

**MECHANISTIC ROLE OF CITRUS FLAVONOIDS  
AND THEIR GLYCOCONJUGATES  
IN THE MANAGEMENT OF TYPE 2 DIABETES**

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**DHANYA. R  
(Reg No: 4155)**

Under the Supervision of  
**Dr. P. Jayamurthy**

**Agroprocessing and Natural Products Division  
NATIONAL INSTITUTE FOR INTERDISCIPLINARY  
SCIENCE AND TECHNOLOGY**



**Council of Scientific and Industrial Research  
Thiruvananthapuram, Kerala, India – 695 019**

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*Dedicated to my family members & all my teachers*

## **Declaration**

I hereby declare that the work embodied in the thesis entitled “**Mechanistic role of citrus flavonoids and their glycoconjugates in the management of Type 2 diabetes**” is the result of the investigations carried out by me, at the Agroprocessing and Natural products Division, National Institute for Interdisciplinary Science and Technology (formerly Regional Research Laboratory), CSIR, Thiruvananthapuram, under the supervision of Dr. P Jayamurthy and the same has not been submitted elsewhere for any other degree. I certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee of the candidate have been incorporated in the thesis.

**Dhanya. R**

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## *Preface*

The incidence of diabetes is rapidly increasing and by 2030 an expected 592 million individuals are projected to be affected (*WHO report*). Hyperglycaemic condition is recognized as the causal link between diabetes and its complications. The chronic hyperglycemia resulting from diabetes brings about a rise in oxidative stress due to overproduction of reactive oxygen species (ROS) as a result of glucose auto oxidation and protein glycosylation. Generation of ROS leads to oxidative damage of the structural components (such as lipids, DNA and proteins) of cells and potentiate diabetes related complications. Oxidative insult in cells is also created by the impairment in functioning of endogenous antioxidant enzymes because of their non enzymatic glycosylation and oxidation. The prolonged exposure of oxidative stress may cause insulin resistance by triggering an alteration in cellular redox balance. Several lines of evidence suggest that oxidative stress occurs in diabetes and could have a role in the development of insulin resistance. The cause and cellular mechanism responsible for this abnormality is not fully understand despite of intense investigative efforts. However it is unknown whether it is the cause or consequence of diabetes. Despite strong experimental evidence indicating that oxidative stress may determine the onset and progression of late-diabetic complications, controversy exists between the cause and associative relationship between oxidative stress and diabetes mellitus.

Disruption of glucose homeostasis is a characteristic feature of Non-insulin dependent diabetes mellitus (NIDDM) and is associated with some complications including cardiovascular disease and renal failure. Glucose transport, the rate limiting step in glucose metabolism, can be activated in peripheral tissues by two distinct pathways. One stimulated by insulin through IRS-1/PI3K,

the other by muscle contraction/exercise through the activation of AMPK. Both pathways also increase the phosphorylation and activity of MAPK family components of which p38 MAPK participates in the full activation of GLUT4.

Insulin exerts its biological effect upon binding with the insulin receptor (IR) thereby activating the downstream signaling that lead to enhanced glucose uptake. In skeletal muscle, it potentiates glucose transport through PI3K mediated or non-PI3K mediated pathways. Alterations or defects in its signal transduction pathway was found in diabetic patients associated with decreased levels of IRb, IRS-1, and PI3K. In the insulin signaling, PI3K is a key molecule and inhibition of PI3K completely abolish insulin stimulated uptake. Akt or Pkb is an important downstream target of insulin stimulated glucose transport and metabolism.

Impairment in fuel metabolism occurs in obesity, and this impairment is a leading pathogenic factor in type 2 diabetes. The insulin resistance associated with type 2 diabetes is most profound at the level of skeletal muscle as this is the primary site of glucose and fatty acid utilization. Therefore, an understanding of how to activate AMPK in skeletal muscle would offer significant pharmacologic benefits in the treatment of type 2 diabetes. Metformin and the thiazolidinedione drugs exert the effects via activation of AMPK. Activation of AMPK occurs in response to exercise, an activity known to have significant benefit for type 2 diabetics. AMPK serves as sensor of energy status whose activity is triggered in response to changes in nutritional status in order to modulate tissue-specific metabolic pathways.

Oral hypoglycemic agents which target on increasing insulin levels, improving sensitivity to insulin in tissues, or that reduce the rate of carbohydrate absorption from the gastrointestinal tract

are used to manage Type 2 diabetes. But these therapies rarely target the real cause of Type 2 diabetes and are found to be responsible for severe adverse effects. Investigators are extensively exploring the efficacy of molecules from natural sources, such as, plants, to address this issue. Various positive leads from these studies suggest several mechanisms for the activity of active principles from plants against diabetes, such as, stimulation of insulin secretion, increase repair/proliferation of pancreatic  $\beta$  cells, thus enhancing the effect of insulin as well as increasing the antioxidant capabilities. To overcome the reported adverse effects of antidiabetic drugs and to suppress the oxidative stress mediated damage in diabetic pathophysiology, a special focus has been shifted towards naturally occurring antioxidants present in food. In the present study, we attempt to gain a better understanding of the therapeutic potential of flavonoids; Quercetin, naringenin, hesperetin & their glycoconjugates in the management of Type 2 Diabetes and other oxidative stress induced impairments by acute and chronic treatment of L6 cell lines followed by induction of oxidative stress by tertiary butyl hydrogen peroxide (TBHP).

Chapter 1 is a general introduction of diabetes, complications associated with diabetes, current treatment and future prospects.

Chapter 2 deals with the antioxidant potential of these flavonoids in skeletal muscle cell line model in which viability of L6 myoblast was measured by means of MTT assay. Cytotoxicity of TBHP and Flavonoids were standardized based on both concentration as well as period of incubation. Intracellular reactive oxygen species (ROS) was estimated by using the fluorescent probe, DCFH-DA as described earlier by Cathcart et al. To investigate the effect of flavonoids on oxidative stress associated with diabetes mellitus we induced stress in L6 skeletal muscle cells by

using TBHP. Pretreatment (acute & chronic) of all the flavonoids and their aglycones were found to reduce the oxidative stress generated within cells. GSH, an abundant and ubiquitous antioxidant, functions mainly as an efficient intracellular reducing agent, protect cells from damage caused by free radical, drugs and radiation. To investigate the effect of Flavonoids in natural antioxidant defence system of L6 cells, GSH levels were monitored after 3 h and 24 h pretreatment of flavonoids (1, 10, and 100  $\mu$ M). On treating with 100  $\mu$ M TBHP, GSH level reduced 66% compared to control. Decreased GSH on TBHP treatment may be due to increased utilization in protecting cells. Pretreatment of L6 myoblast with quercetin (10 & 100  $\mu$ M), rutin (100  $\mu$ M), naringin (100  $\mu$ M) and hesperidin (100  $\mu$ M) actively up regulated GSH level from 50% to 70% and the magnitude of action of these compounds remains the same on both 3 & 24h pretreatment. Intracellular ROS generation, on induction of oxidative stress, catalase activity in L6 myoblast reduced to 40% compared to untreated control (52.30%). Pretreatment of all the compounds nullified the effect of TBHP and even upregulated catalase activity in L6 myoblast.

Chapter 3 deals with the evaluation of antidiabetic potential of the flavonoids. Non-enzymatic glycosylation (glycation) between reducing sugar and protein results in the formation of advanced glycation endproducts (AGEs), which is believed to play important roles in pathogenesis of diabetic complications and elicit oxidative stress. Thus, agents that inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes. Antidiabetic potential of quercetin, naringenin, hesperetin & their glycoconjugates were analysed by 2-NBDG uptake with /without TBHP. GLUT4 upregulation was visualized to determine the end molecular mechanism, antiglycation and triglyceride levels were also monitored in L6 myotubes. All the flavonoids tested, inhibited glycation of proteins. The activity of quercetin,

rutin, hesperidin, hesperetin and naringenin at 10 & 100  $\mu\text{M}$  was much higher than that of positive control ascorbic acid ( $\text{IC}_{50}$  - 30  $\mu\text{M}$ ). Antidiabetic potential of Flavonoids were evaluated by monitoring 2-NBDG uptake in differentiated L6 myoblast. Evaluation of glucose uptake ability in cells plays a significant role in diabetes mellitus. Quercetin and rutin (1  $\mu\text{M}$ , 10  $\mu\text{M}$ , 100  $\mu\text{M}$ ) preincubation significantly increased glucose uptake ( $P \leq 0.05$ ) following acute as well as chronic pretreatment in the presence of oxidative stress induced by TBHP. This increase in glucose uptake by quercetin (100  $\mu\text{M}$ ) and rutin (100  $\mu\text{M}$ ) was remarkably greater than positive control, rosiglitazone for both 3h and 24h pretreatment. Flow cytometry analysis revealed 8%, 8.7% and 30.4% uptake of 2-NBDG in control, TBHP and rosiglitazone treated cells, respectively. TBHP has no significant effect on 2-NBDG uptake in the cells (8.7%). Pretreatment of quercetin (10  $\mu\text{M}$  & 100  $\mu\text{M}$ ) and rutin (100  $\mu\text{M}$ ), naringin (100  $\mu\text{M}$ ), naringenin (100  $\mu\text{M}$ ), hesperidin and hesperetin (100  $\mu\text{M}$ ), for 24 h enhanced the 2-NBDG uptake in L6 myotubes remarkably to 41.8%, 40.9%, 35.9%, 21.2%, 20.2% & 19.3% respectively which was comparable to that of positive control, Rosiglitazone (30.4%) an antidiabetic drug in the thiazolidinedione class of drugs. GLUT 4 levels were monitored by immunoassay with fluorescent labelled secondary antibody at 24 h pretreatment followed by induction of oxidative stress. Quercetin at 10  $\mu\text{M}$  was found to be most effective in up regulating GLUT4 translocation on pre-treatment compared to the positive control group treated with rosiglitazone. Rutin and naringin increased translocation of GLUT 4 that was at par with the positive control. Effect of hesperidin and hesperetin was comparable with positive control. TBHP exposure to cells did not induce any changes in GLUT 4 translocation. The fluorescent intensity was analysed by BD Image Data Explorer software. As these compounds stimulate glucose uptake to a greater magnitude than insulin, suggesting that it



may employ other routes to attain this effect. To investigate whether the compounds stimulated glucose uptake is mediated through PI3K activation, we examined the effects of wortmannin, a selective inhibitor of PI3K and dorsomorphin, an inhibitor of AMPK on flavonoids stimulated glucose uptake. Surprisingly, the effect of these compounds is wortmannin insensitive indicating that the insulin signaling pathway upstream of PI 3-kinase is not involved. Dorsomorphin treatment was marked by a remarkable decrease in glucose uptake especially by quercetin, rutin & naringin.

Chapter 4 explores the molecular mechanism behind the activity of the compounds. The insulin resistance associated with type 2 diabetes is most profound at the level of skeletal muscle as this is the primary site of glucose and fatty acid utilization. Metformin and the thiazolidinedione drugs exert the effects via activation of AMPK. Activation of AMPK occurs in response to exercise, an activity known to have significant benefit for type 2 diabetics. AMPK serves as sensor of energy status whose activity is triggered in response to changes in nutritional status in order to modulate tissue-specific metabolic pathways. Here we found that quercetin, rutin, naringin, naringenin, hesperidin and hesperetin stimulated glucose transport in L6 myotubes. Taking advantage of protein kinase inhibitors, we proved that the effect of these compounds was not through insulin signaling pathway, but through AMPK pathway and its downstream target p38 MAPK. An increase in the cellular AMP: ATP ratio on pretreatment may account for AMPK activation.

Summary and conclusion of all the results are presented in chapter 5.

# **CHAPTER 1**

## ***Introduction***

## **1. INTRODUCTION**

Recent estimates indicate that there are presently 415 million people in the world with diabetes and this is suspected to increase to 642 million by 2040 (International Diabetic Federation, 2015). Diabetes is a condition primarily defined by the level of hyperglycemia giving rise to a risk of microvascular damage (nephropathy, neuropathy and retinopathy) and increased risk of macrovascular complications (Ischemic heart disease, peripheral vascular disease and stroke). Evidence suggest that the average rate of incidence of diabetes in India has reached a value of 9% annually as a result of growing urbanization and changing lifestyle [Zielinska and Ignatowicz, 2008]. The major causes of diabetes includes either defective secretion of insulin (Type 1) due to auto-immune disorder/genetic defects/abnormal physiology or insulin resistance (Type 2) due to down-regulation of receptors (usually in adults due to obesity or other lifestyle factors) leading to elevated blood glucose levels and deleterious effects on multiple organs like kidney, heart, eyes or nerves [Devlin, 1997; Ranjan and Ramanujam, 2002; Kim et al., 2006].

### **1.1. Main events in the history of Diabetes**

Around 230 BC, Apollonius of Memphis used the term "Diabetes", which in Greek means "to pass through". He and his contemporaries considered diabetes as a disease of the kidneys and recommended ineffective treatments.

The first complete clinical description of diabetes appears to have been made by Aulus Cornelius (30 BC - 50 AD). Physicians of India at around the same time developed what can be described as the first clinical test for diabetes. They observed that urine from people with diabetes attracted ants and flies. They named the condition as "Madhumeha" or "honey urine". Indian physicians also noted that patients with " Madhumeha" suffered from extreme thirst and foul breath. Although the polyuria associated with diabetes was well recognized, ancient clinicians could not distinguish between the polyuria due to other condition.

The origin of current understanding of some aspects of diabetes can be traced to discoveries made in Europe between sixteenth and eighteenth centuries. Paracetus (1494-1541), a Swiss Physician observed a white residue on evapourating urine of diabetic patients. He incorrectly

thought that this residue consisted of salt and proceeded to attribute excessive thirst and urination in these patients to salt deposition in the kidneys. In 1670, Thomas Willis in Oxford noticed the sweet taste of urine of patients with Diabetes. Thomas Cawley, in 1788 observed that people with pancreatic injury developed diabetes and suggested the link between the pancreas and diabetes. In 1776, British physiologist Matthew Dobson (1713-1784) showed that the sweet tasting substance in the urine of patients with diabetes was sugar. He also noted the sweet taste of serum in these individuals and thus discovered hyperglycemia. Dobson put forward the theory that diabetes is a systemic disease. In 1815, Eugene Chevreul in Paris proved that sugar in Urine of individuals with diabetes was glucose. Thus, in 19<sup>th</sup> century, glycosuria became accepted criteria for diabetes. The islets of Langerhans were discovered in 1869 by an anatomist named Paul Langerhans. He identified the keys cells in the pancreas that produce the main substance that controls glucose levels in the body. Banting, Best and colleagues went on to purify the hormone insulin from bovine pancreas at the University of Toronto. This led to the effective treatment by insulin injections, and the first patient was treated in 1922, a diabetic teenager in a Toronto hospital named Leonard Thompson. He improved dramatically; his ketonuria and glycosuria were almost eliminated. His blood sugar levels dropped by 77%. A pharmaceutical firm named Eli Lilly and Company, with the University of Toronto, began the mass production of insulin and by the fall of 1923, 25,000 patients were being treated in Canada and United States. For this, Banting and laboratory director John MacLeod received the Nobel Prize in Physiology or Medicine in 1923. Both shared their prize money with others in the team who were not recognized, in particular, Best and Collip. Insulin production and therapy rapidly spread around the world. Insulin was one of the first proteins to be crystallized in pure form in 1926. Insulin became the first protein to be fully sequenced in 1955. That work resulted in a 1959 Nobel Prize for Frederick Sanger. In 1978, researchers from the City of Hope National Medical Center and the biotechnology company, Genentech, synthesized human insulin in the laboratory using a process that could produce large amounts. The important elements of current understanding of diabetes mellitus (DM) can be traced to the nineteenth century when modern scientific disciplines, including Biochemistry, Molecular biology and Experimental Physiology acquired prominence in biological studies.

## **1.2. Other landmark discoveries include:**

- Development of the long-acting insulin, NPH (1940's) by Novo-Nordisk
- Identification of the first sulfonylureas (1942)
- Use of biguanides for Type 2 diabetes (T2DM) reintroduced (1950's)
- Phenformin was withdrawn worldwide (1977) due the side effect, lactic acidosis
- In 1979, metformin was marketed in France
- Sir Frederick Sanger determined the amino acid sequence of insulin. Insulin was the first protein that the amino acid structure was determined
- Rosalyn Yalow and Solomon Berson discovered radioimmunoassay for insulin (gaining Yalow the 1977 Nobel Prize in Physiology or Medicine)
- Dr. Gerald Reaven's identified metabolic syndrome in 1988
- Thiazolidinedione, identified as an insulin sensitizer (1990)

## **1.3. Different types of Diabetes**

Sir Harold Percival (Harry) Himsworth made the first clear distinction between Type 1 and Type 2 diabetes and published in January 1936. Globally, as of 2010, an estimated 285 million people had diabetes, 90% of the cases was Type 2. Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however, expected to occur in Asia and Africa [Wild, 2000].

### **1.3.1. Type I diabetes mellitus (T1DM)**

T1DM is an autoimmune disease marked by the selective destruction of pancreatic beta cells. It is characterized by the slow and progressive destruction of islets and the failure of regeneration. Several features characterize T1DM as an auto immune disease [Narendran et al., 2005] such as the presence of immunocompetent and accessory cells in infiltrated pancreatic islets [Honeyman et al., 2001], presence of islet cell specific autoantibodies association of susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC; human leukocyte antigens HLA) [Ferber et al., 1999], alterations of T cell-mediated immunoregulation, the involvement of monokines and T-Helper cells producing interleukins in the disease process [Herold et al., 2002]. The mechanisms that cause the immune system to

mount a response to this small population of highly specialized cells have been intensively studied [Glandt and Herold, 2004].

#### 1.3.1.1. Contributing Factors

- Caused by the immune destruction of the beta cells of the pancreas
- Antibodies to islet cells and insulin are present at diagnosis
- Insulin secretion gradually diminishes
- May present at any age, but most common in childhood and adolescence
- Insulin by injection is necessary for survival
- Genetic predisposition
- Environmental triggers (infection or other stress)

#### 1.3.2. Type 2 diabetes mellitus (T2DM)

T2DM is caused by a combination of genetic factors related to impaired insulin secretion, insulin resistance and environmental factors such as obesity, overeating, lack of exercise, and stress, as well as aging as shown in Table 1.1. The development of T2DM is clearly associated with a family history of diabetes. The significantly higher concordance rate between monozygotic twins than between dizygotic twins suggests the considerable involvement of genetic factors. The pathogenesis has been assumed to involve a genetic abnormality in the molecules related to the regulatory system of glucose metabolism. The analyzes of candidate genes targeted at glucose-stimulated insulin secretion of pancreatic cells and the molecules comprising the molecular mechanism of insulin action have identified genetic abnormalities that can be independent causes of pathogenesis.

#### 1.3.2.1. Contributing Factors

- T2DM is caused by insulin resistance in the liver and skeletal muscle, increased glucose production in the liver, over production of free fatty acids by adipocytes and relative insulin deficiency. Insulin secretion decreases with gradual beta cell failure.
- Reductions in blood glucose levels often can be achieved with changes in food intake and physical activity patterns, oral medication and/or insulin injections are eventually required.

- Contributing factors include obesity, lack of physical activity, age (onset of puberty is associated with increased insulin resistance), Genetic predisposition, Racial/ethnic background (African American, Native American, Hispanic and Asian/Pacific Islander).

*Table 1.1. Comparison of T1DM and T2DM*

	<b>T1DM</b>	<b>T2DM</b>
Etiology	Auto immune Idiopathic	Genetically linked
Onset	Usually before age 30	Usually after age 30
Genetics	Associated with GLA DR3, DR4 & DQ alleles 40% Concordance in monozygotic twins	Greater heritability than Type 1 Non -HLA associated 80-100 % concordance in monozygotic twins
Pathophysiology	Completely insulin deficient	Abnormal insulin secretion Increased insulin resistance in target tissues, likely due to receptor and post receptor abnormalities Increased hepatic gluconeogenesis.
Risk Factors	History of autoimmune disease increases likelihood of developing DM	Obesity Family history Abnormal glucose tolerance Hypertension Hyperlipidemia Gestational Diabetes Mellitus
Population prevalence	Highest in Finland Rare in Asian, black, Aboriginal and Hispanic	Higher in black, Aboriginal and Hispanic people.
Pharmacological therapy	Insulin required	Combination of oral hypoglycemic agents & insulin therapy.
Circulating Islet cell antibodies	50 - 80%	<10%
Other aspects	Prone to ketoacidosis	Not prone to ketoacidosis but prone to hyperosmolar coma.

### **1.3.3. Other types of Diabetes**

#### 1.3.3.1. Prediabetes

This new term is the name for an intermediate state of glucose intolerance, where blood glucose level is higher than normal yet below the threshold for a diagnosis of diabetes (100-125mg/dl). People who have prediabetes have a high risk of developing diabetes in the future. Depending on how a person's blood glucose level is measured, prediabetes is sometimes called impaired glucose tolerance or impaired fasting glucose.

#### 1.3.3.2. Syndrome X

Also known as insulin resistance syndrome or metabolic syndrome, this condition is associated with an increased risk of cardiovascular disease and diabetes. It consists of a group of symptoms, including insulin resistance, increased blood insulin levels, glucose intolerance, obesity, high blood pressure, and dyslipidemia, specifically low high density lipoprotein ("good") cholesterol levels and high triglyceride levels. Diagnosis generally requires the presence of three of these symptoms or evidence of insulin resistance and two additional symptoms.

#### 1.3.3.3. Gestational diabetes

High blood glucose that occurs during pregnancy is called gestational diabetes. In most cases, blood glucose levels return to normal after delivery, but women who have had gestational diabetes are more likely to develop diabetes later in life.

#### 1.3.3.4. MODY (Maturity Onset Diabetes of Youth)

Maturity-onset diabetes of the young (MODY) is a genetically and clinically heterogeneous subtype of T2DM characterized by an early onset, an autosomal dominant inheritance, and a primary defect in insulin secretion. MODY comprises 2-5% of cases of T2DM. So far, six MODY genes have been identified (MODY1-6): hepatocyte nuclear factor (HNF-4 $\alpha$ ), glucokinase, HNF-1  $\alpha$ , HNF-1 $\beta$ , insulin promoter factor 1 (IPF-1), and neurogenic differentiation factor 1 (NEUROD1). MODY2 and MODY3 are the most common forms of MODY. Mutations in glucokinase/MODY2 result in a mild form of diabetes. In contrast, MODY3 and some of the other MODY forms are characterized by major insulin secretory defects and severe hyperglycemia associated with microvascular complications. About 25% of known MODY is caused by mutations in yet unknown genes and present results suggest that other monogenic forms of T2DM might exist. The diagnosis of MODY has implications for the clinical management of the patient's diabetes.



#### 1.3.3.5. Drug-induced diabetes

Several drugs and chemicals can cause glucose intolerance and diabetes. These include nicotinic acid (used to lower high triglycerides and raise HDL cholesterol), glucocorticoids (such as prednisone), thyroid hormone, thiazides (a type of blood pressure medicines), dilantin (an antiseizure drug), and beta adrenergic agonists (used to treat asthma).

#### 1.3.3.6. Genetic diseases

Several genetic diseases are associated with increased rates of diabetes, including Down's syndrome. There also specific genetic defects in beta-cell function and insulin action that result in diabetes.

#### 1.3.3.7. Infections and other diseases

Examples of diseases and disorders that can damage the pancreas and lead to diabetes include pancreatitis (inflammation of the pancreas), cystic fibrosis, and hemochromatosis (a genetic condition in which iron accumulates in the body). In addition, diseases that disturb levels of hormones such as growth hormone, thyroid hormone, or glucagon can cause insulin resistance or increased glucose production by the liver and potentially diabetes.

### **1.4. Pathways involved in glucose transport**

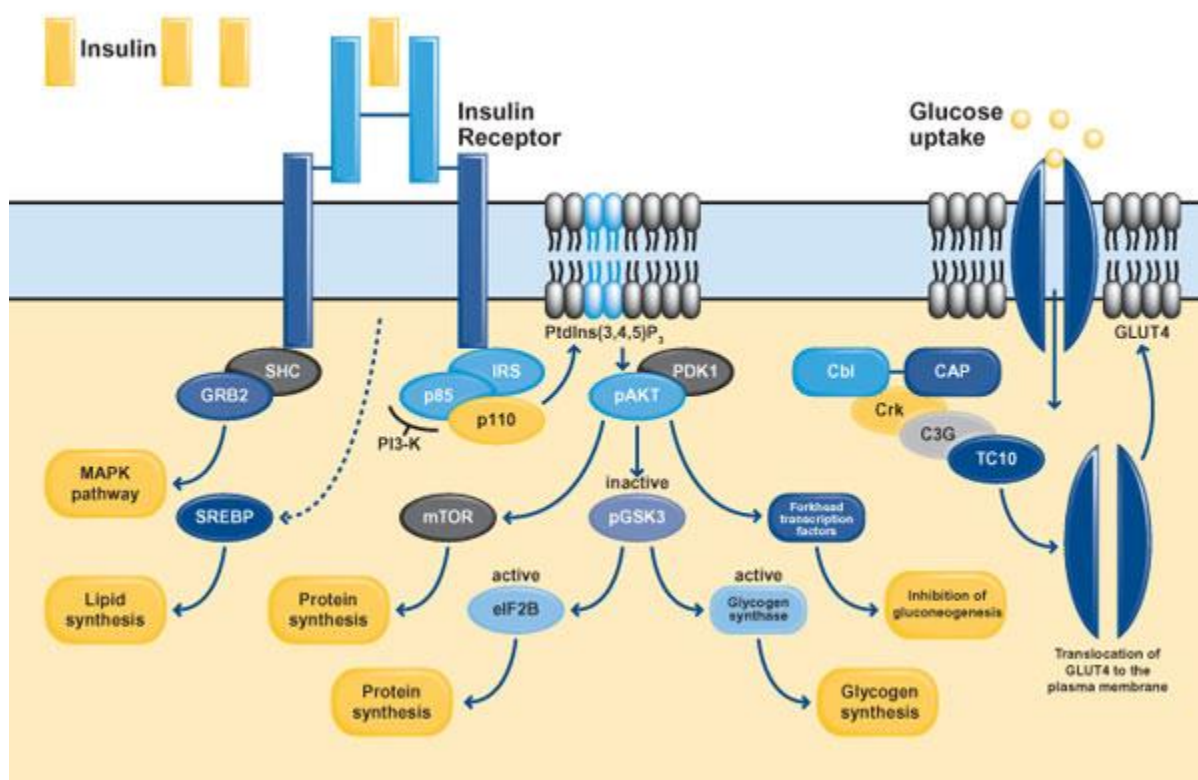
A major metabolic defect associated with T2DM is the failure of peripheral tissues in the body to properly utilize glucose, thereby resulting in chronic hyperglycemia. Glucose transport into most tissues is achieved by the action of molecules called glucose transporters. These molecules transport glucose by facilitative diffusion down concentration gradients, in contrast to energy-dependent uptake of glucose in the gut or kidney. Although glucose movement can be bidirectional across the cell membrane, in general and particularly in metabolically active insulin-sensitive tissues, glucose transport proceeds from the exterior to the interior of the cells.

#### **1.4.1. Insulin signaling pathway**

Maintenance of energy homeostasis during changing demands and availability depends on the concerted action of multiple organs and tissues, including the digestive system, pancreas, brain, liver, muscle and adipose tissue. Together, these tissues sense energy and communicate fuel availability to other organs through the release of metabolites and hormones. Skeletal muscle has a fundamentally important role in the maintenance of normal glucose homeostasis and in

regulating whole body carbohydrate metabolism. Skeletal muscle glucose transport is acutely regulated through the coordinated activation of a series of intracellular proteins. The insulin receptor consists of two extracellular alpha subunits and two transmembrane beta subunits linked together by disulfide bonds (Fig.1.1). Binding of insulin to the alpha subunit induces a conformational change resulting in the autophosphorylation of tyrosine residues present in the beta subunit [Van et al., 2001]. These residues activate phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS) [Saltiel and Kahn, 2001; Lizcano and Alessi, 2002] and the phosphorylated IRS-1 will then dock with SH2 domains of the p85 regulatory subunit of phosphatidylinositol-3' kinase (PI3K), thereby activating the p110 catalytic subunit of this enzyme. Phosphoinositide moieties produced by PI3K can subsequently activate phosphatide dependent kinase (PDK). Activation of AKT also requires the protein kinase 3 phosphoinositide-dependent protein kinase-1 (PDK1), which in combination with unidentified kinase leads to the phosphorylation of AKT (protein kinase B (PKB)), which is recruited to the plasma membrane. Once activated, AKT enters the cytoplasm where it leads to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3) (Fig. 1.1). A substrate of GSK3 is glycogen synthase, an enzyme that catalyzes the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore the inactivation of GSK3 by AKT promotes glucose storage as glycogen. A key action of insulin is to induce glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, from intracellular storage to the plasma membrane. PI3K and AKT are known to play a role in GLUT4 translocation [Lizcano and Alessi, 2002]. PI3K independent pathways may also cause recruitment of GLUT4 to the plasma membrane [Saltiel and Kahn, 2001]. In this pathway, insulin receptor activation leads to the phosphorylation of Cbl, which is associated with the adaptor protein CAP. Following phosphorylation, the Cbl-CAP complex translocates to lipid rafts in the plasma membrane. Cbl then interacts with the adaptor protein Crk, which is constitutively associated with the Rho-family guanine nucleotide exchange factor, C3G. C3G in turn activates members of the GTP-binding protein family, TC10, which promote GLUT4 translocation to the plasma membrane through the activation of as yet unknown adaptor molecules. In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis [Saltiel and Kahn, 2001]. Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation

and dephosphorylation events and also regulates the expression of genes encoding hepatic enzymes involved in gluconeogenesis. Recent evidence suggests that transcription factors, which are excluded from the nucleus following phosphorylation by AKT, play a role in hepatic enzyme regulation by insulin [Schmoll et al., 2000; Barthel et al., 2001].



**Fig.1.1. Scheme of insulin action in glucose uptake.** The insulin receptor is a tyrosine kinase that undergoes autophosphorylation and catalyses the phosphorylation of cellular proteins such as members of the IRS family, Shc and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2 domains, resulting in a diverse series of signaling pathways, including activation of PI(3)K and downstream PtdIns(3,4,5)P<sub>3</sub>-dependent protein kinases, ras and the MAP kinase cascade, and Cbl/CAP and the activation of TC10. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, which results in the regulation of glucose, lipid and protein metabolism. [C. Hooper. Insulin signaling pathways. [http://www.abcam.com/ps/CMS/Images/Insulin\\_Pathway\\_600x480.jpg](http://www.abcam.com/ps/CMS/Images/Insulin_Pathway_600x480.jpg)]

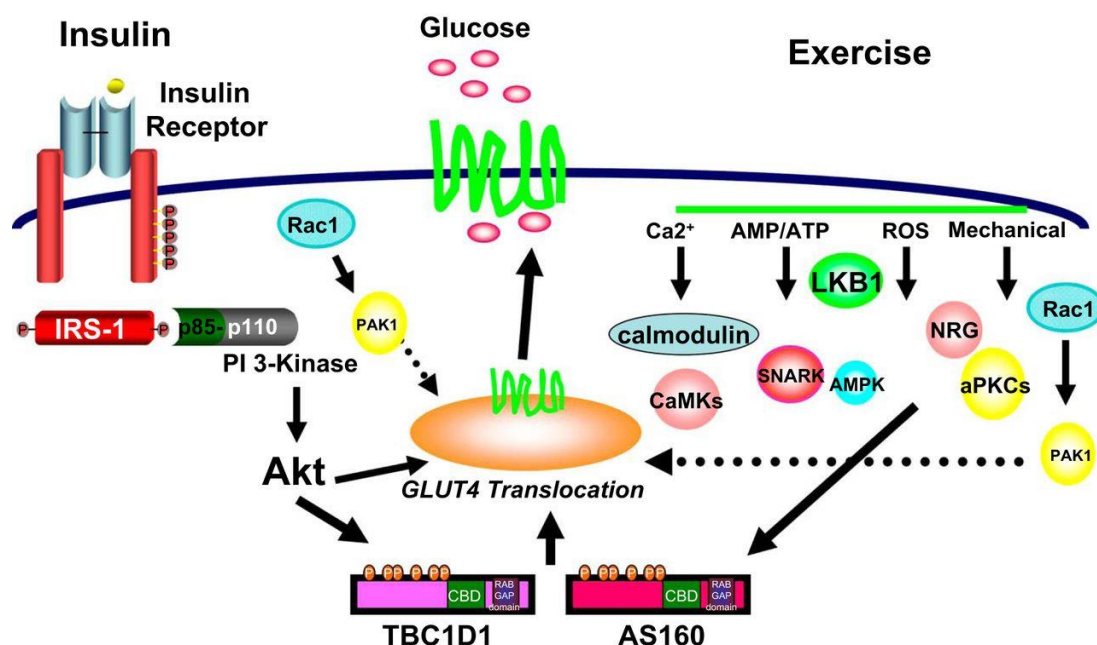
### 1.4.2. AMPK pathway

Involvement of PI3K independent pathways in insulin stimulated GLUT4 translocation has been established [Fryer et al., 2002]. Adenosine monophosphate activated protein kinase (AMPK) is an enzyme that plays a role in cellular energy homeostasis. AMPK, a heterotrimeric protein consisting of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ) [Stapleton et al., 1994;

Woods et al., 1996a]. Isoforms of the three subunits have been identified, including two isoforms of the catalytic subunit,  $\alpha 1$  and  $\alpha 2$  [Woods et al., 1996b]. Earlier studies have shown that AMPK is activated following depletion of cellular ATP together with a concomitant rise in AMP [Corton et al., 1994; Hardie et al., 1999]. AMPK is an important therapeutic target for regulating whole body energy balance. AMPK gets activated when cellular energy is depleted [Corton et al., 1994]. Phosphorylation at Thr 172 of the catalytic subunit of AMPK accelerates ATP generating catabolic pathways, including glycolysis and fatty acid oxidation [Winder and Hardie 1996; Hutber et al., 1997; Vavvas et al., 1997], while simultaneously reducing ATP-consuming anabolic pathways (cholesterol, fatty acid, and triacylglycerol synthesis) [Henin et al., 1995; Makinde et al., 1997]. In addition to its roles in energy homeostasis, activation of AMPK through physiological stimulation, such as muscle contraction or by the pharmacologic activator like 5-aminoimidazole-4-carboxy-amide-1-D-ribofuranoside (AICAR), leads to a significant increase in glucose uptake, which is mediated by translocation of GLUT4 [Ali et al., 2002; Hayashi et al., 1998; Mu et al., 2001]. GLUT4 is highly expressed in skeletal muscle and adipose tissue. GLUT4, the main mediator of glucose removal from the circulation, function as a key regulator of whole body glucose homeostasis [Huang and Czech, 2007]. It has been shown that many compounds with beneficial activity to metabolic syndrome are known to activate AMPK pathway independent of PI3K [Huang et al., 2010]. AMPK is activated by a decrease in phosphocreatine [Ponticos et al., 1998] and an increase in the AMP to ATP ratio through a complex mechanism involving allosteric regulation of AMPK and phosphorylation of AMPK by the upstream kinase AMPK kinase. AMPK is activated during exercise, electrical stimulation producing *in situ* muscle contractions [Stumoll et al., 1995; Hundal et al., 1992; Hundal et al., 2000], and contraction of isolated muscles *in vitro* [Bergeron et al., 1999] as shown in Fig 1.2.

Zhou et al., [2001] demonstrated the activation of AMPK by metformin in hepatocytes and skeletal muscle. Metformin, the most widely used oral drugs for the treatment of T2DM, decreases hyperglycemia and has beneficial effects on circulating lipids, without affecting insulin secretion [Cusi and DeFronzo, 1998; UKPDS group.1998]. The hypoglycemic effect of metformin are attributable to increase in muscle glucose uptake [Inzucchi et al., 1998] and decrease in hepatic glucose production [Hundal et al., 2000]. Activation of AMPK was found to be involved in the decline of glucose production, an increase in fatty acid oxidation in hepatocytes and glucose uptake in skeletal muscle [Hardie et al., 1998]. Skeletal muscle tissue

accounts for the majority, 80%, of insulin-mediated glucose uptake in the post-prandial state and plays an important role in maintaining glucose homeostasis. Therefore investigation of novel compounds that activate AMPK and increase skeletal muscle glucose uptake is of major importance as it may pave way towards the development of new therapeutic targets of insulin resistance and T2DM.



**Fig 1.2. Exercise and insulin regulation of glucose transport**

A proposed model for the signaling pathways mediating exercise and insulin induced skeletal muscle glucose transport is shown. IRS-1, Insulin receptor substrate-1; PAK, P21 protein activated kinase 1; LKB1, Liver kinase B1; PI3K, Phosphatidylinositol 3-kinase; CaMK, Ca<sup>2+</sup>/calmodulin dependent protein kinase; SNARK, sucrose non fermenting, AMP-dependent protein kinase (AMPK) – related kinase; NRG, Neuroglian; aPKCs; GLUT, Glucose transporter; TBC1D1, Tre-2/USP6, BUB2, cdc16 domain family member 1 AS160, Akt substrate of 16 kDa; CBD, Calmodulin-binding domain [Rock KS, Witczak CA, Goodyear LJ. Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. [Rockl et al., 2008]

### 1.4.3. GLUT4, a key determinant of glucose homeostasis

The transport of monosaccharides, polyols, and other small carbon compounds across the membranes of eukaryotic cells is mediated by members of the GLUT family of integral membrane proteins that are encoded by the SLC2 genes and are members of the major facilitator superfamily (MFS) [Augustin, 2010]. Glucose transporters (GLUT) are known to play pivotal roles in energy metabolism. At least six different eukaryotic GLUT isoforms have been

identified. They possess 12 transmembrane helical segments with the N- and C-termini and a large central loop exposed to the cytoplasm. GLUT transporters have distinct antigenic C-terminal regions, and are subject to different metabolic and developmental regulations. They are responsible for the facilitative uptake of D-glucose and its analogues. GLUT 1 is most abundant in erythrocytes and brain microvessels, and is present in most tissues and cell cultures. GLUT 2 is expressed mainly in pancreatic  $\beta$  cells, liver, and enterocytes basolateral membranes. GLUT 3 is found in brain neurons, foetal muscle, skeletal and cardiac myoblasts. GLUT 4 is present in insulin-sensitive tissues, such as brown and white adipose tissues, cardiac and skeletal muscles. GLUT 5 is actually a fructose transporter detected in human enterocyte luminal membranes, adipocytes, skeletal muscle and sperm. GLUT 7 is located in liver microsomes. GLUT 4 is one of the 13 sugar transporter proteins (GLUT 1 to GLUT 12, and HMIT) encoded in the human genome [Hruz and Mueckler, 2001] that catalyzes hexose transport across cell membranes through an ATP independent, facilitative diffusion mechanism. These sugar transporters display differences in their kinetics and substrate specificities, such that GLUT 5 and GLUT 11 are likely fructose transporters. GLUT 4 is extensively expressed in skeletal muscle and adipose tissue. In the case of skeletal muscle, GLUT 1, GLUT 5, and GLUT 12 may significantly contribute to glucose uptake [Stuart et al., 2000, 2006] while GLUT 8, GLUT 12, and HMIT are expressed in adipose tissue [Wood et al., 2003; Wood and Trayhurn, 2003] as shown in Table 1.2. GLUT 4 displays the unique characteristic of an intracellular disposition in the unstimulated state and gets redistributed to the plasma membrane in response to a stimulus [Bryant et al., 2004; Czech and Corvera, 1999]. GLUT 4 contains unique sequences in its N- and COOH-terminal cytoplasmic domains that direct its characteristic membrane trafficking capability. These include a distinctive N-terminal sequence with a potentially critical phenylalanine residue [Piper et al., 1993; Araki et al., 1996; Melvin et al., 1999; Al-Hasani et al., 2002], as well as dileucine and acidic motifs in the COOH terminus [Corvera et al., 1994; Garippa et al., 1996; Shewan et al., 2000; Sandoval et al., 2000; Martinez-Arca et al., 2000]. These motifs likely govern kinetic aspects of both endocytosis and exocytosis in a continuously recycling trafficking system. Changes in GLUT 4 protein levels could translate into alterations in glucose tolerance if the effects observed in transgenic mice apply to normal physiology. Thus, therapeutic strategies based on enhancing GLUT 4 expression may facilitate drug discovery.



Table 1.2: Classification of GLUT transporter

Transporter	Mechanism	Glucose Km	Cellular location	Tissues	Characteristics
GLUT 1	Passive	20 mM	-	Brain, red cells, endothelium, $\beta$ cells	Mediates glucose transport into red cells, and throughout the blood brain barrier. It is ubiquitously expressed and transports glucose in most cells.
GLUT 2	Passive	42 mM	Mobile	Kidney, ileum, liver, pancreatic $\beta$ cells	Provides glucose to the liver and pancreatic cells.
GLUT 3	Passive	10 mM	Apical	Neurones, placenta (trophoectoder)	The main glucose transporter in neurons.
GLUT 4	Passive	2-10mM	-	Skeletal muscle, heart, adipocytes	Primarily expressed in muscle and adipose tissue and regulated by insulin
GLUT 5	Passive	-	Both	Widely distributed	Transports fructose in intestine and testis.
SGLT 1	Sodium dependent	High affinity	Apical	Small intestine, kidney tubules	Contribute to renal glucose absorption.
SGLT 2	Sodium dependent	Low affinity	Apical	Kidney proximal tubule	Majorco-transporter involved in renal glucose reabsorption.

Upstream signaling to GLUT 4 from either the insulin receptor or as the result of muscle contraction appears to be mediated by at least 2 distinct pathways in adipocytes and skeletal muscle. These pathways have been extensively reviewed but are still far from being completely understood [Watson et al., 2004; Rose and Richter, 2005; Thong et al., 2005; Herman and Kahn, 2006]. In adipocytes and skeletal muscle, insulin binding to its receptor results in the dimerization and trans-phosphorylation of the receptor beta subunits, causing the activation of intrinsic tyrosine kinase activity leading to the recruitment and tyrosine phosphorylation of insulin receptor substrate-2 (IRS-2). The tyrosine phosphorylated SH2 domain of IRS-2 then binds to and activates PI3K, leading to the production of phosphatidylinositol 3,4,5 triphosphate (PIP3) in the membrane. Increased PIP3 levels recruit two distinct serine/threonine kinases, PDK1 and mitochondrial target of rapamycin C2 (mTORC2) to the membrane, which then activate the pivotal protein kinase AKT via phosphorylations within its catalytic and hydrophobic domains. AKT exists as a central hub in the regulation of cellular growth, survival,

and fuel metabolism and has several known direct substrates. There are three AKT isoforms and AKT2 appears to mediate, at least in part, the effect of insulin on GLUT 4 translocation in adipocytes via the phosphorylation of AS160 (TBC1D4) [Kane et al., 2002; Eguez et al., 2005].

## **1.5. Etiology of T2DM**

T2DM is a multifactorial disease with genetic and environmental factors playing a key role in its pathogenesis. Central to the etiology is a defect in insulin action, hepatic glucose output, and insulin secretion. Although insulin resistance is frequently the first detectable abnormality in the progression of T2DM, insulin resistance by itself does not cause the disease, which is only manifested when there is a coexisting insulin secretory defect. T2DM typically occurs in middle-aged and elderly people but there is an increasing trend of T2DM occurring in young individuals. The main question yet to be answered is whether T2DM is a group of disorders with hyperglycemia as the end point in the disease pathogenesis. Insulin resistance is common to several other disorders, including ischemic heart disease, hypertension, dyslipidemia, central obesity, and coagulation defects; the clustering of these disorders is known as the metabolic syndrome or the insulin resistance syndrome.

### **1.5.1. Insulin resistance**

One of the features in the etiology of T2DM is the development of peripheral insulin resistance or decreased insulin sensitivity in the insulin responsive tissues. Insulin resistance *in vivo* is caused by abnormalities in the insulin signaling pathway. This pathway is activated by binding of insulin to the cell membrane bound insulin receptor (IR) and activation of intrinsic tyrosine kinase of IR then phosphorylates the  $\beta$ -subunit of IR which stimulates insulin resistance in the insulin signaling cascade through insulin receptor substrate protein-1 (IRS1), PI3K, and AKT, leading to translocation of the glucose transporter (GLUT 4) to the cell membrane and uptake of glucose [Watson et al., 2004]. Abnormalities in this pathway can define insulin resistance. Muscle from subjects with T2DM, or from rodent models of diabetes, commonly show reduced insulin stimulated insulin receptor, IRS1 phosphorylation and PI3K activity [Khan et al., 2002]. A defect in IRS protein function may play a role in the development of insulin resistance. Reduced insulin stimulated IRS1 tyrosine phosphorylation is found in skeletal muscle from T2DM patients, although overall IRS1 expression is unchanged [Bjornholm et al., 1997]



Serine/thr phosphorylation of IRS proteins is closely associated with reduction of signaling. Two potential mechanisms may underlie this phenomenon. First, serine phosphorylation may block the interaction of IRS1 with its target proteins [Paz et al., 1997]. Second, proteasomal mediated degradation of IRS1 may be increased [Pederson et al., 2001]. Obesity is known to be a cause of development of insulin resistance. However, not all obese subjects develop insulin resistance or T2DM, suggesting that the mechanism linking obesity with insulin resistance must be capable of being controlled.

Increased oxidative stress appears to be a deleterious factor leading to insulin resistance,  $\beta$ -cell dysfunction, impaired glucose tolerance, and, ultimately, T2DM [Evans, 2003; Shah, 2007]. Studies have suggested that oxidative stress play a role in systemic inflammation, endothelial dysfunction, and impaired secretion of insulin and glucose utilization in peripheral tissues [Zatalia and Sanusi, 2013]. It has also been shown that neutralization of reactive molecules can significantly inhibit the development of endothelial dysfunction, cardiomyopathy, retinopathy, nephropathy, and neuropathy in patients with DM [Atli et al., 2004; Osawa and Kato, 2005]. Use of transgenic antioxidant enzyme expression or combinations of antioxidant compounds for the inhibition of ROS production induced by hyperglycemia in diabetic mice prevents the development of experimental diabetic complications like retinopathy, nephropathy, neuropathy, and cardiomyopathy. Although several therapies exist for treatment, there are certain limitations due to the high cost and side effects such as the hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity, etc. Based on recent advances and involvement of oxidative stress in complicating diabetes mellitus, efforts are on to find suitable antidiabetic and antioxidant therapy.

### **1.6. Hyperglycemia and stress activated pathways**

In tissues that are independent of insulin for glucose uptake (retina, lens, nerve and endothelium), exposure to elevated glucose level causes an increase in intracellular sorbitol and fructose due to the raised aldose reductase and sorbitol dehydrogenase activity. The depletion of NADPH cell stores by aldose reductase may inhibit the activity of NADPH requiring enzymes including nitric oxide (NO) synthase and glutathione (GSH) reductase. Decreased levels of NO can lead to ROS, polyol pathway, AGEs (Advanced glycated end products), glycated proteins, glucose autooxidation, vasoconstriction and tissue injury, while reduced levels of GSH increase

the susceptibility of endothelial to damage by  $H_2O_2$  [Condell and Tappe, 1983]. More recently, hyperglycemia and ROS has been implicated in the activation of additional biochemical pathways (Fig.1.3) that appear to promote the development of the late complications of diabetes, along with exerting a negative influence on insulin action and insulin secretion. These pathways include hexosamine pathway and stress activated signaling pathways including NF- $\kappa$ B, JNK/SAPK and p38 MAP kinase pathways. The consequence is the production of an array of gene products that, in turn, cause cellular damage and are ultimately responsible for the late complications of diabetes. Furthermore, these pathways are linked to insulin resistance and decreased insulin secretion. ROS and oxidative stress induced by elevation in glucose and possibly free fatty acids (FFA) levels play a key role in causing insulin resistance and  $\beta$ -cell dysfunction by their ability to activate stress sensitive signaling pathways as shown in Fig. 1.3.

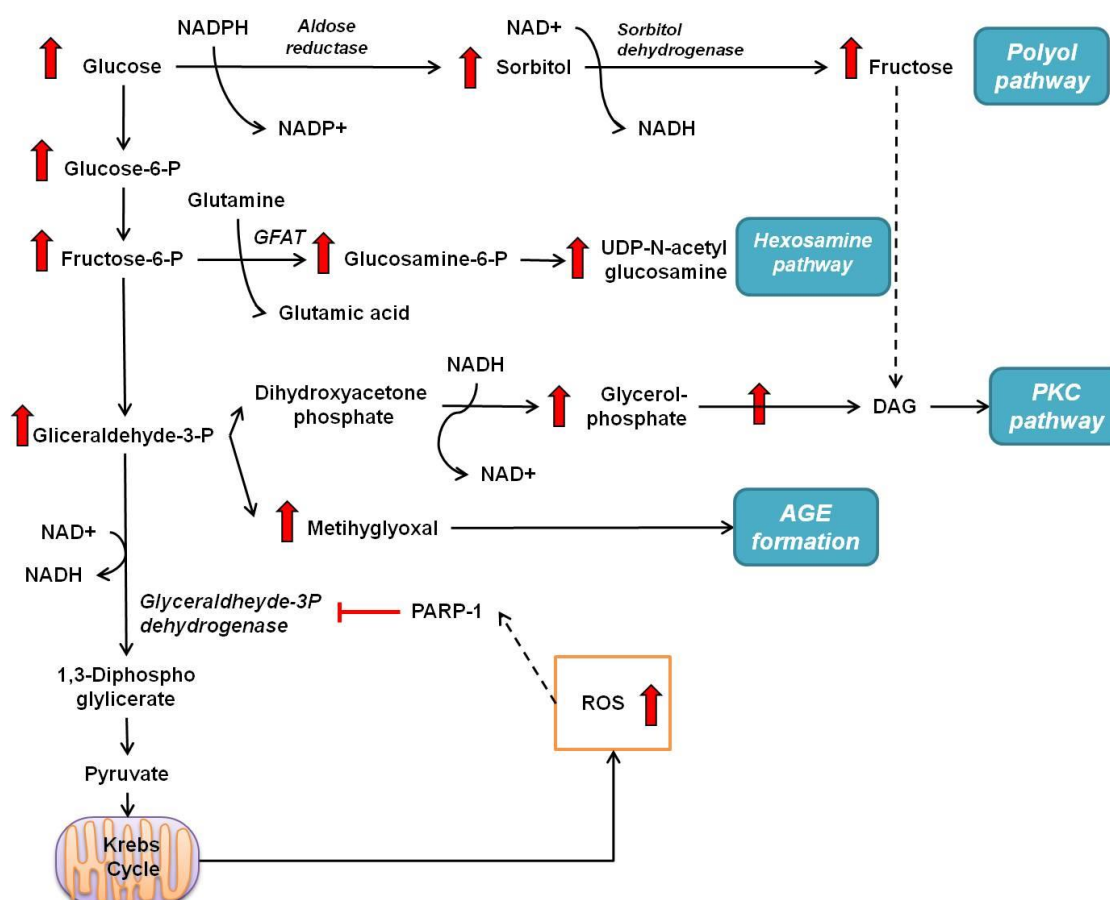


Fig 1.3. Stress sensitive pathways in diabetes mellitus [Maria and Christina 2013]

### **1.6.1. AGEs/Receptors of AGE (RAGE) Pathways**

AGEs formation is enhanced in the presence of hyperglycemia and oxidative stress. AGEs bind to their cognate cell surface receptor, RAGE, resulting in the activation of post receptor signaling, generation of intracellular oxygen free radicals and the activation of gene expression. Retinal expression of VEGF, a mediator of the late complications of diabetes is increased by AGEs-RAGE interaction. Thus, AGEs are not only markers, but also as mediators of late diabetic complications and chronic vascular diseases [Halliwell, 2009]. The AGE precursors diffuse out of the cell modify circulating proteins in the blood, such as albumin. These modified circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology. Studies suggest that accumulation of AGEs on the surface of long lived cells could be responsible in part for the normal turnover of senescent erythrocytes and other cells [Iwata et al., 2004].

### **1.6.2. PKC Pathways**

The concentration of diacylglycerol, an allosteric activator of PKC, is increased in tissues of diabetic complications. As a consequence of the increase in diacylglycerol, several isoforms of PKC are activated. PKC-  $\beta$  is the major isoform that is induced in the vasculature, kidney and retina. Increased PKC activity arises from chronic hyperglycemia and is associated with many processes involved in the pathology of diabetic complications including vascular permeability, blood flow and neovascularization. PKC also synergizes with other kinase pathways [Geraldes and George, 2010].

### **1.6.3. Polyol Pathway**

When intracellular glucose rises, aldose reductase activity is stimulated and catalyses the formation of sorbitol, which can be oxidized to fructose by sorbitol dehydrogenase. Sorbitol accumulates intracellularly, causing cell damage. Stress sensitive signaling pathways including p38 MAPK and JNK are strongly activated by sorbitol. The significance of the activation of the polyol pathway as a cause of diabetic complications has been demonstrated in transgenic mice that overexpression of the aldose reductase gene and by the observations that inhibitors of this enzyme prevent the development of neuropathy, nephropathy, retinopathy and cataract formation

in these animals [Suzuki et al., 1999]. In the process of reducing high intracellular glucose to sorbitol, the aldose reductase consumes the cofactor NADPH and it is an essential cofactor required for the regeneration of reduced glutathione. By reducing the amount of reduced GSH, the polyol pathway increases the susceptibility to intracellular oxidative stress [Horie et al., 1997].

#### **1.6.4. Hexosamine Pathway (HMP)**

Excessive flux of glucose or FFA into a variety of cell types results in the activations of the hexosamine biosynthetic pathway. Over expression of glutamine, fructose-6-phosphate amido transferase (GFAT), the rate limiting enzyme of hexosamine biosynthesis leads to insulin resistance [Brownlee, 2005]. The HMP also functions as a cellular sensor of energy availability and mediates the effects of glucose on the expression of several gene products including leptin. Several products of this important enzyme are able to activate growth and stress sensitive kinases and signaling pathways linked to increased vascular and renal disease including PKC, vascular smooth muscle cell hypertrophy, increased matrix production, and oncogene activation. In addition, the superoxide anion can interact with NO, forming toxic free radicals called peroxynitrites. These reactive nitrogen species impairs the ability of NO to maintain vascular tone and could promote or accelerate the atherosclerotic process. Growth factors appear to play an important role in DM related complications, and their production is increased by most of these proposed pathways. A possible unifying mechanism is that hyperglycemia leads to increased production of reactive oxygen species or superoxide in the mitochondria; these compounds may activate all four of the pathways described earlier. Although hyperglycemia serves as the initial trigger for complications of diabetes, it is still unknown whether the same pathophysiologic processes are operative in all complications or whether some pathways predominate in certain organs. The studies also showed that decreased oxidative capacity and mitochondrial aberrations as a major contributor to the development of insulin resistance and T2DM. Hyperglycemia induced mitochondrial superoxide generation may play an important role in platelet dysfunction observed in patients with diabetes. Superoxide overproduction may thus potentially play an important role in activation of these inflammatory cells as well as platelets, which would also participate in the pathogenesis of accelerated atherosclerosis in diabetes [Etoh et al., 2003]. Excess glucose leads to an oversupply of electrons in the mitochondrial transfer chain that result

in mitochondrial membrane hyperpolarization and the formation of ROS. Recent investigations have demonstrated that uncoupler proteins can prevent mitochondrial ROS formation [Vidal et al., 2000].

### **1.6.5. Glycated hemoglobin (HbA1c)**

HbA1c increase in diabetes was first described in 1969 by Samuel Rahbar and coworkers [Rabbar et al., 1969]. The reactions leading to its formation were characterized by Bunn and his coworkers in 1975 [Bunn et al., 1975]. The use of HbA1c for monitoring the degree of control of glucose metabolism in diabetic patients was proposed in 1976 by Anthony Cerami, Ronald Koenig and coworkers. Of these species, HbA1c, in which glucose is linked to N- terminal valine residues of  $\beta$  chains, is of most important as its formation is increased in diabetic patients with ambient hyperglycemia and is used to monitor clinically for long term control of blood sugar. Data suggests that increased oxidative stress in diabetes mellitus may be due to increased formation of HbA1c [Giugliano, 1996]. Nonenzymatic glycation of hemoglobin has been shown to provide a useful index for measurement of patients with diabetes. The ultimate result of the nonenzymatic glycation and oxidation of proteins is formation of AGEs, whose presence in plasma and tissue has been linked to the development of complications in diabetes. Measurement of HbA1c or other indicators of mild glucose abnormalities in clinical practice may be valuable in identifying patients with an increased risk of subclinical cardiovascular disease [American Diabetes Association, 2005]. Selvaraj et al., [2006] reported that lipid peroxides may have a role to play in glycation of hemoglobin and antioxidants (lipoic acid and taurine) can partially inhibit the formation of glycated hemoglobin by lowering the levels of lipid peroxides. Glycooxidation products affect protein function and activate pro-inflammatory signaling pathways, suggesting that they might play a central role in diabetes complications [Brownlee et al., 1988; Baynes, 1991; Monnier et al., 1991; Brownlee, 2001].

### **1.7. Reactive oxygen species (ROS) production in diabetes mellitus**

ROS are continuously generated in physiological conditions and effectively eliminated of several intracellular and extracellular antioxidant systems [Scott and King, 2004]. When the endogenous antioxidant network fails to provide a sufficient compensatory response to restore cellular redox balance, oxidative stress ensues [Scott and King, 2004]. The most important ROS are the

superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), alkoxyl ( $RO^\cdot$ ), peroxy ( $ROO^\cdot$ ), hydroxyl radicals ( $OH^\cdot$ ) and hypochlorous acid ( $HOCl$ ). Other nonoxygen species existing as reactive nitrogen species such as nitric oxide (NO) and peroxynitrite have also important bioactivity. Hyperglycemia may lead to an increased generation of free radicals via multiple mechanisms. Although the sources of this oxidative stress remain unclear, it has been suggested that the chronic hyperglycemia in diabetes enhances the production of ROS from glucose autooxidation, protein glycation and glycooxidation which leads to tissue damage. Also, cumulative episodes of acute hyperglycemia can be source of acute oxidative stress [West, 2000; Evans et al., 2002]. The magnitude and duration of target tissue exposure to abnormally high levels of blood glucose correlate closely with the extent and rate of progression of complications including nephropathy, neuropathy, retinopathy and macro and microvascular damage [Liuji et al., 2002; Droge, 2002; Babu et al., 2003]. Aldose reductase activation, protein kinase C and AGE in some subtle combination may be responsible for the microvascular complications of diabetes (Fig.1.2) [Kilpatrick et al., 2006]. According to Tiwari [2004], the increased free radical activity has been suggested to play an important role in the lipid peroxidation and protein oxidation of cellular structures causing cell injury and is implicated in the pathogenesis of vascular disease in T1DM and T2DM [Vincent et al., 2003].

### **1.7.1. Biomarkers of oxidative stress associated with T2DM**

Biomarkers are compounds whose variation indicate an alteration in a physiology process and can be used as an early warning against ensuing pathology. Three kinds of oxidative stress markers are classically used to evaluate an oxidative stress [Tiwari et al., 2013]. They are markers of lipid peroxidation, plasma total antioxidants status and specific antioxidant defense systems. The levels of all biomarkers of oxidative stress are modified in diabetic subjects. In T2DM, evidence of lipid peroxidation was observed with high plasma and urine isoprostane levels [Subramanian et al., 2009]. Also, malonaldehyde (MDA) level results were higher than in the normal subjects and correlated with the degree of glycemic control. Lipid peroxidation is probably the most extensively investigated process induced by free radicals. Free radicals can cause the peroxidation of membranes unsaturated fatty acids. It may result in a chain reaction that autopropagates once started, leading to the formation of many lipid peroxide radicals and amplifying the ROS effect. The important compounds are bioactive aldehydes generated by

extensive oxidation of polyunsaturated fatty acids (PUFA). This process generates compounds such as alkanals (hexanal), 4-hydroxyl alkenals (4-HNE), MDA and acrolein. Malondialdehyde is the resultant product formed by autooxidation of polyunsaturated fatty acids. It is broadly defined as oxidative deterioration of polyunsaturated fatty acids or lipids. Use of TBARS as an index of lipid peroxidation was pioneered by Sato et al., [1979] whose group also showed increased plasma TBARS levels in diabetes. Sreekumar et al., [1987] found increased amount of malondialdehyde, a decomposition product of lipid hydroperoxide in the patients of diabetic retinopathy. Griesmacher et al., [1995] observed that T2DM patients exhibited significantly higher plasma TBARS levels than type 1 diabetic patients. According to Sundaran et al., [1996], lipid peroxidation elevated by 80% in early stage of diabetes, and progresses as time passes. According to Abdella et al., [1990] increased lipid peroxidation due to an altered intracellular ratio, between free radical and antioxidant system has been associated with diabetes, and his study suggested that existence of abnormal lipid peroxidation is found in diabetic patients, and free radical play a role in development of lipid peroxidation and arterial dysfunction. According to study of James et al., [2004], nonenzymatic antioxidants would affect lipid peroxidation by scavenging free radicals to produce a less reactive species.

Oxidative stress has been considered by many as an explanation for the tissue damage that accompanies chronic hyperglycemia. Hyperglycemia also promotes glycation and inactivation of antioxidant proteins, such as Cu, Zn-SOD, leading to the inactivation and a reduction in antioxidants defense. Yoshida et al., [1995] suggested that inactivation of glutathione synthesis and thiol transport in diabetic patients increases the sensitivity of the cells to oxidative stress, and these changes may lead to the development of some complications in diabetes mellitus.

### **1.8. Antioxidant protection**

Humans have evolved a highly sophisticated and complex antioxidant defense system to protect the cells and organ systems of the body against reactive oxygen species. It involves both endogenous and exogenous components in origin, that function interactively and synergistically to neutralize free radicals.



These components include:

- Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoic acid.
- Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions.
- Metal binding proteins, such as ferritin, lactoferrin, albumin and ceruloplasmin sequester free iron and copper ions that are capable of catalyzing oxidative reactions.
- Numerous other antioxidant phytonutrients present in a wide variety of plant foods.

### **1.8.1. Dietary Antioxidants**

Vitamin C, vitamin E, and beta carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water soluble antioxidant in extracellular fluids which neutralizes ROS in the aqueous phase before the initiation of lipid peroxidation. Vitamin C has been stated as being capable of regenerating vitamin E [Sies et al., 1992]. Vitamin E, a major lipid soluble antioxidant, is the most efficient chain breaking antioxidant in cell membrane where it protects membrane fatty acids from peroxidation [Russo et al., 1998]. Supplementation with vitamin or dietary free radical scavenger such as vitamin C and E have a potential role in boosting antioxidant related defenses and may be important in mitigating long term complications in patients with diabetes. Hamilton et al., [2004] observed that the increased plasma lipid peroxidation and decreased plasma HDL patients in T2DM mellitus indicated that these may predispose to the development of cardiovascular complications. Beta carotene and other carotenoids also provide antioxidant protection to lipid rich tissues.

### **1.8.2. Phytonutrients**

Many plant-derived substances, collectively termed “phytonutrients,” or “phytochemicals,” are known for their antioxidant capability. Phenolic compounds such as flavonoids are ubiquitous within the plant kingdom, approximately 3,000 flavonoid substances have been identified [Briviba and Sies, 1994]. In plants, flavonoids serve as protectors against a wide variety of environmental stresses while, in humans, flavonoids appear to function as “biological response modifiers”. Flavonoids have been demonstrated to have anti-inflammatory, anti-aging, anti-allergenic, anti-viral, and anti-carcinogenic activity [Cody et al., 1986; Kuhnau, 1976; Havsteen



1983; Middleton and Kandaswami, 1992]. Broad therapeutic effects of flavonoids can be largely attributed to their antioxidant properties. In addition to antioxidant effect, flavonoid compounds may exert protection against heart disease through the inhibition of cyclooxygenase and lipoxygenase activities in platelets and macrophages [Middleton and Kandaswami, 1992].

### 1.8.3. Endogenous antioxidants

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical induced cell damage. The antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase (SOD) metabolize toxic oxidative intermediates and need cofactors such as selenium, iron, copper, zinc, and manganese for optimal catalytic activity as shown in Fig. 1.4. It has been suggested that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms. Consumption and absorption of these important trace minerals are reported to decrease with aging [Duthei and Brown, 1994]. Glutathione, an important water soluble antioxidant, synthesized from the amino acids- glycine, glutamate, and cysteine directly quenches ROS such as lipid peroxides, and plays an important role in xenobiotic metabolism [Percival, 1998].

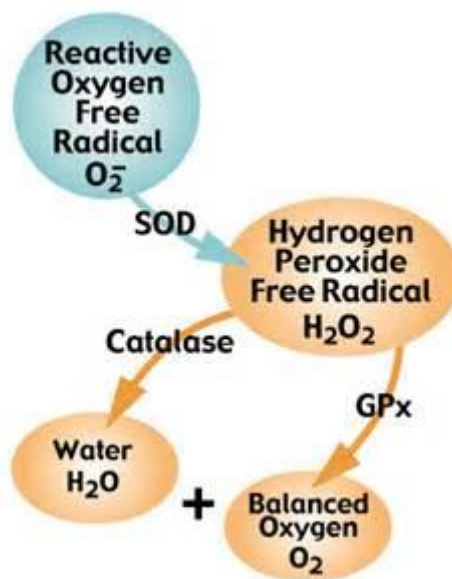


Fig. 1.4 . Role of SOD, catalase & GPx [www.freegrab.net]

Lipoic acid, another important endogenous antioxidant, categorized as a “thiol” or “biothiol,” is a sulfur-containing molecule that is known for its involvement in the reaction that catalyzes the oxidative decarboxylation of alpha keto acids, such as pyruvate and alpha-ketoglutarate, in the Krebs cycle. Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), are capable of quenching free radicals in both lipid and aqueous domains and as such has been called a “universal antioxidant”. Lipoic acid may also exert its antioxidant effect by chelating with prooxidant metals. Animal studies have demonstrated supplemental lipoic acid to protect against the symptoms of vitamin E or vitamin C deficiency [Packer and Witt, 1995].

### **1.9. Current treatment for diabetes**

WHO in 2004 had reported that between 20 - 50% of people with T2DM can control their blood glucose levels by dietary modification alone. Starch based foods fulfill more than 50% of the calorific value of the human diet. Starch contains amylose and amylopectin in the ratio of 1:3. Extensive branching in amylopectin and long linear chains of amylose resist digestion and hence play a role in resistant starch (RS) formation. Mechanisms and processes by which foods, rich in fiber and resistant starch, exert their disease control properties are not well understood. Some of these factors believed to have effects on the rate of digestion and absorption. These include a source of food material, its components, physical nature, the presence of enzyme inhibitors, antinutrients as well as processing methods [Goni et al., 2007]. The diet alone can reduce glucose burden on human body, thereby preventing excessive utility of antidiabetic modalities and delays insulin resistance. However, the physiological response due to glucose homeostasis linked with energy currency attributes, exercise/physical work in the daily schedule is essential to channelize the energy and to revitalize the cells of the body. The combination of these two lifestyle modifications, i.e., exercise and diet, can either reduce or delay the incidence of diabetes by over 50% [Joshi et al., 2008].

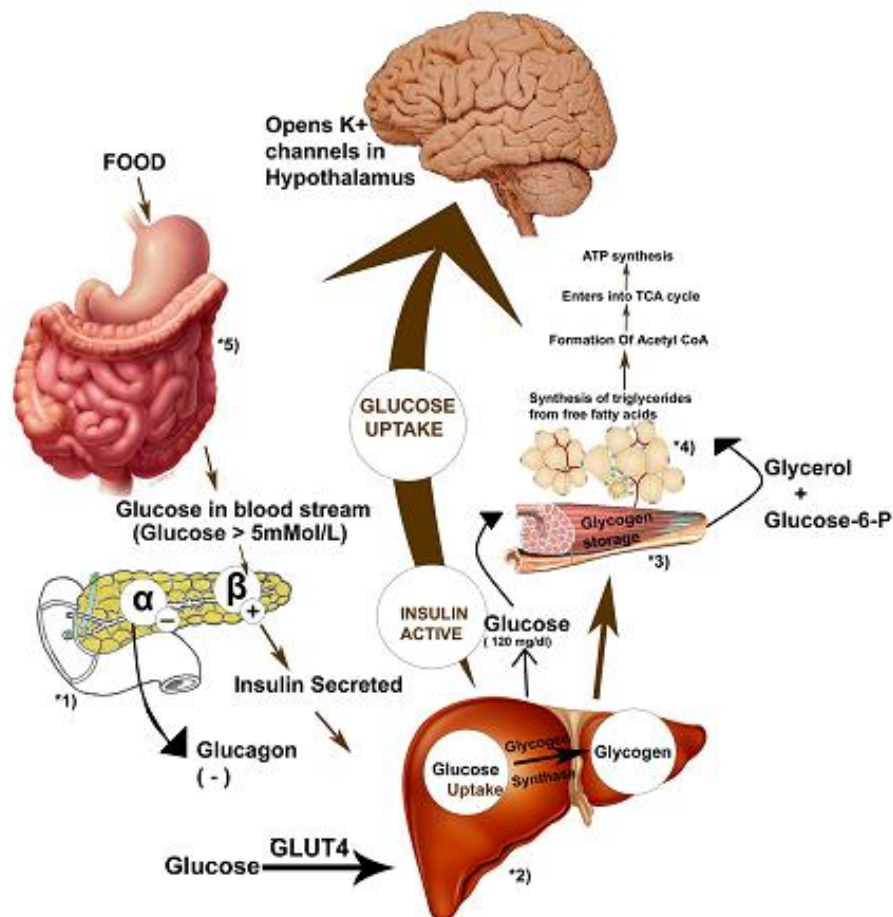
#### **1.9.1. Antihyperglycemic drugs**

The definite therapy for diabetes include target based hypoglycemic drugs or a combination with insulin, based on type and progression of ailment over the years. There are five major classes of oral antihyperglycemic drugs and a wide variety of insulin (short acting / long acting) available for comprehensive management of Diabetes. These five distinct classes of oral

antihyperglycemic drugs include the Sulfonylureas (SUs), Meglitinides, Biguanides, Thiazolidinediones (TZDs)/glitazone,  $\alpha$ -glucosidase inhibitors. These agents are categorized into two, based on mechanism of action, which are “Secretagogues”, or drugs that augment insulin supply (sulfonylurea, non-sulfonylurea secretagogues, and insulin); and “Sensitizers”, or drugs that assist insulin action (biguanides and thiazolidinediones) as shown in Table 1.3 and Fig. 1.5.

*Table 1.3. Oral Hypoglycemics [Palaian et al., 2004]*

<b>Medication</b>	<b>Mechanism of action</b>	<b>Adverse effects</b>	<b>Contraindications</b>
Sulfonylureas Glyburide (Diabeta) Chlorpropamide (Diabinese)	Stimulate release of endogenous insulin	Hypoglycemia Nausea GI discomfort	Hepatic or renal impairment
Meglitinides Repaglinide (Gluconorm)	Stimulate release of endogenous insulin (rapid acting, better post prandial glucose control)	Hypoglycemia (less frequent than with sulfonylureas)	Hypersensitivity, diabetic ketoacidosis (DKA)
Biguanides Metformin (GlucoPhage)	Reduce gluconeogenesis, increase glucose utilization.	Lactic acidosis, anorexia, nausea, diarrhea, GI discomfort	Hepatic or renal impairment, alcoholism, advanced age.
Thiazolidinediones Rosiglitazone (Avandia) Pioglitazone (Actos)	Increase peripheral insulin sensitivity, reduce gluconeogenesis.	Increased TG, Weight gain, hepatotoxicity, anemia	Liver disease, congestive heart failure (CHF)
A-Glucosidase inhibitors Acarbose (Prandase)	Decrease the absorption of carbohydrates (thus decreasing post prandial rise of glucose)	Flatulence abdominal cramping, diarrhea	Hypersensitivity, DKA, inflammatory bowel disease (IBD)



**Fig:1.5. Various physiological targets of synthetic drugs**

(1) Meglitinides, Sulphonylureas [(Glimpiride (Amaryl); Glipizide (Glucotrol); Glipizide-gits (Glucotrol-XL); Glyburide (Diabeta, Micronase); Glyburide Micronized (Glynase); Tolbutamide (Orinase); Chlorpropamide (Diabinese); Tolazamide (Tolinase); Acetohexamide (Dymelor)] & Phenylalanine derivatives; Repaglinide (Prandin) Nateglinide (Starlix) act on pancreas to stimulate insulin secretion by blocking  $K^+$  ions in  $\beta$ -cells, (2)- Biguanide & Thiazolidinediones act on liver to decrease Gluconeogenesis; (3 & 4)- Biguanide Metformin (Glucophage, Riomet) Metformin-XR (Glucophage XR) act on muscle and adipose tissue to augment peripheral glucose uptake & Thiazolidinediones (Rosiglitazone (Avandia) Pioglitazone (Actos) increase insulin sensitivity via activation of receptors;  $\alpha$ -glucosidase inhibitors [(Acarbose (Precose) Miglitol (Glyset)] act intestine to delay glucose absorption. [Raman et al., 2013].

## 1.9.2. Medicinal herbs

Herbal drugs have been used since the inception of human beings on earth. Herbal medicines extracted from aerial or underground parts of plants are widely used in healthcare or dietary supplements. They contain ingredients as complex chemical mixtures. The complications of synthetic drugs have led to a shift towards locating natural resources showing antidiabetic activity. The Indian prehistoric literature reports more than 800 plants with antidiabetic

properties while ethnopharmacological surveys states that more than 1200 plants can be used for hypoglycemic activity [Mishra et al., 2010]. Based on these literatures the most effective antidiabetic Indian medicinal plants are *Acacia arabica*, *Aegle marmelose*, *Agrimonia eupatoria*, *Allium cepa*, *Allium sativum*, *Aloe vera*, *Azadirachta indica*, *Benincasa hispida*, *Beta vulgaris*, *Caesalpinia bonducella*, *Citrullus colocynthis*, *Coccinia indica*, *Eucalyptus globulus*, *Ficus benghalensis*, *Gymnema sylvestre*, *Hibiscus rosasinesis*, *Ipomoea batatas*, *Jatropha cureas*, *Mangifera indica*, *Momordica charantia*, *Morus alba*, *Mucuna pruriens*, *Ocimum sanctum*, *Pterocarpus marsupium*, *Punica granatum*, *Syzigium cumini*, *Tinospora cordifolia*, *Trigonella foenum graecum*. A wide array of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of diabetes. Herbs are also known to provide relief and aid in the prevention of the secondary complications of the disease including cholesterol lowering action. Some of these herbs have also been proven to help in the regeneration of  $\beta$ -cells and in overcoming insulin resistance. In addition to maintaining normal blood sugar level, many of these also possess antioxidant activity. Medicinal plants have been used in traditional medicines, since time immemorial to maintain health or cure ailments from the dawn of civilization [Izzo and Ernst, 2009]. It has been a rich source of new drug discovery. Ayurvedic pharmacology classifies medicinal plants into different groups according to their actions. One of these is the 'Rasayana' group. 'Rasayana' drugs act inside the human body by modulating the neuro-endocrino-immune systems and have been found to be a rich source of antioxidants [Brahma and Debnath, 2003]. The use of herbal medicine for the treatment of diabetes has been authenticated (WHO, 1980) due to the presence of phytochemicals with antidiabetic properties. Approximately 25% of drugs prescribed world wide is derived from plants. There are 252 drugs approved as basic and essential by WHO, of which 11% are of plant origin while the majority of synthetic drugs have plant precursors. Many conventional drugs have been derived from prototypic molecules in medicinal plants. One of the major drawbacks of these herbal medicines is its bioavailability and a possible antagonistic effect of other components. At present herbal formulations attained wide acceptance as therapeutic agents for diabetes. China and India are the largest producers of medicinal herbs and India is called as the botanical garden of the world [Umadevi et al., 2013]. The WHO has listed 21,000 plants, currently in use as medicines around the world. Among these 2500 species are in India, out of which 150 species are used commercially on a fairly large scale.

To date over 400 traditional plant treatments for diabetes have been reported, although only a small number of these have received scientific and medical evaluation to assess their efficacy. The hypoglycemic effect of some herbal extracts has been confirmed in human and animal models of T2DM [Bailey and Day, 1989].

### **1.9.3. Plant derived compounds as drug**

Despite the recent interest in drug discovery by molecular modeling, combinatorial chemistry, and other synthetic chemistry methods, a natural product derived compounds are still proving to be an invaluable source of medicines for humans. The 19th century marked the isolation of numerous alkaloids from plants used as drugs, namely, atropine (*Atropa belladonna*), caffeine (*Coffea arabica*), cocaine (*Erythroxylum coca*), ephedrine (Ephedra species), morphine and codeine (*Papaver somniferum*), pilocarpine (*Pilocarpus jaborandi* Holmes), physostigmine (*Physostigma venenosum*), quinine (*Cinchona cordifolia* Mutis ex Humb.), salicin (*Salix* species), theobromine (*Theobroma cacao*), theophylline (*Camellia sinensis*), and (+)-tubocurarine (*Chondodendron tomentosum*). Following these discoveries, bioactive secondary metabolites from plants were later utilized more widely as medicines, both in their original and modified forms [Samuelsson, 2004; Salim et al., 2008]. Many of the bioactive ingredients isolated from a plant extract that are in use in modern therapy have the same or a similar therapeutic purpose as their original ethnomedical use. In 1805, morphine became the first pharmacologically active compound to be isolated in the pure form, although its structure was not elucidated until 1923. Throughout the century, a number of bioactive compounds were isolated and which are still in use. Discovery of various therapeutic agents from plant sources continued in the 20th century inspired by the discovery and benefits of penicillin. Today about half of the transported drugs are derived from biological sources. Approximately 50% of the new drugs introduced since 1994 were either natural products or their derivatives [Harvey, 2010; Bramachari, 2011; Montaser and Luesch, 2011].

Metformin exemplifies an efficacious oral glucose lowering agent. Its development as a drug was based on the use of *Galega officinalis* to treat diabetes. *Galega officinalis* is rich in guanidine, the hypoglycemic component. As guanidine is too toxic for clinical use, the alkyl biguanides synthalin A and synthalin B were introduced as oral anti-diabetic agents in Europe in the 1920s but were discontinued after insulin became more widely available. However, experience with

guanidine and biguanides prompted the development of metformin. Herbal medicines continue to play a major role in diabetic therapy, particularly in developing countries where there is limited resources and do not have access to modern treatment [Ali et al., 2006].

### **1.10. Flavonoids**

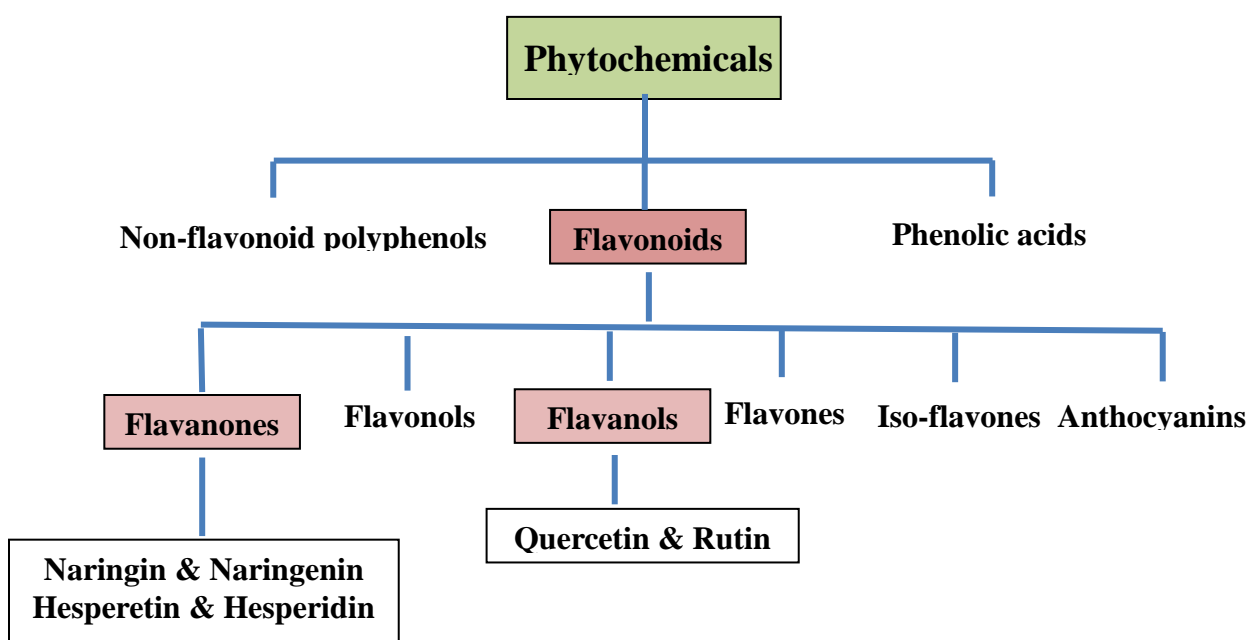
Flavonoids represent one of the largest groups of secondary metabolites, with more than 7000 different compounds. In plant cells, flavonoids exist mostly as glycosides, it appears to be a biological strategy apparently aimed at increasing their water solubility, most likely decreasing their interaction with macromolecules and for their subcellular localization. In the early 1960s, flavonoids were widely viewed as metabolic waste products that were stored in the plant vacuole. In 1936, Nobel Prize winner Albert Szent Gyorgyi reported that flavonoids from paprika and citrus peel restored complete health to scorbutic guinea pigs when vitamin C alone did not. Albert Szent Gyorgyi was also the discoverer of vitamin C. In the early 1990s, Hertog published aspects of correlation between high food flavonoid intake and a lowering in the risk of coronary heart disease and provide estimates of daily intake of flavonoids as 23 mg. Albert Szent Gyorgyi and co-workers at first referred this class of plant compounds to vitamin P. But the chemical diversity of flavonoids precludes their classifications as a single vitamin. Later vitamin P was renamed as flavonoids. Since the discovery, scientists have isolated more than 4,000 flavonoids. Flavonoids, ascorbic acid carotenoids, and tocopherols are the main antioxidants recommended based on the results from experimental models [Pietta, 2000; Middleton et al., 2000]. They have been shown to inhibit ROS production by inhibiting several ROS producing enzymes (i.e. xanthine oxidase, cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione-S-transferase, mitochondrial succinic oxidase, NADH oxidase), inhibiting phospholipases A<sub>2</sub>, and by chelating trace metals. They act by donating a hydrogen atom/electron to the superoxide anion and also to hydroxyl, and peroxy, alkoxy radicals thereby protecting lipoproteins, proteins, DNA molecules against oxidative damage [Gate et al., 1999]. However, free radicals as well as some antioxidative vitamin derivatives (i.e. retinoic acid) are also important regulators of cellular functions including gene expression, differentiation, apoptosis and preconditioning (mitochondrial function) etc [Chertow, 2004]. Although many earlier epidemiological studies have reported lower risk of cardiovascular disease and cancer in populations with higher intakes and higher blood levels of antioxidants, the large scale trials with



antioxidant supplementation have failed to confirm any protective effect of antioxidants on cardiovascular mortality in spite of improving the biochemical parameters of lipoprotein oxidation. Nevertheless, the available evidence does not contradict the advice to increase consumption of fruit and vegetables to reduce the risk of cardiovascular disease in patients with diabetes.

About 8000 phenolic structures are currently known and among them over 4000 flavonoids have been identified [Bravo, 1998; Samuelsson, 2004; Cheynier, 2005]. Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are a rich source of polyphenols. Polyphenols have been classified by their source of origin, a biological function, and chemical structure. Also, the majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at different positions of the polyphenol skeletons. Phenolic acids are non flavonoid polyphenolic compounds that can be further divided into two main types, benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones. While fruits and vegetables contain many free phenolic acids, in grains and seeds particularly in the bran or hull phenolic acids are often in the bound form. Chalcones, though lacking the heterocyclic ring C, are still categorized as members of the flavonoid family. These basic structures of flavonoids are aglycones, however, in plants, most of these compounds exist as glycosides. Biological activities of these compounds, including an antioxidant activity, depend on both the structural difference and the glycosylation patterns. The flavonoid subgroups are the most common, and almost ubiquitous, throughout the plant kingdom. Flavones and their 3-hydroxy derivatives flavonols, including their glycosides, methoxides and other acylated products on all three rings, make this the largest subgroup among all polyphenols. The most common flavonol aglycones, quercetin and kaempferol, alone have at least 279 and 347 different glycosidic combinations [Valant and Wallenweber, 2006; Williams, 2006; Tsao and McCallum, 2009]. The number of flavanones, and their 3-hydroxy derivatives (flavanonols, which are also referred to as dihydro flavonols) identified in the last 15 years has significantly increased. Some flavanones have unique substitution patterns, e.g., prenylated flavanones, furane flavanones, pyran flavanones, benzylated flavanones, giving a large number of substituted derivatives of this subgroup as shown in Fig 1.6.





**Fig. 1.6. Polyphenolic family**

*Polyphenols include several classes of compounds, such as phenols, phenolic acids, flavonoids, anthocyanins, and others, with more complex structures, tannins and lignins. Polyphenols are secondary metabolites produced by plants in response to stress conditions, such as infections, large amounts of UV rays or other factors. [Modified version from [www.gurufitness.com](http://www.gurufitness.com).]*

### 1.11. Bioavailability of flavonoids

The flavonoids although have shown immense health benefits, however, their low bioavailability has been a concern. Flavonoids undergo extensive first pass metabolism, and the chemical forms of flavonoids present in fruits and vegetables, usually glycosides, are quite different from their *in vivo* metabolites. Phase 2 metabolism is known to affect the bioavailability of flavonoids in humans [Manach et al., 2005]. Usually flavonoids undergo sulfation, methylation or glucuronidation in the small intestine and liver [Mullen et al., 2006], and conjugated metabolites can be found in plasma after flavonoid ingestion [Rupasinhe et al., 2010]. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption depending on the structure of flavonoid, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as b-glycosides. Aglycans can be easily absorbed by the small intestine while flavonoid glycosides have to be converted into aglycon form [Moco et al., 2012]. After absorption, the flavonoids gets conjugated in the liver by

glucuronidation, sulfation or methylation or gets metabolized to smaller phenolic compounds [Bravo, 1998]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [Hollman, 2004]. In general, metabolites of flavonoids show reduced bioactivity in comparison to parent compounds but there have been results that reported otherwise as well [Thilakarathna et al., 2013]. Despite the bioactivity expressed in different *in vitro* systems, the bioavailability of flavonoids would be a determinant factor of their bioactivity *in vivo*. Several attempts have been made to improve bioavailability such as the intestinal absorption by the use of absorption enhancers [Shen et al., 2011], novel delivery systems [Zhang et al., 2011] improving metabolic stability [Walle, 2007; Cao et al., 2013], changing the site of absorption from large intestine to small intestine [Nielsen et al., 2006].

### **1.12. Antioxidant potential of flavonoids**

Antioxidants are the compounds that protect cells from the reactive oxygen species (ROS), such as superoxide radicals, singlet oxygen, hydroxyl radicals, peroxy radicals, nitric oxide, and peroxynitrite. Flavonoids are well known for their antioxidant activities. The impairment between the production of ROS and antioxidant defense system results in oxidative stress. Oxidative stress is responsible for cellular damage that is associated with various health deficits such as diabetes, cancer, cardiovascular disorders, and neurodegenerative disorders. Intake of flavonoids via fruits, vegetables, and whole grains increases the level of antioxidants in the body. Flavonoids are earlier reported to be strong antioxidants [Eberhardt et al., 2000; Sun et al., 2002; Chu et al., 2002]. The structural consideration of flavonoids is very important for their antioxidant activity. The structural requirements include the presence of a hydroxyl group at the third position of carbon, a double bond between the second and third position of carbon atoms, a carbonyl group at the fourth position of carbon and polyhydroxylation at both aromatic rings A and B of the basic flavonoid structure. This prevents the damaging effect of reactive oxygen species that includes lipid peroxidation, and oxidation of sulfhydryl and other susceptible groups in protein. Lipid peroxidation is found to be responsible for various diseases such as atherosclerosis, diabetes, hepatotoxicity, and inflammation, along with aging. Quercetin, a flavonoid compound, is well known for its ability to act as an antioxidant. Quercetin seems to be one of the most powerful flavonoids used for protecting the body against reactive oxygen species. Studies have suggested that it helps suppress lipid peroxidation in model systems

[Pavanato et al., 2003]. In addition to quercetin, other flavonoids such as myricetin, quercetrin, and rutin help inhibit the production of superoxide radicals [Rice Evans et al., 1995]. Tea polyphenols also belong to a subclass of flavonoid and a large number of studies have reported their antioxidant capabilities. One of the tea catechins, named epigallocatechin gallate (EGCG), is among the most potent antioxidants. Antioxidant capabilities of tea catechins explain their capabilities to protect the cell components. A study by Nakagawa et al., [1999] suggested that drinking green tea helps in prevention of cardiovascular disorders by increasing the antioxidant capacity of plasma in humans. Citrus flavonoids, similar to tea polyphenols, are also dietary antioxidants that may protect against oxidative stress linked to inflammation and help reduce the risk of macromolecule damage caused by free radicals. For example, studies by Zielinska and Ignatowicz [2008] showed the antioxidant effect of citrus flavonoids such as naringin, naringenin, and hesperidin, by counteracting the effects of ROS on apoptosis via caspase-3 inhibition.

### **1.13. Flavonoids as therapeutics for T2DM**

Plant polyphenols were reported to inhibit  $\alpha$ -amylase and sucrase activity, decreasing postprandial glycemia [Kobayashi et al., 2000]. Individual polyphenols, such as catechin, epicatechin, epigallocatechin, epicatechin gallate, and isoflavones from soya beans also decrease S-GLUT 1 mediated intestinal transport of glucose [Tiwari and Rao, 2002]. Anthocyanins, a polyphenol in bilberries and other berries, may also prevent T2DM and obesity. Anthocyanins from different sources have been shown to affect glucose absorption and insulin level/secretion/action and lipid metabolism *in vitro* and *in vivo* [Jayaprakasam et al., 2005]. Many *in vitro* studies suggest that the anthocyanins may decrease the intestinal absorption of glucose by retarding the release of glucose during digestion [Tsuda et al., 2006; Xia et al., 2006]. Recently, Ganugapati et al., [2012] reported that flavonoids isolated from banana flowers have the potential to activate the insulin receptor tyrosine kinase, and may represent an alternative choice for treatment if T2DM patients with insulin resistance [Ganugapati et al., 2012; Kemertelidze et al., 2012]. Flavonoids, especially quercetin have been reported to possess antidiabetic activity. Vessal et al., [2003] reported that quercetin brings about the regeneration of pancreatic islets and probably increases insulin release in streptozotocin induced diabetic rats. There is mounting evidence that polyphenols can reduce insulin resistance under *in vitro* and *in*

*vivo* studies [Hanhineva et al., 2010; Verma et al., 2012], but data from clinical studies are limited. Several mechanisms have been proposed by which specific flavonoid constituents can reduce biological pathways related to the development of T2DM. Flavonoids also interact with molecular targets and affect signaling pathways with evidence *in vitro* that both the nuclear factor  $\kappa$ -B and mitogen activated protein kinase signaling pathways are modified [Crozier et al., 2009]. In an animal model of T2DM, anthocyanins (i.e., cyanidin 3-glucoside) significantly decreased blood glucose concentrations and ameliorates insulin sensitivity after an insulin tolerance test in male mice [Sasaki et al., 2007]. In addition, GLUT 4 gene expression was upregulated in white adipose tissue, whereas expression of retinol binding protein 4 was downregulated, which resulted in suppression of gluconeogenesis and improved glycemia. Similarly, an anthocyanin rich bilberry extract reduced glycemia and insulin sensitivity in male mice with T2DM accompanied by increased activation of AMP-activated protein kinase and resulted in upregulation of GLUT 4 [Takikawa et al., 2010].

### 1.13.1. Quercetin & rutin

Quercetin and rutin (Fig 1.7) belongs to a group of natural substances with variable phenolic structures and is found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine. The absorption and metabolism of quercetin was not well explored. For many years, it was believed that quercetin was not absorbed as no unchanged compound could be measured in the plasma after oral administration. In the last few years, it has become clear that this bioflavonoid is indeed absorbed but heavily metabolized prior to reaching the plasma. Most of the metabolism is in the form of glucuronidation, or the formation of glucuronide conjugates [Joannou et al., 1995]. Quercetin has a broad range of activities within cells [Bjeldanes and Chang, 1977].

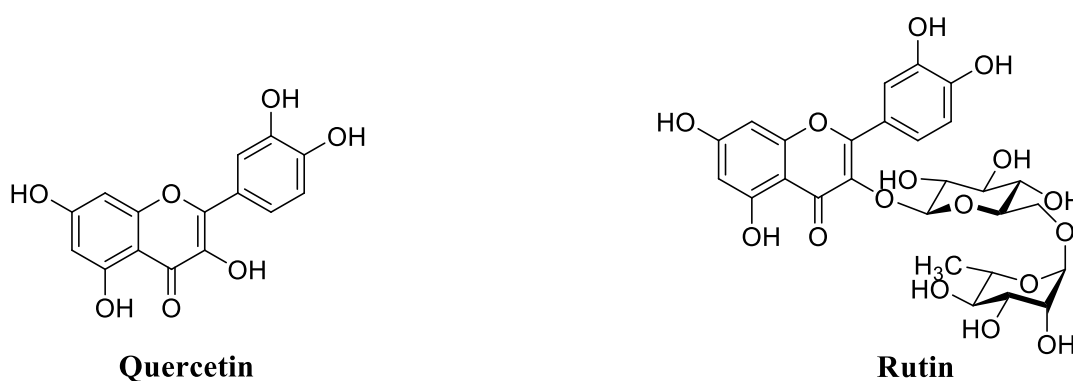


Fig 1.7. Chemical structure of quercetin and its glycoconjugates, rutin.

Scientific studies suggest its antioxidative [Song et al., 2001], antiproliferative [Yoshida et al., 1992], antiinflammatory [Comalada et al., 2005; Dias et al., 2005], anticarcinogenic [Soleas et al., 2006], antihypertensive [Duarte et al., 2001], antidiabetic [Vessal et al., 2003] effect and is able to protect different types of cells against various diseases such as certain forms of cancer, osteoporosis, pulmonary, cardiovascular diseases and aging [Boots and Guido, 2008].

#### 1.13.1.1. Food sources

Bioflavonoid quercetin is found in the edible portion of the majority of dietary plants (e.g., citrus fruits, berries, leafy vegetables, roots, tubers and bulbs, herbs and spices, legumes, cereal grains, tea, and cocoa) [Singleton, 1981]. The best source of quercetin is onion (60-100 mg/g fresh weight). Quercetin is ingested as a major constituent in the diet [Bjeldanes and Chang, 1977]. Content of aglycone form of quercetin, which is absorbed better than quercetin administered in nonglucosidic forms, varies among different food sources (Table 1.4).

Rutin is a citrus flavonoid glycoside (Fig.1.7) found in many plants including buckwheat, the leaves and petioles of *Rheum* species, and asparagus. Tartary buckwheat seeds have been found to contain more rutin (about 0.8-1.7% dry weight) than common buckwheat seeds (0.01% dry weight). Rutin is also found in the fruits and flowers of the pagoda tree, fruits and fruit rinds (especially the citrus fruits orange, grapefruit, lemon, and lime) and apple; berries such as mulberry, ash tree fruits, aronia berries and cranberries. Rutin is one of the primary flavonols found in 'clingstone' peaches.

#### 1.13.1.2. Bioavailability and absorption

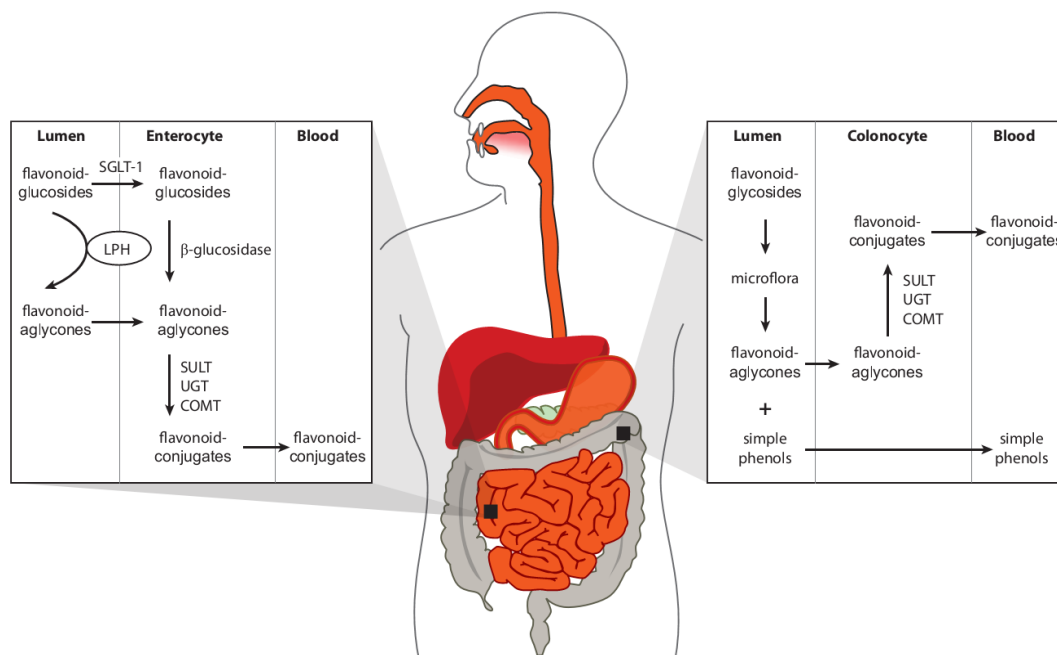
Depending on dietary habits or countries, the daily intake has been estimated between 3 and 70 mg [Hertog et al., 1996]. Quercetin is probably extensively modified before being excreted by kidneys [Ueno et al., 1982]. Quercetin aglycone and its glucosides are absorbed better than quercetin administered in nonglucosidic forms. The bioavailability of two quercetin glucosides, 3-glucoside and 4'-glucoside, do not differ when quercetin and its derivatives are provided for consumption along with their natural sources in which these compounds are dispersed in the matrix, quercetin aglycone is more bioavailable than its glucosides. This finding suggests that in some cases, bioavailability of isolated food components consumed as food supplements could be less than when they are consumed with the food matrix [Olthof et al., 2000].

Table 1.4. Quercetin in different food sources [Erlund 2004]

Forms of Quercetin	Sources	Content of aglycone (mg/kg) and Reference
Quercetin-3,4-glucoside	Onion	284-486 ( Hertog et al., 1992)
Quercetin-3-glucoside		
Quercetin-3-rhamnoglucoside (Rutin)	Black tea	10-25 ( Hertog et al., 1993)
Quercetin-3-galactoside	Apple	21-72 ( Hertog et al., 1993)
Quercetin-3-rhamoside		
Quercetin-3-arabinoside		
Quercetin-3-glucoside		
Quercetin-3-rhamnoglucoside	Black current	44 (Hakkinen et al., 1999)
Quercetin-3-rhamoside		
Quercetin-3-galactoside		
Myrisetin-3-glucoside		

Graefe et al., [2001] in their scientific study observed that the bioavailability of quercetin from onion in which a variety of quercetin glucosides is present, is comparable to the bioavailability of isolated quercetin 4-glucoside. The lipophilic character of quercetin suggests that it can cross enterocyte membranes via simple diffusion [Wiczowski et al., 2008]. Urinary excretion of quercetin seemed to be a small but constant function of quercetin intake [Young et al., 1999]. It is known that humans absorb appreciable amounts of quercetin and that absorption is enhanced by conjugation with glucose as illustrated in Fig 1.8. [Hollman et al., 1995]. Quercetin has a broad range of activities within cells [Bjeldanes and Chang, 1977]. As an antioxidant, it prevents oxidation of low-density lipoproteins and the expression of metalloproteinase 1, thus inhibiting the disruption of atherosclerotic plaques and contributing to plaque stabilization [Song et al., 2001] and also brings about the regeneration of the pancreatic islets and probably increases

insulin release in streptozocin-induced diabetic rats, thus exerting its beneficial antidiabetic effects [Vessal et al., 2003].

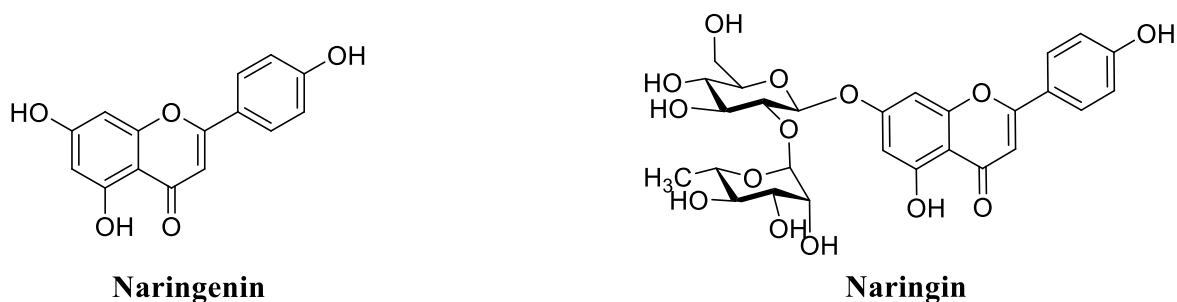


*Fig. 1.8. Metabolism of flavonoids in human body [Massimo et al., 2010]*

Quercetin has also antiinflammatory effects, regulating nitric oxide, interleukin -6, and tumor necrosis factor release [Comalada et al., 2005; Dias et al., 2005; Liu et al., 2005], thereby alleviating oxidative damage in the tissue [Dias et al., 2005] and inhibiting the lipopolysaccharide-induced delay in spontaneous apoptosis and activation of neutrophils [Liu et al., 2005]. It is examined that a single oral daily dose of the bioflavonoid quercetin reduced blood pressure and heart rate, the cardiac and renal hypertrophy, the endothelial dysfunction and the oxidant status in a rat model of spontaneous hypertension, but had no effect on normotensive rats [Duarte et al., 2001]. Protective effect of quercetin against various diseases such as osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases but also against aging was also observed [Boots et al., 2008]. Short-term, high intake of black currant and apple juices had a prooxidant effect on plasma proteins and increased glutathione peroxidase activity, whereas lipid oxidation in plasma seemed to decrease [Young et al., 1999].

### 1.13.2. Naringin & naringenin

Naringin & naringenin are two major flavanones, (Fig 1.9) rich in citrus fruits, with naringin responsible for the bitter flavors of grapefruit. Naringin has been reported to possess antioxidant, anti-diabetic, lipid-lowering, anti-atherogenic, and anti-inflammatory activities [Kim et al., 2004; Goldwasser et al., 2010].



*Fig 1.9 Chemical structure of naringin, and their glycoconjugates, naringenin*

Both naringin and naringenin (the aglycone form of naringin) have been extensively studied in recent years [Erlund et al., 2001; Wilcox et al., 2001; Borradaile et al., 2002; Jung et al., 2003; Seo et al., 2003].

#### 1.13.2.1. Food sources

Naringin is a flavonoid compound found in grapefruit, it gives grapefruit its characteristic bitter flavour. Naringenin is also found in grape fruit, oranges and tomatoes (30-60 mg/g)

#### 1.13.2.2. Bioavailability and absorption

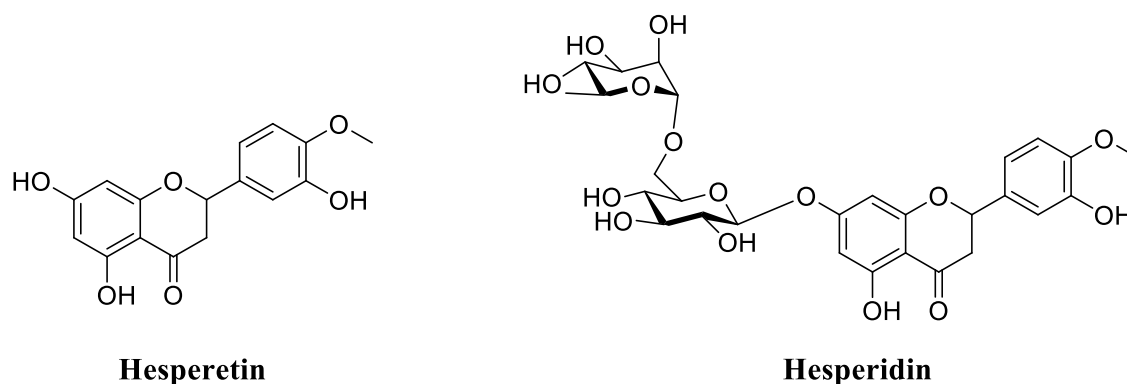
Erlund et al., [2001] studied the bioavailability and pharmacokinetics of flavanones after single ingestion of 400 to 760 mL of orange juice or grapefruit juice. The resulting plasma concentrations were comparatively high (up to 4 mg/L or 151 mol/L), which is not surprising, considering that citrus fruits and juices contain quite high concentrations of the compounds. The plasma half-life of flavanones was relatively short (1-2 h). Furthermore, renal clearance of naringenin appeared to be dose dependent. Similar plasma levels were reported after consumption of 0.5 or 1 L of orange juice [Mouly et al., 1994] and the compounds are bioavailable when citrus fruits and juices are consumed as part of a normal diet (1 glass of orange juice, one half orange, and one half mandarin per day). Hesperetin, and especially naringenin, levels were, however, below the limit of detection of the analytical method in a large part of the plasma samples, which were collected after an overnight fasting. Bugianesi et al.,



[2002] recently made the interesting finding that naringenin is bioavailable from tomato paste, which is a notable source because of its widespread use, despite its low naringenin content.

### 1.13.3. Hesperidin & hesperetin

Hesperidin (hesperetin 7-rutinoside) (Fig.1.10) is a flavanone glycoside, abundantly found in citrus fruits (family Rutaceae).



*Fig 1.10. Chemical structure of hesperetin and its glycoconjugates, hesperidin*

It has shown to possess an antioxidant effect, blood lipid lowering [Galati et al., 1994] and anti-carcinogenic activities [Gaerg et al., 2001] of hesperidin in the diet has been linked with abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps. No signs of toxicity have been observed with normal intake or related compounds. Both hesperidin and its aglycone hesperetin have been reported to possess a wide range of pharmacological properties.

#### 1.13.3.1. Food sources

Hesperidin is isolated in large amounts from the discarded rinds of the ordinary orange *Citrus aurantium L.*, *C. sinensis* [Horowitz and Gentili, 1963] and other species of the genus *Citrus* (family *Rutaceae*) (47 – 50 mg/g). It has been reported to occur in many plants other than *Citrus*, such as in genera *Fabaceae* [Bhalla and Dakwake, 1978], *Betulaceae* [Pawlowska, 1980], *Lamiaceae* [Kokkalou and Kapetanidis, 1988] and *Papilionaceae*. Its presence in the bark of *Zanthoxylum avicennae* and *Z. cuspidatum* (family *Rutaceae*) has been reported [Arthur et al., 1956]. These plants are indigenous to Hong Kong. Hesperidin occurs in crystalline, feather-like aggregates or sphaerocrystalline masses in the cells [Evans, 1996]. Di Mauro et al., [1999] have

recently described a novel procedure for obtaining hesperidin from waste orange peel of the citrus industry based on the adsorption of dilute extracts of hesperidin on a styrene divinyl benzene resin.

#### 1.13.3.2. Bioavailability and absorption

The oral bioavailability of hesperidin is limited most probably due to its crystalline state, this flavanoid is slightly soluble in water (57mg/L), a characteristic which leads to a very low dissolution rate and an irregular absorption of the drug from oral solid dosage form in the gastrointestinal tract. Furthermore, hesperidin also possesses poor transmembrane permeability and is believed to be absorbed primarily by the paracellular pathway. The metabolic fate of hesperidin and hesperetin were studied following oral ingestion in rats. The major metabolic product in the urine was m-hydroxyphenylpropionic acid along with lesser amounts of m-coumaric acid and the aglycones. The aglycones were free as well as conjugated with glucuronic acid. This indicated that absorption had occurred from the intestinal tract followed by dehydroxylation, demethoxylation or demethylation followed by dehydroxylation to yield m-hydroxyphenylpropionic acid. This study also indicated that hesperetin was more readily absorbed than hesperidin in rats, rabbits, as well as in humans [Booth et al., 1958]. When human volunteers ingested hesperidin, a marked difference was observed in the metabolism. 3-Hydroxy-4-methoxyphenylhydracrylic acid was obtained as the major urinary metabolite, indicating that the pyran ring of hesperetin splits to yield this hydracrylic acid. A small amount of the glucuronide of hesperetin was also detected [Booth et al., 1958; Williams, 1964]. Hesperidin was found to be transformed to its aglycone, hesperetin, in the intestine by the bacteria producing alpha-rhamnosidase and beta-glucosidase or endo-beta-glucosidase. It was also found that the anti-platelet activity and cytotoxicity of the metabolite formed in the human intestine was more pronounced than that of the parent compound [Kim et al., 1998]. The average daily intake of hesperidin and hesperetin has been estimated to be 10-28 mg.

### **1.14. Objectives of the study**

Objectives of the present study are:

- (i) To investigate antioxidant potential of the citrus flavonoids and their glycoconjugates under oxidative stress induced by tertiary butyl hydrogen peroxide in skeletal muscle cell line

(ii) Evaluation of the antidiabetic potential of the citrus flavonoids under oxidative stress in skeletal muscle cell line

(iii) Elucidation of its molecular mechanism of action in skeletal muscle cell line

### **1.15. Significance of the study**

Studies on antidiabetic drugs, i.e., metformin, troglitazone and acarbose which were expected to act by abating insulin resistance and secondarily hyperglycemia, found to lower postprandial hyperglycemia by impairing carbohydrate absorption from the intestinal lumen without any direct effect on insulin resistance [Knowler et al., 2002; Buchanan et al., 2002]. Owing to the side effects of currently available drugs, there is a need to look for more efficacious agents with lesser side effects. Some clinical trials have demonstrated that treatment with vitamin E, vitamin C or glutathione, improved insulin sensitivity in insulin resistant individuals [Ceriello, 2000]. Indeed a variety of defects in serum antioxidant status has been reported in diabetic patients compared to healthy subjects [Rakonczay et al., 2003]. In recent studies, some drugs routinely used in the treatment of DM also demonstrated antioxidant effects. Strategies to prevent and ameliorate oxidative stress remain important in the overall treatment of insulin resistance and T2DM. WHO had estimated that approximately 80% of the Earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components owing to their lesser side effects.

Flavonoids though exist naturally as glycoconjugates they are extensively metabolised in humans, resulting in the formation of aglycones, methyl and sulphate derivatives which may have different properties than their parent compounds. Proper evaluation of the biological responses to physiologically relevant forms and concentrations of circulating flavonoids are pivotal for the potential health benefits of dietary flavonoids in humans. Goal of the present study apart from evaluating the antidiabetic potential of flavonoids is to investigate the change in biological activity between a aglycone and its glycosylated form. Currently used antidiabetic agents, such as, troglitazone lower blood glucose levels in patients after 18 h presumably through an increase in glucose transporter synthesis and not by stimulating glucose uptake acutely [Yonemitsu et al., 2011]. The exposure time of these flavonoids to cells would be restricted to 3 and 24 hours because of the much shorter half-life of flavonoids in human plasma [Moon et al., 2008]. A detailed analysis of insulin signalling pathways would reveal whether the antidiabetic

potential of these bioactive flavonoids is dependent or independent of oxidative stress modulation. This study may reveal the importance of citrus flavonoids as a dietary supplement in the management of diabetes and its associated pathophysiology.

### 1.16. Work flow

Schematic illustration (Fig 1.11) of the work is as follows:

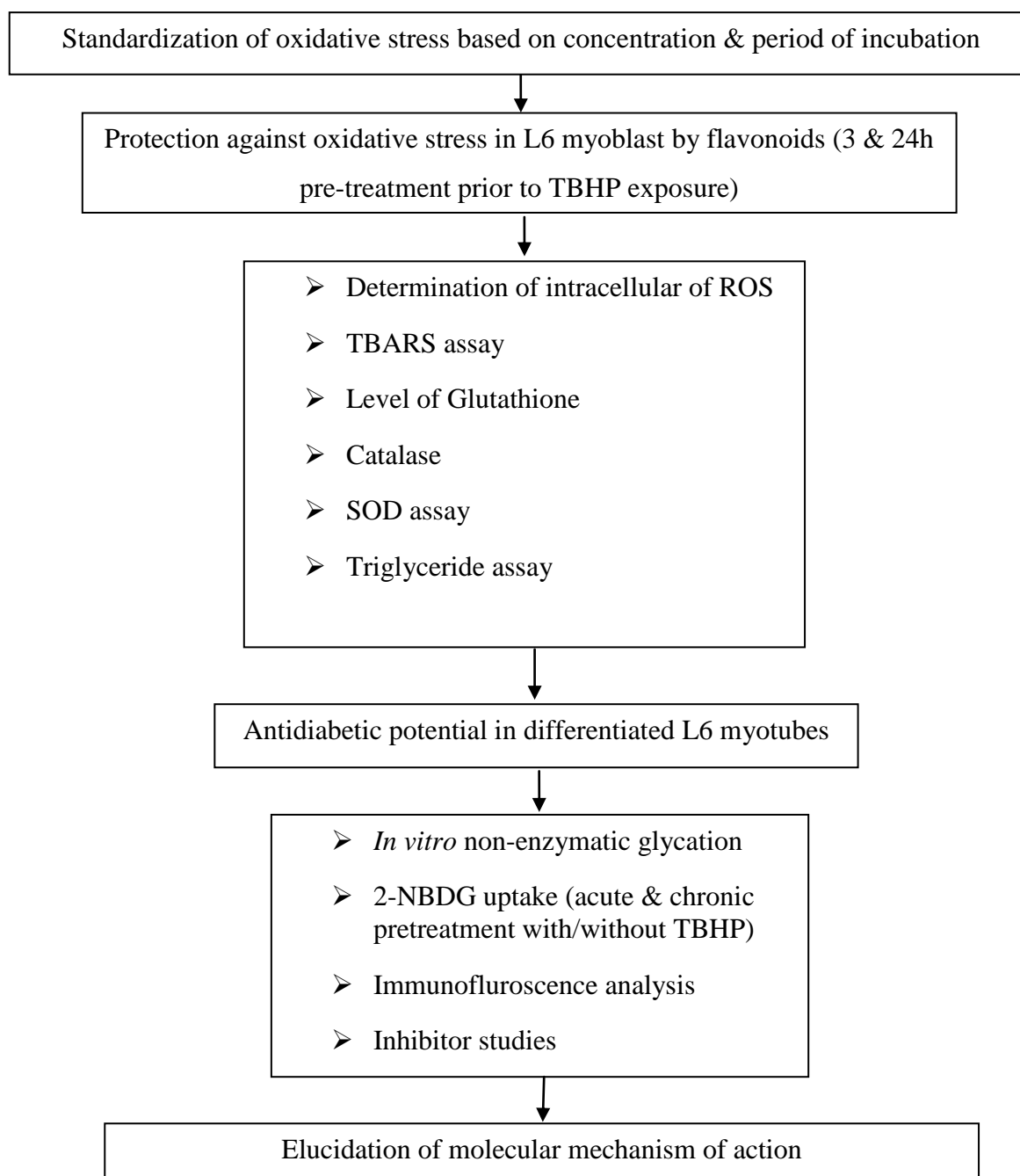


Fig 1.11. Schematic representation of the work flow

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IDF Diabetes Atlas Seventh Edition Update, International Diabetes Federation 2015.

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## **CHAPTER 2**

***Evaluation of the antioxidant effect of citrus flavonoids & their glycoconjugates in skeletal muscle cell line***

## 2.1 Introduction

The chronic hyperglycemia resulting from diabetes brings about a rise in oxidative stress due to overproduction of reactive oxygen species (ROS) as a result of glucose auto oxidation and protein glycosylation [Laaksonen and Sen, 2000]. Generation of ROS leads to oxidative damage of the structural components (such as lipids, DNA and proteins) of cells and potentiate diabetes related complications [Robertson, 2004]. The prolonged exposure of oxidative stress may cause insulin resistance by triggering an alteration in cellular redox balance [Mc Garry, 2002]. Over the past decade, investigators had shown tremendous interest in studying the role of oxidative stress markers in the genesis of diabetes and its associated complications. Defective antioxidant system has been reported in diabetic patients compared to healthy subjects [Likidilid et al., 2010]. Oxidative insult in cells is also created by the impairment in functioning of endogenous antioxidant enzymes because of their non enzymatic glycosylation and oxidation [Chi-Hao et al., 2010]. Absence of compensatory endogenous antioxidant system results in the activation of stress sensitive signalling pathways. This cause cellular damage and are ultimately responsible for diabetic complications. Despite strong experimental evidence indicating that oxidative stress may determine the onset and progression of late-diabetic complications, controversy exists between the cause and associative relationship between oxidative stress and diabetes mellitus [Joseph et al., 2002; Mustafa and David, 2002; Renu and Mamta, 2009; Nweke et al., 2011]. This is partly because measurement of oxidative stress is usually based on indirect and nonspecific measurement of products of reactive oxygen species.

Kazuhiko et al., [2011] found increased oxidative stress as measured by plasma thiobarbituric acid reactive substances (TBARS) at rest and after exercise in young men with Type 1 Diabetes mellitus. TBARS level was significantly increased in T2DM with the duration of disease and development of complications [Rolando and Marisela, 2013]. Induction of oxidative stress may occur through different mechanisms. Glutathione, an important antioxidant agent which detoxifies ROS directly or in a glutathione peroxidase catalyzed mechanism was found to be decreased in different phases of T2DM [Khalid, 2007]. Most studies have also found decreased blood or red cell glutathione levels in T2DM patients. It has been reported that some antioxidants such as vitamins C and E, lipoic acid, antioxidative enzymes, and others reduced hyperglycemia



induced biological changes including cytokine expression and matrix synthesis [Abdulkadir and Thanoon, 2012; Ayman et al., 2012; Ehsaneh, and Ahmad, 2012]. Srinivas and Menon [2003] reported that the antioxidant drug bis-o-hydroxycinnamoyl methane imparts protection to cells against ROS mediated damage by enhancing antioxidants and reduces hyperglycemia in streptozotocin (STZ) induced diabetes. Defect in antioxidant defense system is in close association with induction of lipid peroxidation [Jagetia and Baliga, 2003]. Several lines of evidence suggest that oxidative stress occurs in diabetes and could have a role in the development of insulin resistance [Liang et al., 2002; Lee et al., 2006; Thandavarayan et al., 2009]. The cause and cellular mechanism responsible for this abnormality is not fully understood despite of intense investigative efforts. However it is unknown whether oxidative stress is the cause or consequence of diabetes.

Last decade has witnessed enormous scientific studies on the heterogeneous class of molecules called phytochemicals. They are widely distributed in fruits, vegetables, beverages and in herbal remedies. The wide range of biological activities remains uncharacterized for most compounds. To suppress the oxidative stress mediated damage in diabetic pathophysiology, a special focus has been shifted towards naturally occurring antioxidants present in food. These come mainly from clinical studies, in which potential metabolic effects of pharmacological doses of antioxidants given to healthy diabetic volunteers were evaluated, but failed to conclusively establish a cause and effect relationship between the two. Consequently, the therapeutic potential of antioxidative agents has been raised [Jennalynn et al., 2012; Wei et al., 2012]. Flavonoids are an important component of most edible vegetables and fruits constituting a significant portion of the diet, have emerged as potential alternatives for treating diabetes, hyperlipidemia and oxidative stress, involving multiple signaling pathways. Flavonoids were found to scavenge efficiently the model free radicals of 2, 2-diphenyl-1-picrylhydrazyl and  $\alpha$ ,  $\gamma$ -bisdiphenylene- $\beta$ -phenylallyl [Vincent et al., 2007]. However, its effect on oxidative stress-induced diabetic pathophysiology is not well explored. Flavonoids though exist naturally as glycoconjugates they are extensively metabolized in humans, resulting in the formation of aglycones, methyl and sulfate derivatives that may have different properties than their parent compounds.

Quercetin is a major flavonoid in the human diet and its daily intake with foods is estimated to be 50–500 mg [Claudine et al., 2004]. Though the antioxidant nature of Quercetin and Rutin has been studied extensively [Kim et al., 2011] their beneficial effect on oxidative stress related

diabetic pathophysiology and its complications have been less explored. Naringin treatment is reported to upregulate the gene expression of GPx in high cholesterol fed rat [Jeon et al., 2002]. Hesperidin is an important constituent of citrus fruits and has been isolated from *Citrus aurantium* [Kakadiya et al., 2010]. Hesperidin is reported to have antiallergic, anticarcinogenic, antihypertensive, antimicrobial, and vasodilator properties [Garg et al., 2001]. Although metabolic transformation of hesperidin in humans is well understood, [Nielsen et al., 2006] relatively little is known about the biological activities of Hesperidin and its aglycone, hesperetin.

## **2.2. Materials and methods**

### **2.2.1. Materials**

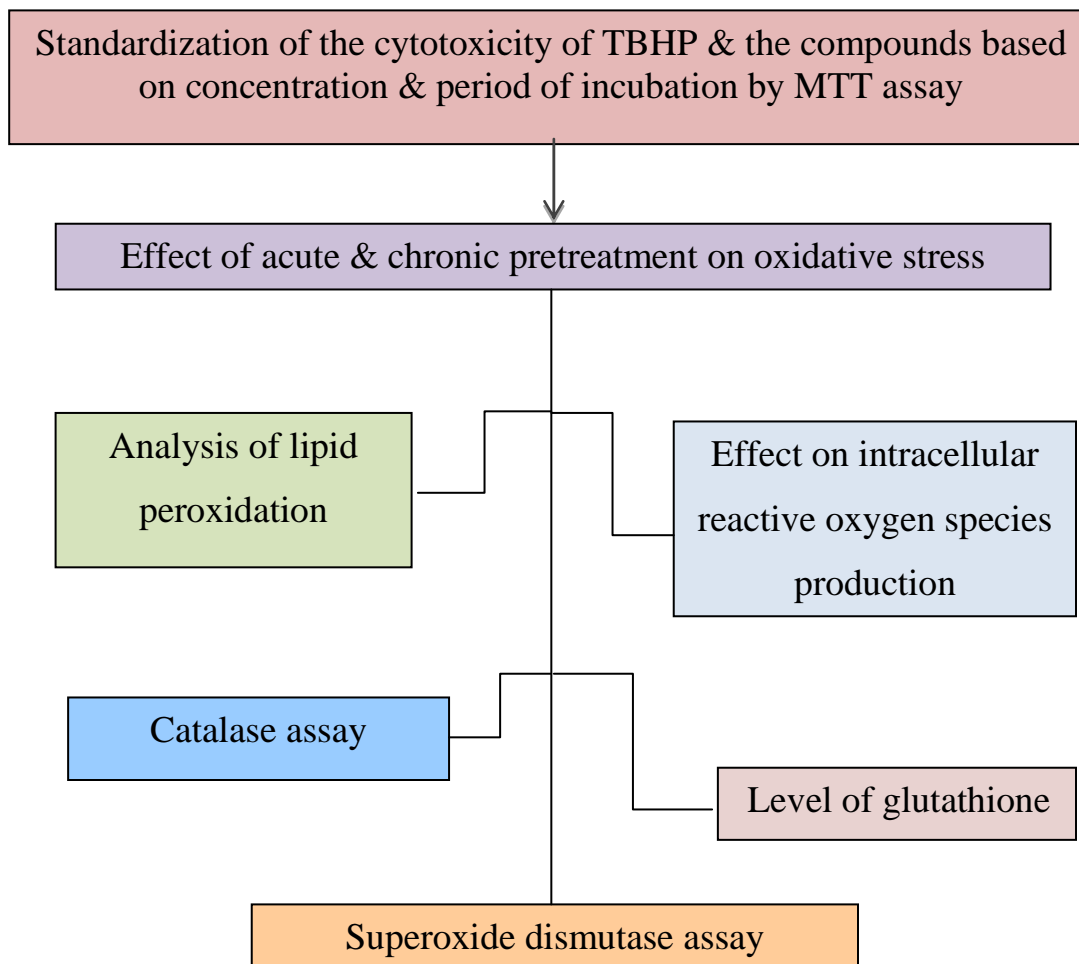
Dulbecco's modified Eagle's media (DMEM), bovine serum albumin, a streptomycin ampicillin–amphotericin B mix, insulin, rosiglitazone, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), thiobarbituric acid, dichloro fluorescence diacetate (DCFH-DA), Phosphate buffer saline (PBS), malonaldehyde, glutathione (GSH), quercetin, rutin, naringenin, naringin, hesperidin, hesperetin, tertiary butyl hydrogen peroxide (TBHP) were purchased from Sigma–Aldrich Chemicals (St Louis, MO, USA); L6 myoblast was obtained from National Centre for Cell Sciences, Pune, India. All other chemicals used were of standard analytical grade.

### **2.2.2. Cell culture and treatment**

Rat skeletal muscle cell lines, L6 myoblasts, were maintained in DMEM supplemented with 10% FBS, 10% antibiotic-antimycotic mix at 37°C under 5% CO<sub>2</sub> atmosphere. Cells were grown at a density of 1x10<sup>4</sup> cells/well on 96-well black plates (BD Biosciences, Franklin Lakes, NJ) and 12-well plates (Costar, USA) for DCFDA assay and other antioxidant assays, respectively.

### 2.2.3. Experimental design

The work flow of this chapter is illustrated in the schematic representation in Fig 2.1.



*Fig 2.1: Schematic representation of Experimental design*

### 2.2.4. Cytotoxicity

Viability of L6 myoblast was measured by means of MTT assay. Cytotoxicity of TBHP and flavonoids were standardized based on both concentration as well as period of incubation. Briefly, cells after incubation with the compounds were washed and MTT (0.5 g/L), dissolved in DMEM, was added to the each well for the estimation of mitochondrial dehydrogenase activity as described previously [Mosmann, 1983]. After 4 h incubation at 37°C in CO<sub>2</sub> incubator, 10% SDS in DMSO was added to each well and the absorbance at 570 nm of

solubilized MTT formazan products were measured after 45 min using a microplate reader (BIOTEK-USA). Results were expressed as percentage of cytotoxicity.

### **2.2.5. Measurement of intracellular reactive oxygen species**

Intracellular reactive oxygen species (ROS) was estimated by using the fluorescent probe, DCFH-DA as described earlier by Cathcart et al., [1983]. Cells were preincubated with different concentrations of Flavonoids for 3 and 24 h followed by TBHP (100  $\mu$ M) for 3 h. After washing with PBS (pH-7.4) cells were treated with DCFH-DA (20 $\mu$ M) for 20 minutes. The cells were imaged after washing with PBS using fluorescent microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (i.e. excitation 490 nm; and emission 525 nm). The fluorescent intensity was analyzed by BD Image Data Explorer software.

### **2.2.6. Lipid peroxidation in L6 myoblast**

The effect of the flavonoids namely quercetin, rutin, naringin, naringenin, hesperidin & hesperetin on lipid peroxidation was analysed in L6 myoblast by the thiobarbituric acid method [Pavlica et al., 2009]. Briefly, after pretreatment of flavonoid, the cells were lysed and centrifuged at 13,000g for 2 min. Supernatant fraction (0.5ml) was mixed with 1ml of 0.67% thiobarbituric acid dissolved in 5% trichloroacetic acid. The mixture was heated at 100°C for 15 minutes. After cooling, the absorbance was read at 532nm. Lipid peroxidation was expressed as nanomoles of malonaldehyde (MDA) per million cells, using the MDA extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### **2.2.7. Determination of intracellular glutathione (GSH) concentration**

Reduced GSH levels in the cells were measured by the method of Hissin and Hilf [1976] using o-phthalaldehyde (OPT) as a fluorescent reagent. The method takes advantage of the reaction of GSH with OPT at pH 8. Cells following pretreatment were lysed in the presence of metaphosphoric acid and centrifuged at 5000 rpm for 10 mins. The supernatant was mixed with 100 $\mu$ l OPT along with 1ml of PBS. After 15 mins, fluorescence was determined at excitation wavelength of 360 nm and emission wavelength of 460 nm. Results were expressed as nanomoles of GSH.

### **2.2.8. Superoxide Dismutase (SOD) assay**

Significant amount of superoxide dismutase (SOD (EC 1.15.1.1)) in cellular and extracellular environments are crucial for the prevention of diseases linked to oxidative stress. SOD also appears to be important in the prevention of other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's Diseases [Liu, 1996; Maier and Chan, 2002]. The reaction catalyzed by SOD is extremely fast having a turnover of  $2 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$  and the presence of sufficient amounts of the enzyme in cells and tissues typically keeps the concentration of superoxide very low. Quantification of SOD activity is therefore essential in order to fully characterize the antioxidant capabilities of a biological system. After respective pretreatment of L6 myoblast, cell lysate was collected and SOD activity (U/ml) was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine following the manufacturer's instruction (Cayman chemical, USA).

### **2.2.9. Catalase assay**

Catalase (EC1.11.1.6) catalyses the decomposition of hydrogen peroxides to give water and molecular oxygen. Catalase activity was determined according to previously reported method [Abei, 1974]. Decomposition of  $\text{H}_2\text{O}_2$  can be followed directly by decrease in absorbance at 240nm. Difference in absorbance per unit time is a measure of catalase activity. Absorbance of cell lysate in substrate solution (10 Mm  $\text{H}_2\text{O}_2$  prepared in 50 mM phosphate buffer, pH 7) were compared with those of a blank. The reaction was initiated by the addition of substrate solution and incubated at 20°C for about 1 min. Catalase activity was expressed as  $\text{mmol H}_2\text{O}_2^{-1}\text{min}^{-1}\text{mg protein}$ .

### **2.2.10. Statistical analysis**

Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ( $n = 3$ ) of three different experiments. Data were subjected to one-way ANOVA and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at  $P \leq 0.05$ .

## 2.3. Results and discussion

### 2.3.1. Standardization of cytotoxicity of TBHP and the flavonoids

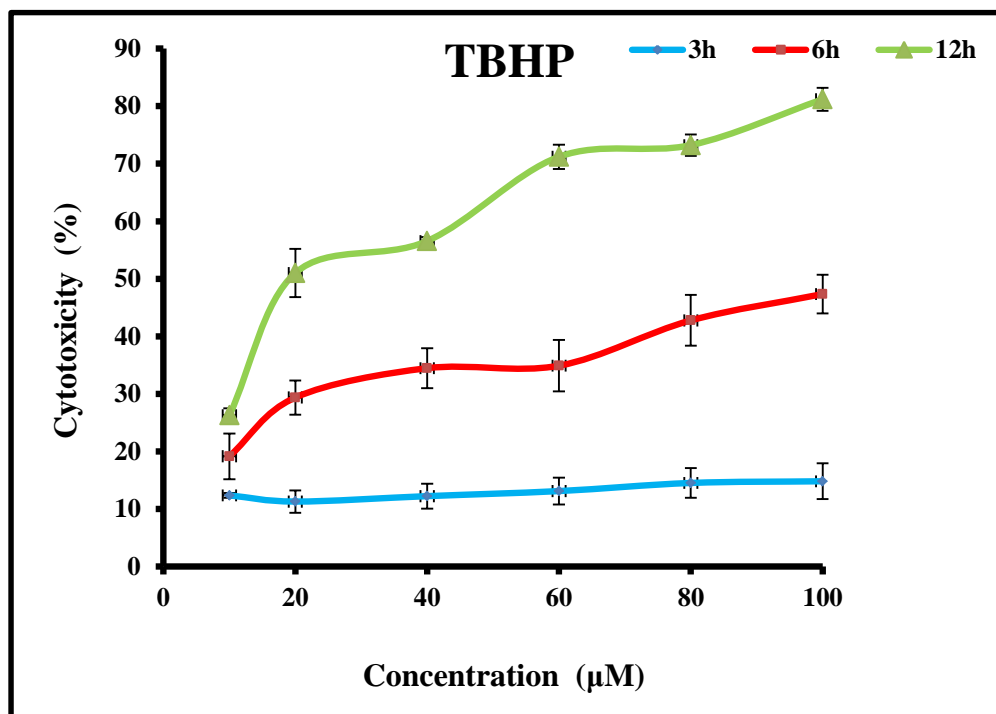
Cytotoxicity of all the six flavonoids and TBHP was standardized by MTT assay based on concentration and period of incubation.

#### 2.3.1.1. Cytotoxicity of TBHP

The cytotoxicity of TBHP was determined by MTT assay as described previously. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO) and absorbance of the released, solubilised formazan reagent was measured at 570 nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The cytotoxicity of TBHP was standardised based on concentration as well as period of incubation. TBHP below 100  $\mu\text{M}$  concentrations were found to be less than 20% toxic for a period of 3 h but on incubating for 12 h the L6 myoblast showed a toxicity of 80% as shown in Table 2.1 and Fig 2.2.

*Table 2.1. MTT based cytotoxicity of TBHP*

Concentration ( $\mu\text{M}$ )	Cytotoxicity (%)		
	3h	6h	12h
10	12.34 $\pm$ 0.41	19.14 $\pm$ 0.31	26.26 $\pm$ 0.40
20	11.27 $\pm$ 0.23	29.36 $\pm$ 0.26	51.01 $\pm$ 0.35
40	12.21 $\pm$ 0.52	34.46 $\pm$ 0.47	56.56 $\pm$ 0.27
60	13.09 $\pm$ 0.33	34.89 $\pm$ 0.36	71.21 $\pm$ 0.31
80	14.50 $\pm$ 0.26	42.76 $\pm$ 0.25	73.23 $\pm$ 0.15
100	14.80 $\pm$ 0.51	47.34 $\pm$ 0.32	81.20 $\pm$ 0.43



**Fig 2.2. Cytotoxicity of TBHP**

Toxicity of TBHP in L6 myoblast was dependent on concentration as well as period of incubation. The results are represented in terms of mean  $\pm$  SD of three determinations.

### 2.3.1.2. Cytotoxicity of flavonoids

All the three flavonoids, namely quercetin, naringenin, hesperetin and their glycoconjugates on incubation for 24 hours showed a toxicity which was less than 15% (Fig. 2.3). The toxicity of quercetin (13.72 %) was around two fold higher than that of rutin (7.2%). The toxicity of naringenin was 12.56 % which was higher than its aglycone, naringenin (10.48%) as shown in Fig 2.4. Cytotoxicity of hesperidin (13.01%) was in par with hesperetin (12.45 %) as illustrated in Table 2.2 and Fig 2.5. Based on the results of MTT assay, all the compounds up to a concentration of 100  $\mu$ M was taken for further studies and the treatment period was fixed to 3h (acute) & 24h (chronic) for the compounds and 3h for TBHP.

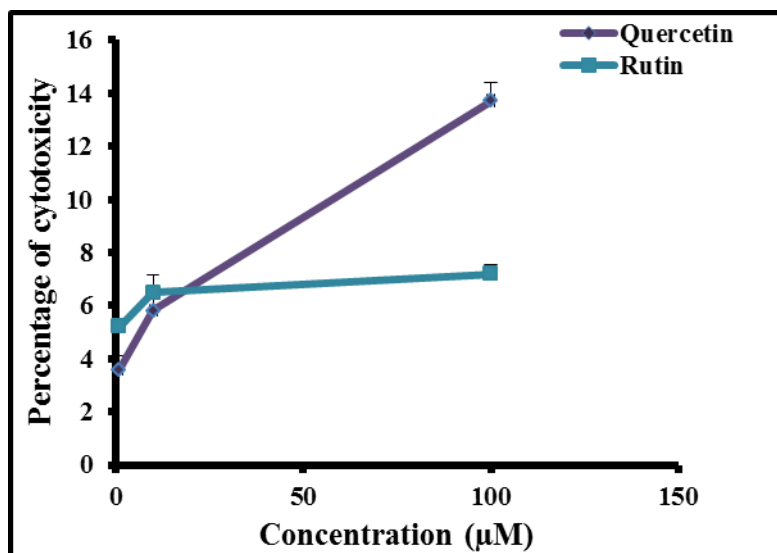


Fig.2.3

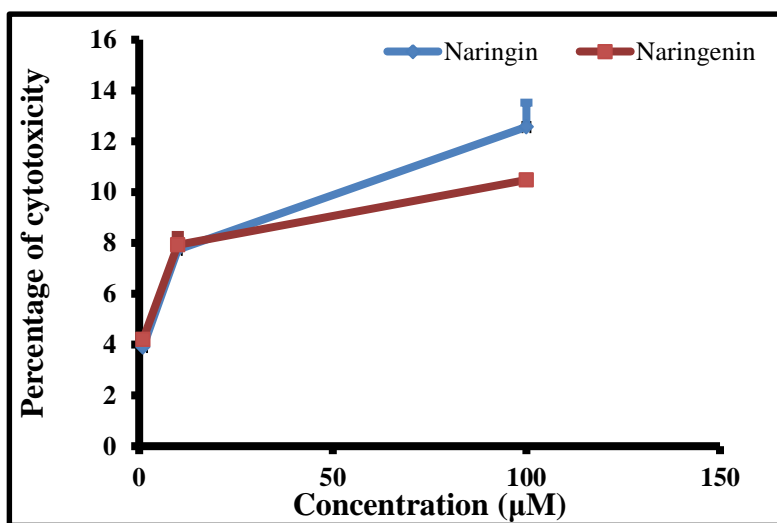


Fig 2.4



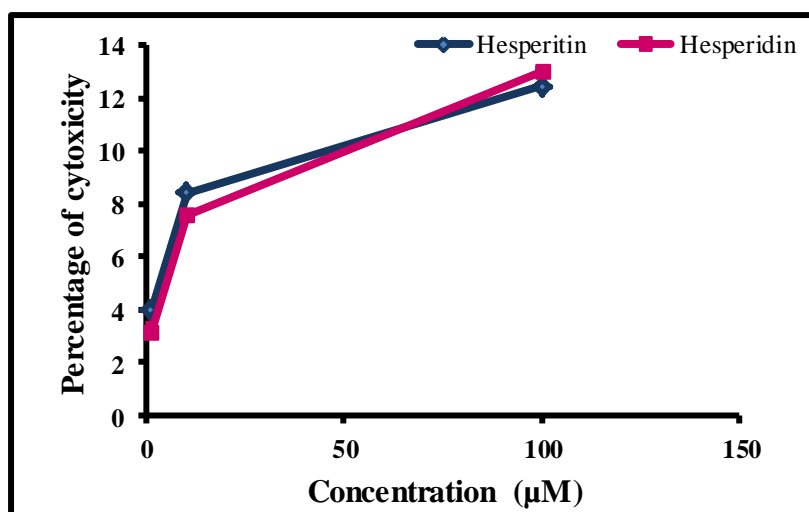


Fig 2.5

*Fig 2.3-2.5. Cytotoxicity of quercetin, rutin, naringin, naringenin, hesperidin & hesperetin on incubation of 24 h, respectively*

*All the flavonoids showed a dose dependent increase in toxicity. The results are represented in terms of mean  $\pm$  SD of three determinations.*

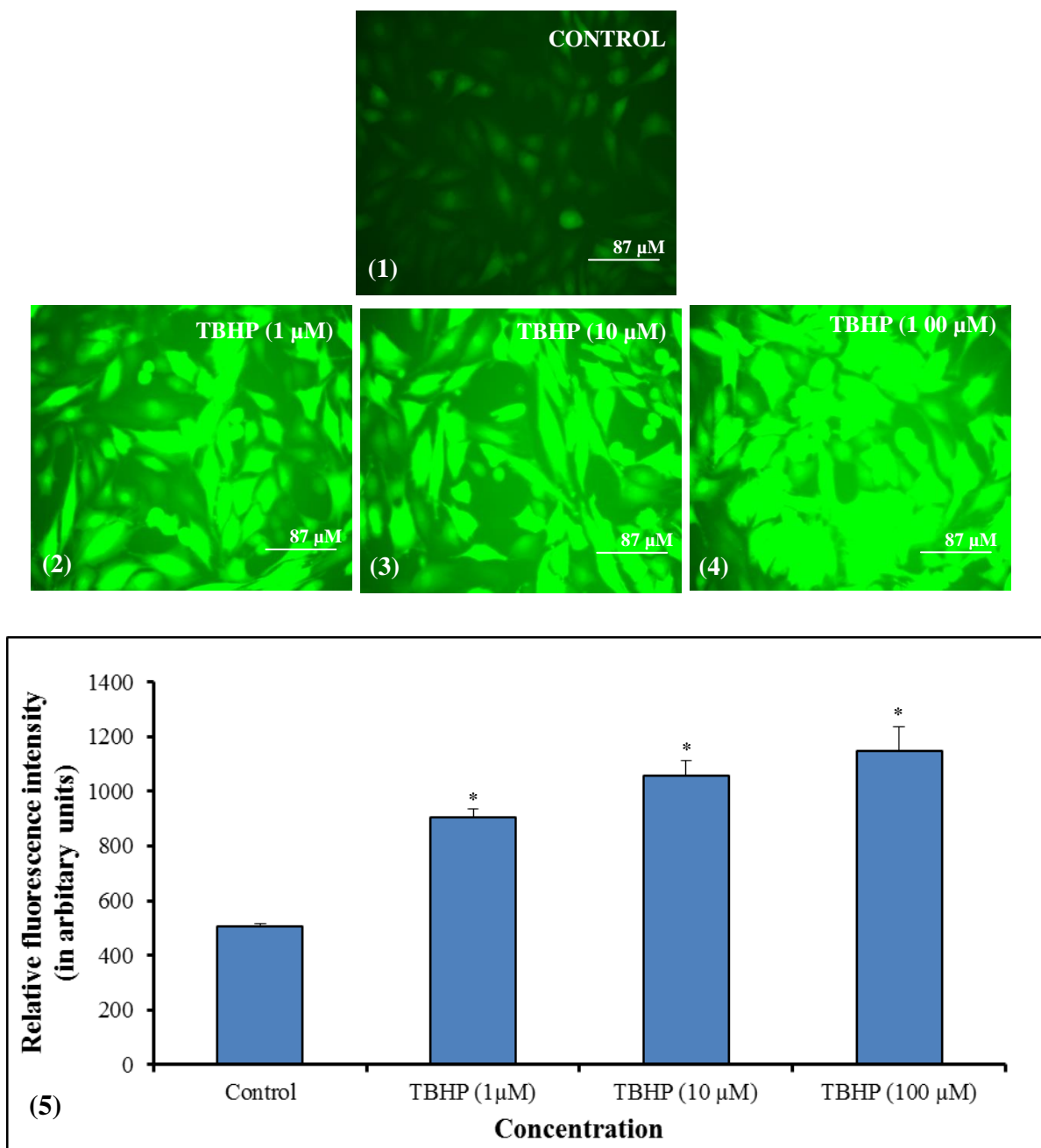
*Table 2.2. MTT based cytotoxicity of flavonoids*

Treatment	Cytotoxicity (%)		
	1	10	100
Concentration (µM)			
Quercetin	3.60 $\pm$ 0.32	6.60 $\pm$ 0.90	13.72 $\pm$ 0.41
Rutin	5.20 $\pm$ 0.24	6.50 $\pm$ 0.54	7.20 $\pm$ 0.53
Naringin	3.90 $\pm$ 0.31	7.92 $\pm$ 0.46	12.56 $\pm$ 0.35
Naringenin	4.20 $\pm$ 0.42	7.92 $\pm$ 0.32	10.48 $\pm$ 0.44
Hesperetin	3.98 $\pm$ 0.53	7.58 $\pm$ 0.51	12.45 $\pm$ 0.95
Hesperidin	3.13 $\pm$ 0.51	7.58 $\pm$ 0.42	13.01 $\pm$ 0.43

### 2.3.2. Determination of intracellular ROS

To establish precise role in antioxidant activity, cellular reactive oxygen species (ROS) was quantified with DCFDA as described by Cathcart et al., [1983]. DCFDA is a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell. After diffusion in to the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' dichloro fluorescein (DCF). DCF is a highly fluorescent compound which can be detected with maximum excitation and emission spectra of 495nm and 529nm, respectively. The two major sources of cellular ROS are complex I (NADH dehydrogenase ubiquinone-ubiquinol reductase) and complex III (ubiquinol cytochrome c reductase), both part of the mitochondrial electron transport chain. These two complexes generate ROS particularly when electron transport is slowed by high mitochondrial membrane potential ( $\Delta\psi_m$ ). The major product of ROS in mitochondrial is in the form of superoxide and hydroperoxyl radical. The exact source is less known and it is believed to be due to electron leakage from its iron-sulphur clusters. Low levels (or optimum levels) of ROS play an important role in signalling pathways. However when ROS production increases and overwhelms the cellular antioxidant capacity, it can induce macromolecular damage (by reacting with DNA, proteins and lipids) and disrupt thiol redox circuits. In the first instance, damage can lead to apoptosis or necrosis. Disruption of thiol redox circuits can lead to aberrant cell signalling and dysfunctional redox control electron transport which allows for the ubisemiquinone anion radical to react with oxygen dissolved in the membrane. ROS generated in cells is usually neutralized by enzymatic and non-enzymatic antioxidant defense system. Decrease in antioxidant defense system is in close association with induction of diabetic complications, atherosclerosis and associated cardiovascular disease.

To investigate the effect of flavonoids on oxidative stress associated with diabetes mellitus we induced stress in L6 skeletal muscle cells by using TBHP. The results from the present study demonstrate that TBHP incubation to the cells at 1, 10 & 100  $\mu\text{M}$  for 3 h generated intracellular ROS in a dose dependent manner (Fig 2.6 (2), (3) & (4)) as compared to control (Fig 2.6 (1)). TBHP at 100  $\mu\text{M}$  for 3 h showed significant increase in intracellular ROS as shown in Fig 2.6 (4) and this concentration and incubation time was used to induce oxidative stress condition for further studies. The intensity of fluorescence was analysed by BD Image Data Explorer software and has been illustrated in Fig. 2.6 (5).

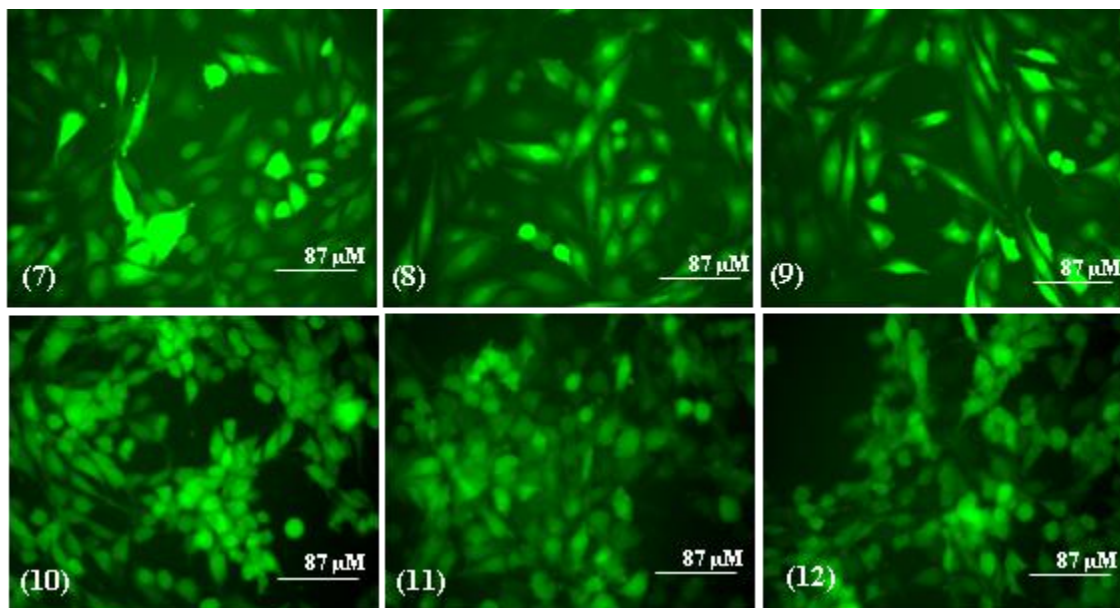
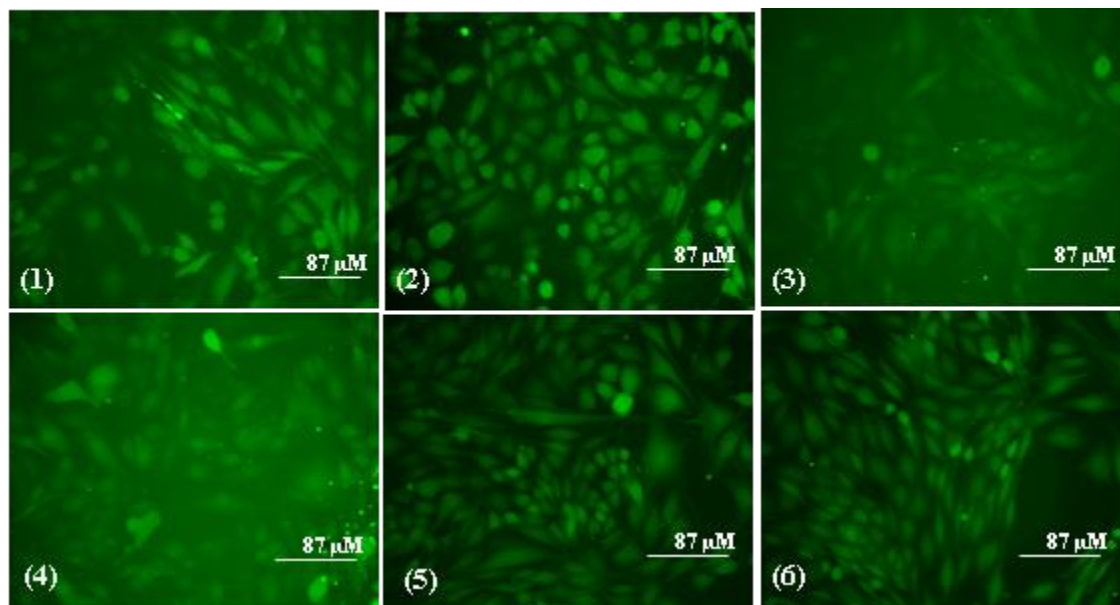


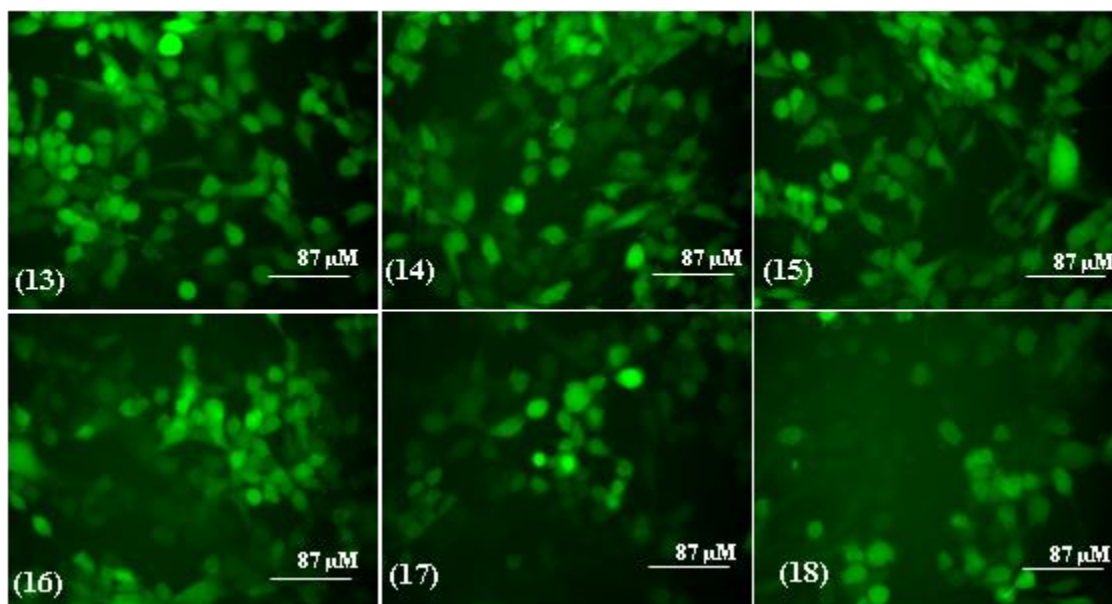
**Fig 2.6. Intracellular ROS production and fluorescence intensity analysis in L6 myoblast**

Control – (1) Untreated cells; (2), (3) & (4) - cells treated with TBHP (1, 10 & 100  $\mu\text{M}$ ) for 3h; (5) Bar diagram shows the relative fluorescence intensity analyzed by BD Image Data Explorer software. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ). Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test. \* $P \leq 0.05$  versus Control.

### 2.3.2.1. Acute pretreatment

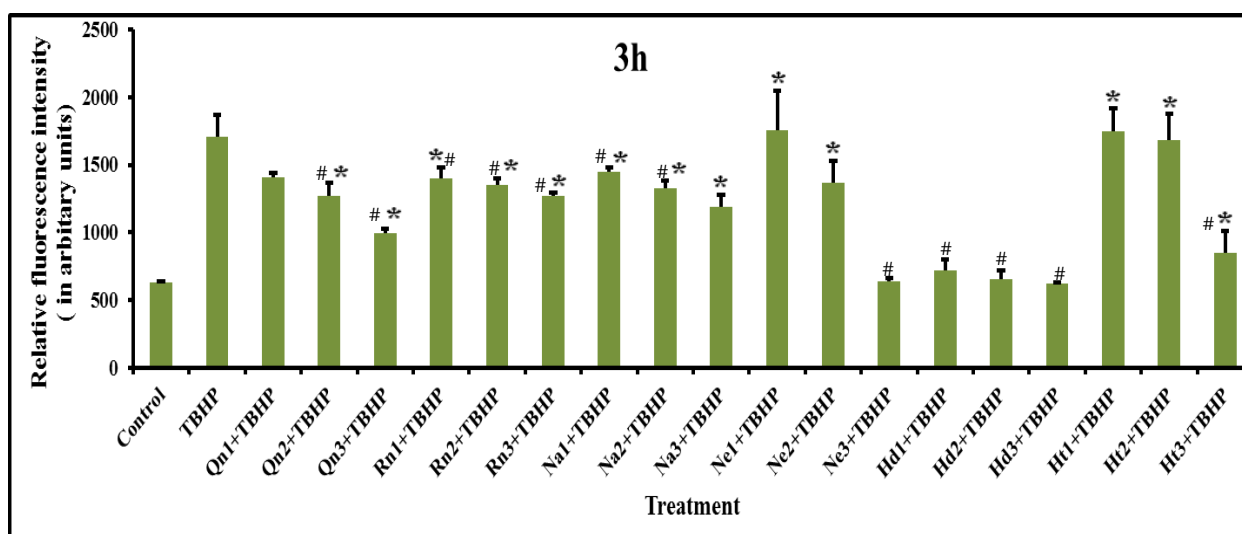
Oxidative stress generated by ROS is known to be the cause of diabetic complications and is linked to various degenerative diseases such as ageing, cancer, atherosclerosis and various inflammatory diseases. Human antioxidant defence system consisting of activity of SOD, catalase, glutathione-S- transferase and GSH [Bandyopadhyay, 1999]. SOD catalyses breakdown of endogenous cytotoxic superoxide radicals to H<sub>2</sub>O<sub>2</sub> which is further degraded by catalase. Thus they play an important role in cellular defense against free radicals [Arivazhagan, 2000]. Over the last decade, medicinal plants as potential sources of naturally occurring antioxidants have been the focus of research. Quercetin and rutin has been explored for its antioxidant activity earlier but here we made a comparative evaluation of antioxidant effect of the flavonoids quercetin & rutin, hesperidin & hesperetin, naringin & naringenin in L6 myoblast induced with TBHP. Pretreatment of all the flavonoids and their aglycones were found to reduce the oxidative stress generated within cells. The oxidative stress induced increase in ROS concentration decreased drastically ( $P \leq 0.05$ ) on preincubation with rutin and its aglycone, quercetin (1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) for 3h in a dose dependent manner as shown in Fig 2.7. Quercetin (100  $\mu$ M), rutin (100  $\mu$ M), naringenin (100  $\mu$ M) & hesperidin (10 & 100  $\mu$ M) showed two fold decrease in fluorescence intensity compared to TBHP induced cells as shown in Fig 2.7 (3), (6), (12), (14) & (15), respectively. Effect of naringin pretreatment (3h) on reduction of intracellular ROS was significantly higher than that of its aglycone, naringenin (Fig. 2.7 (9)). However, the magnitude of antioxidant potential of both hesperidin and hesperetin on pretreatment was comparable. The relative fluorescent intensity was analysed by BD Image Data Explorer software and has been illustrated in Fig. 2.8.





**Fig 2.7. Intracellular ROS production in L6 myoblast on acute pretreatment of citrus flavonoids**

L6 myoblast were pretreated with quercetin, rutin, naringin, naringenin, hesperidin & hesperetin for 3 h followed by induction with TBHP. Fluorescent images (20X magnification) shown above represent the cells pretreated with quercetin (1, 10 & 100  $\mu\text{M}$ ) - (1), (2) & (3); rutin (1, 10 & 100  $\mu\text{M}$ ) - (4), (5) & (6); naringin (1, 10 & 100  $\mu\text{M}$ ) - (7), (8) & (9); naringenin (1, 10 & 100  $\mu\text{M}$ ) - (10), (11) & (12); hesperidin (1, 10 & 100  $\mu\text{M}$ ) - (13), (14) & (15); hesperetin (1, 10 & 100  $\mu\text{M}$ ) - (16), (17) & (18); for 3h and then induced with TBHP. Scale bar corresponds to 87  $\mu\text{M}$ .



**Fig 2.8. Fluorescent intensity analysis by BD Image Data Explorer software**

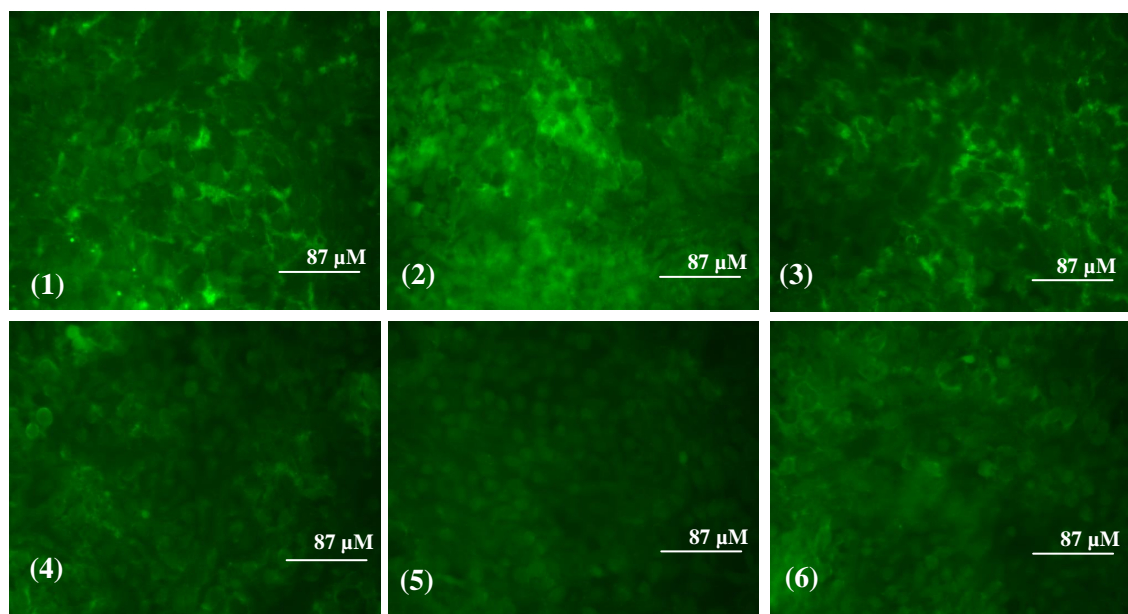
L6 myoblast pretreated with quercetin, rutin, naringin, naringenin, hesperidin & hesperetin for 3 h followed by induction with TBHP. TBHP: Tertiary buty hydrogen peroxide; Qn (1, 2, 3): quercetin (1, 10 & 100  $\mu\text{M}$ ); Rn (1, 2, 3): rutin (1, 10 & 100  $\mu\text{M}$ ); Na (1, 2 & 3): naringin (1, 10 & 100  $\mu\text{M}$ ); Ne (1,2 & 3): naringenin (1, 10 & 100  $\mu\text{M}$ ); Hd (1, 2 & 3): hesperidin (1, 10 & 100  $\mu\text{M}$ ); Hl (1, 2 & 3): hesperetin (1, 10 & 100  $\mu\text{M}$ ).

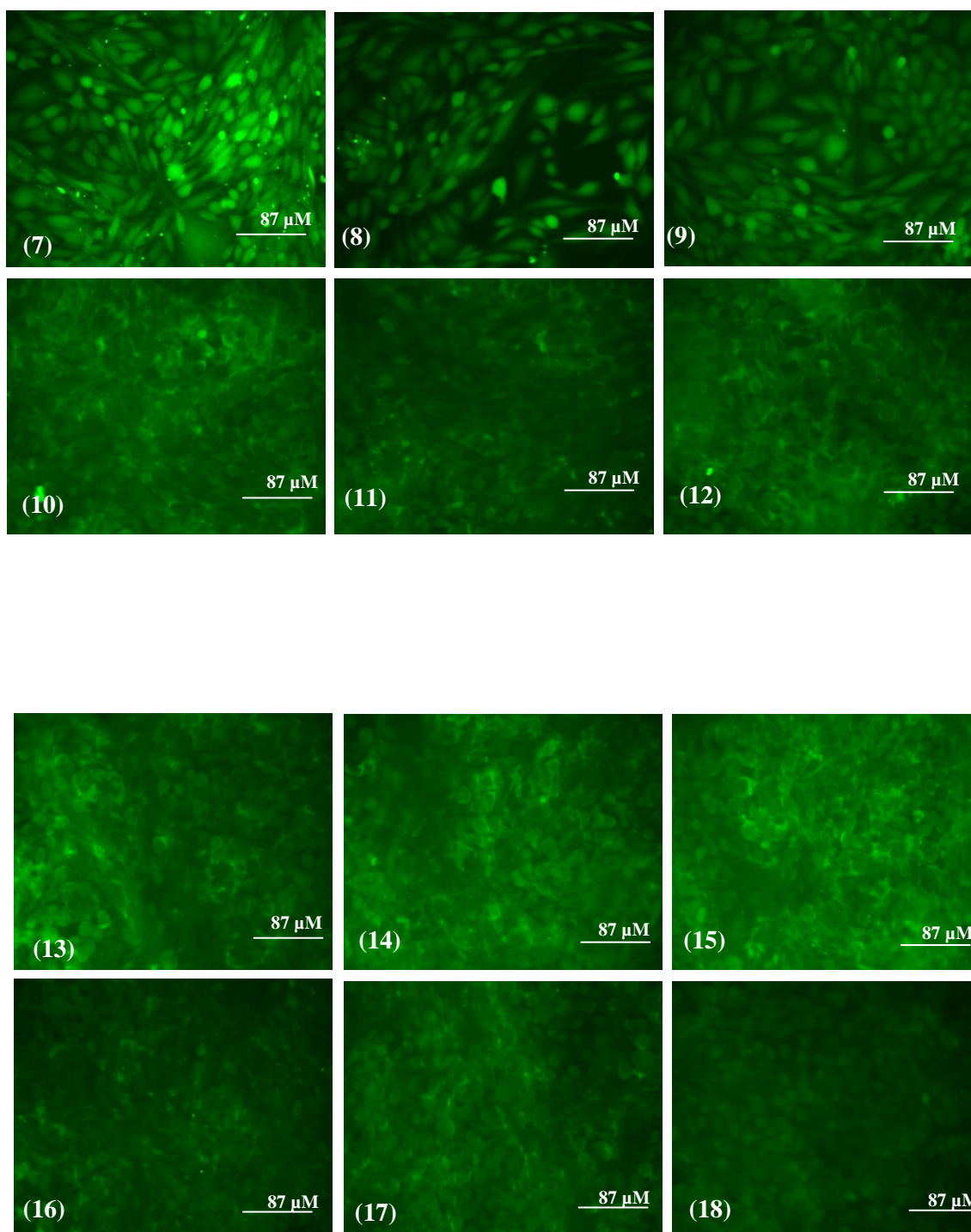


*Hd* (1,2 & 3): hesperidin (1, 10 & 100  $\mu\text{M}$ ); *Ht* (1,2 &3): hesperetin (1, 10 & 100  $\mu\text{M}$ ). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test. \* $P \leq 0.05$  versus Control; #  $P \leq 0.05$  versus TBHP.

### 2.3.2.2. Chronic pretreatment

As the period of pretreatment increased to 24 h, the ROS generation in cells reduced drastically as shown in Fig 2.9. The cells pretreated with all the flavonoids showed a one to two fold decrease in fluorescence intensity at 100  $\mu\text{M}$  as evident in Fig 2.9 (3), (6), (9), (12), (15) & (18). This decrease in fluorescence may be either due to the direct scavenging of free radicals or by the upregulation of antioxidant defence system. The fluorescence intensity was analysed by BDI explorer software and illustrated in Fig 2.10.

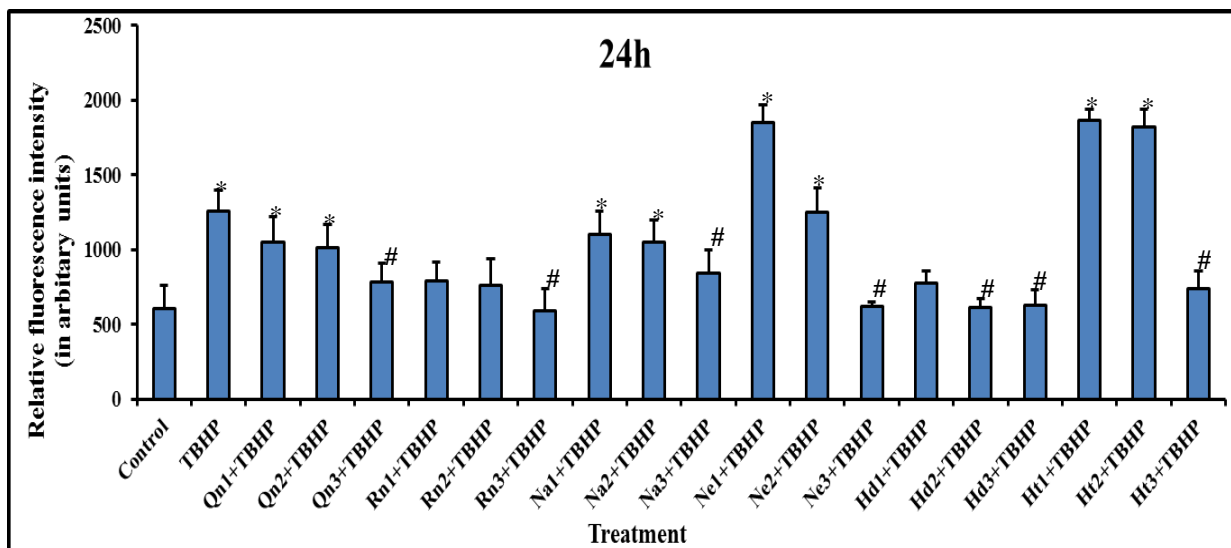




**Fig 2.9: Intracellular ROS production in L6 myoblast on chronic pretreatment of citrus flavonoids**

Fluorescent images (20X magnification) of cells pretreatment with quercetin (1, 10 & 100  $\mu\text{M}$ ) - (1), (2) & (3); rutin (1, 10 & 100  $\mu\text{M}$ ) - (4), (5) & (6); naringin (1, 10 & 100  $\mu\text{M}$ ) - (7), (8) & (9); naringenin (1, 10 & 100  $\mu\text{M}$ ) - (10), (11) & (12); hesperidin, & 1, 10 & 100  $\mu\text{M}$ ) - (13), (14) & (15); hesperetin (1, 10 & 100  $\mu\text{M}$ ) - (16), (17) & (18) for 24h and then induced with TBHP. Scale bar corresponds to 87  $\mu\text{M}$ .





**Fig 2.10. Fluorescence intensity analysis by BD Image Data Explorer software**

L6 myoblast pretreated with quercetin, rutin, naringin, naringenin, hesperidin & hesperetin for 24 h followed by induction with TBHP. TBHP: tertiary buty hydrogen peroxide; Qn (1, 2, 3): quercetin (1, 10 & 100  $\mu$ M); Rn (1, 2, 3): rutin (1, 10 & 100  $\mu$ M); Na (1, 2, 3): naringin (1, 10 & 100  $\mu$ M); Ne (1,2,3): naringenin (1, 10 & 100  $\mu$ M); Hd (1,2,3): hesperidin (1, 10 & 100  $\mu$ M); Ht (1,2,3): hesperetin (1, 10 & 100  $\mu$ M); with TBHP. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \* $P \leq 0.05$  versus Control; #  $P \leq 0.05$  versus TBHP.

### 2.3.3. Influence in lipid peroxidation

Increased oxidative stress as measured by indices of lipid peroxidation has been reported to be increased in both insulin dependent diabetes (IDDM), and non-insulin dependent (NIDDM) even in patients without complications [Cederberg et al., 2001]. Serum MDA levels were higher in patients with newly diagnosed T2DM than in matched controls. Furthermore, MDA formation was tightly correlated with oxidative stress associated with T2DM [Baynes and Thorpe, 2000]. We have observed 49.7% increase in the level of malonaldehyde, a byproduct of lipid peroxidation than that of untreated control on induction of oxidative stress with TBHP for 3 h. Thus the main target of ROS appears to be polyunsaturated fatty acids, the precursor of lipid peroxidation. Kazuhiko et al., [2011] found increased oxidative stress as measured by plasma TBARS at rest and after exercise in young men with T1DM. TBARS level was significantly increased in T2DM with the duration of disease and development of complications [Rolando and Marisela, 2013]. Pretreatment with quercetin at 100  $\mu$ M restricted the percentage production of malonaldehyde to 16.6%. Pretreatment of rutin (100  $\mu$ M) to the cells significantly reduced the

malonaldehyde level than that of control (Table 2.3). Chronic pretreatment of naringin significantly decreased TBARS levels and our results are in accordance with Rajadurai et al., [2009] who stated that naringin ameliorates mitochondrial lipid peroxidation in isoproterenol induced myocardial infraction in wistar rats.

*Table 2.3: GSH, MDA levels in L6 myoblast on flavonoid pretreatment*

Treatment & Dose	MDA (in nmol)		GSH (in nmol)
	24h	3h	24h
<b>Control</b>	0.25±0.099		23.91± 2.13
<b>TBHP</b>	0.53±0.019*		15.78 ±1.75*
<b>Quercetin (1µM)+TBHP</b>	0.32±0.088*#	19.25±0.99*#	26.46±1.07#
<b>Quercetin (10 µM)+TBHP</b>	0.30±0.078*#	21.51±1.01*#	28.29±0.97#
<b>Quercetin (100 µM)+TBHP</b>	0.29±0.055*#	23.49±1.20#	29.78±0.49*#
<b>Rutin (1 µM )+TBHP</b>	0.35±0.082*#	19.42±1.04*#	19.93±1.01
<b>Rutin (10 µM )+TBHP</b>	0.31±0.044*#	21.27±1.13*#	22.82±0.78#
<b>Rutin (100 µM )+TBHP</b>	0.21±0.051*#	23.03±1.11#	27.09±0.92#
<b>Naringin (1 µM)+TBHP</b>	0.39±0.088*#	18.17±0.99*	21.00±1.07#
<b>Naringin (10 µM)+TBHP</b>	0.36±0.078*#	25.00±0.06*#	25.00±0.97#
<b>Naringin (100 µM)+TBHP</b>	0.29±0.055*#	27.00±0.05*#	27.00±0.49#
<b>Naringenin (1µM)+TBHP</b>	0.53±0.012*	19.11±0.05*#	17.01±0.05*
<b>Naringenin (10 µM)+TBHP</b>	0.54±0.038*	23.12±0.05#	18.75±0.02*
<b>Naringenin (100 µM)+TBHP</b>	0.44±0.051*#	19.87±0.06*#	19.25±0.06*#
<b>Hesperidin (1 µM)+TBHP</b>	0.46±0.084*#	17.51±0.03*	16.05±0.07*
<b>Hesperidin (10 µM)+TBHP</b>	0.50±0.025*#	23.08±0.10#	19.25±0.01*#
<b>Hesperidin (100 µM)+TBHP</b>	0.49±0.021*#	23.54±0.09#	27.71±0.02#
<b>Hesperetin (1µM )+TBHP</b>	0.52±0.048*#	16.03±0.06*	18.5±0.081*
<b>Hesperetin (10µM )+TBHP</b>	0.43±0.081*#	19.22±0.02*#	19.39±0.02*#
<b>Hesperetin (100µM )+TBHP</b>	0.36±0.064*#	20.58±0.06*#	22.89±0.06#

*The levels of GSH and MDA were assayed after pretreatment with flavonoids and induction of oxidative stress in L6 myoblast. Each value represents mean ± SD (standard deviation) from triplicate measurements (n=3) and the significance accepted at P≤0.05. \*P≤0.05 versus Control; # P≤ 0.05 versus TBHP.*

#### **2.3.4. Role in GSH metabolism**

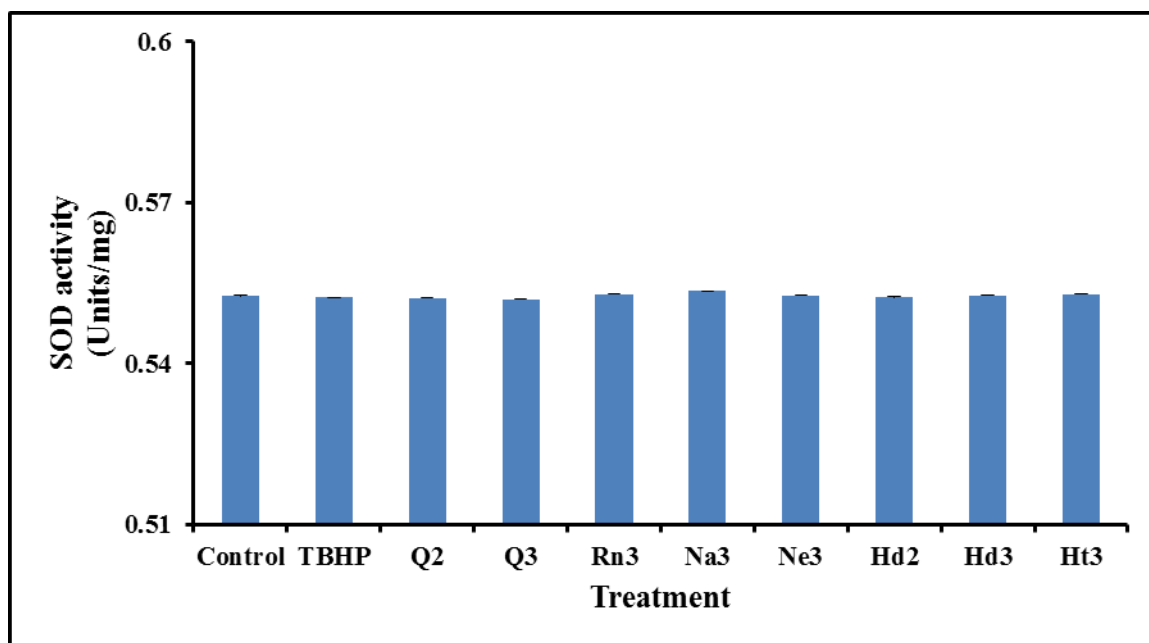
Induction of oxidative stress may occur through different mechanisms. Glutathione, an important antioxidant agent which detoxifies ROS directly or in a glutathione peroxidase catalyzed mechanism was found to be decreased in different phases of T2DM [Khalid, 2007]. Most studies have also found decreased blood or red cell glutathione levels in T2DM patients. It has been reported that some antioxidants such as vitamins C and E, lipoic acid, antioxidative enzymes, and others reduced hyperglycemia induced biological changes including cytokine expression and matrix synthesis [Abdulkadir and Thanoon, 2012; Ayman et al., 2012; Ehsaneh and Ahmad, 2012]. Srinivas and Menon [2003] reported that the antioxidant drug bis-o-hydroxycinnamoyl methane imparted protection to cells against ROS mediated damage by enhancing antioxidants and reducing hyperglycemia in streptozotocin (STZ) induced diabetes.

GSH, an abundant and ubiquitous antioxidant, functions mainly as an efficient intracellular reductant, protect cells from damage caused by free radical, drugs and radiation. To investigate the effect of flavonoids in natural antioxidant defence system of L6 cells, GSH levels were monitored after 3 h and 24 h pretreatment of flavonoids (1, 10, and 100  $\mu\text{M}$ ). On treating with 100  $\mu\text{M}$  TBHP, GSH level reduced by 66% compared to that of control as shown in Table 2.3. Decreased GSH on TBHP treatment may be due to increased utilization in protecting cells against oxidative stress. Pretreatment of L6 myoblast with quercetin (10 & 100  $\mu\text{M}$ ), rutin (100  $\mu\text{M}$ ), naringin (100  $\mu\text{M}$ ) and hesperidin (100  $\mu\text{M}$ ) actively upregulated GSH level from 50% to 70% and the magnitude of action of these compounds remained the same for both 3 & 24h pretreatment as shown in Table 2.3. Naringenin and hesperetin exhibited lesser antioxidant potential compared to their glycoconjugates.

#### **2.3.5. Superoxide dismutase assay**

Oxidative stress has been implicated in the etiology of T2DM. It has been suggested that the increase in serum SOD concentration associated with diabetes may cause decreased binding of the enzyme to the endothelium, resulting in the vascular wall being more vulnerable to oxidative damage. The serum extracellular SOD concentration was suggested to be a marker of diabetic vascular injury [Kimura et al., 2003]. High serum SOD activity is an indication of microangiopathic complications, particularly nephropathy [Mizobuchi et al., 1993].

To elucidate the antioxidant potential of flavonoids in L6 myoblast the activity of superoxide dismutase was analyzed on pretreatment of flavonoids. The results revealed that there was no significant change compared to basal enzyme activity on pretreatment with the six flavonoids. The level of SOD was similar to that of untreated cells as shown in Fig 2.11.



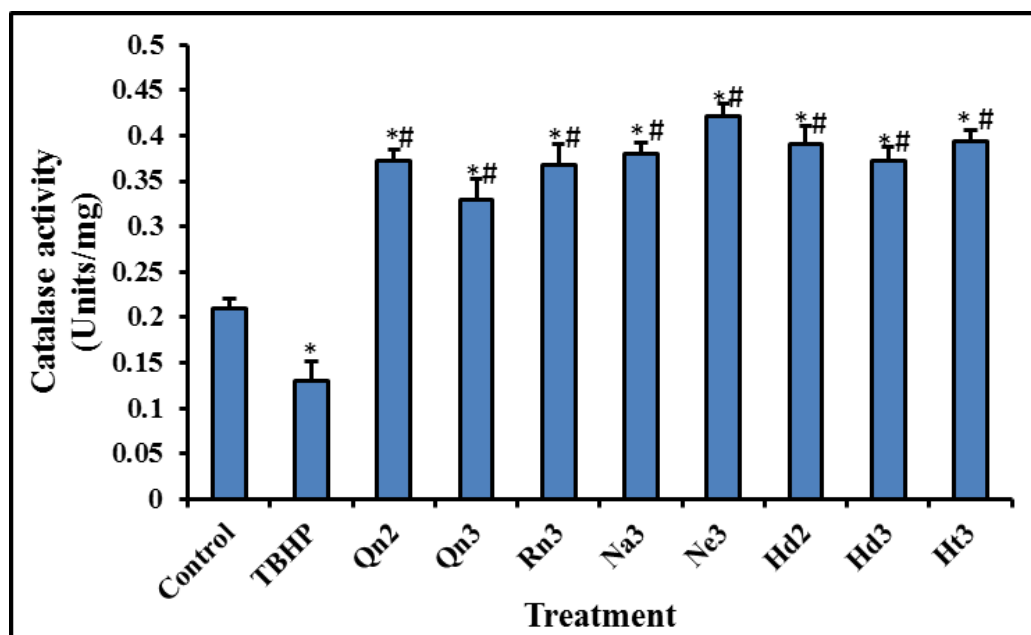
**Fig 2.11** SOD activity in L6 myoblast after pretreatment with flavonoids

The activity of SOD was assayed after treatment with flavonoids and induction of oxidative stress in L6 myoblast. Qn (2&3): quercetin (10 & 100  $\mu$ M); Rn3: rutin (100  $\mu$ M); Na (3): naringin (100  $\mu$ M); Ne (3): naringenin (100  $\mu$ M); Hd (2&3): hesperidin (10 & 100  $\mu$ M); Ht (3): hesperetin (100  $\mu$ M); with TBHP. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3).

### 2.3.6. Effect on catalase activity

On induction of oxidative stress, catalase activity in L6 myoblast reduced to 40% (0.13 Units/mg) compared to untreated control (0.21 Units/mg). Pretreatment of all the compounds nullified the effect of TBHP and even upregulated catalase activity in L6 myoblast as shown in Fig 2.12. Hesperidin (10 & 100  $\mu$ M) and hesperetin (100  $\mu$ M) upregulated the activity to 0.39, 0.373 & 0.39 respectively whereas quercetin (10 & 100  $\mu$ M) rutin (100  $\mu$ M), naringin (100  $\mu$ M) and naringenin (100  $\mu$ M) showed an increase in activity to 0.372, 0.33, 0.368, 0.38 & 0.421, respectively. This modulation in catalase activity may be an early adaptation to ROS. Naringin has been reported to improve the activities of catalase and superoxide dismutase in the heart of isoproterenol induced rats [Sathish et al., 2003]. Evidence states that elevated ROS production

decreased mitochondrial function and promoted insulin resistance in skeletal muscle [Ropelle, 2006; Choi, 2007; Koyes, 2008; Pienheiro, 2010]. Catalase overexpression was reported to improve insulin resistance in muscle cells chronically exposed to fatty acids by modulating mitochondrial function and, consequently, glucose and fatty acid metabolism [Marina, 2013].



**Fig 2.12. Catalase activity in L6 myoblast on pretreatment of flavonoids**

The activity of Catalase was assayed after pretreatment with flavonoids and induction of oxidative stress in L6 myoblast. L6 myoblast were pretreated with quercetin, rutin, naringin, naringenin, hesperidin & hesperetin for 24 h followed by induction with TBHP. TBHP: tertiary buty hydrogen peroxide; Qn (2&3): quercetin (10 &100  $\mu$ M); Rn3: rutin (100  $\mu$ M); Na (3): naringin (100  $\mu$ M); Ne (3): naringenin (100  $\mu$ M); Hd (2&3): hesperidin (10 &100 $\mu$ M); Ht (3): hesperetin (100  $\mu$ M); with TBHP. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) and the significance accepted at  $P\leq 0.05$ . \* $P\leq 0.05$  versus Control; # $P\leq 0.05$  versus TBHP.

## 2.4. Summary

In summary, all the tested citrus flavonoids namely quercetin, rutin, naringin, naringenin, hesperidin and hesperetin possessed significant antioxidant potential as all these compounds significantly reduced oxidative stress generated by TBHP in L6 myoblast. The increased ROS production is the central and major mediator of diabetes tissue damage, causing the activation of five pathways involved in the pathogenesis of diabetes complications, both microvascular and

cardiovascular. The experimental data discussed in this chapter demonstrate the cytoprotective effect of the tested compounds under oxidative stress induced by TBHP. The major findings are illustrated below.

- 📖 All the tested citrus flavonoids decreased intracellular ROS generated by TBHP.
- 📖 Quercetin, rutin, naringin & hesperidin retrieved and even upregulated the glutathione level in L6 myoblast under oxidative stress.
- 📖 The flavonoids remarkably reduced lipid peroxidation induced by TBHP.
- 📖 All the flavonoids increased catalase activity in L6 myoblast.

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## **CHAPTER 3**

*Antidiabetic potential of the compounds in the presence/absence of oxidative stress induced by tertiary butyl hydrogen peroxide*

### 3.1. Introduction

Diabetes mellitus is often defined as a hyperglycemic condition arising due to insulin resistance or impaired insulin secretion. The oxidative stress induced pathways is known to be associated with the onset of diabetes and its complications, which is the real cause of the morbidity and mortality associated with diabetes [Aleksandra et al., 2010]. Under hyperglycemia, the inhibition of ROS generation or its neutralization is reported to prevent the development of diabetic complications [Otero et al., 2004; Atli et al., 2004; Osawa and Kato, 2005; Shen et al., 2006; Vincent et al., 2007]. Extensive studies have shown that oxidative stress induce IRS serine/threonine phosphorylation, disrupts cellular distribution of insulin signaling components thereby decreasing GLUT 4 gene transcription or altering mitochondrial activity [Bloch and Bashan, 2005; Sesti, 2006]. In addition, Evans et al., [2003] had suggested the role of oxidative stress in generating insulin resistance by altering intracellular signaling pathway. Hyperglycemia can also contribute to the advanced glycation end products (AGE) formation. AGE's formed by the non-enzymatic glycation of free amino groups of protein will indirectly lead to the production of ROS. Glycation also inactivates enzymes involved in antioxidant defense system which results in the progression of diabetic complications. Moreover, the condition worsens due to relatively low expression of antioxidant enzymes like glutathione. It is therefore sensible to evaluate the potential usefulness of antioxidants in the treatment of T2DM.

Last decade has witnessed enormous scientific studies on the heterogeneous class of molecules called phytochemicals. They are widely distributed in fruits, vegetables, beverages and in herbal remedies. The wide range of biological activities remains uncharacterized for most compounds. To suppress the oxidative stress mediated damage in diabetic pathophysiology, a special focus has been shifted towards naturally occurring antioxidants present in food. These come mainly from clinical studies, in which potential metabolic effects of pharmacological doses of antioxidants given to healthy diabetic volunteers were evaluated, but failed to conclusively establish a cause and effect relationship between the two. Consequently, the therapeutic potential of antioxidative agents has been raised [Jennalynn et al., 2012; Wei et al., 2012]. Antidiabetic drugs like metformin are known to ameliorate hyperglycemia by lowering carbohydrate absorption without direct effect on insulin resistance [Knowler et al., 2002; Buchanan et al., 2002; Chiasson et al., 2002]. There is a need to look

for more efficacious agents for the management of diabetes, owing to the side effects of currently available drugs.

Flavonoids are an important component of most edible vegetables and fruits constituting a significant portion of the diet and have emerged as potential alternatives for treating diabetes, hyperlipidemia and oxidative stress, involving multiple signaling pathways. Flavonoids were found to scavenge efficiently the model free radicals of 2, 2-diphenyl-1-picrylhydrazyl and  $\alpha$ ,  $\gamma$ -bisdiphenylene- $\beta$ -phenylallyl [Butkovic et al., 2004]. However, its effect on oxidative stress-induced diabetic pathophysiology is not well explored. Flavonoids though exist naturally as glycoconjugates they are extensively metabolized in humans, resulting in the formation of aglycones, methyl and sulfate derivatives that may have different properties than their parent compounds. In the present study, we attempt to gain a better understanding of the therapeutic potential of citrus flavonoids, quercetin, naringin, hesperidin and their glycoconjugates rutin, naringenin and hesperetin on T2DM and other oxidative stress induced impairments by acute and chronic treatment of L6 cell lines followed by induction of oxidative stress by tertiary butyl hydrogen peroxide (TBHP). As pathogenicity associated with diabetes is believed to be due to oxidative damage to the tissues by oxygen free radicals, it is sensible to elucidate the antidiabetic potential of these potent antioxidants in the presence of induced oxidative stress (TBHP).

### **3.2. Materials and methods**

Monoclonal anti-GLUT 4 antibody, secondary anti-mouse immunoglobulin (IgG; Fab specific)-fluorescein isothiocyanate (FITC) antibody produced in goat were purchased from Santa Cruz Biotechnology, USA; Foetal bovine serum (FBS) was purchased from Gibco-BRL (Auckland, NZ); Horse serum was purchased from PAN Biotech (Aidenbach, Germany); 2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA). All other chemicals were of standard molecular biology grade.

#### **3.2.1. Cell culture and treatment**

Rat skeletal muscle cell lines, L6 myoblasts (NCCS, Pune) were maintained in DMEM supplemented with 10% FBS, 10% antibiotic-antimycotic mix at 37°C under 5% CO<sub>2</sub>

atmosphere. Cells were grown at a density of  $1 \times 10^4$  cells/well. For differentiation the cells were maintained in differentiation containing 2% horse serum for 5-7 days.

### 3.2.2. Experimental design

The work flow of this chapter is illustrated in the schematic representation in Fig 3.1.

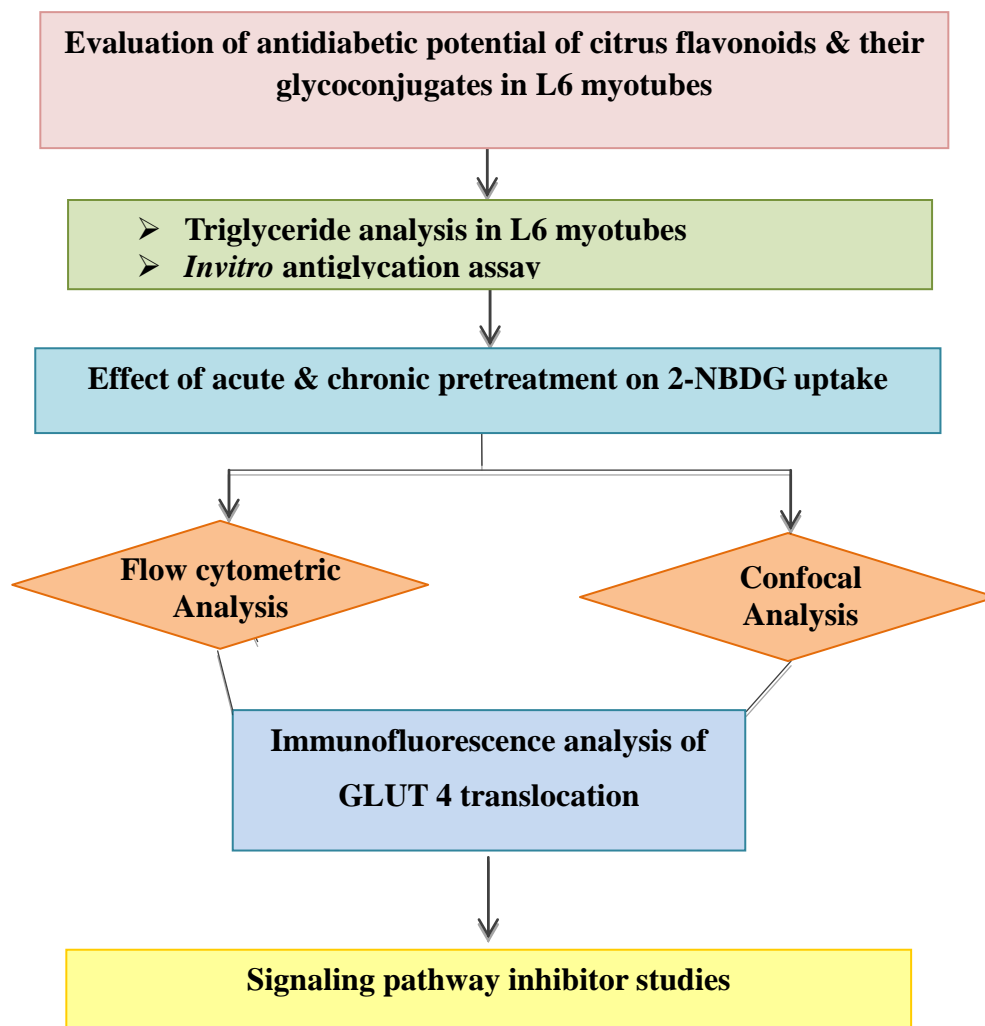


Fig. 3.1. Schematic representation of experimental design

### 3.2.3. Antiglycation assay

Antiglycation assay was performed according to method reported by Arom with slight modifications (Arom, 2005). Briefly about 500  $\mu$ l of albumin (1 mg/ml final concentration) was incubated with 400  $\mu$ l of glucose (500 mM) in the presence of 100 $\mu$ l of compound at different concentrations, the reaction was allowed to proceed at 60°C for 24 h and thereafter reaction was stopped by adding 10 $\mu$ l of 100% TCA. Then the mixture was kept at 4°C for 10 min before centrifugation (Kuboto, Japan) at 10000 g. The precipitate thus obtained was redissolved in 500  $\mu$ l alkaline PBS (pH 10) and immediately fluorescence intensity was read at 370 nm (excitation) and 440 nm (emission). Ascorbic acid was used as positive control. Results were expressed as percentage inhibition relative to control.

### 3.2.4. Triglyceride assay by Oil red O method

Accumulation of triglyceride inside the cells was determined by the Oil red O (ORO) staining of neutral lipids in a 24-well plate (Laughton, 1986). After the appropriate incubation with test compounds, myotube cultures were fixed with perchloric acid. Cells were then washed with distilled water and submerged with ORO dissolved in propylene glycol (2 mg/ml). The wells were washed with PBS three times after overnight incubation at room temperature. ORO was extracted using isopropanol for 10 min at room temperature. The absorbance was measured at 490 nm blanked to cell-free well. Results were expressed as percentage of control.

### 3.2.5. 2-NBDG uptake

L6 myoblast seeded in 96 well black clear bottom plates (BD Biosciences, Franklin Lakes, NJ) were differentiated for 5 days in DMEM containing 2% horse serum. Cell differentiation was evaluated by examining multinucleation. Following preincubation with flavonoids in low glucose DMEM (3 & 24h) and induction of stress, culture medium was removed from each well and replaced with fresh culture medium in the absence or presence of 10mM fluorescent 2-NBDG (Molecular Probes-Invitrogen), a fluorescent derivative of glucose with a 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)] amino group at the C-2 position and incubated for 30

minutes. All the set of cells were co-incubated with insulin. The cells were then washed twice with cold phosphate-buffered saline (PBS) and the fluorescence in cells were acquired by Fluorescent microscope (Pathway 855, BD Bioscience, San Jose, CA, USA) equipped with filters in the FITC range (i.e. excitation 490 nm; and emission 525 nm). The changes in glucose uptake in cells were analyzed using BD Image Data Explorer software.

### **3.2.6. Flow cytometric analysis of 2-NBDG uptake**

The changes in glucose uptake induced by pretreatment of flavonoid were confirmed by flow cytometry analysis. Following preincubation with flavonoid and oxidative stress induction, culture medium was removed from each well and replaced with fresh culture medium in the absence or presence of 10 mM fluorescent 2-NBDG and incubated for 30 min. The cells were then washed twice with cold phosphate-buffered saline (PBS), trypsinized, resuspended in ice-cold PBS and subjected to flow cytometry. Cell acquisition was performed on a FACS Aria II flow cytometer (BD Biosciences, San Jose, CA). 10,000 events were processed for each measurement at FITC range (excitation 490 nm, emission 525 nm band pass filter). The mean fluorescence intensity of different groups were analyzed by BD FACS Diva software and corrected for auto fluorescence from unlabeled cells.

### **3.2.7. Immunofluorescence assay**

Furthermore, to elucidate the mechanism by which flavonoid pretreatment altered glucose uptake, we measured surface GLUT 4 levels under various pretreatment conditions by immunofluorescence assay. Following pre-treatment with the flavonoid and TBHP, cells were washed with PBS and fixed for 5 min with 4% formaldehyde in PBS and quenched with 50 mM glycine in PBS for 10 min. Cells were blocked with 5% BSA in PBS for 1 h and incubated with monoclonal GLUT 4 antibody solution (1:200 dilution in 1.5% BSA in PBS) at 4°C overnight followed by washing with PBS and 1 h incubation at room temperature with FITC-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution, 1.5% BSA in PBS). The cells were then visualized using laser scanning confocal microscope (Nikon A1R, Nikon instruments, Melville, USA) equipped with filters in the FITC range (i.e. excitation 490 nm; and emission 525 nm) and relative fluorescence intensity was analyzed by NIS elements software.

### **3.2.8. Inhibitor studies**

For the evaluation of the effects of kinase inhibitors, fully differentiated L6 myotubes were starved in Serum free (SF) medium for 1.5 h, and then the inhibitors of AMPK (20  $\mu$ M Dorsomorphin) or PI3 kinase (100 nM wortmannin) were added, and cells were incubated for 30 min. Cells were then treated with quercetin (10 & 100 $\mu$ m), rutin (100 $\mu$ m), naringin (100 $\mu$ m), naringenin (100 $\mu$ m), hesperidin & hesperetin (100 $\mu$ m) for 24 h in SF-medium with/without corresponding inhibitors. The glucose uptake assay was initiated by the addition of 2-NBDG uptake. The cells were then washed twice with cold phosphate-buffered saline (PBS) and the fluorescence in cells were acquired by fluorescent microscope (Pathway 855, BD Bioscience, San Jose, CA, USA) equipped with filters in the FITC range (i.e. excitation 490 nm; and emission 525 nm). The changes in glucose uptake in cells were analyzed using BD Image Data Explorer software.

### **3.2.9. Statistical analysis**

Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ( $n = 3$ ) of three different experiments. Data were subjected to one-way ANOVA and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at  $P \leq 0.05$ .

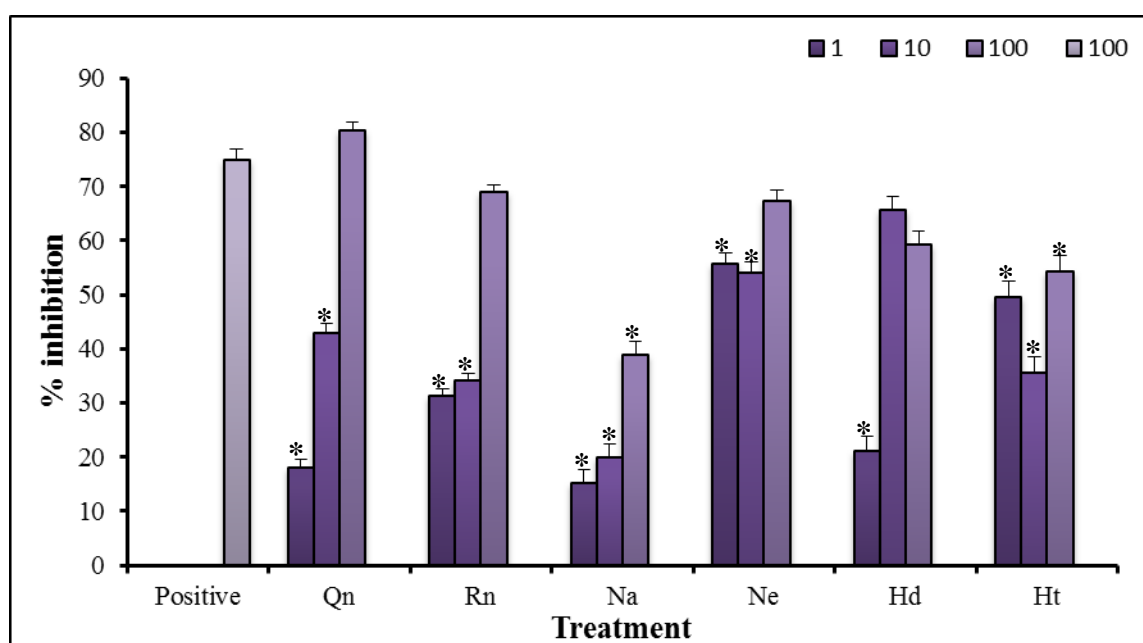
## **3.3. Results and Discussion**

### **3.3.1. Effect on non-enzymatic glycation**

Non-enzymatic glycosylation (glycation) between reducing sugar and protein results in the formation of advanced glycation end products (AGEs), which is believed to play important role in pathogenesis of diabetic complications and elicit oxidative stress. Thus, agents that inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes. AGE inhibitors, including aminoguanidine, improved diabetic complications in both animal models and clinical trials but possess numerous side effects (Thornalley, 2003).



All the flavonoids tested in the present study inhibited glycation of the protein albumin under *in vitro* conditions. Quercetin, rutin, naringin, naringenin, hesperidin and hesperetin showed an inhibition of 80.2, 69.03, 38.94, 67.41, 59.15 & 54.30 % respectively. The activity of quercetin, rutin, hesperidin, hesperetin and naringenin at 100  $\mu\text{M}$  was much higher than that of positive control ascorbic acid ( $\text{IC}_{50}$  - 30  $\mu\text{M}$ ) as shown in Fig. 3.2. Our findings was in accordance with Esmail et al., (2009) who reported inhibitory effect of rutin isolated from *Teucrium polium* on glycation of heamoglobin. Naringenin showed significant antiglycation property at all the concentrations tested whereas the effect of naringin was in a dose dependent manner. The effect of both hesperidin and hesperetin was comparable.



**Fig. 3.2. In vitro antiglycation analysis**

All the citrus flavonoids tested, inhibited glycation of the protein albumin and the effect of quercetin and rutin was higher than the positive control, ascorbic acid. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses positive control.

### 3.3.2. Triglyceride assay

The effect of the most active concentrations of citrus flavonoids on triglyceride levels in L6 myotubes were estimated after 24 h of pretreatment. All the flavonoids and their glycoconjugates significantly reduced the triglycerol level compared to control. Pretreatment with quercetin,

rutin, naringin, naringenin, hesperidin and hesperetin reduced triglyceride level to 18.58, 34.90, 24.42, 10.69, 39.54 & 45.69 % respectively (Fig. 3.3). Quercetin at 10  $\mu\text{M}$  showed a reduction of 14.7% and the effect of both quercetin and hesperidin was higher than that of rosiglitazone. Quercetin was earlier reported to decrease triglyceride in HEPG2 cells and in wistar rats [Vijyaya et al., 2012; Xiuli et al., 2013].

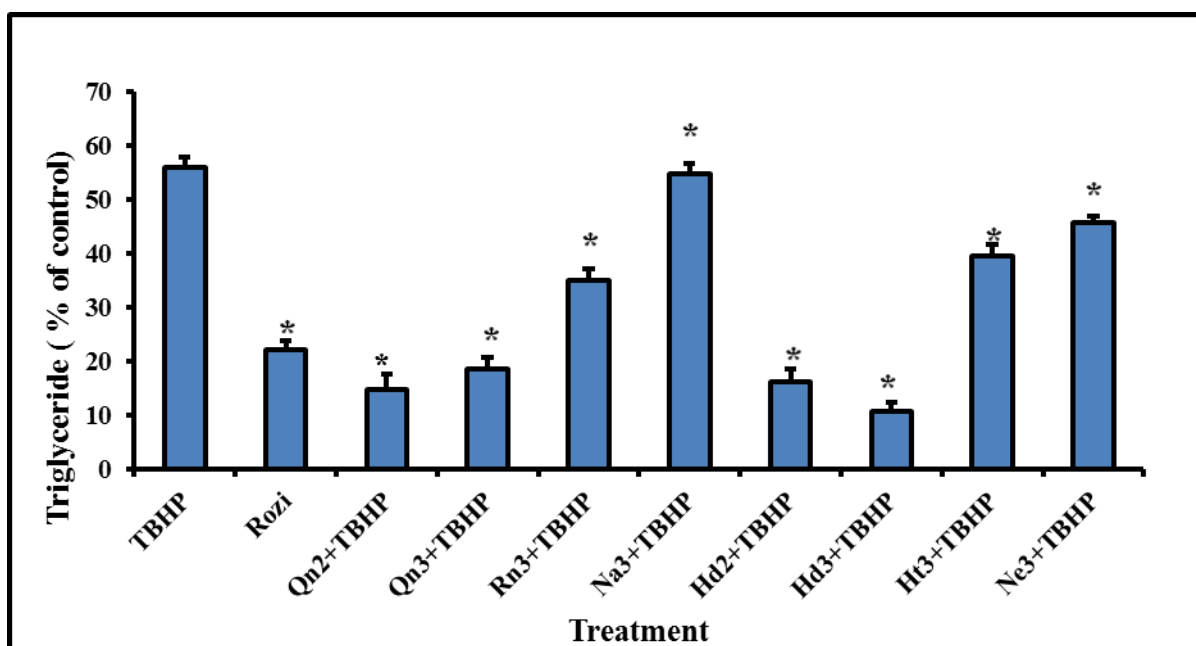


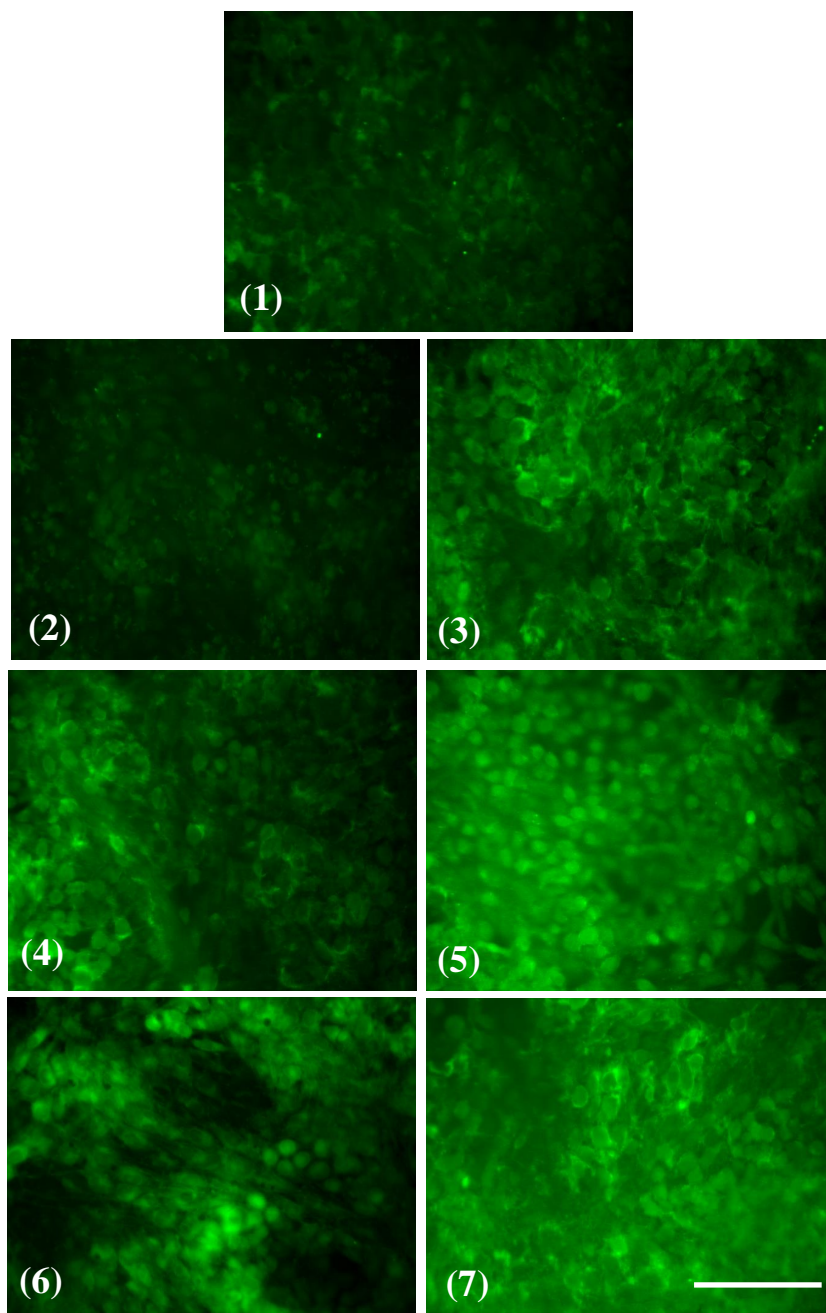
Fig. 3.3. Effect on triglyceride level in L6 myotubes

TBHP: Tertiary butyl hydrogen peroxide (100  $\mu\text{M}$ ); Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu\text{M}$ ); Rn (3): rutin (100  $\mu\text{M}$ ); Na3: naringin (100  $\mu\text{M}$ ); Hd (2,3): hesperidin (10 & 100  $\mu\text{M}$ ); Ht3: hesperetin (100  $\mu\text{M}$ ). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses TBHP.

### 3.3.3. Effect of citrus flavonoid pretreatment on 2-NBDG uptake

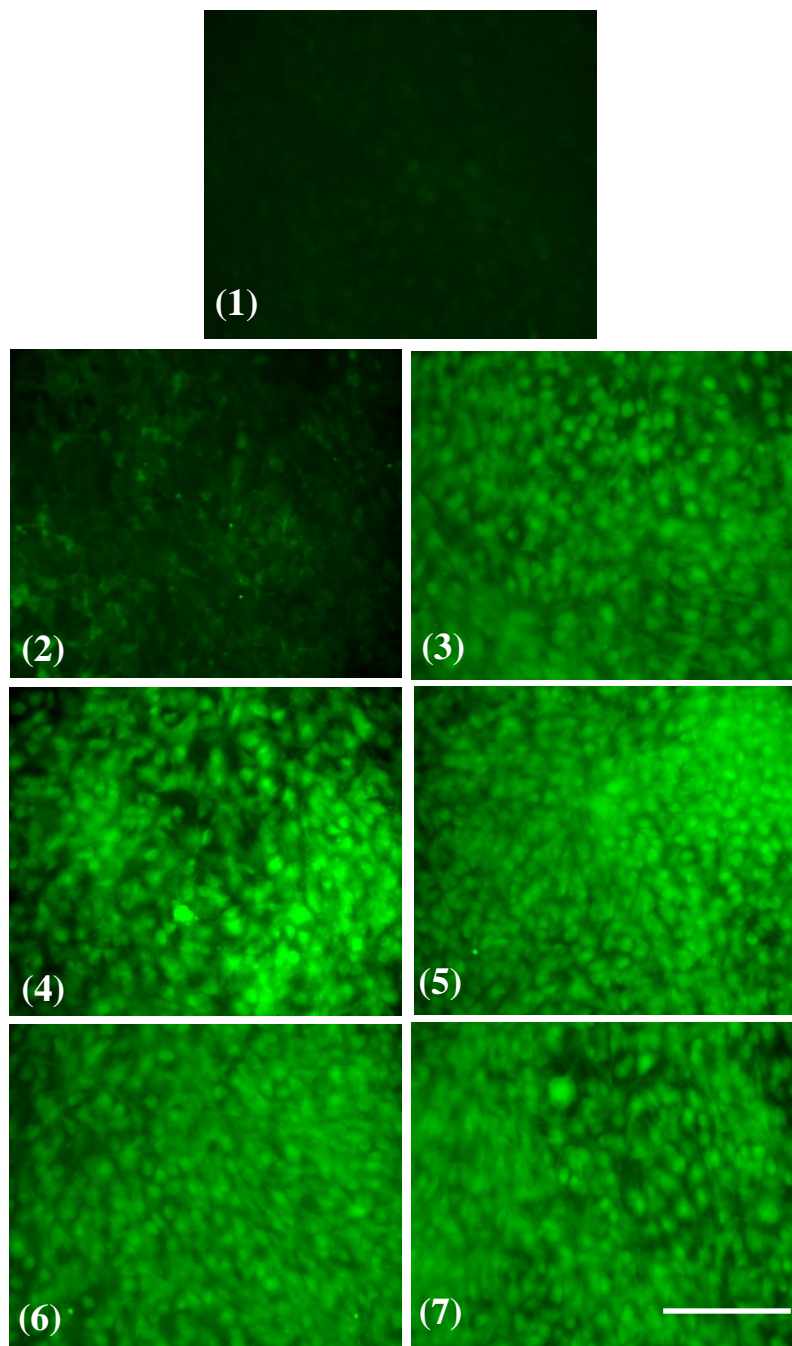
The assay is based on direct incubation of cells with a fluorescent D-glucose analog 2-NBDG. Antidiabetic potential of citrus flavonoids was evaluated by monitoring 2-NBDG uptake in differentiated L6 myoblast. Evaluation of glucose uptake ability in cells plays a significant role in understanding the efficacy of citrus flavonoids in the management of diabetes mellitus. The endocytosis of the fluorescent glucose analog 2-NBDG by rat L6 cells and increased intracellular 2-NBDG fluorescence was visible in distinct groups of cells as shown in Fig. 3.4. Quercetin and rutin (10  $\mu\text{M}$  & 100  $\mu\text{M}$ ) pretreatment enhanced glucose uptake ( $P \leq 0.05$ ) following acute (3h)

(Fig. 3.4 (4), (5), (6) & 7)) as well as chronic pretreatment (24h) (Fig. 3.5 (4), (5), (6) & (7)) and the effect of both the compounds were significantly greater than that of the positive control, rosiglitazone for both 3h (Fig. 3.4 (3) and 24h pretreatment (Fig. 3.4 (3)). The glucose uptake shown by quercetin (100 $\mu$ M) and rutin (100  $\mu$ M) was sustained even in the presence of oxidative stress.



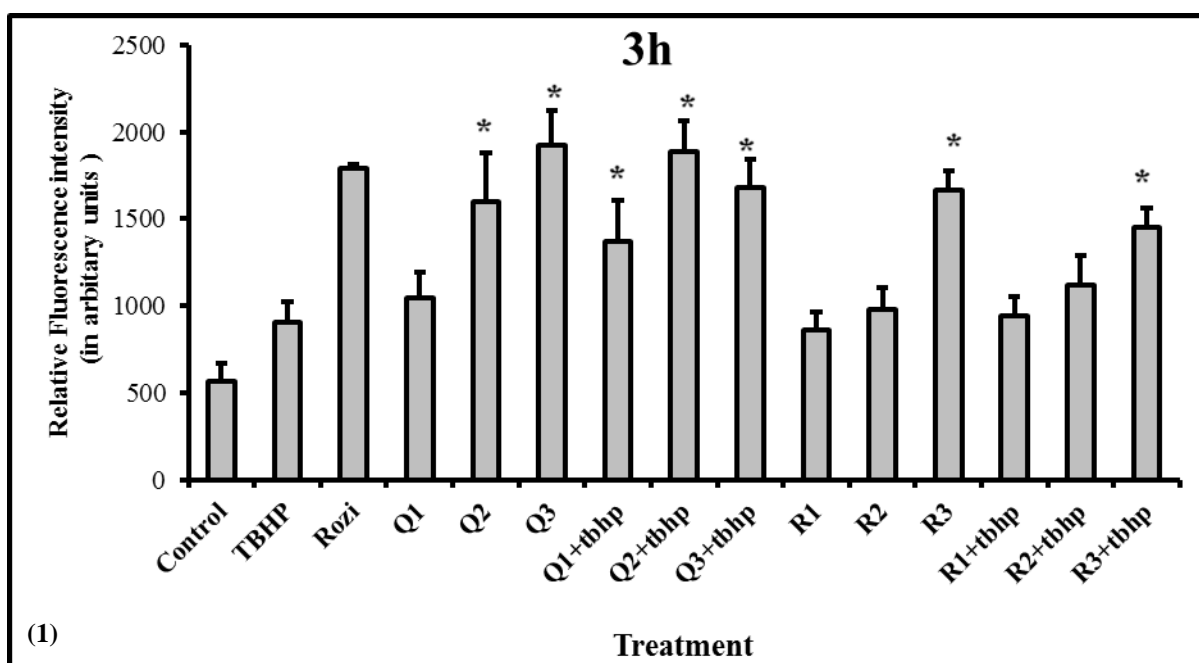
*Fig. 3.4. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification*

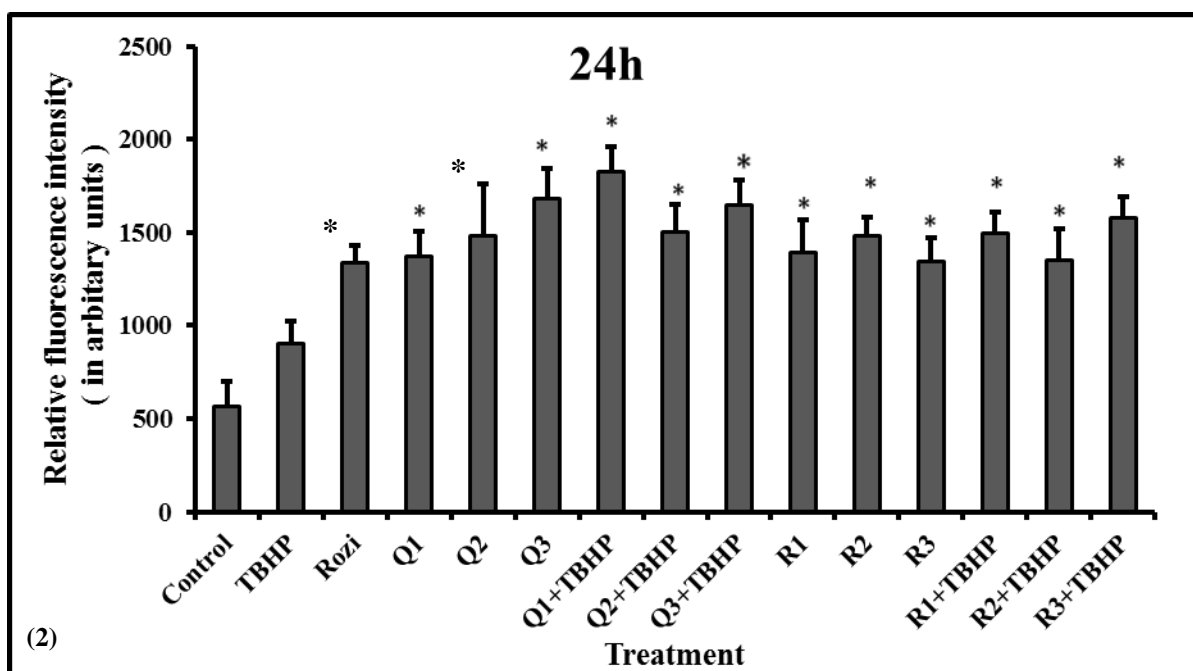
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with quercetin and rutin at 10 & 100  $\mu\text{M}$  concentration for 3h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87  $\mu\text{M}$ .



**Fig. 3.5. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification**  
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with quercetin and rutin at 10 & 100  $\mu\text{M}$  concentration for 24h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87  $\mu\text{M}$ .

The relative fluorescence intensity analysis by BD Image Data Explorer software revealed a two fold increase in glucose uptake on pretreatment of quercetin at 100  $\mu$ M which was higher uptake than its parent compound, rutin, suggesting that the hydrolysis of the glycoside to the aglycone may be required for its action (Fig. 3.6 (1) & (2)). However, the effects of both the compounds were at par with that of the positive control (Fig. 3.4 (3)). Quercetin isolated from *Euonymus alatus* was found to improve glucose uptake in 3T3L1 adipocytes [Xian et al., 2008]. Currently used antidiabetic agents such as, troglitazone lower blood glucose levels in patients after 18 h presumably through an increase in glucose transporter synthesis and not by stimulating glucose uptake acutely [Yonemitsu et al., 2011]. The exposure time of these flavonoids to cells in the present study were restricted to 3 and 24 hours because of the much shorter half-life of flavonoids reported in human plasma [Moon et al., 2008].



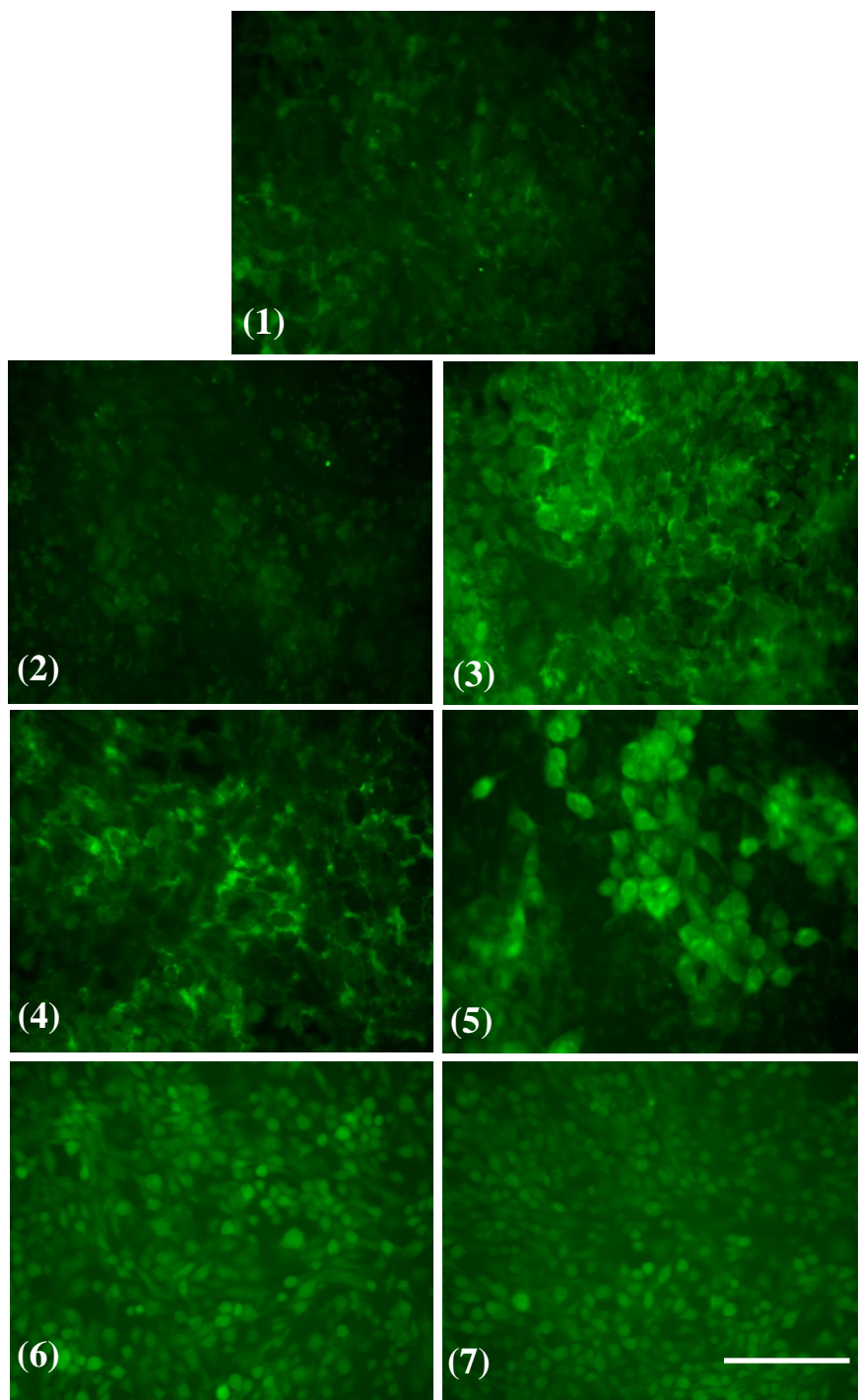


**Fig. 3.6.** Fluorescence intensity analysis of 2-NBDG uptake by differentiated L6 myoblast on 3 h (1) & 24 h (2) pretreatment of quercetin & rutin at different concentrations with or without TBHP by BD Image Data Explorer software

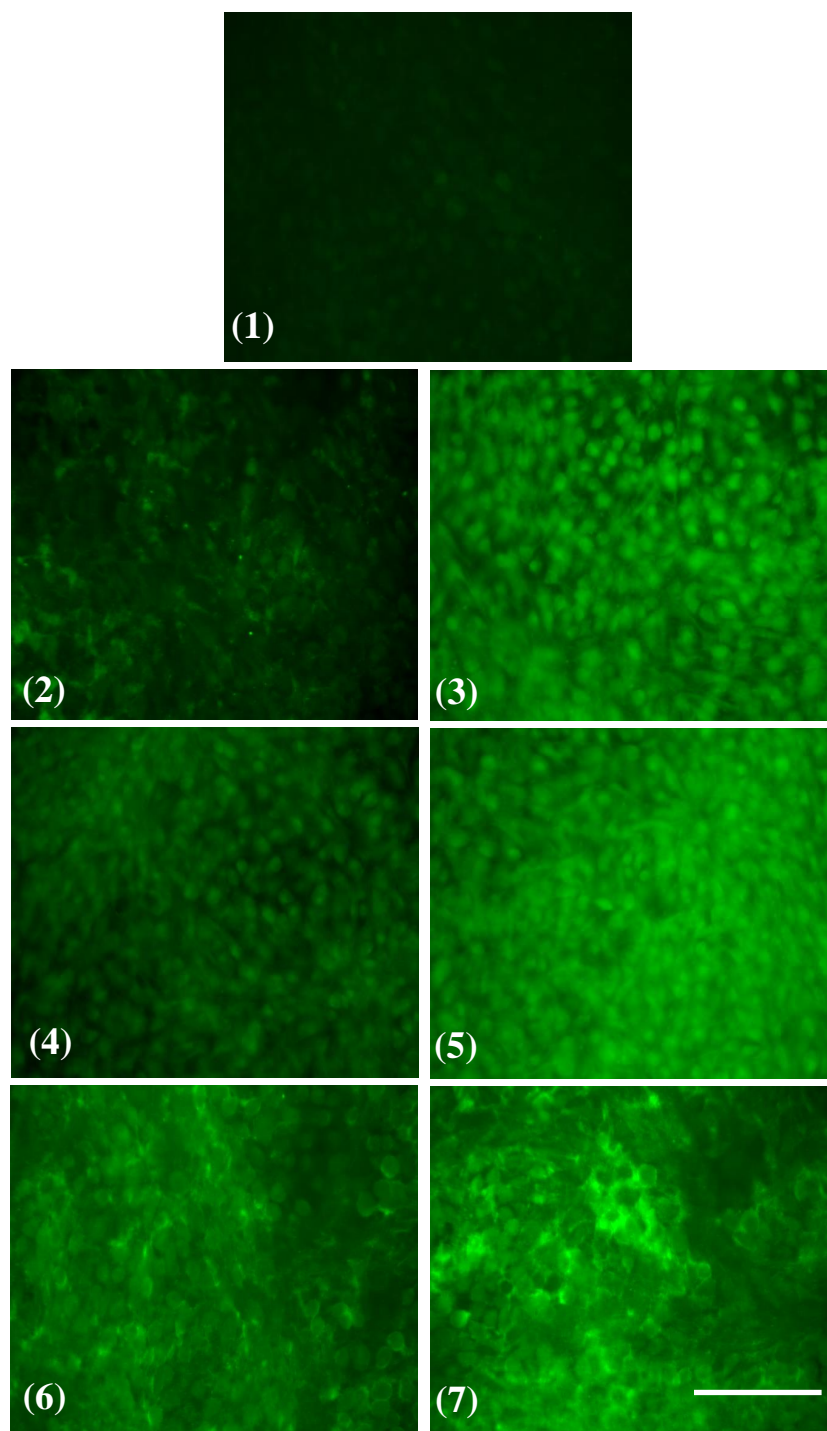
TBHP: Tertiary butyl hydrogen peroxide (100  $\mu$ M); Rozi: rosiglitazone (100 nM); Qn (1, 2, 3): quercetin (1, 10 & 100  $\mu$ M) with/without TBHP; Rn (1, 2, 3): rutin (1, 10 & 100  $\mu$ M) with/without TBHP. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \* $P \leq 0.05$  verses control.

2-NBDG uptake in L6 myotubes on pretreatment of naringin was comparable with the positive control for both 3 and 24h as illustrated in Fig 3.7 & 3.8. Both naringin and naringenin enhanced glucose uptake in a dose dependent manner.





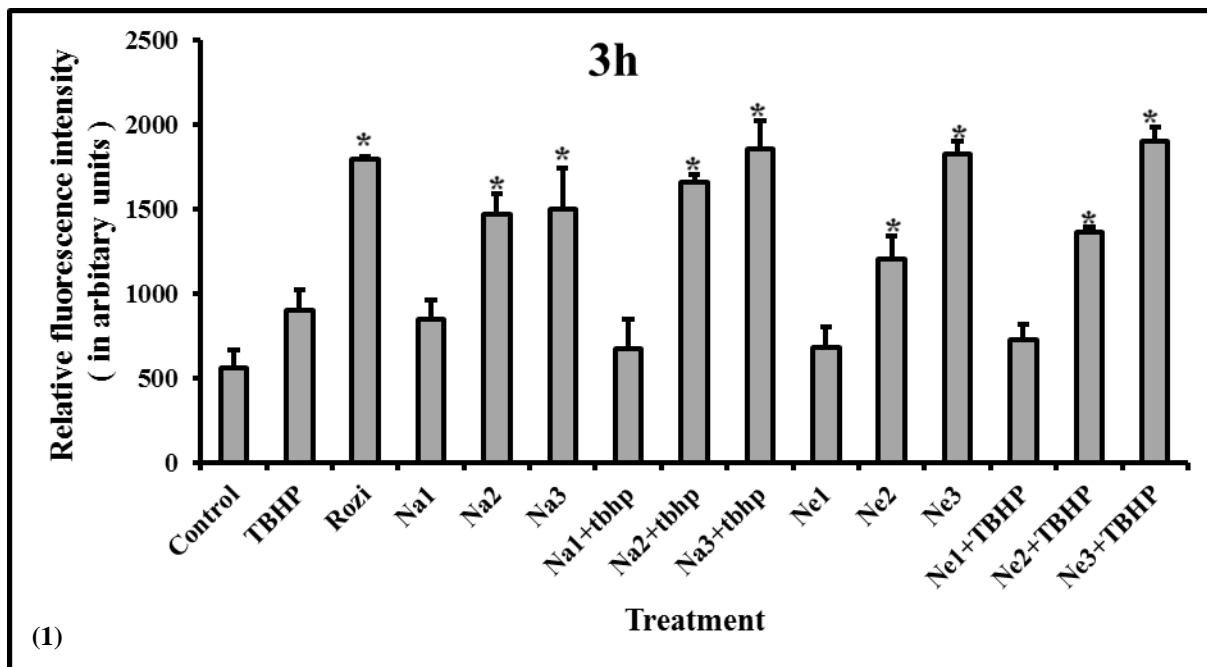
**Fig. 3.7. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification**  
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with naringin and naringenin at 10 & 100  $\mu\text{M}$  concentration for 3 h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87  $\mu\text{M}$ .

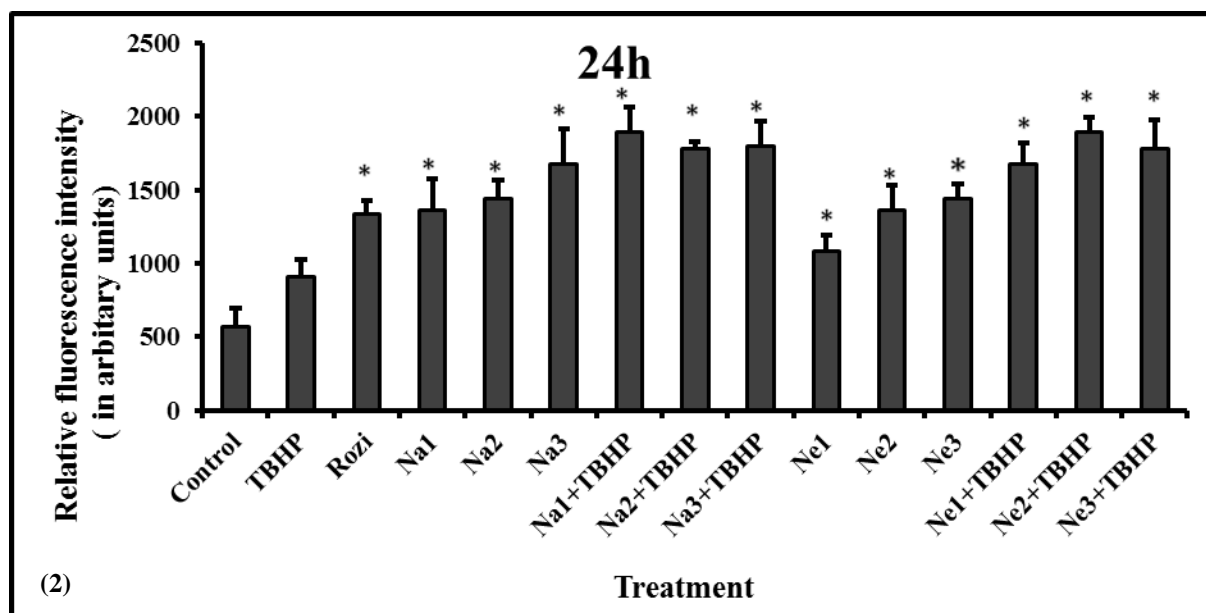


**Fig. 3.8. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification**  
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with naringin and naringenin at 10 & 100  $\mu\text{M}$  concentration for 24h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87  $\mu\text{M}$ .



Relative fluorescence intensity analysis showed a two fold increase in glucose uptake in differentiated L6 myoblast on both acute (Fig. 3.9 (1)) and chronic treatment (Fig. 3.9 (2)) of naringin. Our results were in accordance with Leelavinothan and Selvaraju (2010) who suggested naringin to be an efficient compound in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration by lowering hepatic gluconeogenesis. Mahamoud et al., (2012) reported that hesperidin and naringin administration significantly reversed the increased levels of glucose, glycosylated hemoglobin, MDA, NO, TNF- $\alpha$  and IL-6 in streptozotocin induced rats.

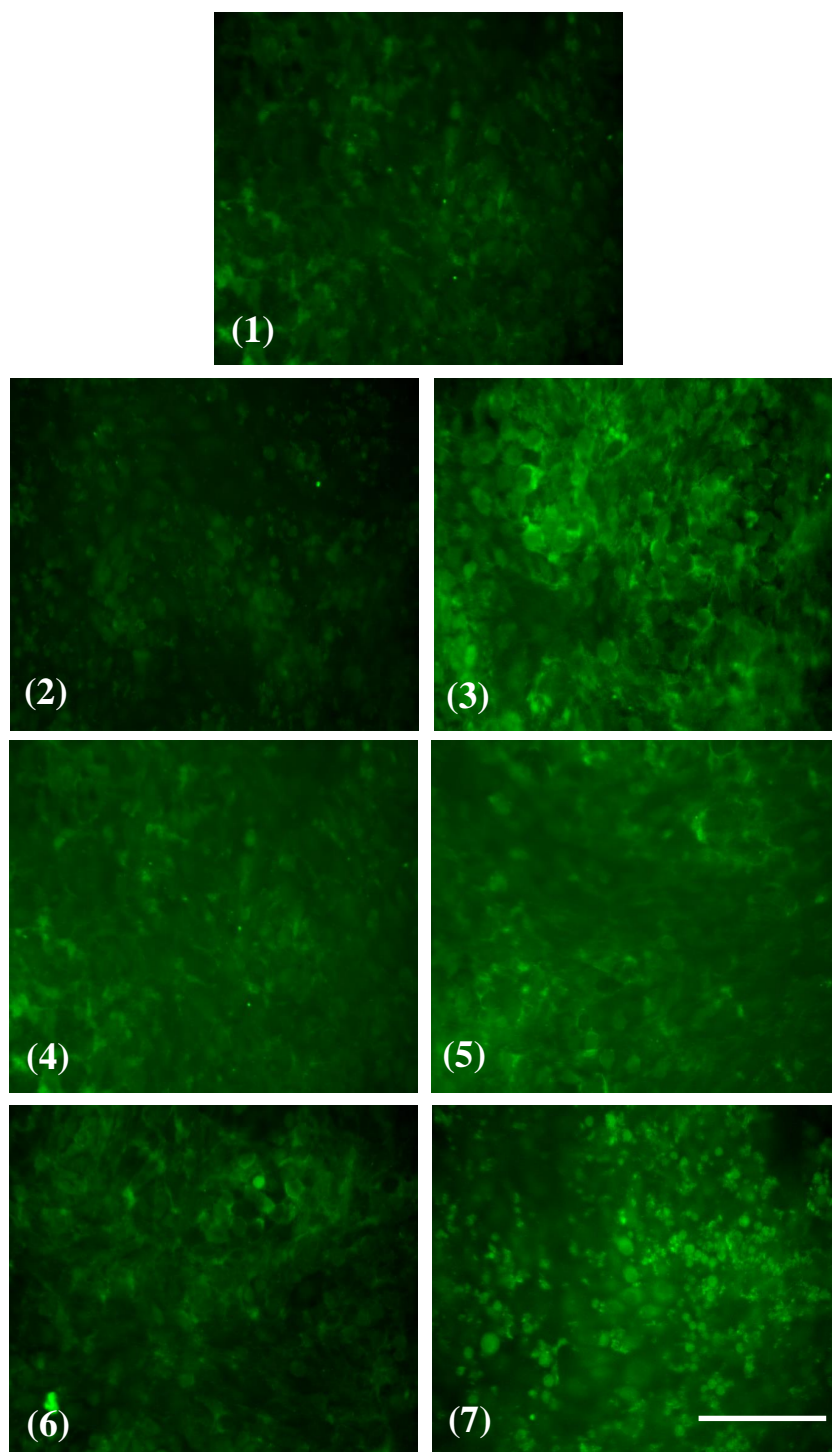




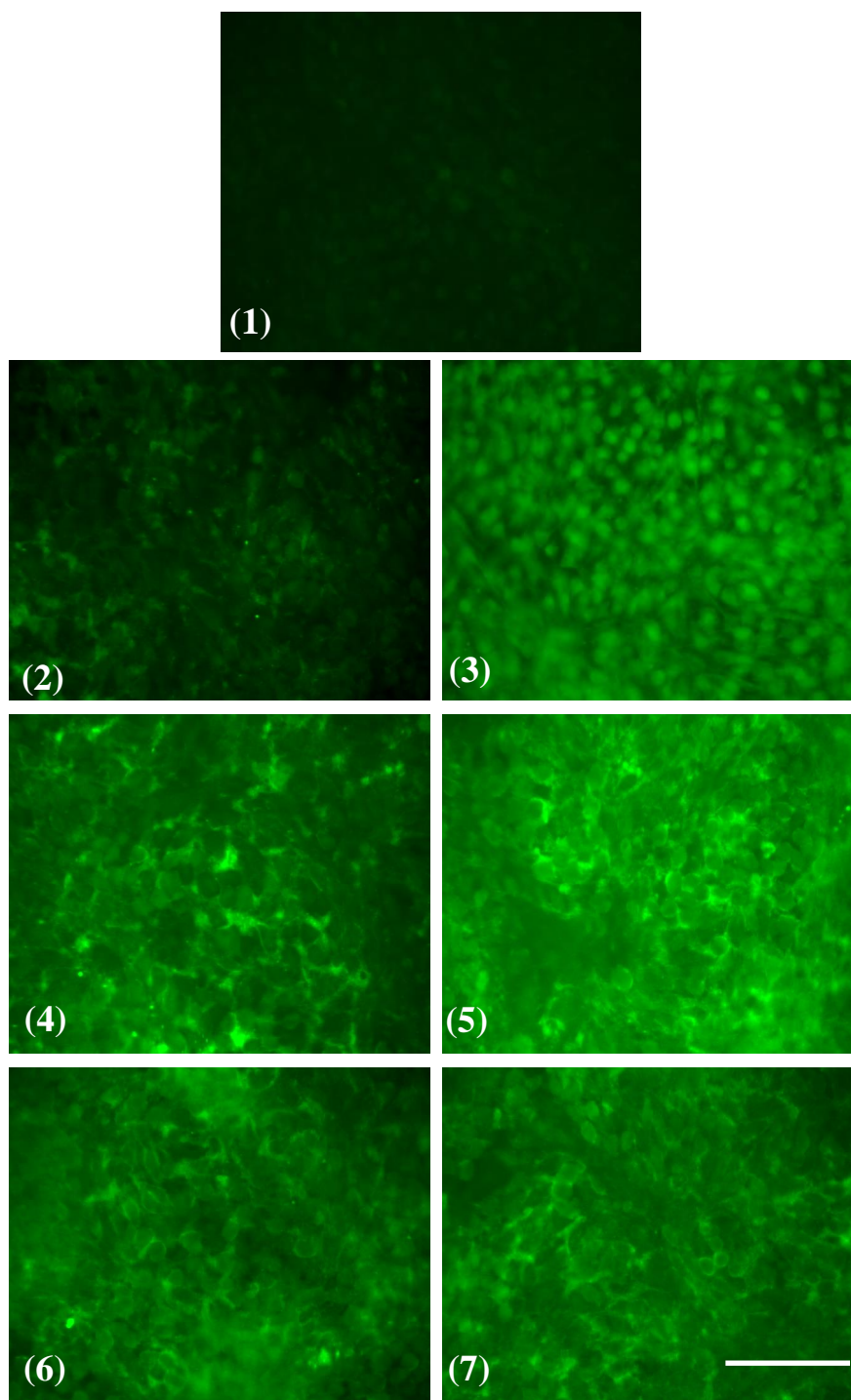
**Fig. 3.9(1-2).** Fluorescence intensity analysis of 2-NBDG uptake by differentiated L6 myoblast on 3 h & 24 h pretreatment of naringin and naringenin at different concentrations with or without TBHP by BD Image Data Explorer software

TBHP: Tertiary butyl hydrogen peroxide (100  $\mu$ M); Rozi: rosiglitazone (100 nM); Na (1, 2, 3): naringin (1, 10 & 100  $\mu$ M) with/without TBHP; Ne (1, 2, 3): naringenin (1, 10 & 100  $\mu$ M) with/without TBHP for 24h. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \* $P \leq 0.05$  verses control.

Pretreatment of hesperidin for 3h stimulated glucose uptake in a dose dependent manner (Fig 3.10). As the period of incubation increased to 24h fluorescence intensity in L6 myotubes also increased indicating enhanced 2-NBDG uptake as in Fig 3.11. At 100  $\mu$ M there was a two fold increase in 2-NBDG uptake compared to control whereas the effect of hesperetin was not remarkable as shown in Fig 3.12.

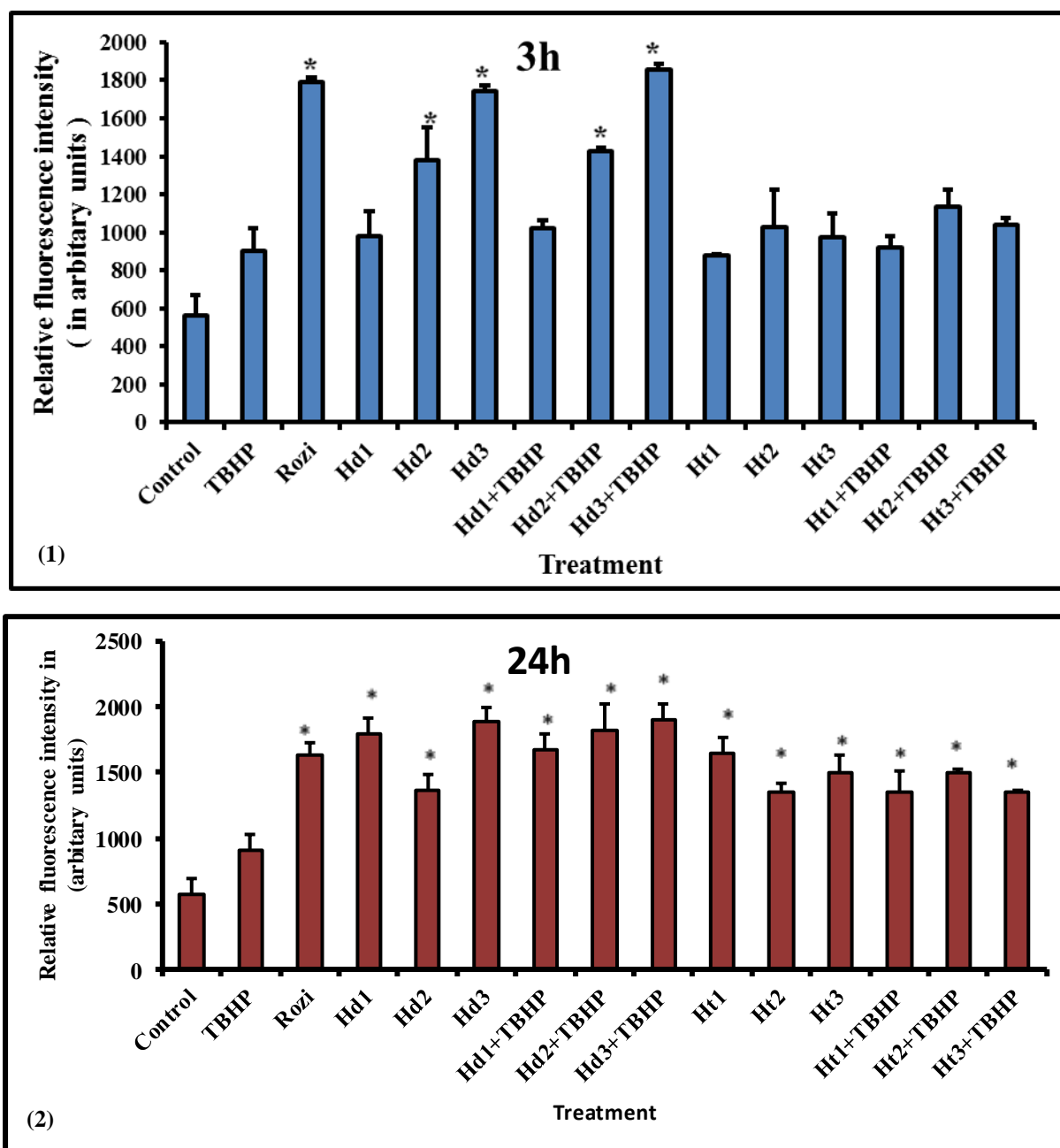


**Fig. 3.10. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification**  
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with hesperidin and hesperetin at 10 & 100  $\mu$ M concentration for 3h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87  $\mu$ M.



**Fig. 3.11. Fluorescent images of glucose uptake in L6 myotubes at 20X magnification**  
(1), (2), (3), (4), (5), (6) & (7) represents Untreated cells, cells treated with TBHP, positive control rosiglitazone, cells pretreated with hesperidin and hesperetin at 10 & 100 μM concentration for 24h. There was a dose dependent increase in fluorescence intensity. Scale bar corresponds to 87 μM.

On relative fluorescence intensity analysis, hesperidin and hesperetin (100  $\mu\text{M}$ ) showed two and one fold increase in 2-NBDG uptake for 24 h pretreatment as shown in Fig. 3.12.



**Fig. 3.12 (1-2).** Fluorescence intensity analysis of 2-NBDG uptake by differentiated L6 myoblast on 3 h & 24 h pretreatment of hesperidin and hesperetin at different concentrations with or without TBHP by BD Image Data Explorer software

TBHP: Tertiary butyl hydrogen peroxide (100  $\mu\text{M}$ ); Rozi: rosiglitazone (100 nM); Hd (1, 2, 3): hesperidin (1, 10 & 100  $\mu\text{M}$ ) with/without TBHP; Ht (1, 2, 3): hesperetin (1, 10 & 100  $\mu\text{M}$ ) with/without TBHP; (1, 2, & 3). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments.

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*Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \* $P \leq 0.0$  verses control.*

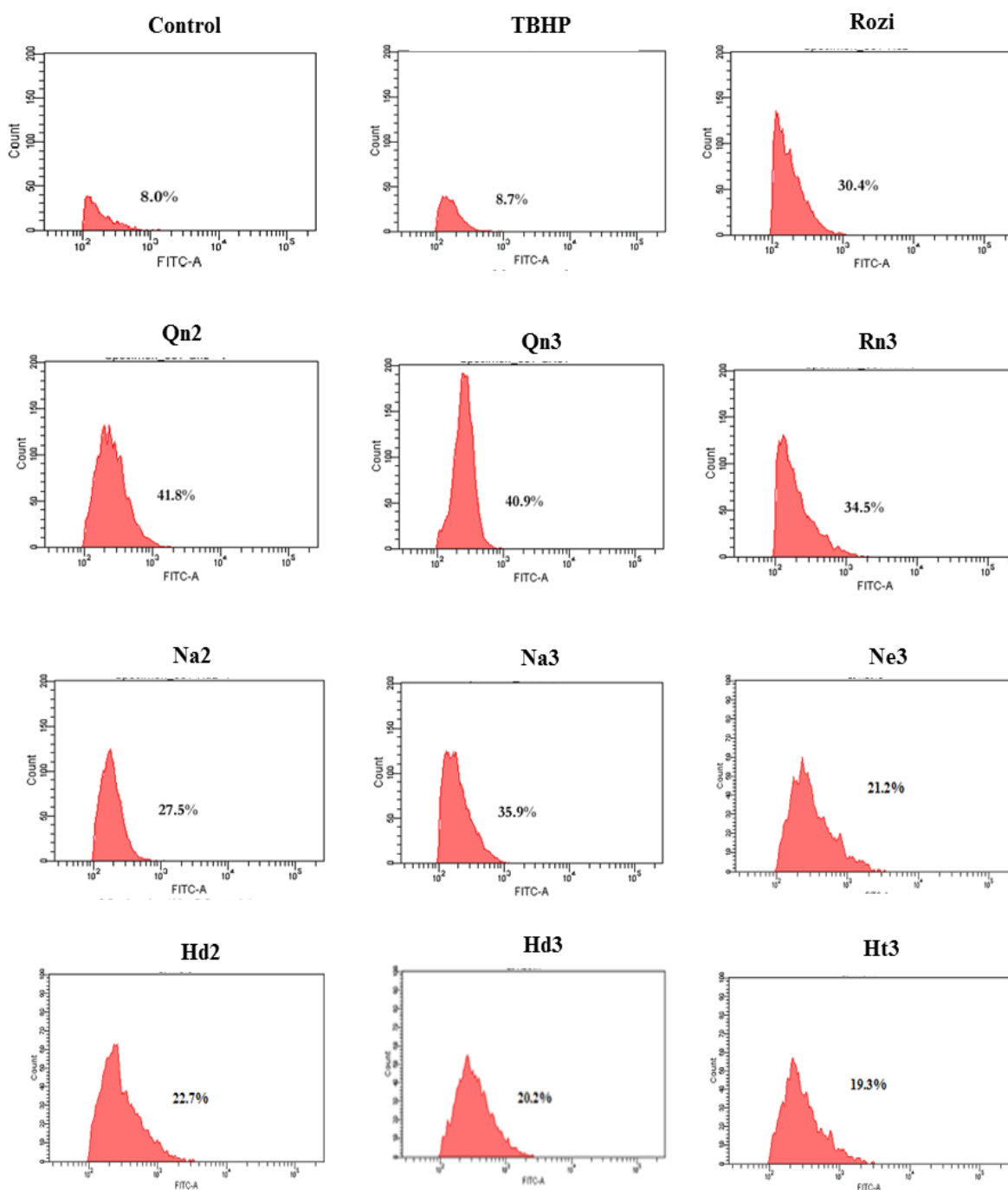
Among all the six flavonoids tested, quercetin, rutin, naringin and hesperidin were most effective in stimulating 2-NBDG uptake and the active concentration was 100  $\mu\text{M}$  for 24h except for quercetin and hesperidin, which was active even at 10  $\mu\text{M}$ . In short, all the tested citrus flavonoids enhanced 2-NBDG uptake in L6 myotubes but their magnitude of action varied. Due to the large proportion of skeletal muscle in the body, inducing glucose uptake in this tissue can have large effects on plasma glucose levels. Moreover, the effect of these compounds was sustained in the presence of induced oxidative stress which was usually generated under physiological conditions of hyperglycemia. Quercetin would be an active 'intracellular metabolite' possessing more antioxidant and antidiabetic activity than its glycoconjugate rutin. Whereas glyconjugate, naringin, poses higher antidiabetic potential than naringenin, similar effect was shown by hesperidin and hesperetin.

### **3.3.4. Flow cytometry analysis**

Flow cytometry analysis was performed by detecting the fluorescence of 2-NBDG within the cells, which was pretreated with the citrus flavonoids at the active concentrations. Flow cytometry analysis revealed 8%, 8.7% and 30.4% uptake of 2-NBDG in control, TBHP and rosiglitazone treated cells, respectively, as shown in Fig 3.13. TBHP was found to have no significant effect on 2-NBDG uptake in the cells (8.7%). Pretreatment of quercetin (10  $\mu\text{M}$  & 100  $\mu\text{M}$ ) and rutin (100  $\mu\text{M}$ ) for 24 h enhanced the fluorescent cell count of remarkably to 41.8%, 40.9% and 34.5% which was much higher than that of positive control, rosiglitazone (30.4%) an antidiabetic drug in the thiazolidinedione class of drugs (Fig. 3.13). Apart from a few isolated studies on the beneficial effects of quercetin and rutin to hyperglycaemic conditions, the scientific evidence is limited.

On 24 h pretreatment of naringin (10  $\mu\text{M}$  & 100  $\mu\text{M}$ ) and naringenin (100  $\mu\text{M}$ ) the count of cells containing 2-NBDG increased to 27.5, 35.9 and 28.2 %, respectively (Fig. 3.13). Naringin is also been reported to modulate hepatic glycolysis and gluconeogenesis [Leelavinothan et al.,

2010]. Hesperetin (1 & 100  $\mu\text{M}$ ) and hesperidin (100  $\mu\text{M}$ ) showed a glucose uptake of 19.3, 22.7 and 20.2 %, respectively (Fig. 3.13).



**Fig. 3.13.** FACS analysis of 2-NBDG uptake in differentiated L6 cells by plotting cell count against FITC. The groups represent untreated cells (Control), cells treated with Tertiary butyl hydrogen peroxide (TBHP treated), Rozi: rosiglitazone (Positive control, 100 nM), Qn2 & Qn3: quercetin (10 & 100  $\mu\text{M}$ ); Rn3: Rutin (100  $\mu\text{M}$ ); Na2 & Na3: naringin (10 & 100  $\mu\text{M}$ ); Ne3: naringenin (100  $\mu\text{M}$ ); Hd2 & Hd3: hesperidin (10 & 100  $\mu\text{M}$ ) & Ht3: hesperetin (100  $\mu\text{M}$ ) respectively.



These active concentrations of citrus flavonoids were used for all further studies. Hesperidin [Choi, 2008] and naringin [Singh et al., 2004; Akiyama et al., 2010 & Pari and Suman, 2010] has been reported to prevent STZ-induced oxidative stress and protect  $\beta$ -cells resulting in increased insulin secretion.

Although our primary focus is on skeletal muscle, it is possible that these citrus flavonoids may also increase glucose uptake in the liver and fat tissue in addition to the effect in muscles.

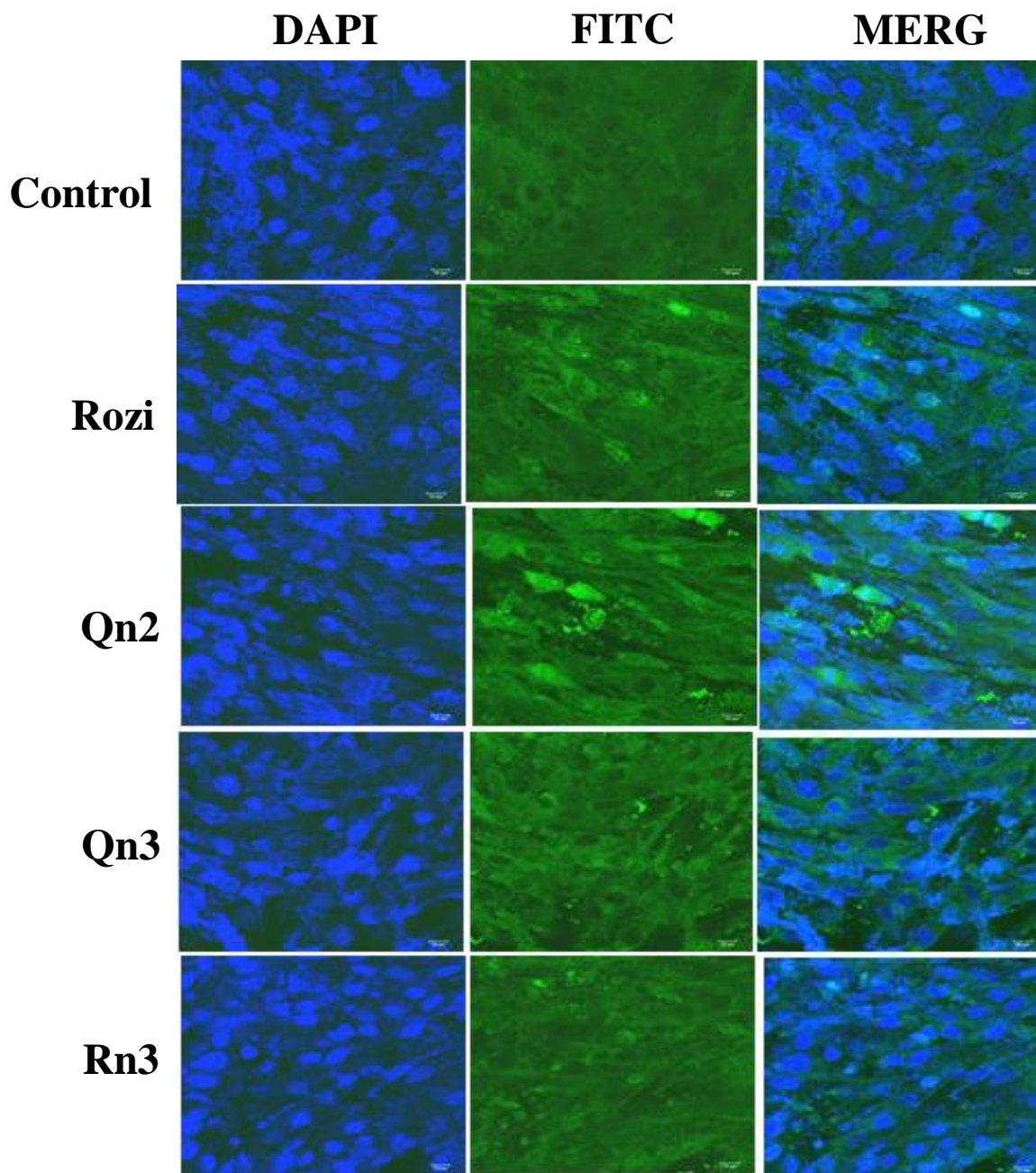
### 3.3.5. GLUT 4 translocation in L6 myotubes

GLUT 4 levels were monitored by immunoassay with fluorescent labelled secondary antibody after 24 h pretreatment with active concentrations of citrus flavonoids. There was an increase in GLUT 4 translocation in the L6 myotubes pretreated with quercetin and rutin. Quercetin at 10  $\mu$ M was found to be most effective in up regulating GLUT 4 translocation on pre-treatment compared to the positive control group pretreated with rosiglitazone. Rutin increased translocation of GLUT 4 that was at par with the positive control as evident from Fig.3.14. TBHP exposure to cells did not induce any changes in GLUT 4 translocation. Our findings are in agreement with the results reported by Kappel et al., [2013] who suggested the involvement of GLUT 4 in the stimulatory effect of rutin on glucose uptake in rat soleus muscle. Translocation of GLUT 4 induced by Rutin was found to be mediated by extracellular calcium and calcium calmodulin dependent protein kinase II [Kappel et al., 2013]. *In vitro* studies using large unilaminar vesicles have shown that quercetin-3-O glucuronide can interact with low, but significant affinity suggesting that an interaction with GLUT 4 vesicles may also be possible [Shirai et al., 2001]. The findings of our study are in line with the reports of Vessal et al., [2003] who showed that quercetin decreased elevated blood glucose concentration and increased insulin release in STZ-induced diabetic rats and the findings of Eid et al., [2010] which reported that quercetin and quercetin 3-O-glycosides are responsible for the antidiabetic activity of *Vaccinium vitis* crude berry extract mediated by AMP-activated protein kinase (AMPK) pathway.

Naringin pretreatment induced translocation of GLUT 4 to same extent than that of rosiglitazone pointing to the molecular end mechanism utilized by naringin in inducing glucose uptake as shown in Fig. 3.15. This may be the first report on the induction of GLUT 4 expression on

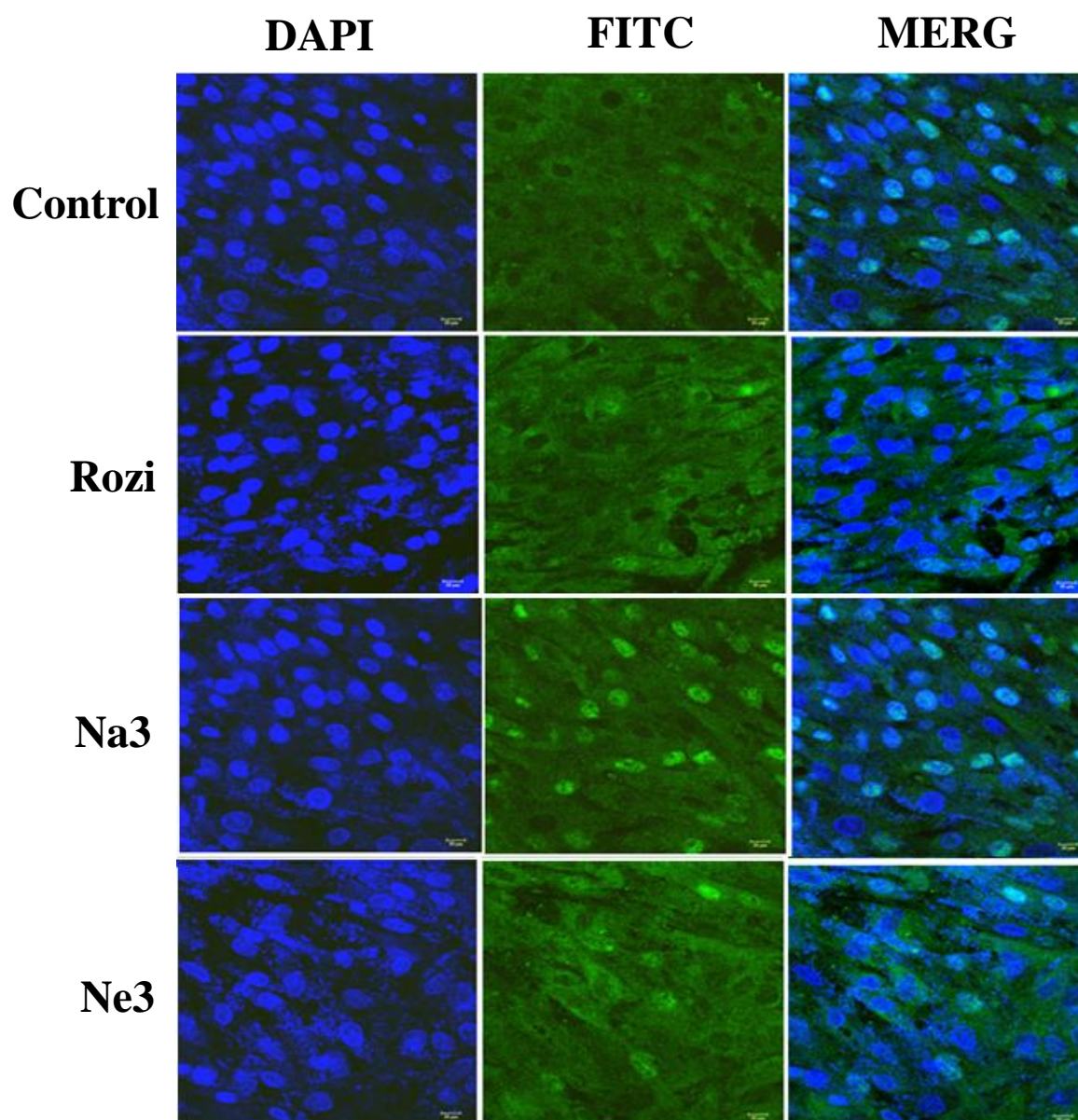


pretreatment of naringin in L6 myotubes. There was no significant effect on cells pretreated with hesperidin and hesperetin as shown in Fig. 3.16.



**Fig. 3.14. GLUT 4 upregulation on flavonoid pretreatment (quercetin & rutin)**

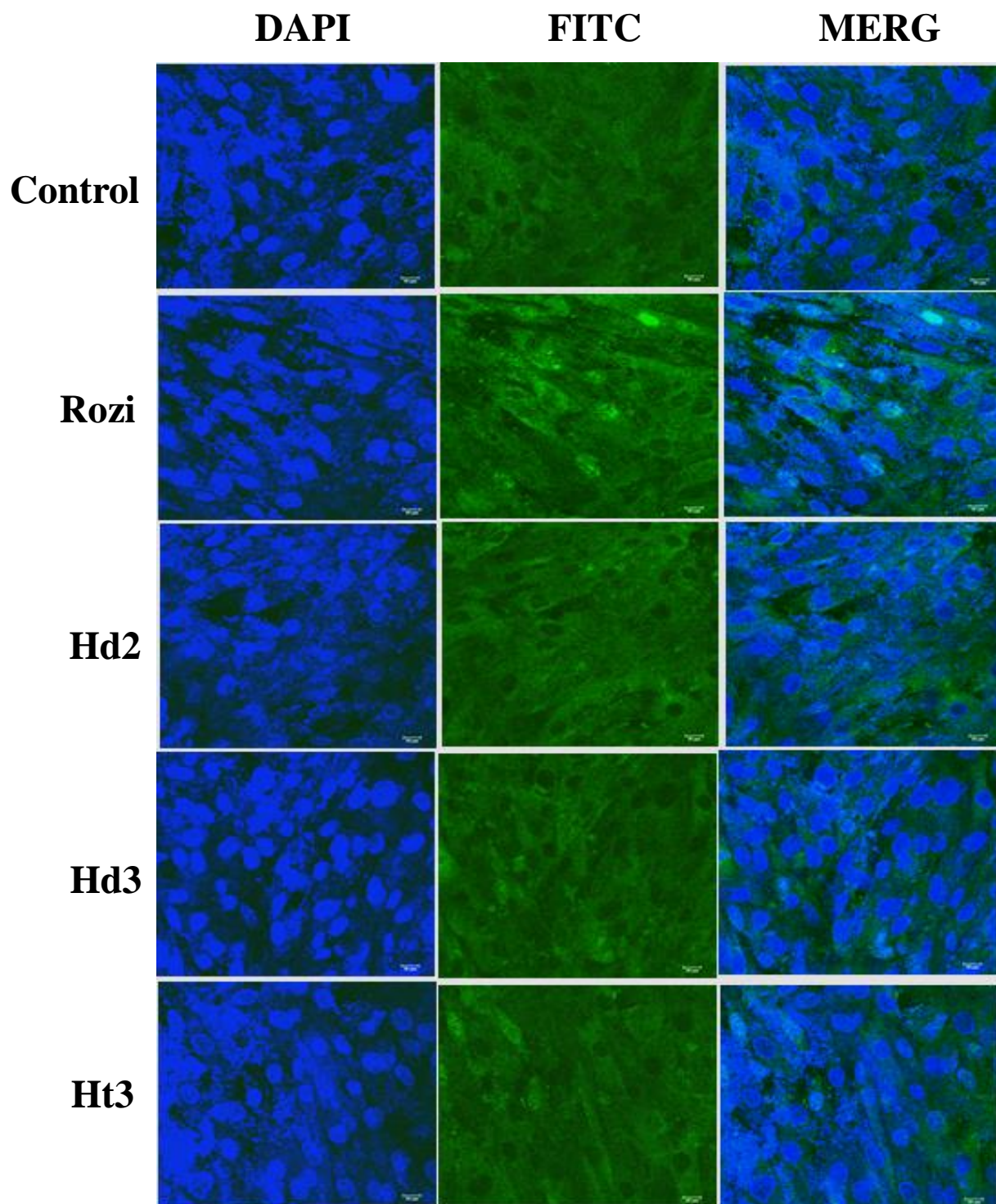
Upregulation of GLUT 4 in differentiated L6 myoblast was visualized by Immunofluorescence assay. High resolution confocal images (40X) of untreated cells (control), L6 myotubes pretreated with flavonoids (quercetin (10 & 100  $\mu\text{M}$ ): Qn2 & Qn3; rutin (100  $\mu\text{M}$ ): rn3) & rosiglitazone (100 nM) for 24 h.



**Fig.3.15. GLUT 4 upregulation on flavonoid pretreatment (naringin & naringenin)**

Upregulation of GLUT 4 in differentiated L6 myoblast was visualized by immunofluorescence assay. High resolution confocal images (40X) of untreated cells (control), L6 myotubes pretreated with flavonoids (naringin (100  $\mu$ M): Na3; naringenin (100  $\mu$ M): Ne3) & rosiglitazone (100 nM) for 24 h.

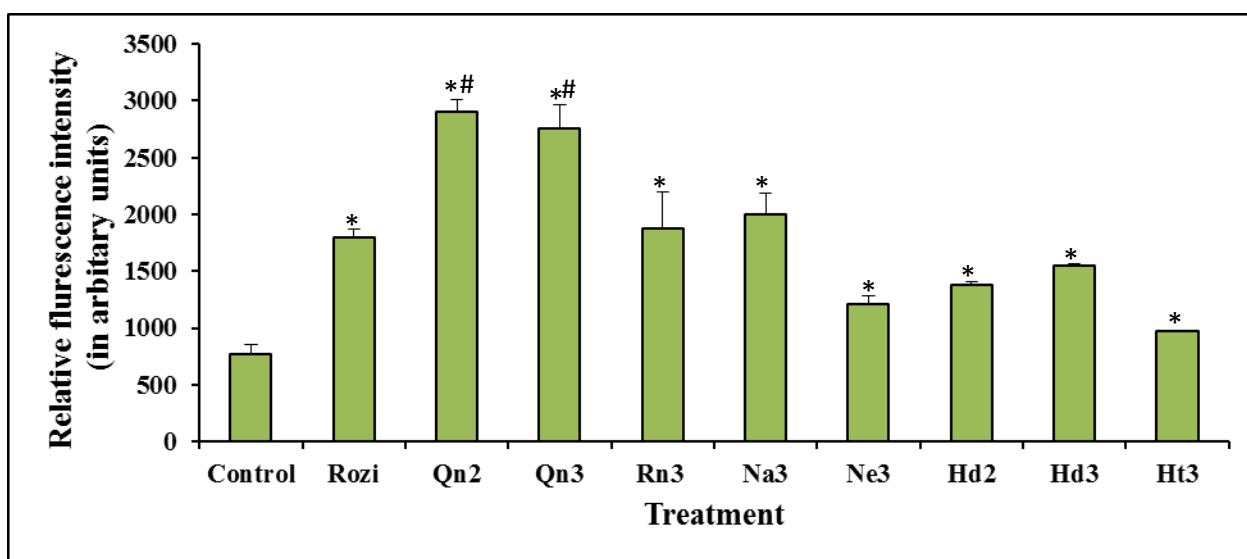




**Fig. 3.16. GLUT 4 upregulation on flavonoid pretreatment (hesperidin & hesperetin)**

Upregulation of GLUT 4 in differentiated L6 myoblast was visualized by Immunofluorescence assay. High resolution confocal images (40X) of untreated cells (control), L6 myotubes pretreated with flavonoids (hesperidin (10 & 100  $\mu$ M): Hd2 & Hd3; hesperetin (100  $\mu$ M): Ht3) & rosiglitazone (100 nM) for 24 h.

The relative fluorescent intensity analysed by BD Image Data Explorer software revealed a two to three fold increase in GLUT 4 in cells pretreated with quercetin (10 & 100  $\mu\text{M}$ ), rutin (100  $\mu\text{M}$ ) and naringin (100  $\mu\text{M}$ ) compared to untreated control as shown in Fig. 3.17. Naingenin, hesperidin and hesperetin showed one fold increase in 2-NBDG uptake. Quercetin was found to be twice more effective than rosiglitazone in the upregulation of GLUT 4 in L6 myotubes. The results obtained by quantifying immunologically labelled GLUT 4 receptors at the surface of intact cells correlates with that of glucose uptake suggesting a positive correlation between the glucose uptake and GLUT 4 translocation.

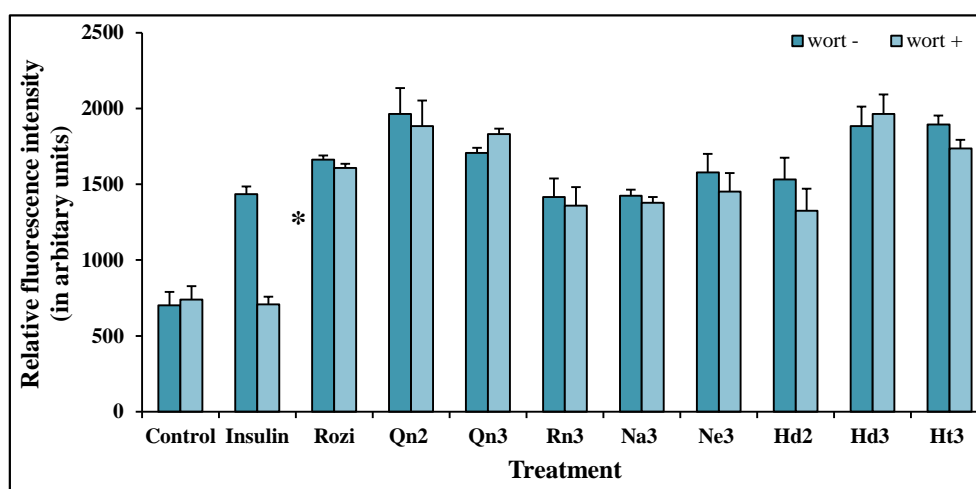


**Fig. 3.17. Fluorescence intensity analysis of immunofluorescence by NIS elements software in L6 myotubes**  
 Control: untreated cells; Rozi: rosiglitazone; Qn2 & Qn3 : quercetin ( 10 & 100  $\mu\text{M}$  ); Rn3: rutin (100  $\mu\text{M}$  ); Na3 : naringin (100  $\mu\text{M}$  ); Ne3: naringenin (100  $\mu\text{M}$  ); Hd3 : hesperidin (100  $\mu\text{M}$  ); Ht3: hesperetin (100  $\mu\text{M}$  ). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus Control; #  $P \leq 0.05$  versus Rozi.

### 3.3.6. Effect of inhibitors on 2-NBDG uptake

The skeletal muscle has a paramount role in energy balance and is the primary tissue for insulin stimulated glucose uptake and disposal. Thus, it is considered as an important therapeutic target tissue for NIDDM and cardiovascular disease. From the fact that quercetin, rutin, naringin, naringenin, hesperidin and hesperetin stimulates glucose uptake in L6 muscle cells, we got a glimpse of evidence on the possible antidiabetic potential. Moreover, the enhanced 2-NBDG uptake shown by the cells was found to be due to the upregulation of GLUT 4 in L6 myotubes.

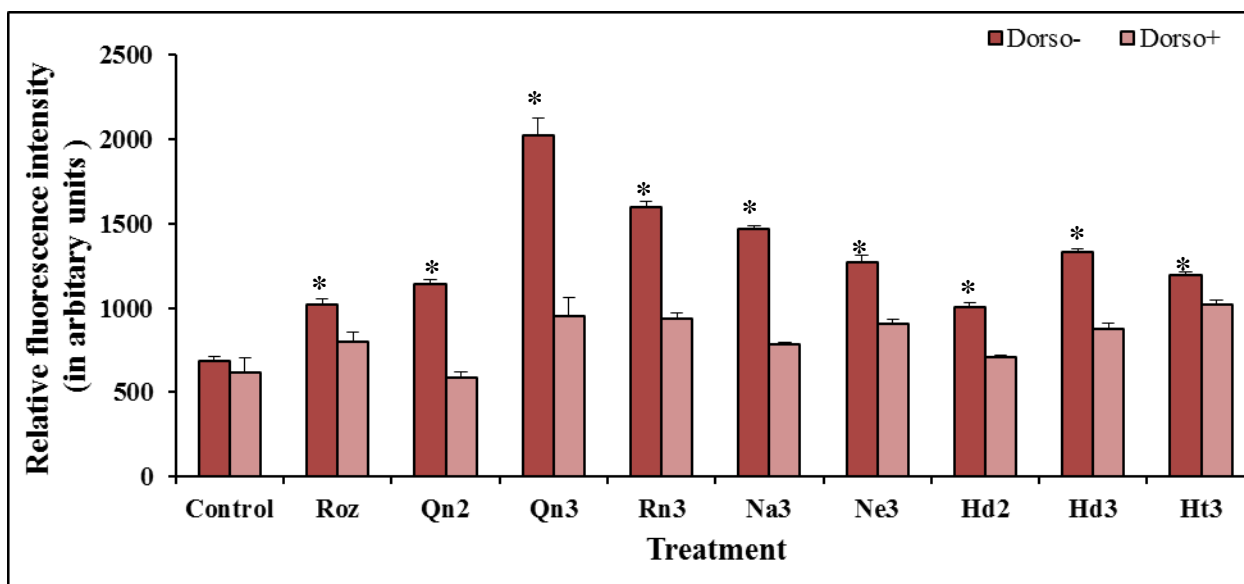
To investigate whether the compounds stimulated glucose uptake is mediated through PI3K activation, we examined the effects of wortmanin, a selective inhibitor of PI3K and dorsomorphin, an inhibitor of AMPK on flavonoids stimulated glucose uptake. As evident from Fig. 3.18, wortmanin had negligible effect on 2-NBDG uptake shown by the tested citrus flavonoids. As the effects of these compounds are wortmanin insensitive, the hypoglycemic actions of these compounds are independent of PI3K, a key molecule, in the insulin signaling pathway. Inhibition of PI3K abolishes insulin-stimulated glucose uptake indicating that the insulin signaling pathway is not significantly involved. As these compounds stimulated glucose uptake to a greater magnitude than insulin, suggesting that it may employ other routes to attain the effect.



**Fig.3.18. Effect of PI3K inhibitor, wortmanin on 2-NBDG uptake in L6 myotubes**

L6 myotubes pretreated with wortmanin followed by co-incubation with Rozi: rosiglitazone (100 nM); Insulin (100nm); Qn (2, 3): quercetin (10 & 100  $\mu$ M) ; Rn (3): rutin (100  $\mu$ M) Na (3): naringin (100  $\mu$ M) ; Ne (3): naringenin (100  $\mu$ M); Hd(3): hesperidin (10 & 100  $\mu$ M) ; hesperetin (100  $\mu$ M for 24h. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus same groups with wortmanin.

On the contrary, the myotubes pretreated with the citrus flavonoids in the presence of dorsomorphin showed a drastic decrease in 2-NBDG uptake especially in cells pretreated with quercetin, rutin, naringin, naringenin and hesperidin as shown in Fig. 3.19.



**Fig. 3.19.** Effect of AMPK inhibitor, Dorsomorphin on 2-NBDG uptake in L6 myotubes

L6 myotubes pretreated with dorsomorphin followed by co-incubation with Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M); Rn (3): rutin (100  $\mu$ M); Na (3): naringin (100  $\mu$ M); Ne (3): naringenin (100  $\mu$ M); Hd(3): hesperidin (10 & 100  $\mu$ M); Hesperetin (100  $\mu$ M) for 24h. Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus same groups with dorsomorphin.

This findings point to the conclusion that AMPK pathway may be involved in the induction of glucose uptake shown by the citrus flavonoids and there may exist a crosstalk between the insulin and AMPK signaling pathways. Polyphenols, such as resveratrol has been reported to stimulate glucose uptake and translocation of GLUT 4 by activating both insulin signaling and AMP activated protein kinase signaling [Miki et al., 2011]. AMPK is a heterotrimeric enzyme that is activated by both allosteric regulation and phosphorylation [Kemp et al., 2007]. It functions as a metabolic switch that phosphorylates key target proteins including enzymes involved in lipid metabolism, lipolysis, fatty acid oxidation and glucose uptake. Activation of AMPK by AICAR stimulates glucose transport in cardiac papillary muscle [Bogoyevitch et al., 1997] skeletal muscle [Taha et al., 1997] and 3T3-L1 adipocytes [Somwar, 2002]. Over expression of constitutively active AMPK in H-2Kb muscle cells indicates that AMPK activation is sufficient to stimulate glucose uptake [Somwar et al., 2000].

### 3.4. Summary

Our data suggest that the biological activities of the metabolites can be neither predicted nor extrapolated from their dietary forms. The experimental data of the present chapter provide significant evidence for citrus flavonoids to be considered as a dietary supplement with potential for the prevention and treatment of T2DM and to suppress oxidative stress mediated damage in diabetic pathophysiology. The dietary supplementation of these flavonoids would also address the diabetic complications arising through oxidative stress following the onset of diabetes. A detailed analysis of its signalling pathways would reveal the molecular mechanism of action of these flavonoids in skeletal muscle cells. The major findings of the chapter are illustrated below:

- 📖 Quercetin, naringenin, hesperetin & their glycoconjugates inhibited glycation of proteins.
- 📖 Flavonoids, especially, quercetin (10 & 100  $\mu\text{M}$ ), rutin, naringin, hesperidin & hesperetin (100  $\mu\text{M}$ ) significantly enhanced 2-NBDG uptake in L6 myotubes.
- 📖 The uptake was sustained even in the presence of oxidative stress mediated through its antioxidant activity.
- 📖 Magnitude of antidiabetic potential of these flavonoids varied. It may be confirming that carbohydrate moiety as an important structural feature and the biological activity is dependent on the chemical nature of the substituent.
- 📖 Upregulation of GLUT 4 expression is the end molecular mechanism behind the increased glucose uptake.
- 📖 AMPK pathway may be involved in the induction of glucose uptake.

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## **CHAPTER 4**

*Elucidation of molecular mechanism of action  
in skeletal muscle cells (L6 myotubes)*

## 4.1. Introduction

Disruption of glucose homeostasis is a characteristic feature of NIDDM and is associated with some complications including cardiovascular disease and renal failure [DeFronzo et al., 1992]. Glucose transport, the rate limiting step in glucose metabolism, can be activated in peripheral tissues by two distinct pathways. One stimulated by insulin through IRS-1/PI 3K, the other by muscle contraction/exercise through the activation of AMPK [Haley, 2013]. Both pathways also increase the phosphorylation and activity of MAPK family components [Sanders et al., 2007; Zhou et al., 2001], of which p38 MAPK participates in the full activation of GLUT 4 [Musi et al., 2003; Xiao et al., 2011].

Insulin exerts its biological effect upon binding with the insulin receptor (IR) thereby activating the downstream signaling that lead to enhanced glucose uptake. In skeletal muscle, it potentiates glucose transport through PI3K mediated or non-PI3K mediated pathways. Alterations or defects in its signal transduction pathway was found in diabetic patients associated with decreased levels of IRb, IRS-1, and PI3K [Haffner et al., 1992]. In the insulin signaling, PI3K is a key molecule and inhibition of PI3K completely abolish insulin stimulated uptake. AKT or PKB is an important downstream target of insulin stimulated glucose transport and metabolism.

Impairment in fuel metabolism occurs in obesity, and this impairment is a leading pathogenic factor in T2DM. The insulin resistance associated with T2DM is most profound at the level of skeletal muscle as this is the primary site of glucose and fatty acid utilization. Therefore, an understanding of how to activate AMPK in skeletal muscle would offer significant pharmacologic benefits in the treatment of T2DM. Metformin and the thiazolidinedione drugs are known to exert the effects via activation of AMPK [Hwang et al., 2009]. Activation of AMPK occurs in response to exercise, an activity known to have significant benefit for type 2 diabetics. AMPK serves as sensor of energy status whose activity is triggered in response to changes in nutritional status in order to modulate tissue-specific metabolic pathways.

AMPK, a heterotrimeric protein consisting of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ). The AMPK- $\gamma$  subunits contain three sites where adenine nucleotides binds and confers the kinase to act as an energy sensor [Xiao et al., 2007; Scott et al., 2004; Lin et

al., 2010]. In normal cells, catabolism maintains the ATP:ADP ratio at around 10:1, and it shifts the adenylate kinase reaction toward ADP synthesis [ $\text{ATP} + \text{AMP} \rightarrow 2\text{ADP}$ ], so that AMP concentrations are very low; the typical ratios of ATP:ADP:AMP in normal cells are around 100:10:1. The  $\gamma$  subunit sites appear to bind AMP, ADP, and ATP with same affinity, but the affinity to ATP is higher compared to MgATP complex [Oakhill et al., 2011]. Since around 90% of ATP is present as magnesium complex, the cellular concentrations of total ADP and free ATP are comparable, allowing these nucleotides to compete for binding to AMPK. Although the concentration of AMP is at least 10-fold lower than that of ADP or free ATP in normal cells, it rises markedly as the ADP:ATP ratio rises during energy stress, due to displacement of the adenylate kinase reaction. Under these conditions, AMP should therefore be able to compete with ATP or ADP at the AMPK- $\gamma$  subunit binding sites. Binding of AMP or ADP to the AMPK- $\gamma$  subunit causes a conformational change that promotes phosphorylation of Thr-172 by upstream kinases while inhibiting dephosphorylation by upstream phosphatases [Xiao et al., 2007; Scott et al., 2004]. Stoichiometric phosphorylation of Thr-172 can cause 100-fold activation although Thr-172 may only be partially phosphorylated *in vivo* even in cells experiencing metabolic stress. The effect of increased phosphorylation is amplified around 10-fold further by allosteric activation, which is caused only by binding of AMP. This tripartite mechanism states that there can be large increases in AMPK activity in response to small increases in the AMP:ATP or ADP:ATP ratios. The calcium/calmodulin-dependent protein kinase kinase- $\beta$  (CaMKK $\beta$ ), a kinase with a more restricted expression in neural tissue, also activates AMPK. AMPK phosphorylation at Thr-172 by CaMKK $\beta$  is triggered by an increase in cytosolic calcium levels. AMPK targets several proteins involved in cellular energy balance, including a regulator of fatty acid biosynthesis and acetyl-CoA carboxylase (ACC). The calcium/CaMKK $\beta$ /AMPK signaling pathway also controls mechanisms relevant to protein degradation by controlling mTOR (mammalian target of rapamycin) signaling and autophagy [Hoyer and Jaattela, 2007]. Indeed, mTOR is a potent repressor of autophagy and is negatively controlled by AMPK [Carling et al., 2008; Hardie, 2007].

Skeletal muscles account for approximately 80% of glucose absorption under insulin-stimulated conditions [Thiebaud et al., 1982], and a reduction in insulin-stimulated glucose uptake in skeletal muscles of T2DM patients has been observed both *in vitro* [Del Prato et al.,

1993] and *in vivo* [Bouzakri et al., 2003]. The L6 myotubes has been widely used to investigate the mechanism of insulin and exercise stimulated glucose transport [Klip and Paquet, 1990; Yaffe, 1968]. Metformin is extensively used in the treatment and management of T2DM. Metformin improves glycemic control primarily via suppression of hepatic glucose production, and to a lesser extent, but still metabolically important, increased peripheral glucose uptake [Zhou et al., 2001; Foretz et al., 2010]. Metformin is also known to activate AMPK and stimulate glucose uptake in isolated rodent skeletal muscle, presumably via the ability of metformin to increase the intracellular AMP:ATP ratio [Musi et al., 2001].

In the present study, we found that quercetin, rutin, naringin, naringenin, hesperidin and hesperetin stimulated glucose transport in L6 myotubes. Taking advantage of protein kinase inhibitors, we proved that the effect of these compounds was not through insulin signaling pathway, but through AMPK pathway and its downstream target p38 MAPK. An increase in the cellular AMP:ATP ratio on pretreatment may account for AMPK activation.

## **4.2. Material and methods**

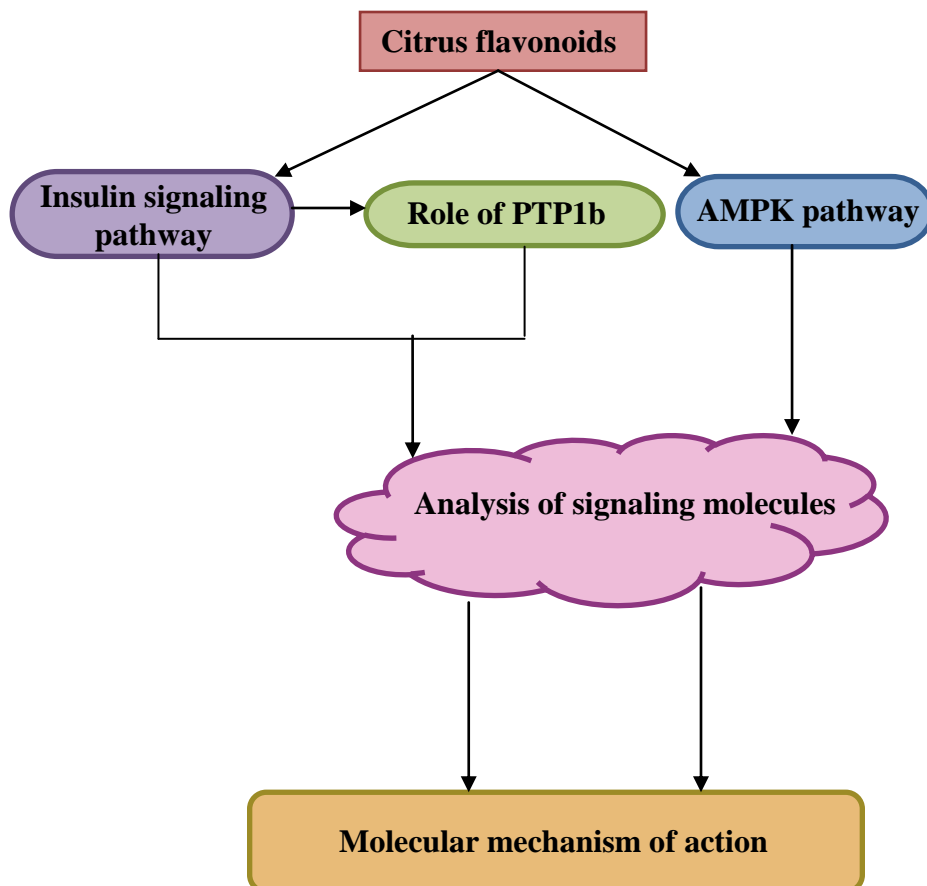
Dulbecco's modified Eagle's media (DMEM), bovine serum albumin, streptomycin ampicillin–amphotericin B mix, insulin, rosiglitazone, quercetin, rutin, naringenin, naringin, hesperidin, hesperetin, para nitrophenyl phosphate, ATP, ADP, AMP HEPES, dithiothreitol, EDTA (Ethylene diamine tetra acetic acid), perchloric acid, wortmanin, dorsomorphin, primers, fura 2AM and JC1 kit were purchased from Sigma–Aldrich Chemicals (St Louis, MO, USA); phospho-specific or pan-specific antibodies against AMPK, P38MAPK, GLUT 4, AKT, IRS1, IRS2. L6 myoblast was obtained from National Centre for Cell Sciences, Pune, India. All other chemicals used were of standard analytical grade.

### **4.2.2. Cell culture and treatment**

Rat skeletal muscle cell lines, L6 myoblasts (NCCS, Pune) were maintained in DMEM supplemented with 10% FBS, 10% antibiotic-antimycotic mix at 37°C under 5% CO<sub>2</sub> atmosphere. Cells were grown at a density of 1x10<sup>4</sup> cells/well. For differentiation the cells were maintained in differentiation containing 2% horse serum for 5-7 days.

### 4.2.3. Experimental design

Schematic representation of Chapter 4 is depicted in the flowchart (Fig 4.1).



*Fig.4.1. Illustration of experimental design*

### 4.2.4. PTP1b assay

PTP1b is a ubiquitous non transmembrane protein tyrosine phosphatase, responsible for negatively regulating insulin signaling by dephosphorylating phosphotyrosine residue of insulin receptor kinase residue and is a potential therapeutic target for treatment of T2DM and obesity. PTP1b activity was assayed on cells collected with 100- $\mu$ l p-nitrophenyl phosphate (PNPP) buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA).

The colorimetric PNPP hydrolysis assay based on the ability of phosphatases to catalyze the hydrolysis of PNPP to p-nitrophenol, a chromogenic product, was used. The intensity of the colour reaction was measured at 410 nm on a microplate reader (BIOTEK, USA). Results were expressed as percentage activity relative to control.

#### 4.2.5. Molecular docking

The compounds were docked into protein tyrosine phosphatase 117 1B (PTP-1B) binding pocket in complex with inhibitor (Drug bank ID: DB08593) using the Autodoc 4.2 program visualized by pyMOL. Binding energy of individual residues was calculated by iGMDOCK. Quercetin (Pubchem CID: 5280343), rutin (Pubchem CID: 5280805), naringenin (ChemSpider ID: 388383), naringin (Chem Spider ID: 390868), hesperidin (Chem Spider ID: 10176.mol), hesperetin (DrugBank:DB01094 (APRD00117, rosiglitazone (Chem Spider ID: 70383) structure were downloaded from Pub Chem (<http://pubchem.ncbi.nlm.nih.gov/>), Drug Bank (<http://www.drugbank.ca/>), Chemspider (<http://www.chemspider.com/>) and converted to PDB file using Chem3D Pro 10. These structures were subjected to docking into the 3D model of PTP1b (PDB id: 2BGE) retrieved from the Brookhaven Protein Data Bank (PDB) (<http://www.rcsb.org/pdb/>).

#### 4.2.6. Adenine nucleotide extraction and measurement

Adenine nucleotide extraction was performed as previously reported [Hahn et al., 2005] with slight modifications. Cells of different groups were trypsinised and counted before centrifugation at 800xg for 3 min. Cell pellets were suspended in 3% perchloric acid and incubated on ice for 30 min. Within 1 h the pH of the lysate was adjusted to between 6 to 8 with 2M KOH. Precipitated salt was separated from the liquid phase by centrifugation at 13000xg for 10 min at 4°C. Aliquoted samples were stored at -80°C until further analysis.

Adenine nucleotide measurements were conducted by HPLC (HP 1100 series) with a Phenomenex Gemini column (5mm, 0.46x15 cm, C18 110A). The nucleotides were detected spectrophotometrically at 259 nm, and eluted at a flow rate of 1.0 ml/min. ATP, ADP and AMP eluted at approximately 5, 7.5 & 12 min, respectively. Internal standards (7.5 µM ATP, ADP and AMP in milli Q water) were used to quantify the samples. The HPLC buffer contained 20 mM  $\text{KH}_2\text{PO}_4$  and 3.5 mM  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  at pH 6.1.



#### **4.2.7. Assay for mitochondrial membrane potential**

Mitochondrial membrane potential was measured using mitochondrial staining kit, JC1. The experiment was done as per the protocol provided with the kit (JC1 kit, Sigma). The kit uses the cationic, lipophilic dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolocarbo-cyanine iodide (JC-1)). In normal cells, due to the electrochemical potential gradient, the dye concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates (JC-1 aggregates). Change in mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers). The cells after treatments were incubated with JC-1 staining solution for 20 minutes at 37°C. The stain was washed off with PBS and examined under fluorescent microscope (Pathway 855, BD Bioscience, USA) and images were collected and fluorescence intensity was also measured. For JC-1 monomers, the fluorescence was measured at 490 nm excitation and 530 nm emission wavelengths, and for JC-1 aggregates, the fluorescence was measured at 525 nm excitation and 590 nm emission wavelengths. Valinomycin (1 µg/mL) was used as positive control for the measurement of dissipation of mitochondrial membrane potential.

#### **4.2.8. Determination of intracellular calcium levels**

Differentiated L6 myoblast (5-7 day) cultured in 96 black well plates were treated with compounds of standardized concentrations for 24 h. Intracellular calcium levels were detected by staining the various groups with Fura-2AM for 20 min at 37°C. Following incubation, the wells were washed off with PBS and visualised under fluorescent microscope (Pathway 855, BD Bioscience, USA) at excitation-emission wavelength of 350 and 510 nm.

#### **4.2.9. Quantitative real time PCR analysis**

Total RNA from pretreated L6 myotubes were isolated using Trizol (Invitrogen Corp., Grand Island, NY, USA) according to the manufacturer's protocol. One microgram RNA was reverse transcribed by SuperScript VILO cDNA Synthesis Kit. The primer sequences for tested genes were as shown in Table 4.1.

Table.4.1. Nucleotide sequences of PCR primers used for quantitative real-time PCR

<b>Gene</b>	<b>Oligonucleotide primers</b>
<b>Ppia</b>	Forward 5'-CAAAGTTCCAAAGACAGCAGAAA-3' Reverse 5'-CTGTGAAAGGAGGAACCCTTATAG-3'
<b>GLUT 4</b>	Forward-5'GTGCCTATGTATGTGGGAGAAA-3' Reverse 5'-TCGTGTGGCAAGATGTGTAT-3'
<b>Akt</b>	Forward 5'-GAGCTGTGAACTCCTCATCAA-3', Reverse 5'-TCTCCATAGTCCTCTGGGTAAG-3
<b>PI3K</b>	Forward 5'- GTGGACAAAGCAGAAGCATTAC-3' Reverse 5'- ACCCTGTGTTCTTTGTCTAGTG-3'
<b>IRS 1</b>	Forward 5'- GAGTTGAGTTGGGCAGAGTAG-3' Reverse 5'- CATGTAATCACCACGGCTATTTG-3'
<b>AMPK</b>	Forward 5'-CCTATGAAGAGGGCCACAATAA-3' Reverse 5'- AGGTCACGGATGAGGTAAGA-3'
<b>CaMKK</b>	Forward 5' - CGCTGGTTCCTACTCTTATC-3' Reverse 5' – GCTCCCTGACTCTTTGCTATT-3'
<b>MAPK12</b>	Forward 5' – CCCAAGGCCAGAAATATGA-3' Reverse 5'- AAGAACTGGCTTGGAGATGG-3'

Quantification was performed using a real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR green. The cycling parameters were as follows: initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Results were presented as levels of expression relative to those of controls after normalization to *ppia* using the  $2^{-\Delta\Delta CT}$  method. Analysis was carried out in triplicates.

#### **4.2.10. Western blotting**

Differentiated L6 myoblast (5-7 day) cultured in 6-well plates were treated with compounds of standardized concentrations for 24 h. L6 cells were homogenized in 1 ml of RIPA lysis buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min on ice and were centrifuged at 12000 x g for 10 min. Supernatants were then stored at -80°C until analysis. Upon thawing, protein content was assayed by the bicinchoninic acid method standardized to bovine serum albumin (Roche, Laval, QC, Canada). Lysates were diluted to equal concentration of total protein and boiled for 10 min at 75°C in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). 30 μL of each sample were separated on 10% polyacrylamide mini gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were blocked for 1 h at room temperature with 5% skimmed milk in TBST (20 mM Tris HCl, pH 7.6 and 137 mM NaCl). Membranes were then incubated overnight at 4°C in blocking buffer with appropriate phospho-specific or pan-specific antibodies against AMPK, P38MAPK, GLUT 4, AKT, IRS1, IRS2 (each at 1: 500 to 1:1000). Membranes were washed 3 times and incubated for 1 h at room temperature in TBST with anti-rabbit HRP-conjugated secondary antibodies at 1:1000 to 1:4000 (Santacruz, USA). Revelation of proteins was performed using diaminobenzidine method (Sigma, USA). Protein band intensities were evaluated by densitometry analysis using image densitometry software (Quantity one-4.6.7, 1D analysis software, Bio-rad, USA).

#### **4.2.11. Statistical analysis**

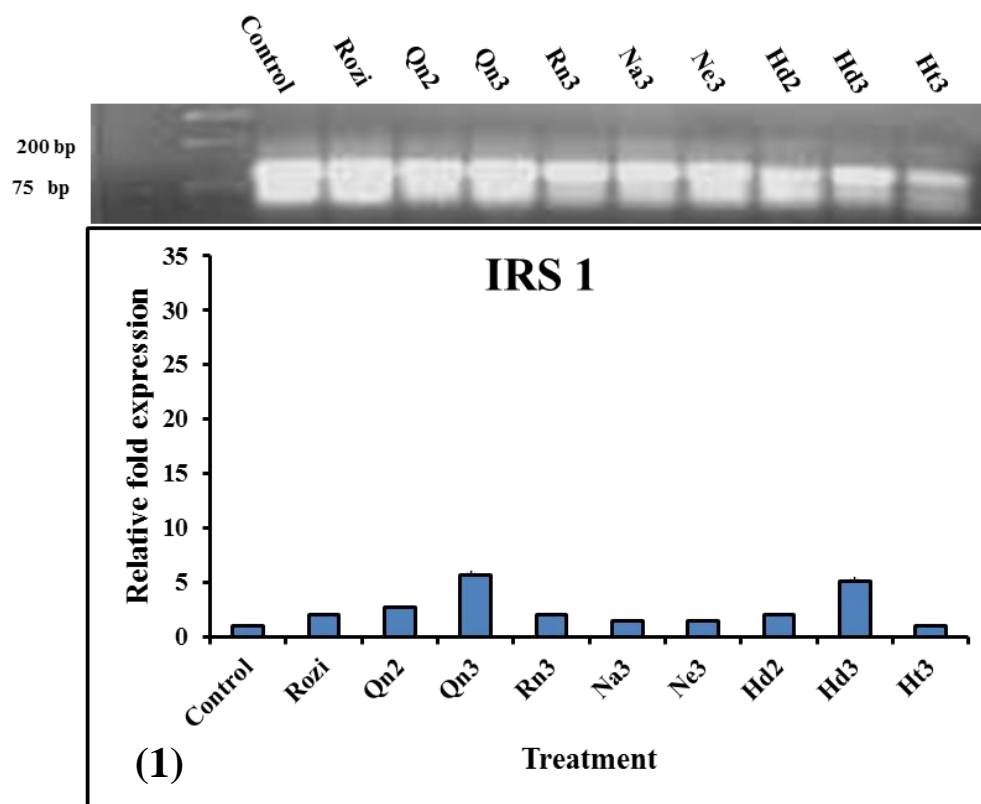
Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements (n = 3) of three different experiments. Data were subjected to one-way ANOVA and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at  $P \leq 0.05$ .

### 4.3. Results and Discussion

#### 4.3.1. Insulin signaling pathway

##### 4.3.1.1. Quantitative analysis of mRNA levels of key signaling molecules

To analyze the effect of the citrus flavonoids on insulin signaling pathway, the mRNA levels of the key signaling molecules such as PI3K, IRS 1 & AKT involved in insulin signaling pathway were evaluated. There was 5 fold increase in IRS 1 expression in cells pretreated with quercetin and hesperidin at 100  $\mu$ M whereas all the other compounds namely rutin, naringin, naringenin and hesperetin caused a feeble upregulation in L6 myotubes as shown in Fig 4. 2 (1). Quercetin (100  $\mu$ M) and hesperetin (100  $\mu$ M) pretreatment in L6 myotubes caused 30 fold increase in the expression of Akt, the key signaling intermediate. Rosiglitazone, rutin (100  $\mu$ M), naringin (100  $\mu$ M) and hesperidin (100  $\mu$ M) increased the Akt expression by 15 fold as shown in Fig 4.2 (2). There was no markable change in PI3K expression. Hesperidin and hesperetin pretreatment even downregulated PI3K expression as shown in Fig 4.2 (3).



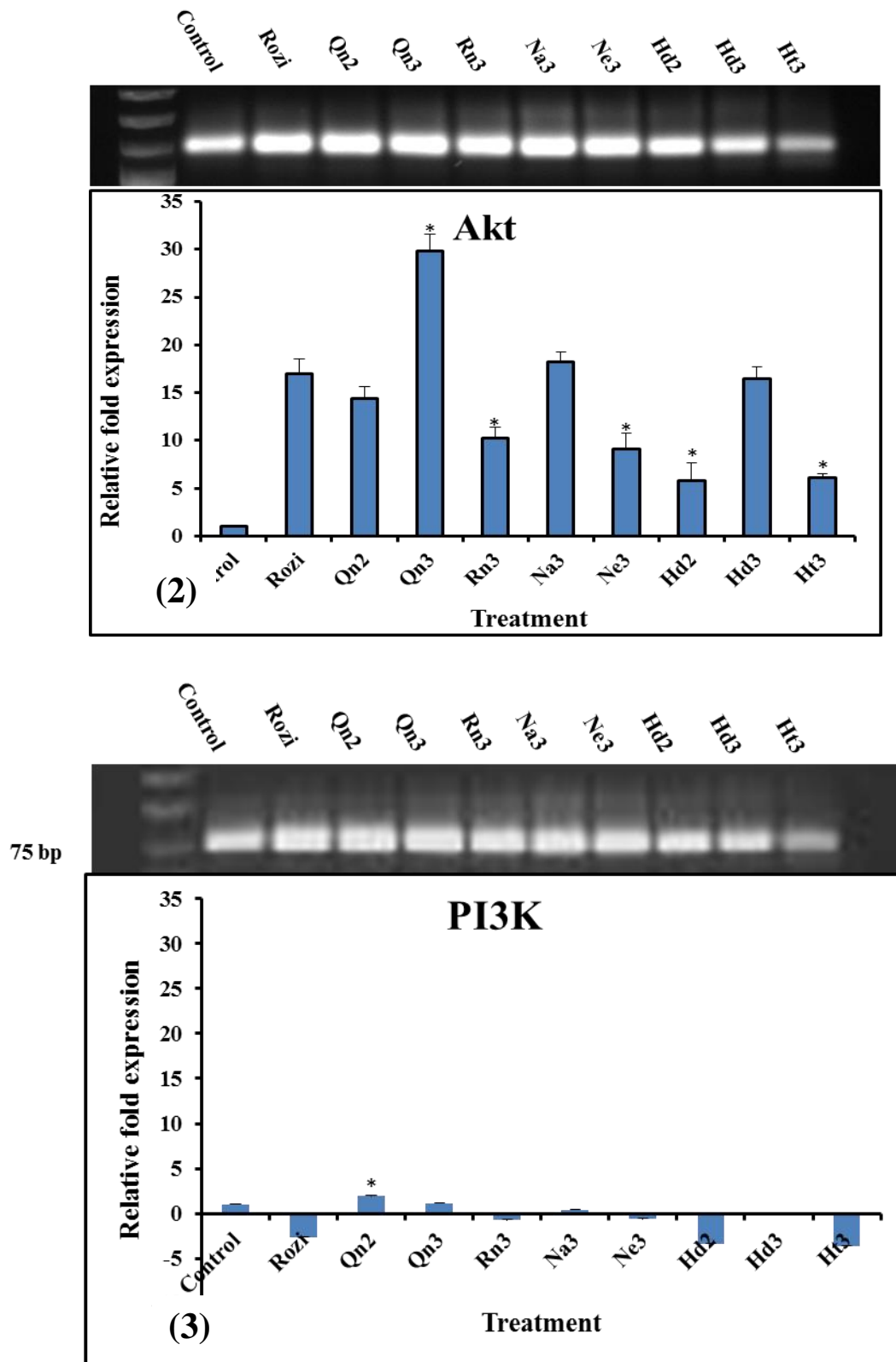


Fig.4.2 (1-3). Bar graphs show the mRNA levels (mean  $\pm$ SE) of IRS 1, Akt & PI3K in L6 myotubes on pretreatment of citrus flavonoids

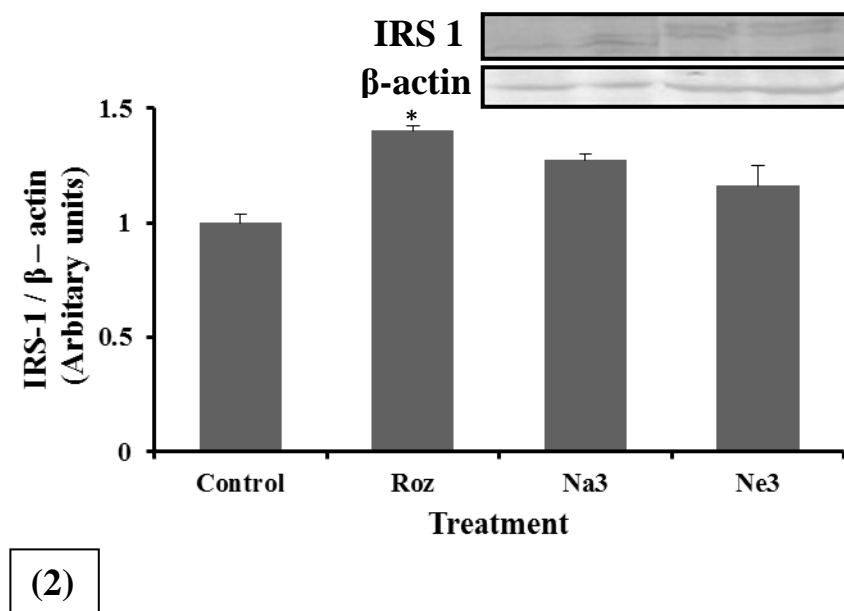
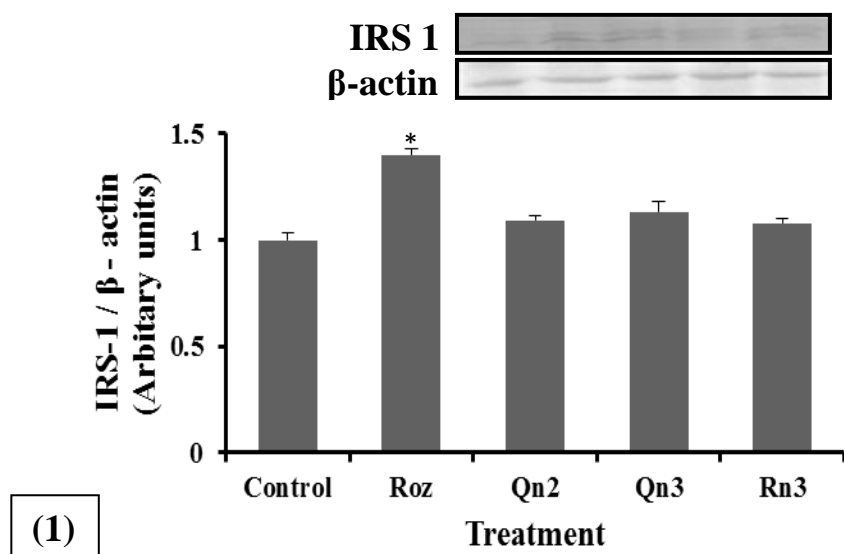
*Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M) ; Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M) ; Ht3: hesperetin (100  $\mu$ M). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses rosiglitazone.*

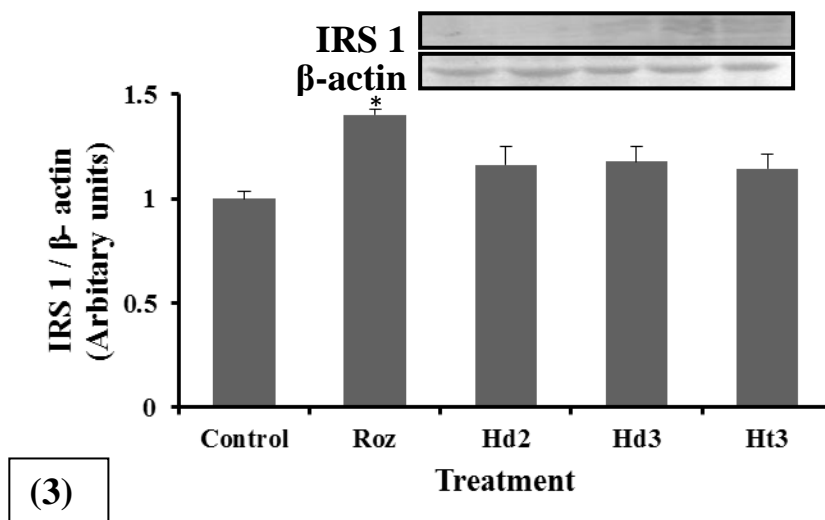
#### 4.3.1.2. Effect of citrus flavonoids on insulin signaling

Our earlier results (Chapter 3) point to the fact that the effect of citrus flavonoids was wortmannin insensitive indicating that the signaling molecules upstream of PI3K were not involved. To elucidate the signaling pathway followed by the citrus flavonoids, the protein expression of IRS1, IRS2, AKT & p-AKT in L6 myotubes on pretreatment were also analyzed. The IRS1 protein is the best characterized of intracellular substrates for IR kinase activity [Sun et al., 1991]. However, IRS2 was identified in mice as an alternative substrate following targeted disruption of the IRS1 gene [Araki et al., 1994; Tamemoto et al., 1994]. This protein is also an important component in the insulin-mediated signal transduction cascade. The IRS2 protein is 100 residues larger than IRS1. The overall amino acid sequence homology between IRS1 and IRS2 is 43% [Sun et al., 1995]. IRS1 and IRS2 propagate the signal to PI3K thereby activating insulin signaling pathway.

The contribution of insulin receptor substrate proteins (IRS1, IRS2, PI3K and AKT) were also investigated on pretreatment of citrus flavonoids. As expected there was a feeble expression of both IRS1 and IRS2 in L6 myotubes pretreated with all the citrus flavonoids as shown in Fig 4.3 (1-3) & 4.4 (1-3). As expected there was no markable change in PI3K expression, in addition hesperidin and hesperetin pretreatment even downregulated PI3K expression. It further confirmed our finding that the mode of action of citrus flavonoids was independent of insulin signaling. The stimulation of glucose uptake by insulin requires the insulin receptor (IRS) mediated activation of tyrosine residues of IRS and subsequent activation of PI3K and AKT [Hers et al., 2005; Welsh et al., 2005; Zaid et al., 2008]. Activation of PI3K was known to be involved in the hypoglycemic action of many bioactive compounds [Jung et al., 2007; Park et al., 2008; Shang et al., 2008]. Ginsenosides and uroslic acid were earlier reported to mimic insulin in stimulating PI3K pathway [Attele et al., 2002; Jung et al., 2007]. Pretreatment of quercetin, rutin and naringin showed one fold increase in the expression of

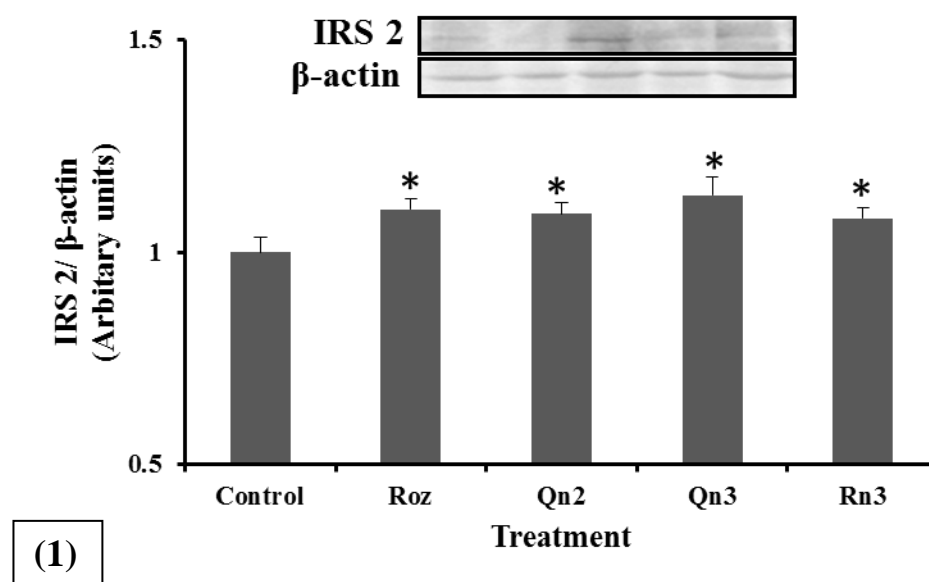
AKT. Since the activation depends on the phosphorylation of the key signaling molecules, we examined the expression of the phosphorylated proteins. Results shown in Fig 4.5 indicated phosphorylation of AKT, on pretreatment of quercetin, rutin and naringin.



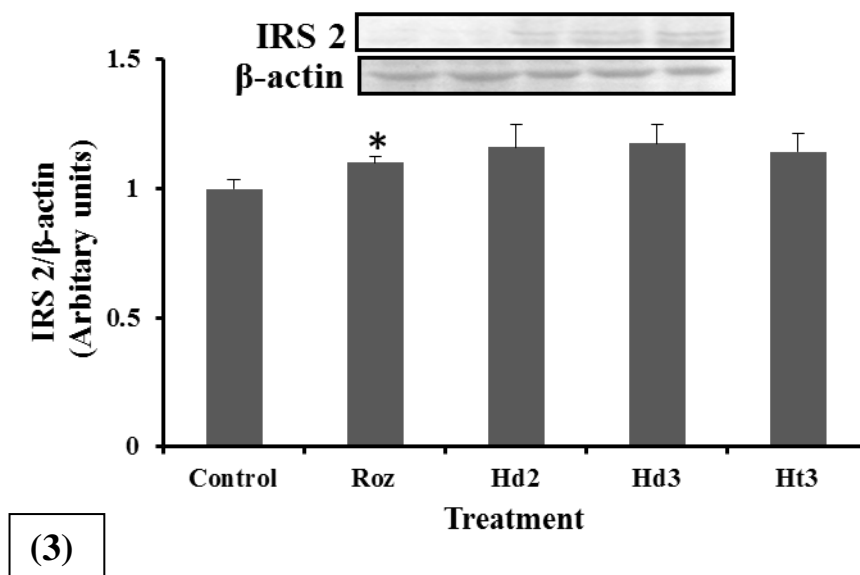
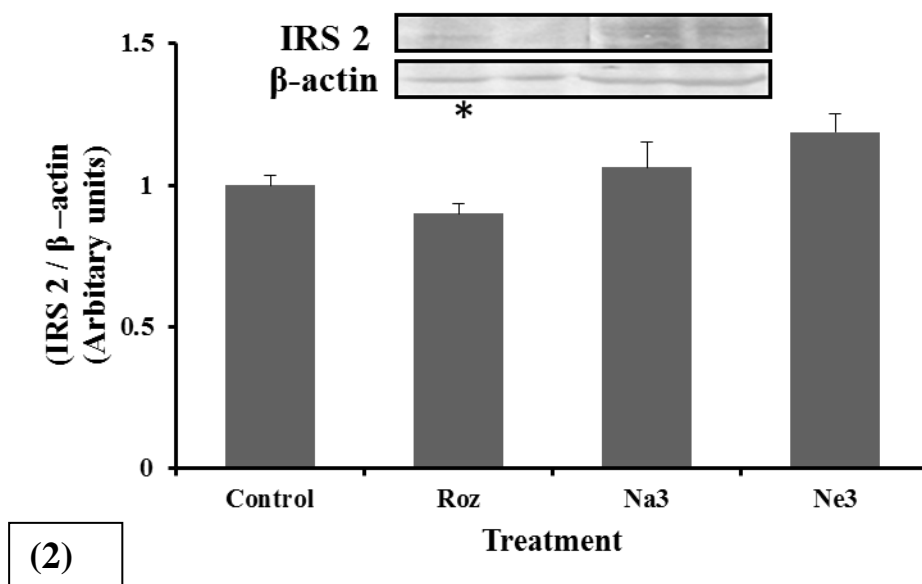


**Fig 4.3 (1-3).** *IRS1* expression in L6 myotubes on pretreatment

The effect of quercetin (10 & 100 $\mu$ M): Qn2 & Qn3, rutin (100 $\mu$ M): Rn3; naringin (100 $\mu$ M): Na3, hesperidin (10 & 100 $\mu$ M) and hesperetin (100 $\mu$ M) on pretreatment for 24h was comparable with that of the positive control rosiglitazone (100 nM). Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses control.

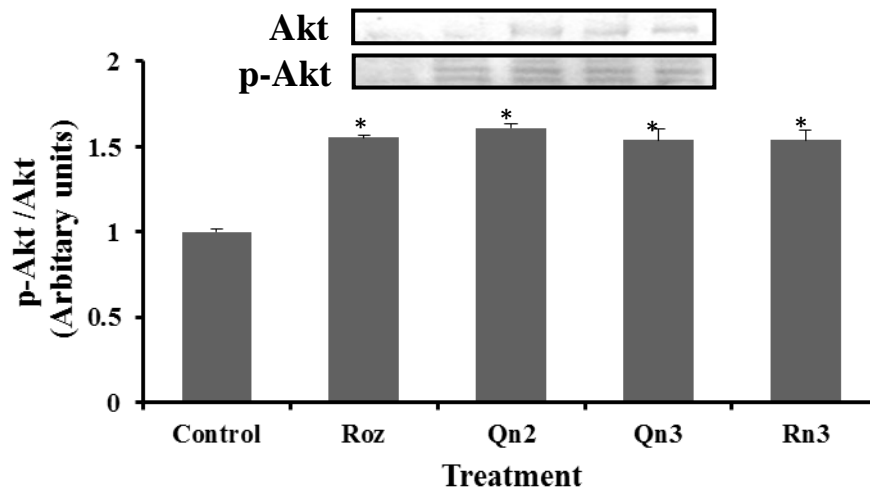




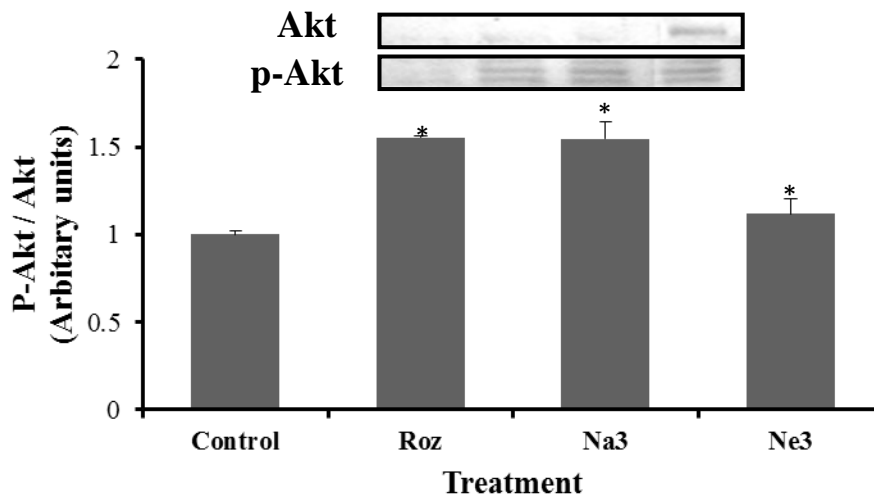


**Fig.4.4 (1-3). IRS 2 expression in L6 myotubes on pretreatment**

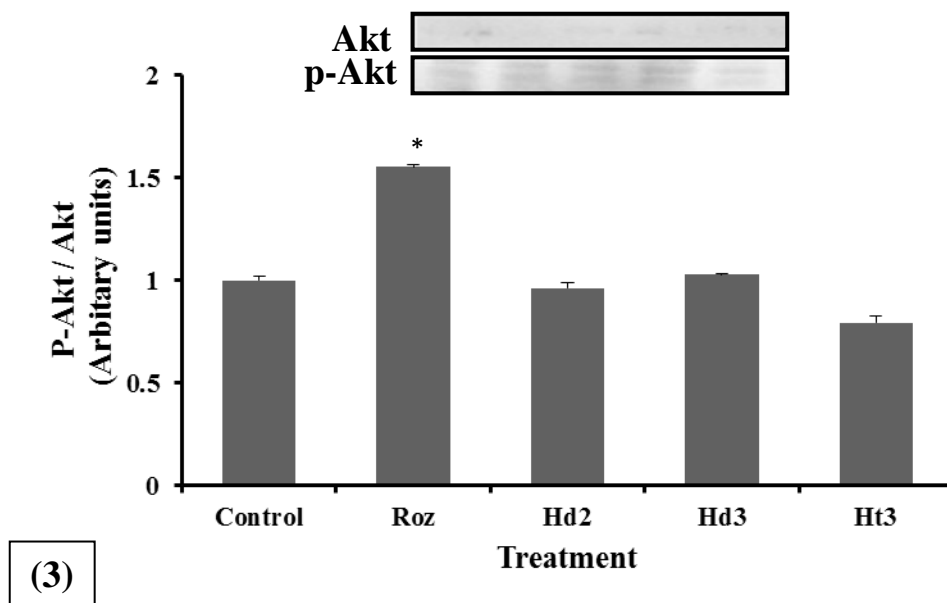
The effect of quercetin (10 & 100 $\mu$ M): Qn2 & Qn3, rutin (100 $\mu$ M): Rn3; naringin (100 $\mu$ M): Na3, hesperidin (10 & 100 $\mu$ M) and hesperetin (100 $\mu$ M) on pretreatment for 24h was comparable with that of the positive control rosiglitazone (100 nM). Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses control.



(1)



(2)



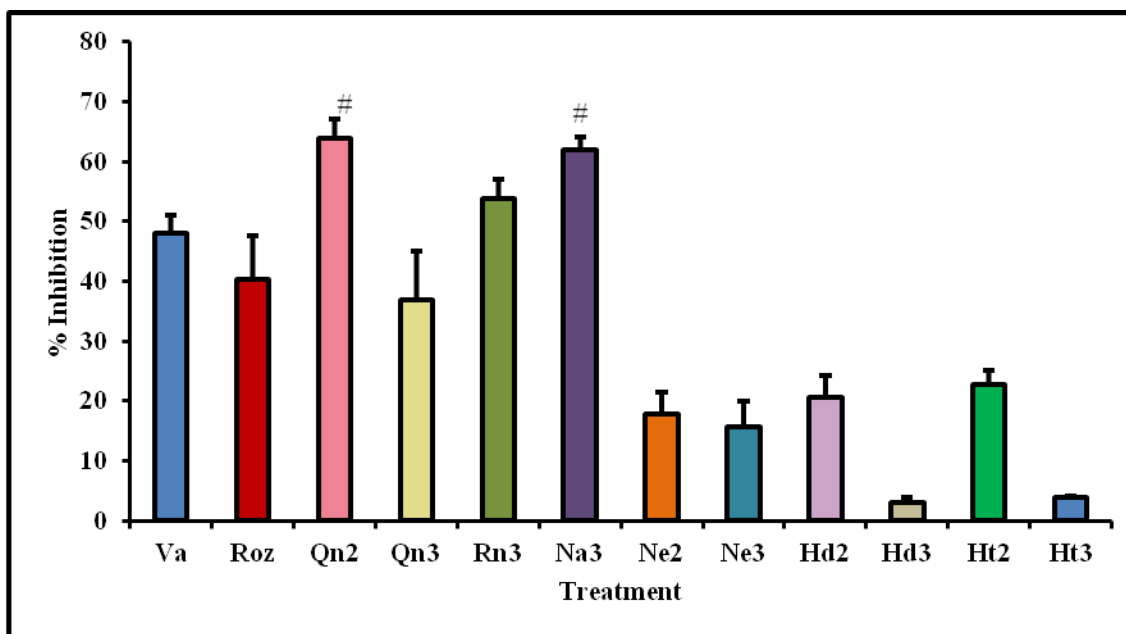
**Fig 4.5(1-3). Akt and p-Akt expression in L6 myotubes on pretreatment**

The effect of Quercetin (10 & 100 $\mu$ M): Qn2 & Qn3, rutin (100 $\mu$ M): Rn3; naringin (100 $\mu$ M): Na3, hesperidin (10 & 100 $\mu$ M) and hesperetin (100 $\mu$ M) on pretreatment for 24h was comparable with that of the positive control rosiglitazone (100 nM). Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus control.

### 4.3.2. Effect on PTP1b activity

PTP1b has been implicated as the negative regulator of the insulin signaling pathway. It dephosphorylates the specific phosphotyrosine residues on the insulin receptor thereby reducing the insulin receptor tyrosine kinase (IRTK) activity. Hence inhibition of this enzyme (PTP1b) would be validated as a therapeutic target in the treatment of NIDDM as well as obesity.

All the compounds were tested for their PTP1b inhibition potential at their optimum concentration. Quercetin (10  $\mu$ M), rutin (100  $\mu$ M) and naringin (100  $\mu$ M) exhibited 63.88, 53.95 and 62.3% inhibition which was higher than that of positive control (Sodium orthovanadate) (51%) as shown in Fig 4.6. The effect of quercetin at 100  $\mu$ M was comparable with that of the positive control. The effect of narigenin (100  $\mu$ M), hesperidin (10 & 100  $\mu$ M) and hesperitin (100  $\mu$ M) were negligible.



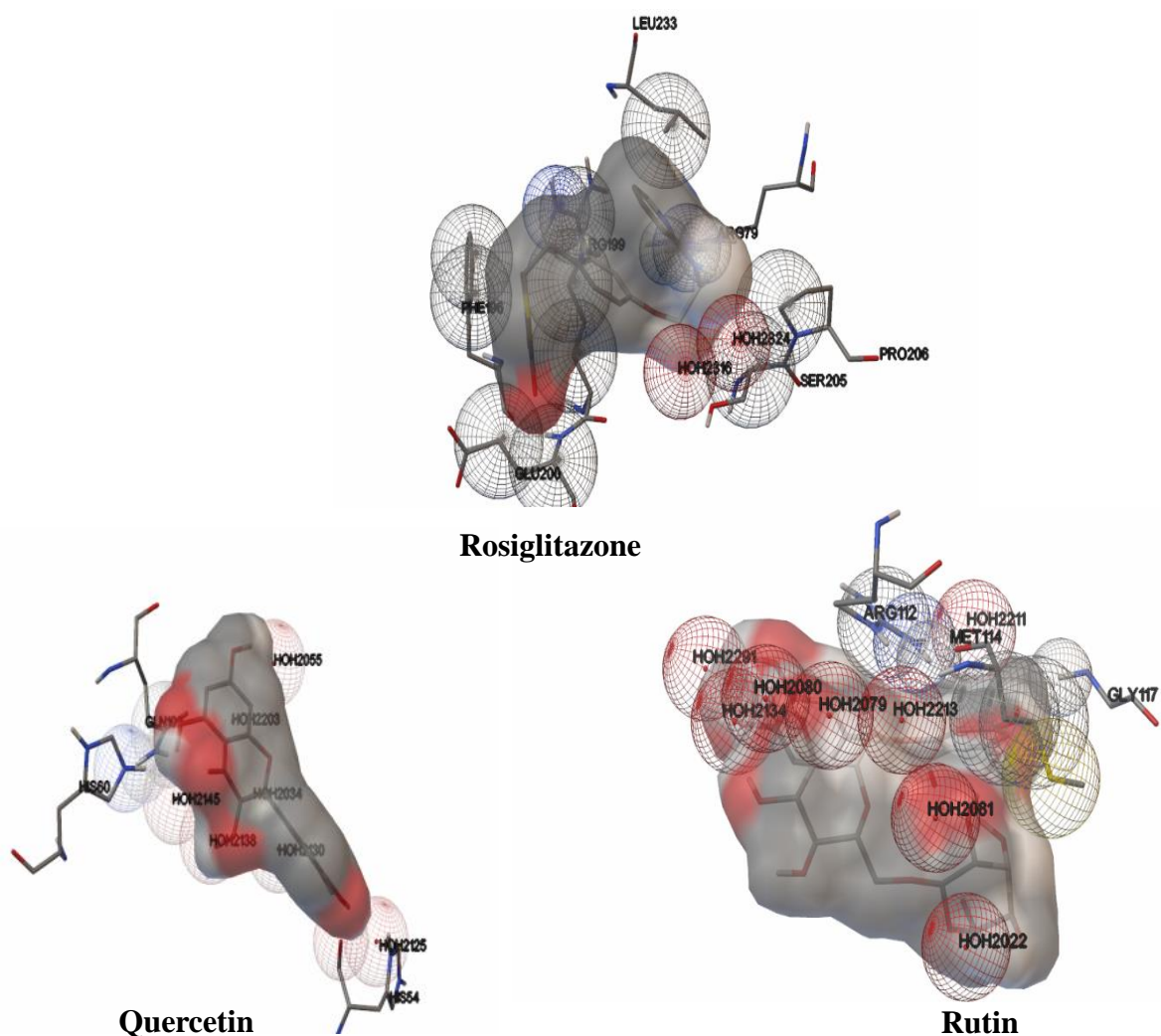
**Fig.4.6. Effect of flavonoids on PTP1B activity**

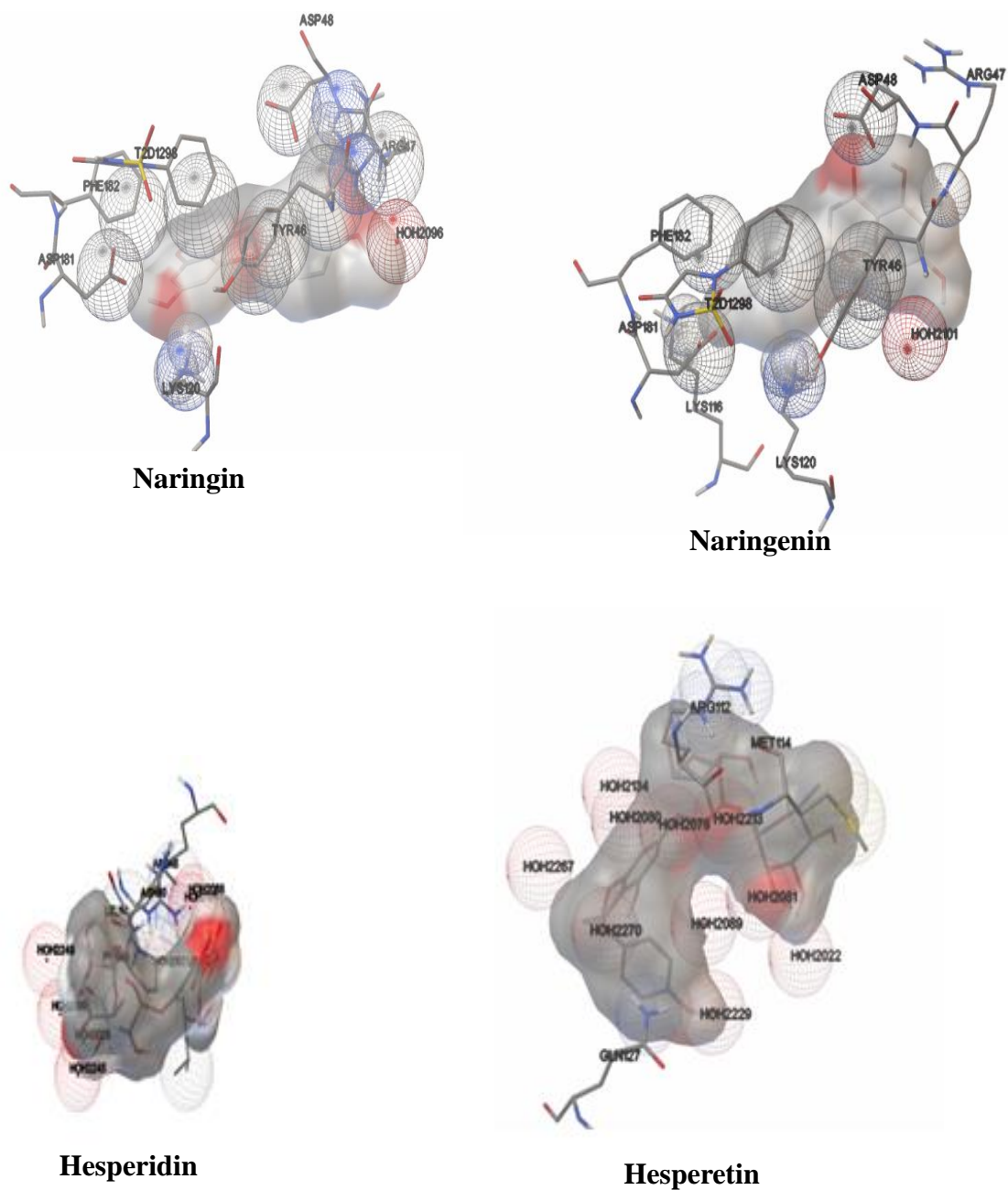
*Quercetin (10  $\mu\text{M}$ ), rutin and naringin at 100  $\mu\text{M}$  exhibited higher percentage inhibition than that of positive control (sodium orthovanadate). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . <sup>#</sup> $P \leq 0.05$  versus sodium orthovanadate.*

### 4.3.3. Molecular docking studies

Molecular docking was performed with autodock 4.2 programme to get insight into the binding modes of all the flavonoids with PTP1b. Docking parameters were validated by the crystal structure of the known ligand 1, 2, 5-thiadiazolidin-3-one-1, 1 dioxide. PTP1b has a catalytic motif phosphate-binding loop (P-loop) characterized by His-Cys-Ser-Ala-Gly-Ile-Gly-Arg from 214 to 221 residues having conserved Cys 215 and Arg 221 amino acids [Tonks, 2003; Zhang et al., 1994]. Other amino acids such as Lys120, Gln262, Val49, Arg47, Phe182 and 397 Tyr46 also contribute to peptide substrate recognition by a combination of hydrogen-bonding interactions, electrostatic and vanderwaals interactions [Tonks, 2003; Zhang et al., 1994]. Ten different poses of each ligand was generated in the binding site of PTP1b. The presented interaction modes of the ligands with the PTP1b binding site were determined from the highest scored conformations visualized using pyMOL. All the six compounds bound to PTP1b either to the active site or inhibitor site as shown in Fig 4.7. The binding energy thus determined revealed that all the compounds had high affinity to the

inhibitor site of PTP1b and the binding energy varied from compound to compound as the binding residue changed as shown in Table 4.2. Overall docking studies showed that the compounds, naringenin, hesperetin, quercetin, hesperidin & naringin possessed the least average binding energy of -5.44, - 5.12, -4.96, -3.54 and -3.37kcal/mol, respectively. Rosiglitazone showed an average binding energy of -3.75 kcal/mol. Overlay of the docked conformation of the ligand with the co-crystallised structure of PTP1b as shown in Fig 4.8 depicted the binding site of the compounds in PTP1b and its orientation. Though sodium orthovanadate was used as a positive control for cell line studies, being a small molecule its docking studies could not be performed. The high resolution model of PTP1b interaction with the compounds has been generated and depicted in Fig 4.9.



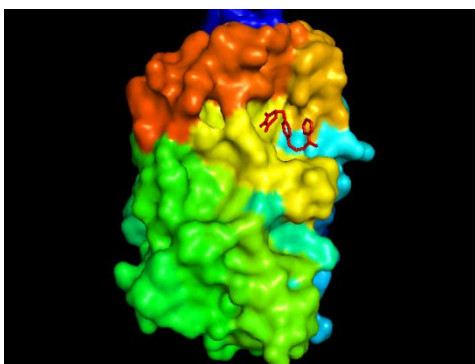


*Fig 4.7 Docking of the citrus flavonoids with PTP1b.*

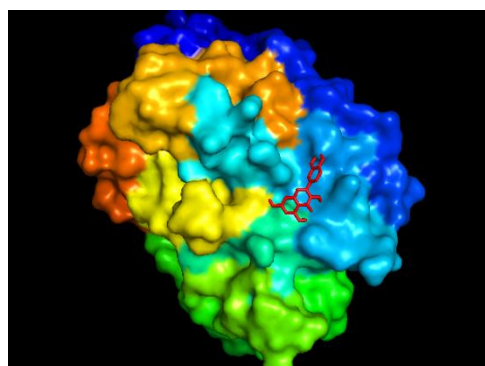
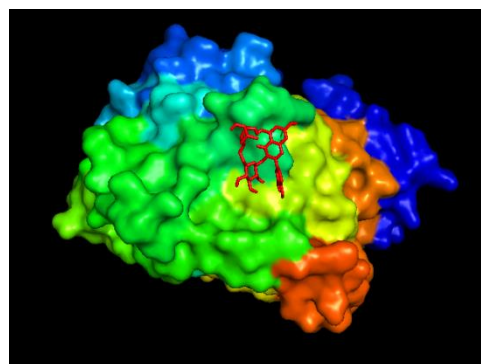
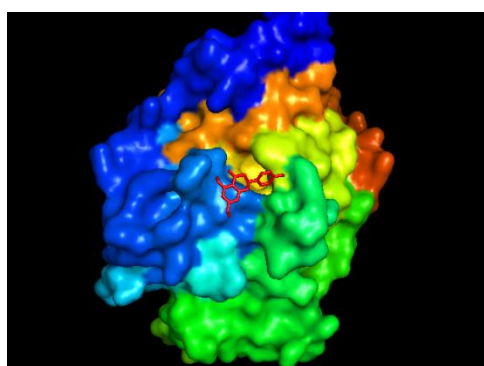
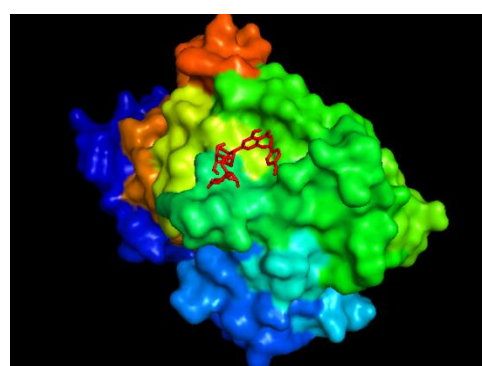
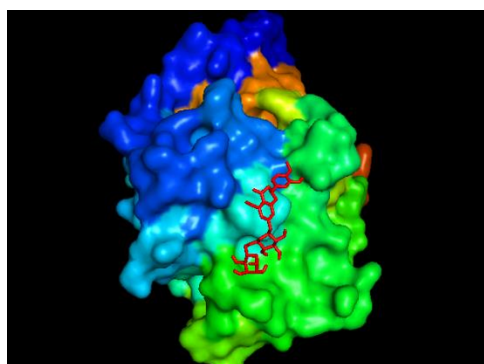
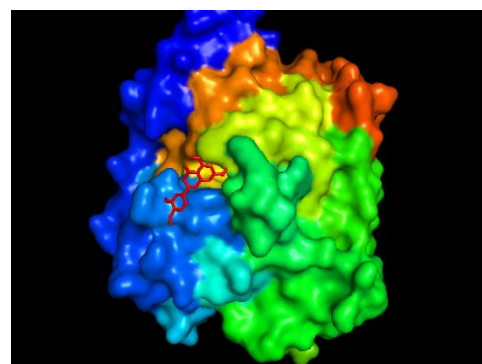
Docking studies shows that the flavonoids form non-covalent interaction with tyr-46, asp-181, phe-182, gln-262 residues of PTP1b which may reflect to the PTP1b inhibitory activities of the citrus flavonoids.

Table 4.2: Binding energy of the flavonoids to the inhibitor site of PTP1b

Molecule	Autodock 4		iGMDOCK			
	Binding Energy (kcal/mol)	Average Binding Energy (kcal/mol)	V-S-TYR-46	V-S-ASP-181	V-S-PHE-182	V-S-GLN-262
Rosiglitazone	-4.93	-3.75	-15.5116	-4.61236	-12.3452	-9.23973
Quercetin	-5.80	-4.96	-22.3287	0	-14.6115	-5.95274
Rutin	-4.27	-2.79	-21.2983	0	-15.3422	-9.7605
Naringenin	-6.02	-5.44	-21.9805	-7.92427	-10.526	-5.18502
Naringin	-4.06	-3.37	-19.1009	-4.16075	-17.7069	-5.76007
Hesperidin	-5.03	-3.54	-22.163	-5.12296	-14.0116	-6.18615
Hesperetin	-6.01	-5.12	-16.3136	-5.29851	-11.62	-4.05931



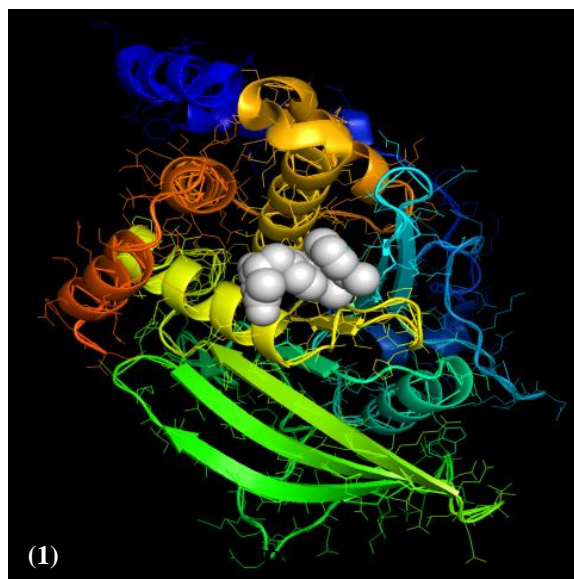
Rosiglitazone

**Quercetin****Rutin****Naringin****Naringenin****Hesperidin****Hesperetin**

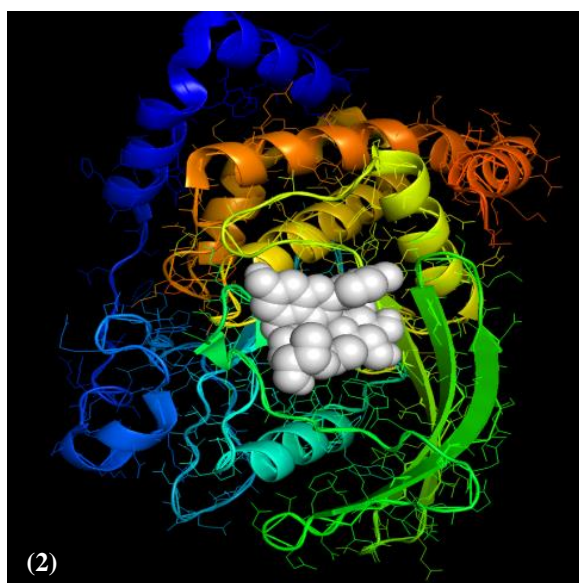
**Fig.4.8.** 3D image of PTP1b interaction with the citrus flavonoids

The citrus flavonoids were docked with PTP1b the 3d image was generated by pYMOL. The citrus flavonoids are coloured red.

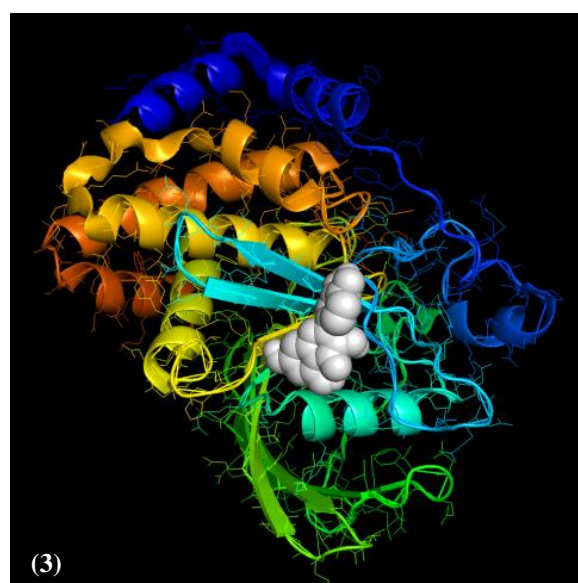




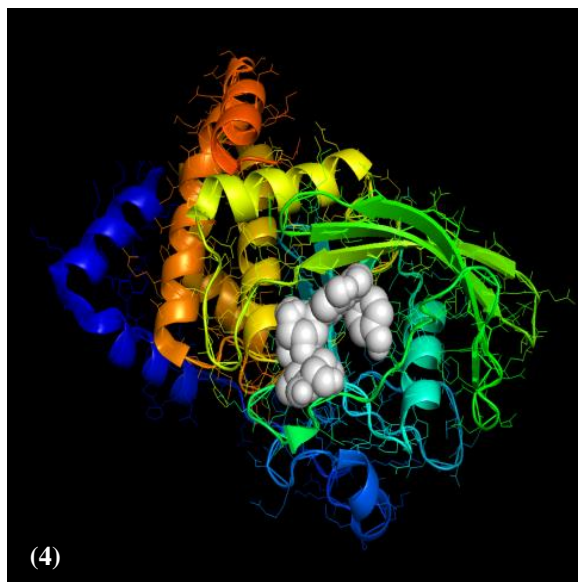
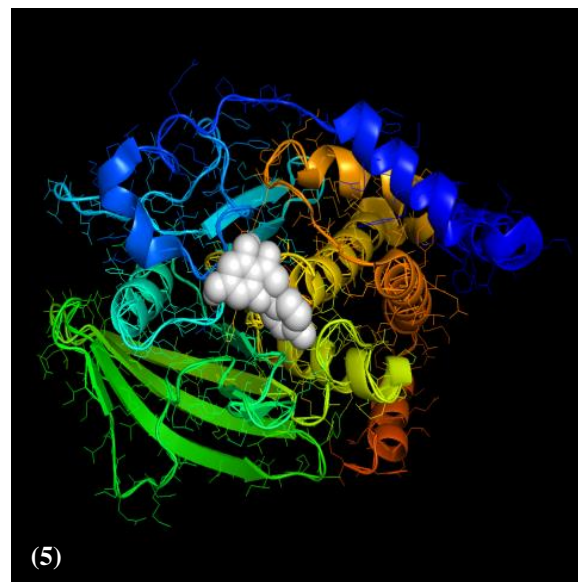
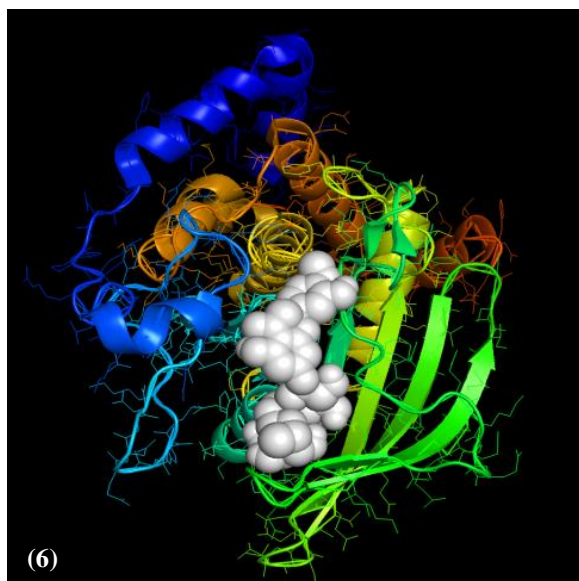
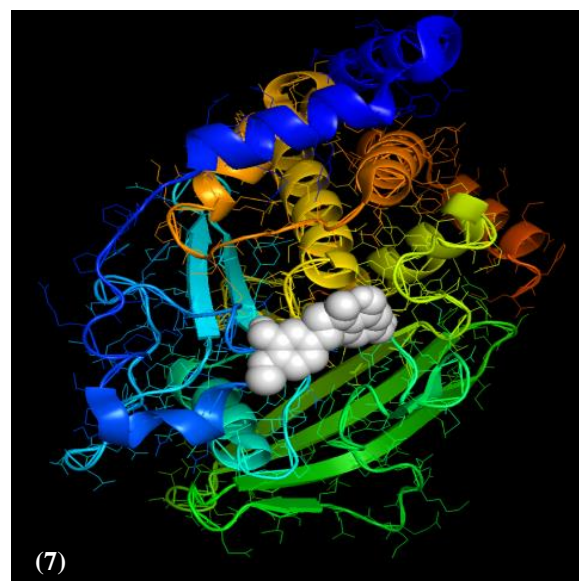
**Rosiglitazone**



**Rutin**



**Quercetin**

**Naringin****Naringenin****Hesperidin****Hesperetin**

**Fig.4.9. High resolution model of citrus flavonoid interaction with PTP1b**

High resolution image of ligand interaction with PTP1b generate by PyMOL. (1), (2), (3), (4), (5), (6) & (7) represents the interaction of PTP1b with the citrus flavonoids quercetin, rutin, naringin, naringenin, hesperidin & hesperetin, respectively. The citrus flavonoids are coloured white.

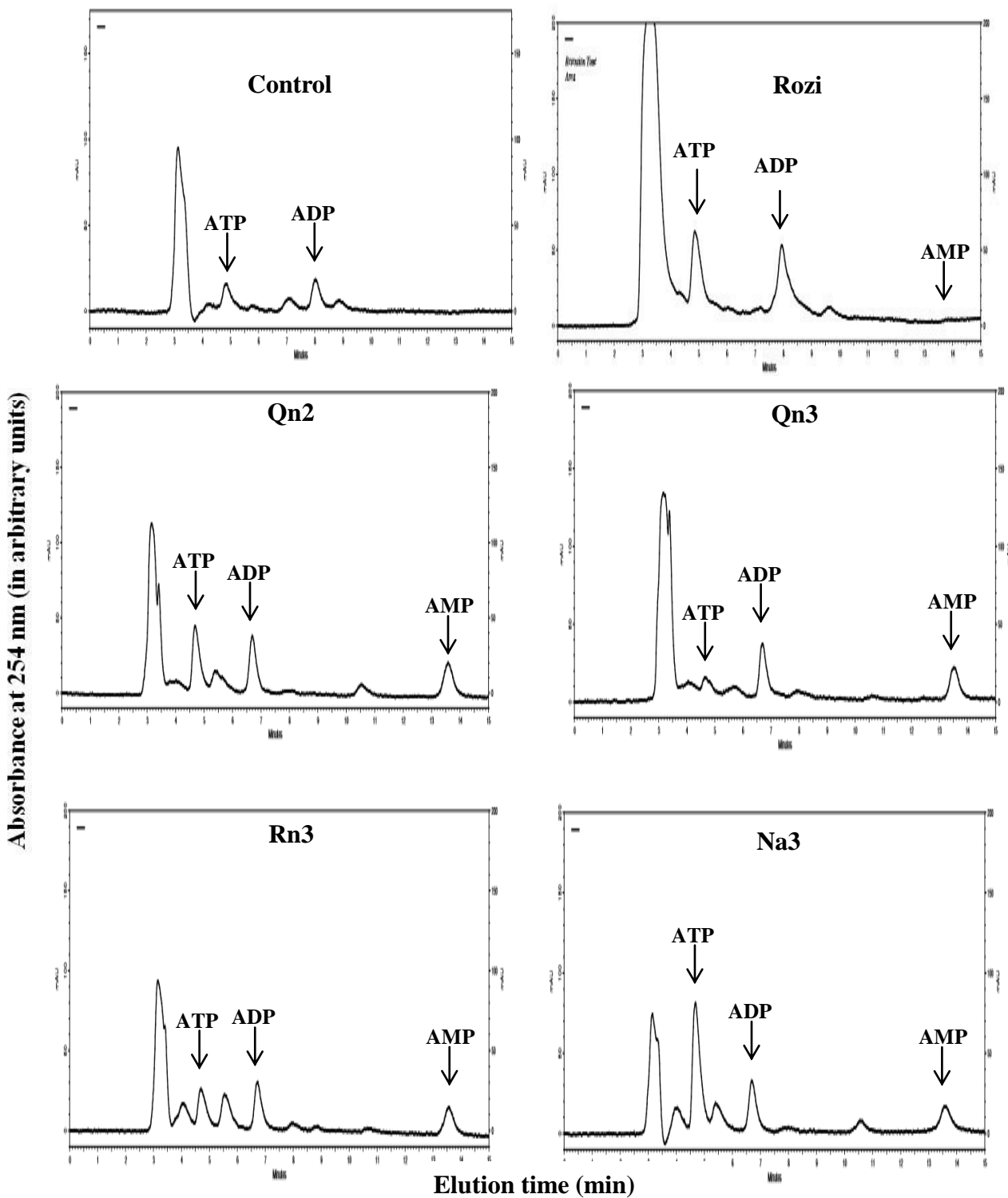
### 4.3.3. AMPK pathway

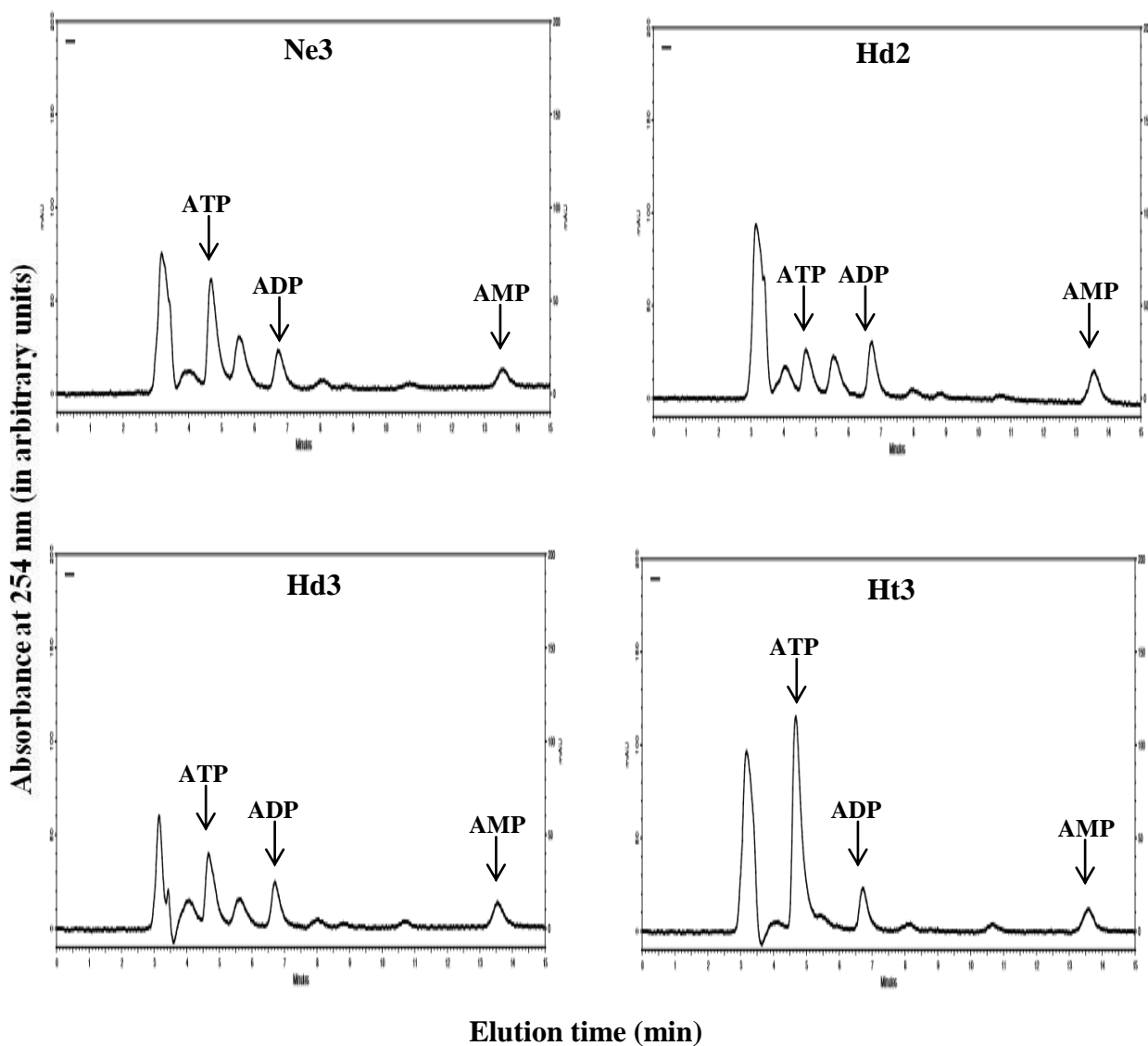
#### 4.3.3.1. Effect of the Flavonoids and glycoconjugates on AMP/ATP ratio

AMP stimulates AMPK phosphorylation by activating the upstream kinases and by inhibiting protein phosphatases. The cellular AMP, ADP and ATP concentrations (Table. 4.3) of L6 myotubes treated with flavonoids are determined by HPLC as depicted in chromatogram (Fig 4.10). And AMP to ATP as well as ADP to ATP ratio was determined as shown in Fig 4.11.

The AMP:ATP ratio is an essential factor for cellular AMPK activation. As the cellular AMP:ATP ratio varies as the square of the ADP:ATP ratio [Hardie and Hawley, 2001], we used the ADP:ATP ratio as a surrogate measure and found that the compounds increased both AMP/ATP as well as ADP/ATP ratio. Quercetin and rutin significantly increased the AMP to ATP ratio as well as ADP to ATP ratio followed by naringin and hesperidin. Narigenin and hesperetin caused a slight increase in AMP to ATP as well as ADP to ATP ratio. This may be the result of increased ATP utilization in these cells. AMPK is activated by various stimuli that cause an increase in AMP to ATP ratio which may be caused by intracellular ATP depletion. This decrease in ATP can originate from other pathophysiological changes like stress, hypoxia, ischemia and muscular contraction during exercise [Michael et al., 2007]. Similar effects of the antidiabetic drug rosiglitazone have been observed in H-2Kb muscles [Fryer et al., 2002]. Since metformin activates AMPK but causes no AMP: ATP ratio change in CHO, H4IIE [Hawley et al., 2002] and H-2Kb [Fryer et al., 2002] muscle cells, other molecular targets may also be involved in AMPK activation. AMPK can also be activated by mechanisms independent of changes in AMP to ATP ratio. AMPK can also be activated 5 fold by the allosteric modification mediated by AMP and 50-100 fold by phosphorylation of  $\alpha$ -subunit (Thr-172) by upstream kinase like tumour suppressor kinase (LKB1) and calcium-calmodulin mediated protein kinase (CaMKK). Many plant derived compounds have been reported to activate AMPK. These include resveratrol from red grapes, ginsenoside from *Panax ginseng*, curcumin from *Curcuma longa*, berberine from *Coptis chinensis* (used in the Chinese herbal medicine Huanglian), epigallocatechin gallate from green tea, the aflavin from black tea [Hwang et al., 2009], and hispidulin from snow lotus, another plant used in Chinese

herbal medicine [Lin et al., 2010]. Many of these compounds are claimed to have favorable effects in T2DM and the metabolic syndrome.





**Fig 4.10.** Analysis of adenine nucleotide levels in L6 myotubes

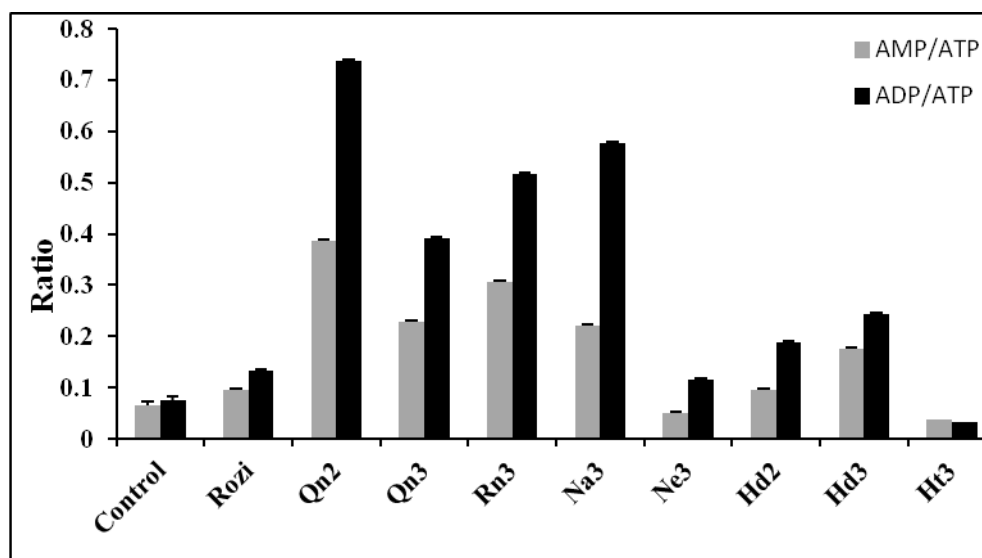
The effect of different treatments (Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M) ; Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M) ; Ht3: hesperetin (100  $\mu$ M)) on cellular nucleotides was determined by HPLC. In each case a representative trace is shown. The position at which ATP, ADP & AMP standards eluted are approximately 5.4, 7.02 & 13.5 min respectively as indicated on each trace by arrows.

Table .4.3. Concentrations of adenine nucleotides in L6 myotubes on pretreatment of citrus flavonoids

Citrus flavonoids	Concentration (pmol /10 <sup>5</sup> cells)				
	AMP	ADP	ATP	AMP/ATP	ADP/ATP
<b>Control</b>	0.037±0.007	1.783±0.001	0.591±0.002	0.064	0.076
<b>Rozi</b>	0.601* ±0.008	0.848±0.001	6.33* ±0.007	0.095*	0.134*
<b>Qn2</b>	1.056* ±0.005	2.015* ±0.002	2.731* 0.008	0.387*	0.738*
<b>Qn3</b>	0.684* ±0.008	1.170±0.008	2.984* ±0.005	0.229*	0.392*
<b>Rn3</b>	1.199* ±0.005	2.018* ±0.001	3.906* ±0.003	0.307*	0.516*
<b>Na3</b>	0.790* ±0.006	2.048* ±0.002	3.544* ±0.002	0.222*	0.578*
<b>Ne3</b>	0.404* ±0.007	3.219* ±0.003	7.108* ±0.005	0.056	0.453*
<b>Hd2</b>	0.610* ±0.007	1.830±0.001	6.327* ±0.008	0.096*	0.289*
<b>Hd3</b>	0.745* ±0.009	1.031±0.002	4.234* ±0.002	0.176*	0.244*
<b>Ht3</b>	0.662* ±0.009	0.580±0.001	6.565* ±0.007	0.100*	0.088*

The areas under AMP, ADP and ATP peaks were integrated to calculate AMP, ADP and ATP concentrations (pmol/10<sup>5</sup> cells). Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100 μM) ; Rn (3): rutin (100 μM); Na3: naringin (100 μM); Hd (2,3): hesperidin (10 & 100 μM) ; Ht3: hesperetin (100 μM). Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the the significance accepted at P≤0.05. \* P≤0.05.verses control.



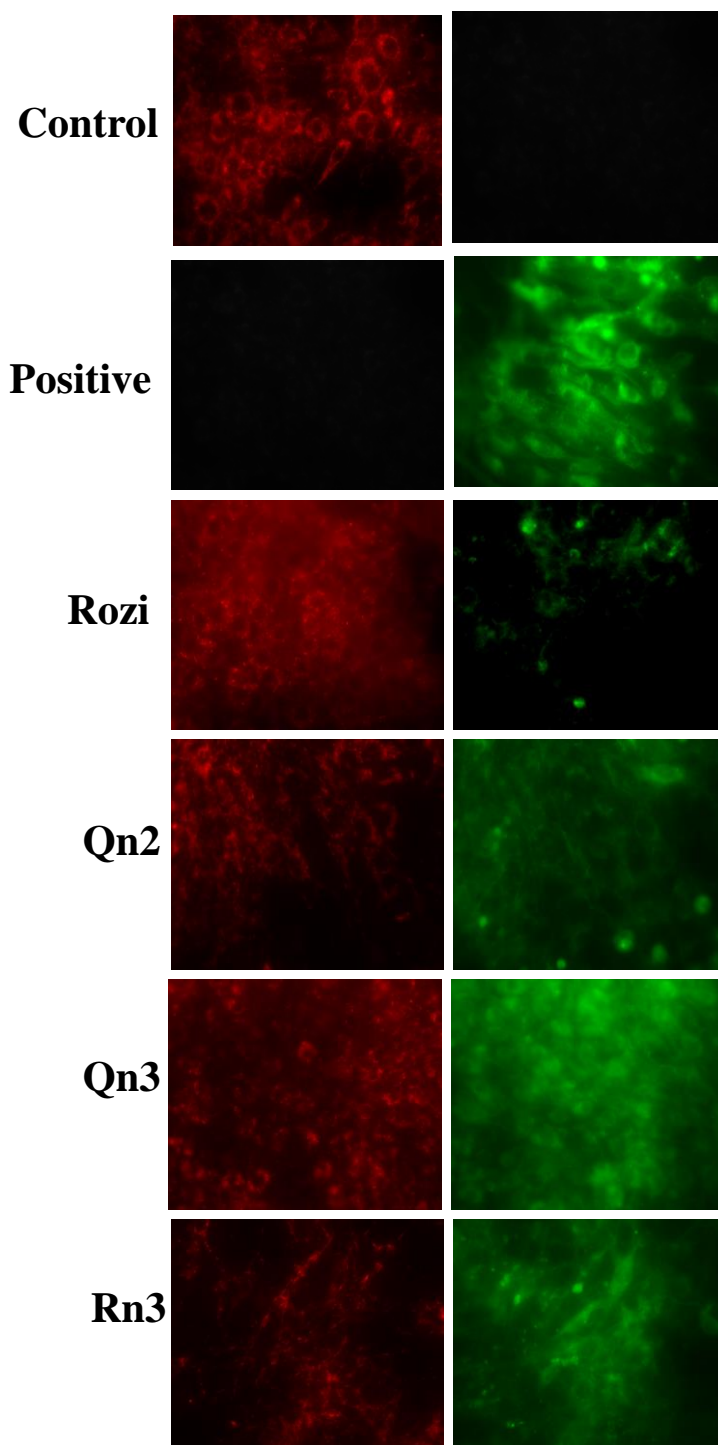


**Fig. 4.11. Adenine nucleotides ratio in L6 myotubes**

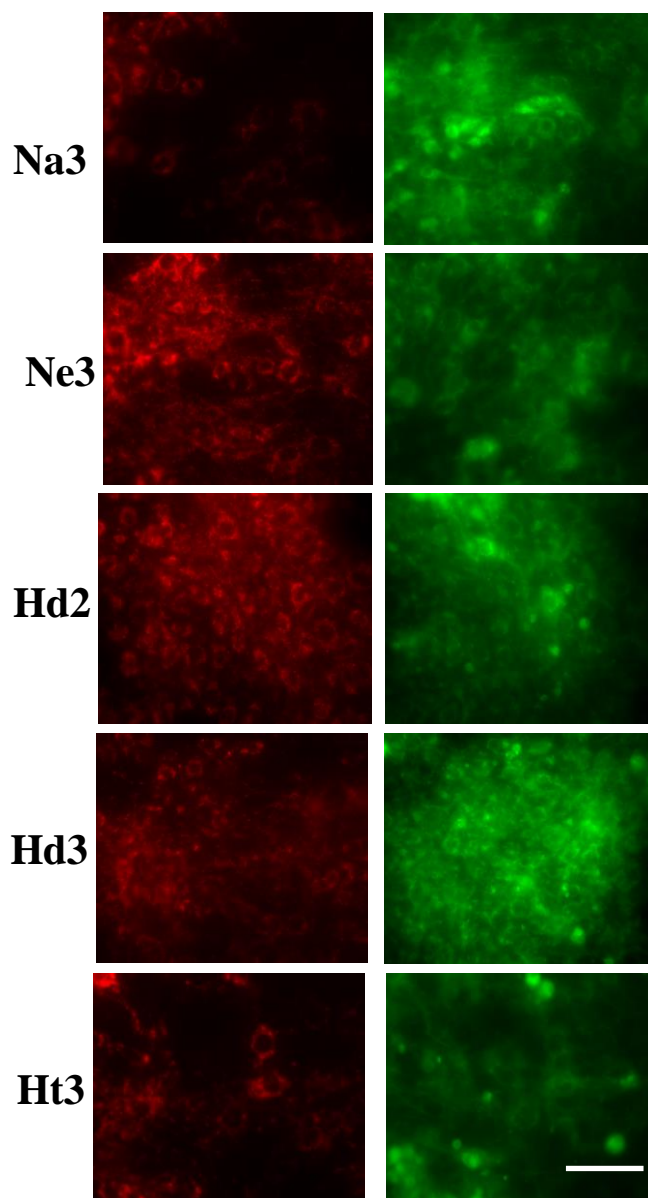
The ratio of AMP to ATP and ADP to ATP in L6 myotubes on pretreatment of citrus flavonoids are depicted in the above bar diagram. Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M); Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M); Ht3: hesperetin (100  $\mu$ M). Data are the mean results of three independent experiments  $\pm$  S.E.

#### 4.3.3.2. Transient mitochondrial depolarization

A transient depolarization was observed in L6 myotubes pretreated with the citrus flavonoids as shown in Fig 4.12, 4.13 & Fig 4.14. Several factors may lead to a mitochondrial depolarization including inhibition of mitochondrial respiration, induction of proton leakage (uncoupling) [Hutchinson et al., 2007] or opening of the mitochondrial permeability transition pore. As we had earlier proved the citrus flavonoids to be non-cytotoxic ( $\leq 10\%$ ) (Chapter 1), we ruled out the possibility of apoptosis or the opening of mitochondrial transition pore. Pretreatment of quercetin increased the AMP to ATP ratio which tightly correlated with its effect on mitochondrial membrane depolarization. This suggested that the citrus flavonoids especially quercetin, rutin, naringin and hesperidin likely inhibited respiration or respiratory complex I in L6 myotubes and therefore activated AMPK. These compounds shared the similar mechanisms with the well-known drug, metformin [Brunmair et al., 2004; Owen et al., 2000], highlighting the prospect of it as a lead compound for the treatment of T2DM.

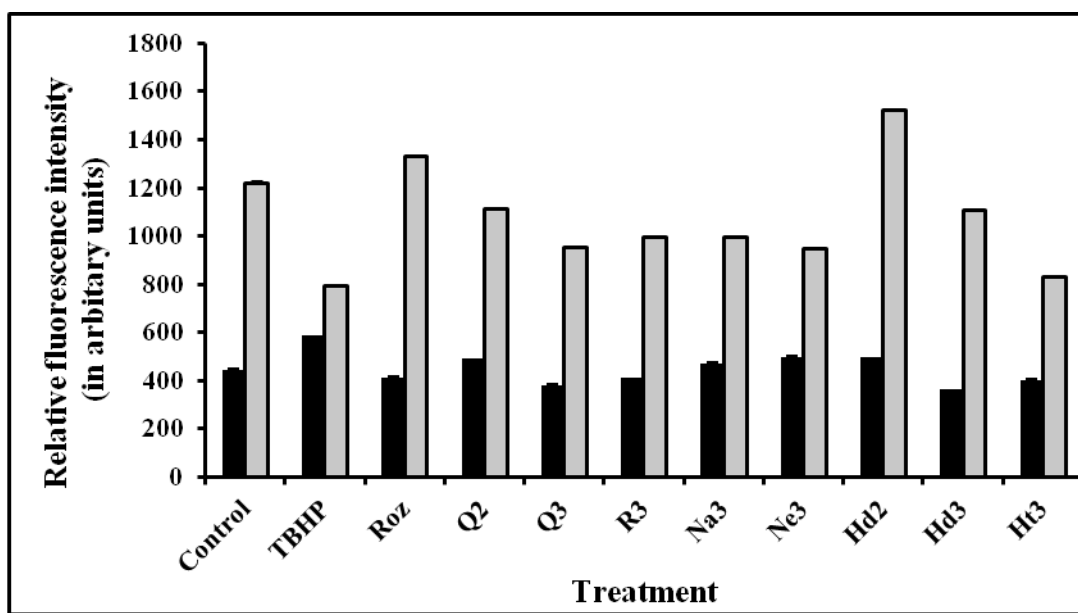






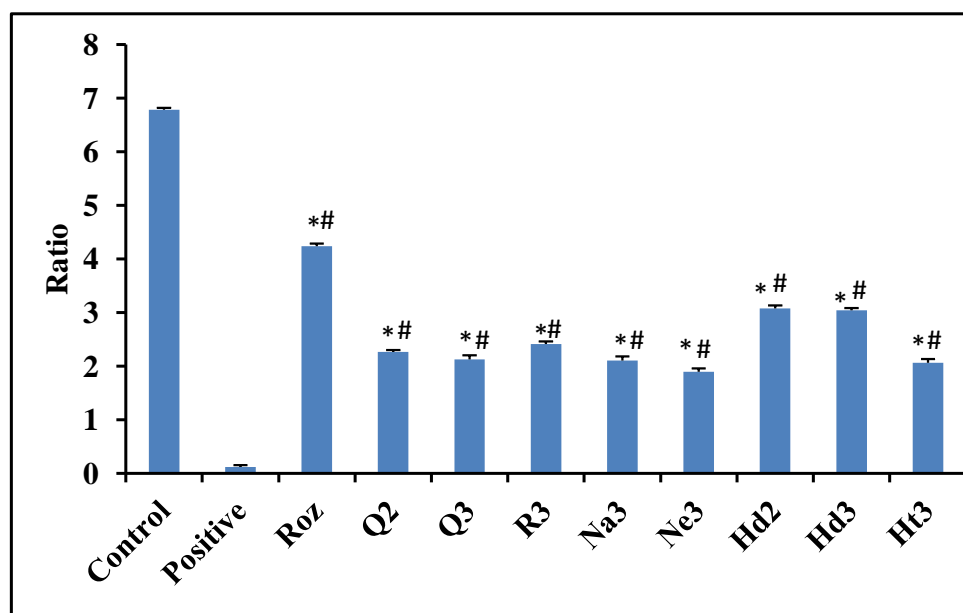
**Fig 4.12.** Change in mitochondrial transmembrane potential in L6 myotubes on pretreatment of citrus flavonoids

Fluorescent images of cells stained with JC1 on pretreatment of untreated cells (Control cells); Positive control valinomycin Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M) ; Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M) ; Ht3: hesperetin (100  $\mu$ M). Scale bar corresponds to 87  $\mu$ M.



**Fig 4.13. Fluorescence intensity analysis of red and green fluorescence**

Relative fluorescence intensity was analysed by BD Image Data Explorer software. There was a shift from red to green fluorescence with all groups indicating a transient change in mitochondrial transmembrane potential. (Control cells); Positive control valinomycin Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M); Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M); Ht3: hesperetin (100  $\mu$ M). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments.



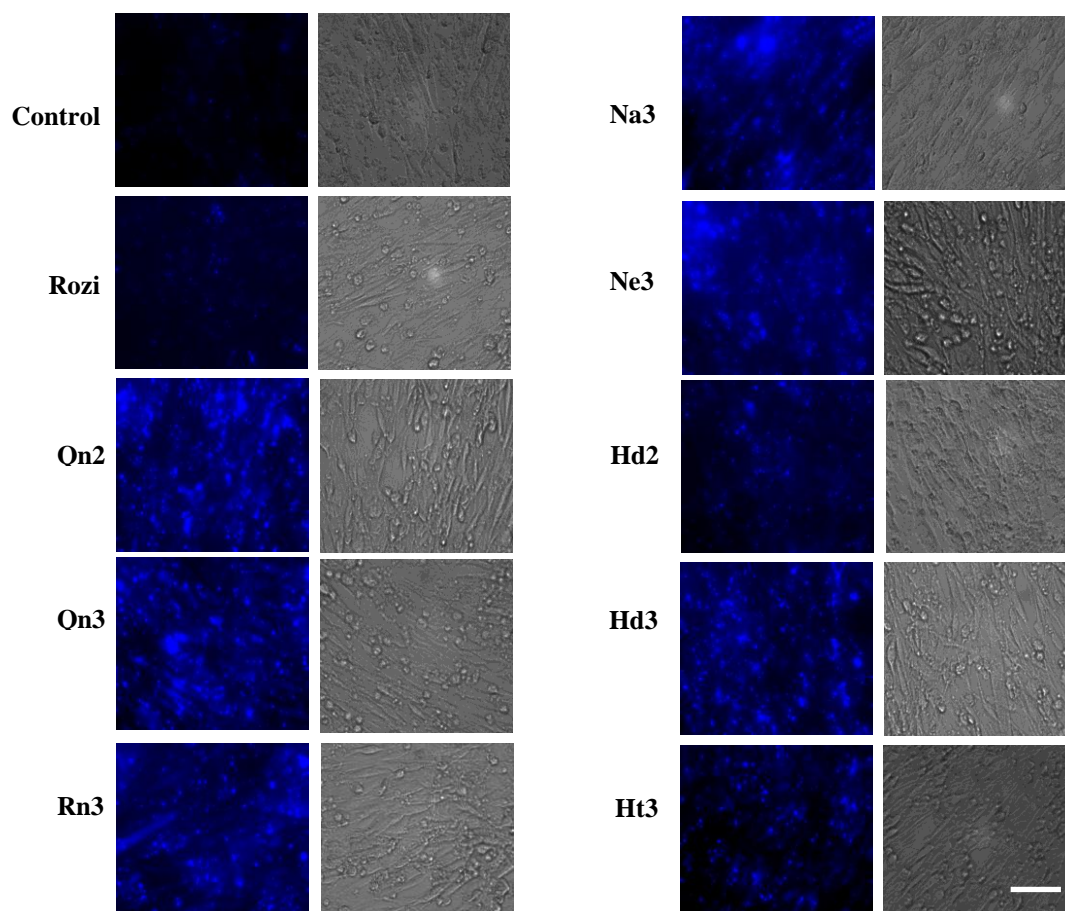
**Fig 4.14. Ratio of J-aggregates (red) to J-monomers (green)**

Bar diagram shows the ratio of red to green fluorescence generated in L6 myotubes on JC1 staining. (Control cells); Positive control valinomycin Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M); Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M); Ht3: hesperetin (100  $\mu$ M). Each

value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$ .verses control; # $P \leq 0.05$ .verses Positive control, valinomycin.

#### **4.3.3.4. Effect on intracellular calcium levels**

To assess the possible change in intracellular calcium levels in treated cells the calcium fluorescent dye Fura 2 was used. Our results showed that the flavonoids impaired calcium homeostasis by significantly increasing cytosolic calcium levels in L6 myotubes. Quercetin, rutin and naringin remarkably increased the intracellular calcium level in treated cells as shown in Fig 4.15, whereas naringenin, hesperedin and hesperetin showed a slight increase. This provided insight into the activation of CaMKK $\beta$  which in turn activated AMPK. CaMKK might have activated Thr 172 residue of the  $\alpha$ -subunit of AMPK, which resulted in the activation of the kinase. Activated AMPK may be responsible for the translocation of GLUT 4 receptors through various intermediate signaling molecules. AMPK is an important protein to provide energy in mammalian cells [Towler and Hardie, 2007] localized in muscles, brain, liver and pancreas, its complete molecular mechanism and function is still uncharacterized. There is hypothesis that it may be activating the transcription factors responsible for the expression of GLUT receptors in cells. Previous reports showed that resveratrol activated AMPK signaling [Miura et al., 2004; Breen et al., 2008] or insulin signaling including AKT [Deng et al., 2012]. Minakawa et al., (2011) proved that resveratrol promoted GLUT 4 translocation through simultaneously enhancing phosphorylation of both AMPK and Akt, resulting in stimulation of glucose uptake in skeletal muscle cells. Although AMPK signaling is independent of insulin signaling, GLUT 4 translocation to plasma membrane is a common final event.



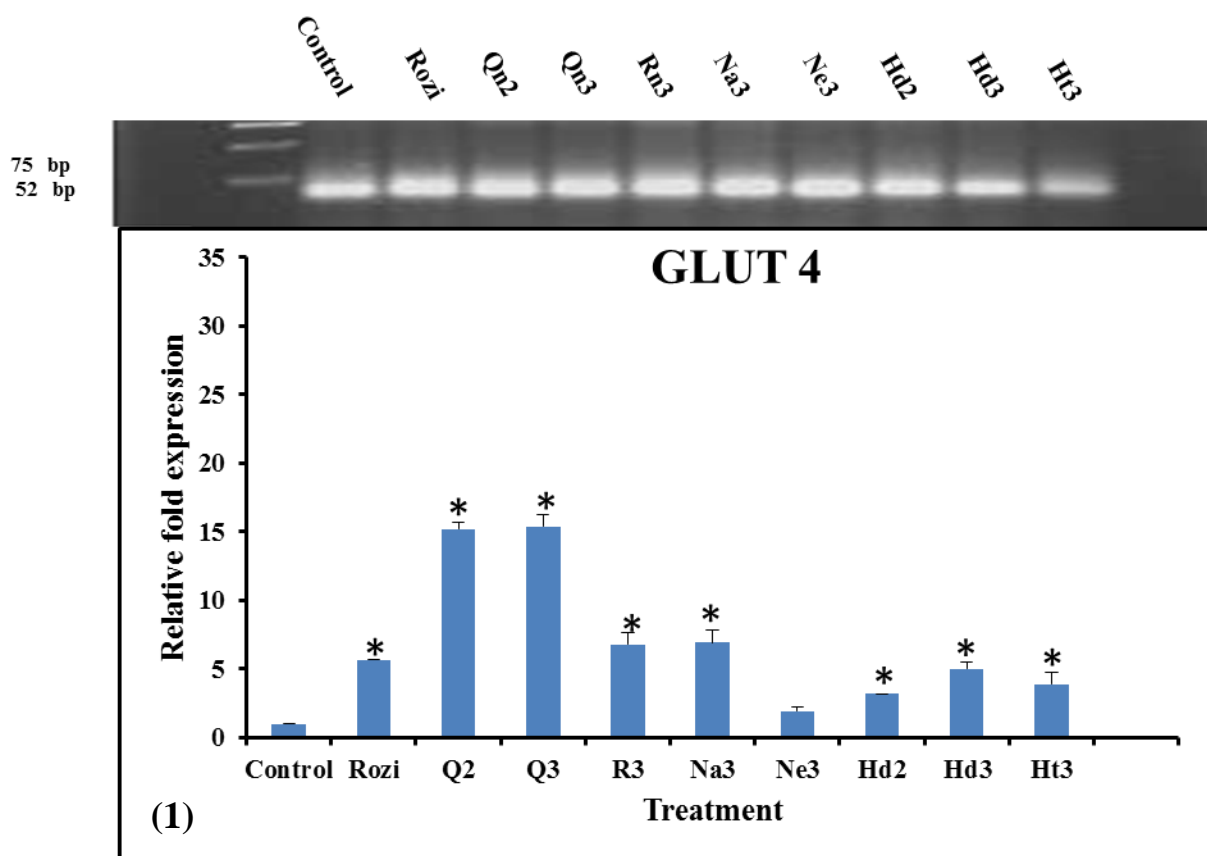
**Fig.4.15. Intracellular calcium levels in L6 myotubes**

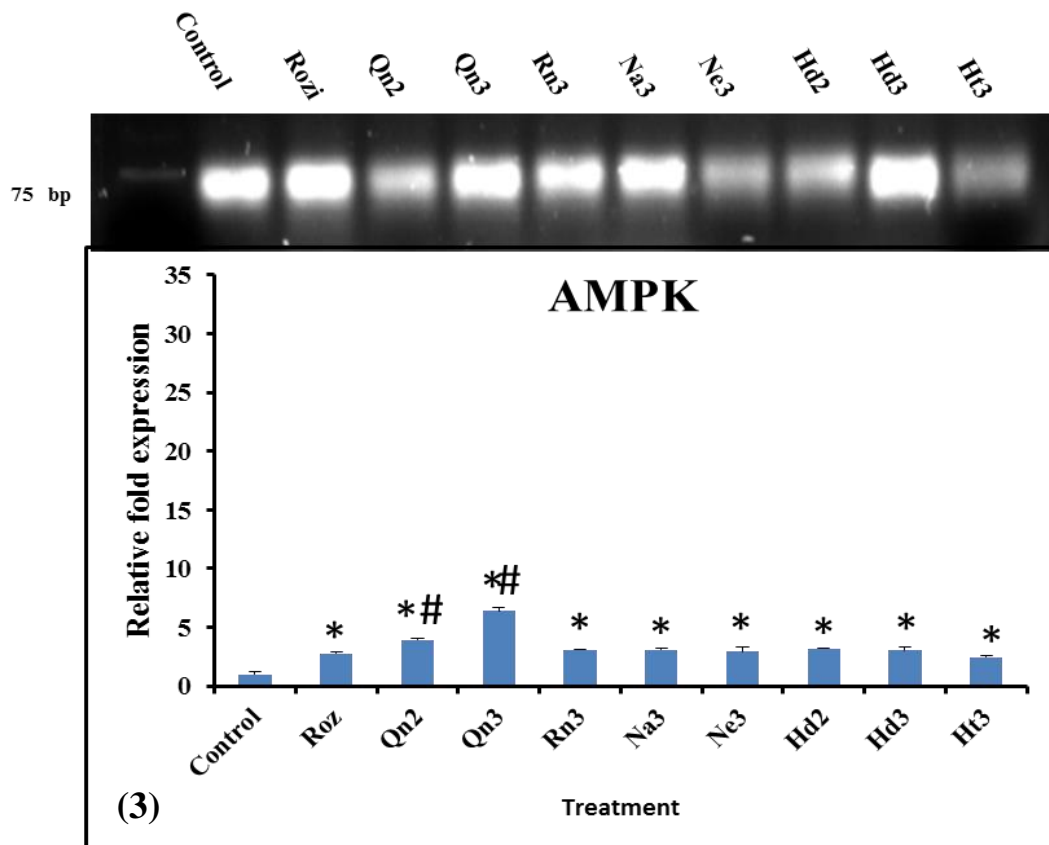
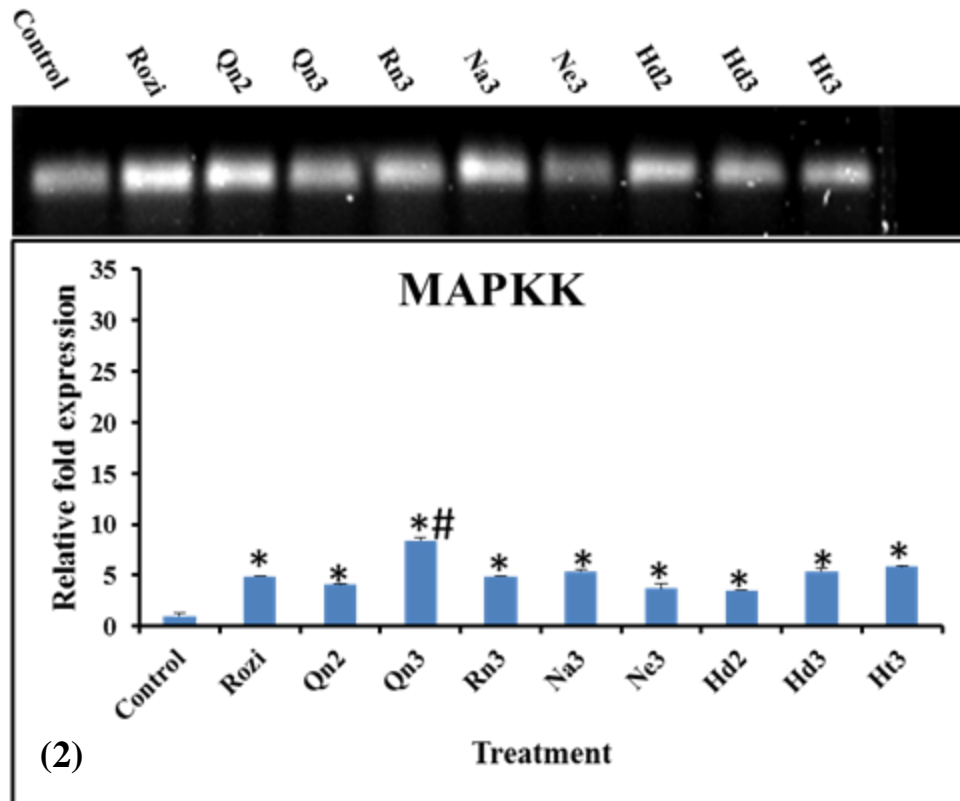
Fluorescent images of cytosolic calcium levels in L6 myotubes were determined by using Fura-2AM. Untreated cells (Control cells); Rozi: rosiglitazone (100 nM); Qn (2, 3): quercetin (10 & 100  $\mu$ M); Rn (3): rutin (100  $\mu$ M); Na3: naringin (100  $\mu$ M); Hd (2,3): hesperidin (10 & 100  $\mu$ M); Ht3: hesperetin (100  $\mu$ M). Scale bar corresponds to 87  $\mu$ M.

#### 4.3.3.5. Quantitative real time PCR

Glucose uptake is mediated by GLUT transporters, especially GLUT 4 in skeletal muscle. To evaluate the role of GLUT 4 in glucose transport induced by the citrus flavonoids in L6 myotubes, GLUT 4 mRNA levels were examined. GLUT 4 mRNA level was steadily and significantly increased on pretreatment of quercetin at active concentrations (10 & 100  $\mu$ M) as shown in Fig 4.16 (1). Quercetin showed a two fold increase in the expression of GLUT 4 compared to the positive control, rosiglitazone. On the contrary, rutin and naringin increased

the expression by one fold. Effect of naringenine, hesperidin and hesperetin was comparable with that of positive control. To analyze the signaling pathway involved, the mRNA levels of AMPK, P38MAPK & CaMKK in L6 myotubes on pretreatment was also evaluated as shown in Fig 4.16 (2-4). AMPK and P38MAPK was significantly upregulated on pretreatment of all the citrus flavonoids. These results demonstrated that the tested citrus flavonoids stimulated glucose uptake and induced upregulation of GLUT 4 by activating insulin independent AMPK pathway. We concluded that there exist a cross talk between insulin signaling and AMPK pathway as there was a significant upregulation of AKT and concomitant partial activation of IRS along with AMPK and P38MAPK.





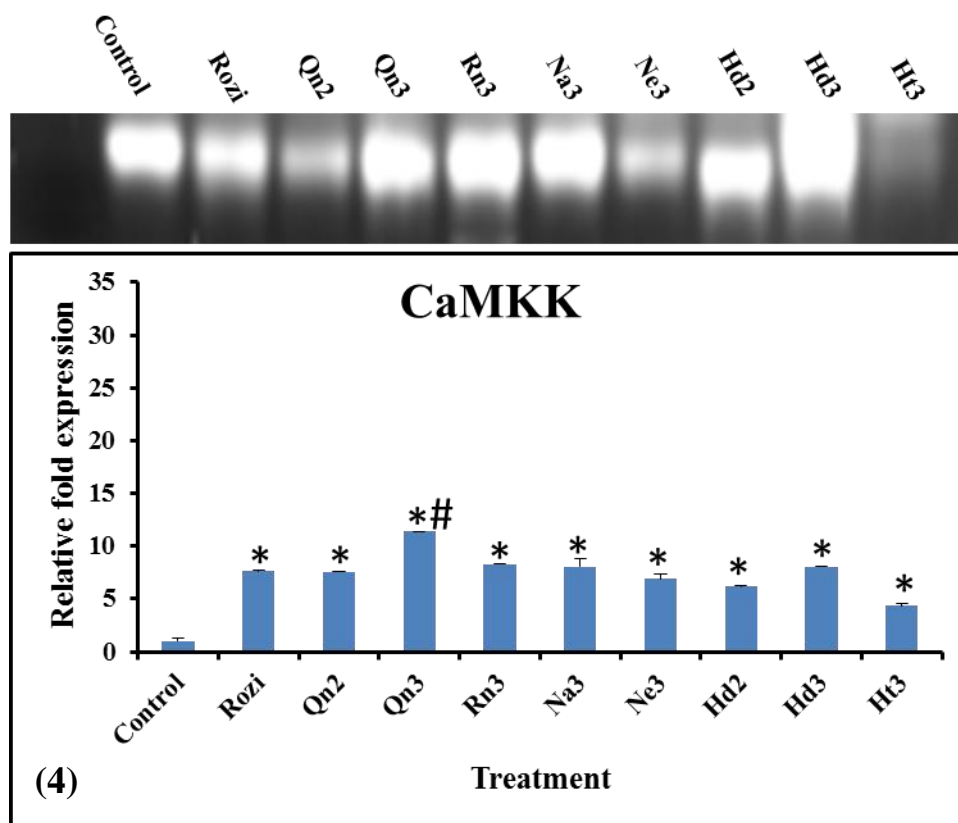


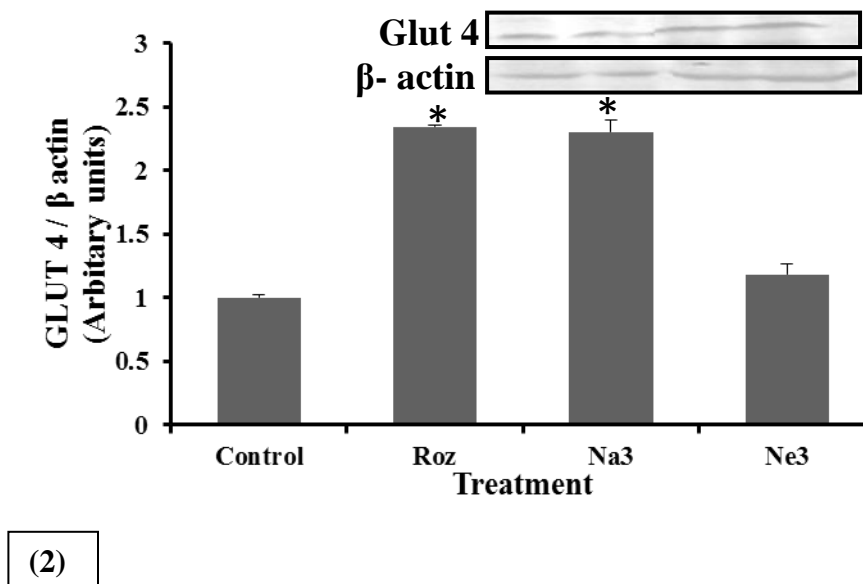
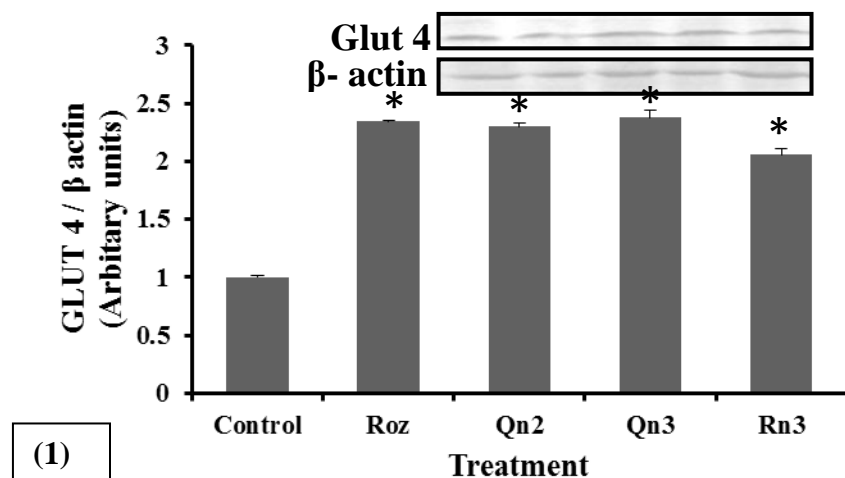
Fig. 4.16 (1-4). Bar graphs show the mRNA levels of GLUT 4, AMPK, MAPKK & CaMKK in L6 myotubes on pretreatment of citrus flavonoids

The mRNA levels (arbitrary units) are expressed in relative to those of control cells. Quercetin (10 & 100 $\mu$ M): Qn2 & Qn3, rutin (100 $\mu$ M): Rn3; naringin (100 $\mu$ M): Na3, hesperidin (10 & 100 $\mu$ M) and hesperetin (100 $\mu$ M). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus control; #  $P \leq 0.05$  versus Rosiglitazone (Rozi).

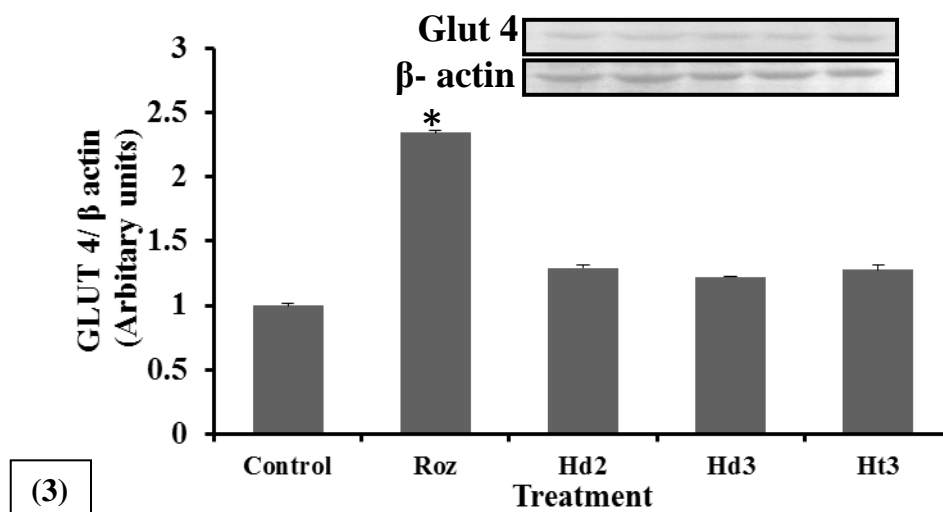
#### 4.3.3.5. AMPK increased glucose uptake through GLUT 4 mediated mechanism

In order to provide insight in to the possible involvement of AMPK pathway in glucose uptake stimulated by the citrus flavonoids, protein expression analysis was carried out by western blotting. The translocation of GLUT 4 from an intracellular location to the plasma membrane and transverse tubules is thought to be the major mechanism by which exercise, AMPK activators and insulin increase skeletal muscle glucose transport in mammals [Rose et

al., 2005]. In the present study, we report that the citrus flavonoids especially quercetin, rutin, naringin and hesperidin stimulated glucose uptake in L6 myotubes via activation of AMPK. The effects of these compounds are similar as that of metformin, the well-known drug against T2DM. Metformin has been shown to increase glucose uptake (5 fold) in rat cardiomyocytes by increasing the retention time of GLUT 4 at the plasma membrane [Yang et al., 2006]. In addition metformin treatment provoked acute increases in the translocation of GLUT 4 to the plasma membrane in mice gastrocnemius muscle and in L6 myotubes [Sajan et al., 2010].





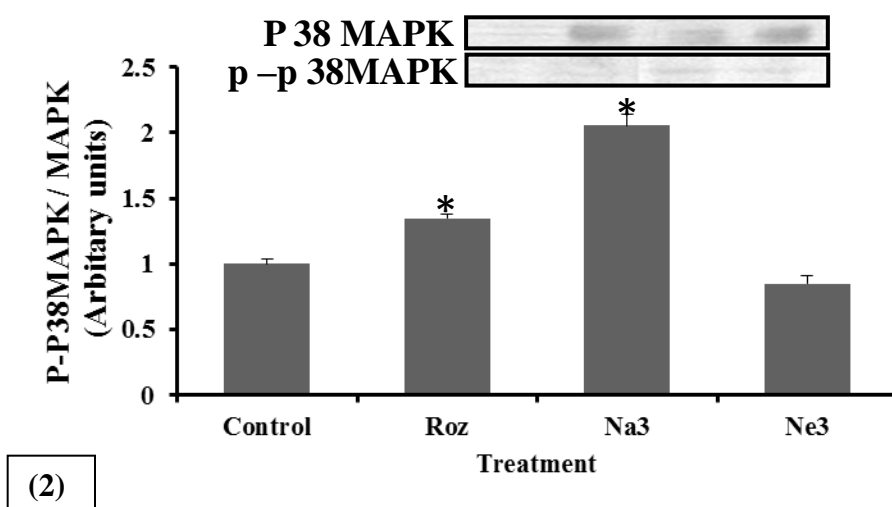
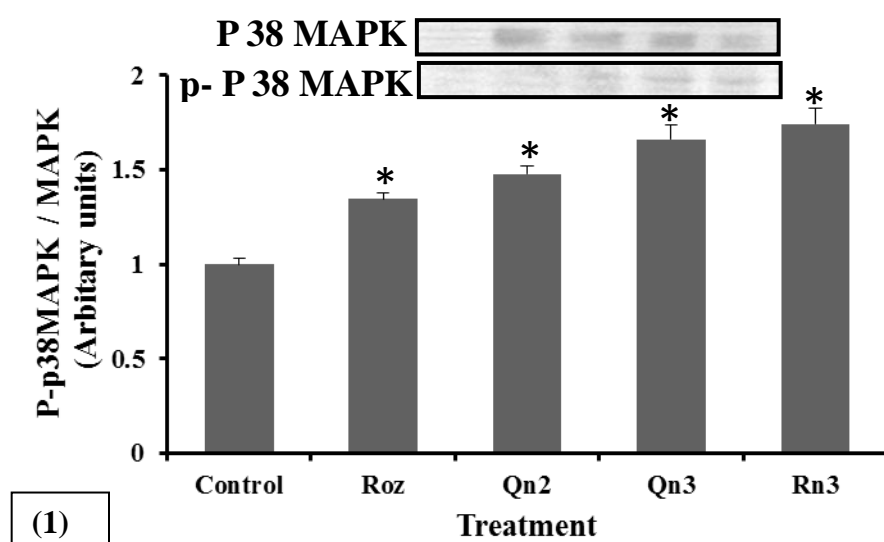


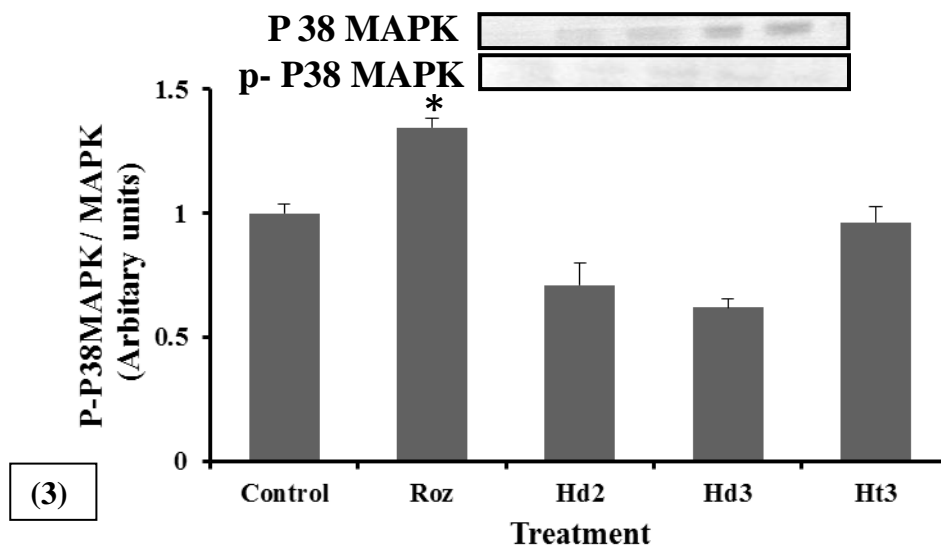
**Fig 4.17 (1-3). GLUT 4 expression in L6 myotubes on pretreatment of the citrus flavonoids**

*Quercetin (10 & 100 $\mu$ M): Qn2 & Qn3, rutin (100 $\mu$ M): Rn3; naringin (100 $\mu$ M): Na3, hesperidin (10 & 100 $\mu$ M) and hesperetin (100 $\mu$ M) pretreatment for 24h enhanced GLUT 4 expression which was compared with the positive control, rosiglitazone (100 nM). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements ( $n=3$ ) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus control*

AMPK activation by AICAR has been mentioned in mammalian muscle cells by similar mechanism involving GLUT 4 translocation. AICAR, an adenosine analogue increase glucose uptake without changing ATP, ADP & AMP levels in muscle cells but it mimics the allosteric effect of AMP in the activation and phosphorylation of AMPK. Whereas metformin alters mitochondrial ATP synthesis and changes AMP to ATP ratio which results in the activation of the enzyme. There was a two fold increase in GLUT 4 expression in L6 myotubes pretreated with the citrus flavonoids such as quercetin, rutin and naringin as shown in Fig 4.17(1-3). Hesperidin and hesperetin also increased GLUT 4 expression pointing to the conclusion that the end molecular mechanism behind the increased glucose uptake was the upregulation of GLUT 4 transporters in L6 myotubes. AMPK is known to stimulate glucose uptake in muscles and adipocytes by insulin independent mechanism [Huang and Czech, 2007]. Many promising compounds or drugs that improve metabolic syndrome are reported to activate AMPK pathway including the well-known antidiabetic drug, metformin [Hwang et

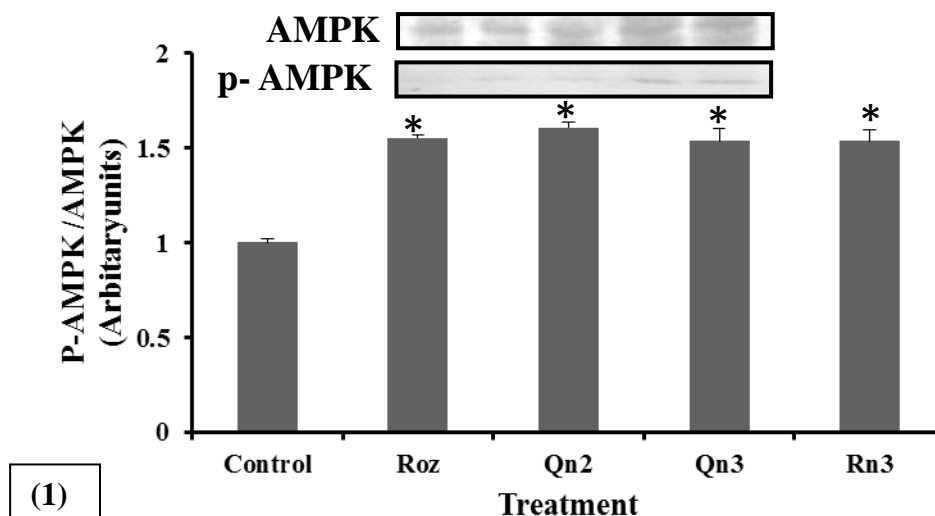
al., 2007; Tan et al., 2008]. In the present study we showed that citrus flavonoids especially quercetin, rutin, naringin and hesperidin increased the phosphorylation of P38 MAPK and AMPK as depicted in Fig 4.18 & 4.19. Pretreatment of quercetin, rutin and naringin showed one fold increase in the expression of P38 MAPK and AMPK. Since the activation depends on the phosphorylation of the key signaling molecules we examined the expression of the phosphorylated molecules. Results shown in Fig 4.18 & 4.19 indicated phosphorylation of P38MAPK and AMPK on pretreatment of quercetin, rutin and naringin.

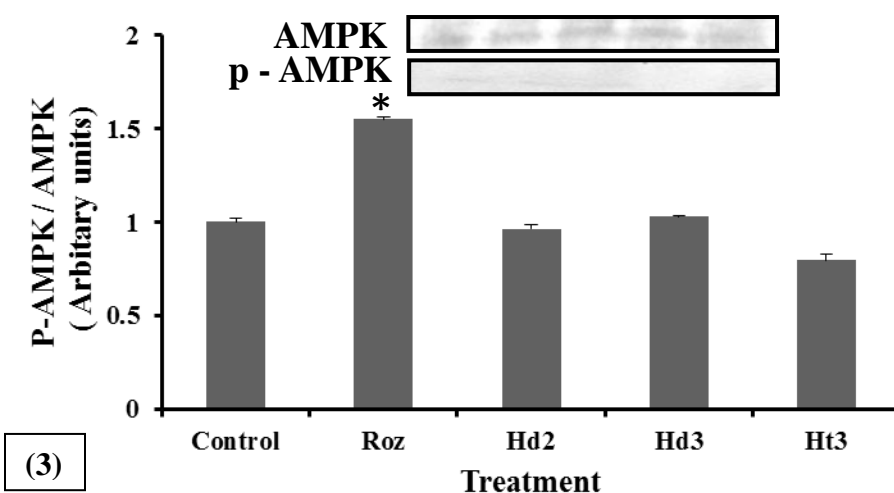
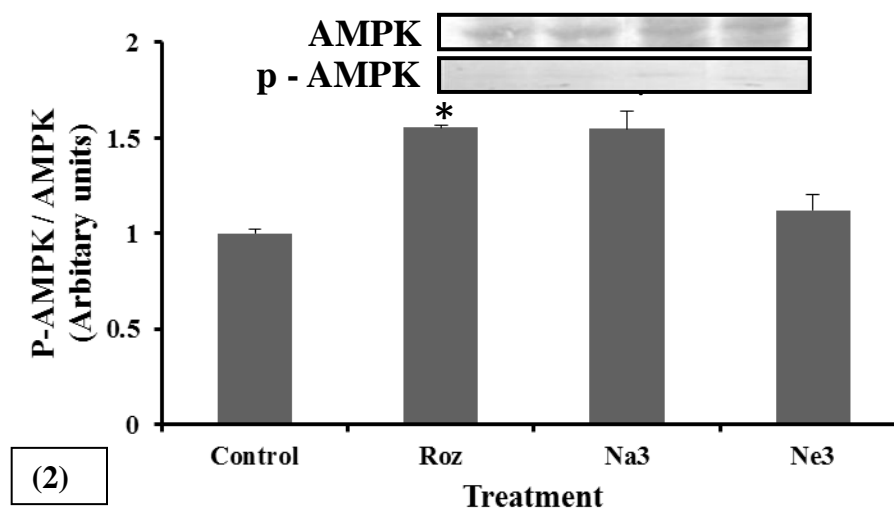




**Fig 4.18 (1-3) p-P38 MAPK expression in L6 myotubes on pretreatment of the citrus flavonoids**

The effect of Quercetin (10 & 100 $\mu$ M):Qn2 & Qn3, Rutin (100 $\mu$ M): Rn3; Naringin (100 $\mu$ M): Na3, Hesperidin (10 & 100 $\mu$ M) and Hesperetin (100 $\mu$ M) on pretreatment for 24h was compared with that of the positive control rosiglitazone (100 nM). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  verses control





**Fig 4.19(1-3). AMPK and p-AMPK expression in L6 myotubes on pretreatment of the citrus flavonoids**

The effect of Quercetin (10 & 100 $\mu$ M):Qn2 & Qn3, Rutin (100 $\mu$ M): Rn3; Naringin (100 $\mu$ M): Na3, Hesperidin (10 & 100 $\mu$ M) and Hesperetin (100 $\mu$ M) on pretreatment for 24h was compared with that of the positive control rosiglitazone (100 nM). Each value represents mean  $\pm$  SD (standard deviation) from triplicate measurements (n=3) of three different experiments. Significance test between different groups were determined by using one way ANOVA followed by Duncan's multiple range test the significance accepted at  $P \leq 0.05$ . \*  $P \leq 0.05$  versus control

In this study, it was noteworthy that the glucose uptake and GLUT 4 expression induced by the citrus flavonoids especially quercetin, rutin and naringin was significantly higher than that of rosiglitazone as shown in Fig. 4.16. Moreover, the mechanistic action of these compounds

was mediated by AMPK pathway which could bypass insulin resistance as it is independent of insulin signaling.

#### 4.4. Summary

This chapter demonstrated the molecular mechanism of the citrus flavonoids involved in mediating antidiabetic potential in *in vitro* model. The tested citrus flavonoids especially quercetin, rutin, naringin and hesperidin, stimulated an increase in skeletal muscle AMPK activity, which resulted in the translocation of GLUT 4 from an intracellular location to the plasma membranes. These findings are in agreement with the hypothesis that AMPK activation may be an early step in the intracellular signaling pathway for contraction-stimulated GLUT 4 translocation and glucose uptake. Increased activation of the AMPK signaling pathway, by either exercise or pharmaceutical agents, may be an effective treatment for T2DM. The AMPK signaling pathway could contribute to correction of insulin resistance through bypassing the insulin-regulated system for GLUT 4 translocation. The major findings of this chapter are illustrated below.

- 📖 The tested citrus flavonoids inhibited PTP1b activity in L6 myotubes by binding to its inhibitor site
- 📖 Citrus flavonoids caused an increase in AMP to ATP ratio and there was a transient change in mitochondrial membrane potential, which may be responsible for the activation of AMPK
- 📖 There was a concomittant increase in intracellular calcium levels, which pointed to the conclusion that CaMKK might have activated Thr 172 residue of the  $\alpha$ -subunit of AMPK, which resulted in the activation of the kinase. Activated AMPK may be responsible for the translocation of GLUT 4 receptors through various intermediate signaling molecules.
- 📖 Citrus flavonoids shared similar mechanisms with the well-known drug, metformin, highlighting it as promising compounds for the mangement of T2DM.

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## **CHAPTER 5**

### ***Summary and conclusion***

## 5.1. Summary and conclusion

The oxidative stress induced pathways is known to be associated with the onset of diabetes and its complications, which is the real cause of the morbidity and mortality associated with diabetes. Several lines of evidence suggest that oxidative stress occurs in diabetes and could have a role in the development of insulin resistance. Oral hypoglycemic agents which target on increasing insulin levels, improving sensitivity to insulin in tissues, or that reduce the rate of carbohydrate absorption from the gastrointestinal tract are used to manage T2DM. But these therapies rarely target the real cause of T2DM and are found to be responsible for severe adverse effects. Targeting therapy to specific macromolecules, tissues and organs of diabetics by specific antioxidants could become a relevant adjuvant pharmacotherapy with improved glycaemic control, protein glycation and management of oxidative stress for the treatment or prevention of progression of micro and macrovascular diabetic complications. Therefore, supplementation with antioxidants as a promising complementary treatment can undoubtedly exert beneficial effects in diabetes.

Last decade has witnessed enormous scientific studies on the heterogeneous class of molecules called phytochemicals. They are widely distributed in fruits, vegetables, beverages and in herbal remedies. The wide range of biological activities remains uncharacterized for most compounds. To suppress the oxidative stress mediated damage in diabetic pathophysiology, a special focus has been shifted towards naturally occurring antioxidants present in food.

The present study explores the antidiabetic potential of citrus flavonoids quercetin, rutin, naringin, naringenin, hesperidin and hesperetin under oxidative stress induced by tertiary butyl hydrogen peroxide in skeletal muscle cell line. Flavonoids though exist naturally as glycoconjugates they are extensively metabolized in humans, resulting in the formation of aglycones, methyl and sulfate derivatives that may have different properties than their parent compounds. Therefore, apart from evaluating the antidiabetic potential of the citrus flavonoids this study attempts to investigate the effect of glycoconjugation on the biological activities of the flavonoids. The exposure time of these flavonoids in cells were restricted to 3 and 24 hours because of the much shorter half-life of flavonoids in human plasma. Results from the present study showed that quercetin (100  $\mu$ M) exhibited 40.95% increase in glucose uptake whereas rutin and naringin showed an increase of 34.5% and 35.9%, respectively, on pretreatment which

was even higher than that of rosiglitazone (100 nM), an antidiabetic drug in the thiazolidinedione class of drugs. The effect of naringenin, hesperidin and hesperetin was comparable with that of rosiglitazone. Taking advantage of protein kinase inhibitors, we proved that the effect of these compounds was not through insulin signaling pathway, but through AMPK pathway and its downstream target p38 MAPK. An increase in the cellular AMP:ATP ratio on pretreatment may account for AMPK activation. Our data suggest that the biological activities of the metabolites can be neither predicted nor extrapolated from their dietary forms. Moreover the tested citrus flavonoids inhibited PTP1b, the key molecule involved in the dephosphorylation of insulin receptors. The experimental data of the present study provide significant evidence for citrus flavonoids to be considered as a dietary supplement for the prevention and treatment of T2DM and to suppress oxidative stress mediated damage in diabetic pathophysiology. Strategies to control oxidative stress have become a relevant pharmacotherapy in the treatment of micro and macrovascular diabetic complications. Antioxidant acting on different molecular targets of diabetes appears to be promising in the management of both diabetes and its associated complications. The major findings of the present study are illustrated below-

- 📖 Quercetin, Naringenin, Hesperetin & their glycoconjugates inhibited glycation of proteins.
- 📖 The citrus flavonoids tested reduced oxidative stress and upregulated antioxidant defense system in skeletal muscle cells.
- 📖 Flavonoids especially quercetin (10 & 100  $\mu$ M), rutin, naringin, hesperidin & hesperetin (100  $\mu$ M) significantly enhanced 2-NBDG uptake in L6 myotubes.
- 📖 Upregulation of GLUT 4 expression was found to be the end molecular mechanism behind the increased glucose uptake.
- 📖 Quercetin, rutin, naringin & hesperidin inhibited PTP1b
- 📖 Activation of AMPK was involved in the induction of glucose uptake. AMPK may be a promising therapeutic target of T2DM as it bypasses insulin signaling.
- 📖 In the case of citrus flavonoids, there exists a cross talk between AMPK and insulin signaling.
- 📖 Quercetin, would be an active 'intracellular metabolite' possessing more antioxidant and antidiabetic activity than their glycoconjugate, rutin. It may be confirming carbohydrate

moiety as an important structural feature and the biological activity is dependent on the chemical nature of the substituent.

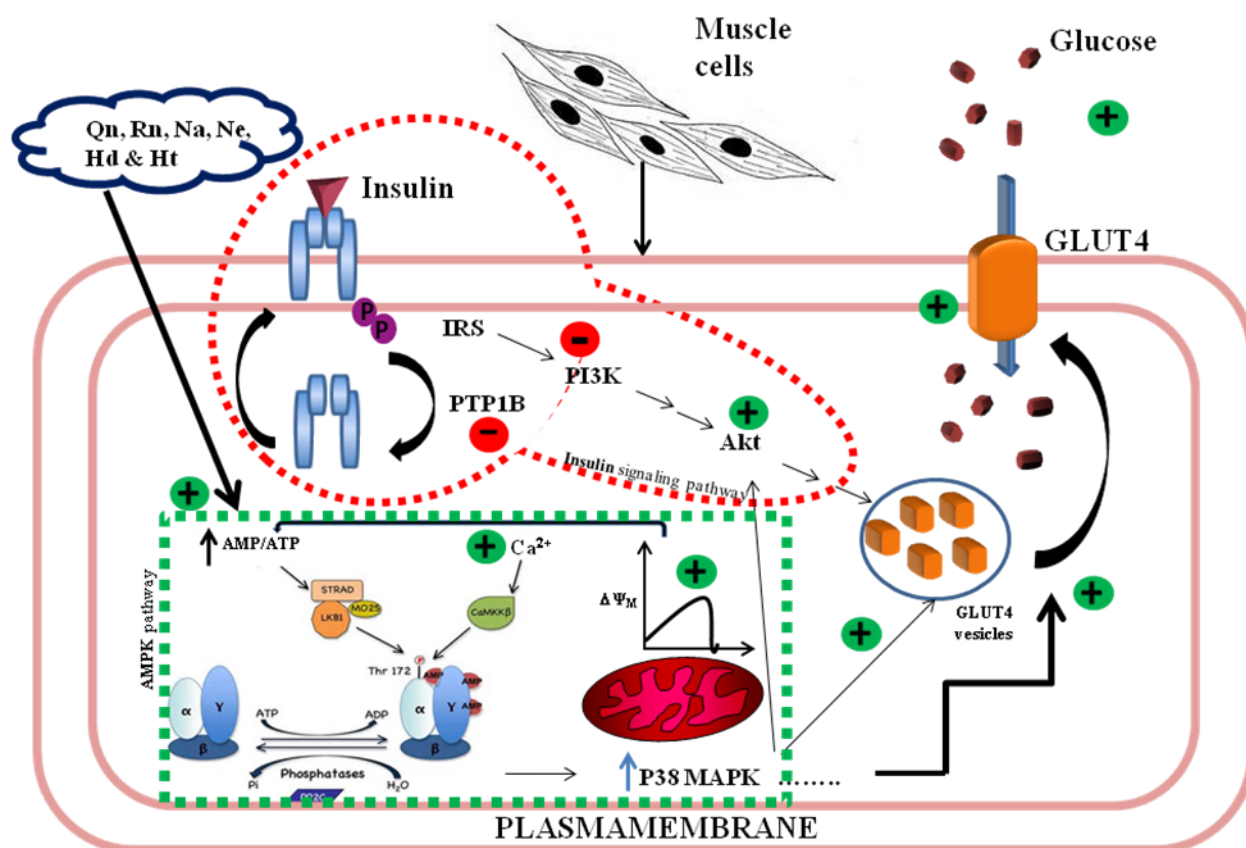
📖 This study provides significant evidence for quercetin, rutin & naringin to be considered as a dietary supplement for the management of T2DM and to suppress oxidative stress mediated damage in diabetic pathophysiology

## 5.2. Proposed mechanism of action

Disruption of glucose homeostasis is a characteristic feature of NIDDM and is associated with some complications including cardiovascular disease and renal failure. Glucose transport, the rate limiting step in glucose metabolism, can be activated in peripheral tissues by two distinct pathways. One stimulated by insulin through IRS-1/PI3K, the other by muscle contraction/exercise through the activation of AMPK. Both pathways also increase the phosphorylation and activity of MAPK family components of which p38 MAPK participates in the full activation of GLUT 4. Insulin exerts its biological effect upon binding with the insulin receptor (IR) thereby activating the downstream signaling that lead to enhanced glucose uptake. In the present study, the tested citrus flavonoids especially quercetin, rutin, naringin and hesperidin was found to act through AMPK pathway. Activation of AMPK occurs in response to exercise, an activity known to have significant benefit for type 2 diabetics. AMPK serves as sensor of energy status whose activity is triggered in response to changes in nutritional status in order to modulate tissue-specific metabolic pathways. Therefore, an understanding of how to activate AMPK in skeletal muscle would offer significant pharmacologic benefits in the treatment of T2DM. Metformin and the thiazolidinedione drugs exert the effects via activation of AMPK.

The proposed mechanism of action of the tested citrus flavonoids is illustrated in Fig 5.1. Our study provide significant evidence that the citrus flavonoids altered AMP to ATP ratio, resulting in a transient change in mitochondrial membrane potential which was associated with a change in intracellular calcium. This provided insight into the activation of CaMKK $\beta$  which in turn activated AMPK. We expected that AMPK may be sensitive to the increase in AMP to ATP ratio. While searching for the upstream signaling molecules we found an increase in intracellular

calcium, then we hypothesized CaMKK as the kinase responsible for the nucleotide dependent activation of AMPK.



**Fig 5.1. Proposed mechanism of action of the tested citrus flavonoids**

The citrus flavonoids pretreatment in L6 myotubes enhanced AMP to ATP ratio which resulted in a transient change in mitochondrial membrane potential. There was also an associated increase in intracellular calcium level, which resulted in the activation of AMPK and its downstream target P38MAPK. Akt was also upregulated pointing to the conclusion that the citrus flavonoids follows AMPK pathway and it overlaps with insulin signaling pathway. Quercetin (Qn); Rutin (Rn); Naringin (Na); Naringenin (Ne); Hesperidin (Hd) & Hesperitin (Ht).

CaMKK might have activated Thr 172 residue of the α-subunit of AMPK, which resulted in the activation of the kinase. The citrus flavonoids activated AMPK, phosphorylating the downstream targets of PI3K, AKT and P38MAPK. P38MAPK may be involved in the translocation of GLUT 4 to the plasma membrane. There exist a crosstalk between insulin and AMPK pathway, as the tested citrus flavonoids activated AMP and downstream targets of PI3K, AKT and P38MAPK. P38MAPK activation in response to various stimuli has been observed, such as hypoxia, hypertonicity [Michelle et al., 2003] and ischemia [Konard et al., 2001]. In muscle cells,

P38MAPK is involved in the activation of GLUT 4 after it translocates to the plasma membrane [Ravingerova et al., 2003; Somwar et al., 2001]. Dominant-negative AMPK mutants abolished P38MAPK activation in Clone 9 cells [Somwar et al., 2001], cardiomyocytes [Pelletier et al., 2005] and L6 muscles [Ha et al., 2006]. Our pharmacological inhibition results further support the notion that p38MAPK is a downstream target of AMPK and a key target involved in glucose uptake. Thus, the AMPK-p38MAPK couple might be a common scheme in the stimulation of glucose uptake by various stimuli that involve AMPK activation. The citrus flavonoids may exert a dual mechanism, AMPK-P38MAPK and AKT activating pathway.

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## Abbreviations

<i>2-NBDG</i>	<i>2-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino-2-deoxy-D-glucose</i>
<i>ADP</i>	<i>Adenosine diphosphate</i>
<i>AGE</i>	<i>Advanced glycated end proteins</i>
<i>AGE</i>	<i>Advanced glycated End products</i>
<i>AICAR</i>	<i>5-ami-noimidazole-4-carboxy-amide-1-D-ribofuranoside</i>
<i>AMP</i>	<i>Adenosine monophosphate</i>
<i>AMPK</i>	<i>Adenosine monophosphate activated protein kinase</i>
<i>ATP</i>	<i>Adenosine triphosphate</i>
<i>CaMKK</i>	<i>Calcium-calmodulin mediated protein kinase</i>
<i>DCFDA</i>	<i>Dichloro fluorescence diacetate</i>
<i>DM</i>	<i>Diabetes mellitus</i>
<i>DMEM</i>	<i>Dulbecco's modified Eagle's media</i>
<i>EDTA</i>	<i>Ethylene diamine tetra acetic acid</i>
<i>FBS</i>	<i>Foetal bovine serum</i>
<i>FFA</i>	<i>Free fatty acids</i>
<i>FITC</i>	<i>Fluorescein isothiocyanate</i>
<i>GFAT</i>	<i>Glutamine, fructose-6-phosphate amido transferase</i>
<i>GSH</i>	<i>Glutathione reductase</i>
<i>GSK3</i>	<i>Glycogen synthase kinase 3</i>
<i>HbA1c</i>	<i>Glycated Hemoglobin</i>
<i>HLA</i>	<i>Human Lecukocyte antigens</i>
<i>HMP</i>	<i>Hexosamine pathway</i>
<i>IDDM</i>	<i>Insulin dependent diabetes</i>
<i>IR</i>	<i>Insulin receptor</i>
<i>IRS</i>	<i>Insulin receptor substrate</i>



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<i>IRS-1</i>	<i>Insulin receptor substrate-1</i>
<i>IRS-2</i>	<i>Insulin receptor substrate-2</i>
<i>IRTK</i>	<i>Insulin receptor tyrosine kinase</i>
<i>JC-1</i>	<i>5,5',6,6'-tetrachloro1,1',3,3'tetraethyl benzimidazolocarboyanine</i>
<i>KOH</i>	<i>Potassium hydroxide</i>
<i>MDA</i>	<i>Malonaldehyde</i>
<i>MHC</i>	<i>Major Histocompatibility Complex</i>
<i>MODY</i>	<i>Maturity Onset Diabetes of Youth</i>
<i>mTORC2</i>	<i>Mitochondrial target of rapamycin C2</i>
<i>MTT</i>	<i>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</i>
<i>NaCl</i>	<i>Sodium chloride</i>
<i>NIDDM</i>	<i>Non-insulin dependent</i>
<i>NO</i>	<i>Nitric oxide</i>
<i>OPT</i>	<i>O-phthalaldehyde</i>
<i>ORO</i>	<i>Oil red O</i>
<i>PBS</i>	<i>Phosphate-buffered saline</i>
<i>PDK</i>	<i>Phosphatide dependent kinase</i>
<i>PI3K</i>	<i>Phosphatidylinositol-3' kinase</i>
<i>PIP3</i>	<i>Phosphatidylinositol 3,4,5 triphosphate</i>
<i>PNPP</i>	<i>Paranitrophenyl phosphate</i>
<i>PTB</i>	<i>Phosphotyrosine-binding</i>
<i>PTP1B</i>	<i>Protein tyrosine phosphate 1 B</i>
<i>PUFA</i>	<i>Polyunsaturated fatty acids</i>
<i>ROS</i>	<i>Reactive oxygen species</i>
<i>SDS</i>	<i>Sodium dodecyl sulphate</i>
<i>SOD</i>	<i>Superoxide dismutase</i>
<i>T1DM</i>	<i>Type I diabetes mellitus</i>

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<i>T2DM</i>	<i>Type II diabetes mellitus</i>
<i>TBARS</i>	<i>Thiobarbituric acid reactive substances</i>
<i>TBHP</i>	<i>Tertiary butyl hydrogen peroxide</i>
<i>TBST</i>	<i>Tris buffered saline – Tween 20</i>
<i>TZDs</i>	<i>Thiazolidinediones</i>
<i>UPS</i>	<i>Uncoupler proteins</i>
<i>WHO</i>	<i>World health organization</i>

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### *List of Publications*

- ❖ **R. Dhanya**, K.B. Arun, H.P. Syama, P. Nisha, A. Sundaresan, T.R. Santhosh Kumar, P. Jayamurthy (2014) Rutin and quercetin enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide. *Food Chemistry*, 158:546–554.
- ❖ H P Syama, S Asha, **R Dhanya**, P Nisha, Syed G Dastagar and P Jayamurthy (2014) Evaluation of underutilized vegetable leaves as a potent source of dietary antioxidant and antimicrobial agent. *International Journal of Food and Nutritional Sciences*, 3.
- ❖ **R Dhanya**, K B Arun, V M Nisha, H P Syama, P Nisha, P Jayamurthy (2015) Preconditioning of L6 muscle cells with naringin ameliorates oxidative stress and glucose uptake. *Plos one*,| Jul 6;10(7)doi:10.1371/journal.pone.0132429
- ❖ K.B Arun, Janu C, **R Dhanya**, K Priya, P Jayamurthy, P Nisha (2015) A comparative evaluation of antidiabetic and antioxidant effect of peel from young and matured potato. *Food Bioscience*, 36-46.
- ❖ **R Dhanya**, S Lekshmy Krishnan, P Nisha, P Jayamurthy (2016) Cytoprotective effect of hesperidin and its aglycone hesperetin in skeletal muscle cells under induced oxidative stress (Communicated)
- ❖ **R Dhanya**, AD Arya, AG Kiran, P Nisha, P Jayamurthy (2016) Quercetin, a lead compound against type 2 diabetes acting via AMPK pathway in skeletal muscles (Communicated).
- ❖ **R Dhanya**, AD Arya, J Elizabeth, P Nisha, P Jayamurthy (2016) Antidiabetic potential of bioflavonoid naringenin under oxidative stress in skeletal muscle cell line (Communicated).
- ❖ V Jamsheena, KS Veena, **R Dhanya**, P Jayamurthy, K Venugopal, L Ravi Shankar, A Ajayagosh. Design, synthesis and application of new 1,2-Dihydropyridine based fluorophores. RSC advances (2016) (Communicated).

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### *Conference papers/posters*

- ❖ *Flavonoids enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide.* National conference on emerging avenues in Food technology for better health and safety. Thangal Kunju Musaliar Institute of Technology, Kollam, 2013 (**Best Paper Award**)
- ❖ *Naringin stimulates glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide.* International Conference on Bioactive Phytochemicals and Therapeutics (ICBPT-2013), Annamalai University, Tamil Nadu, 2013 (Oral presentation)
- ❖ *In vitro evaluation of antidiabetic potential of Hesperidin and its aglycone Hesperitin under oxidative stress in Skeletal muscle cell line.* International symposium on Phytochemicals, Trivandrum, 2015 (**Best Poster Award**)
- ❖ *Rutin and quercetin enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide.* 27<sup>th</sup> Kerala Science Congress, Alappuzha, Kerala, 2015 (**Competed for best paper award**)
- ❖ *Mechanistic role of citrus flavonoids and their glyconjugates in the management of type 2 diabetes.* Dr. Hisham endowment award organised by Kerala academy of science, 2016 (Oral presentation)