Pharmacological Screening of Antioxidant, Hypolipidemic and Antidiabetic Activities of Novel Synthetic Flavonoid in High Fat Fed Followed by Low Dose Streptozotocin Induced Diabetes Mellitus in Rat Model

Thesis submitted for the award of the degree of Doctor of Philosophy in Medical Pharmacology

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I dedicate this research work

to my Parents

Late Smt. Rachamma &Shree Shidramayya Bhixavatimath

And

To my wife Shobha and my daughter Advika whose affection, love, and encouragement made me able to get Success and Honor

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LIST OF ABBREVIATIONS USED

ADA	American Diabetes Association	**C NMR**	Carbon Nuclear Magnetic Resonance
ADA	Adenosine Deaminase	**CO2**	Carbon Dioxide
AGEs	Advanced Glycation End Products	**CPCSEA**	Committee for the Purpose of Control and Supervision of Experimental Animals
AGIs	Alpha-Glucosidase Inhibitors	**DIO**	Diet-Induced Obesity
AIDS	Acquired Immune Deficiency Syndrome	**DM**	Diabetes Mellitus
AMP	Adenosine Monophosphate	**DMEM**	Dulbecco's Modified Eagle's Medium
AMPK	AMP-activated protein kinase;	**DMSO**	Dimethyl Sulfoxide
ANOVA	Analysis Of Variance	**DNA**	Deoxyribonucleic Acid
ATCC	American Type Culture Collection	**DPP4**	Dipeptidyl peptidase 4
ATP	Adenosine Triphosphate	**DPPH**	2,2-Diphenyl-2-Picrylhydrazyl Hydrate
BMI	Body Mass Index	**EDTA**	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay	**FT-IR**	Fourier Transformer Infra-Red
FBG	Fasting Blood Glucose	**GDM**	Gestational Diabetes Mellitus
FBS	Fetal Bovine Serum	**GLP1**	Glucagon-Like Peptide 1
FDA	Food And Drug Administration	**GLUT-4**	Glucose Transporter-4
FOXO1	Forkhead Box O1	**GSH**	Reduced Glutathione
FPG	Fasting Plasma Glucose	**H NMR**	Hydrogen Nuclear Magnetic Resonance
HF	High Fat	**H2O2**	Hydrogen Peroxide
HFD	High fat diet	**HDL**	High Density Lipoprotein
HOMA-B	Homeostasis Model Assessment For Beta Cell	**IR**	Insulin Resistance
HOMA-IR	Homeostasis Model Assessment For Insulin Resistance	**JNK**	Jun N-Terminal Kinase
HRP	Horseradish Peroxidase	**KATP**	ATP-sensitive K+ channels
IAEC	Institutional Animal Ethics Committee	**KRP**	Krebs Ringer Phosphate Solution

IC50	Half-Maximal Inhibitory Concentration	**Kg**	Kilogram
IGF-1	Insulin-Like Growth Factor-1	**LD50**	Median Lethal Dose
IKK	Ikappab Kinase	**LDL**	Low-Density Lipoprotein
IP	Intra-Peritoneal	**PDX1**	Pancreatic And Duodenal Homeobox 1
MAPK	Mitogen-Activated Protein Kinase	**PDX-1**	Programmed Cell Death X-1 Gene
mg	Milligram	**PPARα**	Peroxisome Proliferator-Activated Receptor- Alpha
MODY	Maturity-Onset Diabetes of the Young	**PPARγ**	Peroxisome Proliferator-Activated Receptor- Gamma
MS	Mass Spectrometry	**ROS**	Reactive Oxygen Species
MTT	4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide	**SD**	Standard Diet
MW	Molecular Weight	**SFA**	Saturated Fatty Acid
NMR	Nuclear Magnetic Resonance	**SGLT2**	Sodium/Glucose co-transporter 2
NO	Nitrous Oxide	**STZ**	Streptozotocin
OD	Optical Density	**SUR1**	Sulfonylurea Receptor 1
OECD	Organisation for Economic Co-operation and Development	**T1DM**	Type 1 Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test	**T2DM**	Type 2 Diabetes Mellitus
PBS	Phosphate-Buffered Saline	**TFA**	Trans Fatty Acid
TNF	Tumour Necrosis Factor	**TG**	Triglycerides
Type 2 DM	Type 2 Diabetes mellitus	**TLC**	Thin-Layer Chromatography
TZDs	Thiazolidinedione's	**TLR4**	Toll-like receptor 4
USP	United States Pharmacopeia	**VLDL**	Very Low-Density Lipoprotein
UV	Ultraviolet	**WHO**	World Health Organization

ABSTRACT

Objective: The goal of the current study was to assess the novel synthetic flavonoid's anti-diabetic, anti-hyperlipidemic, and anti-oxidant capabilities in in-vitro and invitro models. **Methods**: A new novel flavonoid compound, NF(3-hydroxy-2-(thiophen-2-yl)-4H-chromen-4-one), was created by condensing hydroxyacetophenone with thiophene carbaxaldehyde reagents and then cycling with hydrogen peroxide. The NF compound and other test flavonoids, including VMF41, VMF43, VMF45, and VMF46, whose synthesis and spectral characterization study have already been completed, were evaluated for invitro radical scavenging activity on hydroxyl, nitric oxide, and superoxide free radicals using invitro spectroscopic methods. Synthetic new flavonoid (NF) was chosen for its anti-diabetic, anti-hyperlipidemic, and antioxidant action in low dosage streptozotocin (STZ 35mg/kg body weight) caused diabetic rats fed on a high fat diet (HFD) during an 8-week period based on its highest antiradical activity. Rats were divided into two main groups for the experimental study: normal control rats given the normal pallet diet (NPD) and diabetic rats given the HFD daily once orally in addition to the normal pallet diet (NPD). The diabetic rats were divided into five new subgroups and treated as follows: DC- diabetic control received Tween 20 alone orally (5 ml/kg); DC + Met- received metformin orally (100 mg/kg); and three test groups, D+NF50, D+NF100, and D+Nf200, were treated with 50, 100, and 200 mg/kg of synthetic novel flavonoid (NF) respectively for 21 days. On the first day of therapy, after STZ, and on the last day of treatment, the FBG and serum lipids from the various treatment groups were assessed. Before and after treatment, serum insulin levels were assessed. By measuring the levels of the antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and lipid peroxide (MDA), the effects of antioxidants were evaluated in the liver

homogenate of experimental rats. Alfa amylase and alfa glucosidase dipeptidyl peptidase IV inhibitory assays were used to assess the test compound's in vitro antidiabetic activity, and the C2C12 cell line study was used to determine the compound's glucose absorption activity. **Results:** The Analysis of Variance (ANOVA) method was used to statistically analyse the findings of this study. The study shows that the treatment group receiving the synthetic new flavonoid (NF) at a dose of 100 mg/kg body weight had significantly higher levels of insulin and lower levels of FBG and serum lipids. With results comparable to those of metformin treatment, animals given the NF compound demonstrated a significant reduction in HOMA IR values and reversed reduced HOMA B values brought on by STZ with HFD. The rats treated with new flavonoid (NF) at 100 mg/kg showed improved differentiation and structural alterations in the liver, according to the histological investigation. In diabetic rats treated with new flavonoid, there was a discernible reduction in the levels of lipid peroxides and an increase in the levels of superoxide dismutase, glutathione, and catalase. The NF compound had shown negligible action in alfa amylase and DPP-IV inhibitory assays, but considerable antidiabetic activity for alfa glucosidase and glucose absorption in C2C12 cell line investigation.**Conclusion:** According to the study's findings, the synthesised new flavonoid (NF) significantly exhibited antidiabetic, hypolipidemic, and antioxidant effects in the aforementioned research models.

Key words: Novel flavonoid, Alfa glucosidase, C2C12 cell lines, Catalase & HOMA-IR,

CHAPTER 1:

INTRODUCTION

CHAPTER 1

INTRODUCTION/BACKGROUND:

Diabetes mellitus is a common chronic complex metabolic disease that develops when the body is unable to govern glucose homeostasis within the cells, when glucose uptake into various tissues is impeded, or when the liver and pancreas are unstable in maintaining glucose levels. This can be brought on by a problem with insulin action, secretion, or both[1]. T2DM is a form of diabetes that affects many people. It is categorised as a metabolic condition with hyperglycemia and is thought to be the main cause of greater morbidity and death. As a result, this condition is regarded as one of the global health emergencies, whose incidence is rising and requires immediate attention[2]. According to the IDF's (International Diabetes Federation) latest Diabetes Atlas, there are currently 537 million individuals (20–70) living with diabetes globally. By 2030 and 2045, that number is expected to climb to 643 million and 783 million, respectively. About 90–95 percent of all instances of diabetes are T2DM, which is one of the major worldwide health issues. India is ranked second in the world, behind China, and has 141 million T2DM patients.[2-4.] Due to the failure of the pancreatic beta cells and insulin resistance, elevated fasting blood sugar of more than 120 mg/dl and a postprandial blood sugar of more than 200 mg/dl are regarded as key diagnostic markers in T2DM. [3,4].

In addition to hyperglycemia, one of the leading causes of death worldwide in diabetes is high lipid levels, which are closely related to atherosclerosis and subsequent cardiovascular illnesses. It has been discovered that higher production of free radicals is linked to persistent hyperglycemia. For its genesis, a number of explanations have been put forth, including the oxidation of glucose, an ongoing rise in the production of advanced glycation end products (AGEs) produced from glucose,

and the breakdown of glycated protein[5–6]. Therefore, uncontrolled hyperglycemia in diabetic individuals increases the risk of developing micro- or macro-vascular problems[7].

Drugs now on the market lower blood sugar levels by either boosting tissue absorption or by raising insulin production. Although there are numerous anti-diabetic medications available on the market, many of them have drawbacks and side effects that are difficult to tolerate, such as liver and kidney failure, hypoglycemia, diarrhoea, and lactic acidosis. Additionally, patient responses to treatment may vary depending on the patient's immune status, secondary complications, and micro and macrovascular damage. Therefore, finding a permanent treatment for diabetes still presents a significant difficulty. So the search for a fresh anti-diabetic medicine that has the greatest effectiveness and the fewest adverse effects continues.

The World Health Organization (WHO) offers 1200 herbal plant species around the world that show potent anti-diabetic activities and could be employed as an alternative therapeutic intervention against prevention of T2DM in light of these medication barriers and diabetes mellitus's widespread prevalence. Natural plants, and flavonoids in particular, are widely distributed in nature and display potent in vitro and in vivo anti-diabetic effects. Therefore, appropriate methods and cutting-edge equipment must be used to create drugs that have fewer harmful effects. Flavonoids, which are natural compounds having -benzopyrone scaffolds, have been extensively studied for the treatment of diabetes and have been shown to act at several stages of glucose use. Additionally advantageous features include their antioxidant capacity and regulation of cell signalling. Flavonoids have reportedly demonstrated therapeutic potential in the management of diabetes and its complications in a number of investigations. However, the medicinal focus has turned to synthetic flavonoids due to

the instability, insolubility, and time-consuming and expensive isolation processes of natural flavonoids. [8-9]

Flavonoids are polyphenolic substances that are found in the majority of edible plant components, such as flowers, fruits, seeds, stems, and roots, among others. They all share the same fundamental structure of diphenylpropane (benzopyrone), which is made up of two benzene rings (rings A and B) connected by a three-carbon chain to create a closed pyran ring (a heterocyclic ring with carbon, oxygen, and a bond with the benzene A ring). Flavonoids are frequently referred to structurally as C6-C3-C6. B rings often connect to C rings in positions 2 and 4, though they can also bind in positions 3 and 4. This makes the flavonoids one of the largest and more varied families of phytochemicals with various pharmacological qualities, along with the structural characteristics of the ring B and the patterns of glycosylation and hydroxylation of the three rings. [10,11]

However, because oxidative stress has been linked to the onset and progression of diabetic problems, several research studies have suggested a close relationship between oxidative stress and type 2 diabetes mellitus[12]. Since the anti-diabetic properties of natural flavonoids are almost always associated with antioxidant activity, synthetic compounds with structurally similar properties but slight modifications to their -benzopyrones by substituting phenyl at position 2 provide better lead molecules in this field[13,14]. In order to produce superior counterparts, we chose to structurally modify -benzopyrone.

In the current study, -benzopyrone containing heteroaryl substituents like thiophene was synthesised and tested in relevant models for potential antioxidant, antidiabetic, and antidyslipidemic properties.

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CHAPTER 2:

AIMS & OBJECTIVES

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2.1 AIM:

In-vitro models of free radical scavenging and antidiabetic potential as well as in-vivo T2DM rat model induced by high fat diet and low dose streptozotocin will be examined and evaluated, along with the antidiabetic, antihyperlipidemic, and antioxidant potential of newly synthesised novel flavonoids.

2.2. THE STUDY OBJECTIVES:

1. To synthesise novel flavonoid and characterise their spectrum properties for FT-IR, 1HNMR, and 13CNMR research.

2. To investigate in vitro antioxidant and antidiabetic activities using the following assays.

    a) To assess the free radical scavenging abilities of synthetic new flavonoids using a variety of conventional biochemical tests, including the superoxide, hydroxyl, and nitric oxide assays.

    b) Investigate and contrast the in vitro antiglycation potential of synthetic Novel Flavonoid (NF) utilising several established techniques, including in vitro C2C12 cell line model, alpha amylase, alpha glucosidase, and dipeptidyl peptidase IV inhibitory assays.

3. To determine whether NF compound has an acute harmful(toxic) effect on Wistar albino mice in accordance with ICH recommendations (OECD-425 guidelines).

4. To assess and contrast the antioxidant, anti-dyslipidemic, and anti-diabetic effects of synthetic Novel flavonoid (NF) in an in-vivo T2DM rat model established by a high-fat diet and low-dose streptozotocin.

5. To examine and contrast the test NF compound's histological effects on the hepatic and renal tissues of diabetic rats brought on by a high-fat diet and low-dose STZ.

HYPOTHESIS FOR RESEARCH

We proposed that, when administered in suitable study models, flavonoids containing benzopyran nuclei would be beneficial in the prevention and treatment of type 2 diabetes mellitus and its associated problems, such as oxidative stress and hyperlipidemia.

CHAPTER 3:

LITERATURE REVIEW

CHAPTER 3:

LITERATURE REVIEW

The literature review for this research thesis has been organised under the following headings:

- **Introduction to Diabetes Mellitus**
- **Diabetes Mellitus relation with oxidative stress**
- **Flavonoid and its importance in diabetes mellitus**

3.1. Introduction:

Hyperglycemia is a symptom of diabetes mellitus, a complicated metabolic disorder of the endocrine system that can be caused by a deficiency in insulin secretion, insulin action, or both[1-2.]This condition is one of the public health problems with a rising prevalence that requires immediate attention. According to the IDF's (International Diabetes Federation) Diabetes Atlas, there are currently 537 million adults (20 to 70 years old) living with diabetes globally. By 2030, that number is expected to climb to 643 million, and by 2045, it will reach 783 million (12.2%). In India, it was predicted that there would be about 74.2 million adults there in 2021 (8.9%), and that number would climb to about 124.8 million adults (12.1%) in 2045-[3-4]. Epidemiological studies conducted in the 1960s and 1970s found that 1-4% of urban populations and 1-2% of rural populations had diabetes mellitus. Urban population DM prevalence is rising quickly, from 2% in the 1970s to 8.2% in the 1980s[5], and then rising to 12-16% in 2001[6-7]. As a result, the high prevalence of DM found among urban residents is quickly spreading to rural areas that are urbanising[8] . Urban areas were predicted to have a greater prevalence (12.1%) than rural (8.3%) areas in 2021. Aging, overpopulation, increased growth rate, growing urbanisation, obesity, high-calorie diets, fast food, etc. are all potential causes of diabetes' rapid rise. In the South East Asian area, India has the second-highest prevalence of diabetes among adults (9.1%). In India, diseases associated with diabetes claim the lives of almost 1.1 million people annually. Diabetes accounts for 12% of all medical expenditures worldwide. A person with diabetes dies every six seconds[3,4] (5.0 million deaths). Therefore, it is crucial to manage diabetes and its side effects in order to reduce suffering in people.

Diabetes Mellitus Subtypes

The type of diabetes is crucial for proper treatment and appropriate glycemic control. The following categorization[9] was suggested in 2018 by the American Diabetes Association (ADA).

1. Type 1 diabetes mellitus (T1DM) is brought on by the autoimmune death of beta cells and typically results in a complete lack of insulin.

2. Type 2 diabetes mellitus (T2DM): characterised by a persistent decline in insulin production from beta cells, typically occurring in the context of insulin resistance.

3. Gestational diabetes mellitus (GDM) is diabetes that was not immediately apparent before becoming pregnant and was discovered in the second or third trimester.

4. Particular types of diabetes brought on by unrelated conditions, such as glucocorticoid use, HIV/AIDS treatment, or organ transplantation; exocrine pancreas diseases like cystic fibrosis and pancreatitis; and monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young (MODY)).

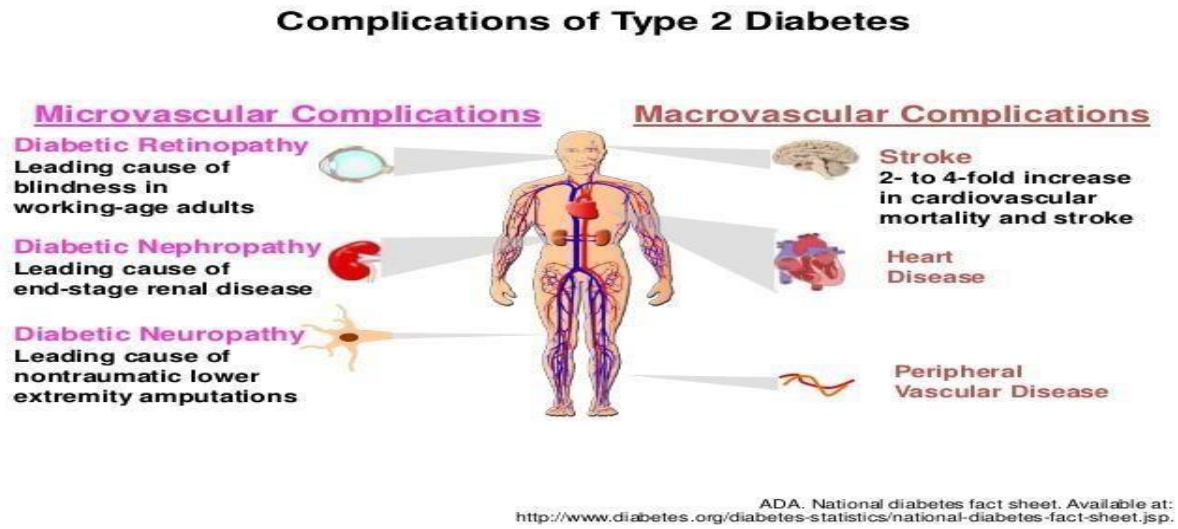
As the study effort primarily focuses on insulin resistance and treatment strategies aimed at resolving the insulin resistance, we have eliminated T1DM and other minor forms of DM from this literature review and concentrated on T2DM.

The most prevalent type of diabetes is type 2 diabetes (T2DM), also known as "non-insulin-dependent diabetes," which accounts for about 90% of cases of diabetes. Type 2 diabetes is characterised by dysregulation of carbohydrate, lipid, and protein metabolism and is caused by impaired insulin secretion, insulin resistance, or a combination of both. It is believed that the combination of glucotoxicity and lipotoxicity, or "glucolipotoxicity," is what causes -cell dysfunction in T2DM[11].

Current research indicates that metabolic overload, oxidative stress, elevated rates of apoptosis, and loss of expression of essential elements of the insulin granule secretory machinery all contribute to -cell failure, but specific genetic mutations that predispose patients with non-MODY T2DM to these events are still unknown[12] . This disorder is influenced by both acquired and inherited defects. When the postprandial blood glucose is greater than 200 mg/dl and the fasting blood glucose is greater than 126 mg/dl, T2DM is diagnosed.

Consequences connected with untreated diabetes mellitus:

Numerous long-term complications are caused by the steady state of hyperglycemia in T2DM, including retinopathy, which may result in vision loss, nephropathy, which can cause renal failure, cardiomyopathy, which can cause heart failure, peripheral neuropathy, which raises the risk of foot ulcers and amputations, autonomic neuropathy, which can result in gastrointestinal, genitourinary, cardiovascular, and sexual dysfunction. The main macrovascular and microvascular side effects of T2DM are shown in Figure 3.1. The activation of protein kinase C (PKC) isoforms via de novo synthesis of the lipid second messenger diacylglycerol (DAG), increased hexosamine pathway flux, increased advanced glycation end products (AGEs) formation, and increased polyol pathway flux are the four main molecular mechanisms implicated in chronic hyperglycemia-induced tissue damage.[13]Renewing interest in the oxidative stress of hyperglycemia as a potential cause for diabetic micro-vascular disease is the well-established relationship between atherosclerosis and lipid peroxidation inside the vascular wall [14,15,16] .



3.1:The major microvascular and macrovascular complications associated with T2DM (Source:www.diabetic.org/diabetes-statistics/national-diabetes-fact-sheet.jsp)

Homeostasis of glucose

The neurological and hormonal components that control glucose uptake and utilisation are referred to as the "glucose homeostasis" system. Despite physiological difficulties such as fasting, the postprandial state, and during vigorous activity, glucose homeostasis keeps the plasma glucose concentration within a specific range [17].

The liver, endocrine pancreas, skeletal muscle, adipose tissue, important regions of the central nervous system such the hypothalamus and brain stem, and the autonomic nervous system work in concert to maintain glucose homeostasis[17]

Correcting insulin action or insulin secretion at the level of muscle, liver, and fat tissue[18] prevents the hyperglycaemic disorders of prediabetes and diabetic states.

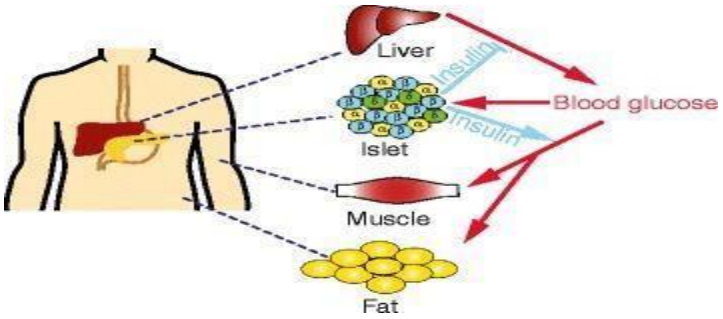


Figure 3.2 Homeostasis (equilibrium) of glucose.

The series of events that occur in glucose homeostasis are as follows:

a) A rise in blood glucose causes the - β-cells (blue) within the pancreatic islets to secrete insulin.

b) Insulin decreases blood sugar by acting on target tissues, reducing the liver's production of glucose, and promoting the absorption of glucose into muscle and fat.

c) δ-cells (green) secrete somatostatin, while d) α--cells (yellow) are the pancreas' glucagon-secreting cells.

Secretion of insulin by pancreatic beta cells.

In the pancreas, beta cells are in charge of secreting insulin in response to increased blood sugar levels in the postprandial period.

The primary nutrient for insulin secretion is glucose.

The ATP-sensitive K+ channels (KATP channels) in pancreatic -cells are open at basal blood glucose levels (figure 3.3), preserving membrane hyperpolarization, Ca2+ channel closure, and preventing insulin production.

The mechanism by which glucose stimulates insulin secretion, also known as glucose-stimulated insulin secretion, needs glucose detection and processing by the beta-cell (Figure 3.4). Glucose is transported into the cell by glucose transporters during the initial stage of insulin secretion (GLUT2 in rodents, GLUT1 in humans). Glucokinase19 then phosphorylates glucose to produce glucose-6-phosphate. The ATP-sensitive K+ channel (KATP)[20] is closed by the production of ATP by glycolysis, the Krebs cycle, and the respiratory chain, allowing sodium (Na+) access without balance. These two occurrences cause the membrane to depolarize and voltage-dependent T-type sodium (Na+) and calcium (Ca2+) channels to open. The influx of Na+ and Ca2+ further depolarizes the membrane, allowing the opening of voltage-dependent calcium channels. This activation raises intracellular Ca2+ levels ([Ca2+]i)[21], which triggers the first phase of insulin secretion and the fusion of secretory granules carrying insulin with the plasma membrane[22–23].

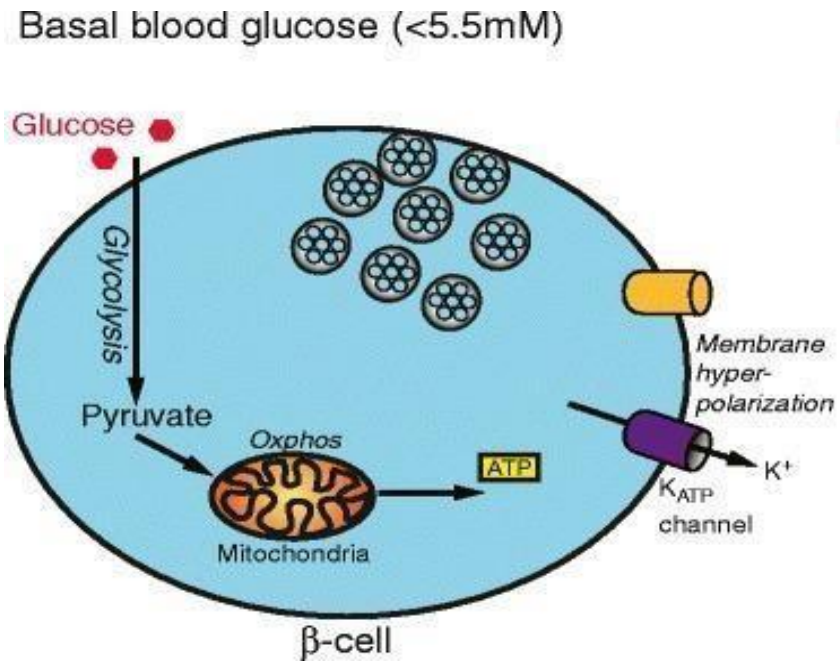


Figure 3.3:ATP-sensitive K+ channel status in pancreatic β-cells at basal glucose level.

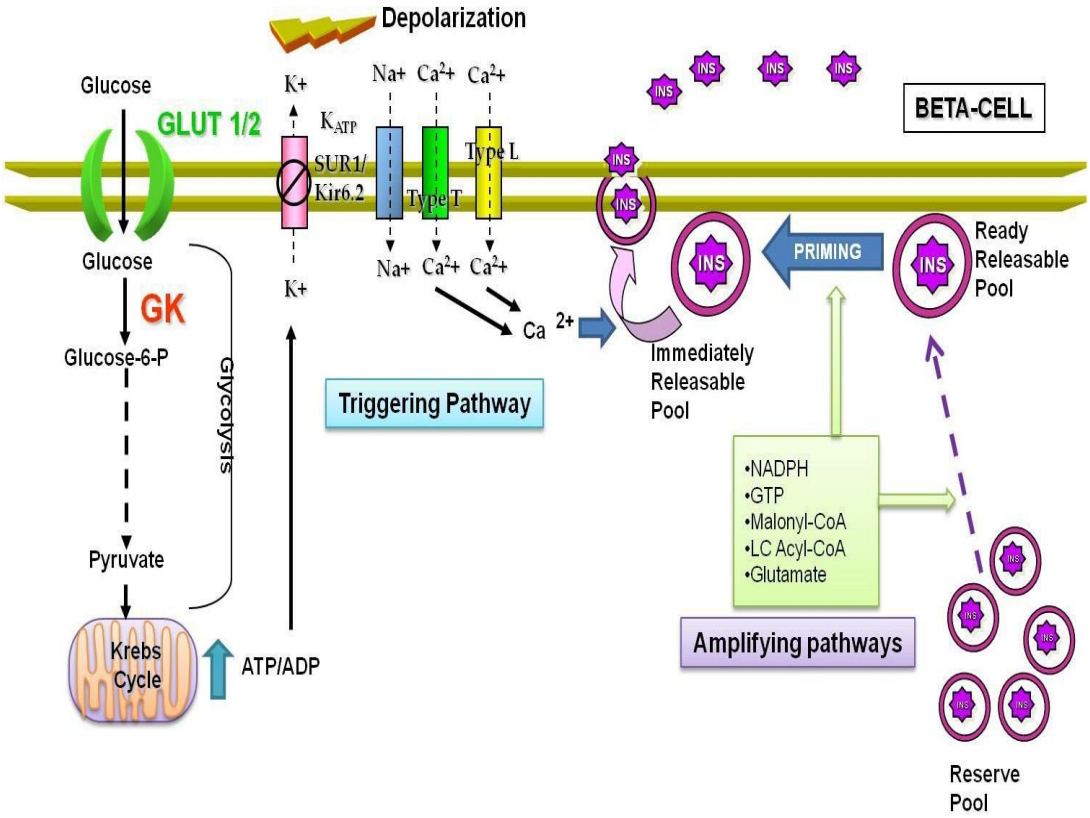


Figure 3.4: **Mechanisms of glucose-stimulated insulin secretion(Source: Cantley J, Ashcroft FM. Q&A: insulin secretion and Type 2 diabetes: why do β-cells fail? BMC Biol. 2015; 13:33)**

Insulin resistance and insulin action:

Insulin is delivered to peripheral tissues after being released into the portal circulation, where it mostly acts anabolically[24].

Insulin binds to the insulin receptor, a transmembrane protein that is a member of the superfamily of protein tyrosine kinase activity receptors and has the ability to autophosphorylate, to begin its action.This starts a chain of processes that involve linking proteins, membrane lipid phosphorylation, and activity of the cytoskeleton[25–26]. The three main signaling pathways activated in response to insulin receptor phosphorylation are 1) PI3K 2)MAPK, and 3) Cb1 The signal from the insulin

receptor is translated into biological actions in the target organs by these pathways, such as glucose transport by delivering GLUT4 vesicles to the membrane, protein, lipid, and glycogen production, mitosis, and gene expression[26.](Figure 3.5).

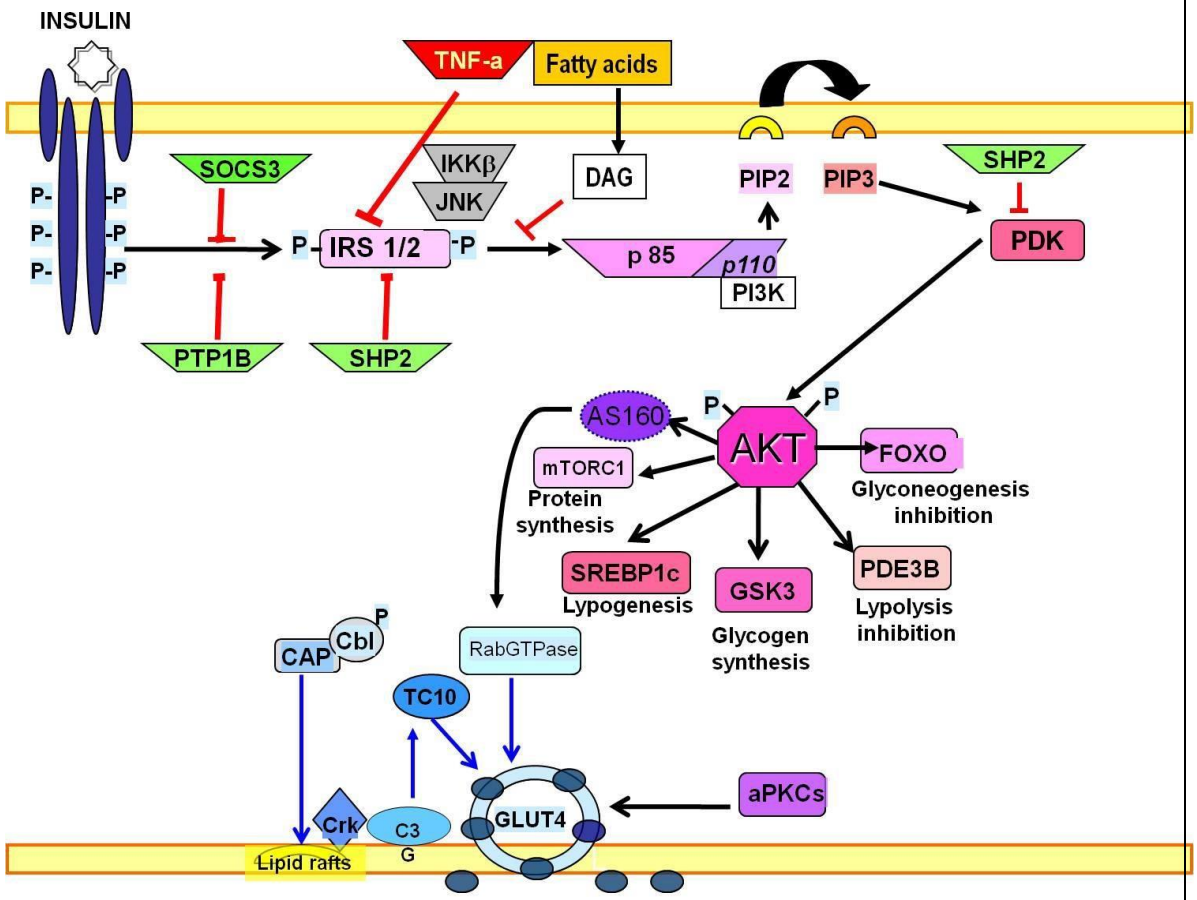


Figure 3. 5: Molecular mechanisms of insulin signaling.

Different phosphatases such as protein-tyrosine phosphatase 1B (PTP1B), Phosphatase and tensin homolog (PTEN), SH2-containing tyrosine- protein phosphatase (SHO2), and suppressor of cytokine signaling 3 (SOCS-3) dephosphorylate and shut down insulin signaling[27]Any change in the insulin pathway, such as ineffective phosphorylation or an increase in phosphatase activity, impairs the ability of insulin to do its job leading to insulin resistance.

3.7.Pathogenesis or Pathophysiology of Type 2 Diabetes

A condition known as pre-diabetes, which occurs when blood sugar levels are higher than usual but not high enough to be categorised as T2DM, can precede T2DM and have a protracted asymptomatic phase. Pre-diabetes will most likely result in the onset of T2DM without pharmacological or non-pharmacological intervention. [28] Figure 3.6 summarises the pathophysiology of T2DM.

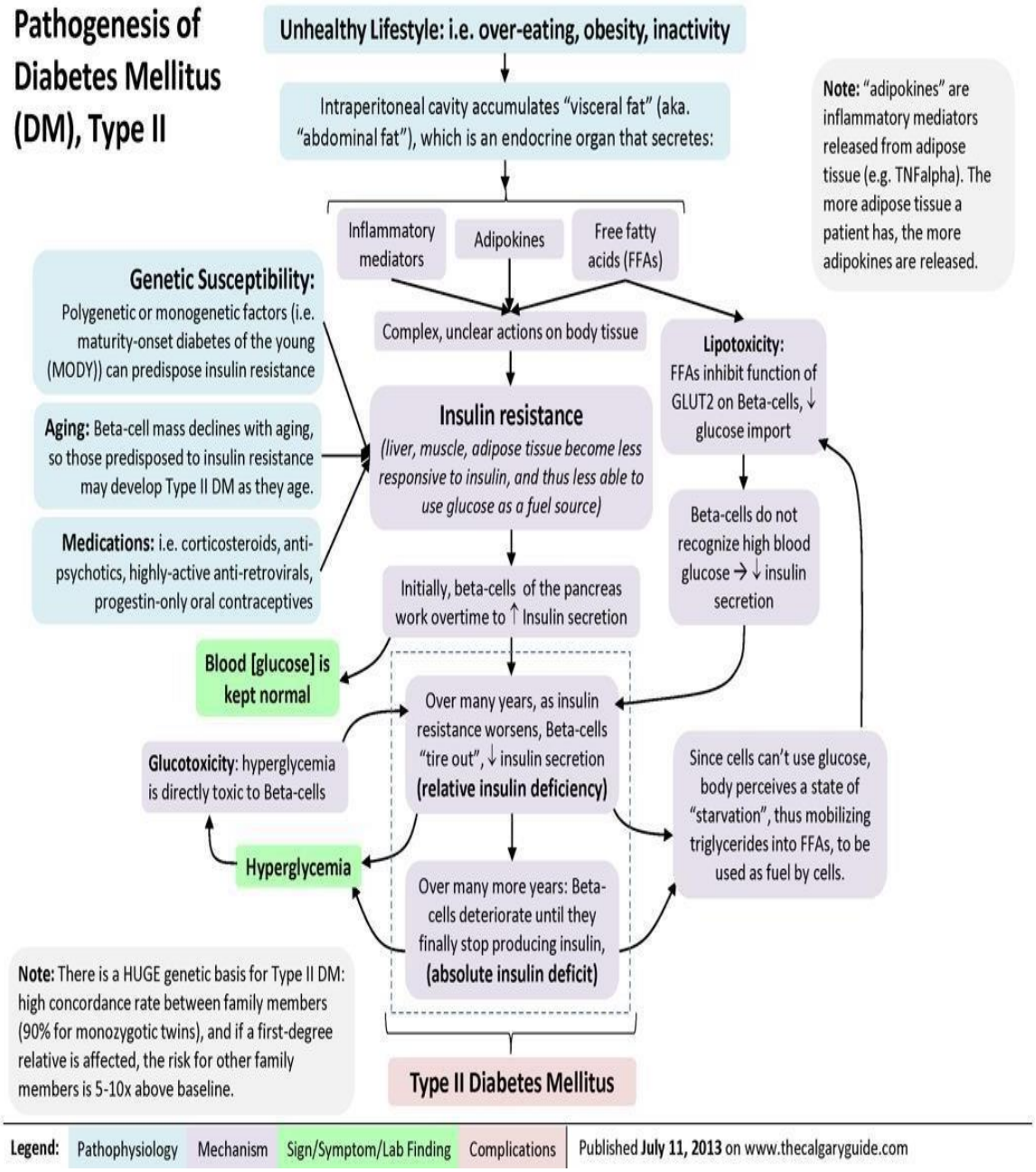


Figure. 3.6: Pathophysiology of T2DM (Source: https://www. thecalgaryguide.com)

T2DM has developed quickly, and the primary factor contributing to this development is reduced insulin production by pancreatic islets against a background of pre-existing insulin resistance in skeletal muscle, the liver, and adipose tissue. [29].

3.8 Risk factors for developing type 2 diabetes: Figure 3.7 shows the risk variables for DM that were substantially linked to age group (45-69 years), marital status, hypertension, obesity, and family history.

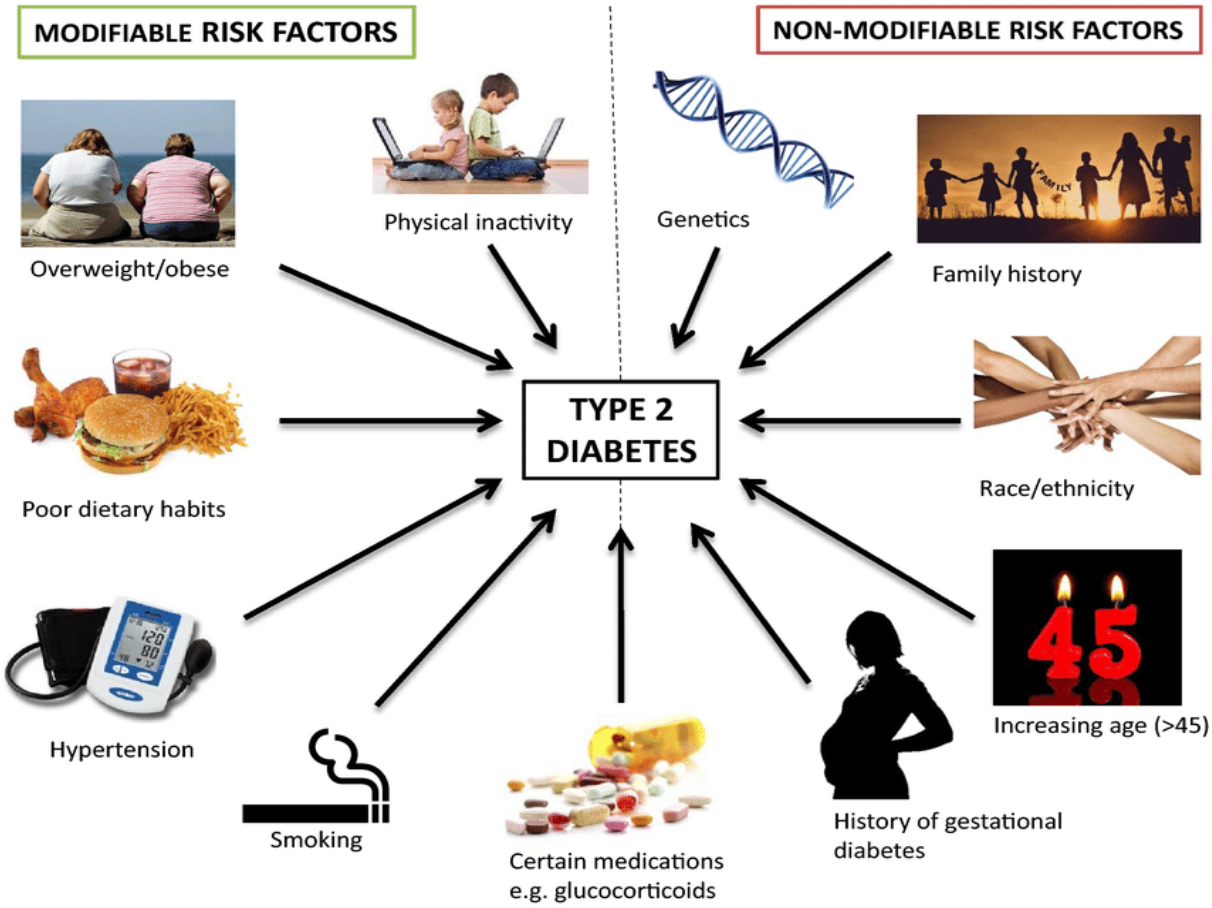


Figure-3.7: High risk factors for DM. https://doi.org/10.1186/s13007-019-0487-8

Based on glycemic high-risk condition that predisposes individuals to T2DM the Diagnostic criteria for T2 DM presented in Table 3.1.

Table3.1: Diagnostic reference values in T2 DM

Parameters	Random blood glucose(RBS)	Fasting blood glucose(FBG)	2-h post OGTT blood glucose(PPBG)	Glycated Hemoglobin (HbA1c)
Normal	<140mg/dl	<110mg /dl	<140 mg/ dl	<6.0%
Prediabetic	140-199 mg/dl	110–125mg/dl	140–199 mg/ dl	6.0–6.4%
Confirmed Diabetic(T2DM)	200 mg/dl or higher with classic symptoms of hyperglycemia	≥126mg/dl*	≥200 mg/ dl*	≥6.5%*

OGTT, oral glucose tolerance test; T2DM, Type 2 diabetes mellitus. *Normal glucose metabolism. World Health Organization.

3.9 The connection between type 2 diabetes and oxidative stress:

When the body's antioxidant defence systems are overwhelmed by an increase in ROS, oxidative stress develops at the molecular level. Increased ROS generation, which can occur either acutely or chronically, varies in intensity across different cellular sites, and was defined as oxidative stress[30.]Numerous studies have shown that T2DM patients have lower antioxidant defence mechanisms and higher levels of oxidative damage in the circulation due to increased ROS generation.[31-34.]

ROS are oxygen-containing molecules that are chemically active and are produced by living systems. All aerobic organisms naturally produce these as byproducts of oxygen metabolism[35]. Superoxide, hydroperoxyl radicals, singlet radicals, hydroxyl radicals, nitric oxide, peroxynitrite, and others are among the main forms of ROS. [36]. Although ROS are largely produced in mitochondria, other processes like as NADPH-

oxidase (NOX), immunological responses, xanthine oxidase, arachidonic acid metabolism, etc. also contribute to their formation[37]. The processes of intracellular signalling, cell activity regulation, apoptotic induction, adaptability to the effects of numerous stimuli, and immunological response are all heavily influenced by ROS[38].

Increased ROS production causes oxidative stress (OS), which damages important biological components such proteins, lipids, and DNA[39]. By reducing the harmful effects of ROS, the antioxidant defence (AOD) system offers crucial defence for the biological system. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase, paraoxanase (PON), and many others are examples of antioxidant enzymes[40]. Ascorbate, tocopherols, retinol, carotenoids, reduced glutathione (GSH), melatonin, polyphenols, ceruloplasmin, carnosine, and other non-enzymatic antioxidants play a significant role in preserving normal ROS levels in addition to enzymatic antioxidants[41]. The redox balance can be off under various disease circumstances, including DM, which has detrimental effects on the cell[42].

3.10 Molecular pathways of oxidative stress in the development of diabetes mellitus

Currently, there is compelling evidence that hyperglycemia directly contributes to the development of DM's vascular problems, with OS coupled with elevated ROS formation playing a key role in their pathogenesis[43]. The primary molecular processes linked to OS in DM have been identified, and they involve lipid and glucose metabolism[44]. In order to stimulate OS development under glycemic conditions, a number of metabolic pathways **(Figure 3.8)** are taken into account, including the glycolytic pathway, increased formation of advanced glycation end products (AGE),

the hexosamine pathway, activation of protein kinase C (PKC), the polyol pathway, and deactivation of the insulin signalling pathway[45].

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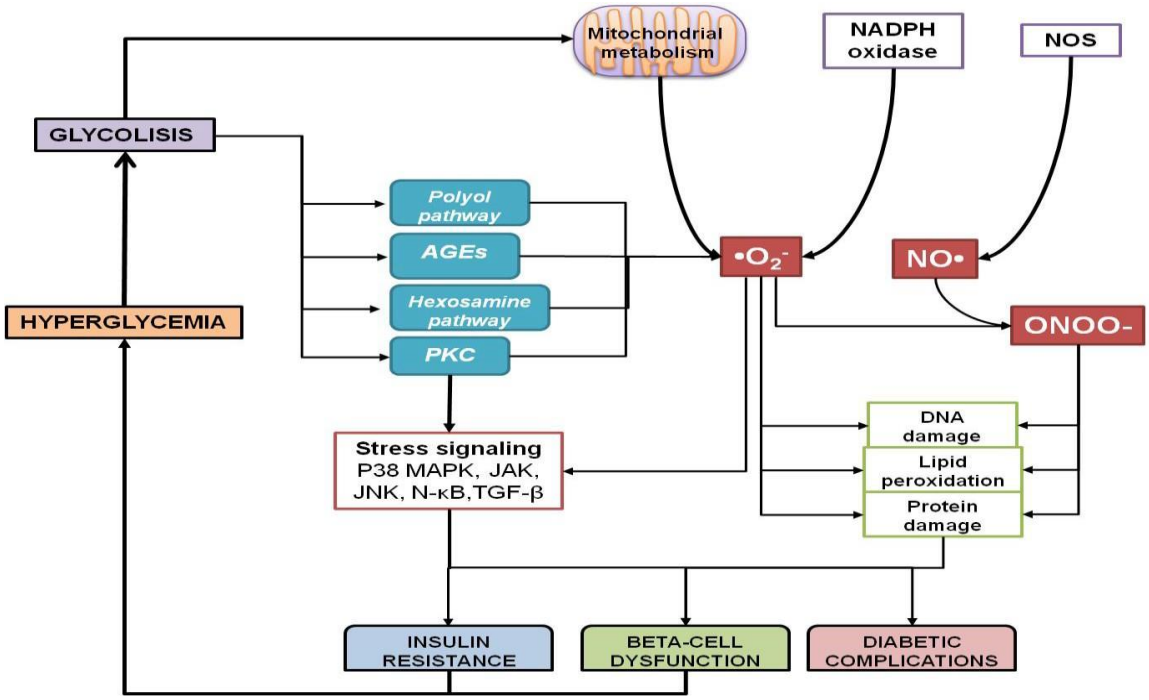


Figure 3.8:Oxidative stress pathways in diabetes mellitus

Increased glucose metabolism results in increased overproduction of NADH and FADH2, which are used by the mitochondria's electron transport chain to produce ATP. The greater proton gradient generation may be due to NADH overproduction. As these electrons are transported to oxygen, more superoxide is created.

In diabetes, oxidative stress can come from nonenzymatic, enzymatic, or mitochondrial mechanisms, among other causes. The oxidative biochemistry of glucose is the source of nonenzymatic oxidative stress. Increased production of reactive oxygen species (ROS) can be directly caused by hyperglycemia. As seen in **Fig. 3.9,** glucose can undergo autoxidation and produce a hydroxyl radical (•OH)

(Wolff and Dean 1987). ROS are produced at various stages of the autoxidation process, which results in the generation of AGEs. A higher rate of glucose metabolism via the polyol (sorbitol) pathway occurs in hyperglycemia, which also increases O2•- generation. O2•- generation during mitochondrial oxidation will occur in small amounts, as illustrated in **Fig. 3.10,** but it will be amplified in chronic hyperglycemia. In other words, the antioxidant defences that are present in the cell are defeated by hyperglycemia (Fig.3.11). Similar to this, nitric oxide synthase (NOS), NAD(P)H oxidase, and xanthine oxidase are the enzymes that contribute to the increased formation of reactive species in diabetes **(Fig. 3.11.)**

Increased oxidative stress seems to be a harmful element that will cause insulin resistance, dyslipidemia, -cell dysfunction, decreased glucose tolerance, and eventually T2DM [27]

a. Glucose autoxidation

One of the main sources of ROS produced by oxidative glycation processes is glucose auto-oxidation. Glucose and its enediol, which can auto-oxidize to create an enediol radical, coexist in equilibrium. This radical produces the superoxide radical by reducing molecular oxygen and then undergoes its own oxidation to become a dicarbonyl ketoaldehyde, which then combines with amino groups in proteins to create a ketoamine **(Fig. 3.9).** . AGE production is aided by ketoamine, which is comparable to Amadori products but more reactive[46]. In the presence of transition metals and without catalase or glutathione peroxidase, the superoxide anion radicals undergo dismutation to hydrogen peroxide, which can produce highly reactive hydroxyl radicals.

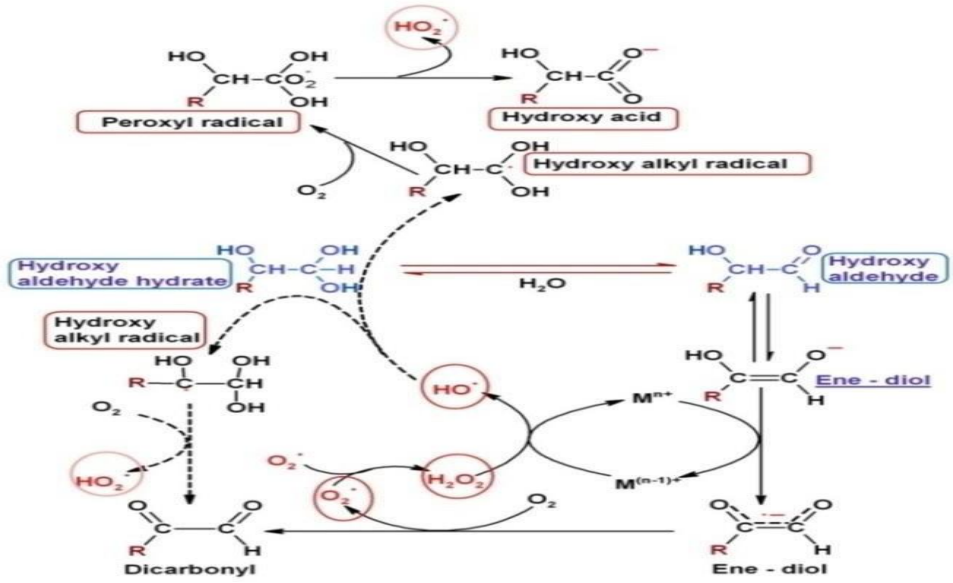
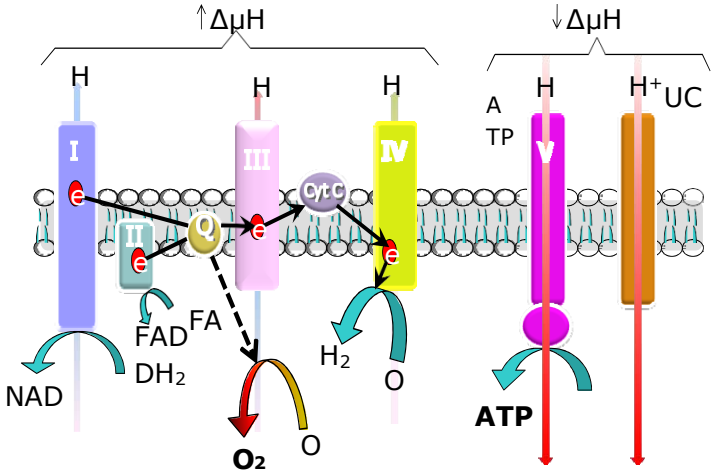


Figure. 3.9: Autoxidation of glucose (as per the reference Wolf and Dean 1987)



Complex I: NADH-Ubiquinone oxidoreductase,

Complex II: Succinate-Ubiquinone oxidoreductase.

ComplexIII:Ubiquinol-Cytochrome-Coxidoreductase ,

Complex IV: Cytochrome-Coxidase.

Complex V: ATP synthase UCP: Uncoupling

Fig. 3.10: Reactive species generation during mitochondrial oxidation

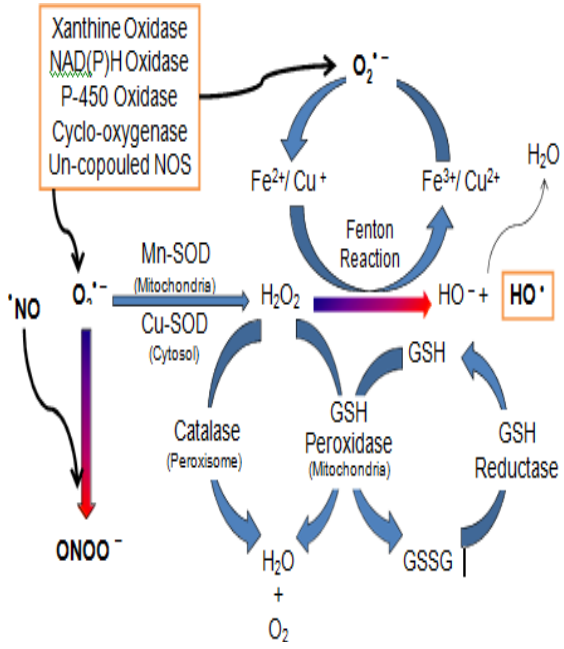


Fig.3.11. Cellular antioxidant protection Key; SOD: Superoxide dismutase; GSH: Reduced glutathione; GSSG: Oxidised glutathione; NO: Nitric oxide; $ONOO^-$: peroxynitrate radical

b. The polyol route(pathway) :

Patients with T2DM who use the polyol route have higher levels of oxidative stress in their blood. There were two enzymes that produced ROS: (1) Aldose reductase uses NADPH in the process to convert glucose to sorbitol. In typical physiological settings, sorbitol production is a modest response. However, the polyol pathway[47] only processes 30%–35% of the glucose under T2DM circumstances. When sorbitol is produced in excess, two things happen: (1) NADPH availability decreases, which has a knock-on effect on glutathione regeneration and NOS synthase activity and results in increased oxidative stress[48]; (2) Sorbitol dehydrogenase oxidises sorbitol to fructose in the second step, which also causes an increase in NADH production[49]. NADH oxidases may consume more NADH to boost superoxide production, which includes mitochondrial overproduction of superoxide.

c. AGE-product formation (carbonyl stress):

In hyperglycemia, carbonyl molecule glyoxal, an AGE precursor, is formed as a result of glucose autoxidation. Additionally, non-enzymatic dephosphorylation of glucose metabolites including GA3P and dihydroxyacetone-3-phosphate results in methylglyoxal, another precursor to AGE. Glyoxal and methylglyoxal cause OS either directly or indirectly by PKC activation [50] by binding to various AGE receptors (AGE-R1, AGE-R2, AGER3, and receptor for advanced glycation end products). The breakdown of the glucose-derived adduct of lysine 1-amino-1-deoxyfructose, also known as the A madori product [51,] yields 3-deoxyglucosone, the third precursor of AGE. Nucleic acids and lipids were discovered to also be able to transform into AGE[52] along with other extracellular matrix components. The link between OS and carbonyl stress is described by the modern notion of OS, which leads to the active creation of carbonyl compounds. Even for DM, this connection is obvious.

d. Oxidation of glucose via the hexosamine route.

Fluctuations in blood sugar cause an increase in the level of fructose 6 phosphate, which is then broken down by glucosamine-fructose aminotransferase into glucosamine-6-phosphate, which is then converted into uridine phosphate-N-acetylglucosamine (UDP-GlcNAc) by the enzyme UDPN-acetylglucosamine-1-phosphate uridy The prooxidant function of the hexosamine pathway in DM is connected to the activation of O-glucosamine-N-acetyltransferase, which is brought on by UDP-GlcNAc accumulation. The hexosamine pathway and this enzyme's activity are linked to alterations in gene expression and increased expression of the transcription factors TGF- and TGF-, which block the mitogenesis of mesangial cells, promote the proliferation of the collagen matrix, and thicken the basement membrane

[53].Due to the inhibition of GA3PDG activity by the PKC activation pathway PARP1, which is triggered as a result of the DNA damage brought on by OS, GA3P and its isomer, dihydroxyacetone-3-phosphate (DHA3P), accumulate. By converting DHA3P to glycerol-3-phosphate in the presence of free fatty acids, the glycerol-3-phosphate dehydrogenase creates diacylglycerol, which binds with the AGE receptor and activates PKC to stimulate OS processes[54].

Relationship between lipid toxicity and oxidative stress in type 2 diabetes

The oxidative situation in T2DM arises from an excess of free radicals that may cause DNA oxidation, organ damage, and interference with cellular function[55]. More calories are produced by excessive dietary intake and are then stored as fat in adipose cells. However, storing too many calories in fat cells for an extended period of time causes hypoxia in adipose tissues, which in turn stimulates the activity of the protein hypoxia inducible factor-1 (HIF-1) [56], as well as the expression of the inflammatory proteins c-Jun N-terminal kinase (JNK) and inhibitor nuclear factor kappa-B kinase (IKK), which causes inflammation in adipose tissues[57]. In order to further increase insulin resistance (IR)[58] and lipolysis[59], cytokines are created as a result of the ongoing inflammatory response. Additionally, inflammatory cytokines can increase inflammation and adipose cellular death while decreasing the activation of the peroxisome proliferator-activated receptor (PPAR)[60]. The development of IR causes insulin's activity to be lipolytic. The breakdown of lipoprotein triglycerides by the hyperinsulinemia may induce lipoprotein lipase. While JNK pathway is active and endoplasmic reticulum (ER) stress is implicated[61]. As a result, a vicious cycle between IR and increased FFA flow in adipose tissues may develop. FFA from fat cells are created in greater quantities, which are released into the bloodstream,

transferred to other organs, and accumulated there to promote lipotoxicity and hasten the systematic IR[62].

Oxidative stress mechanisms and beta cell dysfunction:

Because pancreatic beta-cells have weaker natural enzymatic antioxidant defences than other organs like the liver, they are particularly vulnerable to ROS and RNS. Additionally, they are unable to adjust their low levels of enzyme activity in response to stressors such high glucose or high oxygen levels[63]. Because glucose sensing in the beta-cell is essential for insulin production in addition to delivering energy, glucose enters the beta-cell without the need for insulin. According to some research, hyperglycemia can cause persistent oxidative stress through the glucose oxidation pathway[64], which increases the production of superoxide in the mitochondria and activates uncoupling protein-2 (UCP-2). The beta-cell proton leak caused by this protein affects the ATP/ADP relationship and inhibits insulin secretion[65].

NF-B activity, which may cause beta-cell apoptosis[66], and the JNK pathway, which has been linked to suppression of insulin gene expression, possibly by reducing PDX-1 DNA binding activity, a key regulator of insulin expression[67], are two additional stress signalling pathways that are increased by ROS in beta cells. Activation of the hexosamine pathway in beta-cells has also been found to decrease PDX-1 binding to the insulin(Fig3.12 and other genes involved in insulin production, which may be a factor in the betacell dysfunction seen in diabetes mellitus [68].

NO plays physiologic roles in beta-cells, just like in other cell types. Through s-nitrosilation[69] in the beta-cell, NO may control glucokinase activity and perhaps boost insulin secretion. However, apoptosis may be triggered by excessive NO and concurrent NRS due to caspase-3 activation and a drop in ATP levels[70].

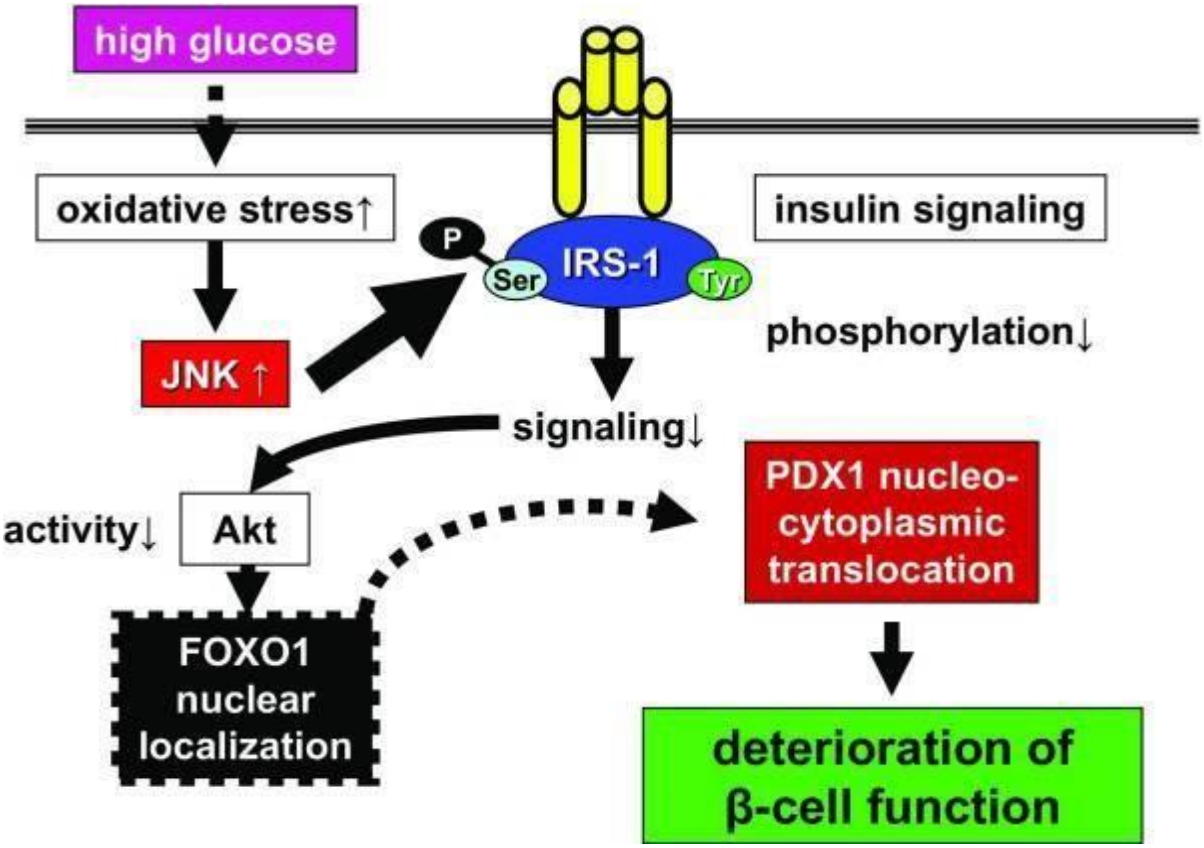


Figure 3.12: Molecular mechanism underlying impaired JNK & PDX1 function in β cells of T2DM (Source: Kawamori D. exploring the molecular mechanisms underlying α- and β-cell dysfunction in diabetes. Diabetol Int. 2017 ;8(3):248-256)

In addition to increasing the production of ROS, excessive mitochondrial metabolism brought on by hyperglycemia in the beta-cell may also change the volume, shape, and behaviour of mitochondria, decoupling K-ATP channels from mitochondrial activity, and changing the way that glucose induces insulin secretion[71]

Processes of oxidative stress in insulin resistance:

The inability of insulin to perform biologically across a broad variety of concentrations is known as insulin resistance. During fasting and in response to an insulin secretagogue, relatively elevated insulin levels are indicators of IR. T2DM frequently manifests as insulin resistance, which can develop years before symptoms do. Additionally, there is a well-known link between obesity and T2DM because so many obese people get the condition. Among other organs, insulin resistance has an

impact on the liver, muscles, and adipose tissue. The key characteristic of T2 DM illness is insulin resistance in the muscle and liver, as well as decreased insulin production by pancreatic cells.

There are numerous methods that might cause cellular insulin resistance. Stress kinases like JNK (Jun NH2-terminal kinase) or IKK are activated in response to unfolded protein response brought on by increased amounts of pro-inflammatory cytokines in sepsis or obesity. The stress kinases interfere with insulin signalling by interacting with IRS (IRS1/2). In addition, oversupply of fatty acids or poor fatty acid oxidation lead to the accumulation of lipid intermediates, which activate new protein kinases C, which in turn act on IRS to block the insulin signalling pathway. Additionally, fatty acids can cause insulin resistance by inducing ER stress or by activating JNK and IKK via toll-like receptors[72]. The insulin resistance is schematically illustrated in Figure 3.13.

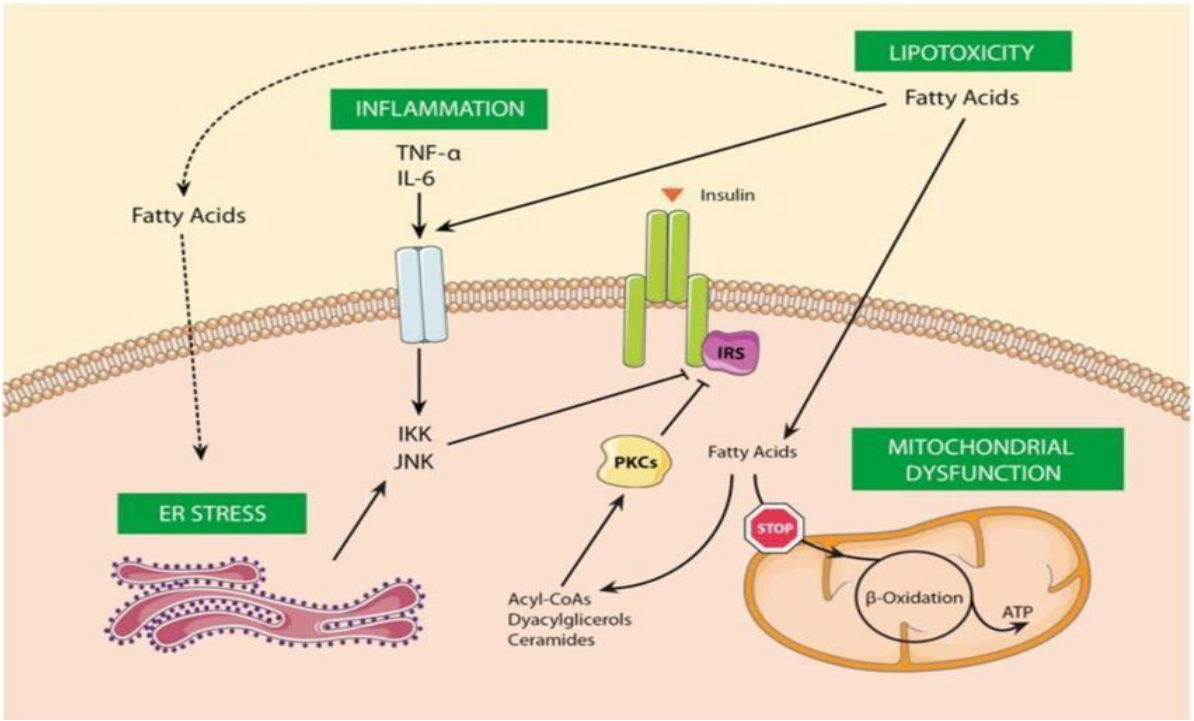


Figure3.13:Schematic of insulin resistance mechanism (Source: Zamora M, Villena JA. Targeting mitochondrial biogenesis to treat insulin resistance. Curr Pharm Des. 2014;20(35):5527-57.)

The insulin signalling cascade is impacted by ROS and RNS[73]. Low levels of ROS have physiological effects on insulin signalling, similar to other ROS actions. NADPH oxidase produces H2O2 after insulin activates its receptor in adipocytes, which in turn reduces PTP1B catalytic activity and raises tyrosine phosphorylation [74].However, insulin signalling may be impaired in diabetes due to oxidative stress brought on by hyperglycemia, which can result in insulin resistance. Numerous responses to excessive ROS in the insulin signalling have been hypothesised, despite the fact that no full mechanisms have been uncovered.

Changes to the cellular redistribution of insulin signalling elements, a process regulated by NF-B [75], may change the insulin cascade. Increases in the phosphorylation of IRS protein in an insulin receptor-independent manner have also been seen, as well as a decrease in the transcription of the GLUT4 gene and an increase in GLUT1 (an insulin independent glucose transporter) (perhaps by the stress kinases). In response to metabolic stress, hyperglycemia and insulin resistance may also result in altered mitochondria and impaired insulin action by cytokines [76–77]. Insulin resistance has also been connected to an increase in the hexosamine pathway. It has also been suggested that this route serves as a cellular sensor for the excess glucose. According to this theory, insulin resistance may be a defence mechanism against the entrance of excessive glucose[78].

According to published research, it has been hypothesised by a number of groups that mitochondrial O2•- triggers an avalanche of oxidative stress that leads to diabetes by increasing the production of ROS and reactive nitrogen species (RNS) through the activation of PKC, NAD(P)H-oxidase, and nuclear factor kappa B (NF-B)-mediated cytokine generation PKC and NAD(P)H-oxidase[79](Fig.3.14).

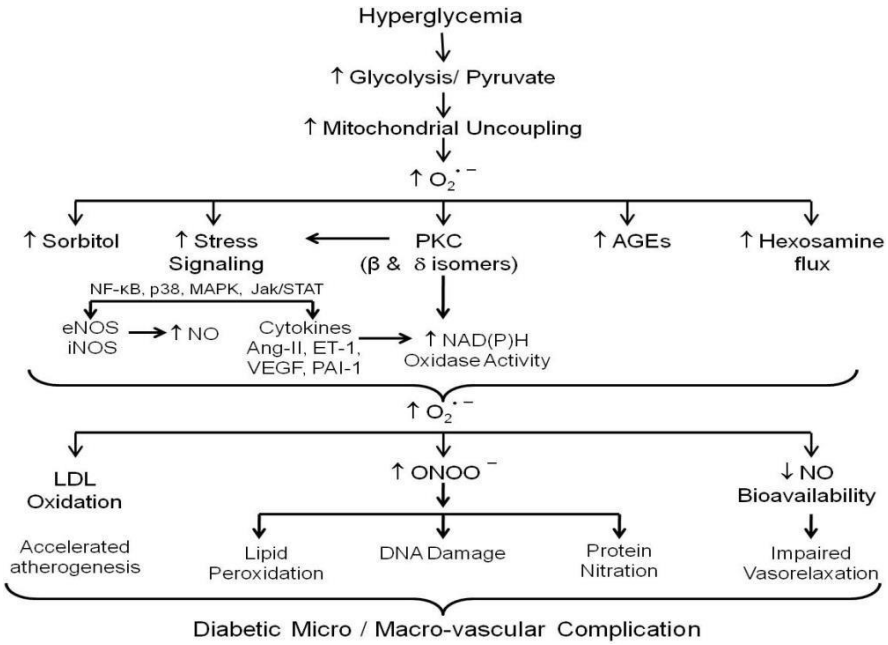


Figure 3.14. Generation of reactive species and downstream targets in diabetes

AGEs: advanced glycosylation endproducts; PKC: protein kinase C; NF-κB: nuclear factor kappa B; MAPK: mitogen-activated protein kinase; NO: nitric oxide; eNOS/iNOS: endothelial/ inducible nitric oxide synthase; Ang-II: angiotensin II; AT-1: endothelin-1; VEGF: vascular endothelium growth factor; PAI-1: plasminogen activator inhibitor-1: LDL: low density lipoprotein; O2•-: superoxide radical; ONOO - : peroxynitrite radical; DNA: deoxyribonucleic acid .

Inhibiting the production of intracellular free radicals would represent a potential therapeutic strategy for the treatment of T2D's oxidative stress and associated vascular problems. Additionally, controlling antioxidant enzymes in target organs like the heart and kidney, which are vulnerable to T2D consequences, may help to prevent and treat heart failure and kidney failure[79].

Clinical trials using antioxidants as a medicinal agent to control diabetes and its consequences.

Numerous investigations on the antioxidant effects of various compounds, including natural antioxidants of plant origin, have been done in light of the fact that oxidative stress plays a substantial role in a number of DM complications. Numerous studies showing the role of various supplements, including (a) vitamins (Vitamin-A, B1, B2, B6, B12, C and E); (b) antioxidants like glutathione (GSH), -lipoic acid, and carotenoids; (c) trace elements like copper, zinc, and selenium; (d) coenzyme Q10 (CoQ10); and (e) cofactors like folic acid, uric acid, etc were revealed the beneficial effect in restoring insulin sensitivity and alter the antioxidant defense system in diabetes[80-81].According to Darenskaya, M. A et al. (2021)[82] -tocopherol, retinol, -cryptoxanthin, ascorbic acid, - and -carotene, lutein and zea- xanthin], and lycopene have also considerably minimise DM complications. It has been discovered that foods and medicinal plants with flavonoid-rich phytochemical components (more than 10,000 different components have been identified) have powerful anti-radical and anti-inflammatory properties, regulate the activities of -glucosidase and lipase, lower blood sugar levels, improve pancreatic function, and work synergistically with hypoglycemic drugs to treat diabetes [83.]

Due to its anti-inflammatory and antioxidant activities, curcumin is deemed suitable for the prevention and reduction of the risk of DM complications [84]. Butein is an anti-inflammatory polyphenol that inhibits the production of NO in vitro, safeguards pancreatic beta-cells from excessive inflammation, and can stop the progression of DM1 [85]. By increasing the expression of numerous β-cell genes and insulin in pancreatic alfa-cells, resveratrol modifies the expression of genes linked to the

development of DM2[86]. Under controlled laboratory conditions, the impact of several antioxidant-active ingredients on the DM course was investigated.

Numerous randomised controlled studies have also assessed the therapeutic efficacy of antioxidants; some of the study findings are listed below in this review.

In T2DM patients (n=84), daily 1000 mg of vitamin C administration demonstrated positive effects by lowering blood glucose and lipids and so lowering the risk of complications, according to Afkhami-Ardekani M and Shojaoddiny- Ardekani's 2007 paper[87]. T2DM patients (n=98) who received alpha-tocopherol (200 mg/day) showed favourable results in lowering blood lipid peroxide concentrations, according to a study by Park S. and Choi SB(2002)[88]. According to K. Chandra, P. Singh, S. Dwivedi, et al. (2019), [89] vitamin C administration at a dose of 2 g/day in newly diagnosed type 2 diabetes (n=90) as an add-on therapy to oral hypoglycaemic agent resulted in improvement in lipid profile to desirable levels, lowering the risk of diabetic complications. While it is well documented that antioxidants should be used in the therapeutic process, despite the fact that numerous studies have demonstrated the usefulness of antioxidants in DM1 and DM2, there is still no proof that the use of a single antioxidant medicine has a fully therapeutic impact[90].

Thus, the dual antioxidant and antidiabetic activity of molecules may be a unique technique for the introduction of fresh chemical entities in diabetes.

Diabetes and the function of flavonoids (benzopyrones) as antioxidant:

With the fundamental ring of benzopyrone (or benzopyran-4-one; Fig: 3.15), flavonoids are naturally occurring polyphenols that are renowned for their antioxidant properties. Due to the complexity of the disease and genetic factors, such chemicals

cannot cure DM, but they help lessen some of the effects of metabolic problems caused by DM. The effects of flavonoids on diabetes have been linked to a variety of processes, including insulinomimetic activity and enhanced skeletal muscle glucose absorption [91,] suppression of –glucosidase. Aldose reductase inhibition, number [92 -93,] activation of AMPK), and [94,] suppression of SGLT-2 [95,] PPAR regulation [96], PTP-1B inhibition [97-98], inhibition of inhibitor kappa-B kinase (IKK)[99], and suppression of AGEs [100] are a few examples of the mechanisms that have been studied. In addition to restoring endothelial functioning, several flavonoids also reduce the severity of diabetes consequences [101]. Additionally, flavonoids are well-known antioxidants and help reduce oxidative stress when a disease develops. The greatest natural flavonoid option for preventing diabetes, quercetin, has been proven to work through a variety of methods. Recent research indicates that quercetin blocks 11-HSD1 [102.] Thus, flavonoids act as insulin secretagogues or insulin mimetics, probably by influencing insulin signalling, to improve the diabetes condition. Flavonoids also regulate the activities of the rate-limiting enzymes in the carbohydrate metabolism pathway and stimulate glucose uptake in peripheral tissues [103].

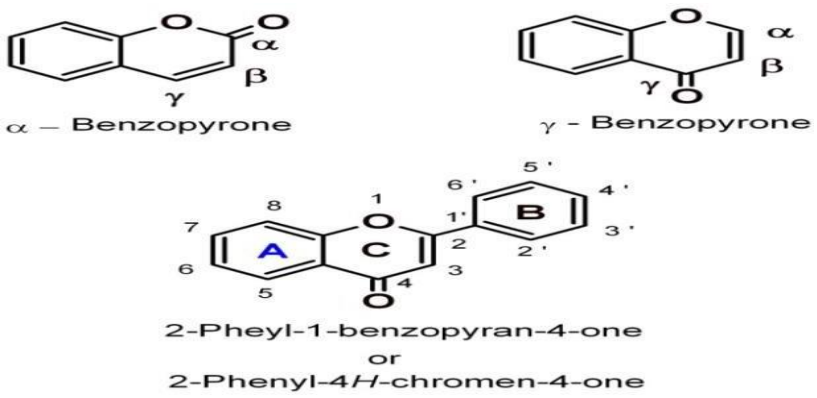


Fig 3.15: Chemical structure detail of flavonoid .

Veerapur et al from 2010a and 2010b research study reported that the flavonoid-rich extract from the plant Dodonaea viscosa (L). Jacq., areolar components demonstrated

antidiabetic efficacy in high fat, low dose streptozotocin (STZ) rat model [104] and fructose-induced insulin resistance in rat model [98,] respectively.

Similar kinds of naturally occurring plant flavonoids have been shown to have anti-diabetic effects in DM. In animal models, flavonoids as kaempferol, baicalein, luteolin, apigenin, diosmetin, genestein, naringenin, chrysin, hesperitin, epicatechin, epigallocatechin, diadzein, myricetin, shamimin, etc. were excellent leads for DM[105] (Fig. 1.7). The cost of extracting these flavonoids from their natural sources, as well as their strength, effectiveness, solubility, and stability, were their main drawbacks. These elements caused several drug discovery teams to change their minds and opt for synthetic alternatives. The benzopyrone (or benzopyran-4-one; Fig. 3.16) ring is shared by all flavonoids, and it is replaced with a phenyl group at position 2 [106]. As a result, we chose the benzopyrone's fundamental molecular skeleton for structural alteration in order to produce analogues of a similar nature that are less poisonous and more effective than naturally occurring flavonoids, which are in short supply. We selected a group of chemicals that could be easily synthesised in just two steps, resulting in higher yields and more affordable synthesis.

In some studies on synthetic flavonoids (benzopyrone analogues), such as Venkatachalam, H. Yogendra, N. Jayashree, B. S. (2012), B. Jayashree, A. Alam, Y.Nayak (2012), P. Bhixavatimath, A. Naikawadi, and Y.Maniyar et al. (2022) [107–109], it was found that these synthetic flavonoids demonstrated antioxidant activity. Because antidiabetic characteristics are known to be linked to antioxidant and antiglycation potential, synthetic -benzopyrone derivatives, which have a structure comparable to natural flavones, are more likely to provide better lead molecules.

We offer the notion that the synthesised lead compounds sharing the -benzopyrone nucleus of powerful flavonoids are therapeutically advantageous in diabetes and related metabolic illnesses based on the evidence given from published literature.

3.16..Currently used T2 DM management techniques:

a) Prevention method.

The non-pharmacotherapeutic strategy will significantly assist T2DM risk patients before they begin genuine medication. There is a lot of data to support controlling obesity with poor glucose regulation with diet and exercise treatments, and to a lesser extent, with metformin and thiazolidinedione pharmaceutical therapy. The American College of Clinical Endocrinologists' recommendations number [110] state that factors like proper nutrition, weight loss, regular exercise, the reduction of cardiovascular risk factors, and the aggressive treatment of hypertension and dyslipidemia play a significant role in the prevention of T2DM at risk.

b) Currently available medications and their status for T2DM are used as a treatment plan.

A. Biguanides: Metformin (chemically known as dimethylbiguanide) is the sole biguanide currently in use in this family of medications. By reducing hepatic gluconeogenesis and slowing intestinal glucose absorption, metformin's main effect is to lower excessive rates of hepatic glucose synthesis. Less frequently, this medication has been shown to enhance extra hepatic tissues' consumption of insulin-stimulated glucose. Insulin levels are either either constant or decreased as plasma glucose levels fall. The medication meatformin [111] has been found to target the enzyme adenosine 5'-monophosphate activated protein kinase (AMPK) (Musi et al. 2002). About 10% of

people using metformin experience diarrhoea and stomach pain as the most frequent side effects. Metformin is one of the most often prescribed oral antidiabetics in the world because of its robust anti-hyperglycemic effects, safe and effective reduction of insulin resistance, numerous ancillary therapeutic benefits, usefulness in combination therapy, and inexpensive cost.

B. Sulfonylureas: These drugs have been in use since the 1960s. They bind to the sulfonylurea receptor (SUR-1) on the surface of -cells and block ATP-dependent K+ channels, which causes I depolarization and Ca2+ influx and the release of insulin into the bloodstream. This class of medications includes tolbutamide, glibenclamide, etc. Reduced hepatic glucose synthesis and increased peripheral insulin sensitivity have effects independent of insulin release.

The limiting aspects sometimes necessitate combined therapy with metformin or peroxisome proliferator-activated receptor gamma (PPAR-) agonists[112] (DeFronzo 1999). This is due to the deterioration in glycemic control over time (known as secondary sulfonylurea failure). Rapid-acting prandial insulin releasers, such as meglitinide, repaglinide, and nateglinide, belong to another closely related class (in terms of their mode of action), the glinides[113], which exert comparable effects by attaching to a different binding site on SUR-1.

C. Thiazolidinediones (TZDs): Also known as agonists of the PPAR-gamma (peroxisome proliferator-activated receptor). Pioglitazone is a key medication in this group. Thiozolidinediones (TZD) activate PPAR- in a range of tissues to increase whole-body insulin sensitivity 114. (Krentz and Bailey 2005). The transcription of a wide variety of genes is modulated by endogenous lipids and prostaglandins, which activate the nuclear receptor PPAR-. TZDs have a variety of metabolic effects in the

liver (decreased glycogenolysis, increased lipogenesis, and glucose uptake), muscle (increased glucose uptake, glycolysis, and glycogenesis), and adipose tissue (increased glucose uptake, fatty acid uptake, lipogenesis, and pre-adipocyte differentiation) [114]. Fluid retention, which exacerbates heart failure, a propensity for myocardial infarction, and weight gain of 1-4 kg are all side effects of TZD medication. Additionally, TZDs cause anaemia, and they should not be used if you have an active liver disease, heart failure, insulin dependence, or are pregnant. The disadvantage is that it takes 6–12 weeks of treatment to achieve the best therapeutic results.

D.Intestinal glucose absorption inhibitors: By preventing the conversion of disaccharides (like sucrose) and oligosaccharides (like galactooligosaccharides) into monosaccharides (like glucose), such as acarbose and voglibose, before absorption, these carbohydrate modulators (-glucosidase inhibitors) delay the absorption of glucose and change the release of glucose-dependent intestinal hormones**[115]**. The most frequent side effects of this class of medications include abdominal discomfort and diarrhoea [116].

E. Incretin stimulants (Exenatide and Sitagliptin):

When a meal is consumed, incretins, which are peptide hormones originating from the gut, are quickly released. GIP (glucose-dependent insulinotropic polypeptide) and GLP-1 are the two main incretins found in humans (GLP-1). Within 10 minutes of a meal, they cause the pancreatic beta-cells to produce insulin. Incretins are also thought to promote beta-cell regeneration, proliferation, and protection from apoptosis, suggesting that therapeutic drugs based on this target may be able to change the course of diabetes naturally. In T2DM, the incretin effect is diminished or

nonexistent, and GIP's insulinotropic effects are eliminated. Due to the enzyme dipeptidyl peptidase-4's quick breakdown of GLP-1, it cannot be used therapeutically (DPP-4). A synthetic GLP-1 analogue that works on GLP-1 receptors is exenatide. It increases appetite suppression, decreases glucagon release, delays stomach emptying, lowers HbA1c levels, and improves glucose-1-mediated insulin secretion. Other medications in this class include liglutide and albiglutide. Adverse symptoms such as nausea, self-limiting vomiting, diarrhoea, and weight loss are common. Haemorrhagic pancreatitis, albeit uncommon, is a dangerous side effect that can be fatal. [117] In people with renal impairment, should be avoided as it may make it worse.

F. Inhibitors of Dipeptidyl Peptidase 4 (DPP-4): An enzyme called dipeptidyl peptidase breaks down incretin hormones including glucagon-like peptide-1 (GLP-1) 117. Sitagliptin increases incretin levels since it is a DPP-4 inhibitor. Currently used medications in this class include linagliptin, saxagliptin, sitagliptin, and alogliptin. Increased insulin release in response to meals and a decrease in erroneous glucagon secretion occur when incretin hormone activity is prolonged.

G. Analogues of Amylin:

The pancreatic beta cells generate the polypeptide amylin. It prevents the release of glucagon, postpones stomach emptying, and reduces hunger. Like amylin [117], the synthetic amylin analogue pramlintide regulates postprandial glucose levels.

H. Inhibitors of SGLT-2:

The sodium-glucose cotransporter-2 reabsorbs a substantial quantity of glucose from the proximal tubule (SGLT-2). The reduction in glucose and salt absorption brought on by SGLT-2 inhibition results in glycosuria. The SGLT-2 inhibitors dapagliflozin,

remogliflozin, canagliflozin, empagliflozin, and sergliflozin have been proven to be helpful in diabetic individuals. When the patients also have hypertension, they are especially helpful [117]. Due to the presence of glucose in the urine, they can result in hypotension and raise the risk of urinary infection. In the case of low GFR, they ought to be avoided.

Despite the large number of therapeutic agents with mechanistic diversity, only partially correction of multiple metabolic defects in non-insulin-dependent diabetes mellitus (NIDDM) happens, with insulin resistance remaining relatively unresponsive to treatment and 63% of T2DM fail to bring down HbA1c levels to the desired level of <7% as advised by the American Diabetes Association. Such patients are at a high risk of developing complications or T2D related metabolic disorders. Therefore, there is still an urgent need for novel antidiabetic agents that can address these limitations with diverse biological targets

3.17. Experimental evaluation of drugs for T2DM:

In vitro testing takes place in a lab and typically entails looking at microorganisms or human or animal cells in culture. By using this technology, scientists can analyse diverse biological events in particular cells without being distracted by or potentially confounding factors from whole organisms. Unlike studies involving animals or people, these can conduct more in-depth assessments and look at biological impacts in a larger number of in vitro individuals. Because these results do not always predict the response of a complete living individual, it is crucial to interpret in vitro data with caution. Nevertheless, even while petri dishes and test tubes offer controlled environments for in vitro testing, they are unable to duplicate the circumstances that exist inside a living thing.

An essential component of medical research generally is in vivo testing, particularly in clinical trials. Studies conducted in living organisms (in vivo) offer useful knowledge about a substance's effects or the progression of a disease. Animal studies and clinical trials are the two main categories of in vivo testing. Animal models are essential for the development, validation, and optimization of novel drugs and therapies as well as their safe use in humans. They serve as a form of bridge between in vitro investigations and human trials.

The advancement of knowledge and clear understanding of the pathology and pathogenesis, as well as the discovery of new therapies, depend on research of diabetes conducted in-vivo and using more modern in vitro techniques. Because they provide fresh perspectives on diabetes, studies of diabetes in various animal models are consequently extremely useful in biomedical research.

For the purpose of screening new anti-diabetic medications, numerous in-vivo and in-vitro techniques are available. In-vivo models frequently use chemicals to cause diabetes, such as streptozotocin, alloxan, etc., whereas in-vitro procedures clearly demonstrate their impact on the cells that cause diabetes in humans. More accurate data and potential diabetic disease mechanisms are provided by in vitro methods. Modern ways for inducing diabetes using viruses have also been developed, and they are proving to be useful tools for assessing antidiabetic medicines.

A. *In -vitro* screening models for diabetes.

Introduction: Though we have many research models for antidiabetic evaluation because of simple steps and easy to perform with good efficacy, only α-amylase , α-glucosidase, dipeptidyl peptidase inhibitory assay based screening models are revived

in this study. And for glucose uptake study in C2C12 cell lines based screening model is discussed here with in invitro models for antidiabetic analysis study.

The enzyme α-amylase and α-glucosidase by breaking the long chain of polysaccharides, disaccharide into monosaccharide play an important role in the digestion of carbohydrate(Fig.3.16). They also suggested that the inhibitory action of these enzymes may reduce the absorbtion of blood glucose in the body [118]. The natural bioactive small molecules with polyphenolic nature have showed a delayed intestinal intake of glucose by inhibiting the pancreatic α-amylase and α-glucosidase enzymes .The inhibition of a-glucosidase enzymes capable of effectively reducing blood glucose level in the management of type 2 diabetes[119].

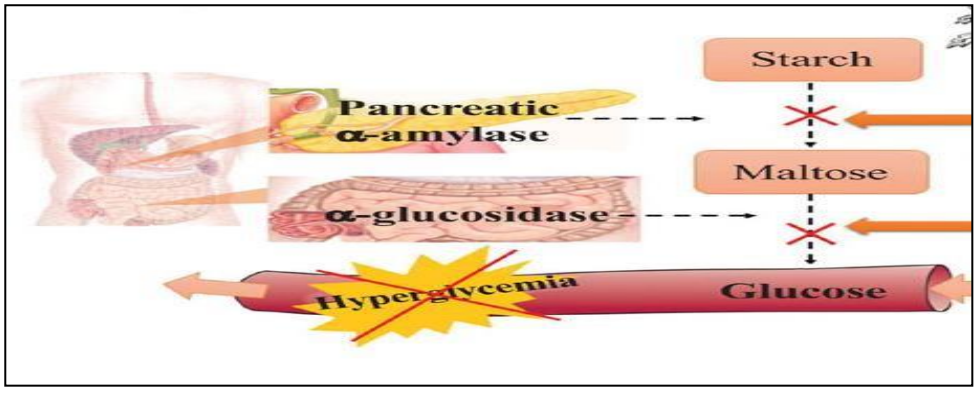


Fig.3.16 Alfa amylase and alfa glucosidase enzyme regulation in carbohydrate metabolism

The resistance of insulin and pancreatic beta cell inactivity are the major detrimental factors to produce chronic diabetes mellitus disease. Therefore enhancers of insulin secretion while protecting beta cells and augmenting insulin function at peripheral tissues represents the important in maintaining glucose homeostasis in T2 diabetes mellitus patients[120]. One of the important incretin hormone such as glucagon like peptide-1 (GLP-1) released from the intestinal cells in response to nutrients absorption, is responsible in maintaining postprandial insulin level in the blood. Because this GLP-1 incretin inhibits glucagon secretion and enhances the

biosynthesis of insulin from pancreas. But endogenous GLP-1 incretin have a very short half life within minutes due to inactivation by dipeptidyl peptidase-4 (DPP-4) enzyme(Fig.3.17)which was first identified by Hopsu-Havuand Glenner[121-123].

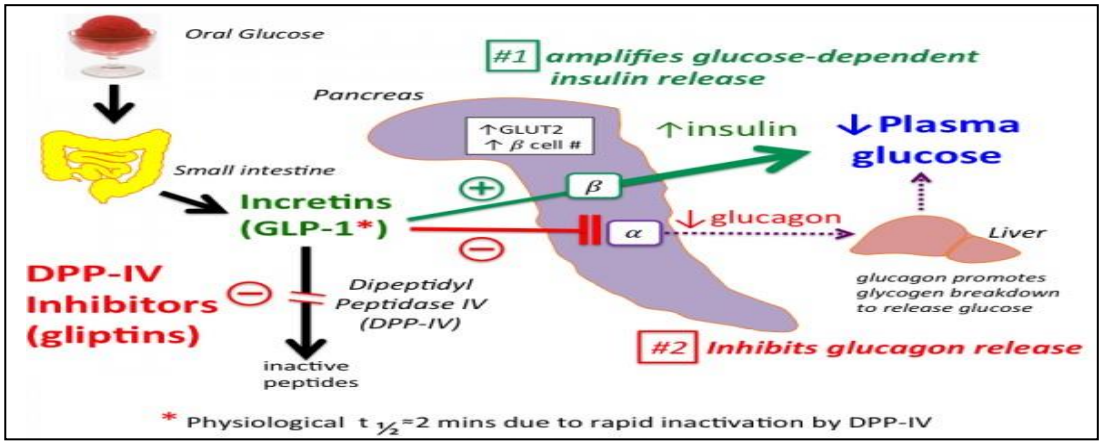


Fig.3.17. Role of Dipeptdyl peptidase -4 (DPP-IV) on incretin mediated glucose homeostasis

Inhibitors of DPP 4 avoids the inactivation of GLP-1 (glucagon-like peptide-1) and GIP (glucose dependent insulinotropic polypeptide) resulting in sustained elevations in plasma levels of intact GLP-1 and GIP and improves the sensitivity of both the α- and β-cells to glucose leading to improved glucose tolerance and reduced FPG. These effects largely explain the improved HbA1c levels without producing hypoglycaemia associated with chronic treatment.

To evaluate the antidiabetic effect of synthetic novel flavonoid, the determination of regulatory actionon α-amylase and α-glucosidase and dipeptidyl peptidase -4 enzymes were considered to be a major carbohydrate regulatory metabolizing enzymes under in vitro conditions. Nonetheless, there were no previous studies have yet been revealed on the inhibition of DPP (IV), alfa -glucosidase assays, and glucose uptake in cell lines and this present research could be regarded as an additional confirmation of antidiabetic properties for this synthetic novel flavonoid(NF).

C2C12 cell lines for glucose uptake study: From the literature it is clear that insulin reduces the glucose level by acting in liver, pancreas, skeletal muscle and adipose tissues. Skeletal muscle and adipose tissues are very important in regulating the glucose utilization mainly by insulin dependent GLUT-4 mediated glucose uptake. Because it is reported that insulin and muscle contraction can stimulate peripheral glucose uptake into muscle and fat cells via eliciting GLUT-4 translocation from the intracellular GLUT-4 storage vesicles (GSVs) to the plasma membrane. In *in-vtro* study it was also revealed that when insulin molecules bind to insulin receptor, tyrosine phosphorylation cascade can be initiated, followed by subsequent increment in the number of GLUT-4 in the perinuclear region of C2C12 cells[123]

In diabetic mellitus, raised blood glucose, and insulin resistance seen in later stage is mainly due to the uncoupling expression of GLUT-4, and drastic impaired GLUT-4 translocation and resulting into insulin insensitivity in tissues. To increase insulin mediated glucose uptake in tissues, increased translocation of GlUT-4 is essential.

To screen the potential drugs for glucose uptake activity in peripheral tissues, mouse C2C12 and rat L6 cell lines have been frequently used in metabolic research field.[124] Although only a limited number of studies made direct comparisons between these two cell lines, studies revealed that the following features, including ATP responses, insulin-induced glucose uptake, insulin sensitivity and responsiveness to muscle wasting, were different.

Firstly, mouse C2C12 have fibronectin and ATP mediated slow K+ current, while rat L6 cells did not express fibronectin and showed relatively less ATP sources[125]. Secondly, the glucose uptake in these cell lines was reported to be insulin-stimulated[126], carrier-mediated and saturable[127].

Rat L6 cells were reported to have lower level of GLUT-1 and GLUT-3 expression, but higher expression of GLUT-4[128]. Mouse C2C12 cells compared to rat L6 cells, have special gene proteins like MYH1 and MYH4 which are involved with muscle development and contraction[129,130] . As C2C12 cells possess features (myosin content, glycogen content) that resemble the differentiated muscles tissue of human myotubes, this enables the use of such cell line for muscle contraction studies that are associated with exercise/ stress[128,131]. Lastly, mouse C2C12 possess the higher rate of oxygen consumption and respiration this enables them for more sustainability in research study when compared to rat L6 cell line[132]. Several drug and natural compounds were reported to influence glucose uptake and insulin sensitivity in C2C12 cells. Overall, insulin-responsive mouse C2C12 cell line is a useful model in investigation of insulin resistance.

Some other research studies have also conducted to understand glucose uptake study by using C2C12 cell lines. Manna P et al (2018)[133] reported that -GLUT-4 translocation and glucose uptake were increased when C2C12 myotubes were treated with either insulin (25 nM) alone or in combination with vitamin D supplement (50 nM). A synergistic glucose uptake effect was demonstrated when insulin was co-administered with vitamin D, thereby insulin-independent signalling pathway may be involved.

Yong Li , Ye Ding (2012)[134] demonstrated a facilitated effect of myricetin on glucose uptake in C2C12 myotubes under normal and insulin stimulated conditions . Furthermore, it was determined that myricetin was the only compound to stimulate lipogenesis and enhance insulin-stimulated lipogenesis among 30 bioflavonoids during a drug screening study. An insulinomimetic effect of myricetin on glucose transport in adipocytes of rats with non-insulin-dependent DM was also observed.

Therefore, the current studies are conducted with an objective to evaluate antidiabetic properties of synthetic novel flavonoid through the in vitro studies by examining the inhibitory activities against alfa-amylase, alfa-glucosidase, and DPP (IV) enzyme along with glucose uptake in C2C12 cell lines.

B. Animal models for T2DM :

Animal models are crucial for the discovery, validation, and optimization of novel medications and therapies as well as their safe use in humans. In experimental drug evaluation for T2D,M non mammalian models, models of large animals, models of non-human primates & rodent models are available currently(fig:3.18)[135].

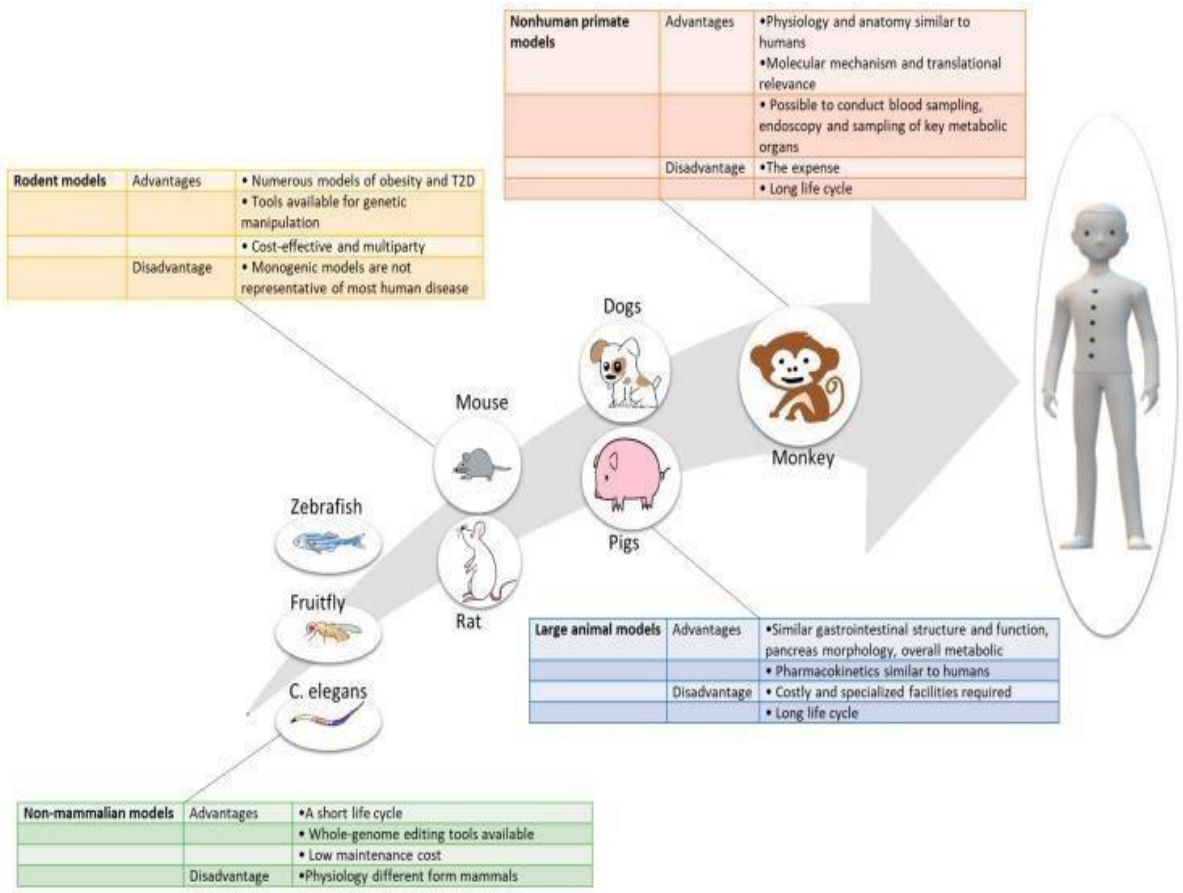


Figure 3.18: Major advantages and disadvantages of different classes of animal models used in T2DM research

a) Non-mammalian models: The worm C. elegans, the fruit fly Drosophila melanogaster, and the zebrafish Danio rerio are often used to develop non-mammalian models for T2 DM in laboratory settings. Non-mammalian models are advantageous because to their low maintenance costs, quick lifespans, and accessibility to long-term gene-editing technologies. However, because of the differences in their physiology from mammals, their translational value is constrained.

b) Models of large animals: Pigs and dogs serve as the main animal models for translational research of T2DM, and it has been shown [136] that dogs can induce diabetic mellitus with pancreatectomy, the administration of alloxan and/or streptozotocin (STZ), or both. The guinea pig is an excellent model for the study of T2DM because it has many similarities to humans, including the anatomy and function of the gastrointestinal tract, the growth and morphology of the pancreas, and general metabolic status. [137]. For the formation of T2DM in guinea pigs, high fat and cholesterol (HFC) diets with or without STZ have been modified [138–139.]

c) Models of non-human primates: The non-human primate model, which includes baboons (Papio species), African green monkeys (Chlorocebus species), cynomolgus monkeys (Macaca fascicularis), hesus macaques (Macaca mulatta), common marmosets, and cynomolgus monkeys, is appropriate for studying T2DM and being used in preclinical trials (Callithrix jacchus) [140]. Islet amyloidosis, IR to β--cell failure, and overt diabetes mellitus, which are all symptoms of T2DM in people, were also seen in diabetic monkeys, suggesting that the causes of islet lesions in both monkeys and people are the same.[141]. Studies in non-human monkey models represent a cost-effective way to research T2DM strategies given the high expense of human clinical trials. However, using nonhuman monkey models is seen to be

exceedingly expensive in biomedical research, and only a small number of labs are set up to handle such studies.

d) T2DM Rodent Models: The many accessible models of T2DM in rats[135] are listed in Table 3.2. The rodent model of T2DM is the most studied and trust worthy model for developing and testing new treatments/drugs for T2DM. The physiology of mice and rats is more similar to that of humans when compared to non-mammalian species because they are mammals.

Srain orMethod	**Species**	**Obesity**	**Hyperglycaemia**	**Insulin resistance**	**T2DM**
$Lep^{ob/ob}$	Mouse	++	+	++	+
$Lepr^{db/db}$	Mouse	++	+	++	
ZDF	Rat	–	+	++	+
As a **Monogenic**					
Polygenic					
C57BL/6J	Mouse	+	–	+	-
C57BL/6N	Mouse	–	+	++	-
$KK\text{-}A^{y}$	Mouse	+	+	++	+
NZO	Mouse	++	+	++	+
TALLYO/Jng	Mouse	+	+	++	+
KK-Ay	Mouse	–	+	++	+
Chemical-induced					
HFD/STZ	Mouse/Rat	+	+	+	+

Table3. 2: In Vivo Rodent Models of T2DM -, absent; +, mild; ++, severe; DIO, diet-induced obesity; HFD, high-fat diet. SD, standard diet; T2DM, Type 2 diabetes mellitus

Chemically-Induced of T2DM model

T2DM is characterised by obesity, IR, and abnormalities in pancreatic beta-cell mass and function, which leads to a disease where insulin's ability to regulate key aspects of glucose, lipid, and protein metabolism is compromised. A chemically produced animal model of diabetes is the high-fat diet/streptozotocin-treated (HFD/STZ) model. [142] In this paradigm, an HFD is used to induce hyperinsulinemia, IR, and/or glucose intolerance, which is followed by the intraperitoneal injection of a low dose (30–40 mg/kg) of STZ, which causes a significant loss in the mass of functional -cells. HFZ/STZ predicted to mirror the pathophysiology of T2DM, albeit on a shorter timescale than that of human disease, in [143–144].

This non-genetic model's main benefit is that it can be tailored to resemble the slow pathogenesis of T2DM that affects the majority of people. This pathogenesis includes the gradual progression from adult-onset DIO to glucose intolerance, IR, the ensuing compensatory insulin release, and finally STZ-induced partial -cell death. The HFD/STZ is an acceptable animal model of T2DM, mostly depicting the latter stage of the disease, depending on the quantity of residual -cell mass, despite its drawbacks and the great diversity of the high-fat feeding regimen and the STZ treatment [144-145].

Model with streptozotocin induction:

Streptozotocin [2-deoxy-2-(3-(methyl-3-nitrosoureido)-Dglucopyranose], an antibacterial and alkylating chemotherapeutic drug, is produced by the fungus Streptomycetes achromogenes. When administered intravenously or orally, it acts as a pancreatic b-cell cytotoxic agent to induce diabetes in mice. It enters pancreatic beta cells via the Gut-2 transporter and permits DNA to be alkylated there. This activates PARP, which causes NAD+ to be depleted, cellular ATP to be reduced, and insulin

synthesis to be inhibited. Additionally, it generates free radicals, which can harm DNA[146].It can result in either severe diabetes (blood glucose levels greater than 200/300 mg/dL) or mild diabetes (glycemia between 120 and 200/300 mg/dL), depending on the dosage, route of administration, and animal strain employed. For severe diabetes induction, STZ is administered intravenously or intraperitoneally at a dose of 40–50 mg/kg body weight throughout adulthood [147]. A single dosage of 45 mg/kg of freshly produced STZ (dissolved in Na+ citrate buffer pH 4.5) administered intraperitoneally (i.p.) every day for four weeks caused IR in SD rats.

Biological models (Gene mutation): Leptin ob/ob and Leptin db/db mouse, Zucker rat, Cohen rats, Goto Kakizaki (GK), New Zealand Obese (NZO), and glucose transporter (GLUT) [134] are among the genetic models of insulin resistance for the evaluation.

Combination of streptozotocin and a high-fat diet:

Even if normal circulating insulin concentrations as determined by IVIGTT are present in male SD rats, a low dose of STZ (35 mg/kg, ip) administered for two weeks after a high fat meal (58 percent of calories as fat) results in frank hyperglycemia. In rats, it was discovered that pioglitazone and glipizide were sensitive. Following low dose streptozotocin (30 mg/kg), 132 Wistar rats were fed a high sucrose-fat diet for 8 weeks that contained 52 percent sucrose, 24 percent fat, 18 percent protein, and 4.8 calories per gramme of chow. These rats exhibited reduced glucose tolerance, obesity, and other signs of insulin resistance. Additionally, they demonstrated that pioglitazone treatment increased the expression of the proteins GLUT4 and IRS-1, while no treatment decreased the expression of the proteins IRS-1 [148.]

- **Reed et al (2000)[145]** repoted that High fat diet fed-low dose streptozotocin induced experimental type 2 diabetes in rats is an ideal animal model as it closely resembles the clinical and metabolic characteristics of human type 2 diabetes and widely used for pharmacological screening.

- **Srinivasan et al (2005)[144]** The HFD-fed rats exhibited significant increase in body weight, basal plasma glucose (PGL), insulin (PI), triglycerides (PTG) and total cholesterol (PTC) levels as compared to NPD-fed control rats. Insulin-resistant HFD-fed rats developed frank hyperglycemia upon STZ(35mg/kg) injection that, however, caused only mild elevation in PGL in NPD-fed rats and significant reduction in plasma insulin level after STZ injection in HFD rats. In addition, the levels of PTG and PTC were further accentuated after STZ treatment in HFD-fed rats.

- **Pallavi Kanthe, Kusal K. Das et al (2017)[149]** Diabetes and dyslipidemia are two main factors which revolve around the pathophysiological effects of abnormal lipid levels and insulin resistance . High fat diet induces a positive fat balance due to the loss of adjustment between fat oxidation and consumption- weight gain. HFD is associated with insulin resistance and reduced insulin secretion by beta cells in the pancreas which may lead to altered glucose homeostasis

- Another study used a high-fat, high-fructose diet for eight weeks before administering a sub-diabetic dose of streptozotocin (35 mg/kg) (HFD-Fr-STZ). Obesity, hyperglycemia, insulin resistance, liver glycogen shortage, and dyslipidemia were all outcomes of the HFD-Fr-STZ diet. Additionally, there was a drop in adiponectin and an increase in serum TNF-a. The symptoms of HFD-Fr-STZ were reduced by mangiferin (20 mg/kg ip) treatment for 28 days starting one week after

STZ, and its activities were similar to those of the typical insulin sensitizer, rosiglitazone [150].

- **Asanaliyar & Nadig (2021)[151]** stated that low dose Streptozotocin (35mg/kg) in 12-week high fat diet-fed rats caused the pancreatic beta cell dysfunction, insulin resistance, hyperglycemia and dyslipidemia. And further stating that it was a working model for type 2 diabetes because standard drug pioglitazone ameliorated all the parameters. High fat diet fed-low dose streptozotocin induced experimental type 2 diabetes in rats is an ideal animal model as it closely resembles the clinical and metabolic characteristics of human type 2 diabetes and widely used for pharmacological screening. Moreover, abnormally high levels of free radicals and simultaneous defects in natural antioxidant defence mechanisms (enzymatic) can lead to the development of insulin resistance

In order to assess the synthetic flavonoid molecule's antidiabetic, antidyslipidemic, and antioxidant properties, we thought about using a combined model of streptozotocin and a high-fat diet. Before the animal investigation began, the potential antidiabetic action of synthetic flavonoids was assessed using in-vitro screening methods for the alpha amylase, alpha glucosidase, dipeptidil peptidase-4 inhibitory, and glucose absorption (in C2C12 cell lines) assays. Numerous oxidative free radicals, including superoxide, nitric oxide, and hydroxyl radicals, among others, have been shown in the literature to have a significant role in insulin resistance and diabetic complications in T2 diabetic mellitus patients. The evaluation of antioxidant property was also carried out along with the antidiabetic activity to assess the antidiabetic potential of synthetic flavonoid as novel antioxidant.

Thus, this work used *in-vitro* and *in-vivo* assay methods to examine the ability of synthetic flavonoids on diabetes screening models.

3.18 Review on Flavonoids with relation to Diabetes mellitus:

a) Chemical structure of Flavonoid:

Flavonoids have a basic skeleton of diphenylpropane (benzopyrone), namely, two benzene rings (ring A and B), linked by a three carbon chain that forms a closed pyran ring (heterocyclic ring containing oxygen, carbon and fusing with benzene A ring. Structurally they are denoted as - C6-C3-C6. In most cases, B ring is attached to position 2 of C ring, but it can also bind in position 3 or 4. The structural features of the ring B and the patterns of glycosylation and hydroxylation of the three rings, makes the flavonoids one of the larger and more diversified groups of phytochemicals with different pharmacological properties[152].Benzopyrones commonly occur in the nature as α or γ-benzopyrones (chapter – 3, Fig. 3.5), but all flavonoids of plant origin have γ- benzopyrone ring as basic moiety. The γ-benzopyrones comprise of various classes of compounds such as flavones, flavonols, flavonones, isoflavones and isoflavonones etc., all of which are collectively called as flavonoids. Naturally occurring coumarins have α-benzopyrone ring. Chemically, flavones are 2-phenyl-γ–benzopyrones, which are also referred to as 2-phenylchromen-4-one or 2-phenyl-1-benzopyran-4-one (chapter–3, Fig. 3.6). The α, β unsaturated ketone group in flavones is believed to be responsible for most of their biological activity.In plants, flavonoids play a vital role in protecting the plant against pathogenic organisms, especially the roots. They also give a bitter taste to the plant and thus protect the plants from threatening by higher animals. In flowers, they give special colour owing to their quinoid structure, which attracts pollinating insects[153].

Flavonoids from natural source have been studied extensively due to their wide distribution, relative low toxic profile and structural diversity.In 1930, a new substance was isolated from oranges, which was believed to belong to a new class of vitamins and was designated as 'vitamin P [154-156]. When it became clear that this substance was a flavonoid, a flurry of research began in an attempt to isolate various flavonoids from plants, along with some synthetic approaches to prepare analogues and to study the possible mechanisms by which they act. Later, in 1950, the term 'vitamin P' was recommended to discontinue[157]. Till now more than 9000 varieties of flavonoids have been identified.[158].

Natural and synthetic analogues of flavonoids and their various biological activities have been major areas of research in the last few decades.

b) Biological role of flavonoids/benzopyrones with special reference to diabetes

Several in-vivo and in-vitro animal evidencesdocumented the promising health promoting effects of dietary flavonoids on glucose homeostasis forthe eradication of diabetes and obesity. Flavonoids perform multiple functions such as carbohydratedigestion, adipose deposition, insulin release, and glucose uptake in insulin-responsive tissues viadifferent cell-signaling pathways [159].

According to Ansari, P et al (2022)[160] , quercetin a flavonoid, based on in-vitro and in-vivo study, improves oral glucose tolerance, as well as pancreatic cell function to secrete insulin. It inhibits the glucosidase and DPP-IV enzymes, which prolong the half-life of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP).

Na et al. (2011)[161], reported that 7 weeks of curcumin, a flavonoid administration in STZ rats fed a high-fat diet produced a decrease in fasting blood glucose and plasma lipid

levels, increased insulin levels and insulin sensitivity by stimulating phosphorylation of AMPK in skeletal muscle.

Very few synthetic benzopyrones are reported in literature. Some reports[162-163] are available but they are targeted for conditions other than diabetes. In 1990's, research by Vlahos et al. 1994[164] on flavonoids revealed that phosphatidylinositol 3-kinase (PI3K) was inhibited by quercetin with an IC50 of 3.8 μM. Various analogues of quercetin were subsequently synthesised and one compound, viz. 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (also known as 2-(4-morpholinyl)-8-phenylchromone, LY294002), was found to completely and specifically abolish PI3K activity (IC50 = 1.40 μM). Wortmannin and LY294002 were also reported to inhibit insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes [165] .The same molecule LY294002, according to Hazeki et al. 2006[166] revealed that, the iNOS and cytokines production in vitro mouse macrophage cell line RAW-264.7 were significantly reduced.

Quercetin was also found to possess affinity for aldose reductase enzyme, which plays a role in diabetes-related complications. Later, several derivatives of quercetin were synthesised in order to target this enzyme [167]. It was subsequently found that the 7-hydroxy-2-substituted-4-H-1-benzopyran-4-one derivatives were better inhibitors of aldose reductase enzyme[168]. In a study conducted by Shukla et al. 2004[169] and Cazarolli et al. 2006[170], the vanadium based flavonoid complexes were designed and studied for their hypoglycemic effect in diabetic rats.

Flavonoids offer multiple drug targets that are typical of metabolic disorders. PPAR modulation subsequently became a popular target for diabetes and metabolic disorders. It was found that the 7-hydroxy-benzopyran-4-one moiety (occurring in flavones, flavanones,

and isoflavones) is the key pharmacophore of these novel molecules, exhibiting similarity to the core structure of both fibrates and thiazolidinediones [171].

Interestingly, α-glucosidase was also targeted by benzopyran-4-one derivatives. A series of 3-[4-(phenylsulfonamido)benzoyl]-2H-1-benzopyran-2-one derivatives were synthesized and evaluated fer α-glucosidase inhibition. Most compounds showed good inhibitory activity with IC50 values ranging from 0.0645 μM to 26.746 μM. The compound 7-hydroxy-6-methoxy-3-[4-(4-methylphenylsulfonamido)-benzoyl]-2H-1-benzopyran-2-one was shown to be the most potent inhibitor[172]. Acording to Y. Nayak, V. Hillemane, V. Daroji et al 2014[173] it was reported that synthetic flavonoids like JY-1, JY-2, JY-3, and JY4 showed significant antidiabetic activity in nicotinamide-STZ-induced diabetes in rats.Quercetin, a natural product with benzopyrone ring, showed significant hypoglycemic activity comparable to glibenclamide. In treatment with Synthetic 3-hydroxyflavone analogues JY-1, JY-2, JY-3, and JY4, the FBG and insulin resistance was significantly alleviated as determined by OGTT, HOMA-IR, and ITT. There was significant normalisation ofliver antioxidant enzymes compared to diabetic rats indicating that all the synthesised benzopyrone analogues are beneficial in reducing oxidative stress and are on par with the standard quercetin and glibenclamide in experimental T2D. The decrease inAGEs and restoration of creatinine levels have clinical relevance in preventing the cardiovascular and renal complications of diabetes.

Natural benzopyrones (flavonoids) were extensively studied and reported in huge numbers. They are well known for their antioxidant activity. Many of these molecules have been reported to possess potent antidiabetic activity[174] (fig.3.19). Literature postulates that antidiabetic activity may be partly mediated through the antioxidant activity of flavonoids. Many studies have demonstrated the hypoglycemic effects of flavonoids in animal models

and in clinical trials. In the following pages, the antidiabetic activity of flavonoids and their molecular mechanism in glucose homeostasis are reviewed.

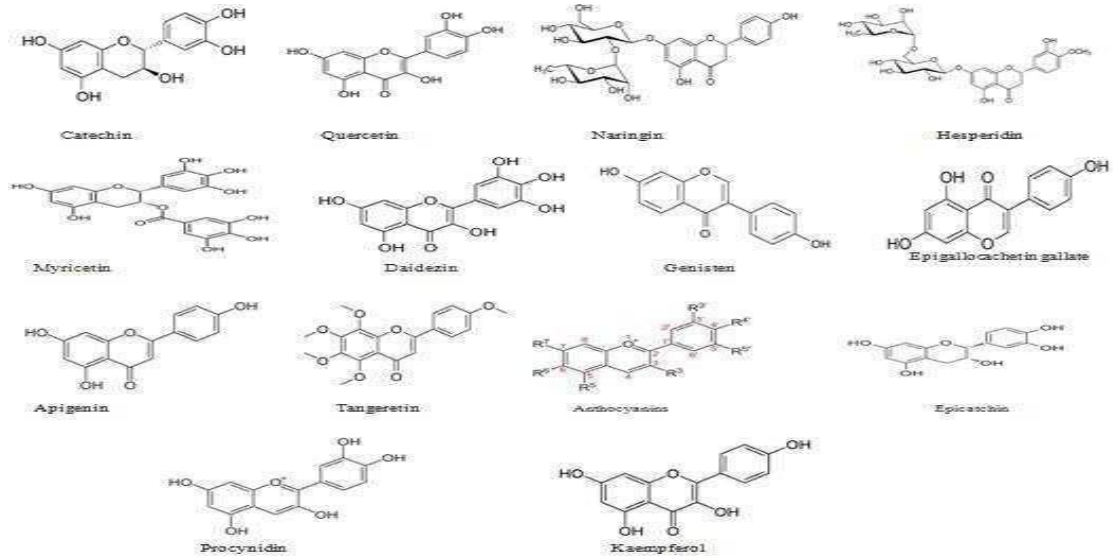


Fig: 3.19: Chemical structures of flavonoids having anti-diabetic properties

It has been observed that cocoa flavanols may promoteglucose homeostasis through mediating carbohydrate function in the gut[175] . Several studiessuggested the ameliorative effects of cocoa treated cells against apoptotic factors promote glucosesynthesis, activate insulin secretion and persuade cellular replication. Therefore, catechin enrichedcocoa flavanol increased glucose triggered insulin secretion, on the other hand, cultured cells treatedwith total cocoa extract or polymeric procyanidin-rich fractions did not show any positive effects atthe level of (0.75 –25 μg/mL)[176] . Ingestion of diet supplemented with 10% cocoa to Zuckerdiabetic fatty (ZDF) rats for 9 weeks declined hyperglycemia, promotes insulin sensitivity andenhanced cell mass function[177] .Moreover, increased glucose induced insulin secretion treated with catechin enriched fractionassociated with improved mitochondrial respiration. It is therefore, indicating the improvement inredox state, enhanced or declined glutathione and nuclear factor erythroid 2-related factor 2 (Nrf2) innucleus for transcription of targeted genes which are responsible for promoting mitochondrial functionand GABPA protein factors[176]. Epicatechin (EC) at the dose of 0.5–

10 μM down regulates theexpression of peroxisome proliferator-activated receptors (PPARs) and reduced DNA targeted bindingin 3T3-L1 adipocytes[178]. Moreover, EC also suppressed tumor necrosis factor (TNF) signalling which contributes in insulin resistance[178]. In-vitro **evidence of inclusion of kaempferol** at (10 μM)improved cell viability, decreased cell apoptosis, and declined caspase-3 activities in β cells andhuman islets exposed to hyperglycemic conditions. Such functions are belonging to enhancedexpression of anti-apoptotic serine/threonine-specific protein kinase (AKT) and B-cell lymphoma-2(Bcl2) proteins, increased cyclic adenosine 3,5-monophosphate (cAMP) signaling, and promotedsecretion and synthesis of insulin in β cells[179]. **Myricetin** treatment in diabetes triggered GLUT4expression[180] and enhanced the phosphorylation of AKT and insulin receptor substrate 1 (IRS1)[179].**Myricetin** at 0.12% supplementation to mice fed on high-fat high-sugar diet lead to reduce bodyweight and promoted hypercholesterolemia and hyper triglyceridemia[181] showing that myricetinmay improve insulin secretion and reduce diabetes and obesity. In 3T3-L1 adipocytes (a cell linederived from mouse), addition of naringenin suppressed glucose uptake[182]and decreased phosphoinositide 3-kinases (PI3K) and Akt phosphorylation naturally potentiated by insulin, hence, itregulating insulin-induced glucose transporter type-4 (GLUT-4) translocation[183]. Apart from that,naringenin also prohibited dyslipidemia and promoted glucose metabolism via reduced level of bloodglucose and lipids through independent channels via fibroblast growth factor-21 (FGR-21)[184].

Moreover, supplementation of pure synthetic daidzein to hamsters at (16 mg/kg bodyweight/day) significantly reduced blood glucose and plasma total cholesterol levels in response tocasein fed rats[185]. A recently investigation on mice revealed that daidzein from soysupplementation diet at 198 ppm and 286 ppm respectively from conception to adulthood increasedlipid peroxidation and glucose metabolism[186]. In addition, daidzein or

genistein supplementation at0.02% may decrease diabetes occurrences and augment glucose homeostasis via intactness ofpancreatic β-cell function in non-obese diabetic (NOD) mice[187].

Green tea has been observed to improve glucose metabolism in healthy humans in oral glucose tolerance tests. Green tea also lowered blood glucose levels in diabetic db/db mice and STZ-diabetic mice without affecting the serum insulin level[188]. Similarly, it improved glucose tolerance and reduced blood glucose levels in normal and alloxan-diabetic rats[189]. The procyanidins from grape seeds have demonstrated a significant ability to reduce glycemic levels together with an increase in insulin secretion,thus showing a supra-additive hypoglycemic effect in rats. There was also an increased expression of GLUT4 in plasma membranes of 3T3-L1 adipocytes when they were incubated with the procyanidins from grape seeds[190].

An isoflavonoid, puerarin has been reported to decrease the plasma glucose levels in normal and hyperglycemic rats[191] . Genistein, an isoflavone from soya, a strong antioxidant, inhibited glucose auto-oxidation-mediated atherogenic modification of low density lipoproteins (LDL)[192]. Chronic treatment with genistein and daidzein in db/db mice and STZ-diabetic rats also ameliorated diabetic conditions. Also, reports suggest that they prevent the onset of diabetes, elevating insulin levels and altering hepatic gluconeogenic and lipogenic enzyme activities in non-obese diabetic (NOD) mice[193]. Similarly, long term treatment with hesperidin and naringin was found to lower the blood glucose level of db/db mice[194]. Hesperidin glycosides were found to alter the expression of genes encoding PPAR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCoA), and LDL-receptor (LDL-R) in Goto-Kakizaki rats [195].

Naringenin, a flavonoid present in large quantities in the citrus fruits and juices, inhibited glucose absorption in the intestine [196]. Epicatechin gallate, myricetin, quercetin, apigenin, epigallocatechin and EGCG were shown to produce marked reduction in glucose absorption from the intestine by competitive inhibition of SGLT-1 [196-198]. Besides reducing glucose absorption, another possible mechanism that controls blood glucose levels is the inhibition of intestinal brush border enzymes such as α-glucosidase and α-amylase. Inhibitory effects on α-glucosidase activity were demonstrated when luteolin, kaempferol, chrysin and galangin were used, both in vitro and in vivo, to study the potential role in the absorption and metabolism of carbohydrates.

Some of the flavonoids have been studied for their effect on renal glucose reabsorption and excretion. Naringenin was shown to inhibit the glucose reabsorption in renal tubular brush border membrane vesicles[196]. Quercetin-fed diabetic rats ameliorate renal dissacharidase activity [199]. Homoisoflavanones isolated from Polygonatum odoratum rhizomes, namely, 3- (4'-hydroxybenzyl) -5,7 –dihydroxy-6 –methyl -8-methoxy- chroman-4-one, 3-(4'-hydroxybenzyl) -5,7-dihydroxy-6,8-dimethylchroman-4-one and 3-(4'-methoxybenzyl) -5,7-dihydroxy -6-methyl -8-methoxychroman-4-one inhibited in vitro AGEs formation more effectively than the positive control, aminoguanidine[200].

c) Molecular mechanism of benzopyrones for antidiabetic activity:

Genistein supplementation increases plasma insulin of the STZ diabetic rats[196] . In vitro studies showed that genistein can increase insulin secretion from mouse pancreatic islets in the presence of glucose by increasing intracellular cyclic adenosine monophosphate (cAMP). The increase in intracellular cAMP is probably the result of enhanced adenylate clyclase activity and activation of protein kinase A (PKA) by a mechanism which does not involve protein tyrosine kinase (PTK). This demonstrates that genistein directly acts on

pancreatic β-cells, leading to activation of the cAMP/PKA signaling cascade and exerts insulinotropic effect[196]. Recently, genistein was reported to induce pancreatic β-cell proliferation through activation of multiple signaling pathways and prevents insulin-deficient diabetes (type 1 diabetes) in mice. This could be because of subsequent phosphorylation of Erk1/2 in both INS1 cells and human islet β-cells. Further it was reported that genestein induced protein expression of cyclin D1, a major cell-cycle regulator essential for β-cell growth[201] . Similarly, genistein reduces the over secretion of extracellular matrix components and transforming growth factor-β (TGF-β) in high-glucose-cultured rat mesangial cells, showing that genistein could be lead molecule for treatment of diabetic nephropathy [202].

Dias et al. 2010[203] reported that quercetin treated normoglycemic and diabetic rats exhibited the increased proliferation of pancreatic islet cells.This effect may be due to increased DNA replication in β-cells. Dias et al. 2010 also found out that quercetin reduced the oxidative stress, abolished the iNOS over-expression in diabetic rat liver and modulates the IKK/ NF-κB signal transduction pathway. This might play a role in blocking the production of noxious mediators involved in the development of early diabetes tissue injury and the evolution of late complications. Quercetin was also found to be an allosteric ligand for GLP-1R. Synthetic flavonoids also have allosteric modulatory activity in Chinese-hamster ovary cells expressing the human GLP-1R. They have also reported that the 3-hydroxyl group on the flavone backbone, i.e. a flavonol, was essential for this activity[204]. Further, inhibition of 11β-HSD1 (another diabetes drug target) by quercetin plays a role in modulating insulin resistance [205]. Kaempferitrin (3,7-dirhamnoside of kaempferol), was reported to enhance the glucose uptake in rat soleus muscle[206]. Kaempferol 3-neohesperidoside showed insulinomimetic effects on the rat soleus muscle[207].

Natural products with γ-benzopyrone scaffolds (flavonoids) are explored greatly for treatment of diabetes and reported to act at different steps of glucose utilization. Their antioxidant potential and cell signalling modulation has shown additional advantage. In several studies it is reported that flavonoids have shown therapeutic potential in the management of diabetes and its complications. However, instability, insolubility and tedious & expensive isolation procedures of natural flavonoids has shifted the therapeutic focus on synthetic flavonoids[208- 209]

Since antidiabetic property of natural flavonoids are almost linked with antioxidant activity, the structurally similar synthetic compounds with slight modification of γ-benzopyrones of phenyl substitution at 2 position, offer a better lead molecules in this field[210.-212] Therefore we have selected γ-benzopyrone for structural modification to obtain better analogues.

In the present investigation, γ-benzopyrone with heteroaryl substituent such as thiophene was synthesised and evaluated for possible antioxidant and antidiabetic &antidyslipidemic activities in appropriate models.

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Chapter 4.

Material and Methods:

Chapter 4.

Material and Methods:

Synthesis of flavonoid (benzopyrone) analogue

Materials: The required chemicals and reagents were obtained from standard commercial sources unless they are indicated.Chemicals like 2-hydroxyacetophenone, 2- thiophene benzaldehyde were purchased from Sigma Aldrich, USA.Solvents such as acetone, dimethylsulfoxide (DMSO), dimethylformamide (DMF), ethyl acetate, absolute alcohol, n-hexane and methanol were of analytical grade and used as such without further purification. The test flavonoid compounds such as VMF41, VMF 43, VMF 45, VMF 46 whose synthesis & characterization study was completed elsewhere[61].Novel flavonoid (NF) was **synthesized** by using Algar-F- Oyamada method. Thin layer chromatography (TLC) was carried out on silica gel plates (Merck 60F) using ethyl acetate – n-hexane (0.5: 9.5) as solvents. Melting point of the synthesised compound was determined using Toshniwal capillary melting point apparatus and was uncorrected. All the glass apparatus used for the present work were procured from standard make (Borosil).

IR spectra of the test compounds were recorded as KBr pellets in the range of 4000 – 400 cm-1 using FT/IR-4700 type A (Shimadzu, Japan). The ^{1}H NMR and ^{13}C NMR spectra of the test compounds were recorded in CDCl3 or DMSO solution at 400 MHz on AMX-400 MHz High Resolution Multinuclear FT-NMR Spectrometer (Brukar) (at the department of chemistry, Shivaji University Kolhapur - India) with tetramethylsilane (TMS) as internal standard. Mass spectra of the test compounds were

recorded on GC-MS-QP5050A (Shimadzu) at quality assurance department, Manipal College of Pharmaceutical Sciences.

The stock solutions of test compounds were prepared by dissolving 10 mg of each in 10 ml of analytical grade DMSO (stock solution) and diluted with pre-saturated n-octanol. Further, 0.5 ml of stock solution was diluted to 50 ml with n-octanol pre-saturated to get a final concentration of each of the test compounds at 10 μg/ml.

Synthesis procedure of flavonoid/benzopyrone (Algar – Flynn – Oyamada synthesis)

By using Algar-F-Oyamada method[1-2], flavonoid containing benzopyran-4-one was synthesised by condensation and cyclisation reaction steps. In the condensation first step, chalcone was synthesised by condensing 2-hydroxyacetophenone with a 2-thiophen benzaldehyde in basic medium following Claisen-Schmidt condensation. In the second step, the synthesised chalcone was cyclised with an oxidizing agent to obtain a required flavone.

First step: In this chalcone intermediate was prepared by dissolving equimolar (5 mmol each/ batch) concentration of 2-hydroxyacetophenone with 2-thiophen benzaldehyde in ethanol fallowed by addition of 20% NaOH solution in conical flask. Then the reaction mixture was stirred by keeping the above reagents of conical flask on magnetic stirrer for more than 12 -14 hours. The progress and completion of reaction was monitored by TLC procedure. The mixture was then suspended in ice-cold water and the resulting solution was acidified with dilute hydrochloric acid. The product which precipitated was collected by filtration, washed with water and dried.

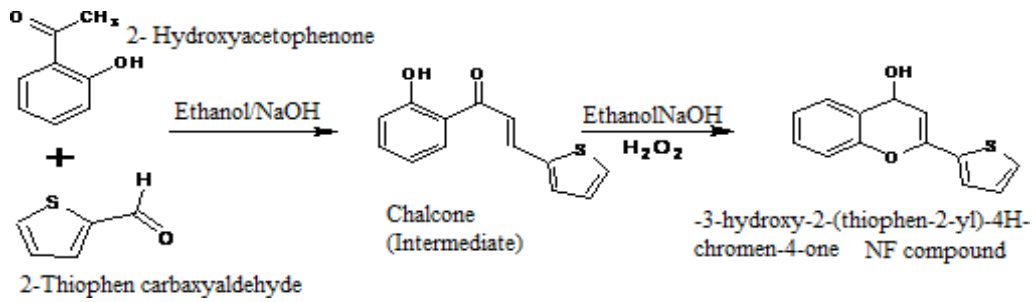


Fig: 4.1. Scheme for Synthesis and Structure of test novel flavonoid (NF)

Second step: Thus the obtained pure chalcone in ethanol was cyclised in presence of 30% pure hydrogen peroxide to give a novel flavonoid (NF Fig. 4.1) molecule. The reaction mixture was then, mixed with ice-cold water and acidified with dilute hydrochloric acid to precipitate flavone. The precipitate thus obtained was filtered, washed and dried. Final compound was purified by recrystallisation with ethanol and its purity was checked by TLC. The % yield was calculated. The structure (Fig.4.1) of the final compound was supported by spectral data such as IR, NMR and Mass spectra.

To sustain identity in the study, the synthesized compound was assigned the code as NF(Novel flavonoid). Along with this NF compound the other test flavonoid compounds such as VMF 41, VMF 43, VMF 45, VMF 46 whose synthesis & characterization study was completed elsewhere have also been selected for evaluation of invitro free radical scavenging activity by different assay methods.

***In-vitro* measurement of free radical scavenging activity of selected synthetic flavonoids (benzopyrones)**

Presently various methods have been used to screen natural or synthetic compounds for their antioxidant activity.The in vitro methods include conjugated DPPH, ABTS radical scavenging, diene assay, hydrogen atom transfer mechanisms such as for the inhibition of ROS and RNS scavenging assays which include superoxide

radical(O^-), hydroxyl radical, nitric oxide scavenging etc. Among these DPPH radical, scavenging method is by far the most frequently used, probably due to its simplicity in terms of time effort, experimental procedure and cheap reagents[3]. However O^-,OH^+& NO^- radical scavenging assays are infrequently used. The in vivo models include microsomal lipid peroxidation and erythrocyte ghost system.

The available information concerning the antioxidant activity of synthetic flavonoids is sparse and the novel flavonoid(NF) compound is first time reporting for its pharmacological activity in this study.

a) Super oxide anion radical scavenging activity [4,5,6]

Superoxide dismutase (SOD) is a metalloenzyme that catalyze the dismutation of superoxide radical into hydrogen peroxide (H2O2) and molecular oxygen (O2) and consequently provide an important defense mechanism against superoxide radical toxicity (Nishikimi et al., 1972). $O2^{*-}$ is one of the most important radicals produced inside human body. Hence, they bear resemblance to biological systems in contrast to DPPH or ABTS which are synthetic radicals

Principle;The principle involved in this assay is the conversion of Nitroblue Tetrazolium (NBT) into NBT diformazan via superoxide radical. Superoxide is generated according to the alkaline reaction as in DMSO(dimethyl sulfoxide) method. The reduction of Nitro Blue Tetrazolium (NBT) by superoxide was be determined in the presence and absence of the test compound. SOD utilizes the highly water-soluble tetrazolium salt and that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD.

At alkaline pH(7.4), the tetrazole ring is disrupted, leading to dismutationwhich

subsequently results in an intense blue insoluble diformazan product, but upon addition of the potential antioxidant compound which reacts with $O2^{*-}$ leading to no blue coloured formazan product . In absence of antioxidant intense blue colour will be observed as above. In the presence of compounds, a low absorption value indicates significant scavenging activity.

Reagent preparation

- 156µl NBT was prepared in 10ml of 100mM phosphate buffer (pH 8).
- 468µM NADH solution was prepared in 10ml of 100mM phosphate buffer (pH 8).
- 60µM PMS (Phenazine methosulfate) in 10ml of 100mM PO4 buffer pH-8.
- 10mg NF test compound in 0.1ml DMSO+ 0.9 ml PO4 buffer.

Working procedure: 1ml of NBT solution, 1ml of NADH solution, 0.1ml of test novel flavonoid (10mg in 0.1ml DMSO and 0.9ml PO4 buffer) and 0.1ml of PMS solution were added together and incubated at 25°C for 5 min. After 5 min the absorbance was read at 560 nm.

- Nitroblue tetrazolium (NBT;150 µM in PO4 buffer pH-8; 1ml) and Nicotinamide adenine dinucleotide (NADH; 234 µM PO4 buffer pH-8; 1ml) were mixed in a series of test tube.
- Various concentrations (10µg, 50µg, 100µg and 150µg) of samples and Ascorbic acid (AA) were added to these test tubes and made up to 3ml with Tris-HCl buffer (16mM; pH 8.0).
- Ascorbic Acid (AA) was used as reference standard for comparison.
- Phenazine methosulphate solution was added(40 µM; 1ml) to each test tube
- The reaction mixture was incubated for 5 min at room temperature.

- A control without the test compound was maintained.
- The absorbance of the samples was measured at 560 nm.
- Super oxide radical scavenging activity was calculated using the followingformula:

% SO radical scavenging activity = [(control OD - sample OD) ×100] / control OD

The percentage scavenging and IC50 values were calculated as given in this method and it was compared with the standard Ascorbic acid.

b) Hydroxyl radical scavenging activity [7-10]

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981).

Principle: HRS assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of test sample. The method used is ascorbic acid-iron-EDTA model of hydroxyl radical generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The addition of antioxidants to attack HO^{*-} radicals by donating an electron to the latter which can consequently quench the radical. Then a pink MDA-TBA chromogen product will not form as shown below.

Reagent preparation:

- Iron-EDTA solution was prepared by mixing 0.13% ferrous ammonium sulphate and 0.26% EDTA.
- 0.018% EDTA was prepared by dissolving 0.018g EDTA in 100ml dist. H2O.
- 1 ml of Dimethyl sulphoxide (0.85% v/v in 0.1 M phosphate buffer, pH 7.4)

- 0.22% ascorbic acid was prepared by dissolving 0.22g ascorbic acid in 100ml dist. H2O.
- 17.5% TCA was prepared by dissolving 17.5g TCA in 100ml of dist.H2O.
- Nash reagent was prepared by adding 15g ammonium acetate, 0.3ml of glacial acetic acid and 0.2ml of acetone to 100ml dist.H2O.

Working procedure :

- Various concentrations (10µg, 50µg, 100µg and 150µg) of samples in DMSO were taken in different test tubes and made up to 250µl with 0.1M phosphate buffer.
- One milliliter of iron-EDTA solution, 0.5 ml of EDTA (0.018%), and 1 ml of Dimethyl sulphoxide (0.85% v/v in 0.1M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. These reaction mixtures were incubated in a boiling water bath at 80 to 90°C for 15 min.
- The reaction terminated by the addition of 1ml of ice-cold TCA(17.5% w/v).
- Three milliliters of Nash reagent was added to all of the tubes and left at room temperature for 15 min for color development.
- The intensity of the yellow color formed was measured spectrophotometrically at 412 nm against reagent blank.
- Ascorbic acid (AA) was used as reference standard.
- The percentage hydroxyl radical scavenging activity was calculated by the following formula:
- % hydroxyl radical scavenging activity = {(Abs of control-Abs of sample) / Abs of control}X100

Where, HRSA is the Hydroxyl Radical Scavenging Activity, Abs control is the absorbance of control and Abs sample is the absorbance of the test flavonoid(NF)/Vit C. The percentage scavenging and IC50 values were calculated as given in this method and it was compared with the standard Ascorbic acid.

In the absence of the MDA-TBA chromogen, the colour of the solution remains pale yellow, indicating good antioxidant activity.

c) Nitric oxide (NO*) scavenging activity [11-12]

Low concentration of NO are sufficient in most cases to produce the physiological function. However chronic, high levels of nitric oxide (NO) radical exposure is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. Nitric oxide (NO) and reactive nitrogen species (RNS) are free radicals are derived from the interaction of NO with oxygen or reactive oxygen species.

Principle :Under aerobic conditions, NO^- can react with O2 to produce nitrate ($NO3^-$) and nitrite ($NO2^-$) as stable products that can be quantified using Griess reagent. To measure the NO^- radical scavenging activity, diazotization assay or Griess reaction was first developed in 1864 by German chemist Johann Peter Griess. Nitric oxide has been shown to be directly scavenged by flavonoids.

In this assay, using sodium nitroprusside (SNP) under acidic medium NO_2^- ion is made to react with sulfanilic acid(SA) to form diazoniumion ion which is subsequently coupled with N-(1-naphthyl) ethylenediamine (NED) to form to form a

water-soluble and red-coloured azo dye (HO3SC6H4-NN-C10H6NH2) that can be measured at a wavelength of ~540 nm.

Reagent preparation:

- Griess reagent : Was prepared by mixing 1 mL of 0.33% SA with 20% glacial acetic acid. It is allowed to react for 5 min at room temperature. Then, 1 mL of NED is added to the resulting acid. It is allowed to react for 5 min at room temperature. Then, 1 mL of NED is added to the resulting solution to form Griess reagent.

Working procedure:

- Various concentrations (10µg,50µg,100µg and 150µg) of samples and Butylated hydroxy anisole (BHA)dissolved in methanol/DMSO were taken in different test tubes and made up to 3ml with 0.1M phosphate buffer (pH 7.2).
- Sodium Nitroprusside (5mM) prepared in buffered saline (pH7.2) was added (1 ml) to each tube.
- The reaction mixture was incubated for 30 min at RT.
- A control without the test compound, but with an equivalent amount of methanol was maintained.
- After 30 min, 1.5 ml of above solution was mixed with 1.5 ml of Griess reagent
- The absorbance of the samples were measured at 546 nm.
- Butylated hydroxy anisole (BHA) was used as reference drug.
- Nitric oxide radical scavenging activity was calculated using the following formula:
- **% NO radical scavenging activity = (control OD - sample OD) ×100./ control OD.**

The percentage scavenging and IC50 values were calculated as given in the Griess reaction method and it was compared with the standard Butylated hydroxy anisole .

In -vitro antidiabetic activity of synthetic Novel flavonoid(NF).

In this study for in-vitro antidibetic evaluation of test sytnetic novel flavonoid, alfa-amylase, alfa-glucosidase, and DPP (IV) enzyme along with glucose uptake in C2C12 cell lines were performed.

a) Alpha-amylase inhibition assay[13-14]

Materials:

DNS solution: 1 g of DNS dissolved in 2N NaOH, 30 g of potassium sodium tartarate was added and whole volume was made up to 100ml.

Alfa amylase (Diastase procured from HiMedia, Mumbai, Cat No. RM 638).

Test samples dissolved in 5% DMSO and prepared stock solution as 1mg/ml concentration. In this study 200, 150 100, 50 and 10 µg of concentration of standard acarbose and novel flavonoids were used. Potato starch, TCA, Folin-Ciocalteau reagents were purchased from SD Fine Pvt. Ltd., Mumbai. All other chemicals used in the study were obtained commercially and were of analytical grade

Methodology: The inhibition assay was performed using the chromogenic DNSA method [Miller, 1959].

- 500 µl of test novel flavonoid and standard acarbose samples in DMSO at different concentrations (10,50, 100 and 150 µg/ml) were taken in separate test tubes.

- 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution was added to the above samples containing test tubes separately and incubated for 10 minutes at 25°C.
- After pre-incubation, 500 µl of 1% (w/v) starch solution in the above buffer was added to each tube and reaction mixtures were incubated at 37°C for 15 min.
- The reaction was terminated with addition of 1ml of DNSA colour reagent, placed in boiling water bath for 5 min, then cooled to room temperature and the absorbance was measured at 540 nm
- The control amylase represented 100% enzyme activity and did not contain any sample of analysis.
- Analysis of Acarbose as standard inhibitor: Acarbose was used as a standard inhibitor and it was assayed at above mentioned test sample concentrations. The assay method was similar to the above mentioned procedure, instead of test samples, acarbose was added. The results were compared to that of test sample

Calculation of 50% inhibitory concentration (IC50)

The concentration of the novel flavonoid(NF) required to scavenge 50% of the radicals (IC50) was calculated by using the percentage scavenging activities at three different concentrations of the test flavonoid. Percentage inhibition (I %) was calculated by I % = [(Ac-As)/ Ac] x 100. Where Ac = Absorbance of the control, As = Absorbance of the sample.

b) Alpha-glucosidase inhibition assay:

Principle: In type 2 DM, inhibition of α-glucosidase therapy is beneficial to delay absorption of glucose after a meal[15]. α-glucosidase plays a role in the conversion of

carbohydrates into glucose. By inhibiting α-glucosidase, glucose levels in the blood can be returned within normal limits [16].

Methodology: The assay of α-glucosidase inhibition activity was performed as described by Li et al., 2005 method[17].

1. ρ-Nitrophenyl-ρ-D-glucopyranoside (PNPG), was used as substrate and prepared by dissolving in 50 mM phosphate buffer (pH 6.5).
2. Test samples(including standard acarbose) were prepared at the concentration of 100, 200, 300, 400 and 500 µg/ml dilution method (in 5% DMSO).
3. 30 µL of each concentration was added with 36 µL phosphate buffer pH 6.8 and 17 µL p-nitrophenyl-α-D-glucopiranoside (5 mM) substrate.
4. The mixture solution was incubated for 5 min at 37°C. To this solution, 17 µL of α-glucosidase 0.15 unit/mL was added after the first incubation, then incubated again for 15 min at 37°C.
5. After the second incubation was finished, 100 µL of Na_2CO_3 267 mM was added into the solution to stop the enzymatic reaction.
6. Solution absorbance was measured with a microplate reader (BIOBASE) at 405 nm.
7. The blank solution was tested by adding Na_2CO_3 right after the first incubation and α-glucosidase after the second incubation. Acarbose was tested as positive control. The inhibition percentage was calculated by following formula.

$$\text{Inhibitory activity (\%)} = \frac{[(\text{OD control} - \text{OD blank}) - (\text{OD sample} - \text{OD sampleblank})]}{(\text{OD control} - \text{OD blank})} \times 100$$

The concentration of samples that inhibited α-glucosidase activity by 50% was also calculated in terms of IC50 value.

Control: DMSO, enzyme and substrate, **Blank:** DMSO

Positive control: NF is replaced by acarbose

The results obtained were tabulated in Table.2 and percentage enzyme inhibitory activity was expressed in graphical representation also (fig.2). The percentage of enzyme inhibition and the IC50 values of the test compounds for α- glucosidase inhibitory activity were also calculated.

c) Dipeptidyl peptidase inhibitory assay.

Materials : DPP-4 screening assay kit from Cayman Chemical (Michigan, USA), (Item no: 700210) was used to assess the test novel flavonoid's (NF) Dipeptidyl peptidase -4 enzyme inhibitory potential . Fluorometer with capacity to measure fluorescence using an excitation wavelength at 350-360nm and emission wavelength450-465nm.

Principle: In this assay to determine DPP-4 activity, the fluorescence substrate Gly-Pro-Aminomethylcoumarin(AMC) was used. Here DPP-4 enzyme by breaking peptide bond in substrate liberates the AMC moiety resulting in fluorescence that can be measured by using an excitation and emission wavelength of 350-360 and 450-465 respectively. In presence of DPP-4 inhibitors the less AMC product will be formed with low fluorescence which intern have less absorbance values indicating DPP-4 blocking property. This assay was performed according to the literature provided along with kit.

Reagent preparation:

1. DPP assay buffer: 3ml assay buffer was added to 27 ml of water and stored at -20^0 C refrigerators.
2. DPP IV h DNA r enzyme: This enzyme in vial was made thaw on ice, then 480 µl of diluted assay buffer was added and made gentle mix to prepare stock solution of diluted enzyme which will be stable for 2 hours on ice.
3. DPP substrate: 120 µl of DPP substrate (containing H-Gly-Pro conjugated with aminomethylcoumarin) was added with 2.88ml of diluted assay buffer and made vortex for proper dissolve.
4. Sitagliptin positive control inhibitor: suspended in 500 µl diluted assay buffer. The addition of 10µl to the assay yields a final concentration of 100 µM inhibitor in the well.

Methodolgy:

1. To the wells containing 10 µl of sitagliptin a positive control inhibitor and 10 µl of test sample NF compound in dimethylsulfoxide (DMSO) at varying concentrations (50, 100, 200, 400 & 800 µg/ml) separately, a 30 µl of diluted assay buffer and 10 µl of DPP (IV) enzyme solutions were added.
2. For 100% initial activity (Control) , a 30 µl of diluted assay buffer, 10 µl of DPP (IV) enzyme and 10 µl of solvent DMSO was taken in separate well.
3. Then the solutions were incubated for about 15 min at 37^0 C.
4. To initiate reaction, 50 µl of the diluted substrate substrate (containing H-Gly-Pro conjugated with aminomethylcoumarin) was added into the mixture of all the wells being used and was incubated for 30 min at 37^0 C.

5. Using an excitation wavelength of 365 nm and emission wavelength at 455nm the fluorescence reading was measured.

The percentage of inhibition was calculated as follows:

% inhibition= (Initial activity- A_{sample} / $A_{100\%\ initial\ activity}$) X 100

Where 100% initial activity is formed by the combination of assay buffer, DPP (IV) and DMSO sovent. Sitagliptin is used as a positive control in this assay. All samples are analysed in triplicate.

Statistical tests: By using Graphpad Prism 5(Demo) software, One way ANOVA followed by Dunnett's multiple comparison test was applied to observe the statistical significance.P value less than 0.05 was considered as statistically significant[18] The IC50 values of test compounds were evaluated from the dose-response curves of each experiment using Microsoft-Excel computer software.

d) In-vitro Glucose uptake study in C2C12 cell lines and estimation of glucose by DNS method[19].

Background

Glucose is a very important fuel source to generate universal energy molecules such as ATP. Glucose level is a key diagnostic parameter for many metabolic disorders. Measurement of glucose can be very important in both research and drug discovery processes. The present work was undertaken to study the effect of test samples on glucose uptake in cell lines like C2C12 cell lines. Glucose estimation by DNS method provides direct measurement of glucose in various biological samples such as, cell lysates, biological fluids and growth medium). The assay is also suitable for

monitoring glucose level during fermentation and glucose feeding in protein expression processes.

Materials: DMEM and FBS were purchased from Invitrogen. Metformin was a generous gift from Merck.

Cell culture

The skeletal muscle cell line C2C12 myoblasts were maintained in Dulbecco's modified Eagle's Medium (DMEM), DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml) in a humidified atmosphere with of 95% air and 5% CO2 at 37ºC until confluent. Differentiation into myotubes was performed as previously described by Kuang et al. 2009[19]. In brief, C2C12 cells were cultured until 75% confluence and differentiation medium (DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 mg/ml streptomycin) was then added and changed every other day. The cells were dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells was checked and centrifuged. Further cells were seeded in a 6-well plate at a density of 2 x 10^5 C2C12 cells/2 ml of media ([DMEM—high glucose(4.5mg/ml), 10% FBS, 1% penicillin/streptomycin, 1% l-glutamine (2 mM)] and incubated in a CO2 incubator overnight at 37°C. Experiments were conducted in well-differentiated C2C12 myotubes for 4 days cultured in differentiation medium.

Preparation of Test Solutions: For in vitro studies, each weighed NF (novel flavonoid) were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Cell viability of NF by MTT assay .

Principle: The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a purple coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The number of cells were found to be proportional to the extent of formazan production by the cells used[20]. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

Method/Procedure:

1. The cells were trypsinized and aspirated into a 15ml centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM medium, such that 200µl of suspension contained approximately 10,000 cells.
2. To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 24 h.
3. After 24 h, the spent medium was aspirated. 200µl of different test concentrations (200, 400, 600, 800 and 1000 µg/ml from stock) of test drugs were added to the respective wells. The plate was then incubated at 37ºC and 5% CO_2 atmosphere for 24 h.
4. The plate was removed from the incubator and the drug containing media was aspirated. 100µl of medium containing 10% MTT reagent was then added to each

well to get a final concentration of 0.5mg/ml and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 3 h.

5. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan.
6. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage cell viability was calculated,by using below formula, and concentration of test drug needed to inhibit cell growth by 50% (IC50) was generated from the dose-response curve for the cell line.

% Cell viable = (Mean OD of sample)/Mean OD of Blank) x 100

Results obtained from this study were tabulated in Table 5.9 and expressed in percentage of viable of C2C12 cells for test NF sample at different concentrations.

Glucose uptake assay of NF compound on C2C12 myoblast cells[21-22]

Glucose uptake activity of NF synthetic flavonoid was determined in differentiated C2C12 cells

. **Method/Procedure for glucose uptake**

1. The cultured C2C12 myoblasts in a 6-well plate at a density of 2 x 10^5cells/2 ml of media [DMEM—high glucose(4.5 mg/ml) supplemented with 10 % FBS, 2 mM glutamine, 2 % horse serum and 1 % antibiotics]were incubated in 5 % CO_2 at 37°C until reaching 90% confluence.
2. After 24 hours, the well differentiated myoblasts were treated with various concentrations of experimental compounds (NF at 20,40 and 80µg and Metformin at 100µg)except in control medium and incubated for 3 hours in 5 % CO_2 at 37°C.
3. At the end of the treatment, remove the medium from all the wells and add 200 µl trypsin and incubate at 37°C for 3-4 minutes.

4. Then 2 ml of culture medium was added and harvest the cells directly into in 12 x 75 mm tubes.
5. Centrifuge the tubes for five minutes at 300 x g at 25°C. Carefully aspirate the supernatant(Media) as set 1 and collect the cell pellet in separate tubes.
6. To the cell pellet add 1ml of 0.1% Sodium dodecyl sulfate(SDS) a detergent and heat it in water bath to lyse the cells.
7. Then centrifuge the cells again to remove the lysed cells and separate the supernatant (Cell lysate) as set 2
8. Both the sets are now taken as the tests samples for the estimation of glucose by DNS method against the standard curve of glucose.
9. The glucose levels (µg/ml) in cell lysates were measured using glucose assay kit. Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls

Glucose estimation Procedures:

Reagents Required

1. Glucose stock solution (100µg/ml)

2. Analytical reagents:

(a) 100 ml of DNS reagent: 1gm of DNS was dissolved in 20ml of NaOH (2M)

(b) 30g Sodium Potassium Tartarate was dissolved in 50ml Distilled water. Then this Sodium Potassium Tartarate solution was slowly poured into the above prepared DNS solution to made100 ml of stock DNS solution by adding distilled water.

Protocol for Estimation of glucose

1. Different dilutions of Glucose solutions were prepared by mixing stock Glucose solution (100ug/ ml) water in the test tube. The final volume in each of the test tubes was 2 ml. The Glucose range was made 5µg to 100µg/ ml.

2. From these different dilutions, pipette out different concentration of glucose solution to different test tubes and the reaction was terminated by adding 2ml of dinitrosalicylic acid (DNS) reagent.

3. The tubes were then incubated in boiling water for 5 min and cooled to room temperature and the absorbance was measured at 540 nm using a Spectrophotometer (Labman)

4. Absorbance values were plotted against glucose concentration to get a standard calibration curve.

In-vivo efficacy analysis of synthetic Novel flavonoid (NF) forantidiabetic, hypolipidemic and antioxidant activity.

A. Materials

a) study protocol Ethical Clearance and Experimental animals Used

The study protocol was approved by the institutional animal ethics committee (Ref. No:SNMC/IAEC/2018-19 dtd-25/06/18). In –bred Wistar Albino rats (150-200g) and Swiss Albino mice (20-25g) of either sex and of approximately the same age were used in this study. These animals maintained at the central animal facility of S.N.Medical college and HSK hospital Bagalkot in sanitized polypropylene cages with paddy husk bedding, were fed with standard pellet diet and tap water ad libitum. The room temperature was maintained at 23±2°C, 35–60% humidity, with 12 h light–dark cycle. All the animals were acclimatized to the laboratory conditions for one week before initiating the experimental protocol. Guidelines laid down by committee for the purpose of control and supervision of experimentation on animals (CPCSEA)[23] were fallowed while handling the experimental animals during the study.

Sample size: According to the equation reported in previous study[24] and as per schedule Y[25] in each group 4-6 animals were maintained in the study. Sample Size= DF/k +1, DF=Degree of Freedom(20-30) k=No. of groups.

b) Drugs and Chemicals: All the chemicals and reagents such as 2-Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), 5, 5'-Dithiobis-2- nitrobenzoic acid (DTNB), 1, 1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl or DPPH), Epinephrine, phenol reagent, cholesterol, streptozotocin(STZ) and Sodium nitroprusside (Qualigens.,Mumbai) were obtained from Sigma Aldrich USA.

Metformin was the procured from Micro Labs Ltd, Bangalore, India. Vanaspati ghee and coconut oil were obtained from local market. All other general chemicals were of laboratory grade and purchased from standard companies. The Novel flavonoid(NF) test compound was synthesized by condensation of 2 hydroxy acetophenone with thiophen carbaxaldehyde fallowed by cyclization reaction with hydrogen peroxide in alkaline medium[26]. The test NF compound and the standard (metformin) were suspended in 0.5%Tween 20 with normal saline solution before feeding to the animals**.**

B. Acute oral toxicity studies:

Swiss albino mice of either sex were used to determine acute oral toxicity of the test novel flavonoid (NF) compound. The OECD guideline 423[27] were used to perform acute toxicity study. A limit dose of 2000mg/kg b.wt, study was done as per the protocol OECD-423. Since The NF compound is new chemical entity and animal safety is not known, 175 mg/kg body weight was used for starting oral dose in this toxicity study.The dose progression factor 3.2 was used to get the next dose till reach the limit dose(2000mg/kg).

Procedure:

The animals were kept fasting for overnight and provide only with water, after which the NF in Tween 20 suspension were administrated orally(Fig: 4.2) one at a time to 3 different animals at 175mg/Kg body weight in 5% Tween 20 vehicle by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administrated was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 500, and 2000 mg/Kg body weight.

. These animals were observed individually over first one hour, then every 6 h, and periodically at every 24 h for any signs of acute toxicity over a period of 14 days. Changes in behavioral parameters such as drowsiness, restlessness, writhing, opisthotonos, catatonia, convulsions and symptoms of acute toxicity such as mortality etc were monitored and noted(fig:4.3).

Fig4.2: Oral administration of drugs in animals

Fig.4.3.Monitoring of animals during acute toxicity study

Thirty min before the test drug administration, individual animal weight was noted and once daily thereafter. Changes in weight were calculated and recorded. Food and

water intake changes were also recorded daily. At the end of the experiment animals were weighed and sacrificed by euthanasia through inhalation of ether over dose.

All test animals were subjected to gross necropsy. All gross pathological changes were recorded for each animal. The following organs (liver, kidney, spleen, heart, lung, and brain) were mopped and weighed separately. The organs were preserved in 10% formalin solution. Since gross observation at necropsy and macroscopic observations showed no evidence of toxicity, hence, only the liver organ was analysed for histopathology.

Procedure for histopathology slide preparation [28]

The procedure was carried out in department of Pathology S,N.M.C and HSK hospital & RC Bagalkot, under the supervision of Dr. S.V. Hiremath MD Prof & HOD pathology.

The preserved specimens were selected and cut into slices. The specimen slices were processed for a period of 12 hours in different solutions at different stages as follows- 80% Alcohol , 90 % Alcohol, 90 % Alcohol, Absolute alcohol (3 stages), Xylene (3 stages) and Paraffin wax (3 stages) respectively. Each specimen slices were then thoroughly infiltrated with wax to form a block.

The specimen was then very carefully orientated in an embedding mould fill with melted paraffin wax. A cassette was placed on top of the mould, topped up with more wax and placed on a cold plate to solidify.

5µ paraffin sections using a semi-automated microtome were made. Dewaxing of the sections were done by keeping it on hot plate. Sections were then stained automatically with haematoxylin and eosin stain (H&E). The stained section was then mounted on a clear glass slide and covered with a thin glass cover slip.

The slide was then viewed under photomicroscope and photographs of slide were taken. An experienced veterinary pathologist was consulted for opinion on the slides.

C. *In- vivo* antidiabetic anti dyslipidemic and antioxidant profiling of NF

a) Animals:

After institutional animal ethics committee approval(Ref. No:SNMC/IAEC/2018-19 dtd-25/06/18) of the study protocol, in-bred male healthy Wistar albino rats(150–200 g) of approximately the same age were used in this study. 7 days prior to experimentation, animals were acclimatized in S..N.M.C central animal facility Bagalkot and maintained at a room temperature of 22–24°C, humidity with a 12-hour light/dark cycle. During acclimatization, the animals were housed in polycarbonate cages with a standard pellet diet and tap water ad libitum. The acclimatised animals were grouped into normal and high fat diet group in where the normal group received normal chow diet and others received high-fat diet of Vanaspati ghee and coconut oil combination (3:1 v/v, 3ml/kg body weight orally per day) along with normal chow diet for 8 weeks. Throughout the study, the animals were cared for food, water intake, and clinical observations.

b) Preparation and composition of high fat diet(HFD)[29.-31.]

The high fat diet was prepared by mixing of Indian Vanaspati and coconut oil in the ratio of 3:1 v/v with 2 g of pure cholesterol powder for 100 ml of preparation and given 3ml/kg per oral/day to each rats in HFD groups for a period of 56 days This mixture of HFD would contain >20% of trans fatty acids and > 60% of saturated fatty acids in Indian Vanaspati ghee and only 9% of trans fatty acid & 90% of saturated fatty acids in coconut oil.

c) Preparation of Streptozotocin- Na citrate solution:

STZ is freely soluble in water and saline, but it is unstable in both. It is stable in 0.1 M citrate buffer (pH 4.5). A solution of STZ was prepared by dissolving weighed quantity of STZ in 0.1 M freshly prepared ice cold citrate buffer pH (4.5) and was administered i.p., in volumes of~2 ml/kg.

Buffering reagents and drug: Trisodium citrate dehydrate , Citric acid, distilled water, STZ distilled water.

Procedure:

Step1. To prepare 100ml of 0.1 Molar solution stock solution of trisodium citrate and citric acid, trisodium citrate dehydrate(2.942gm) and citric acid monohydrate(2.101gm) were dissolved in 100 ml of distilled water separately in separate flasks. Then 44.5ml of 0.1M citric acid monohydrate and 55.5ml of 0.1M trisodium citrate dehydrate were taken in separate flask mixed properly to get 100ml of trisodium citrate buffer solution (pH 4.5).

Step2. Accurately weighed STZ powder of appropriate strength (depending on the required total dose per animal) was transferred into a aluminium foil covered Eppendorf tube to protect from sunlight.

Step3. The contents of Eppendorf tube having STZ were poured into the beaker containing appropriate amount 0.1 Molar Na-citrate buffer, then placed in ice bath to make ice cold.

Step4. Final concentration of 10 mg/ml of STZ was prepared to use freshly intraperitonial administration in volumes of ~2 ml/kg in the study.

d) Test NF drug Dose selection:

Synthetic Novel flavonoid was assessed for acute oral toxicity study in Wistar albino mice according to ICH guidelines(OECD-425guidelines) and was found to be safe and well-tolerated up to the doses of 2000mg/kg and suggesting >2000mg/kg was its LD 50 value. There were no significant changes in body weight, food/water intake observed, and no clinical or histopathological abnormalities were seen during the study except few signs of hepatitis(175mg/kg),and focal fatty changes & glycogenesis features(500mg/kg and 2000mg/kg) on hepatocytes. Hence considering these acute toxicity results in view, this NF test drug was safe to administer up to 2000mg/kg limit dose and no additional information regarding this compound on animal study is available at present, the NF compound's $1/10^{th}$, $1/20^{th}$ and $1/40^{th}$ of safe limit doses that is 200mg,100mg and 50mg/kg body weight have been considered to use in further efficacy study.

e) Administration of drugs:

The 50, 100, 200 mg/kg doses of NF and 100/kg of standard drug (metformin) were prepared as a suspension using 5%Tween 20 gel in purified water as formulation vehicle and administered by oral gavages for 21 days. The normal control group received normal chow diet as regular as daily with 5ml /kg of vehicle(5% Tween 20) daily once where as HFD and HFD with diabetic group rats received high fat diet daily once through oral gavage in addition to regular normal chow diet.

f) Oral Glucose Tolerance Test (OGTT) in normal rats:

All animals were starved at water for 16 hours before treatment. OGTT for non diabetic rats was performed according to the standard method[32].

Study design for OGTT : The rats were divided into six groups comprising of six animals in each (n=6).

Group I stands for normal control group received 5% Tween 20 vehicle.

Group II received only oral glucose (2 gram/kg body weight)

Group III is treated with metformin in vehicle (100 mg/kg b. wt)+ oral glucose solution.

Group IV, V and VI received oral test NF drug in vehicle at 50,100 and 200mg/kg b. wt respectively

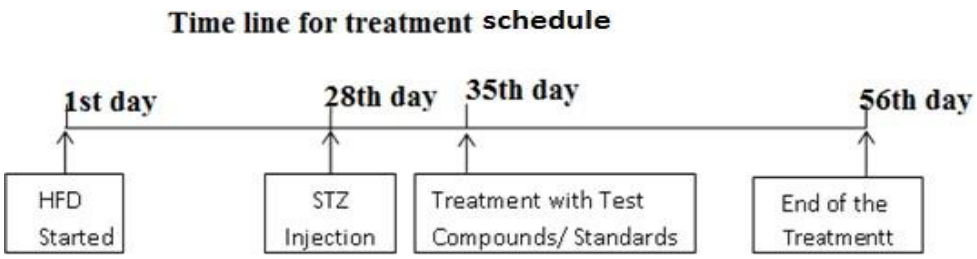
The rats of group III, IV,V and VI were loaded with glucose (2 g/kg, p.o.) 30 minutes after pretreatment with test drugs. Blood samples were collected from tail vein prick and measured at 0, 30, 60, 90 and 120 min after glucose oral load by using blood glucose test strips with glucometer, to evaluate the effect of above drugs on blood glucose levels o the glucose loaded animals.

g) Induction of diabetes and treatment in rats: Asanaliyar M(2021), Munshi R.(2014) and Srinivasan k, et al(2005) [30-33] method with slight modification was used to induce diabetes mellitus in experimentally selected Wistar albino rats allocated in high fat diet group.

Procedure: After 28 days of high fat diet feeding in all HFD group rats, a single dose of freshly prepared (within 15 min) solution of streptozotocin(STZ,35mg/kg from Sigma-Aldrich) dissolved in ice cold citrate(pH 4.5) was injected intraperitoneally, fallowed by overnight fasting. To prevent severe hypoglycaemia in STZ treated rats, 10% glucose and 1% saline in tap water on 1st day and 5% glucose and 1% saline in water for next two consecutive days were allowed drink[34] by replacing their routine water bottles.

After 7 days of STZ injection in these rats, the fasting blood glucose(FBG) was estimated through glucometer (Accu Chek Active) strips by drawing a blood from tail puncture with sterile needle. Rats exhibiting FBG values above 200 mg/dl were considered as diabetic to include in the study. Those rats failed to achieve FBG >200mg/dl, received one more dose of freshly prepared STZ to become diabetic.

Diabetic rats were randomized into different groups (n=6) and were further treated for 21 days according to the following protocol .After diabetic induction, the test NF with 3 different single oral doses (50,100 & 200mg/kg body weight) and metformin (100mg/kg.b.wt) single oral dose as standard drugs were given in experimentally designed rat groups for a period of 21 days during the study. The control rats were injected with an equivalent volume of vehicle (Tween 20 solution) at same time.



Fig; 4.4 Timeline for treatment schedule in rats

Fig; 4.5:**Fasting blood glucose determination through glucostrip.**

h) Experimental design: The animals were randomly divided in to7groups of six each as illustrated below (Fig :4.6).

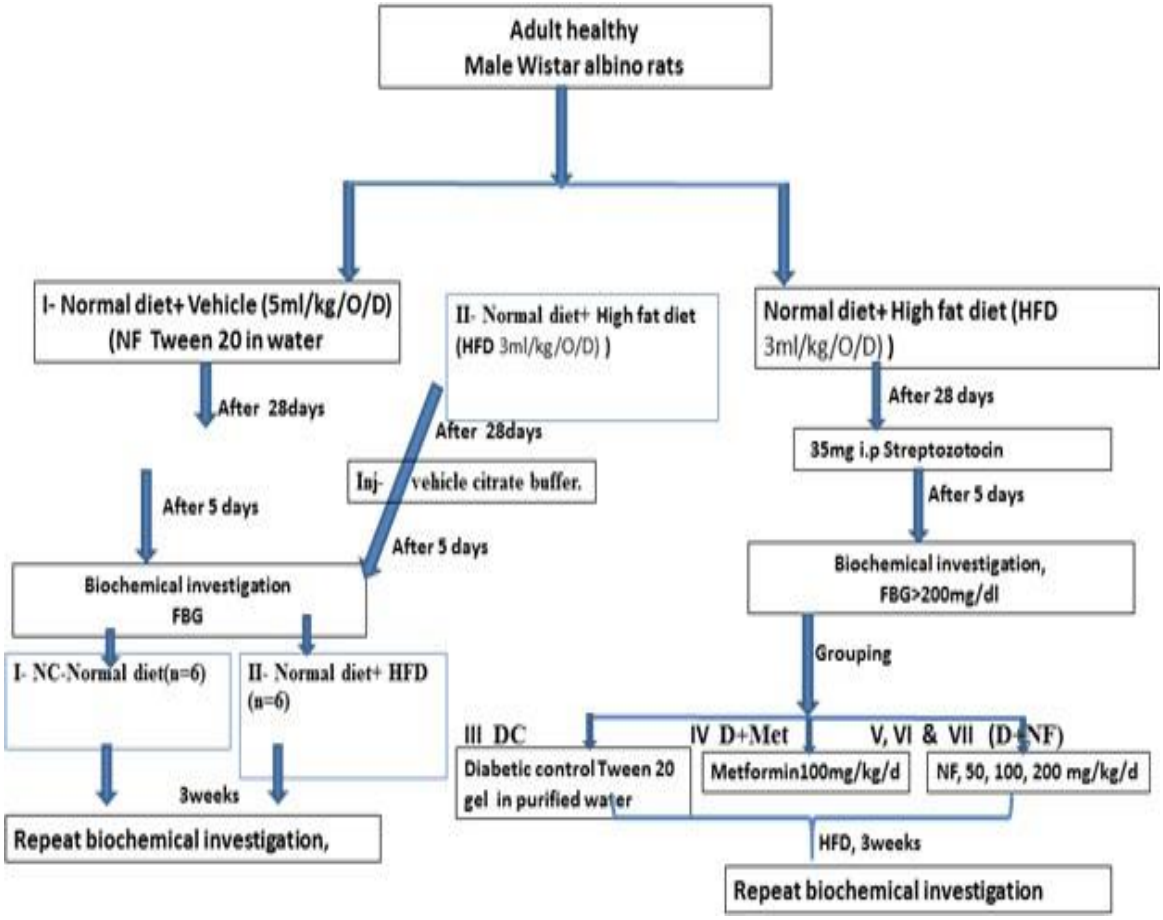


Fig 4.6: Pictorial presentation of animal grouping & Experimental study design

Experimental design protocol for antidiabetic efficacy study

Group 1(NC)- Normal control group received Tween20 as vehicle(5ml/kg) once daily p.o. for 8 weeks

Group 2(HF)-High fat group received HF diet(3ml/kg) once daily p.o for 8 weeks

Group 3(HF+D)-High fat + Diabetic group received HFD once daily for 8 weeks+STZ once on 28th day.

Group 4(HF+D+Met)-diabetic standard group received HFD for 8 weeks, STZ once on 28th day and metformin100mg p.o./day for 21 days.

Group 5 (HF+D+NF50), Group 6(HF+D+NF100) and Group 7(HF+D+NF200) were treated as **diabetic test groups** which received test flavonoid(NF) compound(after STZ induced diabetes) at low(50mg/kg), intermediate(100mg/kg) and high dose(200mg/kg) respectively once daily along with HFD for 21days.

All animals in different study groups were allowed free access to water and pellet diet and maintained at room temperature of 22-26^{0}C. Time line of Treatment schedule was mentained as shown in fig: 4.3 and fig: 4.6.

i) Collection of blood sample through retro -orbital puncture

The animals were observed for one week post diabetic induction for the stabilization of glucose levels. The blood samples were collected on the eighth day following STZ injection through retro- orbital puncture(Fig;4.7) for fasting blood glucose (FBG), serum insulin and lipid profiles.

Figure 4.7: **Blood sample collection through retro orbital-puncture**

j) General parameters observed:

For all rats, body weight was measured before and after the induction of diabetes. Fasting blood glucose level was measured on 1, 7, 14 and 21st day of the study period by tail tip needle prick method. At the end of the experiment, sufficient blood was collected by retro-orbital bleeding from all the animals under mild ether anaesthesia for estimation of haematological and biochemical parameters.

k) Blood / Serum and tissue fluid analysis for biochemical parameters:

The blood samples were centrifuged at 3000rpm for 5 minutes using REMI cooling centrifuge. The serum was kept at -80ºC until analyzed for biochemical parameters such as FBG, lipid profiles (TG,T-Chol,HDL, VLDL and LDL), fasting serum insulin and Glycated hemoglobin as below said methods .

i) Fasting blood glucose(FBG):

It was determined through one touch sugar scan glucometer (Thyrocare Pvt) by glucose oxidase methd[35]

Principle Glucose in sample is oxidized to yield gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a colored quinoneimine complex, with absorbance proportional to concentration of glucose in sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Gl Oxidase}} \text{Gluconic acid and } H_2O_2$$

$$H_2O_2 + \text{phenol} + 4\ \text{AAP} \xrightarrow{\text{Gl Peroxidase}} \text{Quinoneimine Dye} + 2H_2O$$

Reagents required:

Table 4.1. Reagents and their strength reqired in blood glucose estimation

Glucose oxidase	≥ 20000U/L
Peroxidase	≥ 2000 U/L
Phenol	10 mmol/L
Phosphate buffer	200 mmol/L
Glucose standard	100 mg/dl
4-aminoantipyrine	0.5 mmol/L

Procedure:

Blank: 1000µl of enzyme reagent

Standard: 1000 µl of enzyme reagent + 10 µl of standard

Test: 1000µl of enzyme reagent + 10 µl of sample were added in test tubes as mentioned

Mix well after each addition and incubate at 37ºC for 5 mins. Blank the analyser with reagent blank, followed by standard and test.

ii) Percentage reduction in glycemia[36]

% reduction in FBG was calculated with reference to day 7, according to the formula:

% Reduction in glycemia = [(FBG on day 7 – FBG on day 22) / FBG on day 1] x 100 mean of each group was taken for comparisons

iii) Estimation of Fasting Plasma Insulin

Fasting Plasma Insulin was estimated by ELISA using Rat Insulin ELISA kit (Catalog No: BYEK 2755, Lot No.: E23b/2019S Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

Principle of the Assay: The kit is based on sandwich enzyme-linked immune sorbent assay technique. 96 well plates precoated with anti-Insulin monoclonal antibody was used. The HRP conjugated anti-Insulin polyclonal antibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue colour product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the insulin amount of sample captured in plate. OD absorbance was read at 450nm in a microplate reader and then the concentration of insulin can be calculated.

- **Range**: 0μIU/ml-140μIU/ml, **Sensitivity**: <5μIU/ml
- **Kit components**:

1. One 96-well plate precoated with an anti-Rat Insulin antibody

2. Lyophilized insulin standards: 5 tubes (8μIU/ml, 16μIU/ml, 32μIU/ml, 80μIU/ml, 140μIU/ml), **3.** HRP conjugated anti-rat Insulin antibody, **4.** TMB substrate A

5. TMB substrate B, **6.** Stop solution, **7.** Wash buffer

Protocol: • Preparation of sample and reagents

1. *Sample*: Blood was collected in EDTA tube. Centrifuged for 30 min at 1000xg within 30 minutes of collection. Plasma was aliquoted and stored at -20^{0}C for further analysis.

2. *Wash buffer:* The concentrated wash buffer was diluted 25-fold (1:25) with distilled water.

3. *Standard:* Reconstitution of the lyophilized standards: The standards were first equilibrated to room temperature. 0.5ml of double distilled water was added into each vial of the corresponding standard and mixed thoroughly and kept at room temperature for 10 min for use.

- **Assay Procedure:**

1. Standard, test sample and control (zero) wells were set on the pre-coated plate respectively, and then, their positions were recorded. Each standard and sample was run in duplicate.
2. 50µl of 0µIU/ml, 2µIU/ml, 4µIU/ml, 8 µIU/ml, and 12µIU/ml of standard solutions were aliquoted into the standard wells. 50µl of sample diluent buffer was added into control well.
3. 50µl of the properly diluted sample (rat serum) was added into test sample wells.
4. 50µl of HRP conjugated anti-rat insulin antibody was added into above wells (except control wells) at the bottom of each well without touching the side well.
5. The plate was sealed with a cover and incubated at 37^0C for 60 min.
6. The cover was removed and the liquid of the wells discarded, the plate was clapped on the absorbent filter papers and not washed.
7. The plate was washed three times with wash buffer using manual washing. Each well was completely filled with wash buffer and mildly vortexed for 2 min on ELISA shaker. The contents were then aspirated from the plate and the plate was clapped on absorbent filter paper. The whole procedure was done 3 times.
8. 50µl of TMB substrate A was added into each well and then 50 µl of TMB substrate B was added. The plate was gently shaken by hand for 30 sec. the plate was covered and incubated in dark at 37^{0}C for 15 min. The shades of blue were observed in the first 3-4 wells with the highest concentration of rat insulin standard solutions, the other wells showed no obvious colour.
9. 50µl of stop solution was added into each well and mixed thoroughly. The colour changed to yellow immediately.

10. The OD absorbance was read at 450nm in a microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.) within 30 min after adding the stop solution.

Calculations

Relative O.D. $_{450}$= (O.D.$_{450}$ of each well) – (O.D.$_{450}$ of Zero well).

The standard curve was plotted(fig: 4.8) as the relative O.D.$_{450}$ of each standard solution (Y) vs. the respective concentration of the standard solutions (X) . The rat insulin concentration of the samples was interpolated from the standard curve.

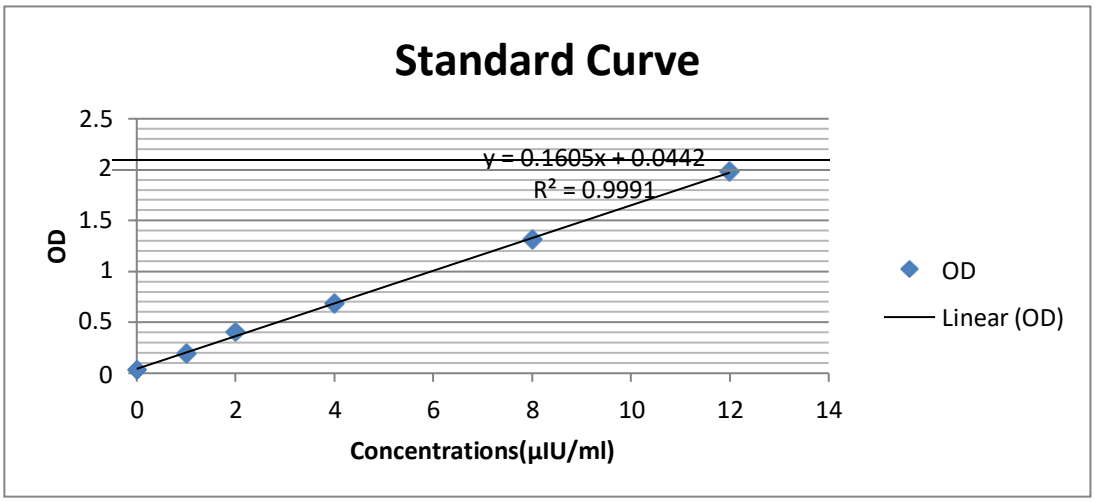


Fig. 4. 8. Standard calibration curve for insulin assay.

iv) Assessment of Insulin resistance & Beta cell function:

Homeostasis model assessment method (HOMA)[37] was used to determine the insulin resistance (IR) and β cell function by using following formula.

HOMA-IR = [fasting plasma glucose (mg/dL)×fasting plasma insulin (μIU/mL)] × (405) $^{-1}$

HOMA-B (β Cell function)=[360×Fastinginsulin(µIU/ml)]/[Fasting glucose (mg/dl) -63.

v) Determination of HbA1C[38] : Fasting Plasma HbA1C was determined by ELISA using Rat HbA1C ELISA kit (Catalog No: BYEK2229, Lot No: E 23 c /201 9S

Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

Test principle; This was based on standard sandwich enzyme-linked immune-sorbent assay technology. The purified anti- HBA1C antibody was pre-coated onto 96-well plates. And the Avidin-Horseradish Peroxidase(HRP) conjugated anti- HbA1Cantibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells subsequently, mixed and incubated, then, unbound conjugates were washed away with wash buffer. TMB substrates (A & B) were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the HbA1C amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of HbA1C can be calculated.

Procedure:

1. Equilibrate kit components for 15-30 min at room temperature.

2. Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions.

3. Add 50 µl of diluted standards (2400 nmol/L, 1600 nmol/L, 800 nmol/L, 400nmol/L, 200 nmol/L) into the standard wells. Add 50 µl of Standard diluent buffer into the control (zero) well. Do not add sample and HRP conjugated antibody into the control (zero) well.

3. For test sample wells, add 40µl of Sample diluent buffer first, then, add 10µl of sample. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.

4. Covered the plate with Plate sealer and incubated at 37℃ for 30 min.

5. Removed the sealer, and washed the plate using manual handling.

6. 50 µl of HRP conjugated anti- HbA1C antibody was added into each well (except control well).

7. Covered the plate with Plate sealer and incubated at 37℃ for 30 min.

8. Removed the sealer, and washed the plate as above.

9. 50 µl of TMB substrate A was added into each well, and then, 50µl of TMB substrate B was added into the above mixture, vortexed gently the plate or shake gently by hand for 30 seconds, and incubated in dark at 37℃ for 15 min. Observed the shades of blue in the wells.

10.By adding 50 µl of Stop solution into each well with proper mixing the raction was stopped . The color changes into yellow was noted.

11. Read the O.D. absorbance at 450nm in a microplate reader within 15 min after adding the stop solution.

Calculations

Relative O.D. $_{450}$= (O.D.$_{450}$ of each well) – (O.D.$_{450}$ of Zero well).

The standard curve was plotted (Fig:4.9) as the relative O.D.$_{450}$ of each standard solution (Y) vs. The respective concentration of the standard solutions (X).

The rat HbA1C concentration of the samples was interpolated from the standard curve.

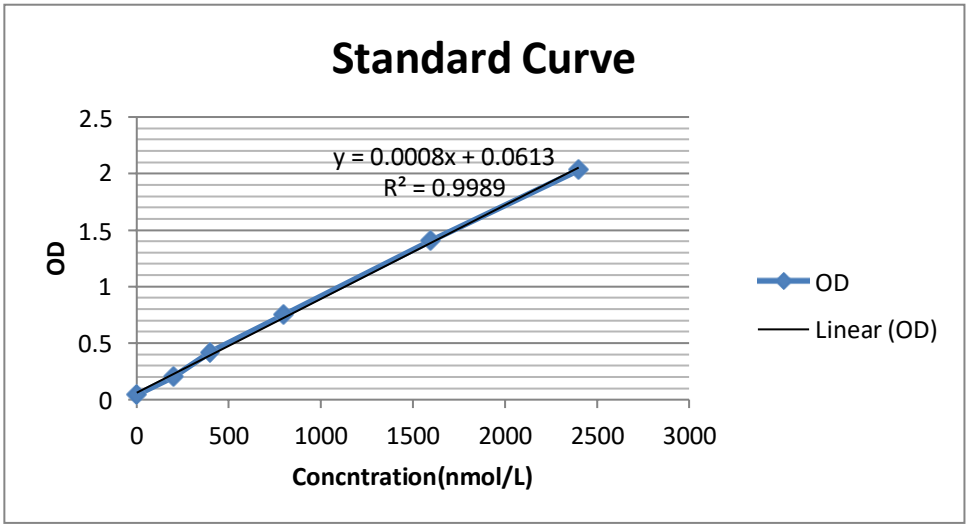


Fig. 4. 9. Standard calibration curve for HbA1C assay.

vi) Measurement of serum Lipid profile[39-42]

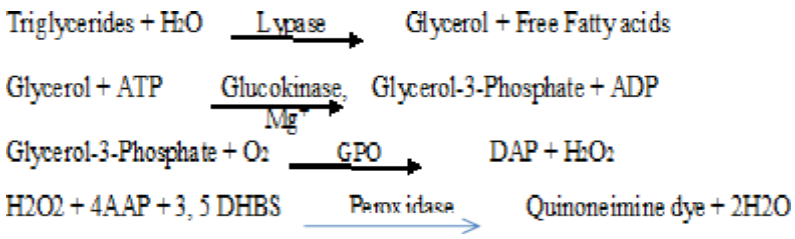
By using Biosystems Diagnostics kit manufacturer's instructions Serum triglycerides (TG) and Total cholesterol (TC) were estimated by enzymatic GPO-POD method[39] and CHOD-POD methods[40] respectively, HDL-cholesterol by phosphotungstate method described by Lopes et. al (1977).[41] and VLDL & LDL cholesterol were calculated by Friedewald's[42] formula

VLDL=TG(mg/dl)/5, LDL=TC-(HDL+VLDL).

Estimation of Triglycerides:

In human nutrition, triglyceride is the most prevalent glycerol esters encountered. They constitute 95% of tissue storage fat are the predominant form of glycerol ester found in plasma. The investigation of triglyceride is part of the overall evaluation of lipids disorders.

Principle: Lipase hydrolysis serum triglycerides to glycerol and fatty acids. The liberated glycerol is converted to glycerol-3-phosphate in the presence of ATP and glycerokinase. Glycerol-3- phosphate is oxidised by glycerol 3 phosphate oxidase to yield dihydroxyacetone phosphate and H2O2. H2O2 thus generated reacts with 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase to form coloured quinoneimine complex. The intensity of colour so developed is proportional to triglyceride concentration and is measured at 505 nm.



Reagent Composition:

Active Ingredient Concentration

- ATP -2.5 mmol/L, Mg^{+2} - 2.5 m mol/L, Aminoantipyrine -0.8 mmol/L

  3,5-DHBS -1mmol/L,Peroxidase ->2000U/L

  Glycerol Kinase ->550U/L, GPO - >8000U/L

  Lipoprotein Lipase ->3500U/L, Buffer (pH 7.0 + 0.1 at 20^0C)- 53mmol

Triglyceride Standard: 200 mg/dl (2.3mmol/L)

Reagent reconstitution: The reagent bottle and AQUA-4 (supplied in the kit) was allowed to attain room temperature (15-30^0C). The amount of AQUA-4 as indicated on the label, was added to the contents of each vial. Swirled to dissolve and allowed to stand for 10 minutes at room temperature.

Table4.2: Reagents prepared in TG estimation as blank, standard & Test

Procedure Pipette in to test tubes marked	Blank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	--	--
Standard	--	10 µl	--
Test	-	-	10 µl

Procedure

1ml of reagent mixed with 10µl of sample and incubated for 5min at 37ºC. Then the absorbance is measured at 505nm.

The absorbance of standard and each test was read at 505 nm on bichromatic analysers against reagent blank with UV visible spectrophotometer (Shimadzu, Model: UV 1800).

Calculations:

Triglycerides (mg/dl) = (Absorbance of test/Absorbance of standard) X Concentration of Standard(mg/dl)

Estimation of serum cholesterol: Serum cholesterol was estimated by cholesterol oxidase – peroxidase enzymatic method (CHOD-POD) (Allain *et al*., 1974) using a commercial Biosystems Diagnostics kit.

Principle : The cholesterol esters and cholesterol present in the sample are acted upon by Cholesterol Esterase to release Cholesterol and Fatty acids. The Cholesterol is oxidized by Cholesterol Oxidase to yield 4-Cholesterol 3-one and hydrogen peroxide as by product. Hydrogen peroxide together with 4-aminoantipyrin and phenolic compound in the presence of peroxidase gives the colored complex. The intensity of the colour is

proportional to the total cholesterol in the sample and is measured at 550nm or with Green filter.

Reagents

1. Reagents

• Good's buffer (50mmol/L), • Phenol (5mmol/L), • 4-aminoantipyrine (0.3mmol/L)

• Cholesterol esterase (>200U/L), • Cholesterol oxidase (>50U/L, • Peroxidase

(>3kU/L), 2. Cholesterol Standard : 200mg/dl.

Procedure: 1ml of enzyme reagent and 10µl of test or standard were mixed well and incubated at 37ºC for 5mins. The absorbance of test and standard were measured at 505nm or using Green filter.

Calculation - Cholesterol conc.mg/dl=

[Absorbance of test/ Absorbance of standard] X conc. of standard (200)

Estimation of HDL Direct

Principle: The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycolmethyl ether (PEGME) coupled classic precipitation method(Pisani et al., 1995) with the improvements in using optimized quantities of PVS/PEGME and selected detergents. LDL, VLDL and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and chylomicron by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzyme selectively reacts with HDL to produce H2O2 which is detected through a Trinder reaction.

Procedure

Step 1 : 200µl of serum and 300µl of HDL ppt reagent were mixed well and allowed to for 10mins. Then the mixture was centrifuged at 3000rpm for 10mins and the supernatant was separated.

Step 2 : 1ml of enzyme reagent and 100µl of supernatant from step A were mixed together and incubated for 5mins at 37ºC. The absorbance is read at 505nm

Calculation: HDL Cholesterol conc.mg/dl =

[Absorbance of test/ Absorption of Standard] X Concentration of Std(mg/dl)

LDL and VLDL levels were estimated by calculation using the Friedwald formula (Friedwald *et al*., 1972)

- LDL mg/dl = Total cholesterol-HDL cholesterol-Triglyceride/5
- VLDL = TG/5

vii) Effect of NF on antioxidant enzymes in liver homogenate:

At the end of drug treatment on 22nd day, all the group of rats were anaesthetized using diethyl ether, then liver was excised out and preserved in 10% formalin after thorough wash with 0.9% ice cold normal saline for further studies. The other part of liver was homogenized with 0.1% ice chilled phosphate buffer(pH 7.4) & at 10000 rpm, centrifuged for 10 min at 4°C to prepare 10% w/v liver homogenate sup ernatant. This homogenate was used for estimation of superoxide dismutase(SOD) catalase(CAT), reduced glutathione(GSH) and lipid peroxidise(TBARS) levels or m alondialdehyde (MDA).

The liver homogenate protein content was estimated according to the modified Lowry's method[43.] This assay is based on the principle that proteins react with Folin's reagent to give a coloured complex. The blue colour so formed is due to the

reaction of the alkaline copper-tartarate with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The concentration of antioxidant enzymes were expressed in terms of protein content in the liver homogenate .

- **Estimation of Superoxide dismutase (SOD)**[44] assay**:** The ability of superoxide dismutase inhibition on auto-oxidation of adrenaline to adrenochrome at alkaline media was used to assay the SOD activity[43]. Briefly, 25 µl of liver homogenate supernatant in carbonate buffer (pH 10.2) was added to 0.1 mM adrenaline and the increase in absorbance(adrenochrome formation) was measured at 480 nm using a UV–Visible double beam spectrophotometer. The activity of the enzyme has been expressed as U/mg protein, where 1U of the enzyme is defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50 % under the conditions of the assay.

- **Estimation of Catalase (CAT)**[45] activity**:** The Claiborne (1985)protocol was used to measure theCatalase activity . Briefly. The reaction mixture contains 1.95.0 ml of H_2O_2 (10mM), in 60 mM phosphate buffer (pH 7.0). By addition of 0.05 ml of homogenate (10% w/v) to above mixture, reaction was initiated. Changes in absorbance were recorded at 240 nm.The extinction coefficient of 0.04 mM^{-1} cm^{-1} was used to determine the specific activity of catalase.A unit of catalase is defined as the quantity, which decomposes 1.0 µmol of H_2O_2 per min at pH=7.0 at 25° C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM. Catalase activity was calculated in terms of units/mg protein.

- **Estimation of Glutathione (GSH)**[46] activity**:** It is based on the reaction between glutathione remaining after action of GSH-Px and dithiobisnitrobenzoic acid (DTNB) to form a complex that absorbs maximally at 412nmThe liver homogenate

after precipitation of proteins with 10% TCA, centrifuged to get supernatant. Then 1 ml of the supernatant was mixed with 6ml of 0.2 M phosphate buffer (pH 8.0) and 1ml 0.6 mM DTNB. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks in spectrometer. The reduced glutathione content was calculated by using the standard plot under same experimental conditions.

- **Lipid peroxidation (LPO) assay activity:** Since malondialdehyde(MDA) is the important oxidative cytotoxic product generated during lipid peroxidation of cellular oxidative reactions, MDA estimation is widely preferred to measure the oxidative stress effects on lipids. TBA can react with MDA and form thiobarbituric acid reactive substances (TBARS) .

In this study, Thiobarbituric acid (TBA) method[46-47] was used to estimate the amount of MDA which is the important cytotoxic by-product of this lipid peroxidation reaction. Briefly, 1 ml of 10% liver homogenate was mixed with 0.5ml of each 15% of TCA and 0.375% of TBA in 5NHCl reagent. The solution was heated at 95°C in water bath for 15 min, then cooled, centrifuged to form supernatant and its absorbance was measured at 532 nm against appropriate blankthat contains all the reagents minus the liver homogenate. The amount of lipid peroxidation was determined byε= 1.56 x 105 M-1 cm-1) and expressed as TBARS (n moles per g of tissue) [48].

l) Histopathological Study[49]

Liver tissues were preserved in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 μ in thickness was cut and stained with haematoxylin and eosin dye, were observed microscopically for histopathological changes.

m) Statistical analysis.

In this study, all the data were presented as Mean± SEM, and results were analysed by one way ANOVA followed by Dunnetts multiple comparison statistical tests using GraphPad Prism 5.0.Statistical significance was considered when a *p* value showed <0.05.

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CHAPTER-5. RESULTS

Chapter-5. Results

Results of general, spectral characterization study of synthesized test drug & interpretation.

Genaral properties of newly synthesized compound 3-hydroxy-2-(thiophene-2yl)-4H-chromen-4-one for the pharmacological activity.

The synthesized compound was appears to be pale yellowish colored crystals soluble in acetone, methanol, ethyl acetate,n-hexane and DMSO. After recrystallization from methanol its yield was around 67%. Its physical properties were as TLC: 0.64{n-haxane;ethyl acetate solvent(9.5:0.5)}; Melting point =196-199ºC; Molecular Formula = $C_{13}H_8O_3S$, Molecular Weight = 244.26582, Elemental analysis= C(63.92%) H(3.30%) O(19.65%) S(13.13%).This novel flavonoid (NF) substitution on nucleus, molecular formulae and other properties ae listed in table5.1. Table 5.2 explains the other test flavonids(VMF41,VMF43,VMF45 and VMF46) general properties which were used for radical scavenging activity comparision with NF compound.

Table.5.1.Different substitution pattern on ring A and B of the synthesized compound along with physico-chemical properties.

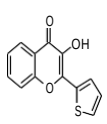
Code	R – H	Elemental analysis	Molecular formula &structure	IUPAC Name	MW	% yield	Melting point (^{0} C)	Rf* Value
NF	R^1–H R^2–H R^3-H R^4-H	C(63.92%) H(3.30%) O(19.65%) S(13.13%)	$C_{13}H_8O_3S$ 	3-hydroxy-2-thiophen-2-yl-4H-chromen-4-one	244	67	196-199	0.63

Table 5.2. The structure, chemical name and physical data of the other benzopyrones (2thiophene-2yl- 3hydroxy-4H-chromen-4-one) VMF41.43,45 and 46 were listed in the below table.

Sl.No	Code	Molecular formula	IUPAC Name	MW	% yield	Melting point (^{0} C)	Rf Value
1	VMF41	$C_{14}H_{10}O_3S$	3-hydroxy-2-(5-methylthiophene-2-yl)-4H-chromen-4-one	258	65	188-195	0.40
2	VMF43	$C_{15}H_{12}O_3S$	3-hydroxy 7-methyl-2-(5-methylthiophene-2-yl)-4H-chromen-4-one	272	50	191-194	0.43
3	VMF45	$C_{15}H_{12}O_3S$	3-hydroxy-6-methyl-2-(5-methylthiophen-2-yl)-4H-chromen-4-one	272	50	198-201	0.35
4	VMF46	$C_{14}H_{10}O_4S$	3-hydroxy-7-methoxy-2-thiophen-2-yl-4H-chromen-4-one	274	45	223-226	0.50

- **Spectral characterization of NF compound:** After synthesis and purification, the test novel flavonoid was assessed for FT-IR(fig-5.1), ^{1}HNMR(fig-5.2) ^{13}CNMR(fig5.3-) and Mass spectral(fig-5.4) characterization. The different chemical shifts/ peaks observed in the above spectral study results were as below.

IR (KBr) 3227, (p, OH), 1610 (C=O), 1560, 1479, 1346, 1294,1120 –(H of Ar)

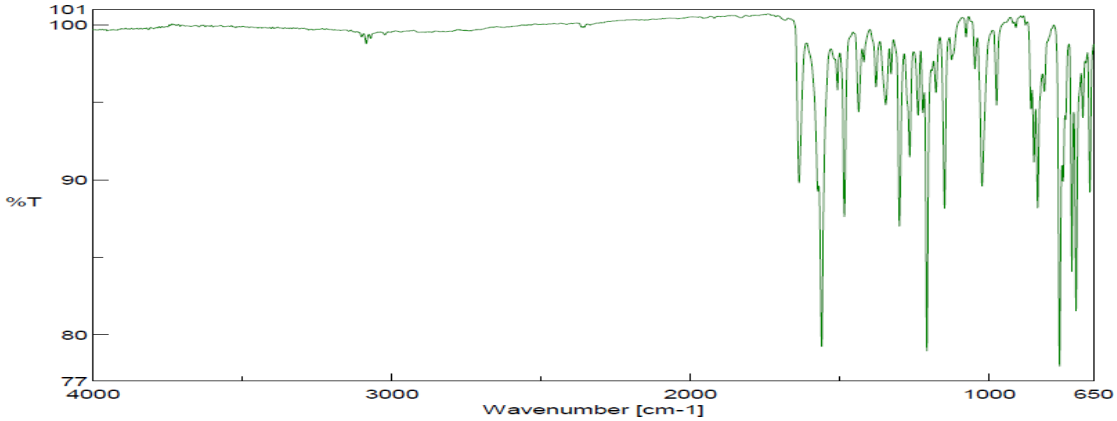


Fig.5.1 FT-IR study of NF compound

IR (KBr) : IR spectrum of the test compound NF exhibited prominent peaks at 3227 which indicates the presence of phenolic hydroxyl group, at 1610 indicating the presence of α and β unsaturated ketone(C=O) and other peaks at 1560, 1479, 1346, 1294,1120 cm^{-1} suggesting the presence of proton of aromatic rings.

^{1}HNMR (400 MHz, $CDCl_3$) δ 12.37 (1H s OH) and 6.98 – 8.10 (m H of Ar)

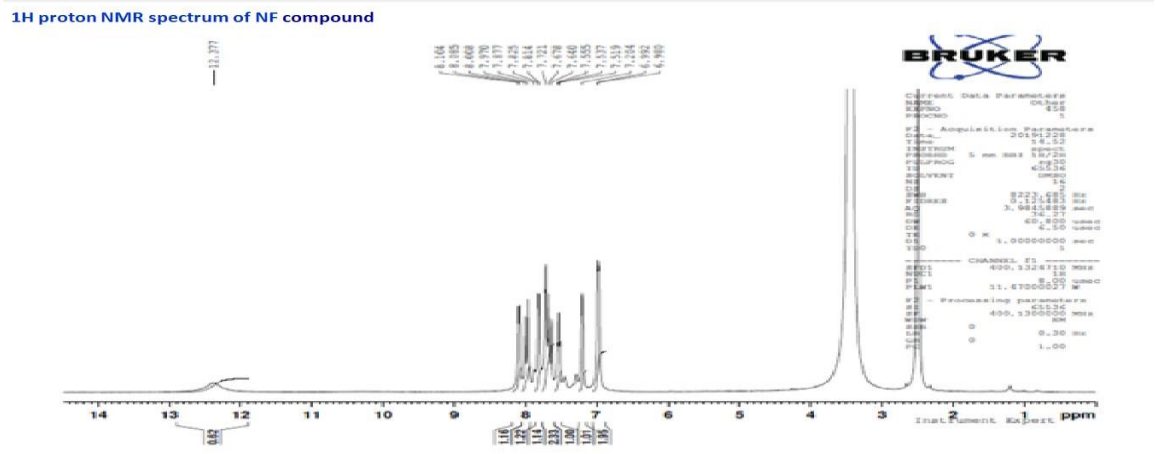


Fig.5.2. ^{1}H (Proton) NMRstudy of NF compound.

[1]H(Proton) NMR study

1) The given [1]HNMR reveals that the presence of proton of phenol group (*s* at 12.37*) and* peaks between 6.98 - 8.10 ppm as multiplets which indicates the presence of aromatic protons(benzene ring)

[13] C NMR (400 MHz, CDCl3) δ 193.17;(C s, C=O) ; and 129-140 ; (C m, of Ar).

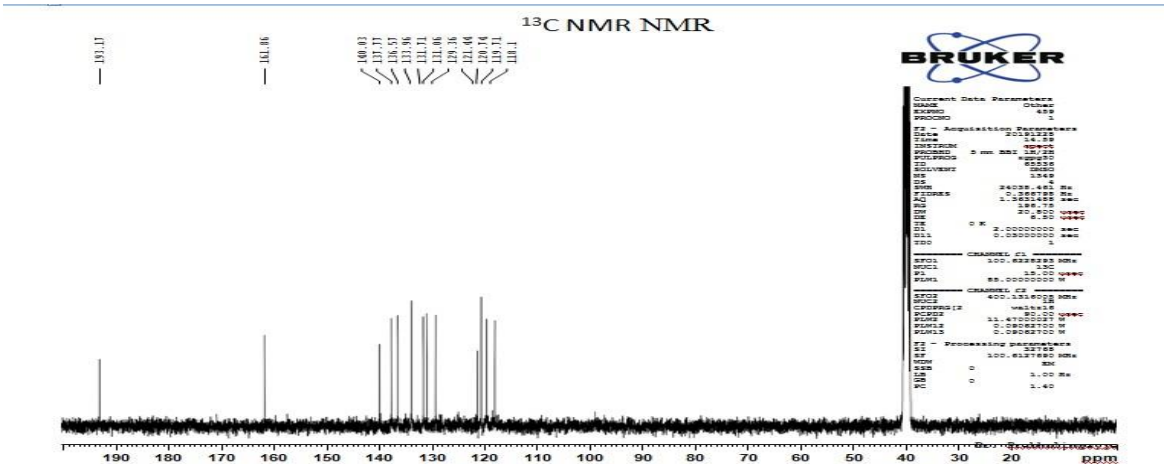


Fig.5.3. [13] C NMRstudy of NF compound

- The given [13C] NMR, peaks at 193.17ppm corresponds to the carbon of α and β unsaturated ketone(C=O) , and multiplet peaks from 129-140ppm represents the carbon of aromatic ring

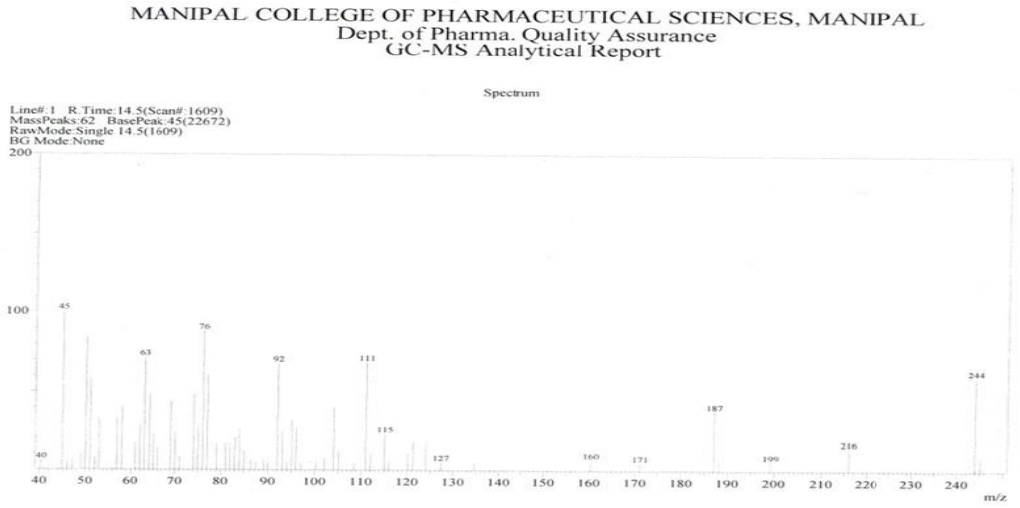


Fig.5.4. Mass Spectralstudy of NF compound

Mass spectral study:

In this spectral study, the compound shows peaks up to 244.26 Da which suggests that the NF compound probably has relative molecular weight of 244.

Mass: *m/z,* 244.26 $(M)^+$.

Results of free radical scavenging activity:

The results of free radical scavenging antioxidant values expressed as percentage inhibition and IC_{50} with different free radicals are shown in fallowing tables 4.1-4.3 and same is represented in graphs 4.1-4.3.

1) Superoxide radical scavenging:

It was observed that the test compounds showed a promising free radical scavenging activity in a dose dependent manner. As shown in Table 5.3, IC50 values of test synthetic flavonoids (SFs) VMF 41, VMF 43, VMF 45, VMF 46 and NF on superoxide radical scavenging activity were 3.44±0.36, 4.52±0.36, 7.76±0.22, 7.49±0.31& 2.32±0.35 respectively. It suggests that NF compound exhibited highest and statistically significant free radical scavenging activity and it is relatively compared with standard ascorbic acid .

Table5.3. Percentage Super oxide scavenging activity of synthetic flavonoids at different concentration with their IC_{50} values

Concentrations	Test Samples and their percentage scavenging activity					
	VMF 41	VMF 43	VMF 45	VMF 46	**NF**	**Ascorbic acid**
10µg	0.8	0.8	1.6	0	36.32	37.36
50µg	24	18.4	8	9.6	45.04	46.88
100µg	40	28	16	15.2	57.76	59.12
IC50	3.44±0.36	4.52±0.36	7.76±0.22	7.49±0.31	2.32±0.35	2.2±0.62

All the values in the table were presented as mean ± SEM(n=3).Data are represented in percentage super oxide radical inhibition

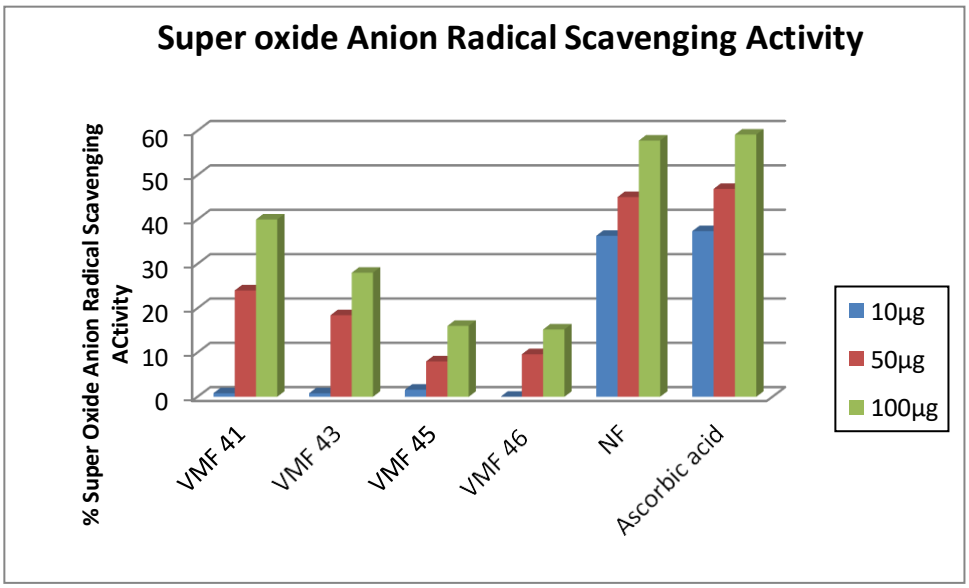


Fig5.5; Effect of synthetic flavonoids on Super oxide scavenging activity

2) **Hydroxyl radical scavenging:** Results were tabulated in Table-5.4. Here the SFs VMF 41, VMF 43, VMF 45, VMF 46 and NF compounds showed a dose dependent hydroxyl radical scavenging activity when tested at 10µg 50 µg and 100µg were tested. Among these VMF 46 and NF compound resulted statistically significant

activity when compared to reference drug. As they have IC 50 values 2.06 ±2.81and 2.55 ±0.82 respectively which are closer to potent standard ascorbic acid.

Table 5.4. Percentage Hydroxyl radical scavenging activity of syntheticflavonoids at different concentration with their IC_{50} values.

Concentrations	Test Samples and their percentage scavenging activity					
	VMF 41	VMF 43	VMF 45	VMF 46	**NF**	**Ascorbic acid**
10µg	8.33	5	4	9	7.67	48.02
50µg	16.17	12.17	19.00	24.67	40.17	69.84
100µg	33.33	41.67	29.50	46.83	59.50	94.84
IC50	4.46±3.27	3.66±0.97	4.55±1.02	2.06±2.81	2.55±0.82	1.11±0.13

All the values in the table were presented as mean ± SEM(n=3).Data are represented in percentage hydroxyl radical inhibition..

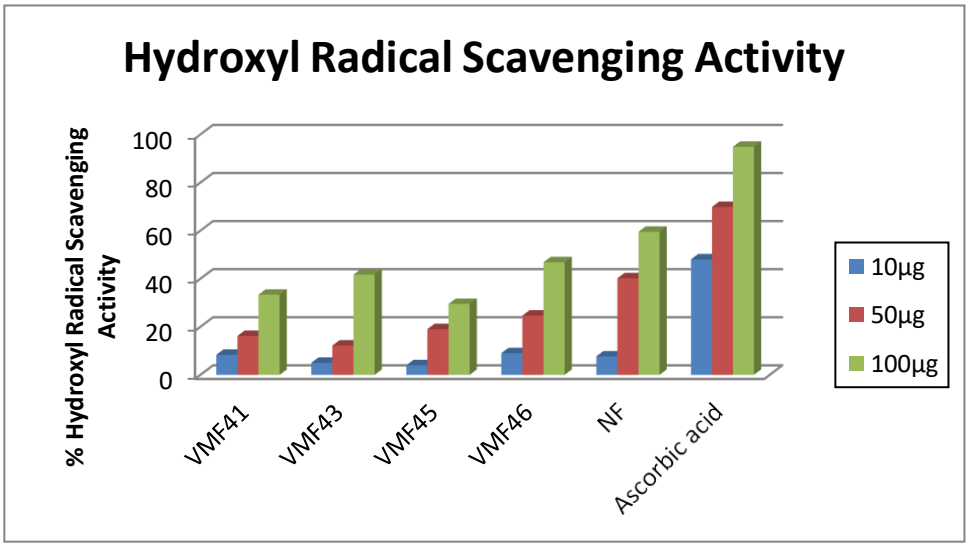


Fig5.6. **Effect of synthetic flavonoids on hydroxyl radical scavenging activity**

3) **Nitric Oxide radical scavenging:** Nitric oxide scavenging activity was performed with five test novel synthetic flavonoid (SFs) compounds and BHA as standard drug. The reductive potential of these compounds exhibited in a dose dependent manner as shown in Table- 5.5. From the IC50 values it is clarified that the NF and VMF 46 synthetic flavonoids showed significant scavenging potential on nitric oxide free radicals and this activity is comparable with the standard BHA drug.

Table.5.5. Percentage Nitric oxide scavenging activity of syntheticflavonoids at different concentration with their IC_{50} values.

Concentrations	Test Samples and their percentage scavenging activity					
	VMF 41	VMF 43	VMF 45	VMF 46	**NF**	**Ascorbic acid**
10µg	5.65	10.00	11.30	5.65	7.39	14.35
50µg	15.65	22.61	18.70	13.04	21.30	34.78
100µg	24.35	26.96	42.61	42.61	41.30	47.83
IC50	5.72±5.12	5.55±0.28	3.65±1.32	3.60±0.13	3.57±0.54	2.91±0.24

All the values in the table were presented as mean ± SEM(n=3).Data are represented in percentage nitric oxide radical inhibition..

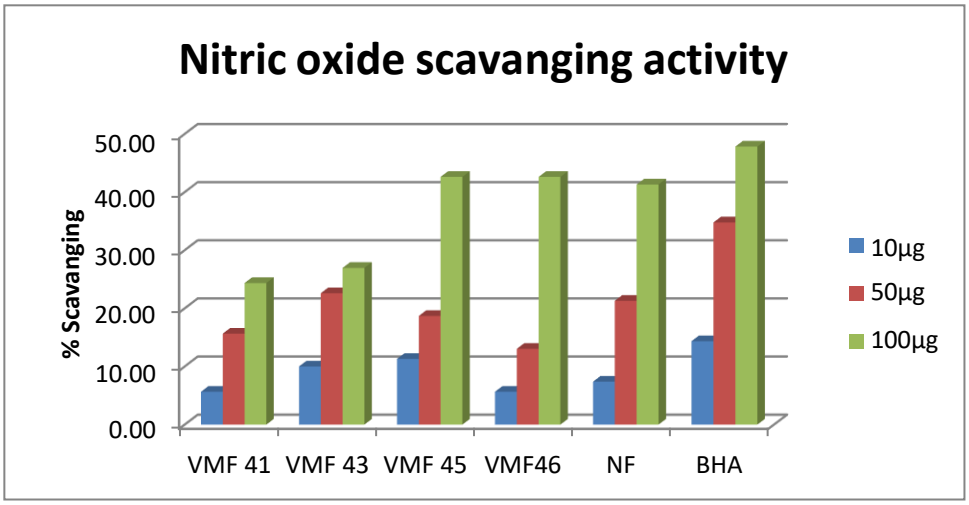


Fig**5.7**; **Effect of synthetic flavonoids on Nitric oxide scavenging activity**

From the free radical scavenging activity assays it is clear that the test NF synthetic novel flavonoid showed statistically significant anion trapping property than the other flavonoids tested in the study and its effects are compared to that of the respective reference drugs tested in the study. Apart from this, the synthesis point of view the easy, availability of regents required to prepare this test drug and good yield during Algar-F- Oyamada method based two step synthetic pathway was also seen, Therefore, we considered to select this synthetic novel flavonoid(NF) for further antidiabetic activity.

5. 3.Results: In-vitro antidiabetic activity of NF

I. Alpha-amylase inhibition activity.

The results of Alpha-amylase inhibition activity of test compounds vs. their respective concentrations were expressed in percentage inhibition on enzyme with their respective IC50 values. These results are shown in Table.5.6. Similarly the graphical representation of the same was given in fig 5.8. The test compounds exhibited dose dependant inhibition of enzyme activity in this study. NF test flavonoid showed maximum inhibitory effect as 6.26% at 200 µg dose which is insignificant when compared to standard acarbose (Fig5.8). Standard acarbose showed 100% inhibition of enzyme from 100 µg on wards and IC50 value of 0.33µg/ml which is statistically significant potent when compared to test NF compound

Table.5.6. Percentage inhibitory effect of NF and Acarbose on Alfa amylase enzyme.

Concentration of samples	% inhibition of Alfa amylase enzyme by test samples		
	Control	NF	Std Acarbose
10µg	0	3.92±0.22[a]	42.6±0.10[***]
50µg		4.72±0.12	99.43±0.03[***]
100µg		5.32±0.05	100±0[***]
150 µg		6.13±0.05	100±0
200 µg		6.26±0.14	100±0
IC50		76.09[b]	0.33[***]

All the values in the table were presented as mean ± SEM(n=3).Data are represented in percentage enzyme inhibition. Values with different superscript in a column and row were significantlydifferent ($p<0.05$).NF- Novel flavonoid.

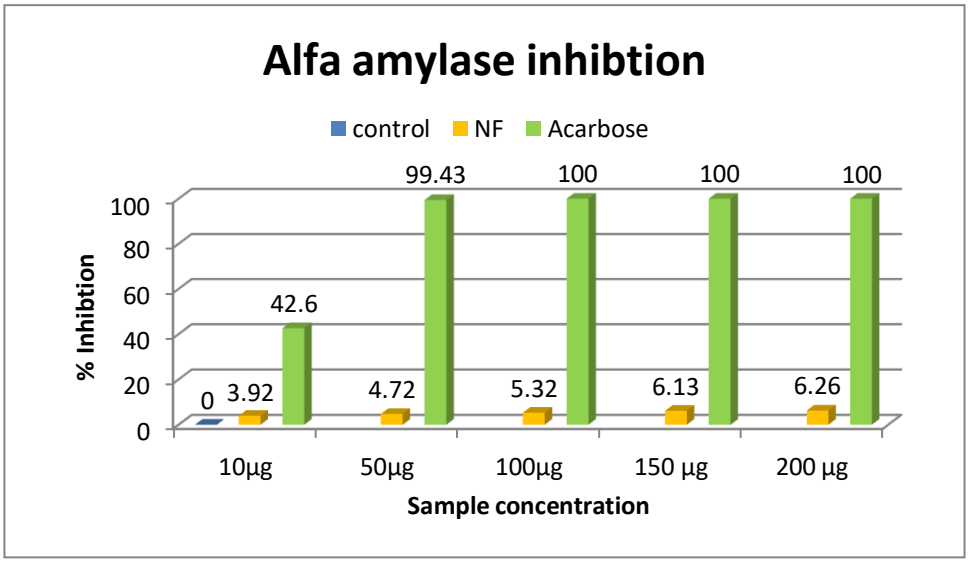


Fig.5.8.Percentage inhibition of NF and Acarbose on Alfa amylase

II. Alfa glucosidase inhibitory activity of NF compound:

The data for alfa glucosidase inhibitory effect has presented in Table5.7. and data were shown as percentage inhibition on enzyme by test compounds. The novel flavonoid when tested at 100 µg, 200 µg,300 µg 400 µg & 500 µg range, has shown dose dependant %inhibition activity on alfa glucosidase enzyme (10.86±0.27%, 12.77±1.23%, 24.34±0.50%, 32.08±0.75% & 32.83±0.99% respectively) and effects are relatively comparable with the positive control acarbose drug. But potency wise acarbose exhibited 62.02µg/ml as IC50 value vs 734.10 µg/m of NF compound showing NF compound as less potent compared to acarbose(fig.9).

Table.5.7. Percentage inhibitory effect of NF and Acarbose on Alfa glucosidase enzyme.

Concentration of samples	% inhibition of Alfa glucosidase enzyme by test samples		
	Control	NF	Std Acarbose
100µg	0	10.86±0.27[a]	48.63±0.59
200µg		12.77±1.23	59.44±0.34
300µg		24.34±0.50	62.80±0.17
400µg		32.08±0.75	66.99±0.43***
500µg		32.83±0.99	68.58±0.50
IC50		**734.10[b]**	**62.02*** **

NF- Novel flavonoid , Data are represented in percentage enzyme inhibition. All the values in the table were presented as mean ± SEM.Data are represented in percentage enzyme inhibition. Values with different superscript in a column and row were significantly different ($p<0.05$).NF- Novel flavonoid.

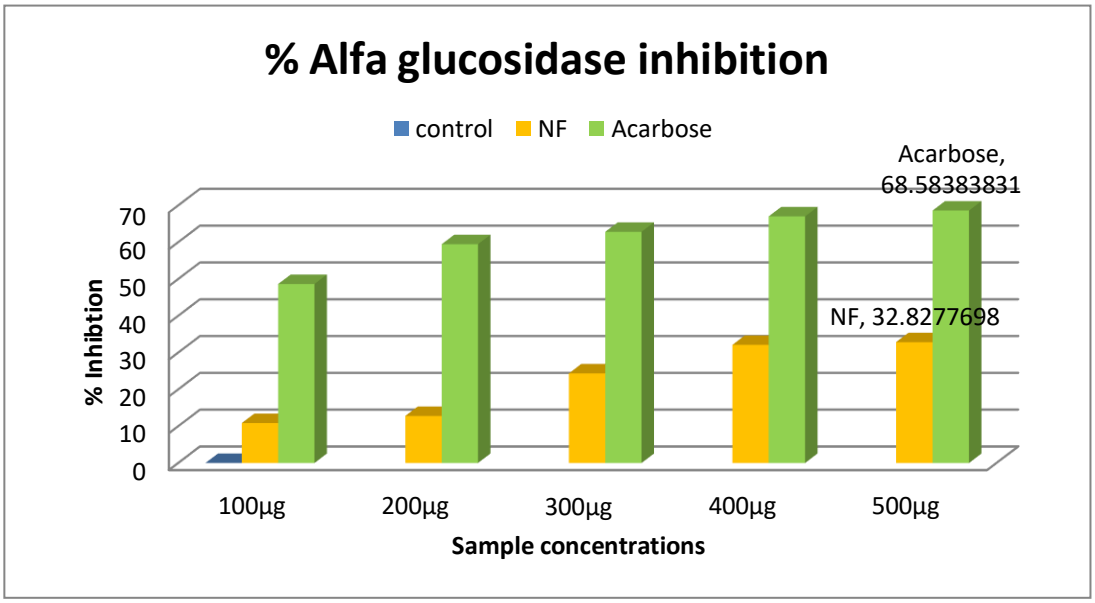


Fig.5.9.Percentage inhibition of NF and Acarbose on Alfa glucosidase

III. DPP-IV Inhibitory assay.

In DPP-4 inhibitory assay, the NF compound showed 17.07±1.34% at 50 µg/ml as minimum and 34.13±0.78% at 800µg/ml as a maximum of inhibitory activity against DPP-IV enzyme (Table. 5.8). However it showed the dose dependant action when compared to standard sitagliptin in the study. The NF compound showed less potent action when compared to sitagliptin. The graphical representation of these results given in fig.5.10

Table.5.8. Percentage inhibitory effect of NF and sitagliptin on DPP-4 enzyme

Concentration	% inhibition of DPP-4 by test samples	
	NF	Sitagliptin
50 µg/ml	17.07±1.35[a]	21.60±1.42
100 µg/ml	22.40±0.83	43.41±3.25
200 µg/ml	27.85±0.89	55.09±2.17
400 µg/ml	29.97±1.02	70.83±2.97
800 µg/ml	34.13±0.79	76.91 ±1.58
IC 50	1564.74[b]	255.32

NF- Novel flavonoid , Data are represented in percentage enzyme inhibition. All the values in the table were presented as Mean ± SEM.Data are represented in percentage enzyme inhibition. Values with different superscript in a column and row were significantly different ($p<0.05$).NF-Novel flavonoid.

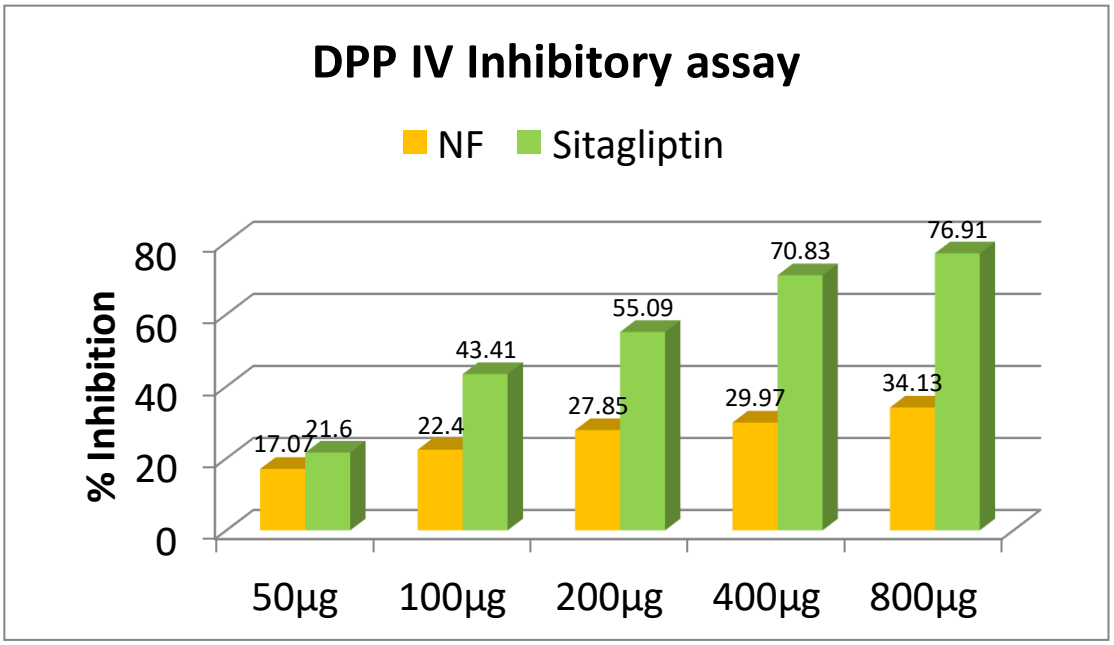


Fig.5.10.Percentage inhibition of NF and Acarbose on DPP-IV enzyme

IV. In-vitro Glucose uptake study in C2C12 cell lines

a) Effect of NF compound on C2C12 viability with MTT assay

The MTT assay was performed with or without NF compound at different concentrations(200, 400, 600, 800 &1000 µg/ml).**on these C2C12 cells for** their viability and compared with cisplatina a cytotoxic drug(Table5.9). Cells were able to have less viable at higher concentrations from 1000 to 600µg/ml as compared to cisplatina standard cytotoxic drug but viability was increased at concentration from 400 to 200 concentrations(Fig.5.9). The NF at 200µg/ml concentration, cells showed 17.13% viable as shown in T**able 5.9** and fig.5.11 This suggests the NF synthetic experimental compound might be existing cytotoxic effect only at higher doses. Based on the above results, the IC50 of NF (fig-)for viability on these cells appears to be lesser than 200 µg/ml and lethal (LD50) effect is greater than 200 µg/ml. Thus assuming that, the NF compound less than 200 µg/ml would be safer for further pharmacological activity(Glucose uptake in C2C12 cell lines), the $1/10^{th}$, $1/20^{th}$& $1/40^{th}$ of the IC50 value(Fig.5.12) of viability result i.e. 20µg, 40µg and 80µg of test NF were considered to use in Glucose uptake assay

Table5.9. MTT assay for C2C12 Cell viability

MTT assay- C2C12 Cell line Vs Sample NF								
	Blank	**Untreated**	**Cisplatin 15µg/ml**	**Test concentrations (in µg/ml)**				
				200	**400**	**600**	**800**	**1000**
Read 1	0.006	1.047	0.047	0.166	0.077	0.037	0.03	0.021
Read 2	0.008	0.964	0.05	0.19	0.08	0.035	0.028	0.026
Mean OD	0.007	1.0055	0.0485	0.178	0.0785	0.036	0.029	0.0235
Mean OD-Mean Blank		0.9985	0.0415	0.171	0.0715	0.029	0.022	0.0165
Standard error		0.0415	0.0015	0.012	0.0015	0.001	0.001	0.0025
% Standard error		4.156234	0.150225	1.201803	0.150225	0.10015	0.10015	0.250376
% Viability		100	4.156234	17.12569	7.160741	2.904357	2.203305	1.652479

Results in this table represents test drugs OD fallowed by % cytotoxic values in SEM(n=2) against their respective different concentrations.

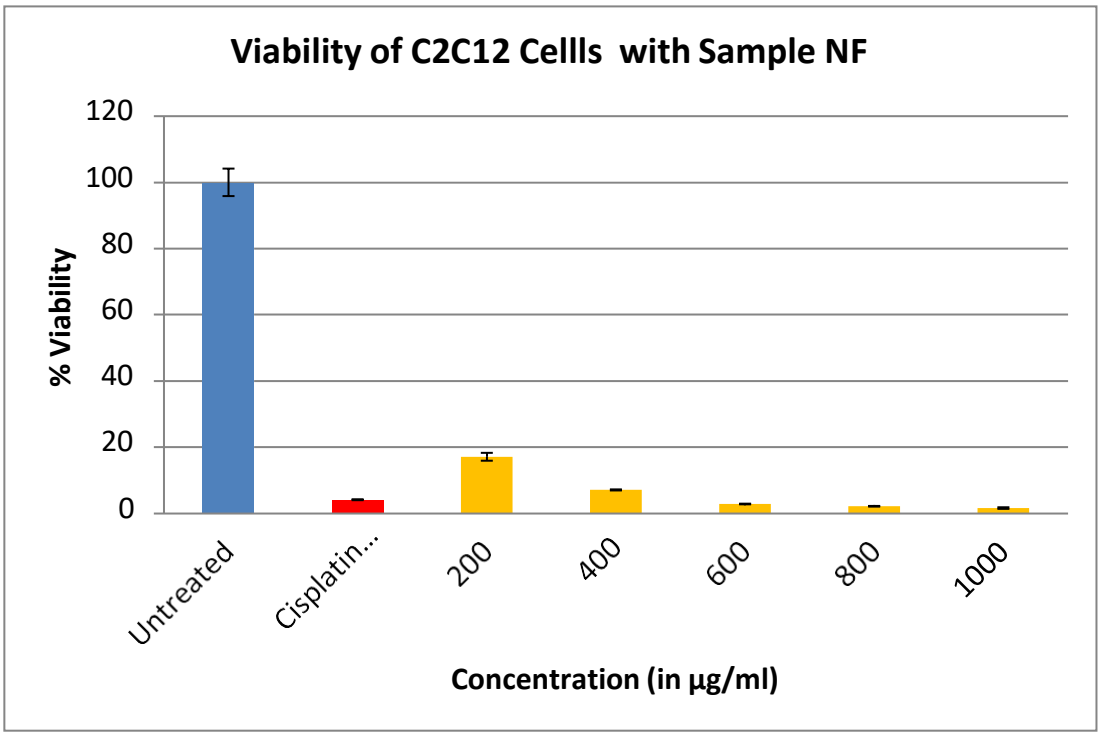


Fig.5.11.Effect of NF compound on C2C12 viability

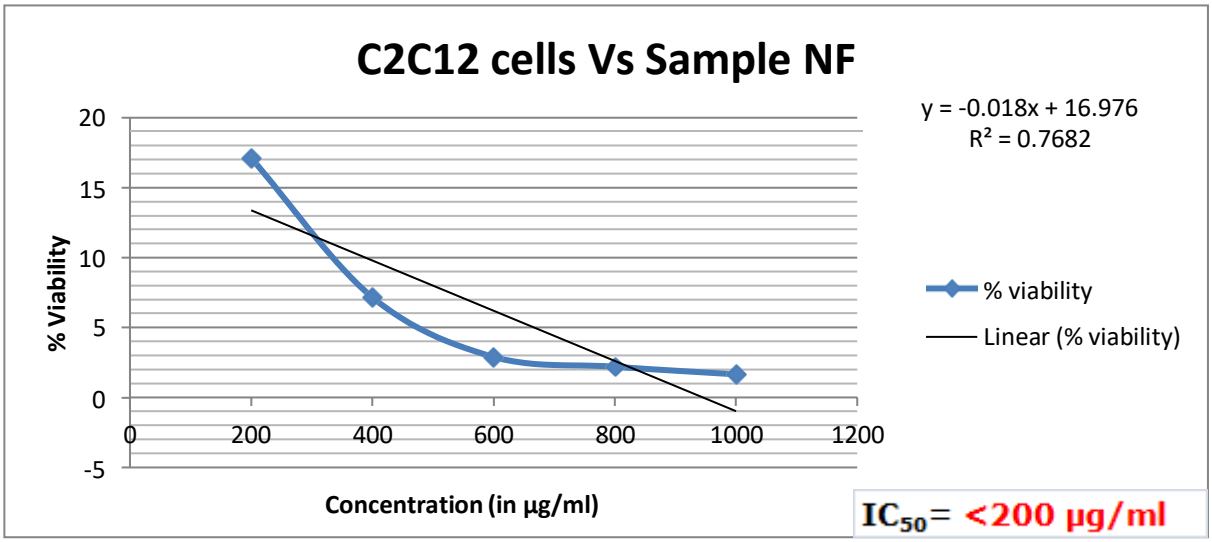


Fig.5.12 Linear graph to determine NF's IC50 for C2C12 cells viability after 24 hour treatment

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b)Effect of NF on glucose uptake in C2C12 myoblast cells.

To assess the impact of synthetic novel flavonoid(NF) on glucose uptake activity in C2C12mouse skeletal muscle cells, the NF compound's 20, 40 and 80 µg/ml various concentrations and 100µg/ml of metformin in DMSO were used. Before the start of glucose uptake assay, the standard calibration curve of glucose was determined quantitatively to establish the linearity as shown in graph Fig 5.13

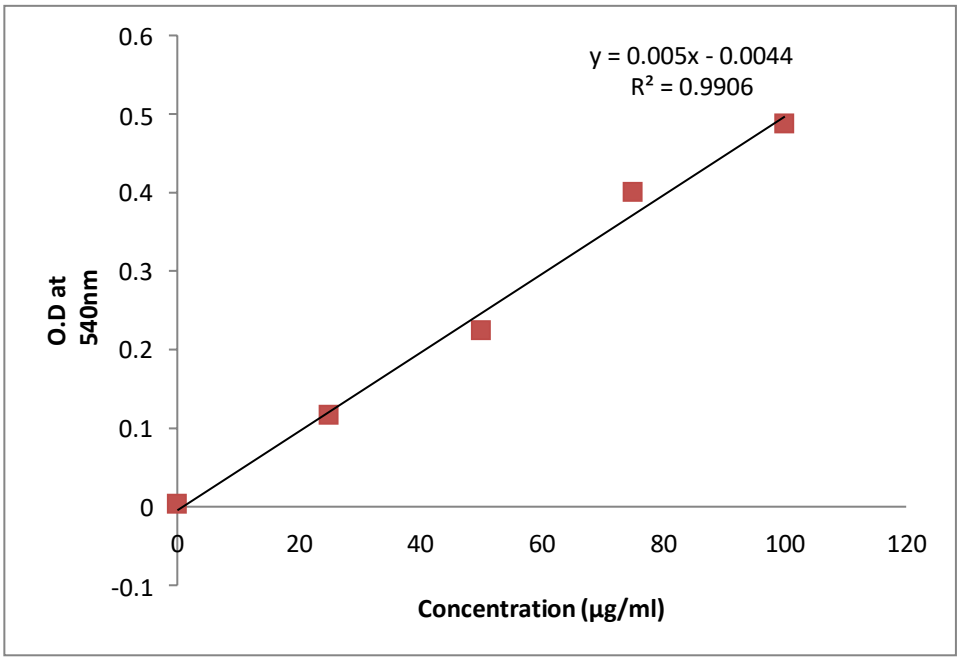


Fig.5.13. Standard calibration curve of Glucose

The results of NF on of glucose uptake assay were tabulated in Table 5.5 and represented in Figure 5.9. In glucose uptake assay experiment, Cells pre-treated with standard metformin showed 1.2 fold stimulation (235.72µg/ml) of glucose uptake when compared to untreated control cells (196.37µg/ml).Metformin as a positive control at a concentration of 100 µgshowed 16.75% glucose uptake while NF showed a concentration dependent increase in the glucose uptake with a maximum activity of 18.04% uptake at 80µg/ml compared to the normal control(7.68%) which is

statistically significant p< 0.05. However the NF compound at dose of 20 µg dose showed insignificant glucose uptake (219.71 µg/ml) in these cells.

Table5.10.. Effect of NF on glucose uptake in isolated C2C12 cells (lysate) of various groups.

Treatment Conc.	Absorbanceat 540 nm			Mean Abs. at 540 nm	Mean Glucose value in Lysate(µg/ml)	% Mean Glucose uptake	SEM	Fold uptake
	n=1	n=2	n=3					
Untreated cells Control	0.9779	0.9774	0.9771	**0.978**	196.37	7.68	0.263	1
Std metformin 100µg	1.1755	1.1741	1.1732	**1.174**	235.72	16.75	0.359	1.2
NF 20µg	1.0941	1.0939	1.0945	**1.094**	219.71	10.62	0.299	1.11
NF 40µg	1.1619	1.1621	1.1632	**1.186**	233.36	16.20	0.263	1.19
NF 80µg	1.186	1.1865	1.1866	**1.162**	238.15	18.04	0.100	1.21

The values indicate mean % glucose uptake± standard error compared to the Metformin (positive control). Each value is a mean of tests done in triplicates. *p<0.05 compared to normal untreated control**p<0.05 compared to positive control.

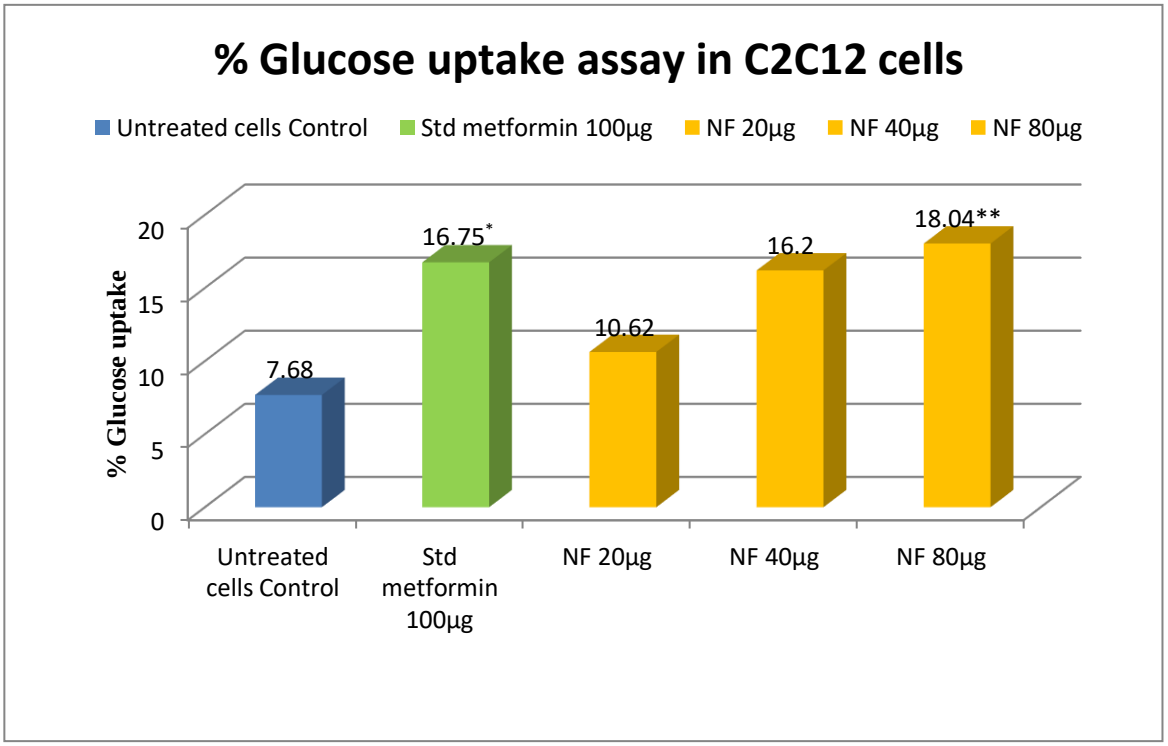


Fig.5.14. Effect of NF on Glucose uptake in C2C12 cells

5. 4. Effect of NF compound on *in-vivo* efficacy parameters

1) Results: In-vivo antidiabetic activity of NF

A) Acute toxicity study

NF compound treated mice did not produced any mortality within 72 hours of observation (Table-11)and no abnormal behavioural symptoms except hepatitis like features at 175mg/kg and fatty changes at 500mgkg and 2000mg/kg b.wt treated animals on histological study during 14days observation time. LD50 was found to be greater than 2000mg/kg body weight. Thus $1/10^{th}$, $1/20^{th}$ & $1/40^{th}$ of limit dose (2000mg/kg) such as 200 ,100 and 50mg/kg was considered to use in the in-vivo efficacy study.NF treated animals.There were no significant changes in body weight after the drug administration except in the animals treated with single oral 175mg /kg

of NF test drug which showed initial decrease in body weight on 3rd day only as shown in Figure5.15.

Table 5.11; Result of NF on behavioural parameters during Ac toxicity

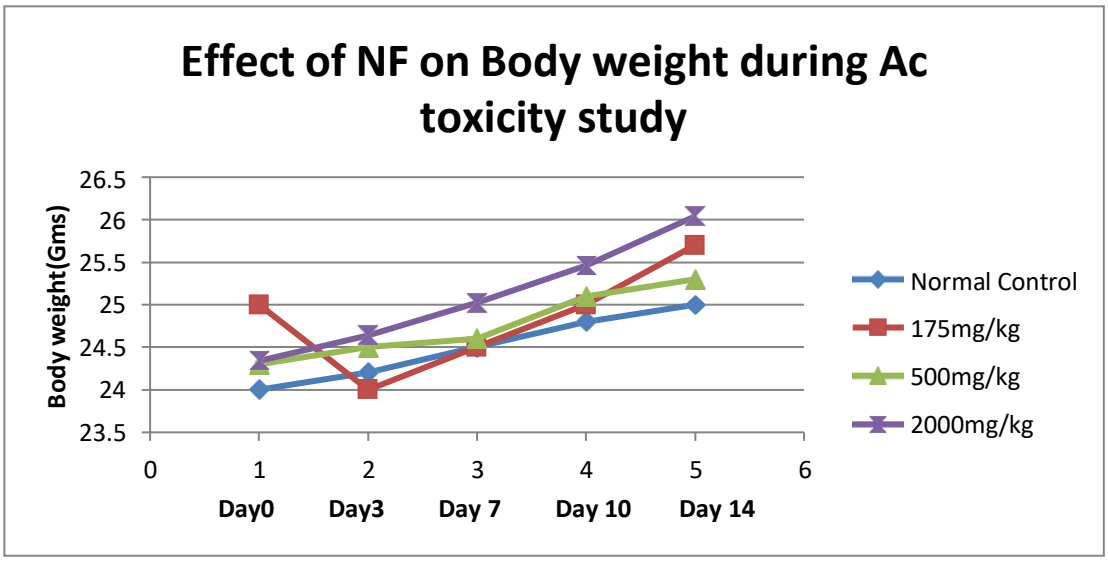
Parameters	Control	NF 175mg/kg	NF500mg/kg	NF2000mg/kg
Vomiting	Nil	Nil	Nil	Nil
Diarrhea	Nil	Nil	Nil	Nil
Salivation	Nil	Nil	Nil	Nil
Lacrimation	Nil	Nil	Nil	Nil
Breathing difficulty	Nil	Nil	Nil	Nil
Lethergy	Nil	Nil	Nil	Nil
Drowsy/ sleep	Nil	Nil	Nil	Nil
Bleeding	Nil	Nil	Nil	Nil
Changes in skin	Nil	Nil	Nil	Nil
Eyes fur	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Urine(color)	Normal	Normal	Normal	Normal
Faecal consistancy	Normal	Normal	Normal	Normal
Skin fur	Normal	Normal	Normal	Normal
Behavior pattern	Normal	Normal	Normal	Normal
Locomotor activity	Normal	Normal	Normal	Normal
Convulsion/Tremors	Nil	Nil	Nil	Nil
Coma	Nil	Nil	Nil	Nil
Mortality	Nil	Nil	Nil	Nil

Table: 5.12. Effect of NF on body weight(Gm) during toxicity study

NF concentration	Effect of NF on body weight(grams) during Ac toxicity study				
	Day 0	Day 3	Day 7	Day 10	Day 14
Normal Control	24±12.4	24.2±17.2	24.5±22.1	24.8±0.84	25±12.25
175mg/kg	25±6.12	24±9.23	24.5±2.24	25±12.8	25.7±13.2
500mg/kg	24.3±7.1	24.5±8.41	24.6±6.2	25.1±0.5	25.3±31.1
2000mg/kg	24.34±6.3	24.64±7.1	25.02±8.1	25.46±24.1	26.04±2.2

Results are expressed as grams of body weight in Mean±SED(n=6)

Fig;5.15.Effect of NF on body weight(grams) during Ac toxicity study on different days



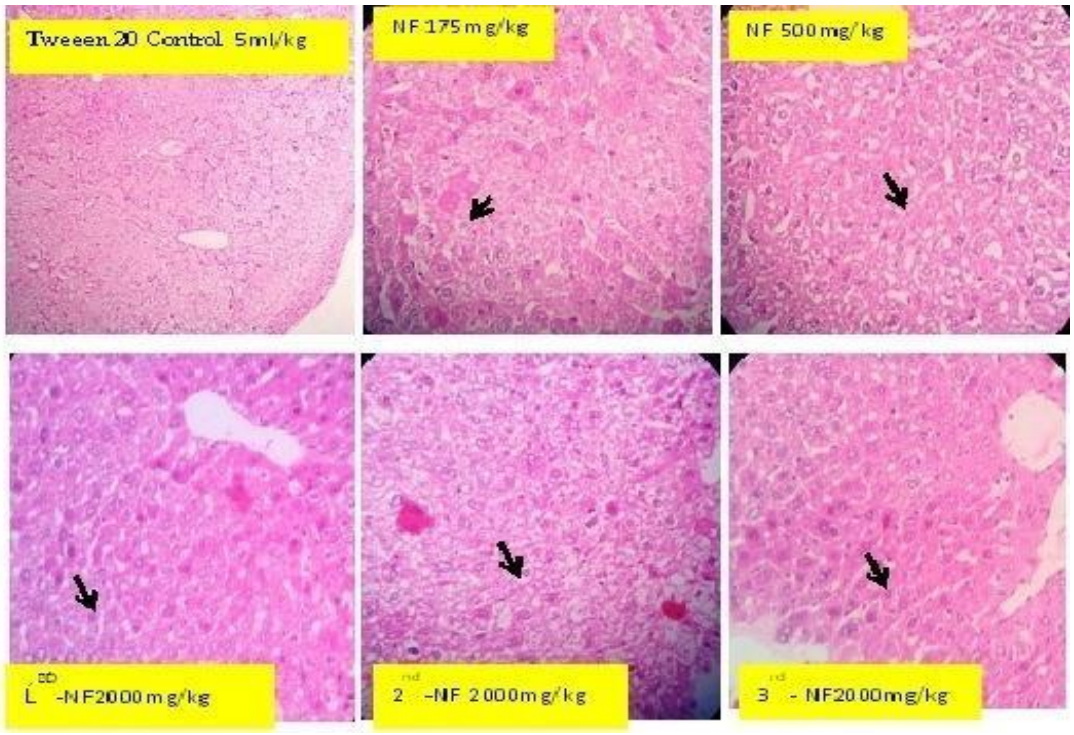


Fig. 5.16; Hepatocytes architecture after oral acute toxicity
Normal architecture of the hepatocytes except signs of hepatitis(175mg/kg),and focal fatty changes & glycogenesis features(500mg/kg and 2000mg/kg) on hepatocites

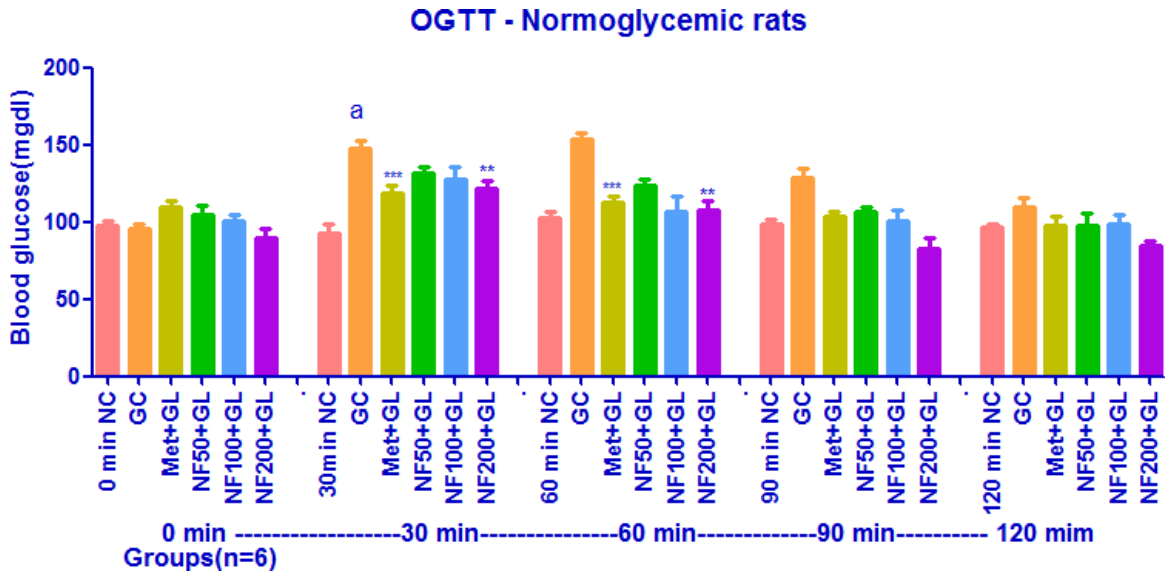
B) Effect of NF on oral glucose tolerance test(OGTT) in normoglycemic rats.

In this study, the hypoglycaemic potential of test NF compound at various dose was tested by oral glucose load(2gkg) in 16 hour fasted normal rats. It was observed that there was increase in sugar level at initial 60 min, then started to decline till 120 min after glucose challenge(Table . It was also observed that, at 30 min after the oral glucose challenge , the blood glucose levels significantly ($P<0.05$) increased rapidly in the glucose control groups against the test drugs treated groups, which showed the glucose induced hyperglycemia was significantly ($P<0.05$) delayed/prevented compared to glucose control group. There was a significant ($P<0.05$) decrease of glucose level as the dose of NF increased from 50 to 200 mg/kg. Rats treated with 100 & 200mg/kg of NF compound showed the significant ($P<0.01$) tolerance to aglucose load which was comparable to the metformin, suggesting that a secretion of insulin in response to hyperglycemia (glucose load) in these treated groups.

Table 5.13.Effect of NF on oral glucose tolerance in normoglycemic rats(OGTT)

OGTT	NC	GC	Met+Gl	NF50+Gl	NF100+Gl	NF200+Gl
0hr	97.33±3.490	95.83±2.587	109.7±4.271	104.5±5.932	100.7±4.248	89.33±6.627
30 min	93.17±5.180	147.5±5.506	118.5±5.123	131.7±4.169	127.7±7.509	121.5±5.123
60min	103.0±3.661	153.3±4.080	112.5±4.288	124.2±3.781	106.5±10.66	107.8±5.902
90min	99.17±2.713	128.7±5.812	104.0±3.011	106.7±2.679	101.2±6.575	82.83±6.755
120min	96.33±1.892	109.3±6.323	98.17±5.492	97.67±7.509	99.17±5.845	84.33±3.630

Each group results represent mean ± SEM(n = 6).NC-Normal control, GC-Glucose control. Met+GL- Metformin + glucose, NF50+ GL- Novel flavonoid+Glucose



Fig;5.17. Effect of NF on OGTT in normoglycemic rats

C) Effect of HFD on FBG, serum insulin and HOMA levels(before STZ)

At the end of four weeks of **HFD feeding** in experimental rats, there was a significant increase in body weight when compared to normal control groups, Apart from this HFD dietary manipulation also raised the basal fasting blood glucose and HOMA-IR index and reduced the HOMA-B levels without much affecting the serum insulin levels when compared to normal control rats(Table-5.14).

Table.5.14.Effect of HFD on FBG, serum insulin and HOMA levels before diabetes induction.

Group	Body weight	FBG	Fasting Serum. Insulin (FSI)	HOMA- IR	HOMA-B
NC	172.7±5.72[a]	83.33± 2.539	**8.65±0.47**	1.78	153.11
HFD	212.8±1.64***	146.47 ± 5.24	**7.95±0.70** [a]	2.88	34.46

Each value represents mean ± SEM (n = 6-8). NC- Normal control, HFD- High fat diet, FBG-blood glucose; FSI-Fasting serum insulin; HOMA-IR-Homeostasis of model assessment for insulin resistance index, HOMA-B- Homeostasis of model assessment for beta cell function.

D. Effect of 21 days treatment of NF in high fat diet and low dose STZ-induced type 2 diabetic rat model for FBG,Insulin, HbA1C, HOMA IR , HOMA B, lipid profile and endogenous antioxidants

1. Effect of NF on Fasting blood glucose(FBG):

The results obtained from this are presented in(Table 5.15 and Fig 5.18). In the present study HFD and STZ received group rats on 35th day of experiment, exhibited a significant (P<0.001) increase in glucose level in contrast to NC(86.50± 3.22) and HF treated group rats(137.8±4.13). Diabetic rats treated with NF compound at 50,100& 200mg/kg doses showed significant decrease in blood glucose levels(159.2±17.33, 104.5±8.55 and 144.3±19.21 respectively) against HFD+ DC group rats (329.7±23.68). These and effect are comparable to HF+D+met treated group rats which is statistically significant(P<0.05).Among different doses of NF, at 100 to 200mg/kg body weight dose showed highest about 125.2% reduction of blood glucose which is almost comparable with standard metformin treated groups(172.48%).

Table 5.15. Effect of 21 days treatment of NF and Metformin on FBG in high fat diet and low dose STZ-induced type 2 diabetic rats.

Groups	1st day	(FBG) 7th day	FBG 22nd day	% Reduction of glycaemia taken from the mean values
1. NC	83.33± 2.539	86.50±3.222	92.50±3.594	--7.2
2. HF	149.2±4.408	137.8±4.135	146.2±2.257	-6.03
3. HF+ D	137.2± 4.151	378.3±43.11[b]	329.7±23.68[c]	35.42
4. HF+D+Met100	144.3±5.226	362.7±45.56	113.8±11.32	172.48
5. HF+D+NF 50	146.8± 5.879	337.5±42.87	159.2±17.33	121.46
6. HF+D+NF 100	149.3±6.042	291.3±20.07	104.5±8.555	125.11
7. HF+D+NF 200	152.0±4.993	334.7±28.20	144.3±19.21	125.26

All values are expressed as mean±SEM (n=6), Group 3 is compared with Group 1. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid

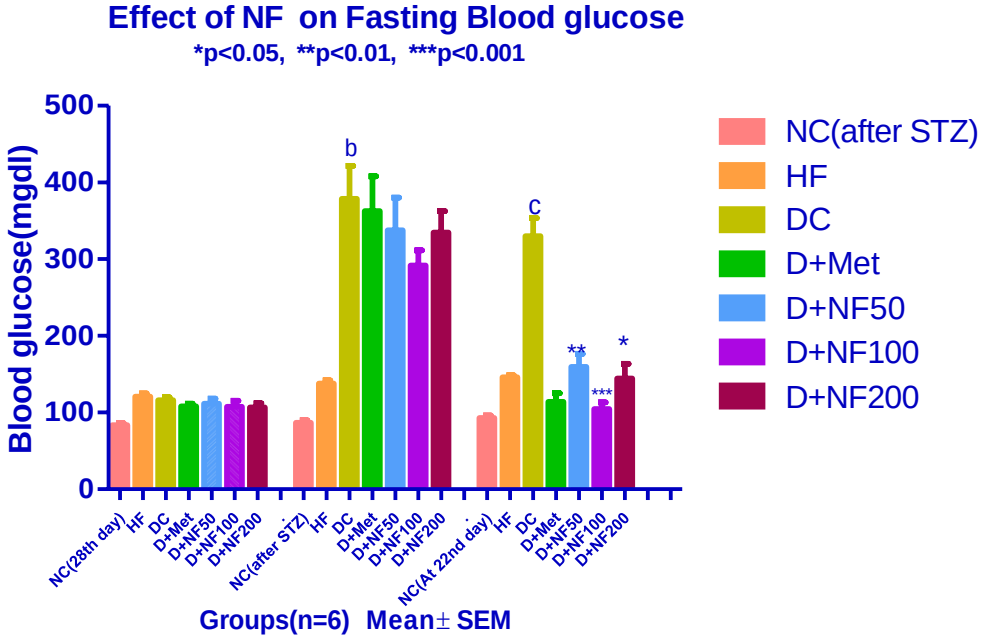


Fig: 5.18. Effect of NF on FBG. Each bar represent the mean ± SEM(n = 6).

2. **Effect of NF on serum insulin, HOMA-IR and HOMA-B**

In the present study, at the end of the treatment on 22nd day, the mean fasting serum insulin levels (mIU/L) of NC, HF, HF+DC, HF+D+Met and HF+D+NF at 50,100&200mg/kg dose groups showed 8.79 ±0.73, 7.85±0.43, 3.02±0.31, 7.89±0.70, 3.86±0.45, 6.69±0.57 & 6.18±0.64 respectively given in Table5.16. The diabetic rats treated with NF at 50, 100&200mg/kg dose for 21 days exhibited significant($P<0.001$) increase in serum insulin levels in contrast to HFD+ STZ treated group rats(3.02±0.31) **(Table 5.16, fig 5.20 & 5.21,)** and this effect was comparable with standard metformin treated groups(7.89±0.70).

Table 5.16. Effect of 21 days treatment of NF on FBG, serum insulin, HOMA-IR and HOMA-B levels in high fat diet and low dose STZ-induced type 2 diabetic rat model.

Study groups	FBG After treatment	FSI	HOMA- IR	HOMA-B
1. NC	92.50±3.594***	8.79 ±0.73	2.00±0.19	121.1±26.38
2. HF	146.2±2.257***	7.85 ±0.43***	2.83±0.13	34.28 ±2.56
3. HF+ D	329.7±23.68a	3.02±0.31b	2.47±0.34c	4.29d±0.67
4.HF+D+Met 100	113.8±11.32***	7.89±0.70***	2.27±0.43	67.12±13.31
5. HF+D+NF 50	159.2±17.33*	3.86±0.45	1.52±0.26	17.39±3.87
6. HF+D+NF 100	104.5±8.555***	6.69±0.57***	1.78±0.27	71.57±14.65**
7. HF+D+NF 200	144.3±19.21**	6.18±0.64**	2.20±0.36	33.30±6.79

All values are expressed as mean±SEM (n=6), Group 3 is compared with Group 1. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.-.

For HOMA-IR results, the mean HOMA-IR index of NC,HF, HF+D, HF+D+Met and HF+D+NF at 50,100&200mg/kg dose group animals after treatment showed 2.00±0.19, 2.83±0.13, 2.47±0.34, 2.27±0.43, 1.52±0.26, 1.78±0.27 and 2.90±0.36 respectively(table 5.16). The rats received only HFD showed increase in insulin resistance index(2.83±0.13) in contrast to normal control animals(2.00±0.19). But the HOMA-IR results in HFD+STZ-diabetic rats treated with metformin and NF at 200mg/kg showed insignificant results(Table5.16). But the animals treated with NF at 50 &100mg/kg dose showed decrease in HOMA-IR index values compared to HF and HFD with STZ control groups**(Table 5.16&fig 5.20)**.

In the study the mean HOMA-B values of NC,HF, HF+D, HF+D+Met and HF+D+NF at 50,100&200mg/kg groups after treatment were found **121.1±26.38, 34.28±2.56, 4.29±0.79 ,67.12±13.31, 17.39±3.87, 82.37±18.72 and 38.31±7.672 respectively(Table 5.16&fig 5.21).** Animals which received only control vehicle exhibited increased pancreatic beta cell mass(HOMA-B) as **121.1±26.38** values in contrast to high fat diet control & diabetic control rats on HFD as **34.28±2.56, 4.29±0.79 respectively**. After 3 weeks of oral treatment with NF at intermediate and high dose significantly($P<0.05$) reversed the reduced HOMA-B values(**82.37±18.72 and 38.31±7.672 respectively)** compared to HF and HF+D control rats and the effect was comparable to standard metformin treated group rats(**67.12±13.31)**.

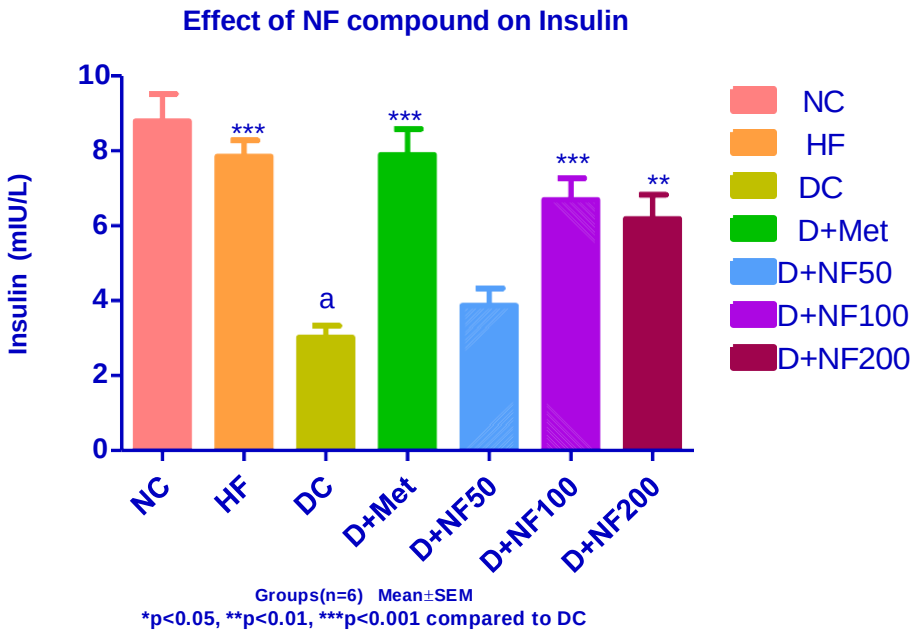


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Fig:5.19: Effect of NF on Fasting serum insulin. Each bar represent the mean ± SEM(n = 6).

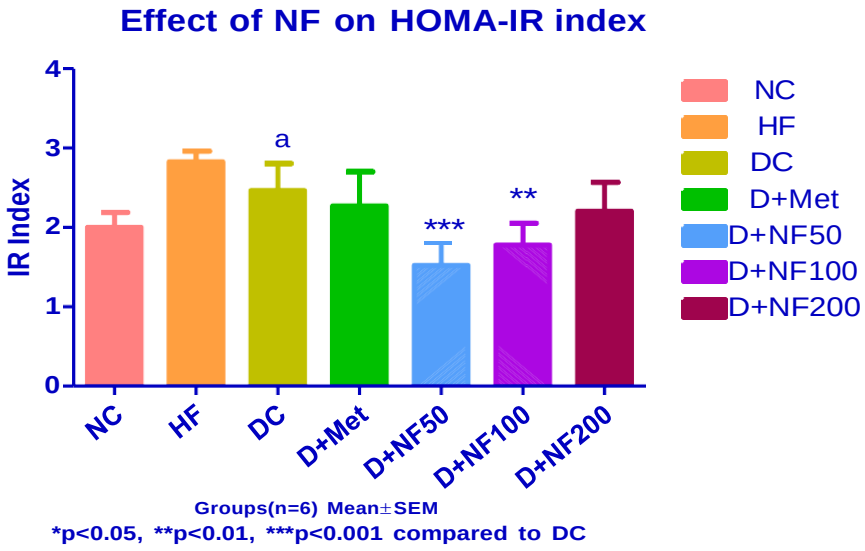

Fig:5.20: Effect of NF on insulin resistance(HOMA-IR). Eachbar represent the mean ± SEM (n = 6).

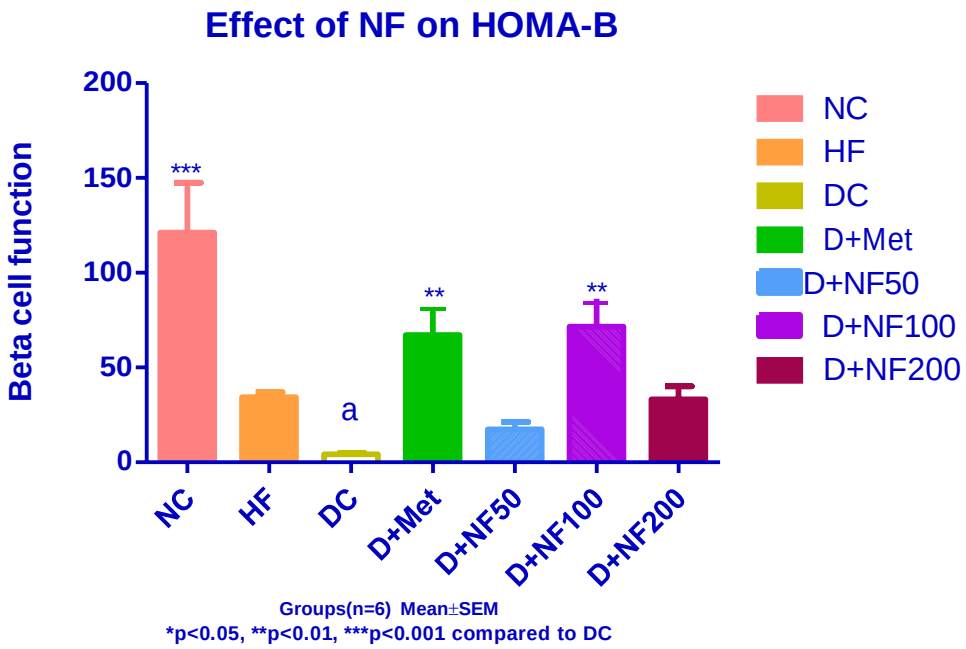

Fig:5.21: Effect of NF on pancreatic beta cell mass(HOMA-B). Eachbar represent the mean ± SEM (n = 6).

3. **Effect of NF on plasma Glycated hemoglobin(HbA1C) levels**

Similarly in the present study, the **Mean**±SEM **% glycated hemoglobin** values are presented in Table.5.17 &Fig5.22. The animals in NC,HF, HF+D, HF+D+Met and HF+D+NF at 50,100&200mg/kg treated groups were showed 6.35±0.25, 8.28±0.52, 12.48±0.9, 7.10±0.78, 9.12±1.15, 7.45±1.24 and 8.10±1.00 respectively. There was a significant (P<0.001) raised glycated hemoglobin(12.48±0.9%) in HFD with diabetic control rats(12.48±0.9) when compared to only HF diet treated and normal control rats. The 100mg/kg and 200mg/kg dose group of NF synthetic novel flavonoid showed a significant reduction (P<0.05) in glycated hemoglobin levels compared to the diabetic control group, and the effect was comparable to standard drug metformin.

Table 5. 17. Effect of 21 days treatment of NF on %Glycated hemoglobin(HbA1C) levels in high fat diet and low dose STZ-induced type 2 diabetic rat model.

Study groups	HbA1C %
1. NC	6.35±0.25***
2. HF	8.28±0.52*
3. HF+ D	12.48±0.91[a]
4. HF+D+Met100	7.10±0.78***
5. HF+D+NF 50	9.12±1.15
6. HF+D+NF 100	7.45±1.24**
7. HF+D+NF 200	8.10±1.00**

All values are expressed as mean±SEM (n=6), Group 3 is compared with Group 1. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.-.

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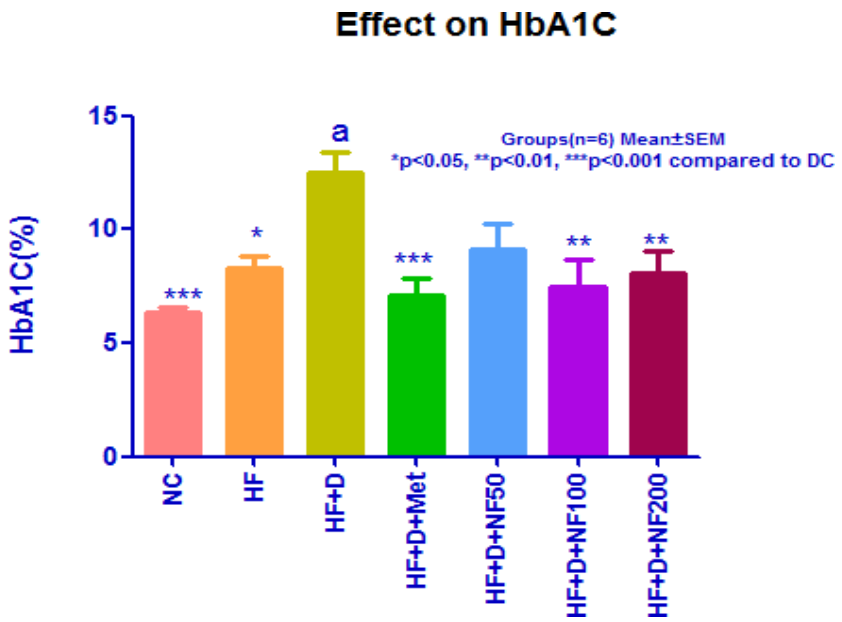

Fig;5.22. Effect of NF on % Glycated hemoglobin(HbA1C) Each bar represent the mean ± SEM (n = 6).

E. Effect of NF on serum lipid parameters :

In the present study, the diabetic control animals showed significant elevations in TG-c, TC, VLDL-c& LDL-c (148.0±3.71, 82.42±2.33, 29.60±0.63 &28.62±2.88 respectively and accompanied with marked decline in HDL(24.20±0.509) relative to the corresponding control groups(Table 5.18 and fig 5.23). Diabetic rats treated with 21 days of NF compound at 100 to 200mg//kg b.wt. dose, showed a significant reduction($P<0.05$) in TG, TC, LDL and VLDL and the effect was comparable to standard metformin. However the results of HDL-c levels in present study indicate that no significant difference was observed between the normal control and diabetic rats treated with NF compound at various doses.

Table. 5.18. Effect of 21 days treatment of NF on lipid profiles.

Lipid prfile	**Triglyceride (mg/dl)**		**Total chol (mg/dl**		**HDL (mg/dl)**		**VLDL (mg/dl)**		**LDL (mg/dl)**	
Days	**Day 1**	**Day 22**	**Day 1**	**Day 22**	**Day 1**	**Day 22**	**Day 1**	**Day 22**	**Day 1**	**Day 22**
1.NC	66.08 ± 5.53[a]	67.94± 5.78	52.08± 2.8[a]	**48.46± 2.13*** **	33.2± 1.69[a]	**29.85± 1.81***	13.22± 1.10	**13.59± 1.15*** ***	5.65± 1.29[a]	**5.02± 1.14*** ***
2.HF	118.32± 0.21	128.22± 2.41	59.23± 0.82	78.26± 3.12	24.92± 1.59	29.60± 0.64	23.66± 0.26	25.64± 1.62	10.65± 0.22	23.02± 28.1
3.HF+ DC	124.6 ± 7.97** *	**148.0± 3.71[a]**	63.59± 1.35[a]	**82.42± 2.33[a]**	29.22± 1.30	**24.20± 0.51[a]**	24.92± 1.59**	**29.60± 0.64[a]**	9.45± 1.53*** *	**28.62± 2.88[a]**
4.HF+ D+Met 100	127.2 ± 6.37*	**67.40± 2.79*** **	60.59± 1.94	**50.27± 2.81*** **	25.77± 0.29	**30.95± 2.00***	25.43± 0.57*	**13.48± 0.56*** ***	9.28± 1.10*** *	**5.835± 0.766** ***
5.HF+ D+NF 50	127.8 ± 2.86*	**102.3± 5.17*** **	60.41± 1.73	**55.12± 2.78*** **	26.02± 1.03	**27.87± 1.48**	25.56± 0.57*	**20.46± 1.03*** ***	9.22± 1.253*** **	**6.80± 1.45*** ***
6.HF+ D+NF 100	123.4 ± 3.29** *	**56.45± 1.94*** **	59.92± 1.58	**46.25± 1.58*** **	25.30± 0.94	**29.15± 1.07***	24.68± 0.66**	**11.29± 0.38*** ***	10.58± 1.27*** *	**5.81± 1.58*** ***
7.HF+ D+NF 200	125.1 ± 2.83** *	**58.22± 0.82*** **	61.43± 1.88	**46.83± 1.96*** **	24.93± 0.46	**30.75± 0.60***	25.02± 0.57**	**11.64± 0.16*** ***	11.48± 1.36*** *	**4.45± 1.47*** ***

All values are expressed as mean±SEM (n=6), Group 3 is compared with Group 1. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.-.

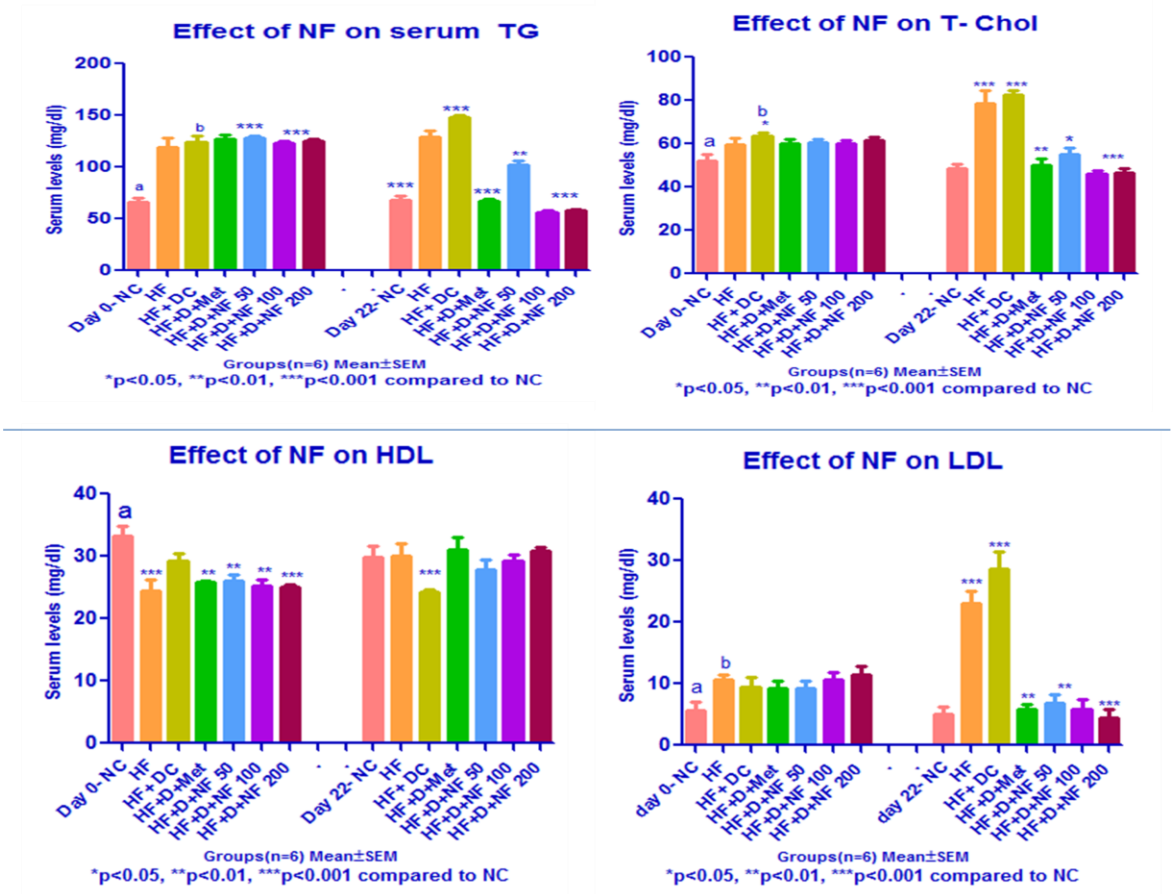

Fig:5.23(a). Effect of NF on lipid profiles.Eachbar represent the mean ± SEM (n = 6).

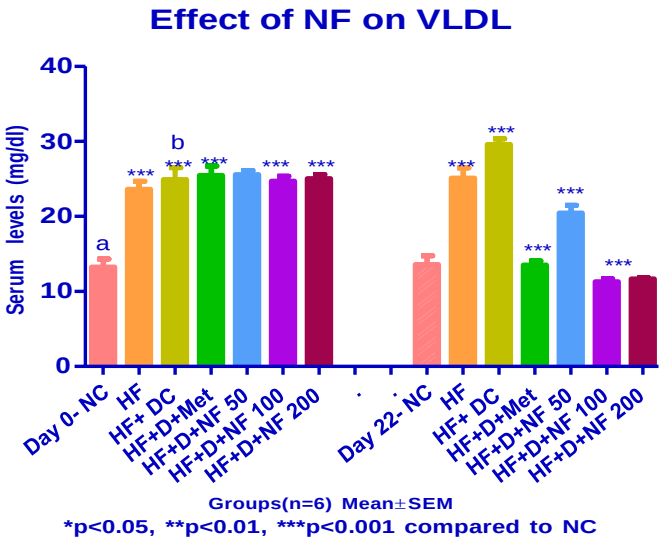

Fig:5.23(b). Effect of NF on lipid profiles.Eachbar represent the mean ± SEM (n = 6).

F. Effect of NF compound on endogenous antioxidant enzyme(SOD,CAT,GSH and LPO)

In this study, HFD + STZ diabetic rats showed significantly increased ($p < 0.05$) in LPO(TBARS) levels(230.0±27.91nmol/g of tissue).Treatment with different doses of NF significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) abolished the increase in TBARS levels induced by HFD + STZ and the effect was comparable with metformin treated groups.HFD + STZ diabetic rats showed significantly decreased ($p < 0.001$) levels of GSH (10.91±1.137 nmol/mg of protein) in comparison to normal control rats. However 3 weeks treatment with NF compound showed significant($p < 0.05$) increase in GSH levels which was comparable to metformin treated groups. In the present study it was observed that. HFD + STZ diabetic rats exhibited significantly reduced ($p < 0.001$) levels of catalase (0.176±0.021 U/mg) and SOD (81.17±5.270 U/mg of protein) compared to normal control rats. However treatment with moderate to higher doses of NF compound showed significantly ($p < 0.05$; $p < 0.01$) increased levels of catalase and SOD.

Table 5. 19. Effect of 21 days treatment of NF on endogenous (liver) antioxidant enzymes in high fat diet and low dose STZ-induced type 2 diabetic rat model.

GROUP	SOD (U/mg of protein)	CAT (U/mg of protein)	GSH (nM/mg of protein)	LPO (nM/mg of protein)
1.NC	125.2±10.85	0.486±0.073	28.48±1.939	63.03±10.05
2.HF	103.5±3.330	0.2488±0.02224	19.89±1.320	196.4±8.533
3.HF+ DC	81.17±5.270 [b]	0.176±0.021[a]	10.91±1.137[a]	230.0±27.91[a]
4.HF+D+Met100	137.0±7.966***	0.516±0.044***	31.55±3.143***	103.0±15.03***
5.HF+D+NF 50	99.03±5.336*	0.257±0.022*	14.80±3.371**	182.3±17.30**
6.HF+D+NF 100	115.1±5.352	0.376±0.047	23.70±2.215	114.4±20.04
7.HF+D+NF 200	128.6±7.770***	0.440±0.039***	27.97±2.095***	115.8±11.96***

All values are expressed as mean±SEM (n=6), Group 3 is compared with Group 1. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.-.

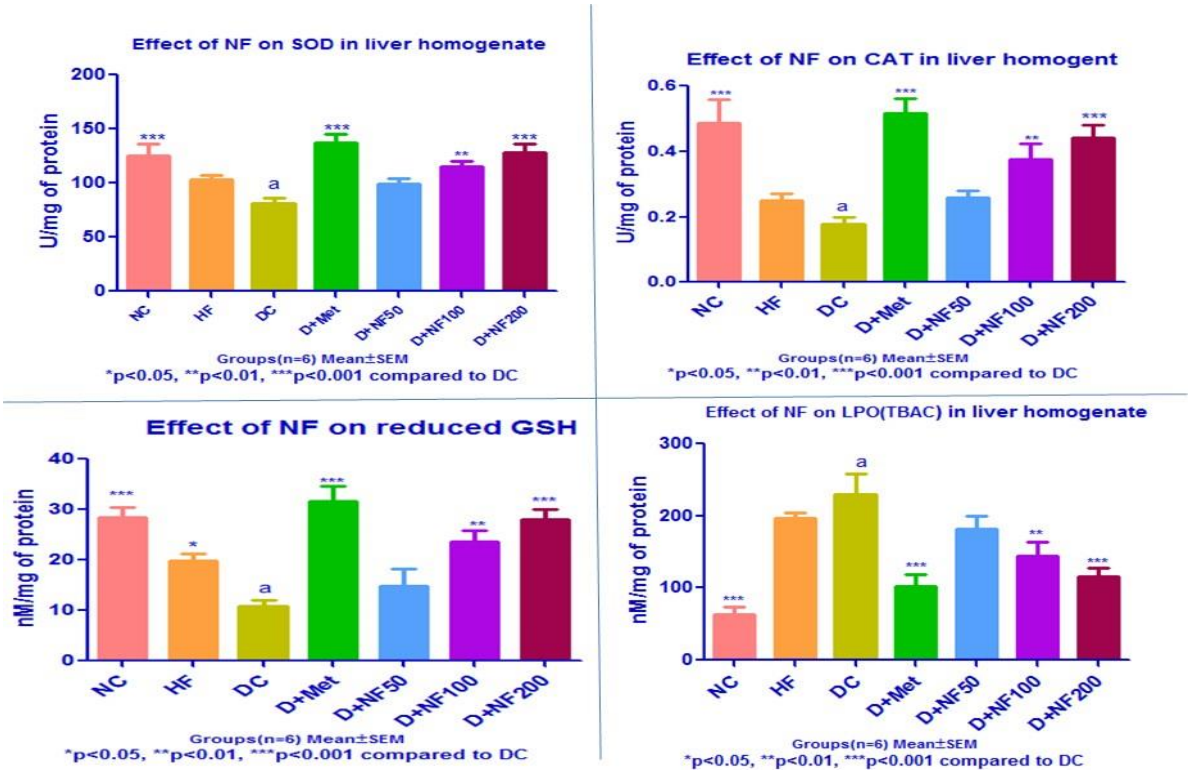

Fig:5.24. Effect of NF on endogenous antioxidants.Each bar represent the mean ± SEM (n = 6).

G. Effect of NF compound on body weight:

In the present study diabetic group rats (HF+D, HF+D+Met, HF+D+NF50, HF+D+NF100& HF+D+NF200) treated with STZ on seventh day, showed significant ($P<0.05$) mean per centreduction in their body weight (-4.46, -1.42, -2.61, -2.99 &-1.75) contrast to normal control(5.04) and HF(8.23) treated animals. HFD-STZ mediated body weight decrease was significantly ($P<0.05$) reversed by the three weeks daily oral treatment withmetformin and test NF drug**. However** diabetic control rats and NF 200 group of rats were continued to lose their body weight significantly when compared to the animals on normal control and HF diet.

Table 5. 20. **Effect of 21 days treatment of NF on body weight in high fat diet and low dose STZ-induced type 2 diabetic rat model**

	1 .NC	2. HF	3. HF+D	4. HF+D+ Met100	5. HF+D+ NF50	6. HF+D+ NF100	7. HF+D+ NF200	
28th day	172.7± 5.72[a]	200.3± 2.50*	212.8± 1.64***	211.2± 3.98***	191.0± 8.80	210.7± 11.2**	211.2± 7.52***	
35th day	181.4± 5.10	216.8± 1.62***	203.3± 4.01*	208.2± 5.56**	186.0± 8.42	204.4± 10.80**	207.5± 2.93**	
% change	5.04	8.23	-4.46	-1.42	-2.61	-2.99	-1.75	
42nd day	189.2± 6.29	229.5± 2.80	191.5± 5.45	229.3± 4.5	188.7± 7.18	223.2± 10.4	224.5± 3.05	
56th day	210.2± 3.655	242.5± 2.03[b]	195.3± 3.403[c]	249.3± 7.2***	208.8± 12.30	222.7± 11.2***	195.2± 3.98	
% change	15.76	11.85	-3.93	19.74	12.25	8.95	-5.92	

All values are expressed as mean±SEM (n=6), and %change in weight. Group 3 is compared with Group 1 and 2. Groups 4, 5,6 and 7 are compared with Group 2 and 3. *p<0.05, **p<0.01, ***p<0.001.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid

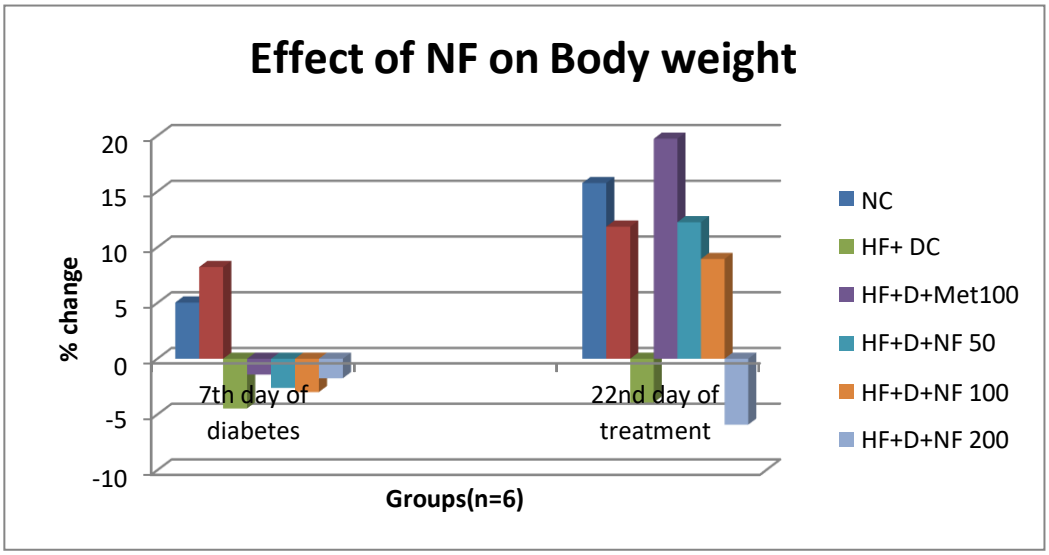

Fig.5.25 Effect of 21 days treatment of NF on body weight

H. Effect of NF on liver and renal histopathology :

In the present study the histology of hepatocytes and renal tissues of the experimental animals has been studied & findings observed were as below. Photomicrograph of liver sections of NC rats showed normal architecture where as HF rats with focal fatty change. In contrast, the liver sections of HF-DC group rats showed moderate focal fatty change, ballooning of cell, and congestion of blood vessels in central vein. Liver sections of NF group rats with 100 &200mgkg showed more glycogen vacuoles, less oedema, and normal structure of central vein and no congestion of sinusoids the effect was comparable with metformin treated group. Photomicrograph of kidney sections of NC andHF rats showed normal architecture of thekidney. In contrast, kidney sections of the HF-DC group showed the glomerular bed congestion, tubular necrosis, inflammation and cloudy degeneration compared to NC group. The flavonoid (NF) and Met treated group rats showed normal glomeruli and more tubular glycogen vacuoles compared to HF-DC group rats.

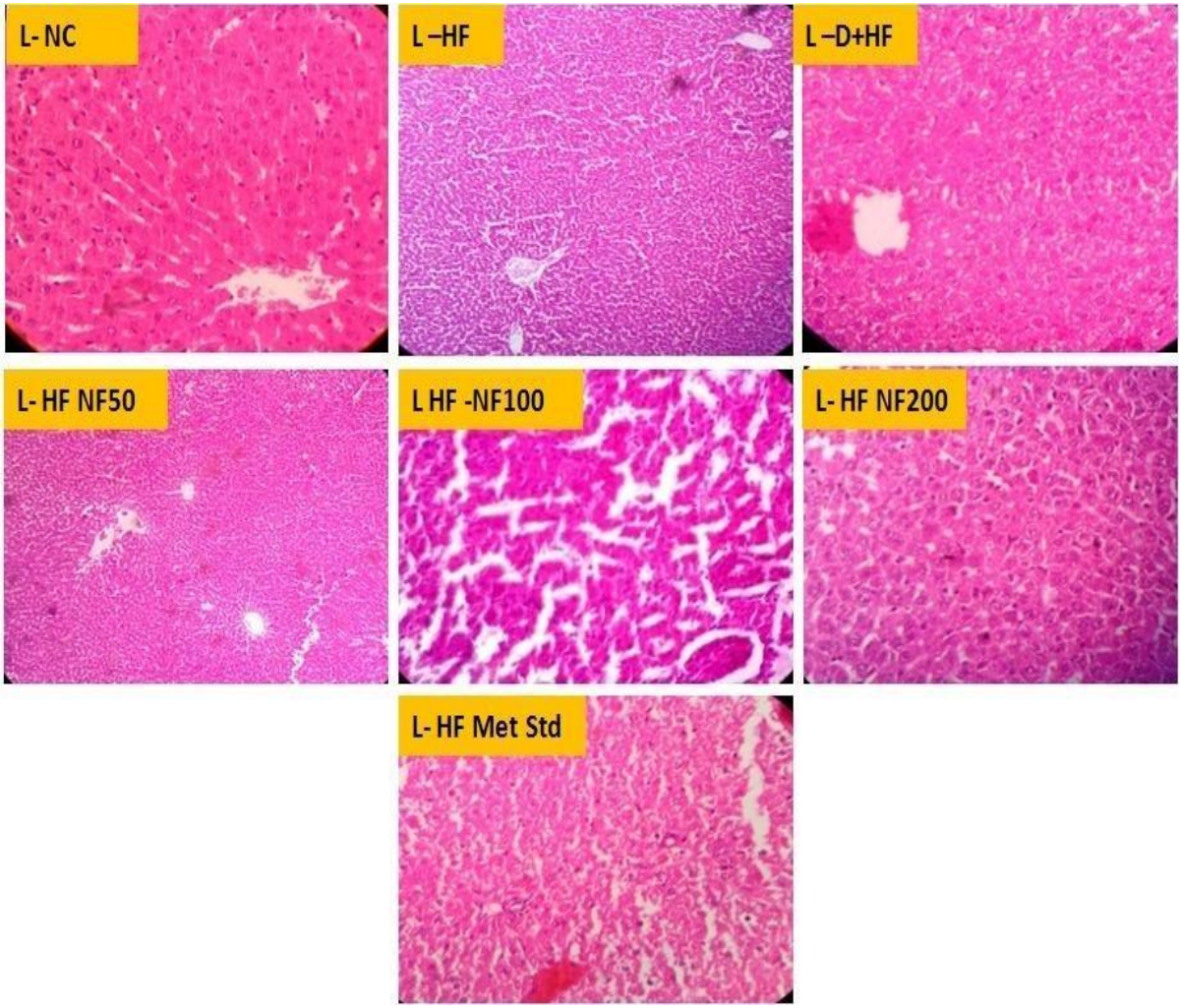

Fig.5.26.Effect of NF on liver histopathology.NC-Normal control, HF-high fat control, HF+ D- High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.

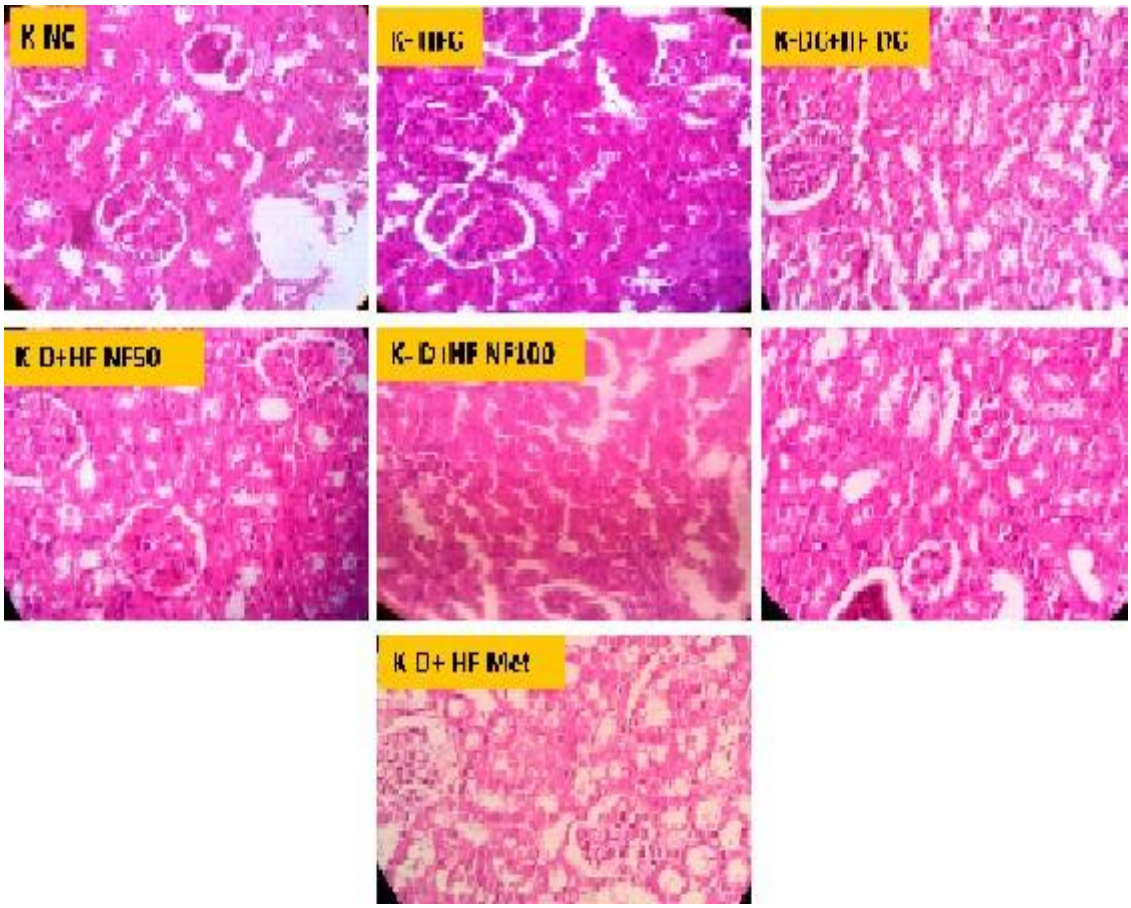

Fig.5.27.Effect of NF on renal histopathology.NC-Normal control, HF-high fat control, HF+ D-High fat with Diabetic control, HF+D+Met – HFD& Diabetic with Metformin100mg, HF+D+NF - HFD& Diabetic with novel flavonoid at 50,100&200mg, NF- Novel flavonoid.

CHAPTER 6.

DISCUSSION:

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The present study was designed to synthesize, characterize and validate the Novel flavonoid compound for in-vitro and in-vivo antioxidant, antidiabetic with antidyslipidemic efficacy in established research models. In literature this work is first time reporting and clinical utility of this synthetic novel compound can only be considered once it is validated in a more robust and permanent T2DM research models.

Synthesis and spectral study: In the present study, due to the instability, insolubility, and tedious & expensive isolation procedure limitations of natural flavonoids, synthetic flavonoids have been used[1]. Algar-F- Oyamada method was used to synthesize newer flavonoid molecule due to the presence of simple two way step and good yield[2]. The synthesized compound after purification, was subjected for elemental analysis and spectral characterization with IR(FT/IR, ^{1}H NMR, ^{13}C NMR and Mass spectroscopic study. The IR spectrum of NF compound showed a prominent absorption band in the region of 1607-1617 cm $^{-1}$(1610 cm $^{-1}$) suggesting the presence of carbonyl group (α and β unsaturated), peaks at 1560, 1479, 1346, 1294,1120cm $^{-1}$these regions gives hint for predence of aromatic double bond –(H of Ar) and a band between 3450-3200 cm^{-1}(has peak at 3227 cm^{-1}) establishes the presence of phenolic hydroxyl group in its chemical structure as present in flavonoids. **The molecular ion peaks**showed in mass spectral study were in consistent with the molecular weight of the compound. Similarly various peaks at 12.37 (1H s OH) and 6.98 – 8.10 (m H of Ar) seen in ^{1}H NMR, represents the presence of hydroxyl group and number of hydrogen atom bond in the phenolic ring and bands at thre region[**193.17;(C *s,* C=O); and 129-140 ; (C *m,* of**

Ar)] in^{13}C NMR spectra suggests the presence of carbonyl group and number of carbon atoms in the structureof the synthesised compound[3-4].

Since the peaks of the test compound at spectral characterization study are in support with the proposed structure and properties of flavonoid given by software, the compound synthesized is belongs to a flavonoid nature, and was assigned the code it as NF for sustained identity in the study.

Free radical scavenging activity: In this study, syntheticcompounds such as VMF41,VMF43,VMF45, VMF46 and NF flavonoids exhibited dose dependant increase in free radical scavenging activity against super oxide, hydroxyl and nitric oxide radicals.This property of these compounds may be due to thepresence of electron donating nature of the substituent's groups such as –OH and -CH3 in benzopyran nucleus. According to Seyoum et al. 2006[5], fromvarious structure–activity relationship (SAR) studies of flavonoids it was reported thatthe number and location of the phenolic –OH groups in their structure are more important in exhibiting the effective radical scavenging activity. These statements are in support with the radical scavenging activity of NF compound in this study.

In the present study, the synthetic novel flavonoid(NF) , since showed highest and statistically significant free radical scavenging activity amongst all, this compound has been selected for further in-vivo and in- vitro antidiabetic screening activity in established models.

Effect on in-vitro antidiabetic enzymes: In the present study, NF compound exhibited insignificant activity on alfaglucosidase and dipeptidyl peptidase IV enzyme inhibition. However the NF compound exhibited dose dependant significant($p<0.05$) inhibition on alfaglucosidase enzyme in in-vitro study and the effect is comparable to acarbose. The

presence of hydroxyl groups on aromatic ring may be responsible for this activity. MTT assay was performed to establish NF compound cell viability activity on C2C12 cell lines. The NF compound exhibited less than 200microgram/ml dose was its IC50 value.At concentration more than 400 µg/ml this compound showed less cell viability. Bjelakovic et al. 2007 explained that mostly oxidative metabolites, formed as sub products during the scavenging of free radicals by antioxidants, are responsible for the adverse effect of these compounds, inducing toxicity at the cellular level. Moreover, the toxicity of these metabolites could worsen oxidative stress.This study supports the cell toxicity and IC50 of NF compound. This MTT test helped to use safest dose ie $1/10^{th}$ of IC50 of NF for glucose uptake study.

In glucose uptake study in C2C12 myotome cells the compound showed remarkable glucose uptake activity and effect is comparable with metformin. This activity may be due to insulin sensitizing activity on peripheral fatty and skeletal muscular tissues.Study by Harley. B, et al.,(2020)[6] demonstrated that the flavanols together with tormentic acid and arjunolic acid due to increased expression of glucose transporter-4 (GLUT-4), stimulated the uptake of glucose above the control in C2C12 myotubes. This flavonoid study on glucose uptake in skeletal muscle is correlating the NF compound's glucose uptake activity in C2C12 cell lines.

Acute toxicity study: In present study, noanimal died at limit dose (2000 mg/kg), this indicates LD_{50} is greater than 2000 mg/kg. However, NF showed moderate toxicity signs like hepatitis(175mg/kg),and focal fatty changes & glycogenesis features(500mg/kg and 2000mg/kg) in hepatocytesliver of rats (Figure-5.16) in comparison to the control vehicle on histopathological study.Thus the therapeutic dose selected for further in -vivopharmacological activity is $1/10^{th}$,$1/20^{th}$& $1//40^{th}$ of limit dose (200, 100 and 50mg/kg b.w) as a safe. Since it induced glycogenesis features in

hepatic cells at acute toxicity pathological reports, explains the presence of compound's hypoglycemic action. The combination of antioxidant and antiglycation(Glycogenesis as showed in this study)activities of the test compound will have complementary benefits in T2D and related metabolic disorders. This evidence supported to use for further analysis of this NF compound in in-vivo diabetic models.

Effect on OGTT:,In present study it was observed that, at 30 min after starting the glucose tolerance test, the blood glucose level increased rapidly in the glucose control groups and continued up to 60 min but in metformin and NF at 50, 100 and 200mg/kg treated groups the glucose induced hyperglycaemia was prevented when compared to control group. This increase in blood sugar after oral glucose load may be direct absorption from the intestinal tract into the blood stream. The prevention of hyperglycemias after glucose challenge in NF treated group may be due to the insulinotropic or insulin sensitizing action in tissues and this effect is consistent with metformin effect in the study.

High fat diet-low dose streptozotocin diabetic model:

Currently people like to consume processed food that is prepared in oil or ghee which consists of a high amount of saturated fats with a meagre amount of polyunsaturated fatty acids (PUFAs).R.P Munshi (2014) et al., reported that the increasing prevalence of diabetes correlates with these faulty dietary habits (like eating more fast food rich in saturated fat content in the form of pizza, burger etc.) and sedentary lifestyle. The nutritional imbalances resulting from excessive dietary intake of fat and/or refined carbohydrates lead to development of insulin resistance.3 It was well established that, high fat containing diet like Indian Vanaspati and coconut oil with appropriate combination would serve as perfect high fat containing diet to develop insulin resistance

as they have high contents of trans and saturated fatty acids which are known to induce insulin resistance. [7]

STZ is antibiotic produced by the soil bacterium Streptomyces achromogenes that exhibits broad spectrum of antibacterial properties and known to cause dysfunction or damage beta cell of pancreases through cytotoxic effect and thus leads to hyperglycaemia[8]. Intraperitoneal injection of high dose STZ alone will degenerate pancreatic beta cells totally but combining of high fat diet feeding with low dose STZ will prevent pancreatic beta cells to become insulin deficient [9].There are various diabetic animal models which can be used to investigate the pathogenesis of diabetes. Many such models are also employed in the screening of new anti-diabetic drugs candidates. However, none of these models are able to reproduce the pathological complexity encountered in human diabetes, especially T2D.Hencecombination high fat diet feeding, and low STZ administration will becost-effective, easy-to-prepare, and rapid onset experimental suitable model for mimicking T2DM and insulin resistance.

Impact of high fat diet on FBG,Insulin and HOMA index in experimental rats: After four weeks of dietary high fat diet manipulation, mild hyperglycemia(146.47 ± 5.24) was induced in rats but not sufficient to sustain diabetes. T2D developed one week after injection of STZ. However, HFD, on its own, could have produced a condition similar to prediabetic, insulin-resistant state in humans. HFD administration for 4 weeks was able to produce insulin resistance(Table;5.14) by decreasing beta cell mass without much reducing the insulin levels.Insulin resistance induced by HFD could be mediated through glucose–fatty acid cycle[10] (Randle et al. 1963). Due to excess fat intake leads to generation of fatty acid oxidation to generate triglycerides. The preferential use of increased fatty acids for oxidation than glucose by different tissues, result in the insulin-mediated reduction of hepatic glucose output and reduces the

glucose uptake or utilization in skeletal muscle. This leads to compensatory hyperinsulinemia, a common feature of insulin resistance[11].In this condition low dose of STZ administration would makes the perdiabetic state into diabetic state resembling human T2 DM[12].

We have successfully demonstrated that administration of low dose streptozotocin (35mg/kg) to 4 week high-fat diet fed rats to made β-cell damage and an insufficient production of insulin, insulin resistance and thus the elevation of fasting blood glucose level leading to increased glycated hemoglobin(HbA1C). We were successful in the induction of diabetes as the animals showed an elevated level of fasting blood glucose level(FBG), elevated HOMA-IR, decreased HOMA-B, slightly elevated HbA1C and increased lipid profile. The standard drug metformin ameliorated all the parameters further demonstrating that it was a working model for T2DM.

Effect of NF on biochemical parameters and HOMA index in High fat diet-low dose streptozotocin diabetic rat model.

Using this HFD-low dose STZ diabetic rat model, we investigated the effect of synthesised NF (benzopyrone thiophene analogue) on biochemical parameters. The administration of these compounds for three weeks resulted in a significant reduction in FBG, serum TG, TC and other dyslipidemic markers along with reversing to near normal level of fasting serum insulin levels, indicating its potential hypoglycemic and hypolipidemic activity. At 100 to 200mg/kg body weight the test NF drug exhibited statistically significant favourable effects in this model of diabetes. Further, supplementation with test compound reduced HOMA-B values and improved glucose tolerance, suggesting a decrease in insulin resistance caused by HFD-STZ in rats. And these effects are comparable with standard metformin. These mechanisms might be due

to like metformin, which could have reduced FBG by a combination of the following mechanisms: (1) sensitizing insulin action in target tissues through diminishing lipolysis in adipose tissue, (2) reducing glucose production in liver, (3) enhancement of insulin-mediated glucose disposal in skeletal muscle.

The positive impact of NF test drug in improving serum insulin concentration may be probably due to regeneration of damaged β -cells of pancreas which is supported with higher HOMA- B index (beta -cell function) exhibited by NF at 100 and 200 mg/kg doses compared to diabetic control group rats (Table-5.16). A reduced HOMA-IR and high HOMA-B index observed with NF at both 100 and 200 mg/kg doses may be due to improved insulin sensitivity and peripheral glucose uptake. This beneficial activity of test flavonoid NF on HOMA B is consistent with the studies conducted by He et al. (2012) that a 15 days of curcumin a isolated flavonoid suplimentation (50 mg/kg/day orally) in High fat diet-fed mice, showed improvement in HOMA-IR and glucose tolerance. Sharma B et al(2012) and Asanaliyar & Nadig (2021) reported[13-14] that the flavonoid containing aqueous seed extract of Eugenia jambolana (Syzygium cumini) significantly stimulated PPAR-γ. The NF compound also reversed the raised glycated hemoglobin in HFD –STZ rats and effects are comparable with the standard metformin. This may be due to enhancing insulin mediated glucose utilization in peripheral tissues through activation of PPAR-γ receptors.

Attenuation of dyslipidemia by NF compound may be due to either from the inhibition of TG synthesis in liver or increased TG clearance in the periphery (by stimulating the enzyme lipoprotein lipase (LPL) and/or inhibition of dietary cholesterol absorption from the intestine (Srinivasan et al. 2005; Colca et al. 1991)[15-16].

Further, in the present study, enzymatic and non-enzymatic antioxidants such as, GSH SOD & Catalase were significantly ($p<0.05$) lowered, whereas MDA(LPO) were increased in untreated HFD-STZ rats in comparison to normal control rats. NF compound restores endogenous antioxidant levels and in turn, reduces oxidative stress in HFD- low dose STZ rat model in dose dependant manner and effects are comparable with metformin. These effects may be due to donation of free radicals or anions to scavenge the harmful endogenous free radical fallowed by protecting the body tissues thereby enhancing the endogenous antioxidant mechanism. Presence of ^{OH*} groups and thiophene substitute on phenyl ring may br responsible to have all these antidibetic and antioxidant property of this test NF compound. Julfikar Ali et al.(2020) reported[17] that three weeks treatment of a flavonoid rich extract TAY increased the SOD,CAT and GSH levels apart from decreasing the raised TBAR. These findings from different researchers on flavonoid support the results of NF compound in oxidative stress.

In the present study, the body weight of rats treated with HFD alone was increased in contrast with the STZ treated ratsin HFD+D control groupwhich showed significant decrease in body weight. This decrease in body weight may be due to the development of hypoinsulinemia mediated lipolytic and proteolytic action. The animals treated with NF compound showed resistance in reduction of body weight induced by STZ and is comparable with metformin. The increased body weight in NF treated group could be attributed to the better utilization of nutrients, glucose, amino acids, fatty acids and other macro-molecular components due to improved insulin secretion by the beta-cells. These results on body weigt are in consistant with the research report performed by Chander et al. 2015[18].

Effect of NF on hepatic and renal tissue pathology in HFD and low dose STZ induced diabetic rat model. **t**he liver sections of HF-DC group rats showed moderate

fatty degeneration, ballooning of cell, less glycogen vacuoles and congestion of blood vessels in central vein when compared to normal control and HF diet treated rats.Liver sections in the HF+D+Met and HF+D+NF treated group rats, showed more glycogen vacuoles, less edema, and normal structure of central vein with no congestion of sinusoids. Hypoglycemia produced by NF and metformin may be the reason for this and also it could be due to increased insulin secretion.kidney sections of the HF-DC group rats demonstrated the glomerular bed congestion, tubular necrosis, inflammation and cloudy degeneration compared to NC group. The flavonoid (NF) and Metformin treated group rats showed normal glomeruli and more tubular glycogen vacuoles compared to HF-DC group rats indicating the renoprotective nature of NF compound against the HF+ STZ inducedglomerular bed congestion, tubular necrosis, inflammation and cloudy degeneration.The HFD + STZ induced fatty changes and damaged histoarchitecture in both tissues were reversed to normal architecture with novel flavonoid treatment.

Hypoglycemia produced by NF and metformin may be the reason for this and also it could be due to increased insulin secretion. Glycogen synthesis in the liver and skeletal muscles of rats gets impaired in diabetes .The liver glycogen content was markedly reduced in diabetic animals, which was in proportion to insulin deficiency. The decrease in hepatic glycogen content in diabetes is due to the lack of insulin which ultimately results in the inactivation of glycogen synthase enzyme[19]. The increased glycogen vacuoles appearance in NF and metformin treated diabetic rat renal and hepatic tissues suggesting the insulin mimetic activity and these actions of NF are in consistent with the above reference articles. All protective effects produced by NF synthetic flavonoid may be due to the presence of hydroxyl group and thiophene analogue in this compound due to which produced the antioxidant activity as reported in previous studies conducted by us

CHAPTER 7

SUMMARY & CONCLUSION:

CHAPTER 7

SUMMARY & CONCLUSION:

- In the present study, the compound is reported for the first time as a synthetic novel flavonoid(NF) moleculefor its in-vitro and in-vivo antidiabetic and antioxidant activities in different research models.
- The NF compound has been shown promising effect against superoxide, hydrogen and nitric oxide free radicals very effectively. This free radical scavenging activity of NF can be attributed to their ability to donate hydrogen, donate/accept electron, quenching of •OH, protection of DNA from Fenton-reagent induced damage and inhibition of lipid peroxidation.
- HFD and STZ induced hyperglycemia, insulin resistance; beta cell dysfunction and raised tissue glycatedhemoglobin have been effectively reduced to near normal values by this test synthetic novel flavonoid (NF) in this study.
- Abnormal raised lipid peroxidation and decreased hepatic SOD,CAT and GSH levels have been effectively reversed to a near normal values by novel flavonoid and metformin treated animals in this study.
- Hepatic and renal tissues damage induced by STZ and HFD in this study have been corrected to some extent in the animals treated with NF and metformin compound in the present study.
- Similarly, the antiglycationand anti dyslipidemicactivity in diabetic research models ismostly because of the high potential antioxidant activity due to presence of thiphene and hydroxyl group in its chemical structure.

- **Research hypothesis** that there is a significant effect of synthetic novel flavonoid(NF) on oxidative free radicals and hyperglycemia causing enzymes or factors at in vitro methods and significant protective effect of synthetic novel flavonoid(NF) on diabetic rat models induced by low dose STZ and high fat diet **has been proved**.
- This flavonoid(NF) may play a significant role in ameliorating diabetes mellitus with limited host toxicity or may be used as lead molecule in the development of newer antidiabetic drug candidates for the treatment of insulin resistant diabetes mellitus.
- However, further studies are required to elucidate the exact mechanism of action and clinical trials are necessary to confirm utilityof this synthetic novel flavonoid in clinical conditions.
- **Scope for future research**: Its radical scavenging action may offer potential target lead molecules for screening & possible therapeutic intervention in other oxidative disorders such as cancer, Alzheimer's diseases etc., also. Hence, the study in this direction is planned.
- **Limitations:** In the present study, the limited in-vitro radical scavenging and in-vitro antidiabetic activity was performed. So the NF compound still needs to be explore for other pharmacological screening activity.
- This compound was studied only for acute toxicity in mice in the present study, this may not be sufficient to prove its potential safety profile. So needs to be carried out for long term toxicity study in different species.
- In silico molecular docking studies could not be done in our set up.

- Needs additional Spectral characterization for identification the specific moieties on it.

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CHAPTER 8-ANNEXURES

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Dr. Prabhulingayya Bhixavatimath. Reg No: 17PHD003**

2. Title of the Thesis: **"Pharmacological Screening of Antioxidant, Hypolipidemic and Antidiabetic Activities of Novel Synthetic Flavonoid in High Fat Fed followed by Low Dose Streptozotocin Induced Diabetes Mellitus in Rat Model"**

3. Department: **Pharmacology**

4. Name of the Guide & Designation: **Dr. Akram Naikwadi, MD, Professor & HOD**

5. Name of the Co-Guide & Designation**:Dr.Yasmeen Maniyar, MD, Professor &HOD**

The above thesis was verified for similarity detection. The report is as follows:

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Similarity Index (%): **Four percent (04%) Total word Count: 36366**

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Name & Designation **Name & Designation**

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Annexure -I

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.....The thesis may be considered for submission to the University. The software report is attached.

Signature of the Guide
Name & Designation
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Prof. & HOD
Dept. of Pharmacology
BLDE (DU) Shri B.M.Patil Medical College Hospital & R.C.

Verified by (Librarian)
Name & Designation
Dr. Prabhulingayya Bhixavatimath
Librarian
BLDE (Deemed to be University)
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Signature of Co-Guide
Name & Designation
Dr Yasmeen Maniyar
Professor & HOD
Dept. of Pharmacology
B. N. Medical College & HSK, Hospital & Research Centre, Navanagar BAGALKOT 587102

Signature of Student
(DR.PS.Bhixavatimath)

Annexure-2.

Ethical clearance certificate

B. V. V. Sangha's
S. NIJALINGAPPA MEDICAL COLLEGE
& HANAGAL SHRI KUMARESHWAR HOSPITAL & RESEARCH CENTRE,
NAVANAGAR-BAGALKOT - 587 103.
KARNATAKA, INDIA
Recognised by Medical Council of India New Delhi No.U.12012/224/2015-ME.1 And
Affiliated to Rajiv Gandhi University of Health Sciences, Bangalore. Karnataka.

Dr. Ashok S. Mallapur M.S. (Gen. Surgery) FAIS
* Dean / Principal
* Prof of Surgery
* Leproscopic Surgeon

Phone : Office - 08354 - 235360, 235340
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Email : snmcmedbgk@yahoo.co.in (Office)
Website : www.snmcbgk.in

Dept. of Pharmacology, S. N. Medical College, BAGALKOT
No. 142/18
Date. 26/6/18

INSTIUTIONAL ANIMAL ETHICS COMMITTEE
S.N. MEDICAL COLLEGE
REG NO: 829/AC/04/CPCSEA

Ref No: SNMC/IAEC/2018-19

Date: 25/06/2018

CLEARENCE CERTIFICATE

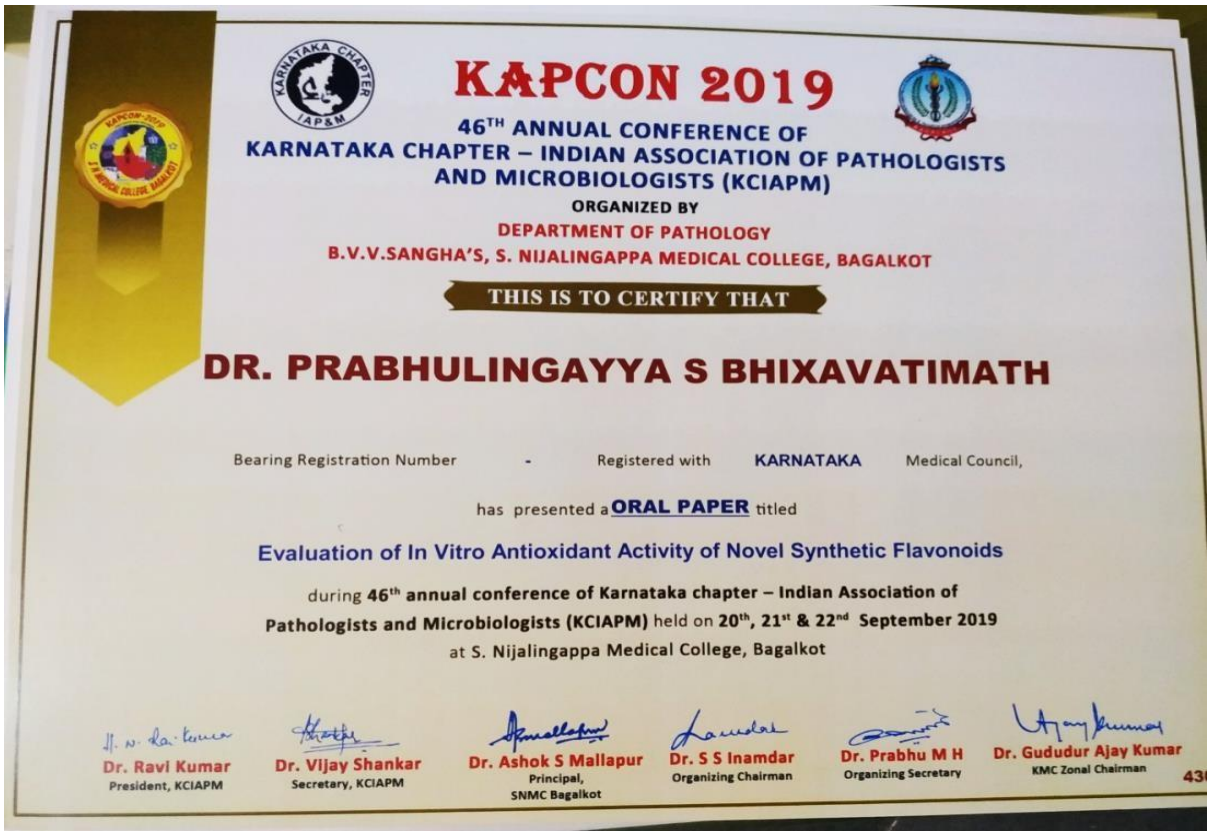
This is to certify that the project titled **"Pharmacology Screening of Antioxidant, Hypolipemic and Antidiabetic Activities of Novel Synthetic Flavonoid in High Fat Fed Followed by Low Dose Streptozotocin Induced Dibetes Mellitus in Rat Model"** by principal Investigator **Mr. Prabhulingayya Bixavatimath** has been approved by IAEC in the meeting held on 25/06/2018

Chairman
Institutional Ethics Committee
S Nijalingappa Medical College
H S K Hospital & Research centre
Bagalkot – 587102

CPCSEA Nominee

: Dr. Ashok S. Mallapur, Principal's Quarters, No: A-3, S.N.M.C.Campus Navanagar,Bagalkot - 587 103 (R) ☎:08354-200219
email : drmallapur@gmail.com

Annexure 3. Paper presentation certificates



KAPCON 2019

46TH ANNUAL CONFERENCE OF
KARNATAKA CHAPTER – INDIAN ASSOCIATION OF PATHOLOGISTS AND MICROBIOLOGISTS (KCIAPM)

ORGANIZED BY
DEPARTMENT OF PATHOLOGY
B.V.V.SANGHA'S, S. NIJALINGAPPA MEDICAL COLLEGE, BAGALKOT

THIS IS TO CERTIFY THAT

DR. PRABHULINGAYYA S BHIXAVATIMATH

Bearing Registration Number - Registered with KARNATAKA Medical Council,

has presented a ORAL PAPER titled

Evaluation of In Vitro Antioxidant Activity of Novel Synthetic Flavonoids

during 46th annual conference of Karnataka chapter – Indian Association of Pathologists and Microbiologists (KCIAPM) held on 20th, 21st & 22nd September 2019
at S. Nijalingappa Medical College, Bagalkot

Dr. Ravi Kumar, President, KCIAPM
Dr. Vijay Shankar, Secretary, KCIAPM
Dr. Ashok S Mallapur, Principal, SNMC Bagalkot
Dr. S S Inamdar, Organizing Chairman
Dr. Prabhu M H, Organizing Secretary
Dr. Gududur Ajay Kumar, KMC Zonal Chairman

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9th ANNUAL INTERNATIONAL CONFERENCE of INDIAN ACADEMY OF BIOMEDICAL SCIENCES

27th, 28th, 29th February 2020

Organized By
DEPARTMENT OF BIOCHEMISTRY
D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR (MAHARASHTRA, INDIA)

A Constituent College of
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Deemed To Be University, Kolhapur-Reaccredited by NAAC with 'A' Grade

CERTIFICATE

Type of Conference-Multi speciality

(CPD Code–MMC/MAC/2020/F–014720)

This is to certify that

Dr/Mr/Ms Prabhulingayya S. Bhixavatimath

has Participated as Delegate / Delivered an Oral / ~~Poster presentation~~ in the 9th Annual International Conference of Indian Academy of Biomedical Sciences.

He / She is granted 4 CPD credit points by Maharashtra Medical Council.

Prof. (MS) Syed Mudassar, President, IABS
Prof. Abbas Ali Mehadi, Secretary, IABS
Prof. Bipin M. Tiwale, Organizing Secretary
Dr.R.K.Sharma, Organizing Chairman
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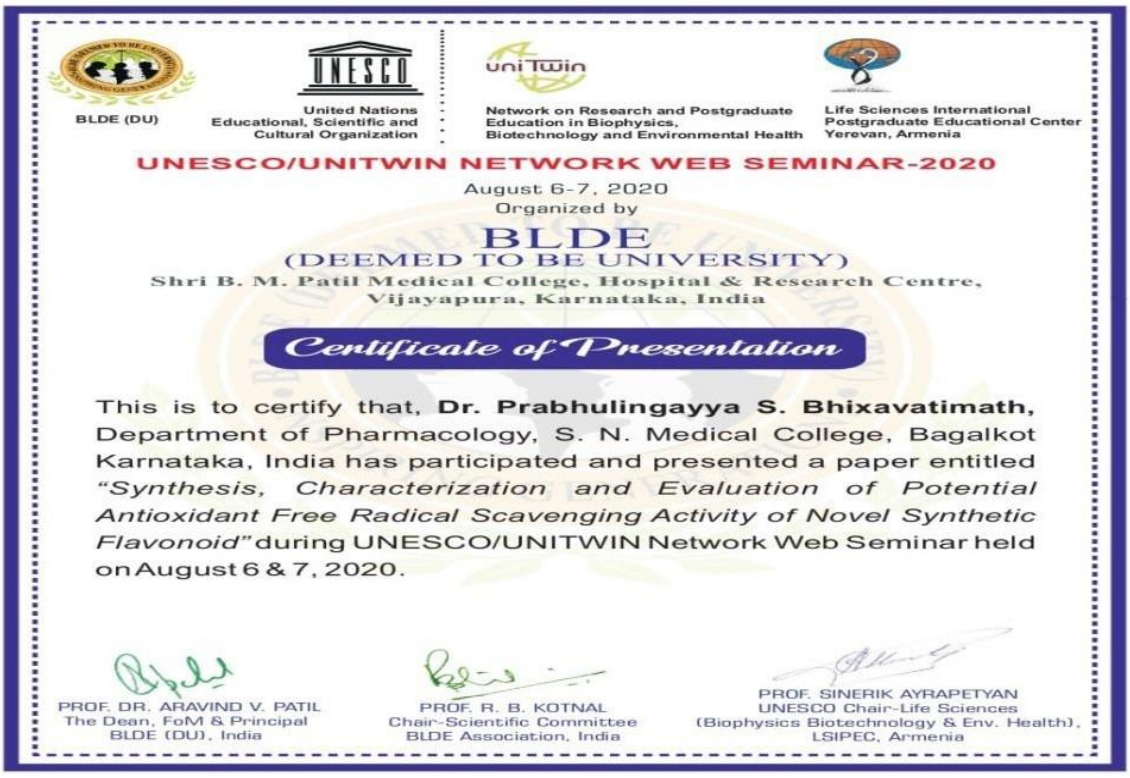

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United Nations Educational, Scientific and Cultural Organization

Network on Research and Postgraduate Education in Biophysics, Biotechnology and Environmental Health

Life Sciences International Postgraduate Educational Center Yerevan, Armenia

UNESCO/UNITWIN NETWORK WEB SEMINAR-2020

August 6-7, 2020

Organized by

BLDE

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Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka, India

Certificate of Presentation

This is to certify that, **Dr. Prabhulingayya S. Bhixavatimath,** Department of Pharmacology, S. N. Medical College, Bagalkot Karnataka, India has participated and presented a paper entitled *"Synthesis, Characterization and Evaluation of Potential Antioxidant Free Radical Scavenging Activity of Novel Synthetic Flavonoid"* during UNESCO/UNITWIN Network Web Seminar held on August 6 & 7, 2020.

PROF. DR. ARAVIND V. PATIL
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PROF. R. B. KOTNAL
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PROF. SINERIK AYRAPETYAN
UNESCO Chair-Life Sciences
(Biophysics Biotechnology & Env. Health),
LSIPEC, Armenia

CERTIFICATE

AWARDED TO

EXPERIMENTAL PHARMACOLOGY LABORATORY

Prabhulingayya S Bhixavatimath

Co-Authors: Yasmeen Maniyar, Akram Naikwadi

For

Poster Presentation

On

"Synthesis and Invitro Antidiabetic Evaluation Of Thiophen Substituted Novel Synthetic Flavonoid"

In

International Webinar on "Pharmacology and Toxicology"
&
40th Annual Conference of Society of Toxicology (STOX), India

STOX-2021

Online Conference on 29th - 30th January, 2021

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Secretary General

Prof. Bikash Medhi
Organizing Secretary

Prof. S S Sharma Joint-
Organizing Secretary

Certificate issued no. STOX-2021/PP/039

Annexure 4. Publications

Research article

Synthesis and characterization of 2-thiophen flavonoid analogue for free radical scavenging antioxidant analysis

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(Received: January 2022 Revised: May 2022 Accepted: June 2022)

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ABSTRACT

Introduction and Aim: Currently research is focussed on the use of antioxidants in preventing oxidative stress induced diseases. Flavonoids present in plant sources gaining more therapeutic importance due to their antioxidant property, but their solubility and some pharmacokinetic concern, diverted the current research study towards the synthesis of these flavonoids for their therapeutic potential. The study was aimed to synthesize and characterize the 2-thiophen flavonoid analogue for free radical scavenging antioxidant activity.

Materials and Methods: The test synthetic compound PNF(3-hydroxy-2-(thiophen-2-yl)-4H-chromen-4-one) a thiophen substituted flavonoid was synthesized from condensation fallowed cyclization reaction in laboratory and DPPH, superoxide, nitric oxide, and hydroxyl radical scavenging activity was determined through established *in vitro* methods.

Results: It suggests that the test flavonoid (PNF) possesses the potent free radical scavenging on DPPH, superoxide, nitric oxide, and hydroxyl radicals with IC_{50} values of 6.89±25µg/ml,4.04µg/ml, 2.44µg/ml and 2.96µg/ml respectively. The radical scavenging potential of test PNF synthetic compound at different concentrations(10µg-150µg) was compared with that of standard antioxidants such as BHA, ascorbic acid used in the study.

Conclusion: Results from this study indicates that the novel flavonoid PNF exhibited the considerable dose dependant *invitro* antioxidant activity. These possible activities could be useful to consider the novel synthetic thiophen derived flavonoid as therapeutic antioxidant agent.

Keywords: Antioxidant; DPPH; flavonoid; hydroxyl; nitric oxide.

INTRODUCTION

In recent years, there is an increase attention towards antioxidants usages in oxidative stress induced diseases, because of their protection over various cells, their organelles and even over metabolic pathways from the harmful oxidative radicals (1, 2). Free radicals are electrically charged unpaired electrons. These radicals being highly unstable, become neutralized when they pick up the electrons from other surrounding biological substances. During this neutralization reactions though earlier radical was deactivated, subsequently newer radical will be produced, leading cascade of radical reaction. Within a fraction of time several free radicals can be generated. Reactive oxygen species (ROS) such as O_2,H_2O_2, peroxyl radicals, OH^- and nitrogen species are most common free radicals usually synthesized during normal cellular metabolism (3). At physiological levels these free radicals play a key role in controlling cell viability, cell signaling, cellular differentiation, protecting cells through killing and degradation of pathogenic organisms (4). But if these free radial levels go beyond normal levels and in presence of weak antioxidant system, leads to the development of oxidative-antioxidant imbalance and oxidative stress induced diseases. Though the body has its own mechanism to combat oxidative stress, when exposure is more than body's antioxidant capacity, the problem aggravates (5) and therefore exogenous antioxidants· which alleviate the harmful effects of free radicals are important (6).Antioxidants are a group of compounds that neutralize or prevent free radicals or reactive species and avoid cell or tissue damage caused by oxidative potential of free radicals (7). Although several indigenous molecules have been proved for their antioxidant activities (8) only few of them have been used clinically. Hence, there is a demand to explore new molecules with antioxidant properties to combat oxidative stress.

Flavonoids are polyphenolic compounds present in herbal food products, are considered as natural antioxidants as they are one of the important constituent present in animal and human diet (9,10).

Though, natural flavonoids being potent antioxidants but have limitation in terms of standardization procedure, instability, solubility concern pharmacokinetic properties (11). Hence a novel test flavonoid was synthesized in laboratory to determine its scavenging antioxidant potential against hydroxyl, nitric oxide, and superoxide anion free radicals in *invitro* methods.

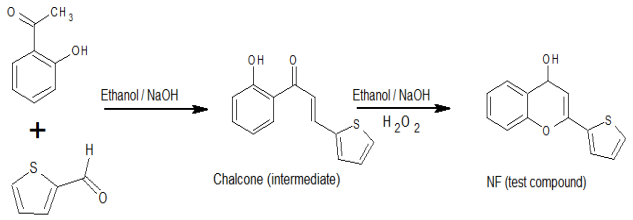
MATERIALS AND METHODS

2-Hydroxyacetophenone and 2-thiophen benzaldehyde(Sigma Aldrich), 1,1-diphenyl-2-picrylhydrazyl, methanol, disodium hydrogen phosphate(Na2HPO4), NADH, EDTA, butylated hydroxy anisole(BHA), nitrobluetetrazolium (NBT), TBA, 2-deoxy-2-ribose, trichloroacetic acid, phenazine methosulphate, potassium ferricyanide and standard ascorbic acid, were obtained from SD fine chemicals and Sigma Loba chemicals.

Synthesis of test flavonoid compound (PNF)

The compound was synthesised by usingAlgar-F-Oyamada method(12)and spectralcharacterization for IR, NMR and Mass spectroscopy was done after recrystalization procedure.

Chemical reaction and proposed structure of test PNF compound drawn from chemsketch software



PNF-3-hydroxy-2-(thiophen-2-yl)-4H-chromen-4-one

Analytical methods

Extent of the reaction and purities of the obtained products were assessed by using TLC plates (Merck 60 F254). The test compound was analyzed for elemental analysis. Open capillaries method was used to determine the melting point in ^{0}C and uncorrected. KBr disc method was employed to get IR spectra through FTIR spectrometer-8300(Shimadzu, Japan) and FTIR 4100.^{1}H-NMR and^{13}C-NMR spectra were recorded on High resolution Ft-MR Multinuclear SpectrometerBrukar) in deuterated chloroform with internal standard tetramethylsilane(TMS), working at 400MHz and 75MHz frequencies, respectively.

Chemical shift readings are presented in δ (ppm)downfield, with respect to an internal standard TMS. GC-MS-QP5050A (Shimadzu) was used to assess the Mass spectral data of the test compound (PNF) in quality assurance department, at Manipal College of Pharmaceutical Sciences Manipal.

Structural and analytical data are presented in Table 1. Spectral data of the synthesised compound is given in Table 2.

In vitro free radical scavenging activity

Various *in vitro* procedures were employed to determine the free radical scavenging property of novel synthetic flavonoid (PNF) as given below,

DPPH radical scavenging activity(13)

The free radical scavenging capacity of the test drug PNF and standard drug Butylated Hydroxy Anisole (BHA) was determined using stable DPPH radical method.

Methanolicsolutions of flavonoid and Butylated Hydroxy Anisole (BHA) in dimethyl sulfoxide (DMSO4) at various concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml) were added to 0.5ml of 0.1 mM methanolic solution of 0.004% DPPHin test tubes separately and were allowed to stand at room temperature for 20 min. A 0.1 mM methanolic solution of DPPH without test was used as control, whereas Butylated Hydroxy Anisole was employed as reference standard. After observing de-colorization of DPPH, the absorbance's of samples were determined at 517 nm. The percentage radical scavenging activity was calculated by using the formula:

DPPH scavenged % = $(A_{Cont} - A_{Sample})/A_{Cont}$ X100;
A_{Cont} and A_{Sample} are the absorbance values at 517 nm. Lower absorbance values suggest high radical scavenging action.

Hydroxyl(OH^*) radical scavenging assay

This was performed according to a method reported by Klein *et al.*, (14**).** To a glass tube containing 1 ml of various concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml)of test flavonoid (PNF) & Ascorbic acid (AA), 1ml of iron-EDTA solution(0.13% ferrous ammonium sulphate 0.26% EDTA), 0.5ml of EDTA solution, 1ml of DMSO (0.85% in 0.1 mol/L phosphate buffer pH 7.4),0.5ml of 0.22% ascorbic acid was added to start the reaction and this was caped tightly & heated in a boiling water bath at 80°-90°C for 15 min. After this ice-cold TCA (17.5%) was added to stop the reaction. Then 3 ml Nash reagent solution was added to the above reaction mixture, and incubated at room temperature for 15 min for colour change.

By using spectrophotometer, the intensity of the yellow colour absorbance was measured at 412 nm against a reagent blank. As reference standard Ascorbic acid (AA) was used. The % hydroxyl radical scavenging activity (%HRSA) was calculated using the formula:

$$\% \text{ HRSA} = [(A_{control} - A_{sample}) / A_{control}] \text{ X } 100.$$

Nitric oxide (NO^*) scavenging assay

The colorimetric Griess reaction method described by Sun J*et al* was used to determine the test flavonoid and standard reference drug's ability to inhibit NO radical generated from sodium nitroprusside (15). The sodium nitroprusside (10 mM, 4 ml)) in phosphate buffer saline (7.4 pH 1ml), was mixed with 1 ml of test compound and standard BHA drugs dissolved in methanol at different concentrations (10µg/ml,50µg/ml, 100µg/ml and 150µg/ml) were incubated at 25ºC for 150 min. After this, 1.5 ml of incubated solution containing nitrate was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and allowed to stand for 30 min in dark place. The absorbance of the chromophore formed during the diazotization of the nitrite with sulphalinamide and the subsequent coupling with napthyethylene diamine dihydrochloride was measured at 546nm. All the tests were performed in triplicate. Percentage NO inhibition was calculated by using the following formula:

% NO radical scavenging activity= (control OD – sample OD) ×100 /control OD

Superoxide anion radical scavenging activity

Superoxide radical inhibition activity was performed using the method described by Jia *et al.*,(16) with slight modification. In this assay, the measurement of nitroblue tetrazolium(NBT)through spectrophotometer is important. The test synthetic flavonoid (PNF) and reference standard ascorbic acid (AA) in phosphate buffer (0.05M and pH 7.8) at different concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml) were added to the respective test tubes containing mixture of 1ml of nitroblue tetrazolium(150 µM)and 1ml of nicotinamide adenine dinucleotide(234 µM)in Tris-HCl buffer 16mM pH 8.0; Then to start the reaction, 1ml of 40 µM PMS (phenazine methosulphate) solution was added to each of the above test tubes containing reaction mixtures and incubated at room temperature for 5 min. The samples optical density was measured at 560 nm. Percentage super oxide radical scavenging activity was calculated by using the following formula: % SO radical scavenging activity = (control OD - sample OD) ×100/ Control OD.

Statistical analysis

One-way analysis of variance(ANOVA), followed by multiple Dunnet comparison tests were used for statistical analysis of the results. $p<0.001$ was considered significant. Data are represented as mean ± S.E.M (n=3).The IC_{50} values for test compound as well as reference standards were calculated using the Microsoft excel.

RESULTS

In the present work, Claisen – Schmidt's condensation reaction between 2 hydroxy acetophenone and thiophen benzaldehyde yielded an intermediate chalcone. Then cyclisation reaction of this chalcone was made with hydrogen peroxide in alkaline medium to get the final product of our interest that is flavone. The progress of reaction and purity of test compound was monitored on TLC plates in appropriate solvent (20% Ethyl acetate in n hexane). The molecular formula, chemical name and experimental data of synthesized test compound were reported in Table1.The purified test compound PNF, characterized by IR, NMR and mass spectroscopy. The spectral data PNF test compound supported the structures and properties of proposed flavonoid compound.

DPPH free radical scavenging activity

It is one of the standard assay among the radical scavenging activity studies as it provides rapid results for the radical inhibition activity of particular compound(17). The results of DPPH scavenging activity of test (PNF) and standard BHA drug are depicted in Fig**.**1.In this study, the novel synthetic flavonoid (PNF) exhibited the significant radical scavenging activity in a dose dependent manner and it was comparable to standard BHA. At 150µg dose the synthetic flavonoid (PNF) and standard BHA showed about 34.12 % and 93.21 % of radical inhibition activity respectively.

Table 1: Substitution pattern moieties on ring A and B structure of the flavonoid compound (PNF)

Code	R-H	Elemental analysis	Molecular formula	IUPAC Name	MW	% yield	Melting point(^{0}C)	Rf* Value
PNF	R^1-H R^2-H R^3-H R^4-H	C (63.92%) H(3.30%) O (19.65%) S(13.13%)	$C_{13}H_8O_3S$	3-hydroxy- 2-thiophen-2-yl-4H-chromen-4-one	244	67	196-199	0.63

Table 2: Spectral data of synthesized compound PNF

Code	IR (KBr)ν(cm-1)	^{1}H NMR(CDCl3)/ δ in ppm	^{13}C NMR(CDCl3) / δ in ppm	Mass spectra
PNF	3227,(b,OH), 1610(C=O), 1560,1479,1346, 1294,1120–(H of Ar)	12.37 (1Hs OH) and 6.98 – 8.10 (mH of ArH)	193.17(Cs,C=O);and 129-140,(Cm, of Ar).	244.26 Da

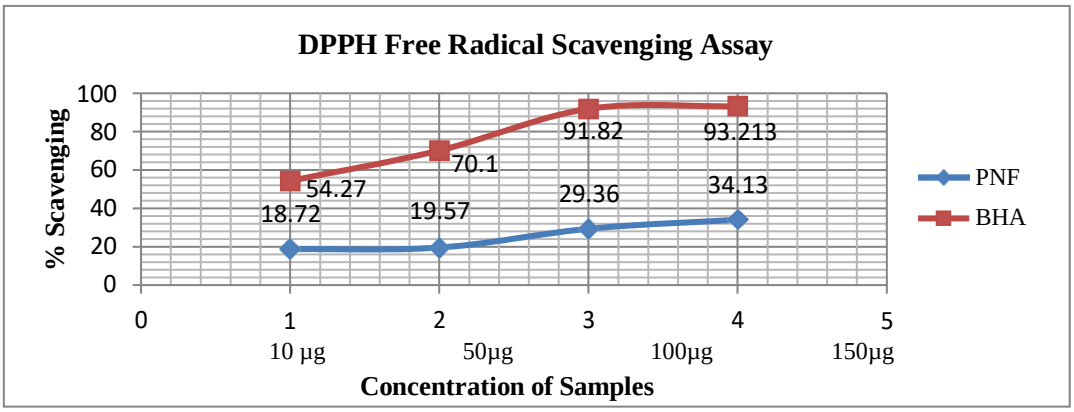


Fig. 1. DPPH free radical percentage scavenging activity of test drugs such as PNF and BHA at different concentrations

The IC_{50} of standard BHA and test synthetic flavonoid (PNF) is around 53±01µg and 6.89±25µg respectively which suggest that the synthetic flavonoid test drug is less potent scavenging antioxidant than the standard drug.

NO radical scavenging activity

The test PNF compound's NO scavenging activity results were summarized in Fig. 2. From the analysis, the synthetic novel flavonoid (PNF) showed the highest (46.08%) radical inhibitory effect at 150µg /mL with the IC_{50}value of 4.04 µg /ml. Whereas, the standard antioxidant BHA at150µg /ml concentration, showed the highest NO radical scavenging activity up to 54.34 % with the IC_{50} of 3.41 µg/ml Fig. 2 explains about the NO radical inhibitory activity of test flavonoid. The test flavonoid (PNF) showed a low to moderate nitric oxide scavenging activity. As the concentration of synthetic flavonoid (PNF) increases, it's percentage radical scavenging activity also increases.Therefore, NO radical scavenging activity of synthetic novel flavonoid (PNF) was quite comparable to BHA at a concentration of 150µg/mL.

Hydroxyl radical scavenging activity

Among the reactive oxygen species, hydroxyl radical is most common, causing severe damage to adjacent biomolecule. By generating OH^- radicals using Ascorbic acid–iron EDTA reaction mixture, hydroxyl radical scavenging activity was determined. Fig.3. shows the hydroxyl radical scavenging activity of various concentration of test PNF and standard ascorbic acid. The PNF at 150µg concentration exhibited highest hydroxyl radical scavenging activity which is comparable to standard ascorbic acid. The IC_{50} values of test synthetic novel flavonoid (PNF) and Ascorbic acid (AA) were 2.96 and 0.88 µg/mL respectively. The test synthetic novel flavonoid (PNF) showed the increasing order of OH^- scavenging potential in a dose dependent manner.

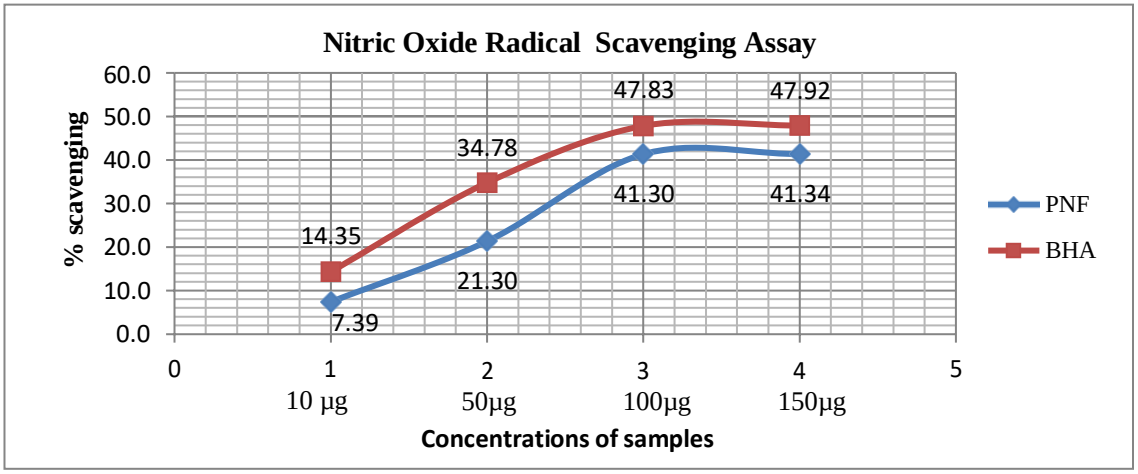


Fig. 2. Percentage nitric oxide free radical scavenging activity of test drugs such as PNF and BHA at different concentration

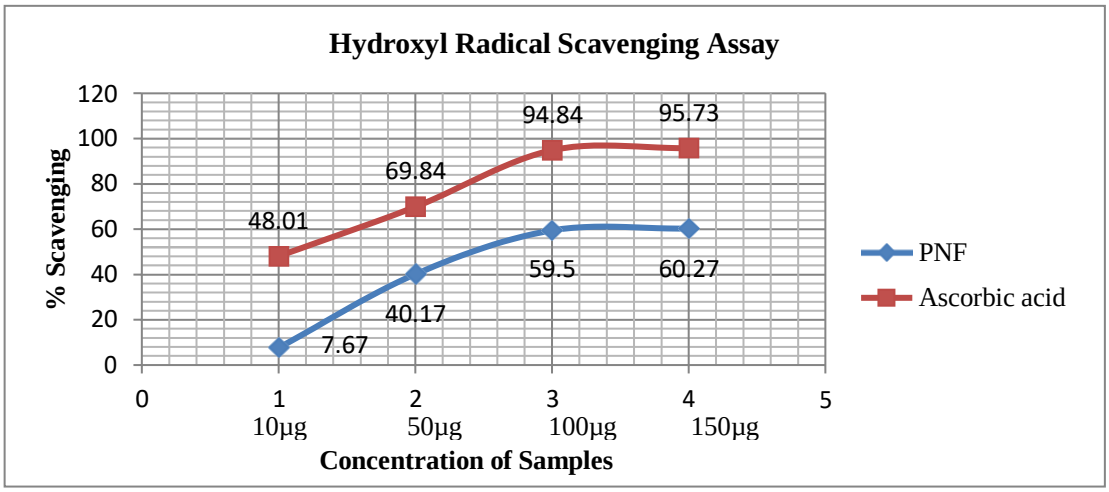


Fig. 3. Percentage hydroxyl free radical scavenging activity of test drugs such as PNF and ascorbic acid at different concentration

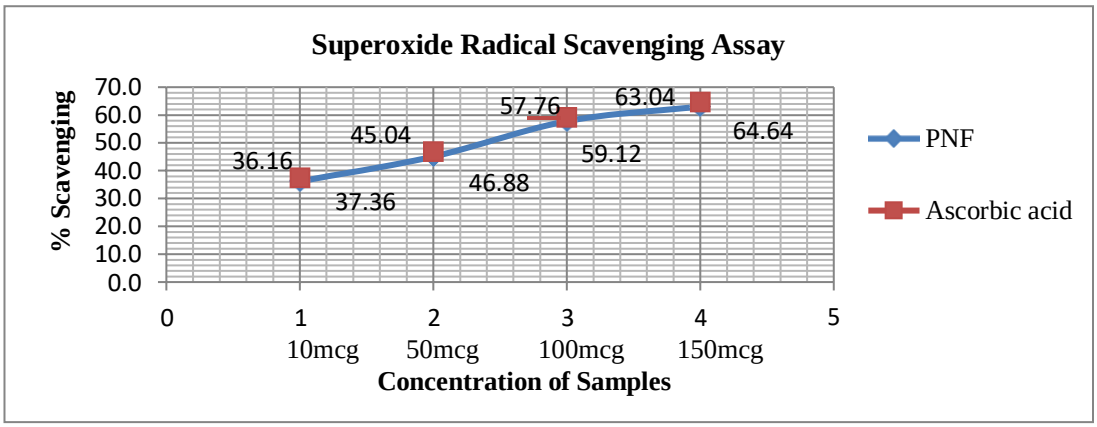


Fig. 4 Percentage superoxide free radical scavenging activity of test drugs such as PNF and ascorbic acid at different concentration

Superoxide radical scavenging activity

In this assay, superoxide anions were formed by using PMS-NADH-NBT system. In this assay, the capturing of superoxide anion and better free radical scavenging potential of the test compounds which were correlated with decrease in absorbance values when observed at 560 nm. The results obtained in this assay, showed that the IC_{50} values of test synthetic novel flavonoid (PNF) and Ascorbic acid (AA) were 2.44 and 2.28 μg/ml respectively, indicating better superoxide (O_2^{*-}) anion radical scavenging activity. In addition to this, these compounds exhibited dose-dependent O_2^{*-} scavenging activity. Fig.4 represents the percentage inhibition of superoxide radical generation at different concentrations of test PNF compound and standard ascorbic acid. PNF exhibited concentration-dependent scavenging activities against superoxide anion radicals generated in PMS-NADH systems. The test novel flavonoid (PNF) showed significant superoxide radical scavenging activity (63.04%), at 150 µg/mL concentration.

DISCUSSION

Capillary tube system of melting point and TLC methods were used to determine the purity of test synthesized compound (PNF). The synthesized compound was further established by IR, 1H NMR, and mass spectral studies. Based on spectral data, it was proved that the synthesized chalcone and flavone derivative meet the standard values of various spectral techniques. Free radicals are highly reactive molecules, associated with oxidative damage where as antioxidants are reducing agents, as they donate electrons to free radicals and preventing them from oxidative damage to biological structures. Due to the instability, insolubility and tedious standardization procedures, the natural flavonoids though highly potent and good antioxidant properties, they are not preferred now a days (11). Hence, study has been undertaken to synthesize the novel flavonoid in laboratory to determine its free radical scavenging antioxidant activities in *invitro* methods.

The diphenyl-picrylhydrazine is unstable, purple colored nitrogen radical and has strong absorption at 517 nm. In this DPPH assay, less stable DPPH radicals are reduced to the more stable yellow coloured DPPH in presence of antioxidants. This method is based on the fact that the hydrogen ion donation by antioxidants in the alcohol solution converts the free radicals such as DPPH to yellow colored non radical reduced form

DPPH-H (18). From the results the test novel flavonoid (PNF) exhibited the dose dependent DPPH scavenging activity this might be due to its proton donating property.

Nitric oxide, apart from its beneficial effects, is also involved in pathological conditions such as inflammation, cancer etc. Nitric oxide was generated from sodium nitroprusside and measured by Greiss reaction. Scavengers of nitric oxide compete with the oxygen, leads to decreased nitric oxide production. Presence of phenolic structure in the test flavonoids as said in previous research reports Hernández *et al*., (19) and Revathi and Rajeshwari (20) play a vital role in NO radical scavenging activity, which might be the reason behind differential inhibitory effect observed in this study. Nitric oxide radical scavenging property of the test PNF compound may be due to the electron donating nature of the substituent's OH and -CH_3 groups present in its benzopyran nucleus.

By the oxidation reaction with the dimethyl sulphoxide (DMSO), hydroxyl radicals were formed along with formaldehyde which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent (21). Results tabulated in table-3 suggests that the synthetic flavonoid(PNF) when used at different concentrations(10 µg/ml, 50 µg/ml and 100 µg/ml)shown to exhibit a dose dependent hydroxyl radical scavenging activity. The presence of hydrogen donating ability of phenolic groups in the test synthetic flavonoid is proposed to be responsible for free radical scavenging activity which supports the work of Pavithra and Vadivukkarasi (22).

In different biological systems, superoxide anions are the most common free radicals generated and under conditions of oxidative stress the concentration of these anions increases(23). Though these superoxide radicals have a weak oxidant, relatively less chemical reactivity, under oxidative stressful conditions, they can produce very dangerous singlet oxygen and hydroxyl radical reactive components which leads to lipid peroxidation(24). The flavonoids are often considered as antioxidants as they have property of donating charged molecules due to the presence of electron donating substituent groups like –OH, -CL and –CH_3 in their chemical benzopyran nucleus(25). Therefore these compounds donated their electrons to the superoxide and scavenge them to prevent their further interaction with NBT followed by inhibition of formation of blue colour formazan product. The presence of OH groups and methyl groups in their chemical structure may be responsible for their radical scavenging power of the test flavonoid(PNF) and standard drug. This action of these test and standard compounds may be explained due to their property of quenching the oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the aromatic ring.

CONCLUSION

From the present study, it is concluded that the test novel flavonoid (PNF) has a significant free radical scavenging activity as compared to standard drugs such as BHA and ascorbic acid. Further study is required to discover its molecular mode of action involved in antioxidant activity. Antioxidant potential of flavonoids may be the major role for their therapeutic implications in oxidative stress diseases such as diabetes, dyslipidemia, Alzheimer's disease etc.,

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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RESEARCH ARTICLE

An *In vitro* Evaluation of Potential Free Radical Scavenging Antioxidant activity of selected Novel Synthetic Flavones

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ABSTRACT:

Background: The study of free radicals nowadays have become more attention as these are evoked continuously due to hazardous environmental conditions and food habits. Endogenously free radicals are introduced in our body due to exposure to different physiochemical conditions or some pathological states by various mechanisms. Oxidative stress occurs due to exposure of excess free radicals to body. Antioxidants act as a major defence against the free radical-mediated injury by scavenging them. Majority of the flavonoids found in plant products are known as antioxidants as they selectively scavenge the free radicals. **Methods:** Synthetic flavones (VMF41, VMF 43, VMF 45 and VMF 46) having different side chains on the**:** 3-hydroxy-2- (5-methyl, 7-methyl, 6-methyl, and 7--methoxy) 2- thiophen-4H-chromen-4-one structure were examined for free radical scavenging potential over nitric oxide, hydroxyl, and superoxide anion radicals by using appropriate *in-vitro* assay methods. **Results and Discussion:** Overall, with few exceptions, all the synthetic flavonoids (SFs) exhibited moderate free radical scavenging activity as compared to the standard drug. The test samples in the study showed dose dependant activity of scavenging activity. In nitric oxide radial scavenging activity, test flavonoids VMF 45 and VMF 46 exhibited significant scavenging activity at 50- 100µg/ml concentrations. The SFs VMF 43 and VMF 46 showed a good scavenging activity for hydroxyl, nitric oxide radicals and compounds VMF41, and VMF 43 showed significant scavenging activity at 50 µg/ml to 100µg/ml concentrations for superoxide radicals. However all the SFs at 10µg/ml concentration showed the low or poor radical scavenging activity. **Conclusion**: It is clear that these synthetic flavonoids (SFs) can be considered as potential antioxidant agents, however needs to be further tested at in vivo experiment to consider them as a lead antioxidant drug candidates.

KEYWORDS: Antioxidant, Benzopyran, Flavonoid, Free radical, Nitric oxide, Superoxide.

1. INTRODUCTION:

Free radicals are highly reactive and unstable molecules containing one or more unpaired electrons, behaves like oxidants or reductants. Because of this property these free radicals attaches very quickly on the defective cell or that area causes a micro crack in the mitochondrial cell, causes cell damage[1]. In modern life we are frequently exposed to free radicals, which can damage any cell in the body with which they come into contact and cause hazardous effects. The excess free radicals can cause a condition called oxidative stress which is mainly responsible for development of many oxidative diseases such as cardiovascular diseases, atherosclerosis, diabetes,

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rheumatoid arthritis, aging, and skin lesions etc, mainly due to harm done to the body by free radicals[2]. Though the body has its own mechanism to combat oxidative stress, when exposure is more than body's capacity, the problem aggravates[3]. There comes the importance of exogenous antioxidants which alleviates the harmful effects of free radicals[4,5]. Antioxidants are a group of compounds that neutralize or prevent free radicals or reactive species and avoid cell or tissue damage caused by oxidative potential of free radicals[6,7]. Many natural substances have been explored for their antioxidant activities[8,9]. There is still need to evaluate new substances for their antioxidant potential to find better alternative sources for treatment of conditions related to oxidative stress caused by free radicals.

Flavonoids are polyphenolic compounds widely distributed and found in plant materials including naturally occurring plant based food products. These are also considered as natural antioxidants as they are known to be the integral part of human and animal diet [10]. Majority of the flavonoids studied, selectively react with free radicals to act as antioxidants[11-12]. Depending on the available information of synthetic flavonoids, the present study was undertaken to evaluate and confirm the radical scavenging antioxidant effects of synthetic flavonoids (dibenzopyrone as a basic nucleus with thiophen substitution) such as VMF41, VMF 43, VMF 45 and VMF 46, using in-vitro radical scavenging activity methods. Nitric oxide, hydroxyl and superoxide anion free radical scavenging methods were used for evaluating antioxidant activities.

2. MATERIAL AND METHODS:

Materials:

The synthetic flavonoids (SFs) VMF 41, VMF 43, VMF 45 and VMF 46, collected from Dr Vijayakumar D Ptofessor and Principal at Cauavery college of pharmacy Mysore Karnataka India, to which synthesis and characterization details have already been reported elsewhere[13]. 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, disodium hydrogen phosphate (Na_2HPO_4), sodium nitroprusside, sulphanilamide, phosphoric acid, nicotinamide adenine dinucloetide (NADH), Ethylene Diamine Tetra Acetate (EDTA), butylated hydroxy anisole (BHA), nitrobluetetrazolium (NBT), thiobarbituric acid, 2-deoxy-2- ribose, trichloroacetic acid, phenazine methosulphate, potassium ferricyanide and standard ascorbic acid, were obtained from SD fine chemicals, Sigma loba chemicals. All other chemicals used were of AR grade and used as received.

2.2. Evaluation of In Vitro Antioxidant Activity:

2.2(a). Nitric oxide radical scavenging assay:

The nitric oxide scavenging activity was estimated according to the earlier described method.[14] Various concentrations (10µg, 50µg and 100µg) of SFs compounds dissolved in DMSO (1mg/ml), as well as Butylatedhydroxy anisole (BHA) a standard compound were taken in separate tubes and the volume was uniformly made up to 3ml with 0.1M phosphate buffer (pH 7.2). Then 1ml of sodium nitroprusside (5mM) at phosphate buffer saline (pH7.2) was added to each tube. At room temperature the reaction mixture was incubated for 30 min. Without the test compound a control with an equivalent amount of methanol was used. After the incubation, 1.5ml of above solution including control was mixed with 1.5ml of Griess reagent (2% phosphoric acid, 1% Sulphanilamide and 0.1% N-1-Naphthylethylene diamine dihydrochloride). By using UV-visible spectrometer the absorbance of chromophore formed was measured at 546nm. The IC_{50} values for each test compounds as well as standard preparation were calculated. Percentage nitric oxide radical scavenging activity was calculated using the following formula:

% NO radical scavenging activity = (Control OD - Sample OD) ×100/ Control OD

The IC_{50} values for each test compounds as well as standard preparation were calculated.

2.3 (b). Hydroxyl radical Scavenging activity (HRS):

The hydroxyl free radical scavenging activity of synthetic flavonoids(SFs) were assayed according to the method[15] described earlier. This assay was used to determine the scavenging activity of test samples at different concentrations in the presence of free hydroxyl radicals which damage the body cells.

The ascorbic acid-iron-EDTA model was used as hydroxyl free radical generating system. In this system, totally aqueous system in which iron - EDTA and ascorbic acid was used that reacts with each other to form hydroxyl radicals. Various concentration of the test SFs (10µg, 50µg and 100µg) in DMSO(1mg/ml), were separately taken in test tubes and made up to 250µl with 0.1M phosphate buffer. The above sample solutions were mixed with 1ml of iron EDTA solution, 0.5ml of EDTA solution, 1ml of DMSO, and then 0.5ml of ascorbic acid was added to start the reaction. The reaction mixture after 15 min of incubation in a boiling water bath, ice-cold TCA was added to stop the reaction. Then 3ml of Nash reagent was added and again the mixture was kept incubation for 15min at room temperature to develop the colour. Then absorbance of the yellow colour formed was measured spectrophotometrically at 412nm against reagent blank. Ascorbic acid (AA) was used as reference standard and percentage hydroxyl radical scavenging activity (% HRSA) was determined by

% HRSA = (Control OD - Sample OD) ×100/ Control OD.

The IC_{50} values for each test compounds as well as standard preparation were calculated using the Microsoft excel.

2.3 (c). Superoxide Anion Radical ($O2^{*-}$) Scavenging Activity:

Scavenging of the superoxide (O2•-) anion radical was measured by the reduction of NBT, according to method which was previously reported[16]. The reaction mixture of 1ml contained phosphate buffer (20mM, pH 7.4), NBT (50µM), PMS (15µM), NADH (73µM), and various concentrations (10, 50 and 100µg/ml) of test samples were incubated for 5 min at room temperature. The blank consisted of pure DMSO instead of alkaline DMSO. The absorbance was measured at 562nm using a UV–VIS spectrophotometer. against blank to determine the quantity of formazan generated. All tests were repeated three times. As positive control ascorbic acid was used. Super oxide radical scavenging activity in percentage was calculated using the following formula:

% SO radical scavenging activity = (Control OD - Sample OD) ×100/Control OD.

The test compounds and standard drug IC_{50} value were calculated by using the Microsoft excel.

3. RESULTS:

Different concentrations ranging from 10, 50 and 100 µg/ml of test novel synthetic flavonoids (SFs) VMF 41, VMF 43, VMF 45 and VMF 46 in DMSO were tested for their free radical scavenging activity with different in vitro methods. It was observed that the test compounds showed a promising free radical scavenging activity in a dose dependent manner. The maximum percentage inhibition with different concentrations of novel synthetic flavonoids for different radical scavenging models is given in the following Tables.

Nitric Oxide radical scavenging:

Nitric oxide scavenging activity was performed with four test novel synthetic flavonoid (SFs) compounds and BHA as standard drug. the reductive potential of these compounds exhibited in a dose dependent manner as shown in Table- 1. From these results, it is clear that the IC_{50} value calculated for SF compounds were found significant as compared to BHA. The IC_{50} of compounds VMF 41, VMF 43, VMF 45 and VMF 46 were 5.72 µg/ml, 5.65µg/ml, 3.65µg/ml and 3.99µg/ml respectively which were found higher than IC_{50} of BHA standard drug. At 100µg/ml concentration the percentage inhibition of VMF 45 and VMF 46 compounds on nitric oxide radicals showed 42.61% which is almost equivalent to that of standard BHA.

Table. 1. Percentage Nitric oxide Radical Scavenging Activity of Synthetic Flavonoids (SFs) compared with Standard

Test Samples and their percentage scavenging activity					
Concentration	**VMF 41**	**VMF 43**	**VMF 45**	**VMF 46**	**BHA**
10µg	5.65	10.00	11.30	5.65	14.35
50µg	15.65	22.61	18.70	13.04	34.78
100µg	24.35	26.96	42.61	42.61	47.83
IC_{50}	5.72± 5.12	5.56± 0.28	3.65± 1.32	3.99± 0.13	3.06± 0.24

Nitric oxide radical scavenging assay. The nitric oxide radical scavenging activity of novel synthetic flavonoid (SFs) and the standard BHA. The data represent the percentage nitric oxide inhibition.

Hydroxyl radical scavenging:

Results were tabulated in Table-3. Here the SFs VMF 41, VMF 43, VMF 45 and VMF 46 at different concentrations (10µg/ml - 100µg/ml) were tested, where they found to exhibit a dose dependent hydroxyl radical scavenging activity.

The SF compounds VMF 43and VMF 46 at 100µg/ml concentration the percentage inhibition of hydroxyl radical were 41.67% and 46.83% respectively. And their IC_{50} values were 3.66µg/ml and 2.06µg/ml respectively where as that of standard ascorbic acid percentage inhibition on hydroxyl radical was 94.84% with IC_{50} value 1.1 suggesting that test flavonoids have less potent than standard drug. The test flavonoids VMF 41 and VMF 45 in this study showed negligible scavenging effect on hydroxyl radicals as shown in the table 2.

Table. 3. Percentage Hydroxyl Radical Scavenging Activity of Synthetic flavonoids (SFs) compared with Standard

Test Samples and their percentage scavenging activity					
Concentration	**VMF 41**	**VMF 43**	**VMF 45**	**VMF 46**	**Ascorbic acid**
10µg	8.33	5	4	9	48.01
50µg	16.17	12.17	19.00	24.67	69.84
100µg	33.33	41.67	29.50	46.83	94.84
IC_{50}	4.46 ± 3.27	3.66 ± 0.97	4.54± 1.02	2.06± 2.81	01.11± 0.13

Hydroxyl radical scavenging assay. The hydroxyl radical scavenging activity of novel synthetic flavonoid (SFs) and the standard ascorbic acid. The data represent the percentage inhibition of deoxyribose degradation.

Superoxide radical scavenging:

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 562nm with the test synthetic flavonoids (SFs) VMF 41, VMF 43, VMF 45 and VMF 46 and the reference compound ascorbic acid indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in Table 1, IC_{50} values of test synthetic flavonoids (SFs) VMF 41, VMF 43, and the standard ascorbic acid on superoxide radical scavenging activity

were 3.44±0.36, 4.51±0.36 and 02.2±0.62 respectively. The IC_{50} value of the synthetic flavonoids were more than that of the standard drug. It suggests that the only test flavnoids VMF 41 and VMF 43exhbited more superoxide radical scavenging activity than other test flavonoids but lesser than ascorbic acid. At 50 µg/ml, the percentage inhibition of the synthetic flavonoids (VMF 41 and VMF 43) were 24%, 18.4%, whereas that of ascorbic acid was 46.88%.

Table. 4 Percentage Super oxide Anion Radical Scavenging Activity of Synthetic Flavonoids (SFs) compared with Standard

Test Samples and their percentage scavenging activity					
Concentration	**VMF 41**	**VMF 43**	**VMF 45**	**VMF 46**	**Ascorbic acid**
10µg	0.8	0.8	1.6	0	37.36
50µg	24	18.4	8	09.6	46.88
100µg	40	28.0	16	15.2	59.12
IC_{50}	3.44± 0.36	4.51± 0.36	7.75± 0.22	7.491± 0.31	02.2± 0.62

Super oxide radical scavenging assay. The Super oxide radical scavenging activity of novel synthetic flavonoid (SFs) and the standard ascorbic acid.

4. DISCUSSION:

Free radicals are highly reactive molecules, associated with oxidative damage where as antioxidants are reducing agents, since they donate electrons to free radicals and preventing them from oxidative damage to biological structures. Otherwise these excess free radicals may leads to various disease conditions, especially degenerative diseases, and extensive lysis[17].

Antioxidants are molecules that prevent the oxidation from other compounds through hydrogen or electron donors as well neutralize free radicals. Recently, many natural antioxidants have been isolated from different plant materials. According to a Afnan E. Abd-Almonuim *et al,*. flavonoids and phenolic compounds possess the antioxidant and radical scavenging properties. Flavonoids are polyphenolic compounds widely distributed and found in plant materials including naturally occurring plant based food products[18]. Majority of the flavonoids studied, selectively react with free radicals to act as antioxidants. Eventhough, natural flavonoids are highly potent, they show certain limitations as far as their stability, solubility characteristics and kinetics are concerned[19].

Therefore, the present study was undertaken to study the free-radical scavenging ability of synthetic flavonoids through invitro assay methods.

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological functions such as vascular homeostasis, neurotransmission, antitumor, and antimicrobial activities etc. However, many reports suggest that, excess concentration of nitric oxide due to unstable nature under aerobic environment, is implicated in the cytotoxic effects observed in various disorders such as AIDS, cancer, Alzheimer's and arthritis etc[20-21]. Drugs which scavenge the nitric oxide compete with oxygen, leading to less formation of nitrite ions. It would be interesting to develop potent and selective inhibitors of NO* for potential therapeutic use[22].

From results shown in Table1, it is clear that synthetic flavonoids VMF 41, VMF 43, VMF 45 and VMF 46 have shown as antioxidants compared to the BHA but lesser than the standard. These compounds can compete with oxygen to react with nitric oxide radical and thus reduce the generation of the nitrite and peroxy nitrite anions. This property of these compounds may be due to the electron donating nature of the substituent's OH and -CH_3 groups of benzopyran nucleus[23].

Hydroxy radical is a highly reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells and is involved in many pathophysiological processes including DNA strand breakage and K+ loss from the cell membrane[24-25]. If any drug scavenges the hydroxyl radical, they may either scavenge the radical or may chelate the Fe2+ ion making them unavailable for the Fentons reaction. Results tabulated in table-3 suggests that the synthetic flavonoids found to exhibit a dose dependent hydroxyl radical scavenging activity when tested at different concentrations (10µg/ml, 50µg/ml and 100µg/ml). Among these test flavonoids only VMF 43 and VMF 46 based on their IC_{50} values, makes clear that of having good hydroxyl radical scavenging activity but lesser than that of standard ascorbic acid which is having 01.11±0.13 as IC_{50} value .

The radical scavenging power of the test flavonoid and standard drug can be explained by the presence of OH groups and methyl groups in their chemical strictures, as they quench the oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the aromatic ring[26].

Superoxide anion is also very harmful to cellular components[27,28]. Robak and Glyglewski[29] reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions.

From results, it was found that the compounds (SFs) VMF 41, VMF 43, VMF 45 and VMF 46 showed potent free radical scavenging activity compared to the ascorbic acid (standard). However the compounds VMF 41 and

VMF 43 exhibited the highest radical scavenging power 40% and 28% respectively than the other test synthetic flavonoids in this study. The flavonoids are often considered as antioxidants as they have property of donating electrons due to the presence of electron donating substituent groups like –OH, -CL and $–CH_3$ in their chemical benzopyran nucleus[30]. Therefore these compounds donated their electrons to the superoxide and scavenge them to prevent their further interaction with NBT followed by inhibition of formation of blue colour formazan product.

5. CONCLUSION:

From the results obtained in this study, it is concluded that the synthetic flavonoids (SFs) VMF 41, VMF 43, VMF 45 and VMF 46 exhibited the radical scavenging activity. For nitric oxide scavenging activity, VMF 45 and VMF 46 test flavonoids (SFs) have showed better antioxidant activity than VMF 41, VMF 43. In case of hydroxyl radical scavenging assay, the compounds VMF 43 and VMF 46, exhibited the highest scavenging power than the other synthetic test flavonoids. Lastly, the compounds VMF 41and VMF 43 synthetic flavonoids represented the good antioxidant than the VMF 45 and VMF 46 compounds in scavenging the superoxide radicals generated in the assay reactions. To conclude based on results in this study; it is clear that these synthetic flavonoids can be considered as a potential antioxidant agents which need to be further explored for proper design of lead antioxidant drug candidates. Furthermore, the in-vivo antioxidant activity of these synthetic flavonoids needs to be assessed prior to clinical use.

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7. CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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