoxicity and neuroprotective effect, which are primary pathways leading to the development of diabetes associated neurological complications. *In-vitro* study concluded that quercetin possesses immense neuroprotective potential and therefore it was further evaluate in mice model of type-II diabetes. It was observed that quercetin treatment significantly prevented hyperglycemia mediated neurodegeneration as evident from histopathological sectioning. Further quercetin treatment enhanced GLUT4 translocation to neuronal membrane which indicates that it may have efficiently reversed insulin resistance in CNS and therefore may aid in preventing neurological complications. Our study concluded that quercetin may a beneficial additive to the therapeutic strategy for the management of diabetic neurological complications.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

S.No.	Abbreviations	Full Form
1.	EtBr	Ethidium Bromide
2.	FBS	Fetal Bovine Serum
3.	DMEM	Dulbecco's Modified Eagle Medium
4.	DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
5.	EDTA	Ethylenediaminetetraacetic acid
6.	DMSO	Dimethyl sulfoxide
7.	PBS	Phosphate Buffer Saline

Chapter 1

Introduction

Diabetes Mellitus (DM) is the chronic metabolic disorder in which is characterized by persistently elevated level of blood glucose due to either insufficient production of insulin or due to insulin resistance. It is considered as most common endocrine disorder from which millions of people are affected. In 2015, 482 million people were diagnosed with DM out of which 5 million lost their life. India is home to 69.2 million diabetic people and globally stands second after China in terms of diabetic burden. Global diabetic burden is expected to up by 54% to 642 million by year 2040, in which India is projected to share 123.5 million patients. Apart from this, few other statists will clearly indicates that DM will become epidemic in coming years is that in 2015 WHO reported that 52% cases of DM remain undiagnosed, 382 million people are suffering from impaired glucose tolerance (prediabetic) and in India 21.4% population is overweight, 4.7% is obese and 12.1% population in physically inactive. All these factors are key elements for development of DM and will intensify diabetic load in near future. In terms of annual health care expenditure, 12% of global health care expenditure is spent on diabetes and despite of that we are unable to halt the development and progression of DM and its associated complications (International Diabetic Atlas, 2015). DM can be classified into 3 major catagories:

1. **Type I Diabetes Mellitus (T1DM)**:

   It is also known as insulin dependent diabetes or early onset diabetes. Insulin is a hormone which is required for the translocation of glucose from outside of the cell to inside of the cell. The insulin hormone is synthesis in pancreas. The β-cells of pancreas secrete insulin hormone to move the glucose into the cell where it can used as an energy source. T1DM is an autoimmune disorder in which body produces antibodies against its pancreatic β-cells thereby lowering the person's ability of secrete insulin. Thus the body produce little or no insulin and the plasma blood glucose level increases which leads to various complications. The production of these auto-antibodies can be of viral infection which stimulates the production of auto-antibodies. The genetically susceptible individual has higher chances of having Diabetes mellitus II. The polymorphism in HLA genes are major factor which makes a person susceptible to this disease. The insulin therapy is best suited for T1DM patients.

2. **Type II Diabetes Mellitus (T2DM)**:

   It is also known as insulin independent diabetes or late onset DM. It occurs due to insulin resistance in which insulin fails to exert its blood lowering effect and consequently blood

glucose rises. The molecular mechanism behind the insulin resistance is still unclear. But it is said that an increase in the amount of intracellular fatty acid metabolite activates a serine kinase cascade which leads to down regulate the insulin signalling pathway (Saini, 2010). It is found out that the many molecules like IRS-2 (insulin receptor substrate) get dysfunction (Qiao et al, 2002) which leads to the increase in the blood glucose level. Poorly controlled Diabetes mellitus II leads to various microvascular and macrovascular complications. Unlike the patients of type 1 diabetes, T2DM patients are not dependent on insulin. The obesity and inactivity among children leads to increase the cases of Diabetes mellitus II in younger generation. This diabetic condition is basically is a complex interactions between environment and genetic factors. It is also characterized by insulin resistance as well as inadequate insulin supply to body. Genomic factors also play a major role in Diabetes mellitus type-II. Some of the SNPs were found in various genes which lead to increase the chance of diseases. One of the genes is PPARG which associated with fat metabolism. As the chances of diabetes induced complications were increase in case of Diabetes mellitus II. Cognitive dysfunction is also seen as a major diabetic induced complication.

Major risk factors of Diabetes mellitus, some of them are as follows:

- Obesity
- Age (but now it is affecting the younger population also)
- Family history of Diabetes mellitus II in parents and siblings
- History of gestational diabetes in mother

3. **Gestational Diabetes**

It is a form of diabetes which is seen in pregnant women with onset around 24 weeks of pregnancy. This DM is harmful to both mother as well as baby since both of them are highly succeptible for the development of type 2 diabetes in near future. Further, babies have born from gestational diabetic mothers show several neurodevelopmental disorders, which limit their healthy and normal life in future. But as there is change in life-style and increase in obesity there is increase in gestational diabetes (Ben, 2004). Women with a history of gestational diabetes have higher risk of having Diabetes mellitus II as well as their children have higher risk of having Diabetes mellitus II and obesity (Hapo study Cooperation Research group, 2002). In normal pregnancy it is seen that the tissues become insensitive to insulin. It may be because of hormone secreted by placenta, obesity or pregnancy related factors which are still not fully understood (Kampmann et al, 2015). There is 50% decrease in

insulin mediated glucose disposal and in order to maintain the homeostasis state the insulin secretion must increase by around 200 to 250% (Barbour et al, 2007). Gestational Diabetes develops when body do not produce sufficient amount of insulin to compensate this insulin resistance state. The glucose is transfer through the placenta to fetus. But when there is condition of maternal hyperglycaemia stimulates the fetal hyperinsulinaemia to counter the excess glucose transferred through the placenta. The higher insulin level in fetus leads to stimulate fetus growth which results in macrosomia in fetus (Pedersen, 1968).

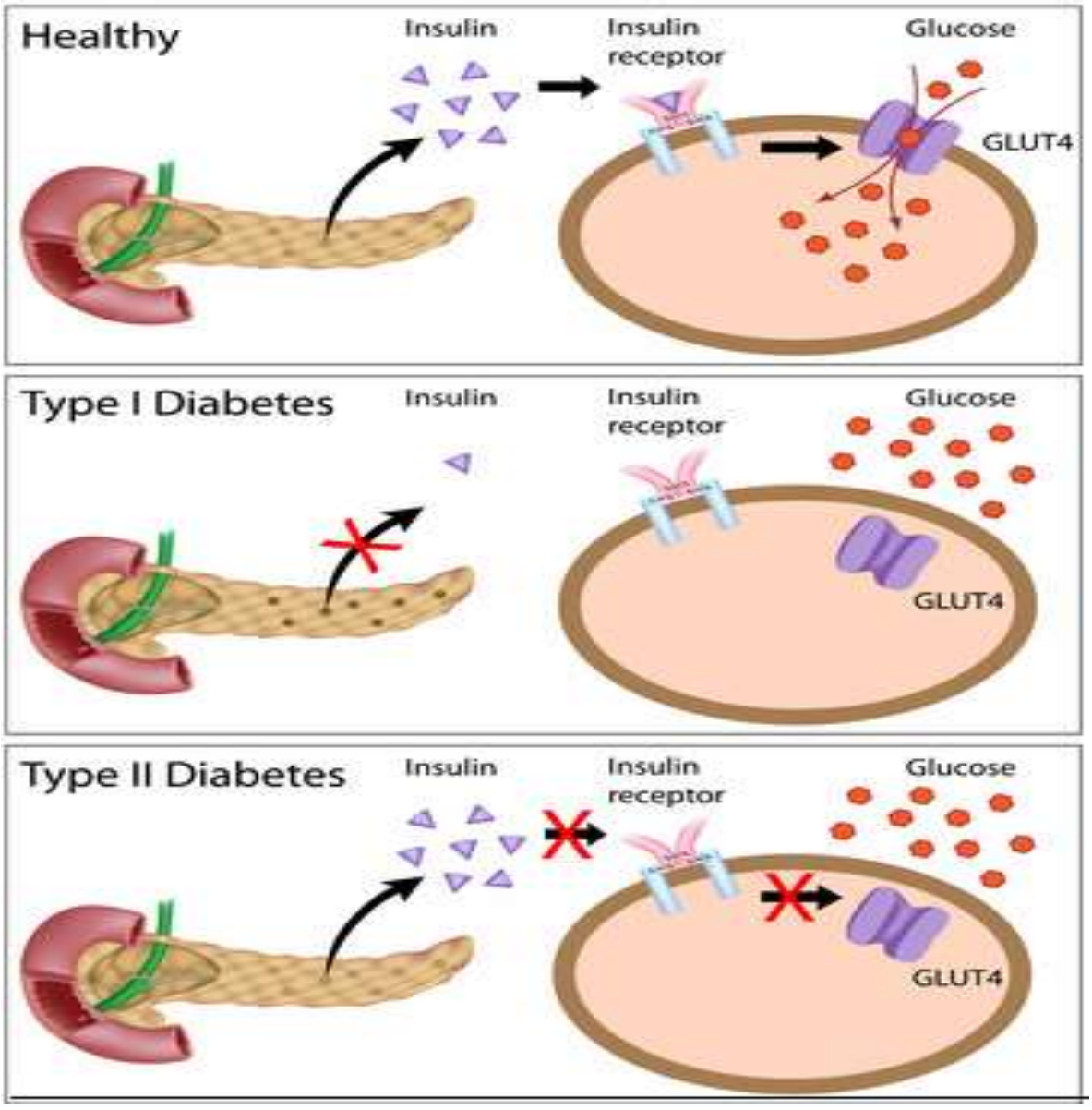


Figure 1: Types of Diabetes mellitus (AlilaMedicalMedia.com)

Insulin

Insulin is a 51 amino acid peptide hormone which was discovered by Banting and Best in 1921. It was the first protein that was sequenced by Sanger. It has two peptide chains, A and B, having 21 amino acids and 30 amino acids respectively. These chains were linked together by disulphide bridges. The N-terminal of A-chain has helix which is linked to anti-parallel present in C-terminal. In body, insulin is synthesized as 110 amino acid protein complex known as pre-pro-insulin. This pre-pro-insulin undergoes subsequent modification to release 22 amino acid chain and leaving behind single chain of 86 amino acid known as pro-insulin In pro-insulin there is a 35 amino acid connecting peptide chain which links the N-terminal of A to the C-terminal of B chain. This C-peptide chain is proteolitically cleaved to release insulin, which is biologically active (Fig. 2) (Wilson, 2005). Insulin hormone is responsible for maintaining normal blood glucose levels. It is the regulator hormone for the glucose uptake in the cells. For diabetic patients doctors gave them insulin therapy in Diabetes mellitus type-I and even in cases of insulin resistance or Diabetes mellitus type-II.

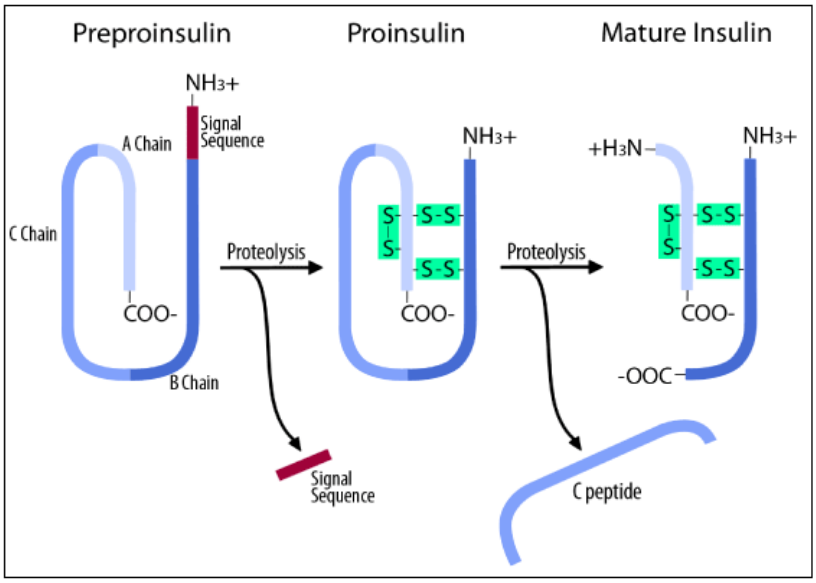


Figure 2: Processing of preproinsulin (omicsonline.org)

- **Mechanism of Insulin Secretion**

  Insulin is stored inside beta-pancreatic cells in vesicles. Whenever the glucose level increases in the blood, it enters into β-cells of pancreas with the help of glucose transporter-2. Inside pancreatic cells this glucose is metabolized and glucokinase is activated for this process which in turn activated glycolysis cycle. Glycolysis leads to the excessive production of ATP and thus ATP/ADP ratio increases inside pancreatic β-cells. This in turn closes the ATP

sensitive K^+ channels resulting in membrane depolarization. Membrane depolarization activates voltage gated calcium channels and high inflow of calcium ions occurs. Calcium ions mobilize insulin storing vesicles to cellular membrane. These vesicles fuses with cellular membrane and drain out its content into the blood. The calcium increase in the cells works as a signal for the secretion of insulin (Soria et al, 2004). Pancreas secretes 0.25-1.5 units of insulin per hour during fasting state.

- **Mechanism of action of Insulin in glucose metabolism**

  Insulin exerts its physiological effect through its interaction with the insulin receptor, which belongs to protein kinases family of receptors. The insulin receptors is a heterotetramer having 4 subunits, 2-α and 2-β. The α-subunit is the binding domain for insulin and β-subunit has catalytic domain. When insulin binds to insulin receptor resulting in conformational change to the β-subunit which leads to phosphorylates the β-subunit conferring tyrosine kinase activity. This leads to tyrosine phosphorylation of intracellular protein called insulin responsive substrate (IRS). Then the IRS molecules bind to other downstream signalling molecules leading to the phosphorylation and activation of Akt/protein kinase-B which activates insulin responsive genes. This is followed by transcription and translation leading to synthesis of glucose transporters. These transporters are then transported to the cellular membrane and then through these transporters glucose enters cells and its level in the circulation is reduced.

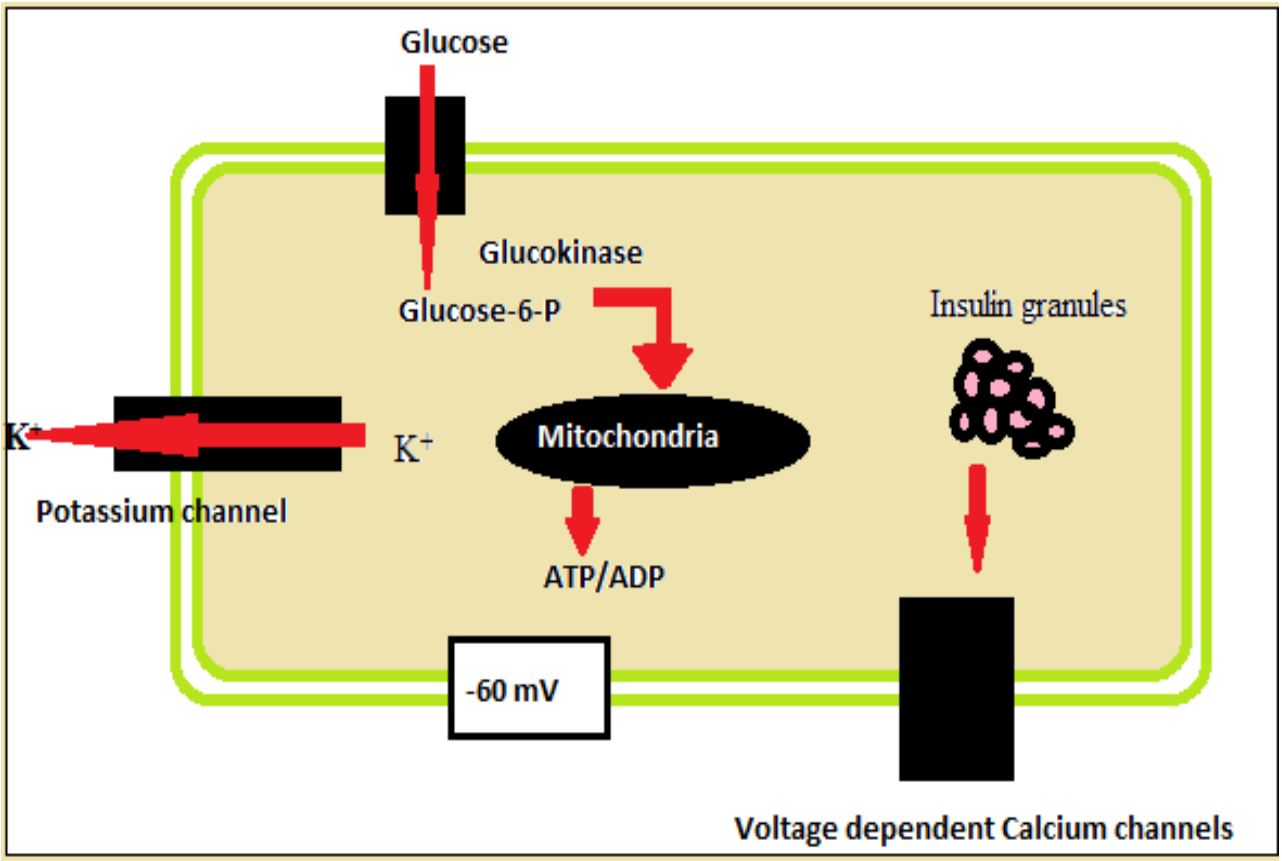


Figure 3: Insulin Secretion by β-cells of pancreas

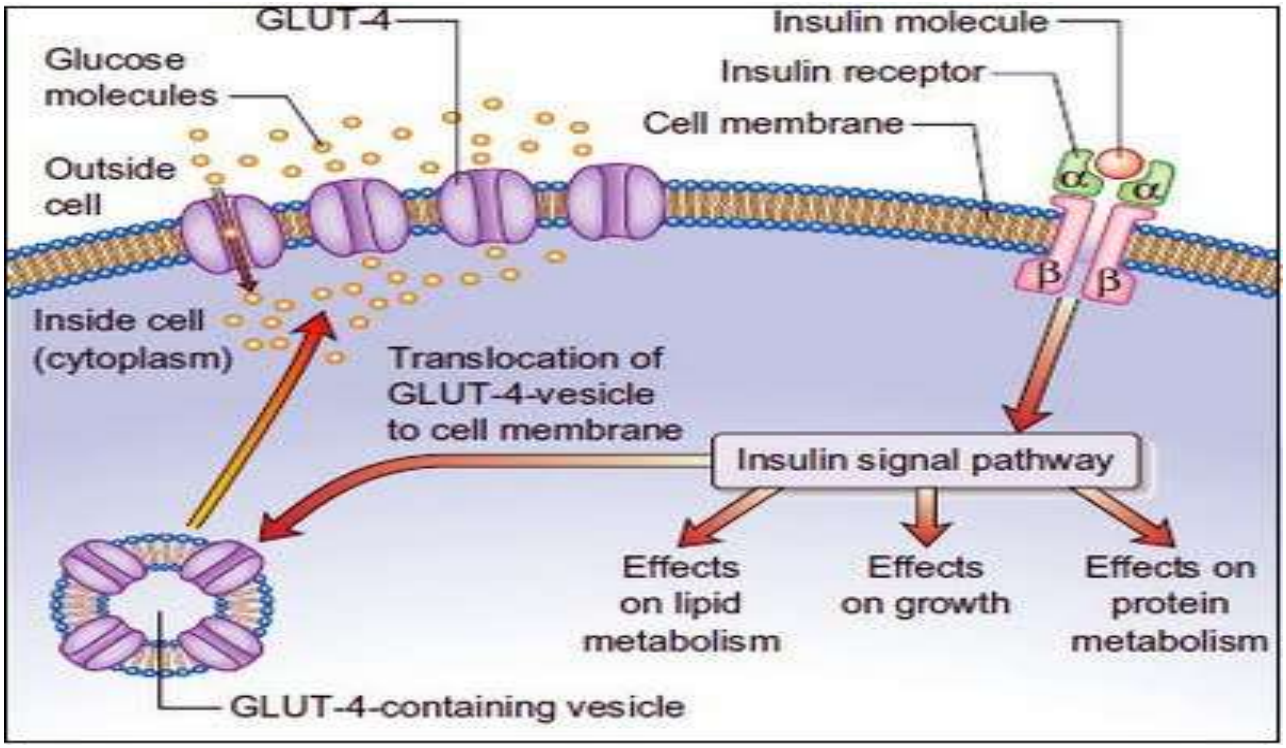


Figure 4: Mechanism of Insulin in Glucose Uptake (Chabhara N, 2012)

Diagnosis

Diabetes mellitus can diagnose by the kit available in the market. A drop of blood is added onto the strip having glucose oxidase enzyme which metabolized glucose to gluconic acid and hydrogen peroxide. Level of hydrogen peroxide is measured and outcome is received in term of electric signal. The other blood is to check the level of glycated haemoglobin in blood which gives the information about the blood glucose level in the body.

The following methods are used now days:

- *Fasting blood glucose level*

  It is specific screening test to check blood glucose level. Person does not eat before this test for at least 8-12 hr. The small blood drop is added onto the strip which has glucose oxidase enzyme converts the glucose into gluconic acid and H_2O_2. The released H_2O_2 is measured by the sensor. Normal range of fasting blood glucose level is 80-100 mg/dl. If person blood glucose level is 100-125 mg/dl the person is considered as pre-diabetic and if blood glucose level is more than 126 mg/dl is considered as diabetic.

- *Glucose Tolerance test*

  It is used to detect how efficiently body metabolize the glucose. It is similar as of fasting blood glucose test except in this after checking the blood glucose level, the patient is given a measured dose of glucose to drink and then the glucose level is checked at different interval. This test is also tell us about that person has which type of diabetes. As in insulin resistance state the body do not efficiently metabolize sugar after different intervals of time.

Table No. 1: Glucose Tolerance Test Chart

Normal		**Pre-Diabetic**		**Diabetic**	
Fasting blood glucose (mg/dl)	Glucose load after 2 hours (mg/dl)	Fasting blood glucose (mg/dl)	Glucose load after 2 hours (mg/dl)	Fasting blood glucose (mg/dl)	Glucose load after 2 hours (mg/dl)

<100	<140	100-125	140-199	>126	>125

- *HbA1C test:*

  This test provides information about the long term glucose level in glucose by glycation of haemoglobin by the glucose. In healthy person the level of glycosylated haemoglobin is 3-6% which increases in diabetic person. The blood drop is added onto the strip contain monoclonal antibody against the glycated haemoglobin.

Table No. 2: A1C Test Chart

Normal		**Pre-Diabetic**		**Diabetic**	
A1C %	Fasting blood glucose (mg/dl)	A1C %	Fasting blood glucose (mg/dl)	A1C %	Fasting blood glucose (mg/dl)
<5.6	<100	5.7-6.4	100-125	>6.5	>125

Complications of Diabetes mellitus:

Diabetes mellitus is associated with several other complications which originates because of hyperglycaemia and therefore are known as secondary diabetic complications. It is seen that if the blood glucose level rises it leads to the various complication due to the increase in the oxidative stress, inflammation and many other reasons which are not clear yet. But the main player is oxidative stress. The oxidative stress plays a major role in the diabetic induced complications (Brownlee, 2001). Oxidative stress is also used by cell against a defence mechanism but when oxidative stress increases it leads to the release of reactive oxygen

species. There are several molecular pathways are involved in oxidative stress due to high glucose level. The polyol pathway is the most common one in which the aldo- keto reductase enzyme catalyses the reduction of wide array of carbonyl compounds. The affinity of this reductase enzyme is low when blood glucose level is normal. But when it increased results in increased enzymatic conversion of polyalcohol sorbitol, thus leads to decrease in the NADPH (Brownlee, 2001). The NADPH acts as a cofactor for many antioxidant enzymes thus there is a decrease in the antioxidant mechanism in the body (Giacco et al, 2010). This pathway also leads to the PKC activation. The AGE (advanced glycation end product) produced from auto-oxidation of glucose. It modified intracellular proteins thus these proteins bind to the AGE receptor on cells like macrophages, vascular endothelial cells. Therefore induces the production of ROS.

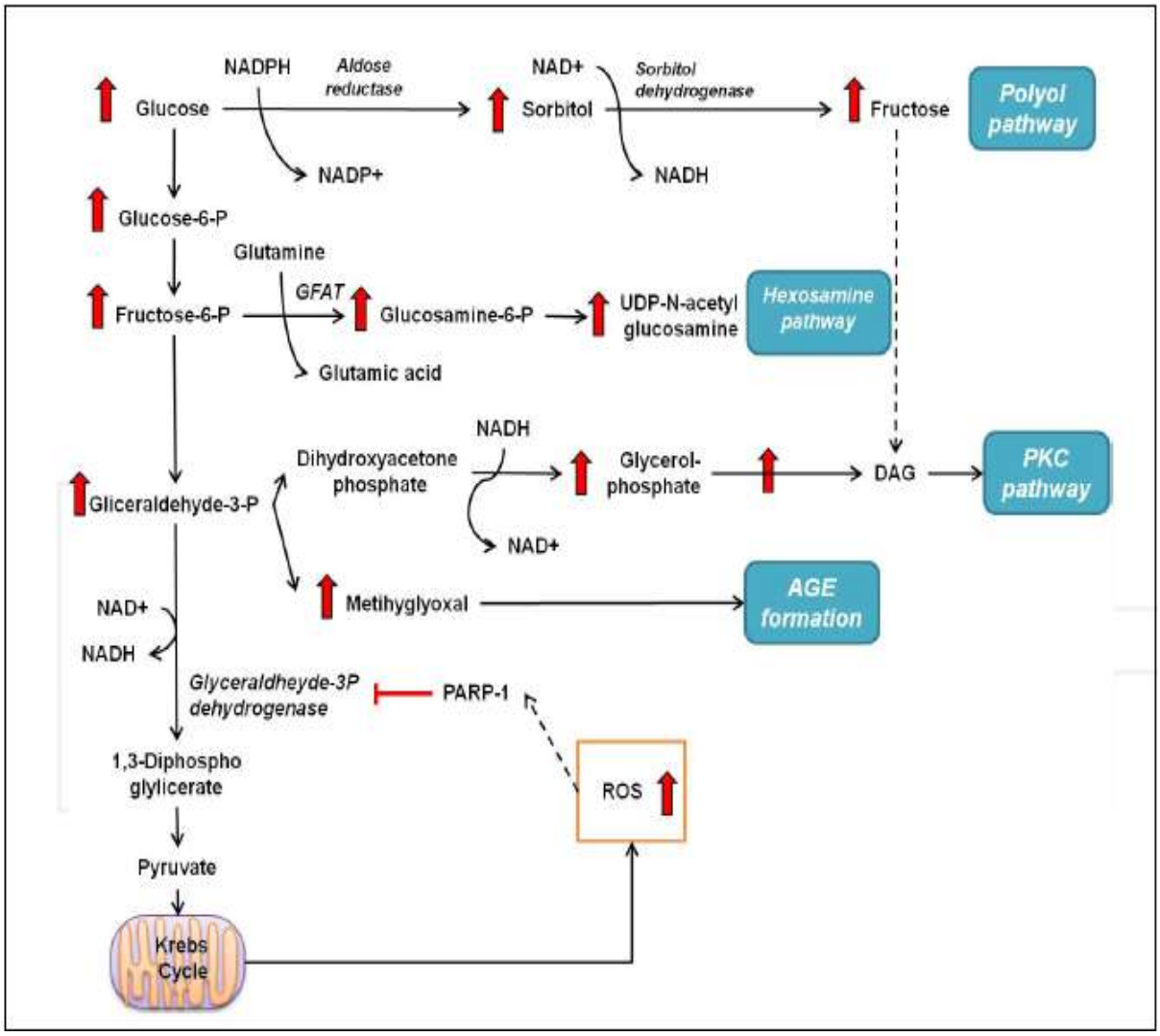


Figure 5: Oxidative stress- related Pathways linked with glucose metabolism (Monrey et al, 2013)

Now the oxidative stress plays a major role in the progression of diabetic complications. It leads to microvascular complications and macrovascular complication. The microvascular complications lead to the macrovascular complication. So here some of the microvascular complications:

- *Diabetic Nephropathy:*

  It is seen that in around 30% diabetic people developed nephropathy which leads to renal failure (Brennan et al, 2013). It is occur due to the development of proteinuria which results in decline in the glomerular filtration rate (Forbes J et al). The initially microalbuminuria is developed which is usually reversible with insulin administration (Bogdanovic, 2008). It is seen that the nephropathy is closely linked with the onset of neuropathy. The development of uremia along with high oxidative stress leads to damage of the neurons and develop peripheral neuropathy (Boltan, 1980). In the Uremic neuropathy the most common form is distal symmetric polyneuropathy in the lower limb due to the axonal degradation and loss of myelin sheaths (Pirzada and Morgenlander, 1997). It remains asymptomatic until the renal function reduced by 75%. Therefore it leads to more damage.

- *Diabetic neuropathy:*

  50% of diabetic patients are suffering from peripheral neuropathy. It is mainly occur due to the oxidative stress leads to damage of the neurons thus it results in the slow down the function of motor and sensory nerves (Moore, 2009). But as the disease progress it leads to more severe results, there is an increase the motor deficits, muscle weakness and foot deformities (Tesfaye et al, 2010). The prevalence of the disease is based on the disease duration. Diabetes is also linked with various neurological complications. The diabetes and depression is interlinked which is confirmed by many reports. The occurrence of depression is higher in diabetic people (Campayo et al, 2010). The mechanisms behind both of these diseases are associated with the Hypothalamic-pituitary-adrenal axis dysfunction (Stetler and Miller, 2011). A diabetes and Alzheimer disease is also considered to be associated with each other. The glucose intolerance and impairment of glucose secretion also increase the risk of dementia. One of the study indicated that the alteration in insulin pathway also considered being the missing link between two diseases (Takeda et al, 2010).

- *Diabetic Retinopathy:*

  It is seen that 40% of diabetic patient is suffering from diabetic retinopathy. It generally appears in the patients after 10-15 year. It is seen that a small like dot is formed in the retina and fat deposition is also occur. It is seen that there is a formation of new blood vessels on retina cause haemorrhage and finally leads to blindness (Brownlee, 2001).

- *Diabetic cardiovascular compplications*

  Cardiovascular complications associated with diabetes are leading cause of mortality due to diabetes. Hyperglycaemia results in elevated blood fatty acid contents, causes stiffness of blood vessels resulting in hypertension, heart attack, stroke etc. Diabetes increase risk to cardiac failure, stroke and heart attack by 3 times.

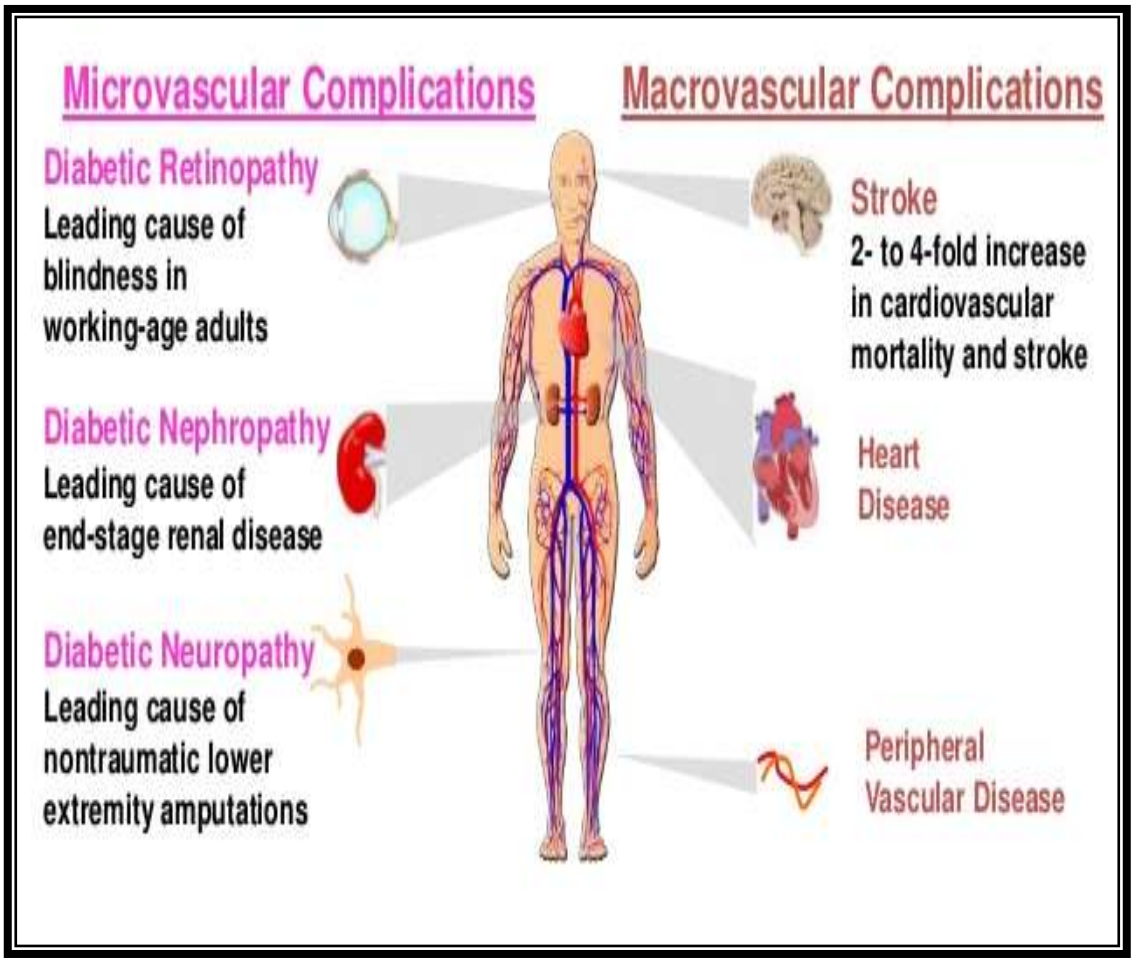


Figure 6: Complications of Diabetes Mellitus-2 (Diabetes-statistics)

Oral Hypoglycemic Agents for Diabetes mellitus treatment:

There are many oral anti-diabetic drugs are available in the market. The Diabetes mellitus type-I is still can be controlled by giving insulin injections. But the management of Diabetes mellitus is quite difficult. As the reasons of Diabetes mellitus type-II is vary from person to person. Thus the various oral drugs are given to the patients. Here are some of them:

- *Sulfonylureas:*

  This is a major class of drug which is used for diabetic patients. It helps in stimulating the insulin release from pancreas and it also helps in improving of insulin resistance. The sulfonylureas therapy lowers down the blood glucose level by in fasting plasma glucose concentration by 60-70 mg/dl and glycosylated haemoglobin by 0.8 to 2.0% (Luna B, 2001).

  The worst side –effects of drug is hypoglycaemia in patients. It is also seen that it leads to increase the weight thus makes a worst choice for the obese people. Examples of sulfonylureas: Glimepiride, Glimizide etc.

- *Meglitinides:*

  These are new agent which action is quite similar to sulfonylureas but it has short half life and short period of action (15-30 min). It also helps in release of insulin. The biggest advantage related to drug is that there are decreases chances of hypoglycaemia and decrease in postprandial glucose. The decrease in blood glucose level and reduction in glycosylated haemoglobin is quite similar to the sulfonylureas.

  Example of meglitinides: repaglinide.

- *Biguanides:*

  These are an important class of anti-diabetic drug. They work as reducing the hepatic glucose output and also increase insulin sensitivity in liver and peripheral tissues. Its other effects also includes decreased the plasma triglycerides levels and cholesterol. It lowers down the fasting plasma glucose concentration by 50-70mg/dl and glycosylated haemoglobin by 1.5-2%. It has side-effects but it is seen that these side-effects were seen in starting of the therapy. This drug do not associated with hypoglycaemia but if it used with some other drug then hypoglycaemia is also one of the side-effect. The worst side-effect of this therapy is lactic acidosis but it is very rare. The cases of lactic acidosis are 3

out of 100,000 in patients taking this drug (Stumvoll et al, 1995). It is very unusual drug as it leads to weight lose in patients even in obese people.

Example of biguanides: Metformin

- *Thiazolidinediones:*

  These are known to improve insulin sensitivity in muscles and liver cells. In some extent drugs belong to this category also inhibits the glucose production from liver. It is reduced the fasting plasma glucose by 25-50mg/dl and 0.5-1.5% in glycosylated haemoglobin. It is also studied that it also alters the lipid profile in diabetic patients. It doo not affects the insulin secretion thus the hypoglycaemia is not a problem in the therapy. The weight gain is associated with the drug. It is safe for the patients who have renal problems as it can metabolize by the liver. Example of thiazolidinediones: Pioglitazone, Rosiglitazone

Table No. 3: Oral drugs and their advantages and disadvantages

Drugs	Advantages	Disadvantages
Sulfonylureas	Reduce fasting plasma glucose, low cost	Hypoglycaemia, weight gain
Meglitinides	Reduce risk of hypoglycaemia	High cost
Biguanides	No weight gain, no hypoglycaemia	Lactic acidosis, high cost
Thiazolidinediones	No hypoglycaemia	High cost

All these therapeutics currently used in the market use the following biochemical processes:

- *Glucose Absorption:* Dietary polysaccharides converted to glucose before entering to gastrointestinal tract by the enzymes present in the saliva. Therefore inhibition of these enzymes leads to lower down the glucose absorption in the body. Thus reduction in blood glucose level leads to activate the glycogenolysis and lipolysis which leads to weight loss in obesity.

- *Glucose Metabolism:* Glucose is oxidised and used in the body as energy source. When glucose increases in body, it converted to sorbitol by Aldose reductase and used NADPH. The excessive sorbitol leads to change the osmotic pressure and damage the microvasculature. Thus inhibition of this enzyme significantly slower down the complications caused by the excessive glucose. But it does not affect the blood glucose level hence do not stop the progression (Sharma and Sharma, 2008).
- *Glucose excretion:* Kidney filters around 180g of glucose per day which again reabsorbed with the help of transporter like sodium glucose co- transporter. There are drugs which inhibit this transporters thus reduce the glucose absorption. Thus these transporters gained attentions to lower down the blood glucose level (Tehrani et al, 2013).

- *Oxidative stress induced damage:* the high blood glucose level increases the oxidative stress as well as reduced the amount of natural antioxidant enzymes. Thus all these factors lead to various complications like neuropathy, nephropathy (Fisher et al, 2006 and Johansen et al, 2005).

Photochemicals and antioxidant activity:

Phytochemicals are the compounds extracted from the plant. These phytochemicals have many roles and many health benefits. One of the health benefits is that help in scavenging the reactive oxygen species thus protect the body cells from these harmful free radicals. Therefore these are called the antioxidants.

They play a major role in the disease prevention as it is believed that the reactive oxygen species leads to the many diseases. Reactive oxygen species is believed to damage the tissue (Packer and Glazer, 1990). However the protective mechanism is present in our body to fight

against these oxygen species. Like SOD (sodium dimutase) convert the O_2 free radices into H_2O_2, and then this hydrogen peroxide is degraded by catalase. Glutathione reductase, Glutathione peroxidase etc also leads to act antioxidant enzymes. These phytochemicals act as a coenzyme for these antioxidant enzymes. For an example riboflavin is a cofactor for Glutathione reductase. These phytochemicals act by scavenging these radicals or inhibits the oxidation of lipoproteins. In phytochemicals flavanoids plays a major role in the antioxidant effects. Many studies provide a justification of their activities. These flavanoids also has insulin mimetic and insulin signalling modifying activity apart from antioxidant. Therefore these can be used a therapeutics for the diabetes related complications.

Here are the lists of flavanoids having different activities:

Table No.4: Flavonoids having Antioxidant activity

S. No.	Flavanoids	Mode of Action
1.	Quercetin	Inhibition of advanced glycation end product (AGE), improve the activity of antioxidant enzymes
2.	Sulfuretin	Inhibit AGE formation
3.	Astilibin	Inhibit AGE formation
4.	Luteolin	Increase the level of antioxidant enzymes thus reduce the level of reactive oxygen species

5.	Eriodictyol	Reduce the lipid peroxidation, reduce blood glucose level
6.	Disomin	Activate the antioxidant enzymes, increases the vitamin C and E level
7.	Fisetin	Reduce lipid peroxidation
8.	Hesperidin	Elevate the level of antioxidant enzymes
9.	Naringin	Elevate vitamin C level
10.	Rutin	Reduced TBARS
11.	3,4,2′,4′- tetrahydroxy-3-geranyldihydrochalcone	Improve the functioning of mitochondria

12.	Pelargonidine	Elevate the level of sodium dismutase in brain
13.	Coumestrol	Inhibit AGE formation
14.	Apigenin	Inhibit AGE formation
15.	Puerarin	Inhibit the production of reactive oxygen species and NADPH oxidase activity

Table No. 5: Flavanoids having insulin and insulin signalling modifying activity

S. No.	Flavonoids	Mode of Action
1.	Myricetin	Increase the expression of GLUT transporter
2.	Epigallocatechin-3-O gallate	Enhance the phosphorylation of AMPK pathway
3.	Rutin	Reduce glucose from plasma and increase the insulin

4.	Apigenin	Increase glucose uptake
5.	Karanjin	Translocation of GLUT transporter
6.	Puerarin	Increase insulin level
7.	Tangeretin	Improve insulin signalling and Glut translocation
8.	Biacalein	Improved glucose intolerance
9.	Morin	Activate and phosphorylate insulin receptor
10.	Kaempferol	Increase the phosphorylation of β-subunit of insulin receptor

Need for Natural Therapeutics for the Management of Diabetes:

The drugs for diabetes are available in the market but they have many side-effects as well as they do not control the complication of diabetes. Thus there is a need for new therapeutics which does not cause any side-effects as well control the complications of diabetes. Therefore the phytochemicals is the attractive choice it controls the complication (studied in animal models) as well do not cause any side-effects. Just that it need more research to find out how it will interact with the various enzymes and proteins to reverse or stop the diabetes induced complications.

Chapter 2

Review of Literature:

Diabetes is a metabolic disease which now does not considered as an epidemic now as it turned into a pandemic disease. An India has second highest number of diabetic patients and 85-90% is suffering from diabetes type 2. It is found out that the in Diabetes the occurrence and severity of the complications are depending on duration of diabetes. The most of diabetic patients do not show any symptoms especially in type 2 diabetes in the early stages (Kharroubi et al, 2015) which leads to increased the chances of having diabetic induced complications. If we see statistics around 60-70% population is suffering with the diabetic induced neuropathic complications (Sapllone et al, 2011). The mechanism behind these complications is still unclear but it is a multifactorial which includes life-style, environment and genetic predisposition. Diabetic induced neuropathy complication is also due to oxidative stress. This neuropathy leads to major complications like depression, Parkinson's Alzheimer's etc. The researchers are trying to find out the link between diabetic neuropathy and these major CNS complications.

Some of the studies suggested that there are changes in CNS due to diabetes. The actual reason behind it is not clear but blood brain barrier considered being the main contributor for these changes (Mooradian, 1997). Changes in plasma glucose level are the main reason of which altered blood brain barrier transport function, integrity and oxidative stress in CNS microcapillaries (Prasad et al, 2014). The microvascular pathogenesis leads to breakdown of blood brain barrier thus it causes the leakage of serum-derived components into brain parenchyma leads to neuronal dysfunction (Serlin et al, 2011). In Diabetes generation of reactive oxygen species also activates the process leading to DNA damage. These DNA single strand breaks are a feature of neuronal cell death (Singh et al, 2003). It is also find out that in elderly people suffering of Diabetes II there is ventricular enlargement reflecting cerebral atrophy. These atrophy changes are primarily in the frontal lobe can occur as early as in one year after clinical diagnosis of Diabetes II (Lee et al, 2013). The hyperglycemia also associated with the progression of cerebral ischemia and thus leads to increase the chances of having secondary brain injury.

The effect of diabetes on CNS leads to cognition dysfunction and cerebrovascular disease. In animals which were suffering from spatial learning, memory and cognition occur in the association with distant changes in hippocampal region of brain which is important for memory and learning and very sensitive to change in glucose homeostasis. The pathogenesis

related to this is not yet fully understood but apoptosis play a crucial role in diabetes related neuronal loss in hippocampus (Sadeghi et al, 2016).). In Diabetes II, metabolic stress and proinflammatory signals leads to alter insulin signalling and decrease the response of cells towards the insulin. This insulin resistance state it affects the ability of cell to maintain energy homeostasis. The Alzheimer brain also shows similar abnormalities (Felice et al, 2014). It is observed that in diabetes II the patient have lower level of cognitive function and the risk of having dementia increases as compared to non-diabetic (Alzheimer's Association, 2015). The previous studies clearly documented that the association of Diabetes with dementia, Alzheimer's diseases and vascular dementia. The chances of having the dementia and Alzheimer in Diabetic patients are 73% and 56% respectively (Gudala et al, 2013). One study of 2013 indicates the relation between high blood glucose level and Alzheimer's. They found out that high blood glucose level due to type 2 diabetes make Alzheimer's plaque more toxic to the brain cell.

The patients show that due to insulin resistance in brain leads to decline in ability to use glucose as a fuel for brain functions. It is documented that the insulin influences the functions of cerebral. It acts as a neuromodulator affects the release and uptake of neurotransmitter and improving the memory and learning. Hyperglycemia due to diabetes leads to increase the peripheral utilization of insulin thus reduces the level of insulin into the brain. Therefore the defective insulin makes neurons more vulnerable to energy deficient and impairs synaptic plasticity (Bosco et al, 2011).

In a journal Diabetology study published in 2007 reported the CNS complications related with diabetes and their relation with HbA_{1c} (Gold et al, 2007). They tested the cognitive skills, memory of diabetic patient as well in the non-diabetic people. The diabetic patient in less than10years had memory problems. Then using MRI and brain imaging they found that the there is a decrease in the hipocampal size. So they concluded that hipocampal size is correlated with the HbA_{1c} level.

As we discussed so many studies which were suggested that the Diabetes mellitus II can develop the CNS complication after some duration of the onset generally it takes 7-10 years in most of patients. Therefore we urgently need some therapies which can help in reducing these CNS complications due to diabetes. Most of these complications occur due to increase in the glucose level due to insulin impairment, oxidative stress, inflammation and many more. We need some of the compounds or medications which will manage the glucose level in

blood as well improve or slow down these cognition dysfuntion. Some of antidiabetic has these effects.

In 2013, one study claims that these drugs have a potential to treat mild cognitive impairment (Alagaikrishnan et al, 2013). People who take metformin have a lower risk of having dementia. One more study on Australian population was done to see the effect of diabetic treatment on the change of specific cognitive domain over the 4 years. They found out that except metformin no other drugs shows any significant effect. Metformin effects were seen in verbal learning, working memory, and executive function domain (Herath et al, 2016). The rosiglitazone and glyburide therapy had shown to improve the glycemic control on fasting plasma glucose (Ryan et al, 2006). The antidiabetic drugs show some effect in reducing the CNS complications. But these can only help in the early stages of the cognitive dysfuntions. It is seen that drug treatment for diabetes helps in glycemic control, thus reduce the occurrence of diabetic complications (Wolever et al, 2008). But many studies also show that the widely prescribed insulin-sensitizing drug, metformin affects β-amyloid precursor protein metabolism and β-amyloid generation and it did not show any effect on the β-amyloid degradation. A 2008 study shows that the anti-diabetic drug when used as a monotherapy has harmful consequences in elderly people but if it is used with insulin it increases the insulin effect in reducing β-amyloid (Chen et al, 2008). It is demonstrated that the metformin activates AMPK (AMP-activated protein kinase) which significantly activated the generation of β-amyloid peptide. But still most of the data available on the antidiabetic effects on cognition dysfunction and dementia shows that these anti-diabetics have a potential to improve cognition function. Thus these anti-diabetic drugs have potential to improve the neurological effects induced due to diabetes.

The insulin therapy also has potential to improve the diabetic induced neuropathy. Insulin combined with oral antidiabetic drugs showed to slowing down the cognitive decline in patient of Diabetes mellitus II suffering from mild to moderate Alzheimer diseases (Plastino et al, 2010). The exendin-4, an antagonist of glucagon-like peptide-1 receptor also ameliorates the severity of diabetic polyneuropathy (Himeno et al, 2011). In Diabetes mellitus II the progressive microvascular complications disrupt the ability of cerebral vessel to supply blood to brain thus increase the risk of dementia. One vasodilator, resveratrol can enhance the systemic circulation thus it can also improves the cerebral vasodilator improvement in Diabetes mellitus II (Wong et al, 2016).

The antidiabetic drugs as well as some other compounds show some effect in reducing the CNS complications. But these can only help in the early stages of the cognitive dysfuntions. It is seen that drug treatment for diabetes helps in glycemic control, thus reduce the occurrence of diabetic complications (Wolever et al, 2008). But in most of the cases patient is asymptomatic for diabetes 2. Therefore we need additional therapy to reduce the incidence of diabetic induced complication.

The most attractive alternative is the phytochemicals. Many phytochemicals has been reported to show anti-diabetic drugs. Many plants are rich source organic compounds such as flavonoids, Tannins, polyphenols, steroids and terpenoids. These organic compounds can be used to develop new anti-diabetic drugs.

Many studies show that the phytochemicals can help in lowering down the effects of increase blood glucose level. The use of plant to cure a disease is a practice from a long time. Diabetes mellitus was known from the ancient ages and different medicinal plants were used (Gupta et al, 2007). In ancient times people were not aware of the mechanism behind these plants they just know there benefits. But now people are working on different components of plants which show the anti-diabetic effects using different analytical techniques.

Many studies show that the phytochemicals can help in lowering down the effects of increase glucose level. Cohort studies also showed that phytochemicals improved metabolic homeostasis and development of Diabetes mellitus II and its complications was prevented and slowed down by frequent consumption of wholegrain food (Zhang et al, 2015). One of the study showed that *Vaccinium vitis* stimulates or improves the glucose uptake. The active compound in this plant is quercetin-3-O-glycosides which has glucose uptake activity by mediating AMPK (Eid et al, 2010)

These phytochemicals also show antioxidant properties and as we already discussed plays a major role in diabetes. As increase in blood glucose level increases the reactive oxygen species. Thus these phytochemicals can helps in the reducing the free radicals which leads to these diabetic complications. In 2016 one study showed that one of the seaweed *Ulva fasciata* has anti-diabetic activity. The reason behind its anti-diabetic activity is due to its antioxidant, hypoglycaemic activity which requires further in-vitro and in-vivo mechanism to find out the exact mechanism behind its activity (Mohopatra et al, 2016). In 2007 *Annona squamosa* contain quercetin-3-O-glucoside which appears to be anti-diabetic has insulin stimulating (Davis et al, 2012).

These phytochemicals constitute the non-enzymatic defence against oxidative stress. They scavenge the free radicals and oxidized themselves. Then the oxidized from of phytochemicals are regenerate by glutathione (Monroy and Mejia, 2013). But the proper mechanism behind these antioxidants is not clear. Several studies are done to find out the correlation between phytochemicals and their antioxidant activity. Some studies documented the decrease in these plasma antioxidants such as ascorbic acid, tocopherol, lutein, zeaxanthin during diabetes leads to the diabetic complications (Polidori et al, 2000). One recent study of 2016 suggested that the flax seed also delay the development of Diabetes mellitus II. Flax seeds contain SDG (Secoisolariciresinol diglucoside). SDG has the antioxidant and hypoglycaemic effects which decreases the risk of Diabetes mellitus (Prasad and Dhar, 2016). It is also concluded that the flax seed has a beneficial effects against diabetes induced glucotoxicity by modulating glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase and glutathione-S-transferase (Gok et al, 2015). As garlic is important ingredient of the Indian kitchen. One study shows that the aged garlic extract has shown beneficial effects in diabetic mice which required further support to use it as a possibly supplementary treatment in Diabetes mellitus (Thomson et al, 2016). Palm oil and ground nut oil supplement also has anti-diabetic effect as because of antioxidant activity. The ground oil has higher antioxidant components as compared to ground nut oil (Adewale et al, 2016). But further studies are required to find out the exact active ingredient which has antioxidant activity. The other natural compound rutin also shows antioxidant, antidiabetic and anti-inflammatory activities thus can be used as attractive candidate for diabetic complication. The studies suggest that the rutin has beneficial effect on the diabetic neuropathy (Tian et al, 2016). Rutin also reduce the plasma glucose and Nrf2 signalling.

One more active ingredients quercetin also shows anti-diabetic effects. One new study of 2016 concluded that the quercetin and its major metabolite isorhamentin trap methylglyoxal, a precursor of advanced glycation end product which is a major cause of diabetic complications. Quercetin also increases the expression of glyoxalase I and II expression and glutathione/ glutathione disulfide ratio to promote the detoxification of methylglyoxal (Zhao et al, 2016).

In other studies people are also working on the role of phytochemicals and gene expressions of different genes regulating the glucose transporters, insulin secretion and antioxidant effect. In 2004 one studies found the role of quercetin and gene expression via NF-κβ pathway (Moskaug et al, 2004).

The anti-diabetic effects of phytochemical are documented. But the actual mechanism is still not known which needs further investigation. But one thing is clear that phytochemicals have an attractive choice to use against complications of Diabetes mellitus.

Chapter 3

Aims and Objectives:

To study diabetes mediated CNS complications in mouse model of type 2 diabetes.

- To screen various natural compounds for genotoxicity preventive potential.
- To compare the anti-genotoxicity activity of compounds with commercial available drugs.
- To screen herbal compounds for their ability to enhance glucose uptake in neuronal cells.
- To evaluate diabetes induced neurodegeneration in hippocampus of mice and the effect of quercetin treatment on it.
- To evaluate protein expression level in hippocampus of diabetic and quercetin treated mice.

Materials:

Table No.6: List of all chemicals, drugs and natural compounds

S.No.	Materials	Company	S.No.	Materials	Company
1.	Ascorbic acid	Loba chemie	**20.**	Agarose	Loba chemie
2.	Gallic acid	Loba chemie	**21.**	EtBr	Sigma
3.	Quercetin	Merck	**22.**	FBS	HiMedia
4.	Ellagic acid	Loba chemie	**23.**	DMEM	HiMedia
5.	Cinnamic acid	Loba chemie	**24.**	Goat IgG sc1606	Santa-Cruz Biotech.
6.	Caffeine	Loba chemie	**25.**	Donkey anti-goat IgG sc2033	Santa-Cruz Biotech.
7.	Piperine	Loba chemie	**26.**	Hematoxylin	Loba chemie
8.	Rosiglitazone	Sigma	**27.**	Eosin	Loba chemie
9.	Metformin	Sigma	**28.**	Xylene	Merck

10.	Glimipride	Sigma	**29.**	DAPI	HiMedia
11.	Trypsin	HiMedia	**30.**	DPX	Loba chemie
12.	Hydrogen peroxide	Fisher scientist	**31.**	Sodium Chloride	Loba chemie
13.	Disodium phosphate	Merck	**32.**	Potassium Chloride	Loba chemie
14.	Potassium diphosphate	Merck	**33.**	EDTA	Merck
15.	Tris	Merck	**34.**	Triton X-100	Loba chemie
16.	DMSO	Merck	**35.**	Sodium Hydroxide	Loba chemie
17.	Hydrochloric acid	Emplura	**36.**	Paraformalde -hyde	Loba chemie
18.	Paraffin	Loba chemie			
19.	Tween 20	Loba chemie			

TABLE No.7: List of all instruments:

S.No.	Instruments	Company
1.	Electrophoresis unit	Medox
2.	Centrifuge	Remi R-24
3.	Fluorescence microscope	Nikon
6.	Microtome	Unicon

Methods

1.1 To screen various natural compounds for genotoxicity preventive potential Using Comet Assay:

For comet assay human blood was used. 14 slides were dipped in high melting agarose. Wiped one side of all the slides and allowed agarose to air dry into thin film. All the slides were labelled. The 8µl of blood was collected in eppendorf and labeled them from 1 to 12. The 2µl of plant compounds and drugs were added and incubated for 10 min. After 10 min 16µl of H_2O_2 were added in eppendorf and incubated for 20 min. The eppendorf were centrifuged at 7000rpm, discarded the supernatant and washed the pellet with PBS of pH 7.4. The eppendorf then transferred to water bath at 45°C. The low melting agarose was melted and cooled down at room temperature. The pre-treated slides were kept in oven for 1 min. The 100µl of low melting agarose was added in all eppendorf and immediately placed in water bath after proper mixed with pipette. The100µl of low melting agarose and cell suspension were added onto the pre-coated slides. The slides were covered with other slides to make thin smear of low melting agarose containing cell suspension. The slides were incubated in lysis buffer for 1 hr at 4°C in a dark place. Then the slides were places in running buffer for 20 min at 4°C. After 20 min run the electrophoresis unit at 25mV for 25 min in ice-cold condition. The slides were removed and neutralized in neutralizing buffer for 4-5 times. The slides were stained at EtBr and incubated for overnight. The slides were observed in fluorescence microscope next day.

Table No. 8: Lysis Buffer Composition (pH-10)

Ingredients	Grams/250ml
NaCl (2.5M)	36.25
EDTA (100mM)	7.306
Triton X- 100 (1%)	2.5
DMSO (10%)	25

Table No. 9: PBS (Phosphate Buffer Saline) Composition (pH- 7.4)

Ingredients	Gram/l
Sodium chloride	8
Disodium phosphate	1.44
Potassium chloride	0.2
Potassium diphosphate	0.24

Table No.10: Neutralizing buffer Composition (pH-7.5)

Ingredients	Gram/100ml
Tris (0.4M)	4.8
Water	90

Electrophoresis Buffer:

- 10M NaOH (200g/500ml H_2O)
- 200mM EDTA (14.89g/200ml)

15ml NaOH and 2.5 ml EDTA were dissolved in 500ml distilled water.

Staining solution:

1ml EtBR(10X) was mixed in 9ml distilled water.

1.2 To compare the anti-genotoxicity activity of compounds with commercial available drugs using CASP software:

The pictures were uploaded in Casp_1.2.3b1 software for analysis. The analysis was done by clicking on assay button. The results were come in a table having percentage of DNA in tail and head. The results were compared to find out which compound is showing the best anti-genotoxicity effect.

1.3 To screen herbal compounds for their ability to enhance glucose uptake in neuronal cells using immuofluorescence:

The fetal brain were isolated and incubated in artificial cerebrospinal fluid at 4°C. The hippocampus was dissected using dissection microscope. The brain were triturated at 0.25% trypsin at 4°C and then centrifuged at 1000 rpm for 5-10 min. The supernatant was discarded and 1ml DMEM media & 10% FBS to the pellet and resuspended into the media. The number of cells were counted and cultured into the culture plate. After 14 days, the drugs were added into the fully grown cells in following fashion:

Table No.11: Different Treatment of Drugs and GLUT transporter on Brain Cells

S. No.	Treatment 1	Treatment 2
1.	Control	Glut antagonist
2.	Ascorbic acid	Ascorbic + Glut antagonist

3.	Gallic acid	Gallic acid+ Glut antagonist
4.	Ellagic acid	Ellagic acid+ Glut antagonist
5.	Quercetin	Quercetin+ Glut antagonist
6.	Cinnamic acid	Cinnamic acid+ Glut antagonist
7.	Caffeine	Caffeine+ GLUT antagonist
8.	Piperine	Piperine+ Glut antagonist
9.	Metformin	Metformin+ Glut antagonist
10.	Rosiglitazone	Rosiglitazone+ Glut antagonist
11.	Glimipride	Glimipride+ Glut antagonist
12.	Insulin	Insulin+ Glut antagonist

The culture plates were incubated for 2 hr, centrifuged it and discarded the supernatant. The cells were fixed with 4% fomaldehyde in two separated rows (one row contained GLUT antagonist). The primary antibody was added and washed after incubation. Then the secondary antibody labeled with FITC dye were incubated for 2 hrs at 37°C. After washing the DAPI was added and washed it. The images were taken with the help of fluorescent microscope.

Primary Antibody:

1.2µl of primary antibodies was dissolved in 600 µl of PBS (1:500).

Secondary Antibody:

0.3µl of secondary antibody was dissolved in 300µl of PBS (1:1000)

1.4 To prepare the paraffin-embedded brain tissue sections:

The brains were fixed in 4% formalin for 1 hour 50 minute at 35°C. Then the brains were dehydrated in different concentrations of ethanol in ascending order as follow:

Table No.12: Different Concentration of Ethanol in Dehydration step

S.No.	Ethanol Concentration	Incubation Time	Temperature
1.	50% Ethanol	1 hour	35°C
2.	70% Ethanol	1 hour	35°C
3.	80% Ethanol	1 hour	35°C
4.	100% Ethanol	1 hour	35°C

The brains were then incubated in 4 times in xylene for 30 min each at 35°C. The brains were dipped into the molten wax for at-least 3 hours @ 65°C. The moulds were kept at -4°C overnight. The paraffin embedded brains were cut into thin sections using microtome. The thin sections were then placed in water of 37°C. Then the sections were placed on slides.

1.5 To evaluate diabetes induced neurodegeneration in hippocampus of mice using Hematoxylin & Eosin staining:

The thin tissue sections were washed with the xylene for 15 min. The tissue sections were rehydrated in ethanol concentration for 2 min at decreasing concentration of 100%, 80%, 70% and 50%. Sections were stained with Hematoxylin for 15-20 min at room temperature. Then the sections were washed in running water for 2 min. 1% acid alcohol were added on the tissue sections. The tissue sections were stained with counter stain eosin for 1 min. The tissues sections were then washed with the ethanol of different concentrations in ascending order of 50%, 70%, 80% and 100 % for 2 min each. The slides were mounted with DPX and observed in fluorescence microscope.

1.6 To evaluate protein expression in hippocampus of diabetic and quercetin treated mice using immunofluorescence:

The paraffin embedded tissue sections placed in 60°C and washed with xylene for 3 times 2min each. The slides were washed with distilled water. The tissue section where rehydrate with PBS for 4min. The tissue sections were permeabilized with PBS + 0.1% triton X-100 for 10mins and washed with PBS. The slides were blocked with 2-3% BSA for 45 min. The primary antibodies were prepared in PBS in 1:500, vortex gently, spins down for 5 min at 10,000-15,000 rpm. The 100 µl of primary antibody were added onto the slides. The slides were incubated overnight at 4°C in humidity chamber. After incubation the slides were washed with PBS+ 0.05% tween 20 for 15mins. The secondary antibodies were prepared the same way as primary in ration of 1:1000 in PBS. The 100µl of secondary antibody were added onto the slides. The slides were incubated for 60min at 37°C. The sections were washed with PBS + 0.05% tween 20 to remove BSA as it affects the binding of DAPI. The DAPI were added onto the slides 1-2 min. The slides were washed with PBS thrice for 5 min each. The slides were mounted in DPX, incubated overnight at 4°C. The slides were observed in fluorescence microscope.

BSA (Bovine Serum Albumin):

30 mg of BSA were dissolved 1ml of PBS.

Primary Antibody:

1.2 µl of primary antibodies was dissolved in 600µl of PBS (1:500).

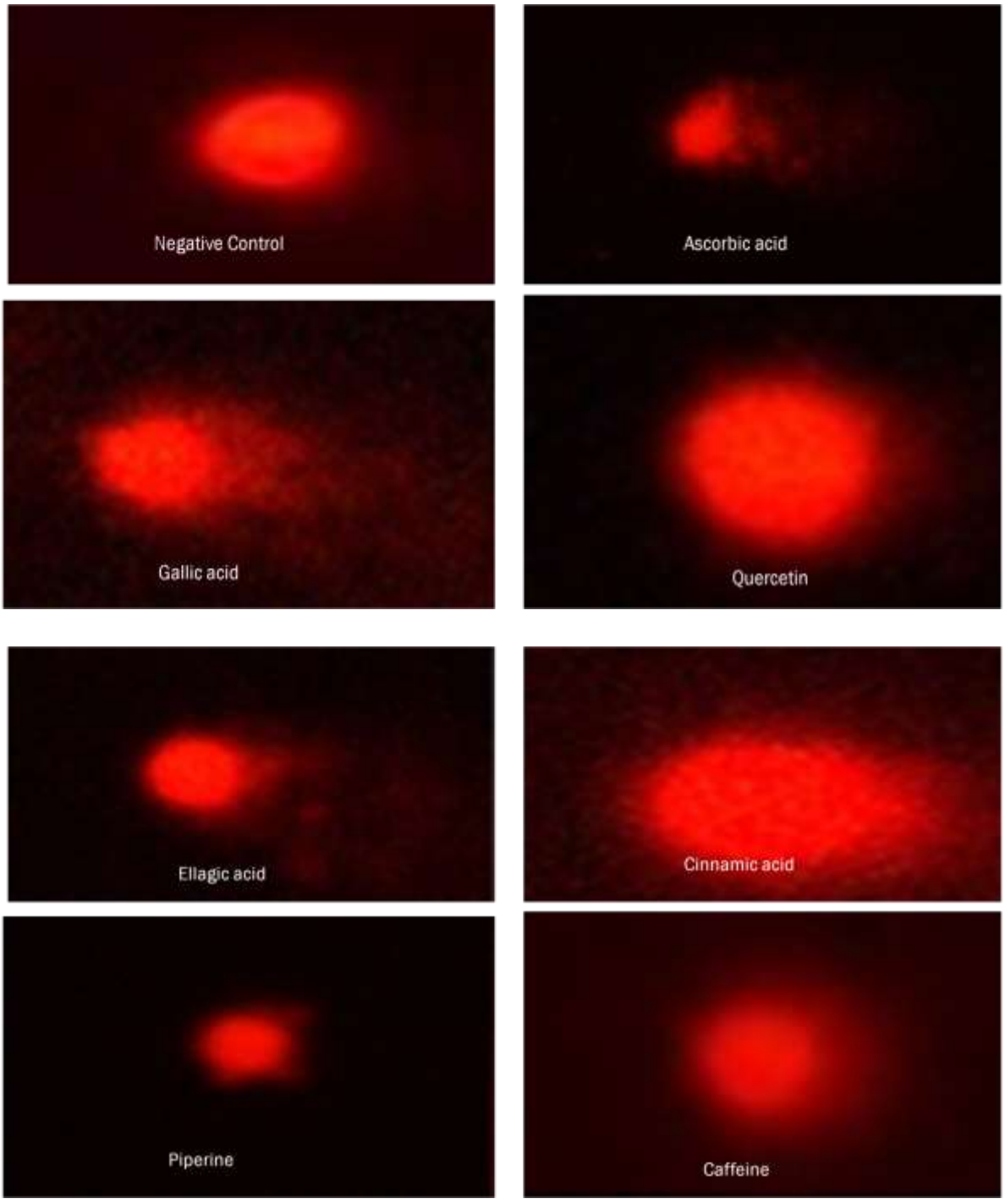
Secondary Antibody:

0.3 µl of secondary antibody was dissolved in 300µl of PBS (1:1000)

Chapter 4

Results:

1.1 To screen various natural compounds for genotoxicity preventive potential Using Comet Assay:



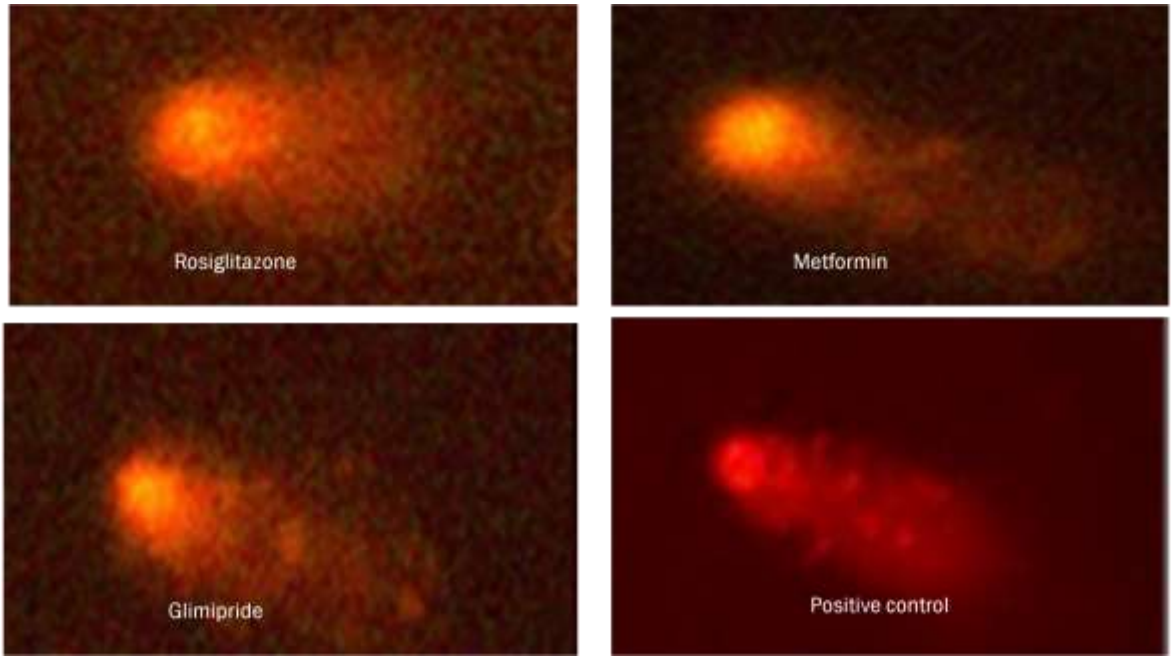
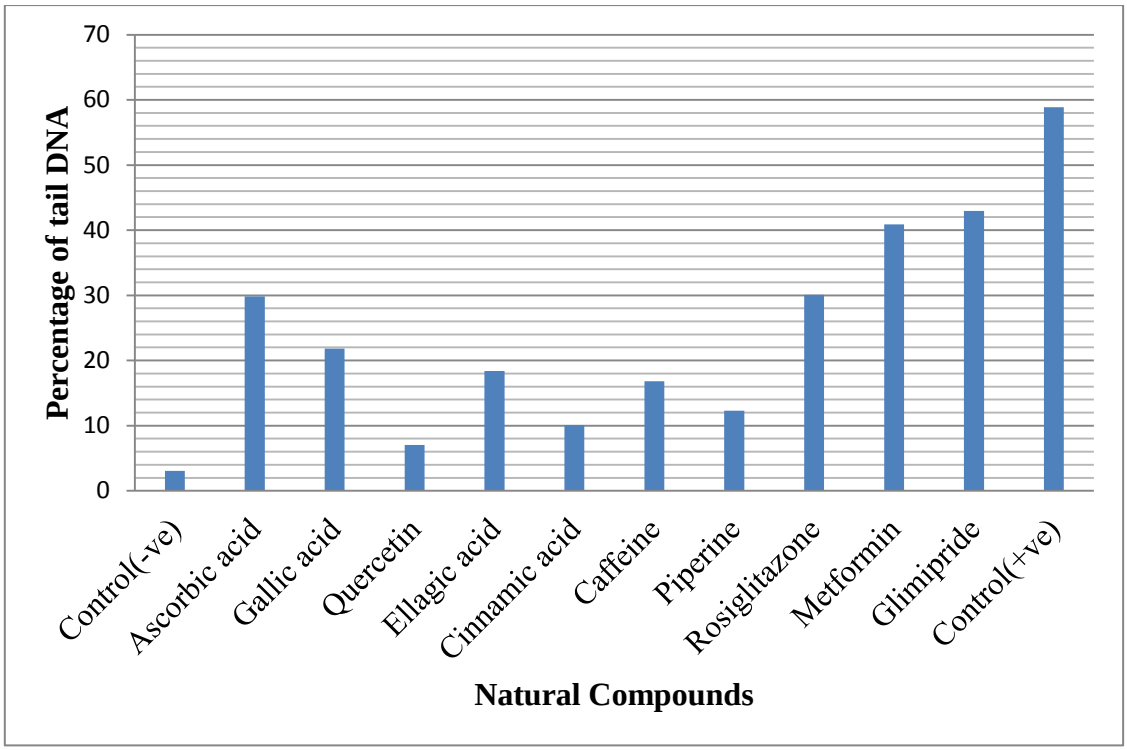


Figure 7: Images of Comet Assay (Captured by fluorescence microscope)

1.2 To compare the anti-genotoxicity activity of compounds with commercial available drugs using CASP software:

Table No. 13: Head and Tail DNA % of various Natural Compounds

Compounds	**Head DNA (%)**	**Tail DNA (%)**
Control (-)	96.94	3.055
Ascorbic acid	70.17	29.82
Gallic acid	78.15	21.85
Quercetin	92.75	7.027
Ellagic acid	81.615	18.38
Cinnamic acid	89.13	10.08
Caffeine	83.19	16.08
Piperine	87.52	12.27
Rosiglitazone	70.24	30.079
Metformin	59.12	40.87
Glimepiride	57.06	42.93
Control (+)	41.13	58.86

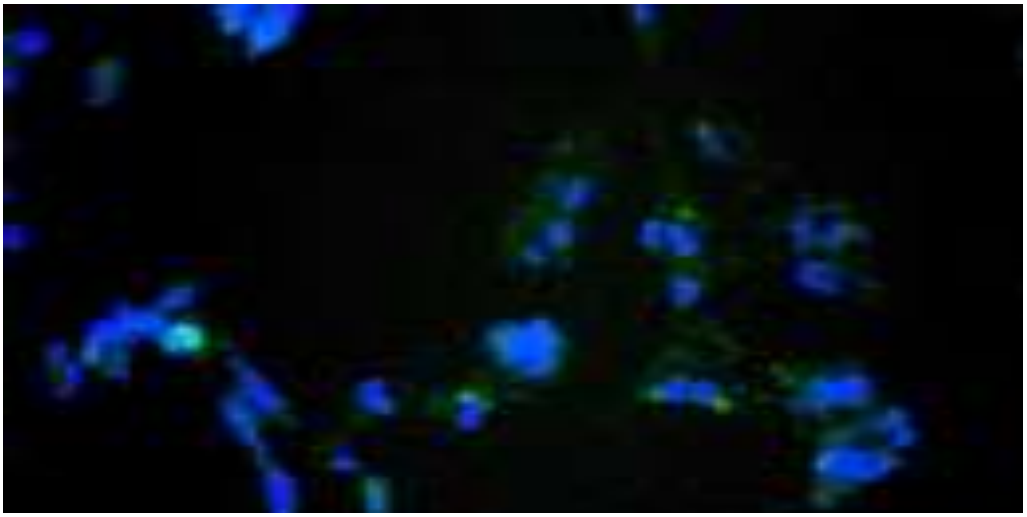


Graph: Effects of various compounds on the genotoxicity of hydrogen compounds

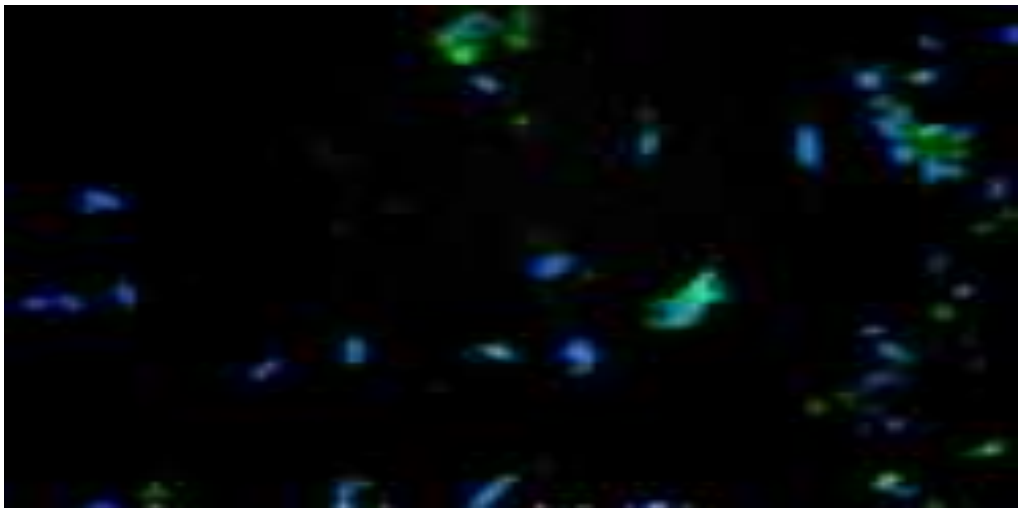
The comet assay was used to detect the anti-genotoxicity activity of natural compounds. The head DNA indicates the intact DNA and tail DNA indicates the fragmented DNA. The smaller the percentage of tail DNA more the compound has anti-genotoxicity effect. As the images table were clearly depicted the anti-genotoxicity activity of natural compounds when compared to the positive control.

1.3 To screen herbal compounds for their ability to enhance glucose uptake in neuronal cells using immuofluorescence:

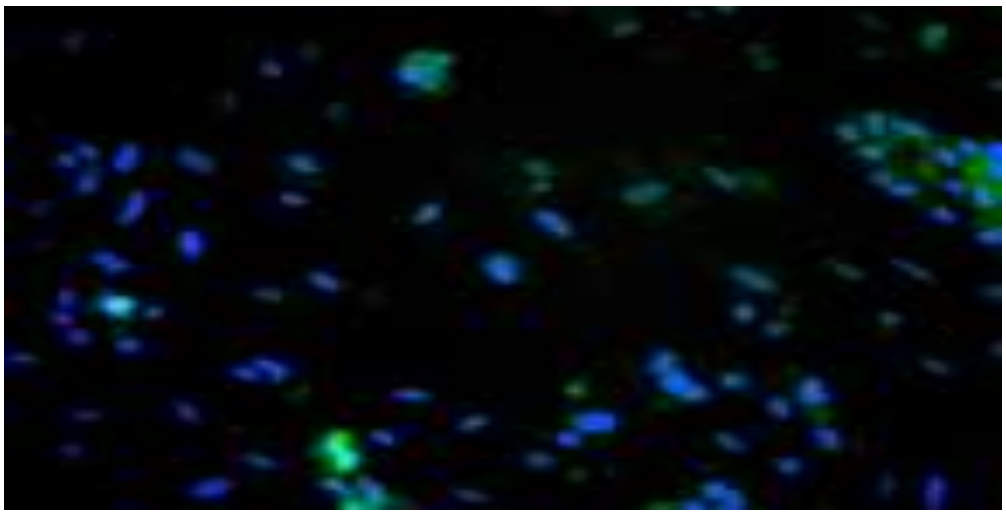
(A) The GLUT transporter proteins were used to uptake the glucose into the cells. The antibodies against the GLUT transporters were used to detect whether the herbal compounds increased the glucose uptake. Most of the herbal compounds increased the glucose uptake thus increase the translocation of GLUT transporters.



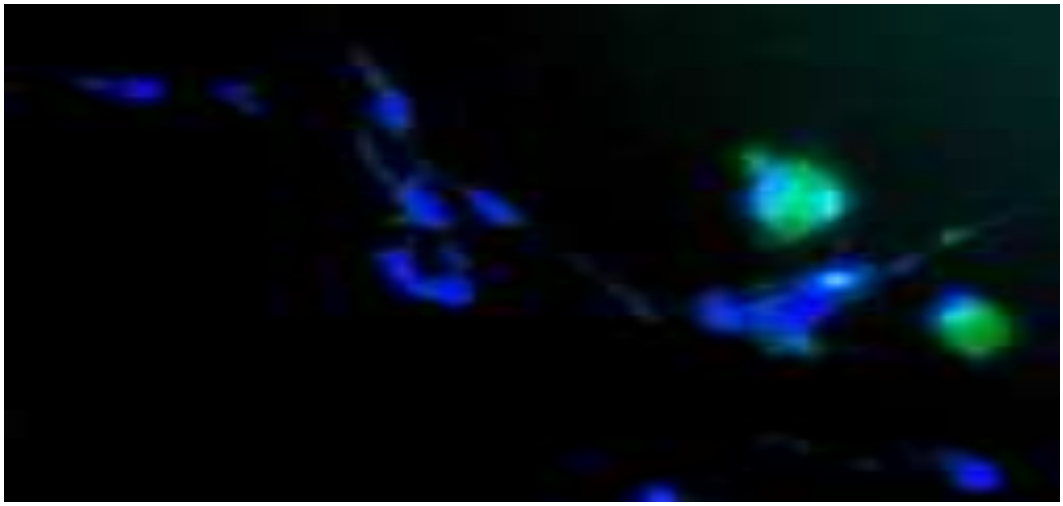
Control (A)



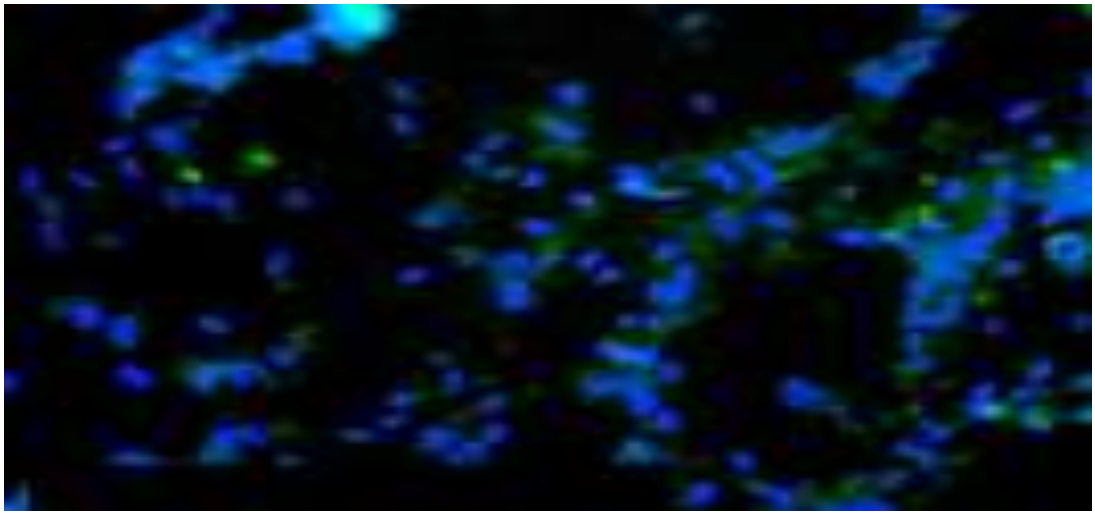
Ascorbic acid (A)



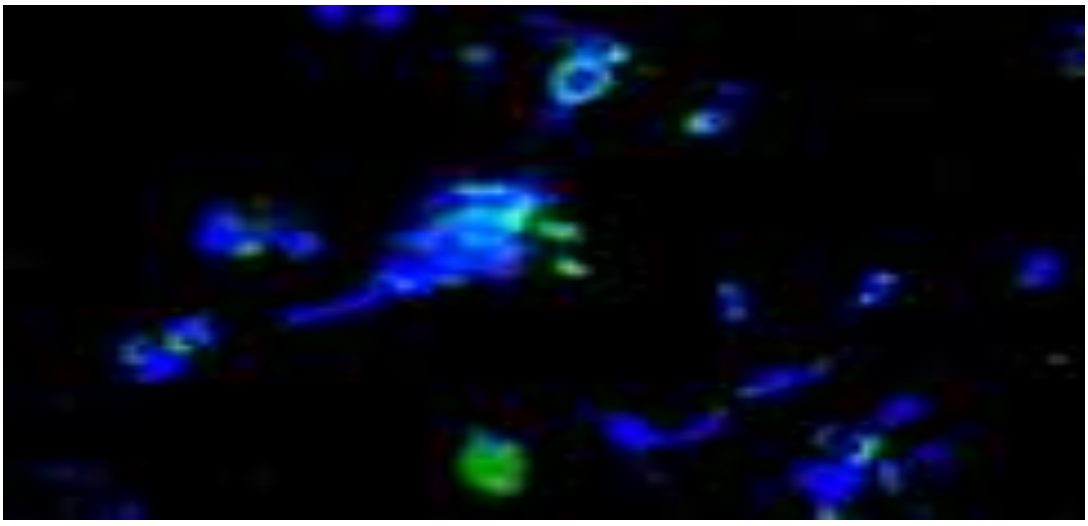
Gallic acid (A)



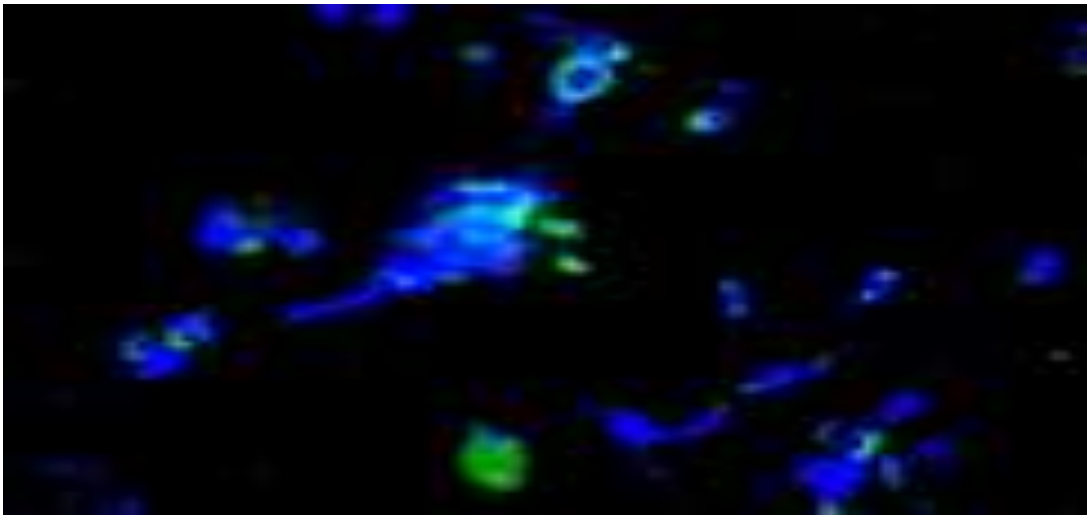
Ellagic acid (A)



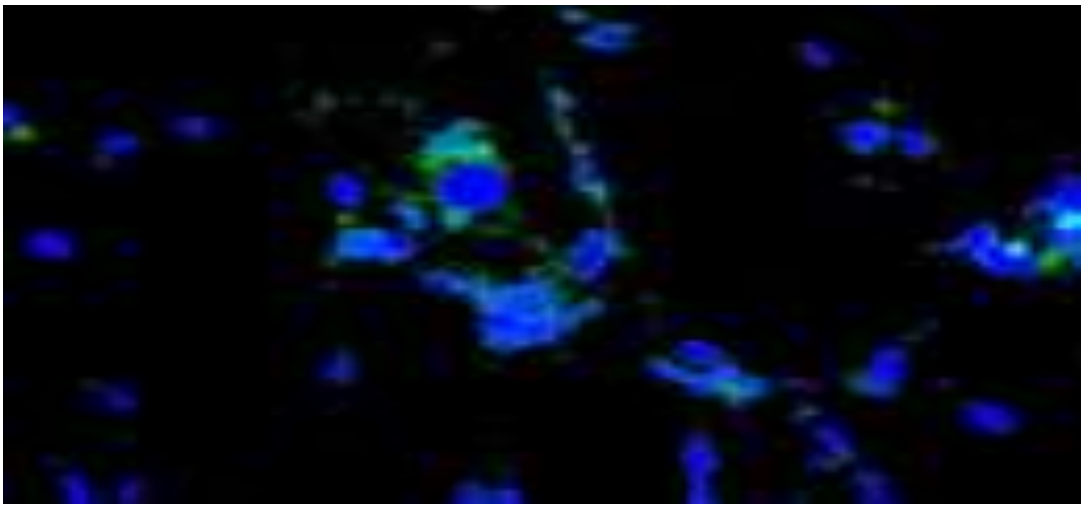
Quercetin (A)



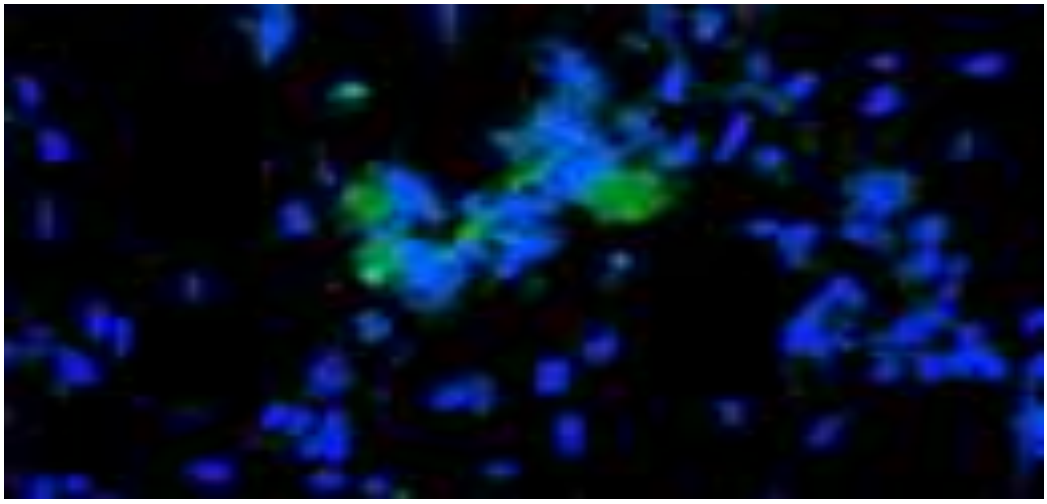
Cinnamic acid (A)



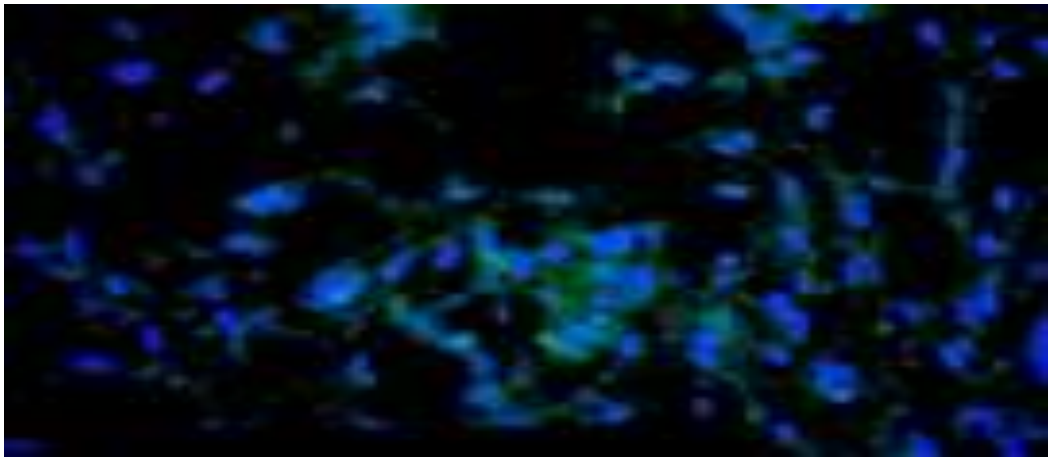
Caffeine (A)



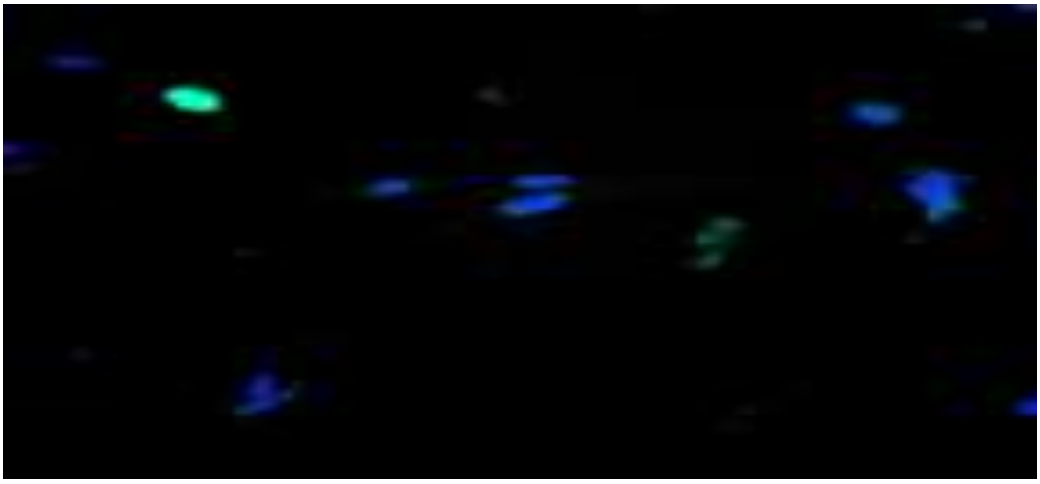
Piperine (A)



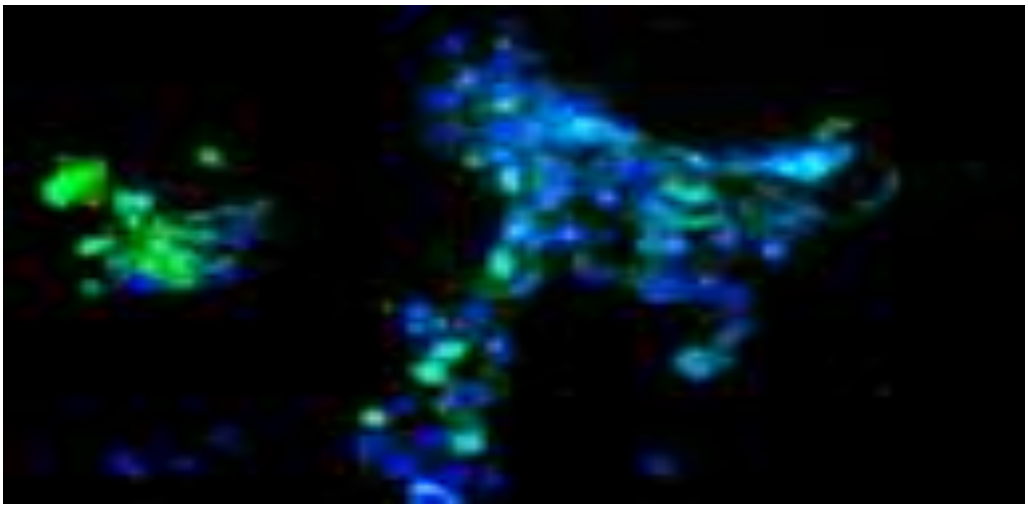
Metformin (A)



Rosiglitazone (A)



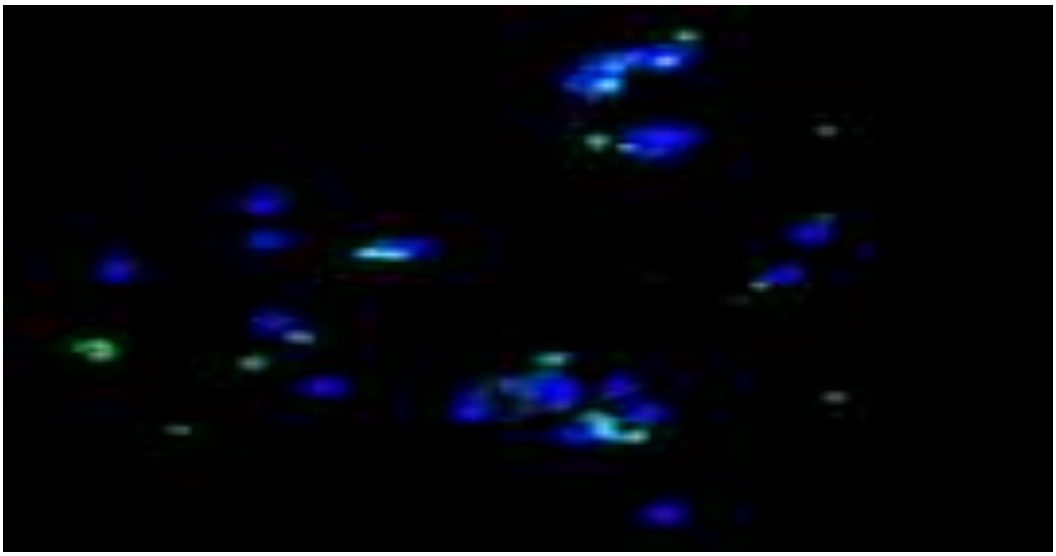
Glimepiride (A)



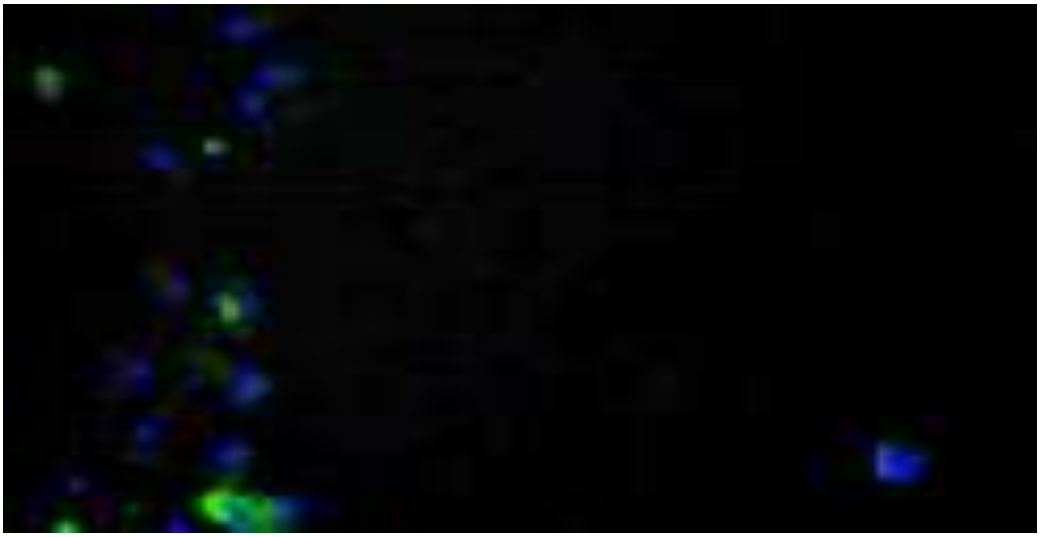
Insulin (A)

Figure 8: Ability of Herbal Compounds and drugs on Glucose uptake in Neuronal Cells

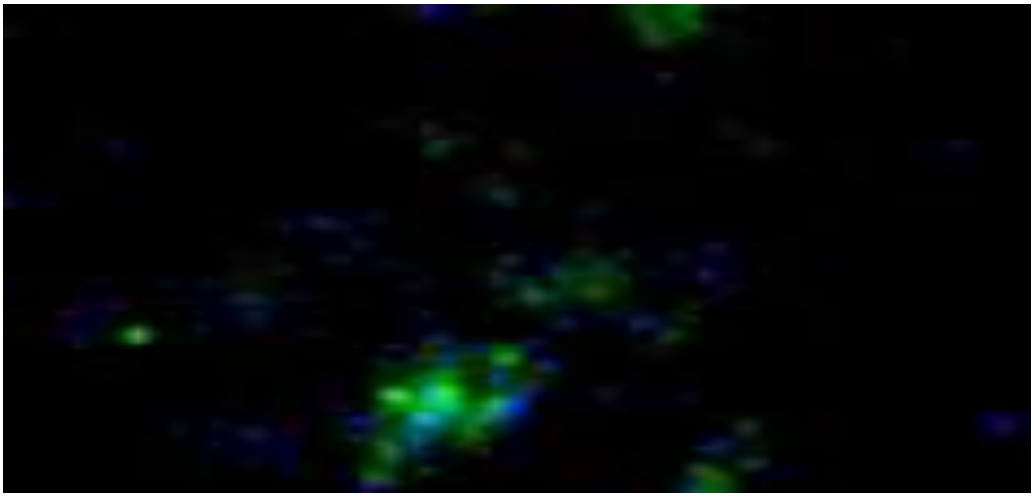
(B) In Experiment B also the translocation of GLUT transporters were seen in the presence of GLUT antagonist. The GLUT antagonist blocks the activity of GLUT transporters. It is seen that very few herbal compounds shows it activity for glucose activity in the presence of GLUT antagonist.



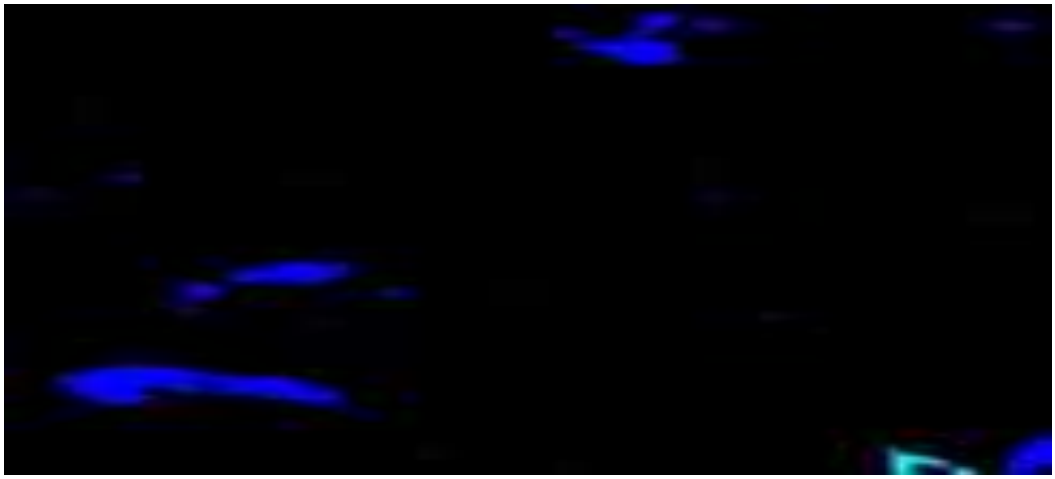
Control (B)



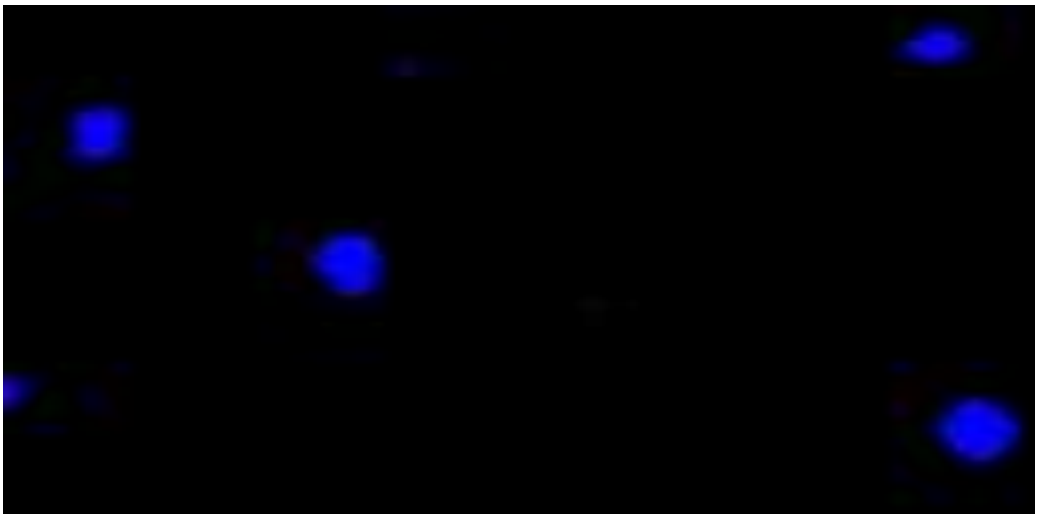
Ascorbic acid (B)



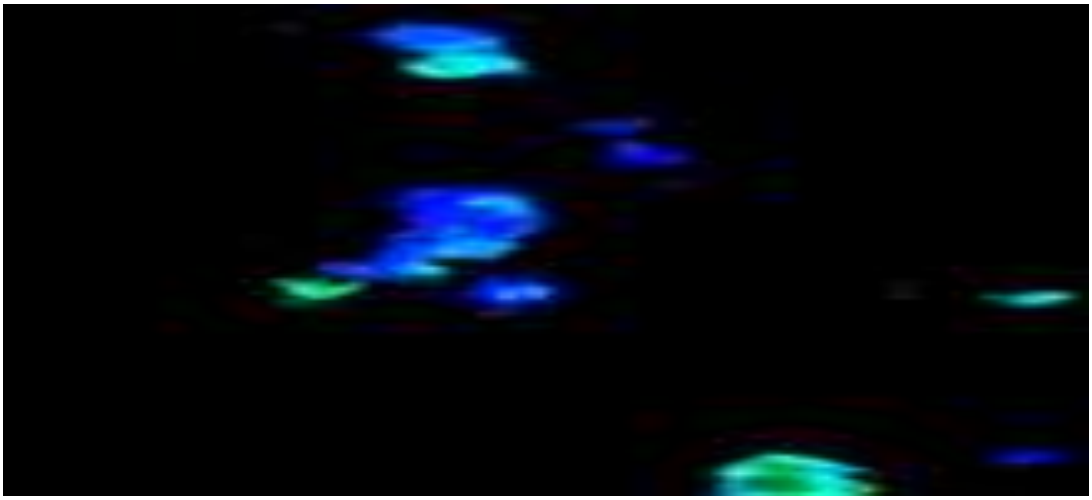
Gallic acid (B)



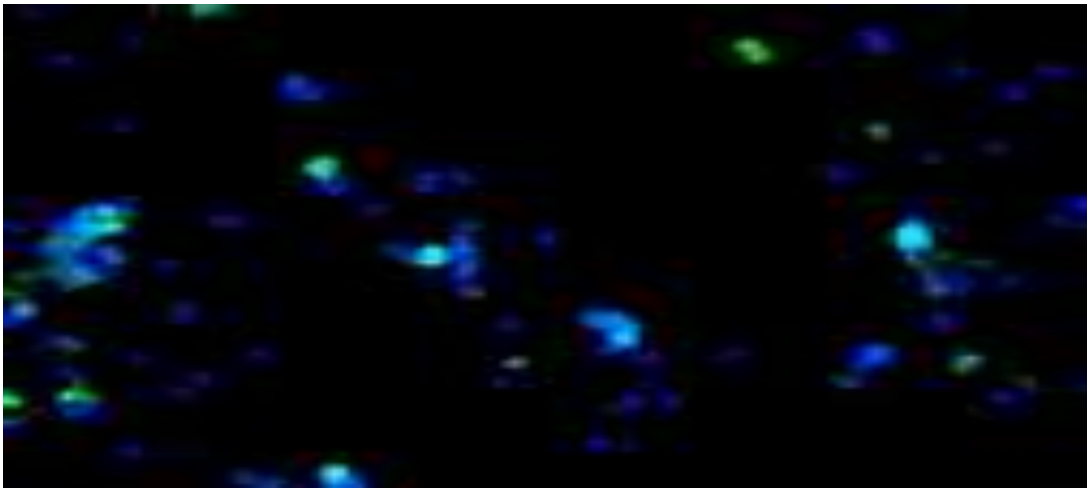
Ellagic acid (B)



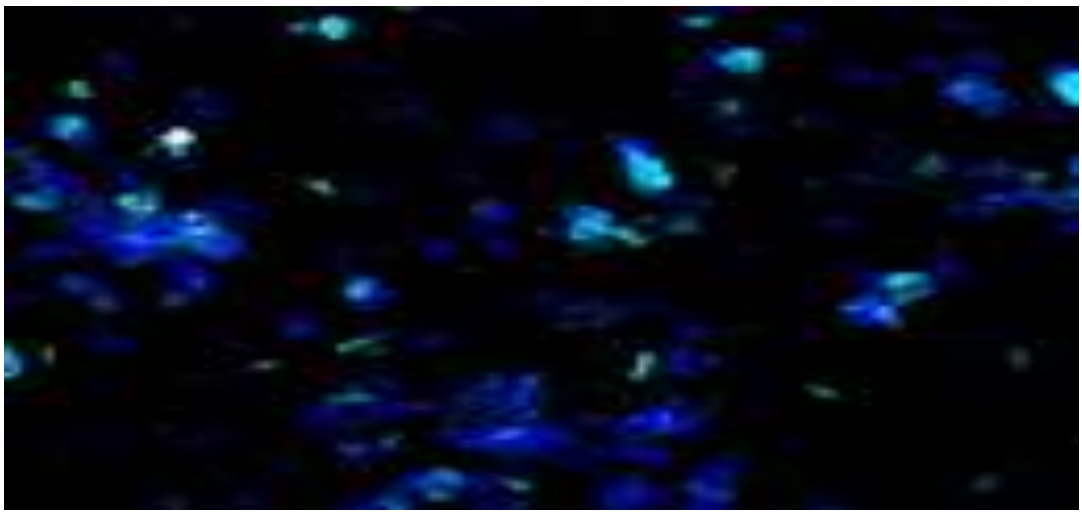
Quercetin (B)



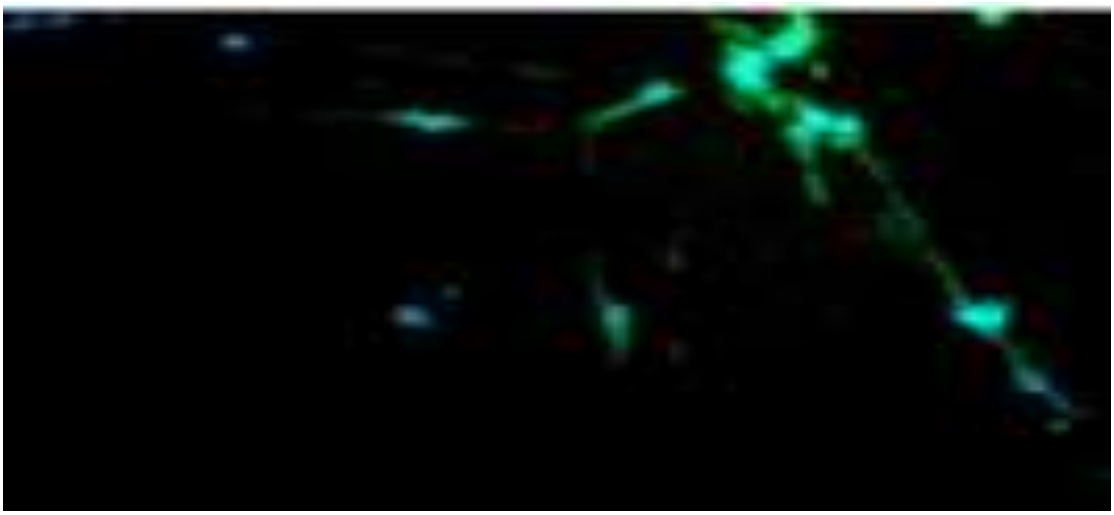
Cinnamic acid (B)



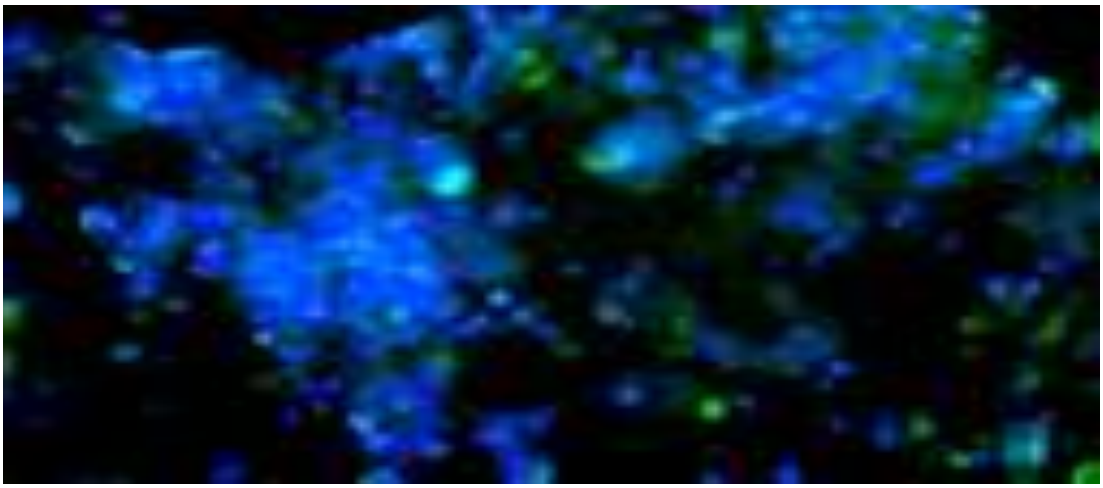
Caffeine (B)



Piperine (B)

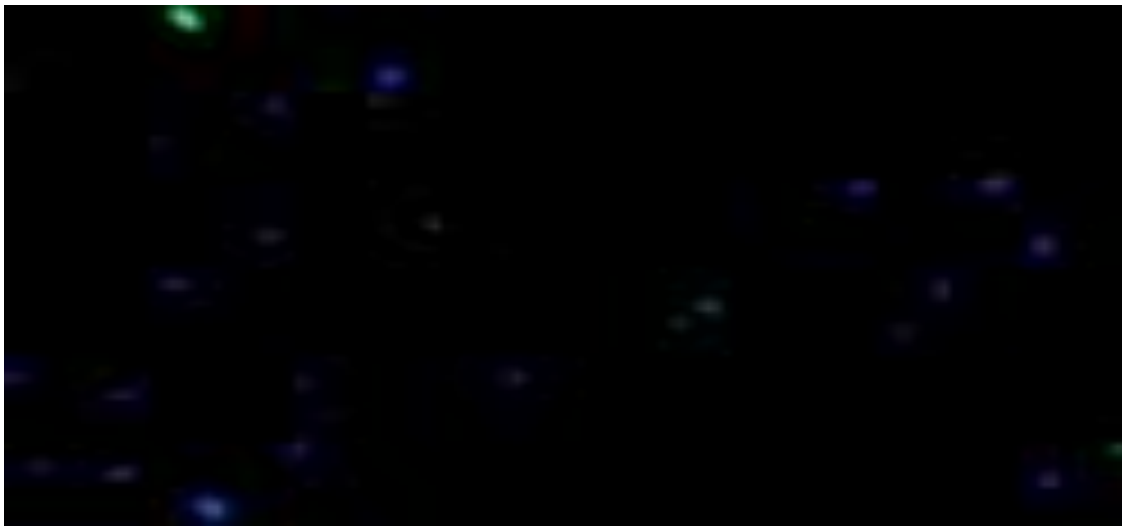


Metformin (B)



Rosiglitazone (B)

Glimipride (B)

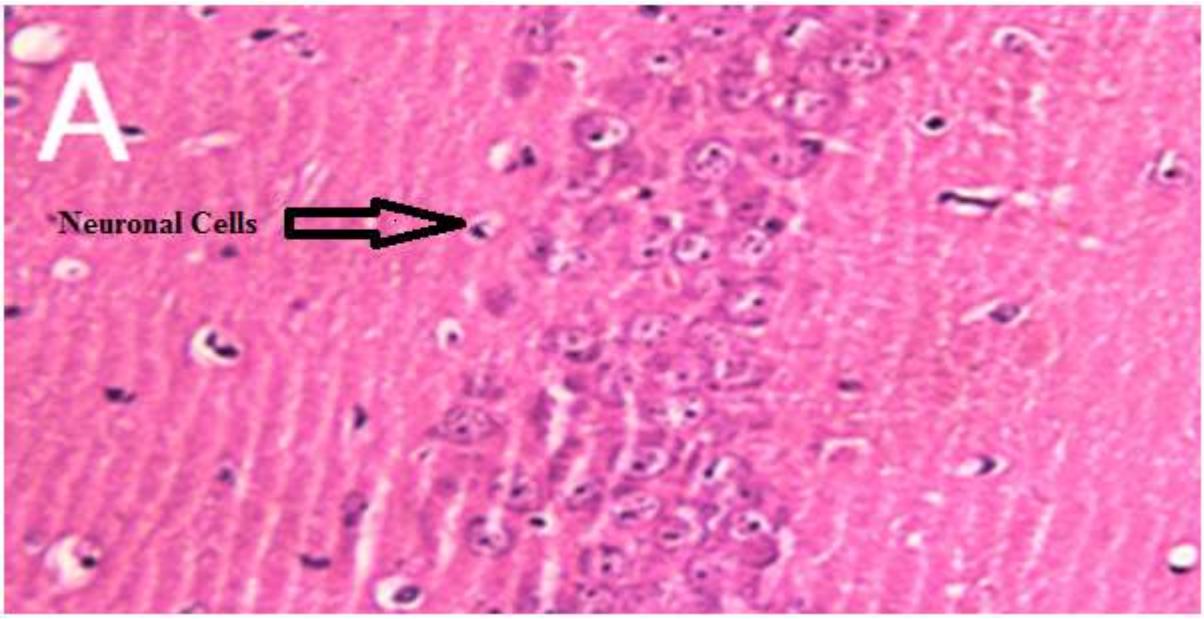


Insulin (B)

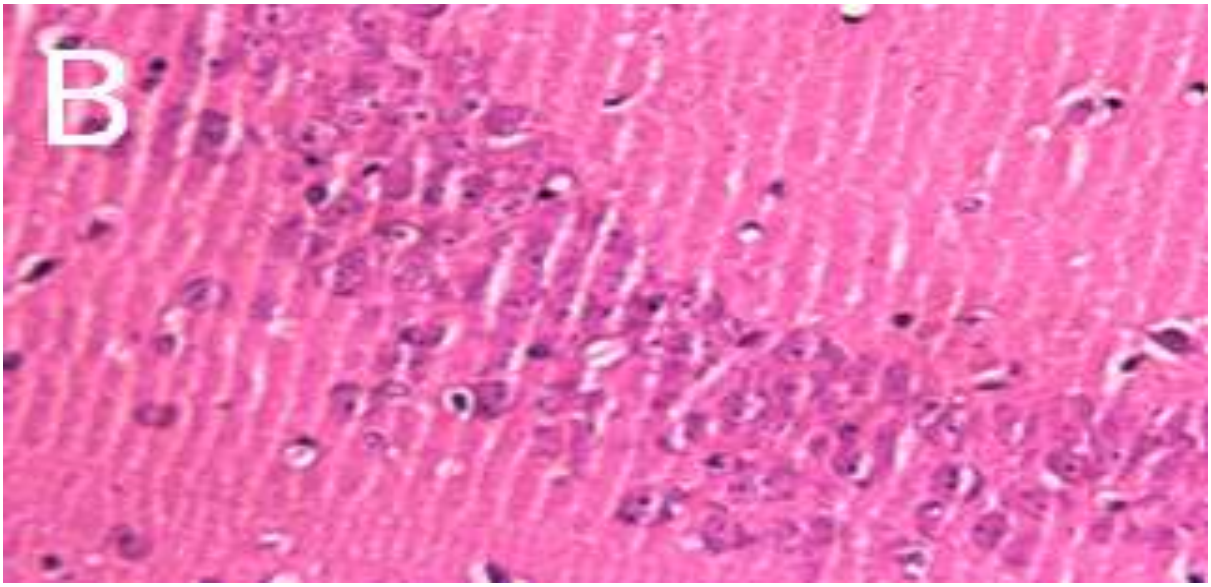
Figure 9: Ability of Herbal compounds and Drugs on Glucose uptake in Neuronal Cells (GLUT antagonist)

1.4 To evaluate diabetes induced neurodegeneration in hippocampus of mice using Hematoxylin & Eosin staining:

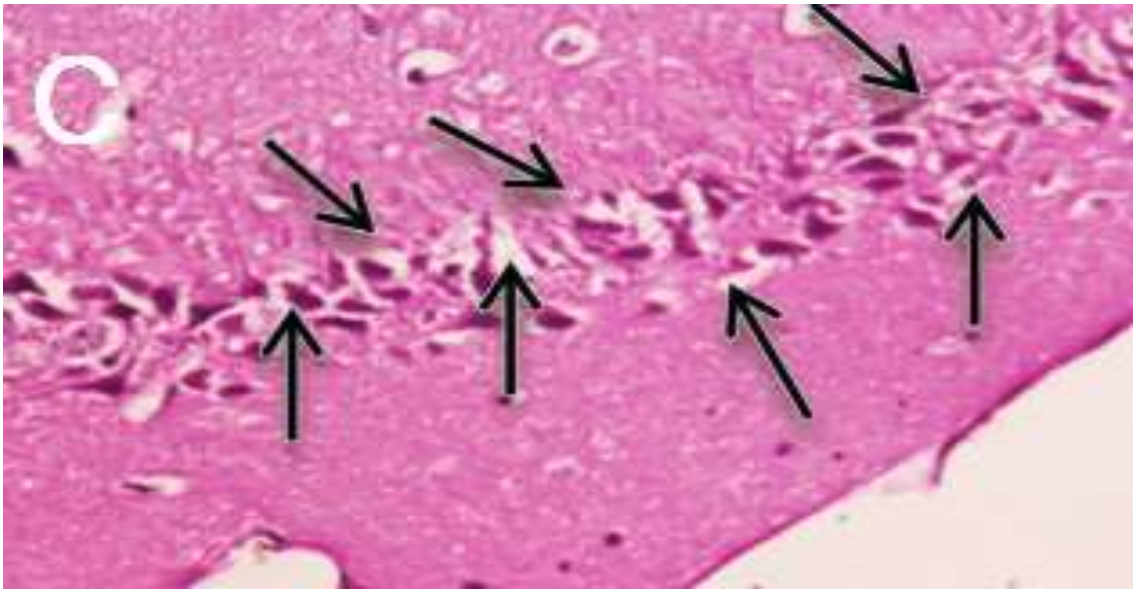
The images A, B, C D were the brain sections of mice. The images clearly depicted that the quercetin treatment given to the diabetic mice controls the neurodegeneration in hippocampus region.



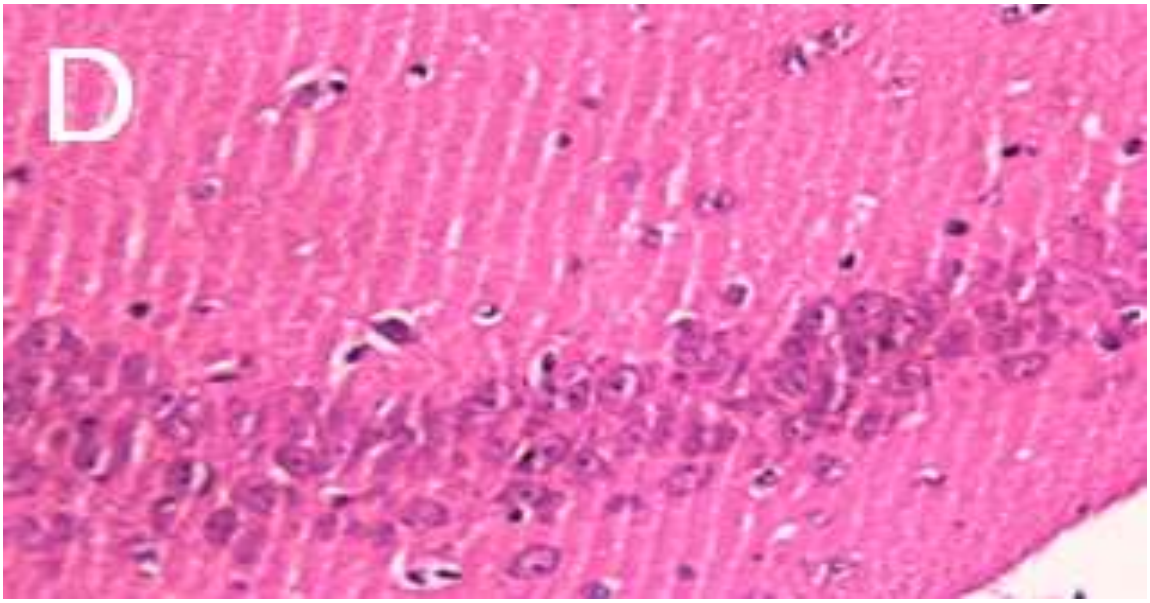
Normal (Brain section of non-diabetic mice)



Normal+ Quercetin (Brain section of non-diabetic mice)



Streptozotocin (Diabetic mice brain section without treatment)

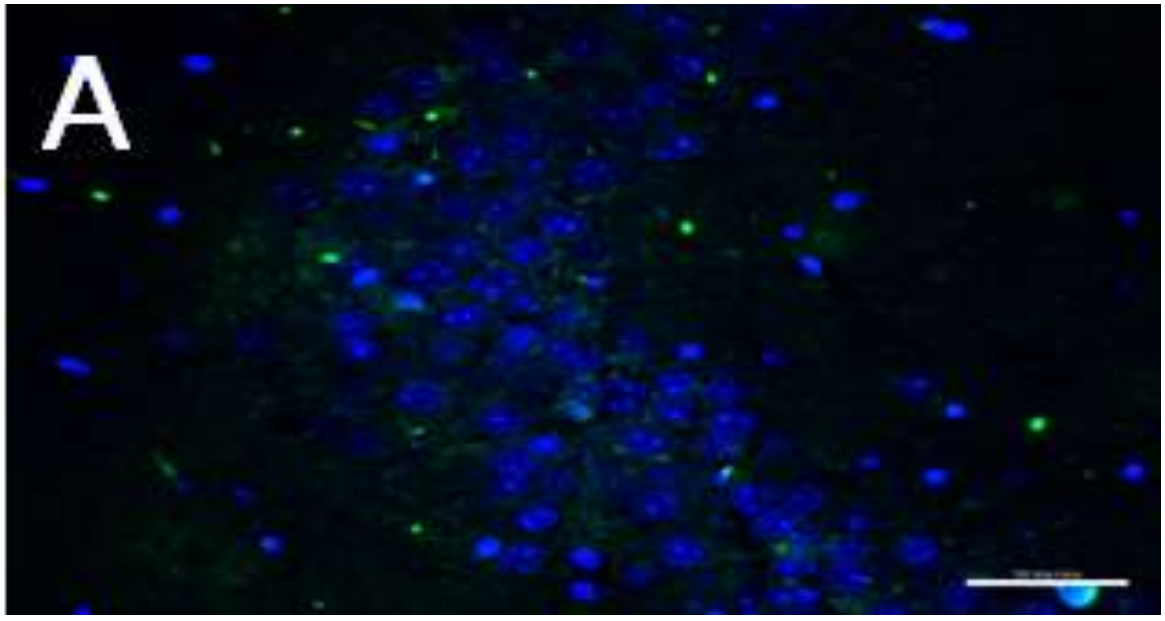


Diabetic mice brain section (Quercetin treatment)

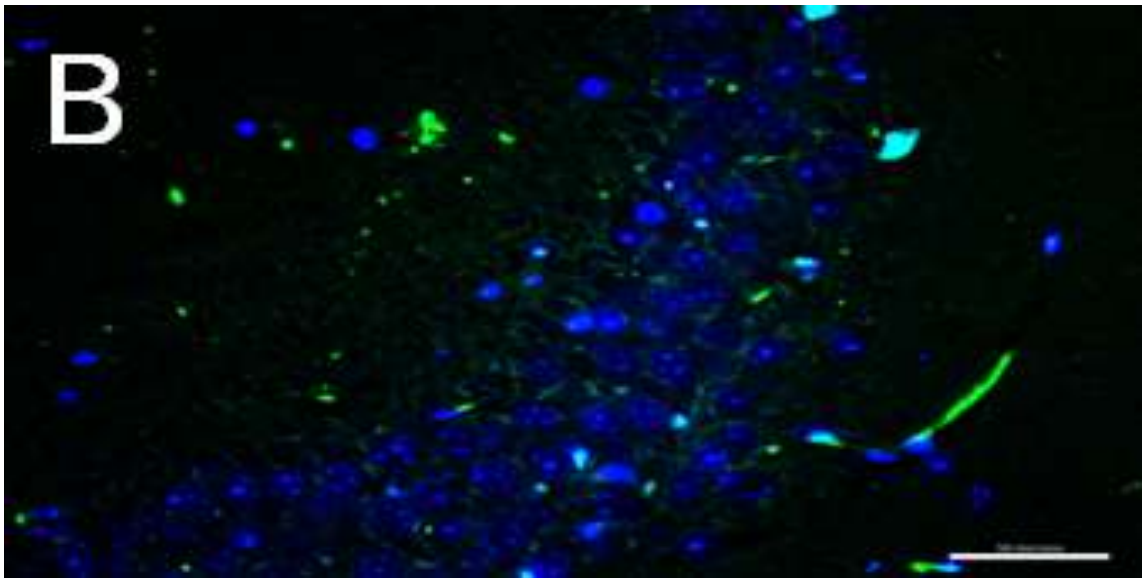
Figure 10: H&E Staining of Neuronal cells in Hippocampus

1.5: **To evaluate protein expression in hippocampus of diabetic and quercetin treated mice using immunofluorescence:**

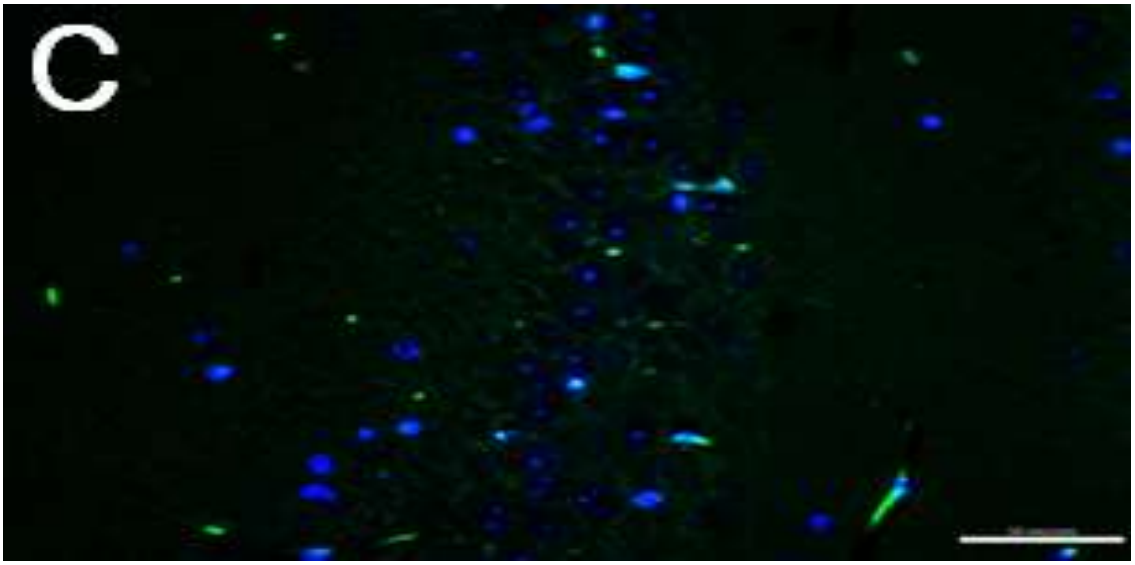
The images of tissue sections normal, normal+ quercetin, diabetic without treatment and diabetic with quercetin treatment of mice brain depicted that the quercetin treatment improved the GLUT expression in the brain as well as protected the cells from neurodegeneration.



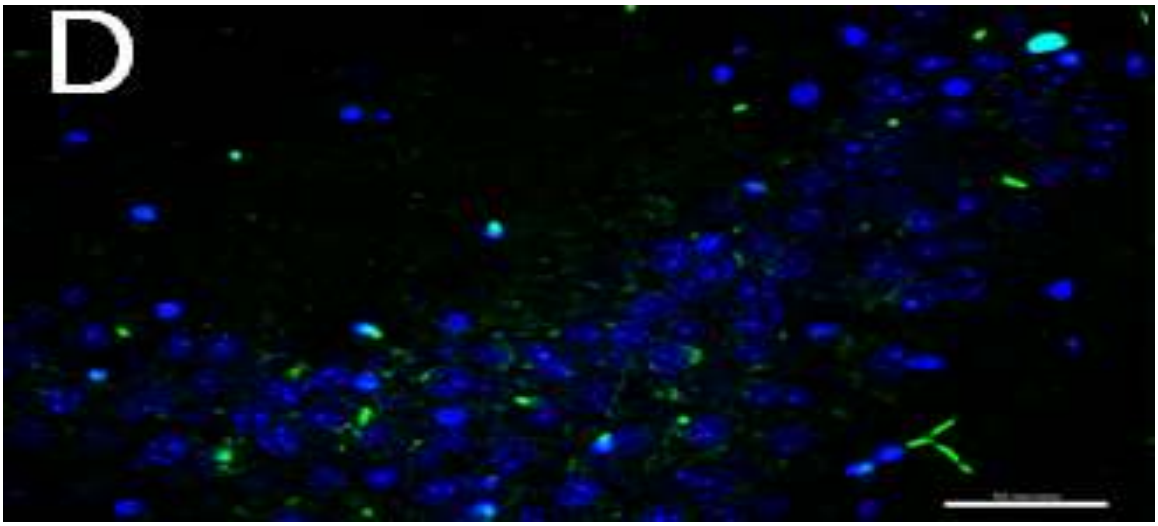
Normal (Brain section of non-diabetic mice)



Normal+ Quercetin (Brain section of non-diabetic mice)



Streptozotocin (Diabetic mice brain section without treatment)



Streptozotocin+ Quercetin (Diabetic mice brain section)

Figure 11: GLUT transporter expression in Neuronal cells in Hippocampus

Chapter 5

DISCUSSION

Genotoxicity is one of the major secondary complication of hyperglycaemia which arise due to the interaction of highly reactive oxygen free radicles with DNA resulting in impaired DNA functioning. During hyperglycaemia, excessive free radicals are generated due to excessive availability of glucose therefore genotoxic effects are common and severe. The anti-genotoxicity of the natural compounds was done by comet assay. It is basically a combination of two techniques, the DNA gel electrophoresis and fluorescence microscopy to visualize the migration of DNA. Comet assay detected the breaks in the DNA. If DNA contains breaks in it, then the supercoils DNA were relaxed and the fragmented DNA migrates towards the anode. The intact DNA donot migrate because of its large size. Therefore the relative amount of DNA migrated is way to measure the number of breaks in the DNA. The cell containing DNA looks like comet. The tail contains the broken fragments of the DNA and head contain the high molecular intact DNA which does not migrate during electrophoresis (Olive and Banath, 2006). In our study we used this method to evaluate whether natural compounds are capable of preventing genotoxicity or not in H_2O_2 induced genotoxicity model and compared them to marketed drugs. Control reaction showed that H_2O_2 induced severe levels of DNA fragmentation as observed from tail length and %age of DNA in the tail of comet. The natural compounds significantly prevented DNA fragmentation and thus provided efficient protection against genotoxicity. As the percentage of DNA in the tail was less as compared to hydrogen peroxide control or positive control. The compound that shows the best anti-genotoxicity was quercetin followed by cinnamic and piperine . These results depicts that natural molecules protect the DNA from degradation by hydrogen peroxide and therefore may be efficient in preventing hyperglycaemia induced genotoxicity and associated complications.

During diabetes mellitus type-II, insulin resistance develop in brain and as a result of that neurones are deprived of required amount of glucose. This neuronal starvation leads to neuronal death or neurodegeneration which adds on the development and progression of diabetes associated neurological complications. Therefore we performed an *in-vitro* assay to evaluate the potential of natural molecules to enhance glucose utilization by neurones and therefore their ability to protect neurones against neuronal starvation. The glucose uptake by the neuronal cells was increased in the presence of natural compounds which were detected through the immunofluorescence. The glucose uptake by the cell is mediated by the GLUT4 transporters. Therefore if a compound increases the glucose uptake it leads to increase the

expression of the GLUT4 transporters which were detected by using the antibodies specific for GLUT4. We further incubated neurones with GLUT4 membrane translocation antagonist, LY294002, and observed that antagonist significantly reduced GLUT4 expression. Treating cells with natural compounds revealed that quercetin, caffeine and ascorbic acid significantly elevated GLUT4 expression in neuronal membrane indicating that they might utilize insulin signalling to enhance GLUT4 translocation. Therefore, these compounds may protect neurons from insulin resistance mediated starvation and may provide protection against neurodeneration and associated complications.

The haematoxylin and eosin staining was used to detect the neurodegeneration in the brain sections of diabetic mice, diabetic mice get quercetin treatment, non-diabetic mice and non-diabetic mice get quercetin treatment. The diabetic mice without the quercetin treatment had neurodegeneration in hippocampus region which was evident from the fewer number of healthy neurones and large number of damaged neurones. While the diabetic mice treated with quercetin did not show the sign of neurodegeneration and those sections resembled to that of normal animals. In normal animals treated with quercetin there was no signs of any toxicity or degenerative effects and here also sections were similar to normal animals. Thus the quercetin protected neuronal cells from the degenerative stress inflicted by hyperglycaemia and therefore may protect neurodegeneration mediated complication associated secondary to hyperglycaemia.

Further, we evaluated GLUT4 expression in the hippocampus of animals to evaluate the effect of diabetes and quercetin on it through immunofluorescence technique. In our study we observed that diabetic mice showed lower expression of GLUT4 on neuronal membrane which was significantly reversed in quercetin treated animals. Quercetin treated animals increased GLUT4 expression which was observed to be similar to that observed for normal and normal animals treated with quercetin. These results demonstrate that quercetin is very efficient in improving neuronal glucose utilization during insulin resistant state and therefore have protective effect against hyperglycaemia induced neuronal death and thus, associated neurological complications.

Chapter 6

Conclusion

Diabetes mellitus type-II has leads to various neurological complications which severely impairing healthy state of living. The current therapeutics is not effective in preventing or reversing the progression of secondary diabetic complications and therefore it is necessary to find out alternative therapeutic strategies that not only prevent these complications from progressing further but also reverse the damage that have already been set in. In our study we evaluated natural compounds for their ability to interfere with pathways that leads to the development of neurological complications. This study concluded that the phytochemicals, especially quercetin, caffeine and ascorbic acid are very efficient neuroprotective agents as they enhanced glucose utilization and GLUT 4 translocation to neuronal membrane. Further, these molecules efficiently prevented genotoxicity/DNA fragmentation, which intensify the severity of diabetic complications. In *in-vivo* experimentation revealed that quercetin significantly prevented hyperglycaemia mediated neurodegeneration and efficiently improved neuronal GLUT4 expression, giving indication that it might have reversed insulin resistance in CNS. In a nut shell it can be concluded that quercetin can be an efficient therapeutic additive to anti-diabetic therapeutics and may aid in halting the development and progression of neurological complications associated with type-II diabetes mellitus.

Chapter 7

References:

- Adewale OF, Isaac OO, Tunmise MT and Omoniyi OO (2016), Palm oil and ground nut oil supplementation effects on blood glucose and antioxidant status in alloxan-induced diabetic rats, *Pakistan Journal of Pharmaceutical Science,* Vol-29, 83-87.
- Alagiakrishnan K, Sankarlingam S, Ghosh M, Mereu L and Senior P (2013), Antidiabetic drugs and their potential role in treating mild cognitive impairment and Alzheimer's disease ,*Discovery Medicine*, Vol-16(30): 277-286.
- Andersen JK (2004), Oxidative stress in neurodegeneration: cause or consequence?, *NATURE*, Vol-10, 18-25.
- Aguilar CMJ, Baena GJ, Rodriquez BR et al (2015), Maternal diabetes mellitus and its impact on child neurodegeneration, *Nutricion Hospitalaria,* Vol-32(6), 2484-2495.
- Barbour LA, McCurdy CE, Hernandez TL, Kirwan JP, Catalano PM and Friedman JE (2007), Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes, *Diabetes Care*. 2007, Vol-30(2), 112–119.
- Bastaki S (2005), Diabetes mellitus and its treatment, *International Journal of Diabetes and Metabolism*, Vol-13: 111-134.
- Ben HA, Yogev Y and Hod M (2004), Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes, *Diabetic Medicine*, Vol-21(2), 103-113.
- Blazque E, Velazquez E, Carneiro VH and Albusac JMR (2014), Insulin in the brain: its pathophysiological implications for states related with central insulin resistance, type 2 diabetes and Alzheimer's disease, *Frontiers in Endocrinology,* Vol-5, 1-21.
- Bogdanovic R (2008), Diabetic nephropathy in children and adolescents, *Pediatric Nephrology*, Vol-23(4):507–525.
- Boltan CF (1980), Peripheral neuropathies associated with chronic renal failure, *Neurological Science*, Vol-7: 89-96.
- Bosco D, Fava A, Plastino M, Montalcini T and Pujia A (2011), Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis, *Journal of Cellular and Molecular Medicine*, Vol-15, 1807-1821.
- Brownlee M (2001), Biochemistry and molecular biology of diabetic complications, *Nature*, Vol-414(6865): 813-820.
- Campayo A, de Jonge P, Roy JF et al (2010), Depressive disorder and incidence diabetes mellitus: the effect of characteristics of depression, *The American Journal of Psychiatry,* Vol-167(5), 580-588.

- Chhabra N (2012), Insulin Biosynthesis, Secretion and Action, *Biochemistry for Medics.*
- Chen Y, Zhou K, Wang R, Liu Y et al (2009), Antidiabetic drug metformin (Glucophage^{R}) increases biogenesis of Alzheimer's amyloid peptides via up-regulating *BACE1* transcription, *Proceeding of the National Academy of Science,* Vol-106(10), 3907-3912.
- Davis JA, Sharma S, Mittra S, Sujatha S et al (2012), Antihyperglycemia effect of *Annona squamosa* hexane extract in type 2 diabetes animal model: PTP1B inhibition, a possible mechanism of action?, *Indian Journal of Pharmacology,* Vol-44(3), 326-332.
- Eid HM, Martineau LC, Saleem A, Muhammad A et al (2010), Stimulation of AMP-activated protein kinase and enhancement of basal glucose uptake in muscle cells by quercetin and quercetin glycosides, active principles of the antidiabetic medicinal plant *Vaccinium vitis-idaea, Molecular Nutrition & Food Research, Vol-54(7), 991-1003.*
- Etxeberria U, Garza AL, Campión J, Martínez JA and Milagro FI (2012), Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase, *Expert opinion on therapeutic targets,* Vol-*16*(3), 269-297
- Felice FGD, Lourenco MVand Ferreria ST (2014), How does brain insulin resistance develop in Alzheimer's disease?, *Alzheimer's & Dementia*, Vol.-10(1), 26-32.
- Fisher A, Zhang Q, Geoffrey J and Steven D (2006), NADPH and NADPH oxidase, *Encyclopedia of Respiratory Medicine*, 77-84.
- Giacco F and Brownlee M (2010), Oxidative stress and diabetic complications. *Circulation Research,* Vol-107(9): 1058-1070.
- Gok M, Ulusu NN, Tarhan N, Tufan C et al (2016), Flaxseed Protects Against Diabetes-Induced Glucotoxicity by Modulating Pentose Phosphate Pathway and Glutathione-Dependent Enzyme Activities in Rats, *Journal of Dietary Supplements,* Vol-13(3), 339-351.
- Gold S, Sweat D, Tirsi S, Rogers K, Bruchi H, Tsui W, Richardson S, Javier E and Convit A (2007), Hippocampal damage and memory impairment as possible early brain complications of type 2 diabetes, *Diabetologist,* Vol-50: 711-719.

- Gudala K, Bansal D, Schifano F and Bhansali A (2013), Diabetes mellitus and risk of dementia: A meta-analysis of prospective observational studies, *Journal of Diabetes Investigation*, Vol- 4(6), 640-650.
- Gupta R, Bajpai KG, Jhori S and Saxena AM (2007), An Overview of Indian Novel Traditional Medicinal Plants with Anti-Diabetic Potentials, *African Journal of Traditional, Complemetary and Alternative*, Vol-5(1): 1-17.
- HAPO Study Cooperative Research Group (2002), The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study, *International Journal of Gynecology & Obstetrics*, Vol-78, 69–77
- Herath PM, Cherbuin N, Eramudugolla R and Anstey KJ (2016), The Effect of Diabetes Medication on Cognitive Function: Evidence from the PATH Through Life Study, *Biomed Research International*, Vol-2016, 1-7.
- Himeno T, Kamiya H, Naruse K et al (2011), Beneficial effects of exendin-4 on experimental polyneuropathy in diabetic mice, *Diabetes*, Vol-60(9), 2397-2406.
- International Diabetic Federation: The Diabetes Atlas, (2015).
- Johansen JS, Harris AK, Rychly DJ and Ergul, A (2005), Oxidative stress and the use of Antioxidants in diabetes: linking basic science to clinical practice, *Cardiovascular diabetology*, Vol- 4(1), 5.
- Kahn CR and White MF (1988), the insulin receptor and the molecular mechanism of insulin action, *Journal of Clinical Investigation*, Vol-82, 1151-1156.
- Kampmann U, Madsen LR, Skajaa GO, Iverson DS, Moeller N and Ovesen P (2015), Gestational diabetes: A clinical update, *World Journal of Diabetes*, Vol-6(8), 1065-1072.
- Kharroubi A, Darwish H, Akkawi M, Ashareef A, Almasri Z, Badar K and Khammash U (2015), Total antioxidant status in type 2 diabetes patients in Palestine, *Journal of Diabetic Research*.
- Kiec AD, Mykkanen O, Wilk BK and Mykkanen (2008), Antioxidant phytochemicals against type 2 Diabetes, *British Journal of Nutrition*, Vol-99, 109-117.
- King H, Aubert R and Herman W (1998), Global burden of diabetes; prevalence, numerical estimates and projections, *Diabetes Care*, Vol-21: 1414-1431.
- Krishnaiah D, Sarbatly and Bono A (2007), *Biotechnology and Molecular Biology Review*, Vol-1(4): 97-104.

- Lee JH, Yoon S, Renshaw P.F et al (2013), Impact of hypoglycemia and diabetes on CNS: Correlation of mitochondrial oxidative stress with DNA damage, *PLos One*, Vol-8(4), e60515.
- Lipson KL, Fonseca SG, Ishigaki S et al (2006), Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1, *Cell Metabolism*, Vol-4, 245-254.
- Luna B (2001), Oral Agents in the Management of Type 2 Diabetes Mellitus, *American Family Physicians*, Vol-63(9), 1747-1757.
- Mehta V and Malairaman U (2016), Flavonoids: Perspective strategy for management of diabetes and its associated complications, Handbook of research on Advancing Health Education through Technology.
- Mohopatra L, Bhattamisra SK, Panigraphy RC and Parida SK (2016), Evaluation of the Antioxidant, Hypoglycaemic and Antidiabetic Activities of Some Seaweed Collected from the East Coast of India, *Biomedical & Pharmacology*, Vol-9(1), 365-375.
- Mooradian AD (1997), Central nervous system complications of diabetes mellitus-a perspective from the blood-brain barrier, *Brain Research*, Vol-23(3): 210-218.
- Moore D, Gregory J, Crystal Y and Simmons J (2009), Mitigating micro- and macro-vascular complications of diabetes beginning in adolescence, *Vascular Health and Risk Management*, Vol-5: 1015-1031.
- Monrey M and Mejia F (2013), Oxidative Stress in Diabetes Mellitus and the Role of Vitamins with Antioxidant Actions, 210-232.
- Morino K, Petersen KF and Shulman GI (2006), Molecular mechanism of insulin resistance in Humans and their Potential Links with mitochondrial dysfunction, *Diabetes*, Vol-55(2), 9-15.
- Moskaug J, Carlsen H, Myhrstad M and Blomhoff (2004), Molecular imaging of the biological effects of quercetin and quercetin rich foods, *Mechanism of Aging and Development*, Vol-125: 315-324.
- Olive PL and Banath JP (2006), The Comet Assay: a method to measure DNA damage in individual cells, *Nature*, Vol-1(1), 23-29.
- Packer L and Glazer AN (1990), Oxygen radicals in biological systems, *Method of Enzymology, Vol* 186, and New York: Academic Press.

- Panda S and Kar A (2007), Antidiabetic and antioxidative effects of Annona squamosa leaves are possibly mediated through quercetin-3-O-glucoside, *Biofactors*, Vol-31, 201-210.
- Pedersen J (1968), The pregnant diabetic and her newborn-Problems and management, *Archives of Disease in Childhood*, Vol-43, 391
- Pirzada NA and Morgenlander JC (1997), peripheral neuropathy in patient with chronic renal failure: a treatable source of discomfort and disability, *Postgraduate Medical Journal*, Vol-102: 249-50.
- Plastino M, Fava A, Pirritano D, Cotronei P et al (2010), Effects of insulinic therapy on cognitive impairment in patients with Alzheimer disease and Diabetes Mellitus type-2, *Journal of Neuroscience*, Vol-288, 112-116.
- Polidori MC, Mecocci P, Stahl W, Parente B et al(2000), Plasma levels of lipophilic antioxidants in very old patients with type 2 diabetes, *Diabetes/Metabolism Research and Review*, Vol-16(1): 15-19.
- Prasad K and Dhar A (2016), Flaxseed and Diabetes, *Current Pharmaceutical Design*, Vol-22, 141-144.
- Prasad S, Sajja RK, Naik P and Cucullo L (2014), Diabetes Mellitus and Blood-Brain Barrier Dysfunction: An Overview, *Pharmacovigilance*, Vol- 2(2), 125.
- Qiao LY, Zhande R, Jetton TL, Zhou G and Sun XJ (2002), In vivo phosphorylation of insulin receptor substrate 1 at serine 789 by a novel serine kinase in insulin-resistant rodents, *The Journal of Biochemistry*, Vol-277: 26530-26539.
- Ryan CM, Freed MI, Rood JA, Cobitz AR, Waterhouse BR, Strachan MW (2006), Improving metabolic control leads to better working memory in adults with type 2 diabetes, *Diabetes Care*, Vol-29(2), 345-351.
- Sadeghi A, Hami J, Razavi S et al (2016), The Effect of Diabetes Mellitus on Apoptosis in Hippocampus: Cellular and Molecular Aspects, *International Journal of Preventive Medicine*, Vol-7.
- Saini V (2010), Molecular mechanisms of insulin resistance in type 2 diabetes mellitus, *World Journal of Diabetes*, Vol-15: 68-75.
- Spallone V, Ziegler D, Freeman R, Bernardi L, Frontoni S, Pop-Busui R, Stevan M, Kempler R, Hilsted J, Tesfaye S, Low P et al (2011), Cardiovascular autonomic neuropathy in diabetes: clinical impact, assessment, diagnosis and management, *Diabetic/Metabolic Research and Review*, Vol-27(7): 639-653.

- Serlin Y, Levy J and Shalev H (2011), Vascular Pathology and Blood-Brain Barrier Disruption in Cognitive and Psychiatric Complications of Type 2 Diabetes Mellitus, *Cardiovascular Psychiatry and Neurology.*
- Sharma S and Sharma N (2008), Epalrestat, an aldose reductase inhibitor, in diabetic neuropathy: An Indian perspective, *Annals of Indian Academy of Neurology,* Vol-11(4), 231.
- Shulman GI (2000), Cellular mechanism of insulin resistance, *The Journal of Clinical Investigation,* Vol-106, 171-176.
- Singh P, Jain A and Kaur G (2004), Impact of hypoglycemia and diabetes on CNS: Correlation of mitochondrial oxidative stress with DNA damage, *Molecular and Cellular Biochemistry*, Vol-260(I), 153-159.
- Soria B, Quesada I, Ropero AB, Pertusa JA, Martin F and Nadal A (2004), Novel players in pancreatic islet signaling: from membrane receptors to nuclear channels, *Diabetes*. Vol-53(1), 86–91.
- Stetler C and Miller GE (2011), Depression and Hypothalamic-pituitary-adrenal activation: a quantitative summery of four decades of research, *Psychosomatic Medicine,* Vol-73(2), 114-126.
- Stumvoll M, Nurjhan N, Perriello G, Dailey G and Gerich JE (1995), The metabolic effects of metformin in non-insulin-dependent diabetes mellitus, *The New England Journal of Medicine*, Vol-333, 550–554.
- Tahrani, AA, Barnett AH, and Bailey CJ (2013), SGLT inhibitors in management of diabetes. *The Lancet Diabetes & Endocrinology*, Vol-1(2), 140-151.
- Takeda S, Sato N, Yamada KU et al (2010), Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Aβ deposition in an Alzheimer mouse model with diabetes, *Proceedings of the National Academy of Science,* Vol-107, 7036-7041.
- Tesfaye S, Boulton AJ, Freeman R, Horowitz M, Kempler P, Lauria G, Malik RA, Sapllone V, Vinik A, Bernardi L and Valensi P (2010), Diabetic neuropathies: updates on definition, diagnostic criteria, estimation of severity and treatment, *Diabetes care,* Vol-33(10): 2285-2293.
- Thomas M, Qattan KK, Divya JS and Ali Muslim (2016), Anti-diabetic and anti-oxidant potential of aged garlic extract (AGE) in streptozotocin-induced diabetic rats, *BMC Complementary and Alternative Medicine,* Vol-16, 17.

- Tian R, Yang W, Xue O, Gao L et al (2016), Rutin ameliorates diabetic neuropathy by lowering plasma glucose and decreasing oxidative stress via Nrf2 signaling pathway in rats, *European Journal of Pharmacology*, Vol-771, 84-92.
- Wilson G (2005), Insulin and insulin resistance, *The Clinical Biochemist Reviews*, Vol-26(2), 19-36.
- Wolever TM, Brand MJC, Abernethy J et al (2008), Measuring the glycemic index of foods: interlaboratory study, *American Journal of Clinical Nutrition*, Vol-87(1): 247-257.
- Wong RHX, Nealon RS, Scholev A and Howe PRC (2016), Low dose resveratrol improves cerebrovascular function in type 2 diabetes mellitus, *Nutrition, Metabolism & Cardiovascular Diseases*, Vol-26, 393-399.
- Zhang YJ, Gan RY, Sha L, Zhou Y et al (2015), Antioxidant Phytochemicals for the Prevention and Treatment of Chronic Diseases, *Molecules*, Vol-20(15), 21138-21156.
- Zhao Y, Wang P, Chen H and Sang S (2016), Dietary quercetin inhibits methylglyoxal-induced advanced glycation end products formation in mice, The *FASEB Journal*, Vol-30, 692-694.