INVESTIGATION OF RESVERATROL BASED COMPOUNDS FROM DIPEROCARPACEAR FAMILY FOR THE ANTI-DIABETIC POTENTIAL - A MOLECULAR MECHANISTIC APPROACH

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Dedicated to my family and my respected teachers

Declaration

I hereby declare that the PhD thesis entitled **'Investigation of anti-diabetic potential of Resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae Family - A Molecular Mechanistic Approach'** submitted to University of Kerala is an original, independent work carried out by me under the supervision of Dr P Jayamurthy, and it has not been submitted to any other university or institution for any other degree, diploma or title.

Lekshmy Krishnan S

28/03/2022

Trivandrum

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TABLE OF CONTENTS

Content	Page no
CHAPTER 1 Introduction	1
1.Introduction	3
1.1. History of Diabetes	5
1.2. Early Treatments	7
1.3. Types of Diabetes Mellitus	8
1.3.1. Type 1 Diabetes Mellitus	8
1.3.1.1. Epidemiology	8
1.3.1.2. Risk Factors	9
1.3.1.3. Management of blood glucose: Monitoring and Treatment	9
1.3.2. Type 2 diabetes mellitus	9
1.3.2.1. Epidemiology	10
1.3.2.2. Correlations with influencing factors on T2DM	10
1.3.3. More categories of diabetes mellitus	11
1.4. Pathways involved in insulin production, insulin transport, insulin dependent and insulin independent signaling	13
1.4.1. Insulin secretory pathway	13
1.4.1.1. Structure of pancreas	13
1.4.1.2. Insulin	14
1.4.1.3. Biochemical mechanism of insulin secretion	15
1.4.1.4. Carbohydrate metabolism and insulin secretion	16
1.4.1.5. Glucose Regulation	19
1.4.2. Insulin signaling	20
1.4.2.1. Insulin Receptor	20
1.4.2.2. Insulin Receptor Substrates	20
1.4.2.3. Phosphatidylinositol (3,4,5)-Triphosphate and Phosphoinositide 3-Kinase	21
1.4.2.4. Activation of downstream kinases	22

1.4.2.5. Other actions of insulin downstream from PI3-K	*23*
1.4.2.6. GLUT4- the key thespian for glucose uptake	*24*
1.4.3. AMPK Pathway	*25*
1.5. Current treatment for diabetes	*27*
1.5.1. Lifestyle measures	*28*
1.5.2. Anti-diabetic drugs	*28*
1.5.3. Natural compounds	*31*
1.6. Objectives of the study	*32*
1.7. Significance of the study	*32*
CHAPTER 2 ***Screening Of The Resveratrol Oligomers Isolated From Different Plants Belonging To Dipterocarpaceae Family For Their Anti-Diabetic Potential***	*35*
2.1. Introduction	*37*
2.2. Dipterocarpaceae family	*37*
2.3. Resveratrol oligomers	*37*
2.3.1. Ampelopsin F	*39*
2.3.2. (-) Hopeaphenol	*43*
2.3.3. Vaticaphenol A	*46*
2.3.4. α-Viniferin	*49*
2.3.5. NIIST C1 (Novel compound)	*52*
2.3.6. 2,4,8-trihydroxyphenanthrene-2-O-glucoside (THPG)	*55*
2.3.7. Resveratrol hexamer (Vaticanol M)	*58*
2.3.8. ε-Viniferin	*61*
2.4. Objectives	*64*
2.5. Materials and methods	*66*
2.5.1. Materials	*66*
2.5.2. Cell culture and treatment	*66*
2.5.3. Cytotoxicity studies by means of MTT	*66*
2.5.4. Glucose (2-NBDG) uptake in L6 myoblasts	*67*

2.5.5. 2-NBDG uptake analysis by flow cytometry	*67*
2.5.6. Immunofluorescence assay	*68*
2.5.7. Insulin Secretion Assay	*68*
2.5.8. Statistical analysis	*69*
2.6. Results and discussion	*69*
2.6.1. MTT assay	*69*
2.6.2. 2-NBDG uptake analysis by confocal microscopy	*72*
2.6.3. Flow cytometry analysis	*75*
2.6.4. Immunofluorescence assay	*79*
2.6.5. Cytotoxicity in Beta-TC-6 cell lines	*82*
2.6.6. Insulin secretion studies	*85*
2.7. Summary	*87*
CHAPTER 3 ***Elucidation Of Molecular Mechanism Of The Resveratrol Oligomers In Insulin Secretion***	*89*
3.1. Introduction	*91*
3.2. Objectives	*92*
3.3. Elucidation of molecular mechanism of the resveratrol oligomers in insulin secretion	*93*
3.3.1. Material and methods	*95*
3.3.1.1. Cell culture and treatment	*95*
3.3.1.2. Determination of mitochondrial membrane potential in Beta-TC-6 cell lines	*95*
3.3.1.3. Determination of calcium efflux using Fura-2, AM	*96*
3.3.1.4. Western blotting	*96*
3.3.1.5. DPP4 activity assay	*97*
3.3.1.6. Statistical analysis	*97*
3.3.2. Results and discussion	*97*
3.3.2.1. Determination of change in mitochondrial membrane potential	*97*

3.3.2.2. Intracellular calcium efflux	*101*
3.3.2.3. Western blot analysis	*104*
3.3.2.4. DPP4 activity assay	*107*
3.3.3. Summary	*109*
3.4. Elucidation of molecular mechanism of the resveratrol oligomers in insulin signaling	*111*
3.4.1. Materials and methods	*113*
3.4.1.1. Cell culture and treatment	*113*
3.4.1.2. Western blotting	*113*
3.4.1.3. PTP 1b assay	*114*
3.4.1.4. Statistical analysis	*114*
3.4.2. Results and discussion	*115*
3.4.2.1. Western blot analysis	*115*
3.4.2.1.1. IRS -1 expression analysis	*115*
3.4.2.1.2. PI3-K expression analysis	*116*
3.4.2.1.3. AKT expression analysis	*117*
3.4.2.1.4. GLUT4 expression analysis	*119*
3.4.2.2. PTP 1b Inhibition assay	*122*
3.4.3. Summary	*124*
CHAPTER 4 ***Elucidation Of Molecular Mechanism Of The Resveratrol Oligomers In Insulin Independent Signaling Pathway***	*127*
4.1. Introduction	*129*
4.2. Objectives	*130*
4.3. Materials and methods	*133*
4.3.1. Cell culture	*133*
4.3.2. Inhibitor analysis	*133*
4.3.3. Determination of Intracellular Calcium Levels	*134*
4.3.4. Inhibitor analysis with STO-609	*134*
4.3.5. Western Blotting	*134*

4.3.6. Determination of mitochondrial membrane potential	*135*
4.3.7. Determination of mitochondrial content	*135*
4.3.8. Measurement of nitrite content	*135*
4.3.9. Statistical analysis	*136*
4.4. Results and discussion	*136*
4.4.1. Inhibitor studies	*136*
4.4.2. Calcium dependent AMPK signaling pathway	*141*
4.4.3. Change in mitochondrial membrane potential in AMPK signaling pathway	*148*
4.4.4. LKB1 Expression analysis	*153*
4.4.5. AMPK expression analysis	*155*
4.4.6. Downstream events followed by post AMPK activation	*157*
4.4.6.1. p38 MAPK protein expression after AMPK activation	*157*
4.4.6.2. Post mitochondrial alterations after AMPK activation	*159*
4.5. Summary	*165*
CHAPTER 5 ***Summary And Conclusion***	*167*
5.1. Screening of the resveratrol based compounds for their anti-diabetic potential	*169*
5.2. Anti-diabetic potential of Ampelopsin F and (-) Hopeaphenol in insulin secretory pathway	*170*
5.3. Anti-diabetic potential of Ampelopsin F and (-) Hopeaphenol in PI3-K/AKT dependent insulin signaling pathway	*171*
5.4. Anti-diabetic potential of Ampelopsin F in AMPK signaling pathway	*172*
5.5. Anti-diabetic potential of (-) Hopeaphenol in AMPK signaling pathway	*174*

LIST OF FIGURES

CHAPTER 1	***Introduction***
Fig 1.1.	*Global fact of Diabetes Mellitus according to IDF atlas 10th edition, 2021*
Fig 1.2.	*Regional fact sheet of South East Asia according to IDF atlas 10th edition, 2021*
Fig 1.3.	*Anatomy of pancreas*
Fig 1.4.	*Insulin Secretion Pathway*
Fig 1.5.	*Insulin signaling pathway via PI3-K/AKT activation*
Fig 1.6.	*AMPK activation by the upstream kinases*
Fig 1.7.	*Different targets of anti-diabetic drugs*
CHAPTER 2	***Screening of the resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family for their anti- diabetic potential***
Fig 2.1.	*The conditions of resveratrol oligomers in polymerization*
Fig 2.2.	*Structure of Ampelopsin F*
Fig 2.3.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Ampelopsin F*
Fig 2.4.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Ampelopsin F*
Fig 2.5.	*Structure of (-) Hopeaphenol*
Fig 2.6.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of (-) Hopeaphenol*
Fig 2.7.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of (-) Hopeaphenol*
Fig 2.8.	*Structure of Vaticaphenol A*
Fig 2.9.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Vaticaphenol A*
Fig 2.10.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Vaticaphenol A*
Fig 2.11.	*Structure of α-Viniferin*
Fig 2.12.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of α-Viniferin*
Fig 2.13.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of α-Viniferin*
Fig 2.14.	*Structure of NIIST C1*
Fig 2.15.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of NIIST C1*
Fig 2.16.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of NIIST C1*

Fig 2.17.	*Structure of 2, 4, 8 - trihydroxyphenanthrene-2-O-glucoside*
Fig 2.18.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of 2, 4, 8-trihydroxyphenanthrene-2-O-glucoside*
Fig 2.19.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of 2, 4, 8-trihydroxyphenanthrene-2-O-glucoside*
Fig 2.20.	*Structure of Resveratrol hexamer*
Fig 2.21.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Resveratrol hexamer*
Fig 2.22.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Resveratrol hexamer*
Fig 2.23.	*Structure of ε-Viniferin*
Fig 2.24.	*^{1}H NMR spectrum (500 MHz, Acetone-d_6) of ε-Viniferin*
Fig 2.25.	*^{13}C NMR spectrum (125 MHz, Acetone-d_6) of ε-Viniferin*
Fig 2.26.	*Flow chart showing the experimental design for screening of resveratrol oligomers in L6 myoblasts and Beta-TC-6 cell lines*
Fig. 2.27.	*MTT cytotoxicity assay of resveratrol based compounds.*
Fig 2.28.	*2-NBDG uptake assay by fluorescent microscopy in L6 rat myotubes*
Fig 2.29.	*Fluorescence intensity analysis graph*
Fig 2.30.	*Flow cytometry analysis of fluorescent labelled 2-NBDG uptake in differentiated L6 skeletal muscle cell lines*
Fig 2.31.	*Modulation of GLUT4 expression*
Fig 2.32.	*MTT cytotoxicity assay of resveratrol based compounds in Beta-TC-6 cell lines*
Fig 2.33.	*The insulin secretion potential of the compounds determined by ELISA*
CHAPTER 3	***Elucidation of molecular mechanism of the resveratrol oligomers in insulin secretion***
Fig 3.1.	*Schematic representation of experimental design in Beta-TC-6 cell lines to elucidate the mechanism of action*
Fig 3.2.	*Determination of mitochondrial membrane hyperpolarization in Beta-TC-6 cell lines*

Fig. 3.3.	*Fluorescence intensity analysis of JC-1 aggregated(red) and JC-1 monomers(green)*
Fig 3.4.	*Determination of ratio of red to green fluorescence in Beta-TC-6 cell lines*
Fig 3.5.	*Determination of intracellular calcium efflux*
Fig 3.6.	*Intracellular calcium levels in Beta-TC-6 cell lines*
Fig 3.7.	*GLUT2 protein expression in Beta-TC-6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 3.8.	*Glucokinase protein expression in Beta-TC-6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 3.9.	*Determination of decrease in DPP4 activity*
CHAPTER 3b	***Elucidation of molecular mechanism of the resveratrol oligomers in insulin signaling***
Fig 3.10.	*Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action of resveratrol oligomers*
Fig 3.11.	*IRS-1 protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-)Hopeaphenol*
Fig 3.12.	*PI3-K protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-)Hopeaphenol*
Fig 3.13.	*AKT protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-)Hopeaphenol*
Fig 3.14.	*GLUT4 protein expression modulation in L6 myotubes on pretreatment with Ampelopsin F and (-)Hopeaphenol*
Fig 3.15.	*PTP1b inhibition assay on L6 myotubes*
CHAPTER 4	***Elucidation of molecular mechanism of the resveratrol oligomers in insulin independent signaling pathway***
Fig 4.1.	*Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action in AMPK signaling*

Fig 4.2.	*Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action downstream to activation of AMPK*
Fig 4.3.	*Inhibitor analysis in L6 myotubes by flow cytometry*
Fig. 4.4.	*Inhibitor studies in the presence and absence of inhibitors wortmannin and dorsomorphin*
Fig 4.5.	*Determination of cytosolic calcium levels in L6 myotubes pretreated with resveratrol oligomers*
Fig 4.6	*Fluorescence intensity analysis of cytosolic calcium levels in L6 myotubes*
Fig 4.7.	*Inhibitor studies using a specific CaMKK inhibitor, STO-609*
Fig 4.8.	*CaMKKβ protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 4.9.	*Determination of mitochondrial membrane potential using fluorescence microscopy*
Fig 4.10.	*Determination of Mitochondrial membrane depolarization using JC-1 dye by means of flow cytometry analysis*
Fig 4.11. a).	*Determination of red to green fluorescence intensity in L6 myotubes*
Fig 4.11. b).	*Determination of ratio of red to green fluorescence*
Fig 4.12.	*LKB1 protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 4.13.	*AMPK protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 4.14.	*p38 MAPK protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol*
Fig 4.15.	*Determination of mitobiogenesis using Mitotracker Red in L6 skeletal myocytes*
Fig 4.16.	*Fluorescence intensity analysis of mitobiogenesis in L6 myotubes*
Fig 4.17.	*Determination of nitrite using Griess Reagent in L6 myotubes*

Fig 4.18.	*PGC-1α protein expression in L6 myotubes*
CHAPTER 5	***Summary and Conclusion***
Fig 5.1.	*The proposed molecular mechanism of action of Ampelopsin F and (-) Hopeaphenol in Beta-TC-6 cell lines*
Fig 5.2.	*The proposed molecular mechanism of action of Ampelopsin F in L6 cell lines*
Fig 5.3.	*The proposed molecular mechanism of action of (-) Hopeaphenol in L6 cell lines*

LIST OF TABLES

CHAPTER 1	***Introduction***
Table 1.1.	*Top countries of South East Asia with diabetes in 2011 and 2021 according to IDF atlas 10th edition, 2021*
Table 1.2.	*Classification of glucose transporters*
Table 1.3.	*Anti-diabetic drugs, mechanism and its adverse effects*
CHAPTER 2	***Screening of the resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family for their anti-diabetic potential***
Table 2.1.	*Resveratrol oligomers and the plants in which it is isolated*
Table 2.2.	*MTT based cytotoxicity assay of resveratrol oligomers (expressed in percentage) in L6 myoblasts*
Table 2.3.	*MTT based cytotoxicity assay of resveratrol oligomers (expressed in percentage) in Beta-TC-6 pancreatic beta cell lines*
Table 2.4.	*Insulin secretion assay using Insulin ELISA in Beta-TC-6 cell lines*

CHAPTER 1

Introduction

Introduction

Diabetes is a condition leading to elevated levels of blood sugar when the body does not produce sufficient insulin or is not able to respond to insulin properly. Hence, diabetes mellitus can be labeled as a metabolic disease associated with impaired insulin secretion, insulin resistance or both resulting in prolonged hyperglycemia (Diabetes, 2014). According to IDF diabetes atlas 2021, diabetic complications account for 6.7 million annual mortality around the world (Fig 1.1). In 2021, approximately 537 million people within the age group of 20 to 79 years (1 in 10 adults) were suffering from diabetes, and 783 million is the expected global projection by the year 2045. As per the reports in 2019, 463 million people were suffering from diabetes (IDF, 2019), which was increased by around 74 million in 2021. Nearly 541 million individuals were at a higher risk of developing diabetes mellitus in 2021 when compared to 374 million in 2019. Three quarters of people suffering from diabetes live in low and middle income countries, and two thirds of diabetic people live in urban areas (IDF, 2021).

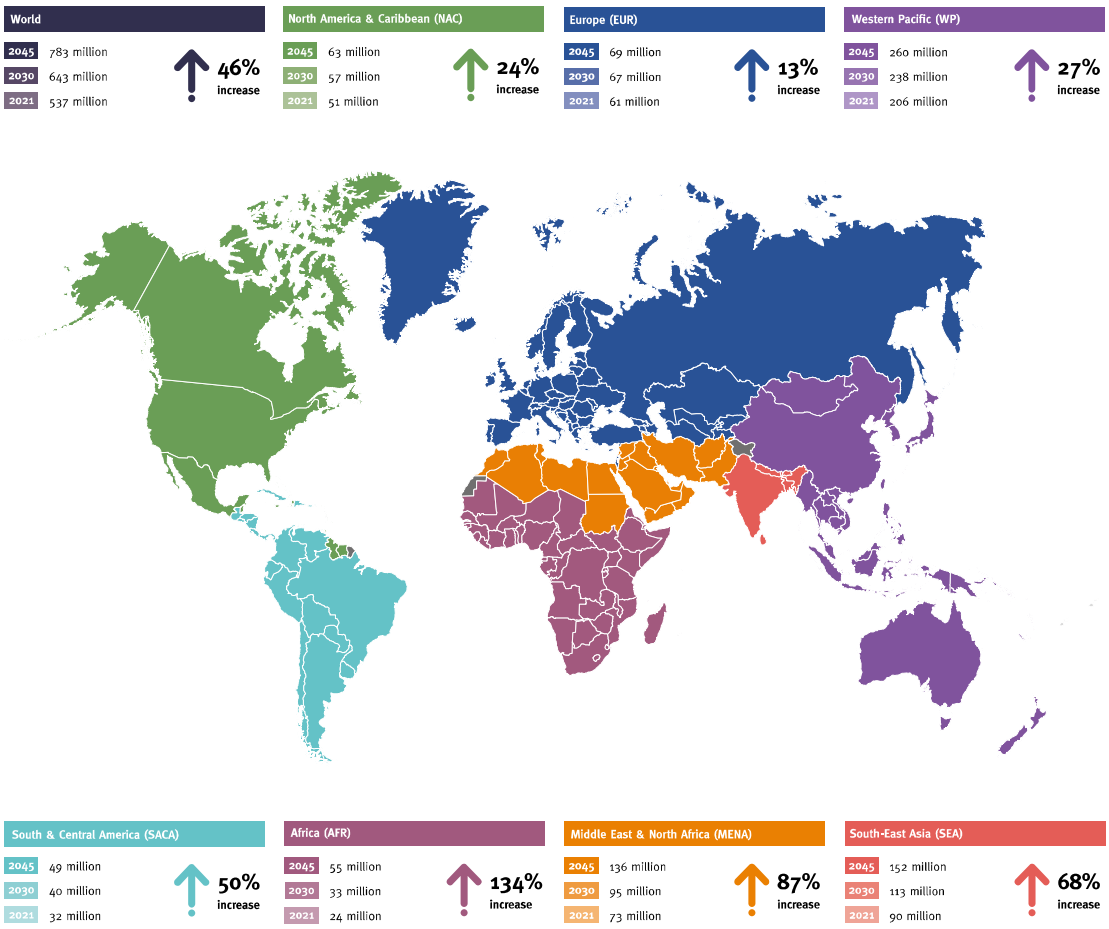


Fig 1.1. Global fact of Diabetes Mellitus according to IDF atlas 10th edition, 2021

Around 90 million adults (1 in 11) are diabetic in South East Asia, out of which one in seven adults comes from India (Fig 1.2). An estimated rise of 151.5 million people with diabetic by 2045 is expected in South East Asia. As per the statistics of IDF 10th edition 2021, about 74.2 million diabetics belong to India (Table 1.1), and 747,000 fatalities in the South East Asian region are caused by diabetes. Around 51.2% of people remain undiagnosed and are at a greater risk of developing detrimental complications (IDF, 2021).

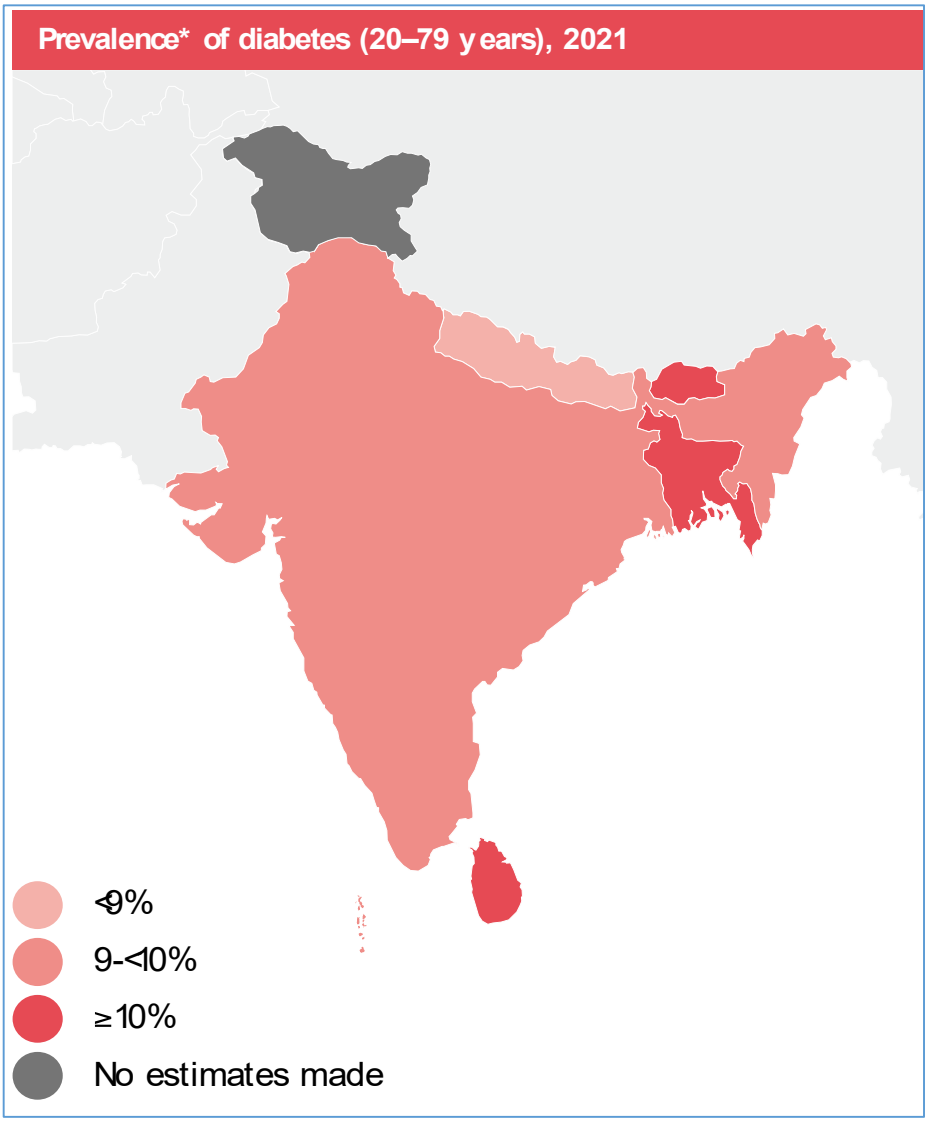


Fig 1.2. Regional fact sheet of South East Asia according to IDF atlas 10th edition, 2021.

Table 1.1. Top countries of South East Asia with diabetes in 2011 and 2021 according to IDF atlas 10th edition, 2021.

	2011	2021
Top 5 countries for number of people with diabetes (20–79 years)		
India	61.3m	74.2m
Bangladesh	8.4m	13.1m
Sri Lanka	1.1m	1.4m
Nepal	488,200	1.1m
Mauritius	138,200	250,400

Diabetes is a rising challenge in India. The incidence of diabetes is exponentially growing in urban and rural areas of India, affecting the poor and middle-class population. The development of this pandemic occurred predominantly in the age group of 45-64 years, which creates negative imprints on the economy of this developing country. The expenditure of health care for diabetic patients in India was estimated at 8750 per person according to IDF 2021 and probably extensible to a total of 1,26,0000 crore by 2025 (Bansode et al., 2019). The restructuring of health care and the investment in research for developing counter drugs is thoroughly required to meet the economic constraints.

1.1. History of Diabetes

Ancient Egyptians stated an ailment featuring extreme urine output, dehydration, and loss of weight. A discovery by ancient Hindus from India utilized ants for testing diabetes. The ants were attracted to the sugar content in the urine which was a sign of diabetes. So, they called this condition madhumeha which means honey urine (Sanders et.al, 2002). The Hindu physicians Charaka, Susruta and Vaghbata described it as polyuria and glycosuria. The initial reference on diabetes was endorsed in the Ebers papyrus, a first-rate record associating the ancient practice of therapy scripted around 1550 BCE, derived from centuries-old series of books. It states solutions for the management of polyuria. The prescribed mixture comprised a measuring tumbler filled with water from the Bird pond, Elderberry, asit plant fibers, Fresh milk, Beer-Swill, cucumber, and Green Dates. Rectal injections of olive oil, honey, sweet

beer, sea salt, and wonder fruits seeds were remedied for urinary dilemmas in the grownups. Although the father of medicine, Hippocrates did not remark particularly on diabetes in his writings, there are interpretations consistent with the signs and symptoms of the disease. He encouraged the concept of preventive medicine and promoted lifestyle modification with diet and exercise. Galen, one of the disciples of Hippocrates and an ancient Roman physician mentioned diabetes but noticed merely two people suffering from it, henceforth diabetes was considered a rare disease in those days. A contemporary of Galen, Aretaeus specified the initial exact depiction of diabetes symptoms. Apollonius of Memphis during the third century BCE coined the term "Diabetes".

The natives in India and China by the fifth century BCE had found out a difference between type 1 and type 2 diabetes and noticed the group with type 2 diabetes was common in obese wealthy individuals who ate more and were less active. Avicenna and Moses Maimonides were two noticeable physicians who contributed to the understanding of diabetes in the 11^{th} and 12^{th} centuries. Maimonides claimed to have found more than 20 cases of diabetes but with no authentic proposition.

The observations made by Thomas Willi in 1674 and the experiments done by Matthew Dobson in 1776 established diagnosing diabetes by the appearance of saccharine in blood in addition to the urine. According to an article published in the journal 'Medical Observations and Enquiries' in 1776, Matthew Dobson confirmed the sweet taste in the urine of individuals with diabetes by quantifying and confirming the scenario of high glucose in people with diabetes. He also observed the fatal and chronic condition of diabetes among people elucidating the disparities between type 1 and type 2 diabetes. People assumed that diabetes was an ailment of the kidneys owing to frequent urination, but in the late 18^{th} century a physician belonging to Britain observed this condition in individuals who experienced an injury in the pancreas. There were no figures in the early 19^{th} century regarding the diagnosis of this disease.

By the first half of the 19^{th} century, Claude Bernard discovered that a substance is released by the liver which can affect blood sugar levels. In 1857, he isolated glycogen as the internal secretion of the liver which manifested the role of the liver in diabetes. Paul Langerhan's historical finding in his doctoral dissertation in 1869 was later named as 'islets of Langerhans'. Joseph Von Mering and Oskar Minkowski in 1889 spotted the development of

diabetes after eliminating the pancreas from dogs leading to its death. This finding eased scientists to realize the function of the pancreas in regulating blood sugar levels. In 1910, Sir Edward Albert Sharpey- Schafer noticed a chemical lacking in the pancreas during the development of diabetes, and termed it insulin which means 'island' as it is produced by islets of Langerhans in the pancreas. In 1921, Frederick Banting and Charles Best transplanted healthy pancreatic islets from dogs onto dogs possessing diabetes leading to a reversal in the condition. This finding marked the discovery of insulin followed by insulin purification from cows leading to the development of the first-ever diabetes treatment. In January 1922, Leonard Thompson, a 14-year-old boy was injected with insulin to treat diabetes and he survived a further 13 years with the condition, however, he later died of pneumonia. John Jacob Abel, in 1926, purified insulin and identified its crystalline structure. In 1936, Sir Harold Percival Himsworth theorized diabetes as an outcome of insulin resistance and insulin deficiency in which insulin resistance is one of the aspects leading to the development of type 2 diabetes. In 1958, Frederick Sanger was awarded the Nobel prize for the exact amino acid sequencing of insulin. The therapeutic application of insulin was remarkable progress in diabetes treatment.

In 1979, Cullen, one of Britain's leading clinicians, consultants, and educators, was first to distinguish between Diabetes mellitus and Diabetes insipidus. However, Diabetes insipidus is not linked to Diabetes mellitus. Though it does not disturb the body's insulin production or usage, it points to thirst and frequent urination resulting from a problem with vasopressin, a hormone produced by the pituitary gland.

1.2. Early Treatments

The early Greek physicians believed in physical activities to reduce the need for excessive urination, henceforth recommended exercise, preferably on horseback. Other treatment options included fenugreek, lupin, and wormseed powder, easy digesting foods include veal and mutton, barley water boiled with bread, rancid animal food, green vegetables, fasting, etc. These Greek physicians suggested a diet including 65% fat, 32% protein, and 3% carbohydrate and instructed to avoid fruits and garden harvest (Wu et al., 2019). Several medical experts suggested various chemicals and medicines, including some metallic salts and suggested to improve the lifestyle actions such as exercising, warm clothing, getting massages, and avoiding stress. However, these methods didn't contribute to managing diabetes particularly and effectively.

1.3. Types of Diabetes Mellitus

Diabetes mellitus is characterized by high blood glucose owing to insufficient and inefficient insulin response. The chronic condition of this disease directs to prolonged-term impairment and collapse of different organs (Hajiaghaalipour et al., 2015). Diabetes mellitus can be primarily classified into the following two types:

1.3.1. Type 1 Diabetes Mellitus

Type 1 diabetes is one of the most common long-term ailments affecting kids and adolescents globally with an initial peak at 4-6 years and a second at 10-14 years which indicated a bimodal distribution. It appears in those with genetic predisposition and exposure to environmental factors. Viral infections, immunizations, diet, and vitamin D deficiency are some so-called factors but none are proven (Kalathil et al., 2018). Type 1 diabetes is a heterogeneous condition that arises due to pancreatic beta-cell damage leading to severe damage in insulin secretion and ultimately complete insulin deficiency (Diabetes, 2005). Majorly, an autoimmune-based invasion on beta cells happens, while in a few, idiopathic ruin arises (Maahs et al., 2010). It can also be defined as a T-cell mediated autoimmune response that causes insulin deficiency due to the destruction of pancreatic beta cells which ultimately leads to an increase in blood glucose and a tendency to ketoacidosis (Kahanovitz et al., 2017). Polydipsia, polyphagia, and polyuria along with hyperglycemia persist in the investigative symptoms in kids and adults. A lifetime treatment is required with an immediate requirement for exogenous insulin substitution in Type 1 Diabetes (Atkinson et al., 2014).

1.3.1.1. Epidemiology

Facts from large epidemiologic studies globally stated that the occurrence of T1D has been growing by 2–5% universally (Maahs et al., 2010). Several significant projects aimed at childhood diabetes were implemented like the Diabetes Mondiale Project (DIAMOND), The Epidemiology and Prevention of Diabetes (EURODIAB), and the SEARCH for Diabetes in Youth (SEARCH) (Dabelea et al., 2007). In 1990, WHO initiated the project DIAMOND to account for type 1 diabetes (T1D) in kids as its main objective. Liese and his co-workers (2006) mentioned the reports from 2000, that in 50 countries during 1990-1994, 4.5% of children ≤ 14 years of age had suffered from diabetes.

1.3.1.2. Risk Factors

Age: Globally, most of the diabetic cases (more than 85%) have been stated with less than 20 years of age, and its incidence peak amplified in between 10-14 years worldwide (Mehers et al., 2008).

Gender: Generally, women are more prone to most autoimmune disorders, but, both genders were equally affected in the young population with Type 1 Diabetes (Virtanen et al., 2003).

Genetics: As a polygenic disorder, approximately forty loci influence the proneness of Type 1 diabetes. It is suspected that the insulin-dependent diabetes mellitus 1 (IDDM1) locus that is the HLA region on chromosome 6 provides partial vulnerability to the risk of the disease. HLA class II displays a potent connection to the disease in which the haplotypes DRB1*0401-DQB1*0302 and DRB1*0301-DQB1*0201 deliberate the highest vulnerability, and DRB1*1501 and DQA1*0102-DQB1*0602 offer prevention of the ailment. Class I MHC, independent of Class II molecules, appears to stimulate the risk for this disease. The immune responses that are thought to associate with the risk of type 1 diabetes support the involvement of genetic influences (Howson et al., 2011).

1.3.1.3. Management of blood glucose: Monitoring and Treatment

Insulin therapy is the basic remedy for those with type 1 diabetes to imitate typical physiological insulin secretion forms. Short-acting and long-acting insulin combinations are frequently used because plasma insulin levels normally vary generally throughout the day with the low level of insulin during fasting and overnight and increasing rapidly in the post-prandial period. Continuous subcutaneous insulin infusion or insulin pump therapy is regularly used for juveniles with type 1 diabetes.

1.3.2. Type 2 diabetes mellitus

Type 2 diabetes, the most predominant form, accounting for over 90% of cases is a metabolic syndrome characterized by sustained hyperglycemia, lack of insulin sensitivity, and relative insulin deficiency. Genetic, environmental, and behavioral risk factors contribute to the epidemiology of this chronic disorder (Chen et al., 2011). Those with this ailment are particularly susceptible to different forms of both long and short-term complications leading to premature death. Its secret onset and late recognition especially in developing countries increase the morbidity and mortality of this disease (Azevedo et al., 2008).

1.3.2.1. Epidemiology

A projected increase in the number of people suffering from type 2 diabetes mellitus is expected in the future, and the frequency changes particularly from one geographical area to the other. Indians, Pacific Islanders, and Americans are at a greater risk of developing the disease. As per the reports from IDF atlas 2021, the three nations with the most number of diabetic patients are China (140.9 million), India (74.2 million), and Pakistan (33 million). This tendency is likely to persist in 2045, with China (174.4 million), India (124.9 million), and Pakistan (62.2 million) expected to retain the most number of people with diabetes (IDF, 2021). This is due to environmental and lifestyle risk factors where the majority are aged between 45-64 years of age (Tabish, 2007).

1.3.2.2. Correlations with influencing factors on T2DM

a) Genetic correlation

There is a major genetic component that overwhelms Type 2 diabetes. Nearly 40% of first-degree relatives of type 2 diabetes may develop type 2 diabetes.

b) Susceptibility loci

Since early 2007, genome-wide association studies have been used to discover the susceptibility loci of type 2 Diabetes mellitus. KCNJ11 is one of the susceptible loci which encodes islet ATP- sensitive potassium channels. The other genes include TCF7L2, IRS 1, MTNR1B, PPARG2, IGF2BNP2, HHEX, etc. Some of them are targets of various classes of drugs widely used for the treatment of type 2 diabetes mellitus.

c) Lifestyle factor correlation

Sedentary lifestyle, sudden urbanization, lack of physical activity resulting in weight gain, alcohol consumption, tobacco usage are some of the major lifestyle factors leading to the development of type 2 diabetes mellitus. According to WHO (2016), around 90% of diabetic patients developing this condition are strongly associated with obesity. Furthermore, an unhealthy diet is considered an adaptable risk factor for type 2 diabetes mellitus (WHO, 2019).

d) Gut metagenome correlation

Some studies showed gut metagenome as a factor to develop type 2 diabetes mellitus. Some studies reported that the pathogenesis of diabetes mellitus could be a result of specific pathogens, and also the metabolites like bile acids secreted by the gut microbiota (Wanping et al., 2018).

1.3.3. More categories of diabetes mellitus

Prediabetes

This word has been coined for the medium condition of glucose intolerance, in which the blood sugar status seems elevated than standard level nevertheless under the point for the detection of diabetes (Tuso, 2014). "Prediabetes" refers to the threat of developing diabetes and its complications besides impairment in the fasting of glucose and glucose tolerance and the increment of glycated hemoglobin (HbA1c) in the blood. According to IDF, the diagnosis for noticing prediabetes comprises increased fasting plasma glucose (100–125 mg/ dL), glycated hemoglobin (HbA1c) value ranging from 5.7 - 6.4 %, or high plasma glucose level resulting after an oral glucose tolerance test (140–199 mg/dL) (Diabetes, 2011). Those who are prediabetic are at a high risk of developing diabetes in the future.

Syndrome X

This state was otherwise named insulin resistance syndrome and likely increases the possibility of evolving diabetes. The present symptoms comprise elevated blood insulin levels, insulin resistance, glucose intolerance, hypertension, dyslipidemia, etc. Diagnosis of syndrome X includes the appearance of any set of three of the above symptoms or lack of insulin sensitivity along with any two added manifestations.

Gestational diabetes

Gestational diabetes mellitus is a usual complication during pregnancy that develops a spontaneous increase in blood glucose levels. Predominantly, this rise in blood glucose levels is the result of impaired glucose tolerance owing to dysfunction in pancreatic beta cells which might have developed as a result of chronic insulin resistance. According to IDF (2019), around 14% of pregnancies globally experienced gestational diabetes which is known to disturb one out of four childbirths through hyperglycemia. Overweight and obesity, maternal age, westernized diet, deficiency in micronutrients, family history, etc., comprise the risk

factors for gestational diabetes. This type of diabetes generally resolves after childbirth. Conversely, the chance of developing enduring physical issues in the mother prevails (Plows et al., 2018).

Maturity Onset Diabetes of the Young (MODY)

MODY is a monogenic or a medically heterogeneous subdivision of type 2 diabetes and results in a hereditary, young-onset insulin-independent form of diabetes in addition to the defect in insulin secretion. Fourteen classified MODY genes besides three new and unclassified genes linked with MODY have been identified. And these have been predominantly hammered in the pancreatic islet cells in which the nearly familiar ones identified include HNF1A, HNF 1B, HNF4A, and GCK genes (Yahaya et al., 2021). The mutations occurring in these genes result in the dysfunction of beta cells which leads to the underproduction of insulin consequently resulting in hyperglycemia. In comparison with type 1 diabetes, type 2 diabetes, and other forms of diabetes mellitus, MODY genes have been reported with diverse mechanisms of action and phenotypic presentations. The diagnosis of MODY has associations with the clinical management of a patient's diabetes.

Drug induced diabetes

Glucose intolerance could be induced by several drugs and chemicals which ultimately results in diabetes mellitus. Some of them include nicotinic acid, glucocorticoids, thyroid hormone, thiazides, Dilantin, and beta-adrenergic agonists. Nicotinic acid is being used to lower high triglycerides and raise HDL cholesterol (Shah et al., 2013). Thiazides are a kind of medication used to treat hypertension and Dilantin is served as an anti-seizure pill.

Genetic diseases

Numerous diseases associated with genetic impairment are involved in the raised frequency of becoming diabetic. Down Syndrome is one such genetic disorder. In addition, the abnormalities in the pancreatic islet cells and subsequently the impaired activity of insulin lead to the development of diabetes mellitus.

Infections and other diseases

Pancreatitis (inflammation of the pancreas), cystic fibrosis, and hemochromatosis (a genetic condition with iron accumulation) are some of the diseases or disorders which can damage the pancreas leading to diabetes mellitus. Alterations in the levels of various

hormones were associated with decreased insulin sensitivity. Moreover, the increment in hepatic glucose release likely leads to the onset of diabetes mellitus.

1.4. Pathways involved in insulin production, insulin transport, insulin dependent and insulin independent signaling

1.4.1. Insulin secretory pathway

Any alteration in the homeostatic regulation of fuel metabolism in the body can result in diabetes mellitus, cardiovascular disease, stroke renal disease, and other metabolic syndrome. The polymeric form ingested is converted into its absorptive form, glucose, the primary metabolic fuel source, and the subsequent elevation of blood glucose levels after digestion is modulated by two pancreatic hormones insulin and glucagon. The targets of these hormones include muscle, adipose tissue, and liver and subsequently maintaining blood glucose levels within narrow limits (Roder et al., 2016). Any dysregulation occurring in these metabolic processes may result in chronic hyperglycemia, dyslipidemia, etc. These changes antagonistically affect different cells including, beta cells, myocytes, adipocytes, hepatocytes, etc., ultimately leading to the development of diabetes mellitus (Czech, 2017).

1.4.1.1. Structure of pancreas

The pancreas is an important mixed gland that lies in the upper abdomen behind the stomach, associated with the Gastro-Intestinal Tract. It majorly includes two parts- an exocrine part and an endocrine part. The exocrine region comprises a significant portion of the pancreatic mass (around 95%) involved in the production of digestive juices into the small intestine. The endocrine region produces hormones and releases them into the circulatory system to regulate the metabolism. This includes the pancreatic islets that secrete insulin, glucagon, somatostatin, and pancreatic polypeptide into the blood.

Islets vary in size and lie predominantly in the main and interlobular ducts of the pancreas. Most of them are spherical and ellipsoidal, but sometimes some irregularities in shape could be seen owing to reflecting the pressure of an adjacent structure, often a duct (Longnecker; 2021).

Various islet cell types are located in the islets, including beta cells, alpha cells, delta cells, and pancreatic polypeptide (PP cells) (Fig 1.3). The majority of the islets of Langerhans

comprises beta cells (75- 80 %), followed by alpha cells (approximately 15%), delta cells comprise about 5% of the islet population and very few pancreatic polypeptide cells could be found (Xavier, 2018).

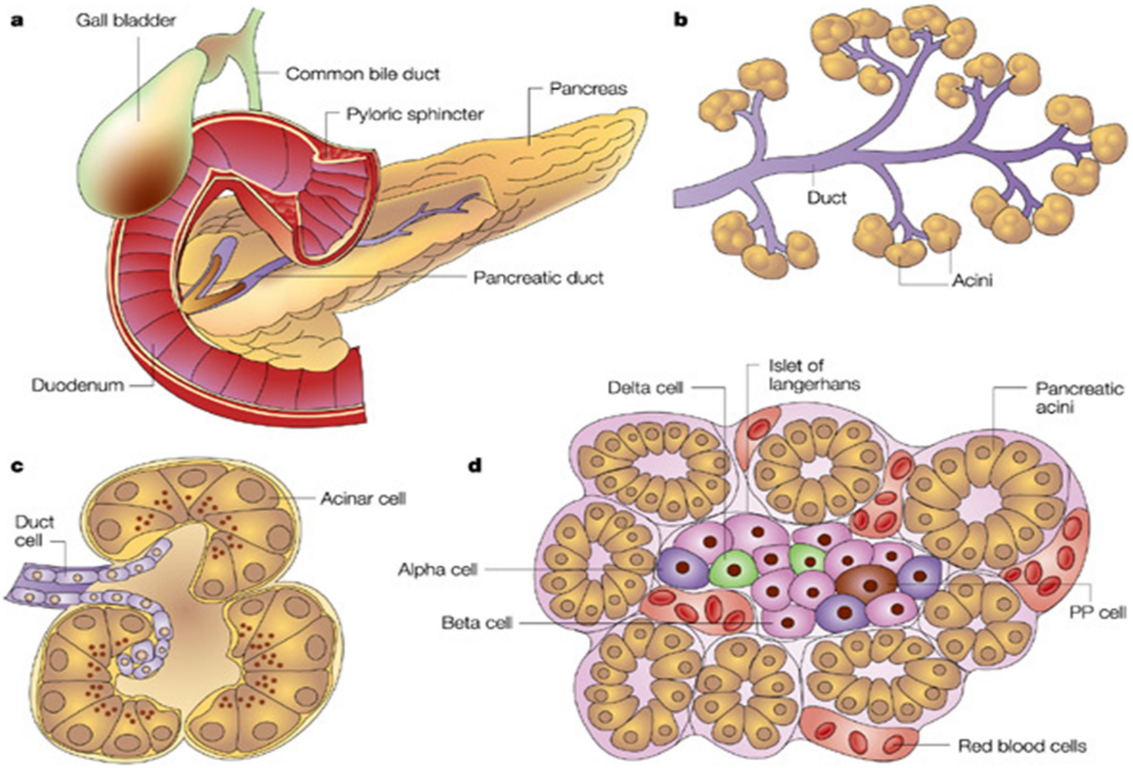


Fig 1.3. Anatomy of pancreas; Bardeesy and DePinho, 2002.

1.4.1.2. Insulin

The first peptide hormone discovered was insulin and it plays an important role in regulating human metabolism. This hormone is secreted by the beta cells present in the islets of Langerhans of the pancreas. It is a 51 residue anabolic protein containing two chains, A and B (21 residues A chain linked by a 30 residue B chain) associated via a disulfide linkage. Insulin is the product after the post-translational modification of the prototype, defined as pro-insulin. The mRNA of the insulin gene codes for pre-pro-insulin. This single-chain polypeptide includes proinsulin elongated at the amino-terminus by a 24 residue signal peptide of hydrophobic residues, which is the characteristic feature of the proteins entering the secretory pathway. This pre-pro-insulin undergoes translation and is consequently relocated in ER via various interconnections to signal recognition particle (SRP) and its receptor. A signal peptidase found on the luminal side of the rough endoplasmic reticulum

membrane mediates the cleavage of signal peptide resulting in the formation of pro-insulin. Pro-insulin encounter rapid folding and disulfide linkage and hence helped in generating the native tertiary structure, which is the direct precursor of insulin. This pro-insulin finally translocated to Golgi bodies and wrapped into granules, and altered into insulin along with C-peptide (Weiss et.al., 2014).

1.4.1.3. Biochemical mechanism of insulin secretion

Insulin is reserved in huge dense-core vesicles and released through exocytosis. It could be likely by transporting and fusing the insulin-containing vesicles with the cell membrane There are many factors to foster the release of insulin including nutrients, other hormones, and neurotransmitters. These are in association with the depolarization of the plasma membrane of the beta cell and subsequently the release of insulin (Quinault et al., 2018).

Elevation of glucose concentration results in beta cell depolarization. Glucose enters into the beta cell through the glucose transporter GLUT1 and gets metabolized by the enzyme glucokinase via entering into the glycolytic pathway, TCA cycle following substrate-level phosphorylation and electron transport chain in mitochondria. Eventually, mitochondria generates ATP, which results in the alteration of ATP: ADP ratio, which ultimately leads to the closure of K^+-ATP- sensitive channels. This directs the inflow of Ca^{2+} via voltage-gated calcium channels and ultimately leads to the merging of insulin vesicles with the cellular membrane, leading to insulin export. This kind of stimulation by glucose is accountable for the first phase of insulin secretion (Fig 1.4). But this lasts for 5 to 10 minutes, the more sustained phase of insulin release for the second phase is over a period of 30 to 60 minutes which is entirely dependent on metabolic stimulus secretion. Gembal et al., (1993) experimentally proved the release of insulin even in the presence of glucose when K^+-ATP-sensitive channels remained open by the addition of diazoxide. This mechanism termed K^+-ATP independent GSIS is processed by various courses of action like TCA intermediates and associated products, phospholipase C/ PKC signaling, modifications in intracellular lipid levels, and increase in cAMP levels, enhance cytosolic Ca^{2+} flux and hence exocytosis. In addition, various reports suggested the role of multiple coupling factors such as NADPH, NADH, glutamate, and malonyl CoA in amplification of K^+-ATP independent Glucose Stimulated Insulin Secretion (GSIS) (Fridlyand and Philipson, 2010).

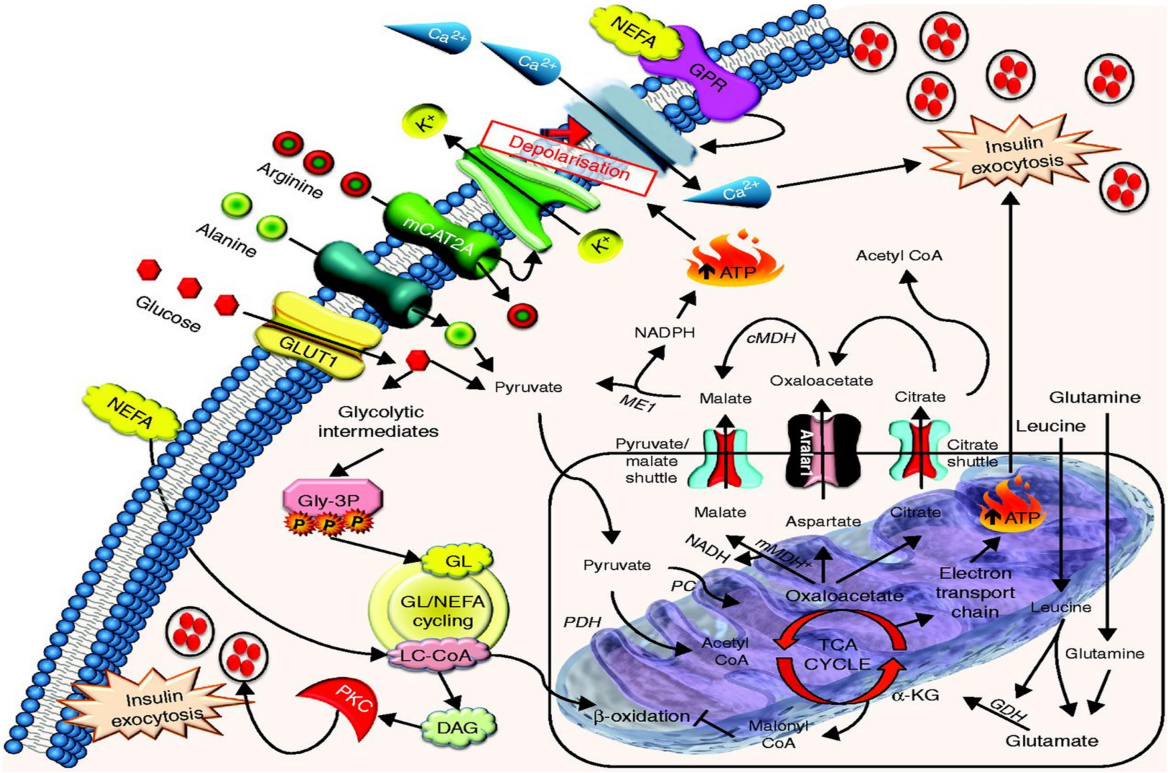


Fig 1.4. Insulin secretion pathway; Newsholme et al., 2014.

1.4.1.4. Carbohydrate metabolism and insulin secretion

Beta cells are precisely drafted for insulin release upon dietary nutrients stimulation particularly glucose. The fuel-sensing adaptations allow throughout monitoring of plasma load coupled with instant oxidative metabolism. This converts the high glucose sign to maximize ATP production to release insulin. The aforementioned qualities comprise the capability for fuel sensing especially glucose in the physiological range with high K_M and the enzyme glucose transporters and the enzyme glucokinase (2-20 mmol/L), decreased expression of lactate dehydrogenase (LDH), regeneration of reducing equivalents by increased expression of redox shuttles, high expression of pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH), which ensure productive oxidative metabolism in hyperglycemic condition (Mac Donald et al., 2005).

GLUT membrane carrier proteins are involved in the regulation of nutrient influx. Generally, the glucose transporter expressed in humans is GLUT1 and GLUT2 in rodent beta cells. (Table 1.2). The transporters have high K_M for glucose- 6mM for GLUT1 and 11 mM

for GLUT2 (Zhao and Keating, 2007). Hence these transporters are active probably in postprandial conditions. Following the uptake of glucose into the cell, the generation of ATP followed by glycolytic degradation to pyruvate is a predominant stimulus-secretion coupling factor.

Table 1.2. Classification of glucose transporters

Trans-porter	**Mecha-nism**	**Substrate**	**Gluco-se K_M**	**Tissues**	**Characteristics**
GLUT1	Passive	Glucose	3 mM	Brain, red cells, endothelium, ß cells	Involved in the transportation of glucose into RBC. It is expressed in most cells and mediates the transport of hexose sugar extensively.
GLUT2	Passive	Glucose	17 mM	Kidney, ileum, liver, pancreatic ß cells	Provides glucose to the liver and pancreatic cells.
GLUT3	Passive	Glucose	1.5 mM	Neurons, placenta	The main glucose transporter in neurons.
GLUT4	Passive	Glucose	5 mM	Insulin sensitive tissues like Skeletal muscle, heart, adipocytes	Predominantly located in skeletal and fat tissue and directed by insulin
GLUT5	Passive	Fructose	6 mM	Small bowel, sperm	Transports fructose in intestine and testis.
GLUT6	-	-	-	Brain, spleen, peripheral leucocytes	-
GLUT7		Glucose	0.3 mM	Small and large intestine	
GLUT8		Glucose	2 mM	Testis	

GLUT9		Urate	0.9 mM	Liver, kidney	Renal urate reabsorption
GLUT10		2- Deoxy glucose	0.3 mM	Heart, Lungs	
GLUT11		Glucose	0.1 mM	Muscle, heart, adipose tissue, kidney	
GLUT13		Myoinosi-tol	0.1 mM	Brain	Proton- coupled myoinositol transport in the brain
GLUT14		Glucose		Testis	
SGLT1	Sodium depen-dent		High affinity	Small intestine, kidney tubules	Contribute to renal glucose absorption.
SGLT2	Sodium depen-dent		Low affinity	Kidney proximal tubule	Major co-transporter involved in renal glucose reabsorption.

The hexokinase enzyme expressed in liver and pancreatic cells are glucokinase (GCK). It can also act as a glucose sensor as it possess high K_M for glucose (6mM) (Matschinsky and Wilson, 2019). It can maintain high glycolytic flux under hyperglycemic conditions as it is not inhibited by the product glucose-6-phosphate, unlike other hexokinases and hence coupling carbohydrate sensing to beta cell insulin secretion. The alterations in the action of predominant enzymes, glucokinase, and phosphofructokinase involved in glycolysis play a part in regulating glucose-stimulated insulin secretion (GSIS). This ultimately leads to impairment in glucose oxidation and production of insulin. Several significant glucose metabolizing beta cell gene expression including SLC2A2, GCK (Glucokinase), Ca^{2+} channels and insulin transcriptional factors, Pdx 1, Neuro D1, Maf A could be negatively regulated under chronic hyperglycemic conditions similar to type 2 diabetes mellitus (Bae et al., 2010). Downregulation of the above-mentioned genes in diabetes directs to impairment in nutrient utilization by beta cells and subsequently insulin-responsive cells. Hence the

reduction in glucose oxidation leads to elevated plasma glucose concentration resulting in glucotoxicity.

1.4.1.5. Glucose Regulation

There are distinct glucose transporters to ease the sugar transport in the tissues to lower glucose concentration in blood. This glucose would be utilized by the body or else stored as glycogen. There are majorly two types of glucose transporters: Sodium (Na+) dependent and Na+ independent. Insulin responsive isoform includes Na+- independent transporters.

The Na^+-dependent glucose transporters act by absorbing glucose against the concentration gradient by coupling the movement of glucose along with Na^+ into cells. This transporter depends on the intracellular and extracellular sodium ion concentration in which the movement of Na^+ depends on the electrochemical gradient. When this Na^+ moves down the electrochemical gradient, this energy generated is utilized to transport glucose into the cells, all these processes are maintained by a Na^+/ K^+- ATPase ion pump. This type of transporter family has been identified particularly in the small intestinal epithelium (SGLT1), renal proximal tubule (SGLT2), and other kidney tubule cells.

The sodium-independent glucose transporters which, exist in different isoforms, allow the entry of glucose through the cell membrane. Glucose transporter 4 (GLUT4), is the one expressed more in insulin-sensitive tissues like skeletal muscles, cardiomyocytes, and adipose tissue. GLUT4 and to a minor extent GLUT1 permit the uptake of glucose into the cells and thereby lowering the blood glucose concentrations. The swift conversion of glucose to glucose-6-phosphate results in lower intracellular glucose concentration and this favors the entry of glucose into the cells in the presence of active transporters.

Upon insulin stimulation, the increase in expression of glucose transporters results in the enhanced glucose uptake in the target cells (Navale and Paranjape, 2016). The stimulation of insulin in these cells translocate these transporter molecules from intracellular compartments towards the plasma membrane where it fuses with the membrane to permit the uptake of glucose into the cells.

1.4.2. Insulin signaling

Insulin signaling modulates the energy homeostasis via glucose and lipid metabolism predominantly in the liver, adipocytes, and skeletal muscle (Boucher et al., 2014). The explicit regulation of this pathway is essential for the biological process as the individual shift from the fed to the fasting stage. A well-coordinated biological response upon insulin stimulation in different tissues has been regulated by positive and negative modulators which might be acting at different steps of the signaling pathway. New drug development to treat diabetes relies on a better understanding of these pathways.

1.4.2.1. Insulin Receptor

Insulin receptors are highly homologous tyrosine kinase receptors and the biological effects of insulin were exerted upon insulin receptors. This insulin receptor consists of two alpha polypeptides and two beta polypeptides bonded by a disulfide linkage. These subunits are produced via proteolytic cleavage from a huge precursor. There exist two isoforms of the insulin receptor, isoform A (IR-A) and isoform B (IR-B). IR-A has a higher affinity for insulin and is primarily expressed in fetal tissues and the brain, and IR-B is predominantly expressed in the liver (Belfiore et al., 2019).

1.4.2.2. Insulin Receptor Substrates

Upon ligand binding to the alpha subunits, a conformational change occurs inducing the kinase activity in beta subunits resulting in the transphosphorylation among beta subunits. Insulin receptor substrate (IRS) family of proteins, simply IRS-1 is the best-characterized substrates for insulin. IRS proteins are recruited to the membrane. It engages in tyrosine phosphorylation and activates the substrate for effector particles which include Src-homology 2 (SH2) domains. (Chang et al., 2004).

These binding sites contain structurally identical tyrosine phosphorylation residues, but functional disparities were reported. Knock-out studies were conducted to analyze this functional modification. IRS 1 knockout (KO) shows growth hindrance and impaired action of insulin, particularly in muscle but lacks glucose intolerance (Farese et al., 2007). IRS 2 knockout mice show growth retardation only in particular tissues like neurons and islets, but develop impairment in hepatic insulin signaling. Some defects in differentiation at the cellular level were spotted in IRS-1 KO preadipocytes however IRS-2 KO preadipocytes differentiate normally. IRS-2 KO preadipocytes develop impairment in insulin-stimulated

glucose transport. Similarly, IRS-1 is essential for skeletal muscle differentiation and energy consumption wherein IRS-2 is required for fat metabolism. A controlled tissue distribution pattern was observed with IRS 3 and IRS 4. IRS 3 is mostly expressed in adipocytes, liver, and lung in rodents whereas, in humans, IRS-3 represents a pseudogene. IRS-4 is also expressed in skeletal muscle, liver, heart, brain, and kidney but the IRS-4 knock-out mice show a diminutive growth hindrance and lack of glucose tolerance. IRS-5 and IRS-6 are comparatively poor substrates of IR and devour restricted expression in tissues (Sesti et al., 2001).

Insulin-stimulated IRS tyrosine phosphorylation and the synthesis of DNA could be prevented by the dominant interference of IRS PH and IRS PTP domains. But this has no substantial influence on GLUT4 translocation. IRS PH and IRS PTP domains act as a negative regulator of insulin tyrosine phosphorylation and are henceforth dispensed as a potential target in the treatment of diabetes mellitus.

1.4.2.3. Phosphatidylinositol (3,4,5)-Triphosphate and Phosphoinositide 3-Kinase

Phosphoinositide3-kinase (PI3K) and AKT pathway is the critical pathway associated with IRS proteins to devour the metabolic actions of insulin. The class 1a PI3-kinases comprise a catalytic subunit and a regulatory subunit in which each exists in numerous isoforms. The phosphorylated IRS proteins stimulate these heterodimers via binding to the regulatory subunit of PI3-K. This could activate the catalytic subunit of PI3-K, which further phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) resulting in the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3), which is a lipid, second messenger. This messenger molecule aids in the recruitment of AKT to the cell membrane and further phosphorylates to stimulate downstream signaling. Three distinct genes encode for distinctive isoforms of the regulatory subunit of PI3-K comprising Pik3r1, Pik3r2, and Pik3r3. Pik3r1 codes for approximately 65% -75% of entire regulatory subunits expressed predominantly as p85α and in addition the splice variants p55α and p50α. Pik3r2 codes for around 20% of the regulatory subunits expressed as p85β. Pik3r3 codes for p55γ which possess structural similarity to p55α, but shows limited expression in most of the tissues (Liu et al., 2009).

The three distinctive catalytic subunits codes from three distinct genes are p110α, p110β, and p110δ. The stability of the catalytic subunit has been increased upon binding to the regulatory subunit and henceforth upholding a state of inhibition (Soberg, 2018). The

activation of the particular protein was followed by relieving the regulatory subunit from the catalytic subunit, which occurred by binding the regulatory subunit to phosphotyrosine motifs present in IRS proteins.

Numerous studies proposed the activation and/or targeting of the enzyme to a specific intracellular site via the interaction of IRS with PI 3-kinase, possibly involving its association with GLUT4 vesicles.

Though PI3-kinase activity is essential for the insulin-responsive uptake of glucose, the translocation of GLUT4 could be induced by additional signals. Some studies reported the PI-3 kinase activation stimulated by IL-4 or the assignation of definite integrins but failed for GLUT4 translocation. But two genetic modifications in the IR stood entirely efficient in stimulating PI3-K nonetheless failed in stimulating the translocation of GLUT4 (Krook, et al., 1997). Some data disclosed zero effect on GLUT4 translocation by the addition of a cell-permeable analog PI (3, 4, 5) P3. However, the treatment of adipocytes with insulin and wortmannin- a PI3-kinase inhibitor along with this PI (3, 4, 5) P3 analog improve the uptake of glucose. Although the PI3-kinase pathway is indispensable for insulin-stimulated glucose uptake, there is at least a single supplementary pathway that is independent of triggering PI 3-kinase (Isakoff et al., 1995).

1.4.2.4. Activation of downstream kinases

Downstream of PI 3-kinase includes two classes of serine/threonine kinases which comprise isoforms of AKT-the serine/threonine kinase, also called protein kinase B (PKB). There is a structural similarity among AGC kinase (protein kinase A, G, and C) family members and means of stimulation through phosphorylation of two serine and threonine residues. PDK-1 (3-phosphoinositide-dependent protein kinase 1), stands as the main upstream protein accountable for activating AGC kinase members and the initiation controlled by PI3K. PDK-1 activates via binding to membrane-bound PIP3. This further triggers the activation of AGC protein kinases via phosphorylation at serine/threonine motifs, like Thr-308 for the activation of AKT. Three different isoforms of AKT encoded by distinctive genes acquire a PH domain for the interaction with PIP3 in which Akt2 is the most profuse isoform found in the insulin-sensitive tissues and hence its shortage appears to have a prevalent role in developing diabetes mellitus (Hassan et al., 2013).

The upregulation of the phosphorylated AKT ensures improved glucose transference and continual translocation of glucose transporter to the cell membrane in 3T3L1 adipocytes. (Kohn et al., 1996). Contrarily, the expression of a dominant-interfering AKT mutant hinders insulin-dependent localization of GLUT4 (Wang et al., 1999). Similarly, the activation of PKCζ stimulated by the formation of polyphosphoinositides, and consequently, the expression of PKCζ or PKCλ are also conveyed to persuade the translocation of GLUT4, whereas translocation of GLUT4 could be hindered by dominant-interfering PKCλ (Tadahiro et al., 1998).

Several other multiple substrates of AKT include GTPase- activating protein TBC1D4 also termed as AKT substrate 160 kDa (AS160), and its homolog TBC1D1. These substrates tangled in insulin-mediated glucose uptake as well as with contraction-mediated uptake of glucose.

The inactivation via phosphorylation of glycogen synthase kinase 3 by AKT results in the activation of glycogen synthase and subsequently the accumulation of glycogen in the liver. Gluconeogenesis and fatty acid oxidation were prevented by AKT-dependent phosphorylation of PGC-1α. The activation of phosphodiesterase 3B (PDE3B) by its phosphorylation by AKT results in a fall in cyclic AMP levels consequently inhibiting lipolysis in adipocytes and β cell insulin secretion (Huang et al., 2018).

1.4.2.5. Other actions of insulin downstream from PI3K

Many other insulin actions have been mediated by AKT including the regulation of expression and its activity of some enzymes, transcription factors, apoptosis and survival proteins, cell cycle regulatory proteins, etc. Tumorigenesis could be initiated by inhibiting p53 mediated apoptosis through phosphorylating Mdm2 (Murine double minute 2) by AKT. Sometimes cell cycle inhibitors like p21 Cip1/WAF1 and p27Kip1 have been phosphorylated by AKT resulting in cellular growth and inhibition of apoptosis. Cell survival could be promoted by phosphorylating Bax, Bad, and caspase-9 resulting in its inhibition. The inhibition of IkB kinase (IKK) via phosphorylation by AKT leads to the activation of NF-κB. AKT can phosphorylate endothelial nitric oxide synthase (eNOS) leading to its activation and hence producing the vasodilator and anti-inflammatory molecule nitric oxide (NO), which plays a major role in linking insulin resistance and cardiovascular disease. SGK (Serum- and glucocorticoid-induced protein family of kinases) family of proteins are potentially highly

homologous to AKT. This family of proteins is activated in a PI3-K-dependent manner by means of double phosphorylation by PDK-1 and mTORC2.

1.4.2.6. GLUT4- the key thespian for glucose uptake

The members of the GLUT family of integral membrane proteins mediates the transport of monosaccharides & other small carbon compounds. These proteins are encoded by SLC2 genes (Augestin, 2010). These GLUT informs contains 12 transmembrane helical segments and has a N- terminus & C- terminus. It has a large central loop exposed to the cytoplasm. GLUTI is abundantly found in red blood cells and brain micro vessels whereas GLUT2 predominantly found in beta cells of pancreas, hepatocytes and enterocytes. GLUT3 is expressed in neuronal cells in the brain, foetal muscle skeletal myoblasts and cardiomyocytes GLUT4 is predominantly expressed in insulin- sensitive tissues like adipocytes, skeletal muscle cells and cardiocytes. GLUT4 catalyzes hexose transport across cell membranes through a facilitative diffusion process (Fig 1.5). The major difference found among these GLUT isoforms include the kineties and specificity to substrates for e.g.: GLUT5 is a fructose transporter (Zwarts et al., 2019). The unique feature of GLUT4 is intracellular disposition under unstimulated stage.

Though GLUT4 disposes intracellularly under unstimulated stage which makes it unique, it gets translocated to the plasma membrane upon receiving the stimulus via means of exocytosis. It is proposed that both insulin and contraction stimulations play prominent roles in regulating GLUT4 translocation.

Skeletal muscle plays a predominant role in regulating glucose homeostasis. Approximately 75% glucose disposal accounts here following post prandial conditions. Both physical activity and insulin synergistically enhance glucose disposal in skeletal muscle. GLUT4 is the superabundant GLUT isoform found in the skeletal muscle and appraised to be the rate limiting for the uptake of glucose and metabolism.

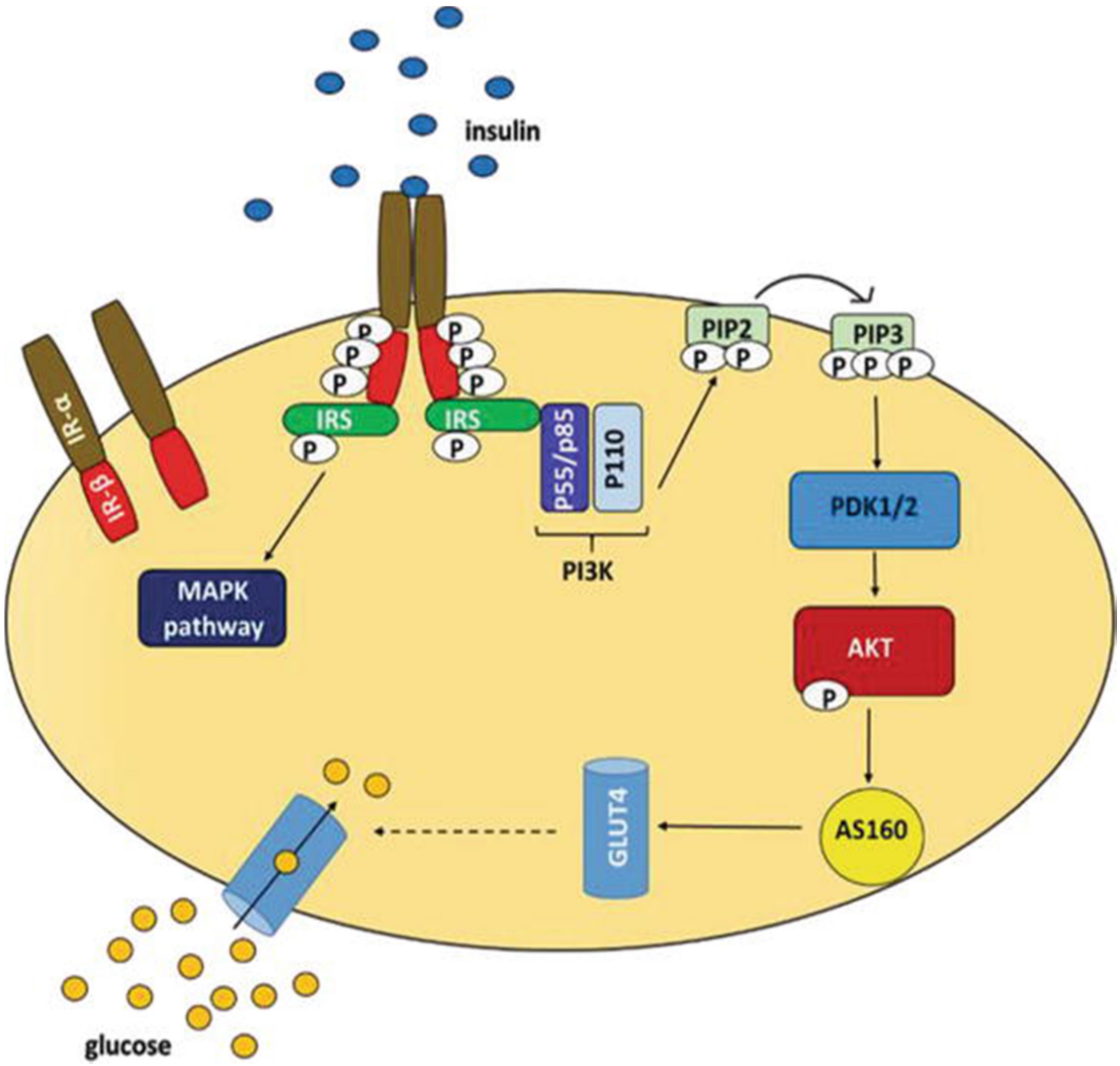


Fig 1.5. Insulin signaling pathway via PI3K/AKT activation; Swiderska et al., 2018

1.4.3. AMPK Pathway

The downregulation of AMPK leads to the development of insulin resistance as well as type 2 diabetes mellitus; hence studies focusing on the regulation of the AMPK pathway in the management of diabetes mellitus is gaining momentum. Insulin-stimulated GLUT4 translocation is diminished in type 2 diabetes. Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein that devours a central role in cellular energy homeostasis. It has a catalytic subunit (α) and two regulatory subunits (β and γ). Isoforms of subunits of AMPK have been identified and the changes in AMPK α1 and α2 activity during exercise suggested its various physiological roles (Stapleton, 1996). It is possible with a total of twelve heterotrimeric combinations encoded by separate genes. The activation of AMPK is

regulated allosterically as well as by post-translational modifications. Most importantly, the allosteric activation of AMPK by binding of AMP and/or ADP to γ subunit, resulting in the phosphorylation of α subunit at Thr_{172}. The depletion of ATP results in a rise in AMP, in which ATP can competitively inhibit the binding of AMP or ADP. Hence an increment in AMP/ADP or ADP/ATP ratio results in the activation of AMPK (Coughlan et al., 2014). In skeletal muscles, the uptake of glucose could be stimulated by AMPK mediated by the translocation of the glucose transporter GLUT4.

The AMPK activation majorly depends on an increment in the intracellular AMP: ATP ratio coupled with the phosphorylation of Thr_{172} by an upstream kinase LKB1 (Liver Kinase B1) on the α subunit and by the activation of calcium-dependent protein kinase CaMKKβ (Fig 1.6). It is also known to be activated by transforming growth factor-activated protein kinase -1 (TAK1). It was reported with an inhibitory site at Ser485 on the α subunit which could be phosphorylated by AKT, PKA, or the autophosphorylation in different cell types like adipocytes, cardiocytes, resulting in reduced AMPK activity.

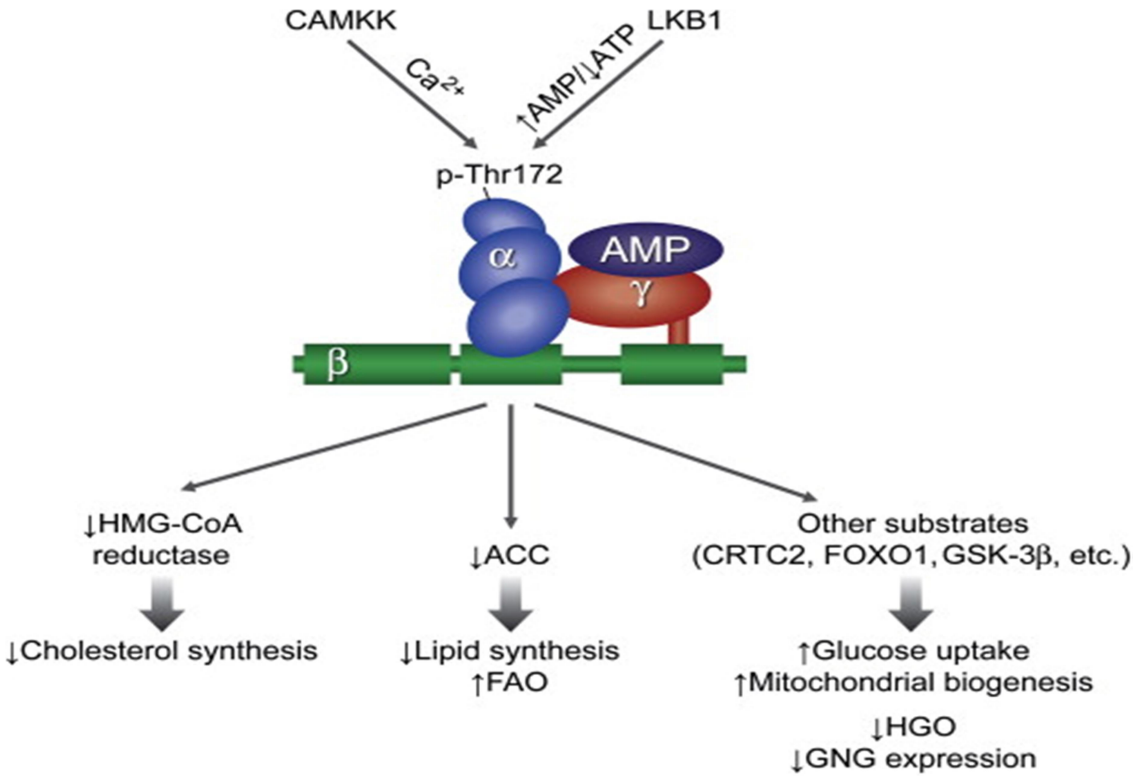


Fig 1.6. AMPK activation by the upstream kinases; Zhang et al., 2009.

AMPK has been exhibiting dual roles in regulating the generation and consumption of ATP by upregulating the catabolic pathways including glycolysis and fatty acid oxidation and downregulating anabolic pathways (cholesterol, fatty acid, and triacylglycerol synthesis) respectively (Hardie, 2008).

The γ subunits (γ_1, γ_2, and γ_3) contain four CBS domains (Cystathionine beta synthase) also called as Bateman domain where three of the four CBS domains bind adenine nucleotides like AMP, ADP, and ATP. Studies reported that the mutations in these domains lessen the activation of AMPK due to lack of binding of AMP proving the Bateman domains are the regulatory binding sites of AMPK (Soga et al., 2014). Under normal physiological conditions, these domains are being bounded by ATP. Upon increment in the AMP: ATP ratio, ATP is replaced by AMP in the CBS domains resulting in the allosteric activation of AMPK. There is a competitive inhibition with a high concentration of ATP which resists the binding of AMP and ADP to the γ subunit and hence opposes the activation of AMPK (Towler and Hardie, 2007).

Some basal phosphorylation of Thr_{172} resulting in the activation of AMPK has been found in cells in which LKB1 is not expressed. Here, the activation of AMPK increased by the efflux of Ca^{2+} ionophore in which the upstream kinase is responsible for the phosphorylation of Thr172 was found to be Ca^{2+}/calmodulin-dependent protein kinase (CaMKK), especially the CaMKKβ (CaMKK2) isoform in skeletal muscle cells (Zhang et al., 2009).

As the improper functioning of AMPK plays a lead role in the progress of insulin resistance and subsequently type 2 diabetes mellitus, regulation of AMPK seems interesting to study as the target of type 2 diabetes mellitus (Jeon, 2016). Hence, AMPK activation by either physiological or pharmacological factors which can increase skeletal muscle glucose uptake is of great interest to prevent or ameliorate insulin resistance and type 2 diabetes mellitus and may pave way for the development of therapeutic targets (Musi et al., 2001).

1.5. Current treatment for diabetes

Diabetes can be metabolically controlled for a longer period via a combination of lifestyle modifications and pharmacological treatments. For lifestyle modifications, proper nutritional diet and physical activity contribute as the basic thing for the treatment of diabetes mellitus (Penalver et al., 2016).

1.5.1. Lifestyle measures

One of the cost-effective measures in preventing and managing diabetes mellitus is lifestyle modifications. Dietary intake and physical exercise are the two leading factors of energy equilibrium. It is preferable to consume carbohydrates from berries, vegetables, pulses, grains, dairy, etc. Another key factor considered for diabetics is to prevent the consumption of alcohol and the incorporation of fibrous foods in the diet. Physical activity improves insulin sensitivity, glycemic control, lipid profile, blood pressure as well as the psychological well-being of the diabetics.

1.5.2. Anti-diabetic drugs

Good glycemic control remains the foremost base for the treatment of type 2 diabetes mellitus and this approach aids in preventing or delaying the development of diabetes complications. The explicit therapy for diabetes comprises anti-hyperglycemic medications or combinatorial drugs with insulin, considering the kind and development of illness over time. The main categories of oral anti-hyperglycemic medicaments comprise biguanides, sulfonylureas, meglitinide, thiazolidinedione (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors, and α-glucosidase inhibitors (Table 1.3). But a rise in HbA1C level up to 7.5% while on medication or if the early HbA1C is ≥9%, it is recommended for combination therapy with two oral agents, or plus insulin (Padhi et al., 2020).

Table 1.3. Anti-diabetic drugs, mechanism and its adverse effects

Medication	Mechanism of action	Adverse effects	Risk of hypo-glycemia	Contra-indications
1) Sulfonylureas: Glyburide Cholorpropamide Glimepiride	Stimulate secretion and release of endogenous insulin	Nausea GI discomfort, increased cardiovascular risk	Prominent	Hepatic or renal impairment

2) Meglitinide: Repaglinide	Stimulate insulin release (rapid acting, better post prandial glucose control)		less frequent than with sulfonylureas	Hypersensitivity, Diabetic ketoacidosis (DKA)
3) Biguanides: Metformin (Glucophage)	Insulin sensitizer, Reduce Gluconeogene-sis, increase glucose utilization	Lactic acidosis, anorexia, nausea, diarrhea, GI discomfort, Vitamin B12 deficiency	None	Hepatic or renal impairment, alcoholism , advanced age
4) Thiazolidine-diones: Rosiglitazone (Avandia) Pioglitazone (Actos)	Increase insulin sensitivity, reduce gluconeogenesis	Cardiac failure, pedal oedema, Increased TG, Weight gain, hepatotoxicity, anemia		Hepatic disorder, heart failure, bladder cancer
5) Alpha Glucosidase inhibitors: Acarbose (prandase)	Lowers the absorption of starch (hence lowering post prandial rise of sugar)	Boasting, peritoneal pain, dysentery		Tenderness, hyperosmotic hyperglycemic nonketotic state, ulcerative colitis
6) Dipeptidyl peptidase 4 (DPP4) Inhibitor: Sitagliptin, Saxagliptin	Inhibiting the degradation of incretins	Pancreatitis, Upper RTI infection	Low	Decreases postprandial lipemia

7) Sodium-glucose cotransporter (SGLT2) inhibitor: Canagliflozin, Dapagliflozin	Melituria owing to hindrance in dextrose reabsorption in proximal convoluted tubules	Genital mycosis, may increase LDLc	Low	Bone fractures
8) Insulin	Activating insulin receptors and downstream signaling in various insulin sensitive tissues	Lipoatrophy and lipohyper-trophy at the injection sites, Allergy	Prominent	Heart failure if used in combination with thiazolidine-diones

Most drugs expended for nursing diabetes causes hypoglycemia as a consequence. Traditional medicinal plants could be able to serve as an ideal candidate in treating obesity and type 2 diabetes and can act as a better alternative for the treatment of metabolic disorders. The increasing prevalence of diabetes, its connection with various signaling pathways, highlight a critical requirement for a molecule with various actions. Various anti-diabetic drugs are available in the market with different cellular targets (Fig 1.7). Even though the currently available drugs seem effective to the patients, several drugs have adverse side effects including hepatotoxicity, weight gain, and cardiovascular issues (Chaudhury et al., 2017). Hence, safer and more effective anti-diabetic drugs are still being explored. Nature has gifted innumerable structurally diverse and biologically functional scaffolds that are nowadays undergoing considerable appreciation by the scientific consensus owing to the petite lethality and increased bioavailability. Therefore, the exploration of novel hypoglycemic leads from nature for the better management of diabetes is on surge globally.

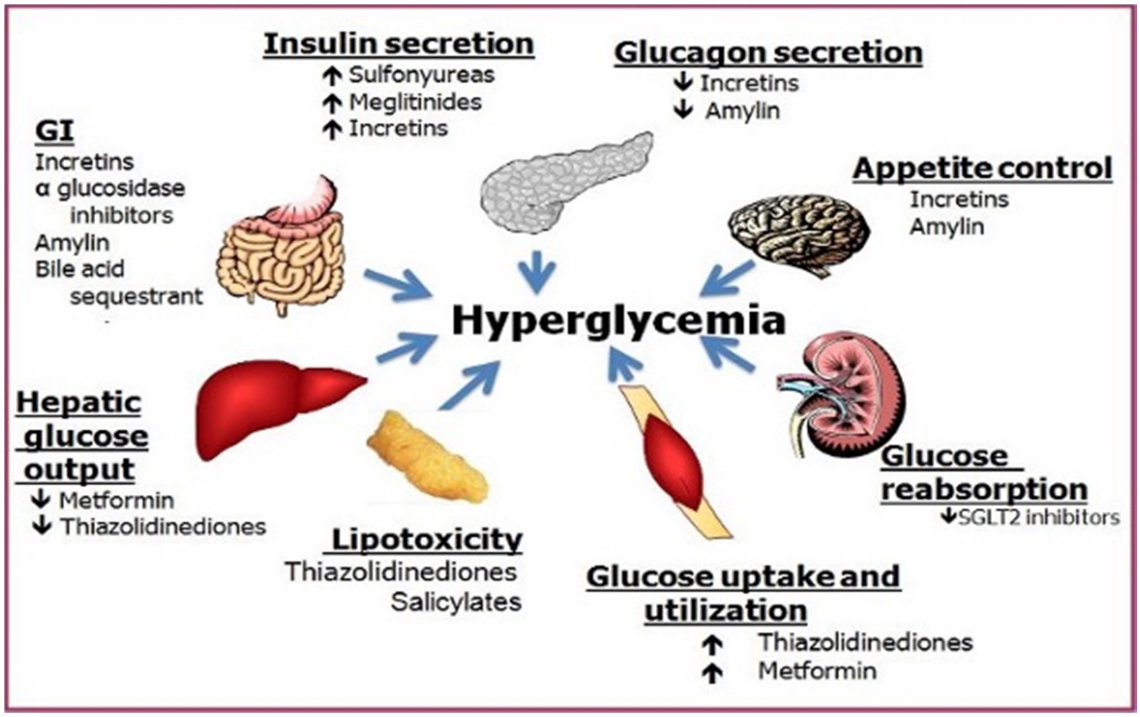


Fig 1.7. Different targets of anti-diabetic drugs; Feingold, 2021.

1.5.3. Natural compounds

Phytomedicines extracted from various portions of plants/trees comprising flowers, fruits, leaves, berries, roots, bark, etc are reported in the management of several diseases. (Pan et al., 2014). The practice of using herbal medicine as a prescription in curing and healing of illnesses has begun since 2600 B.C. The usage of phytomedicines is still persistent in this modern era and it has been projected that about a quarter of prescription medicines globally originated from plants. According to the WHO report, in most countries, traditional plants are being used as primary health care. The increased side effects and cost of treatment of allopathic medicines compelled many patients to depend on complementary and alternative medicinal therapies rather than conventional therapies owing to their unsatisfactory outcomes. The herbal formulations of one or more plants are being used or sometimes they have been used in combination with allopathic medicines. Herbal medicines or herbal formulations are used to treat cancer, memory impairedness, GI disorders, hypertension, cardiovascular disease, skin infections, inflammation, liver diseases, etc (Choudary et al., 2018).

Innumerable common herbs claimed to possess hypoglycemic effect but the choice of herbs should depend on diverse factors like disease stage, accessibility and safety of herbs, types of associated comorbidities. Certain herbal supplements are being expended as an adjuvant for diabetes mellitus (Telapolu et al., 2018). Certain marketed polyherbal formulations in diabetes are Diabecon by Himalaya, Glyoherb, Diabeta Plus, etc. Henceforth the potential of herbs in managing diabetes by regulating the secretion of insulin and decreasing insulin resistance has become a standard therapeutic strategy for the conventional treatment of diabetes mellitus. Hence the scientific world is trying to explore the bioactive scaffolds from natural cradles and extended the laboratory research to the bedside of patients for clinical trials. Proper validation is an instant prerequisite for the evaluation of active molecules through the clinical trials for determining their improved efficacy and safety (Panda et al., 2013)

1.6. Objectives of the work

The objectives of the current study are

- ***To evaluate glucose uptake potential of the resveratrol oligomers using L6 myotubes.***
- ***To evaluate the insulin secretion potential of the resveratrol oligomers using Beta-TC-6 cell lines.***
- ***To explore the possible mechanism of action of the resveratrol oligomers in both cell lines.***

1.7. Significance of the study

Polyphenols provide defence against the development of various chronic disorders comprising diabetes, malignancy, cardiovascular disease, etc. As stated in some recent studies, the biological activity of some polyphenols showed significant protection against diabetes mellitus and related complications. Polyphenols including resveratrol-based compounds varying from dimers to octamers are discovered richly in plants belonging to the Vitaceae, Dipterocarpaceae, Leguminosae, and Cyperaceae families. Various biological activities like anti-microbial, anti-HIV, anti-inflammatory, and anti-tumor properties label resveratrol a probable pharmaceutical lead. Henceforth the investigation of the molecular mechanism implicit in the biological activities of resveratrol-based compounds would provide a considerable indication in developing novel leads.

Resveratrol-based compounds isolated from the stem bark of several trees belonging to the Dipterocarpaceae family had never been explored for their anti-diabetic potential. This study is the first of its kind to explore the anti-diabetic potential of resveratrol oligomers isolated from Dipterocarpaceae family through two different cellular models and enzyme based assays.

In addition, the thesis in detail explores the possible mechanism of action of these compounds in insulin secretion and insulin signaling pathways.

CHAPTER 2

Screening of the resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family for their anti-diabetic potential

2.1. Introduction

Traditional medicinal plants serve as a complementary medicine for recovering from various metabolic disorders (Prathapan et al., 2012). Currently, several drugs have been established from plant sources or from synthetically improved plant-based natural products (Katiyar et al., 2012). The exploration for pharmacologically significant natural products is an exciting area to the scientific community. Majority of the novel drug leads are inspired by the natural products.

2.2. Dipterocarpaceae family

One of the dominant plant family found in South East Asia is Dipterocarpaceae and it comprises a total of 470 species. Shorea, Hopea, Dipterocarpus &Vatica comprise the largest genera of Dipterocarpaceae family. The other genus of Dipterocarpaceae family includes Anisoptera, Cotylelobium, Dryobalanops, Marquesia, Monotes, Neobalanocarpus, Parashorea, Pseudomonotes, Stemonoporus, Upuna, Vateria and Vateriopsis. One of the peculiarities of the family is the two-winged fruit in which the family's name emerged (Di- two, ptero–wing, karpos fruit). This family species is particularly used for timber trade and in addition it provides essential oils, resins etc. and they are a rich source of plywood. Some products derived from this family possess medicinal values. A gum resin 'Indian copal' is used as a medicine, which is isolated from *Vateria indica.*

Plants belonging to this particular family are a rich source of resveratrol based compounds, produced after the successive condensation of resveratrol. Discovery of the biological significance of resveratrol in the health of humans gave an impetus scientific interest in exploring the resveratrol oligomers. Resveratrol oligomers are reported for their involvement as pathway intermediates resulting in the formation of other condensed components. Many resveratrol oligomers are reported to be optically active (Ito, 2020).

2.3. Resveratrol oligomers

Resveratrol is an extensively recognized polyphenolic stilbenoid. This compound is commonly identified in grapes, blackberries and numerous other plants. This stilbenoid is extensively used for the prevention of enduring inflammatory diseases, like diabetes, cardiovascular disease, obesity, and neurodegenerative diseases, estrogen receptor agonist

(Gehm et al.,1997) etc. Resveratrol oligomers (oligostilbenoids) are derived from the oxidative combination of C6-C2-C6 units discovered in some plant families. The polymerization of resveratrol results in structurally distinct resveratrol oligomers ranging from dimers to octamers (Fig 2.1) (Shen et al., 2017). A structurally miscellaneous organic pool is constructed after the consecutive regioselective and stereoselective oligomerization of resveratrol (Ito, 2020).

Resveratrol oligomers are reported to have various biological activities comprising anti-microbial (Zetterstrom et al., 2013), anti-cancer (Empl et al., 2014), anti-HIV (Dai et al.,1998), anti-inflammatory (Annabi et al., 2012) etc. Owing to the pharmaceutical importance of resveratrol & its oligomers, investigation to explore the possible mechanism underlying its biological activities would provide a substantial clue in the development of new drug leads.

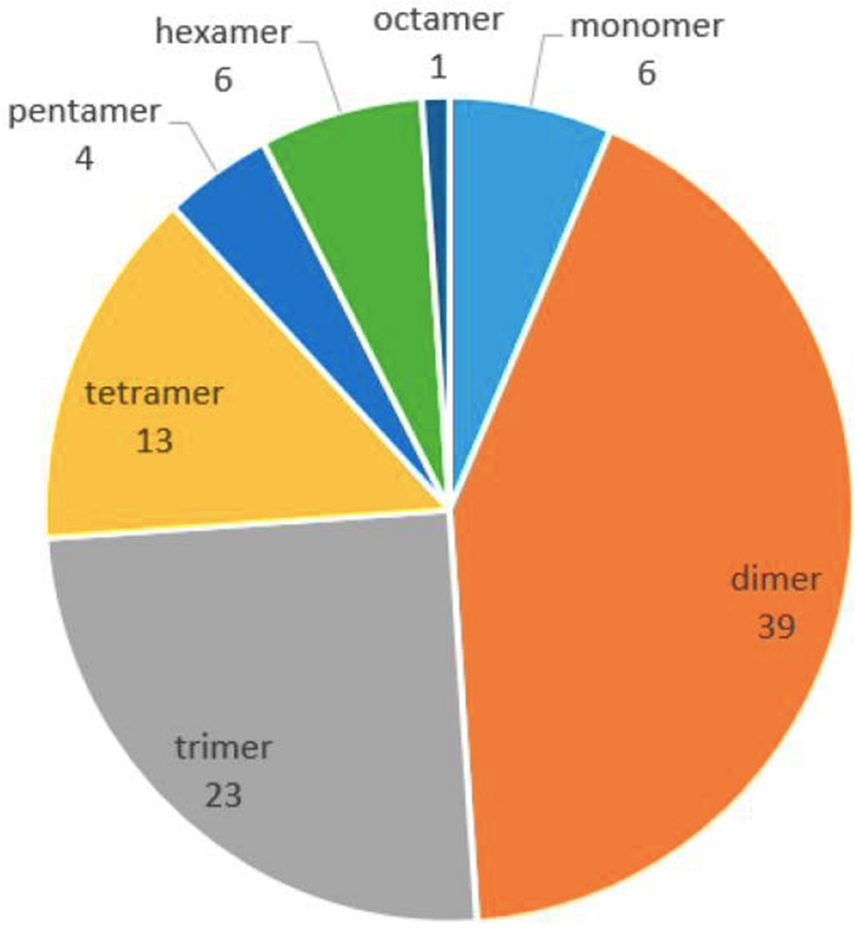


Fig 2.1. The conditions of resveratrol oligomers in polymerization; Shen et al., 2017

The current study focused to explore the anti-diabetic potential of eight resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family. The eight resveratrol-based compounds isolated from different plants belonging to Dipterocarpaceae family is depicted below in the Table 2.1.

Table 2.1. Resveratrol oligomers and the plants in which it is isolated

Sl. No:	Compounds	Plants in which it is isolated
1	Ampelopsin F	*Vatica chinensis*
2	(-) Hopeaphenol	*Vateria indica*
3	Vaticaphenol A	*Vatica chinensis*
4	α-Viniferin	*Hopea ponga*
5	NIIST C1	*Vatica chinensis*
6	Trihydroxyphenanthrene glucoside	*Hopea ponga*
7	Resveratrol Hexamer	*Vatica chinensis*
8	ε-Viniferin	*Vatica chinensis*

2.3.1. Ampelopsin F

Ampelopsin F is a resveratrol dimer (Fig 2.2) that was isolated from the stem bark of *Vatica chinensis* L. It contains a dibenzobicyclo (3,2,1) ictadione skeleton (Zhang et al., 2016). This compound is widely distributed in Vitaceaeous plants and reported to exhibit anti-microbial activity (Kou and Chen, 2012) and anti-malarial activity (Indriani et al., 2017). The NMR spectra of Ampelopsin F is depicted in Fig 2.3 and Fig 2.4.

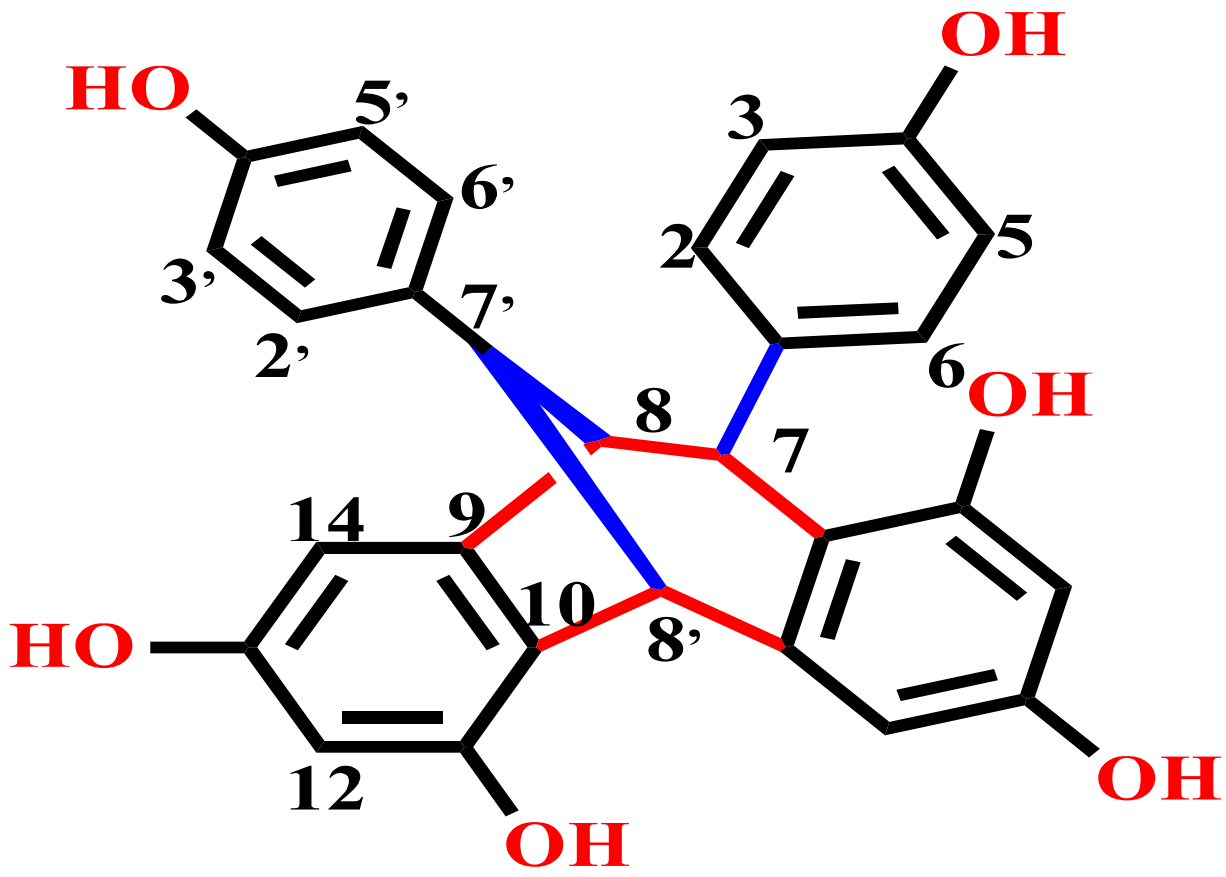


Fig 2.2. Structure of Ampelopsin F

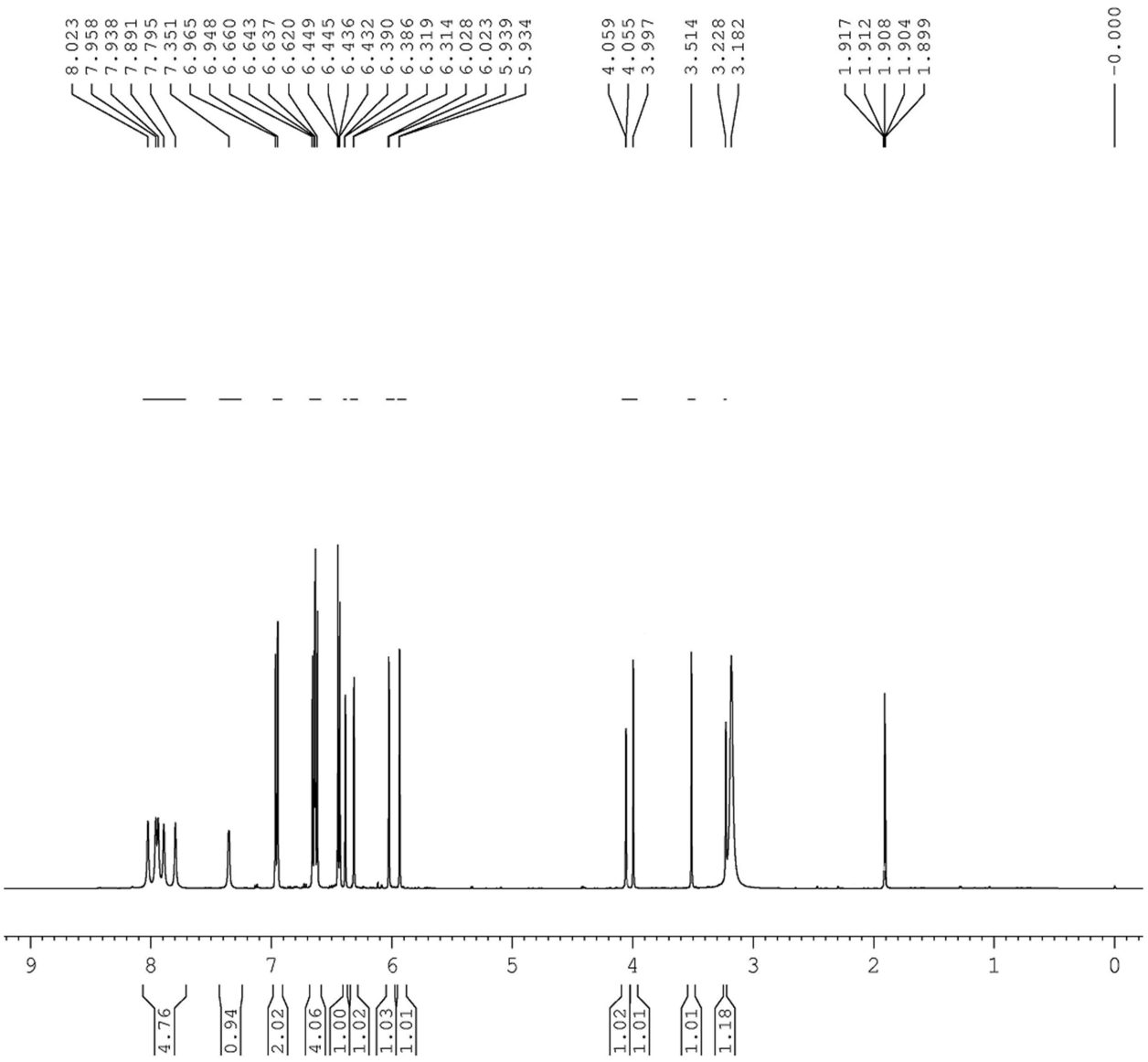


Fig 2.3. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Ampelopsin F

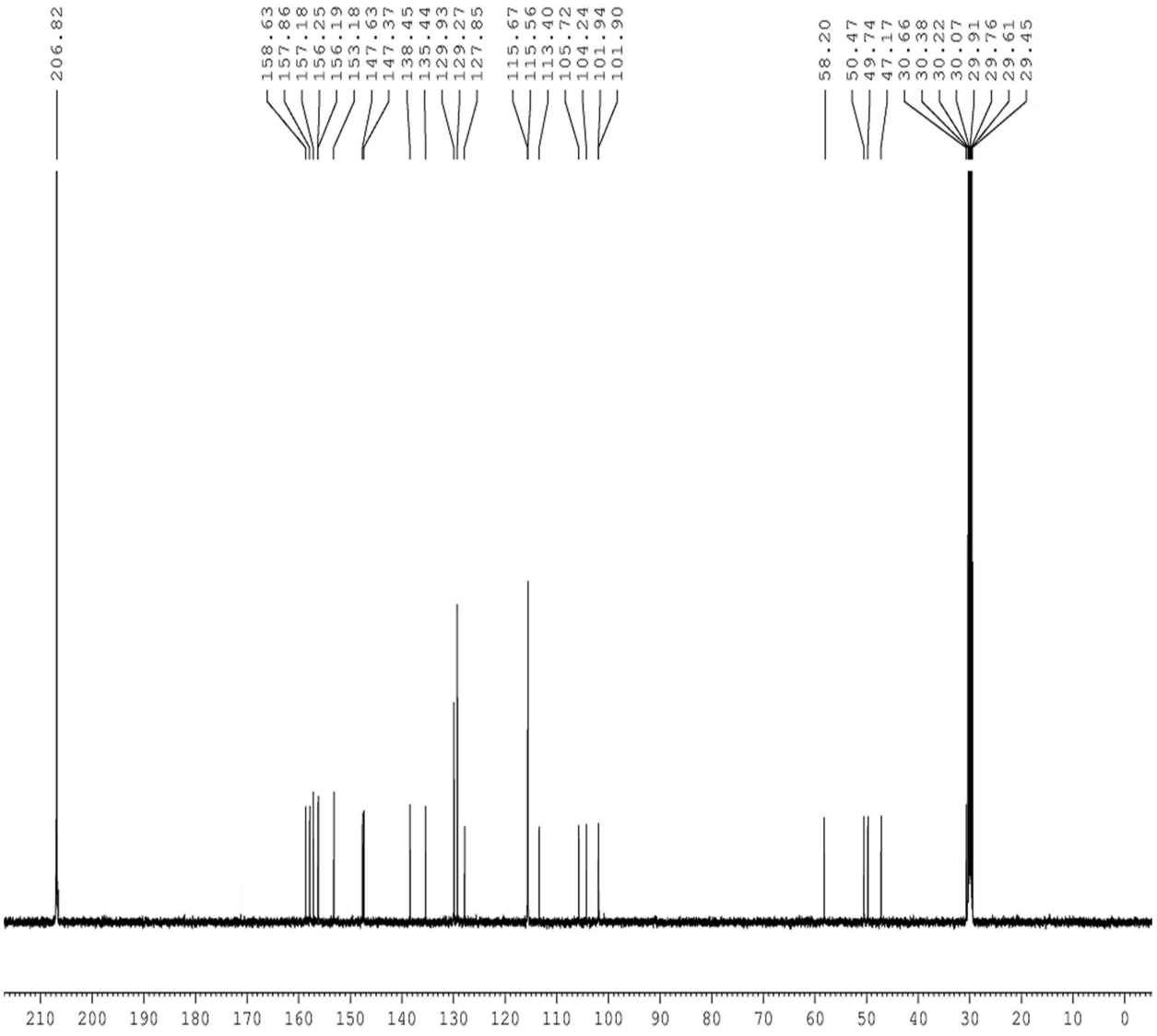


Fig 2.4. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Ampelopsin F.

2.3.2. (-) Hopeaphenol

(-) Hopeaphenol is a resveratrol tetramer (Fig 2.5) isolated from the stem bark of *Vateria indica*. The dimerization of resveratrol dimers results in the formation of resveratrol tetramers. Hopeaphenol is one of the most abundant resveratrol tetramer found in Dipterocarpaceaeous plants (Ito, 2020). Hopeaphenol was reported for various biological activities such as anti-bacterial (Zetterstrom et al., 2013), anti-oxidant (Subramanian et al., 2015) activity etc. The NMR spectra of (-) Hopeaphenol is depicted in Fig 2.6 and Fig 2.7.

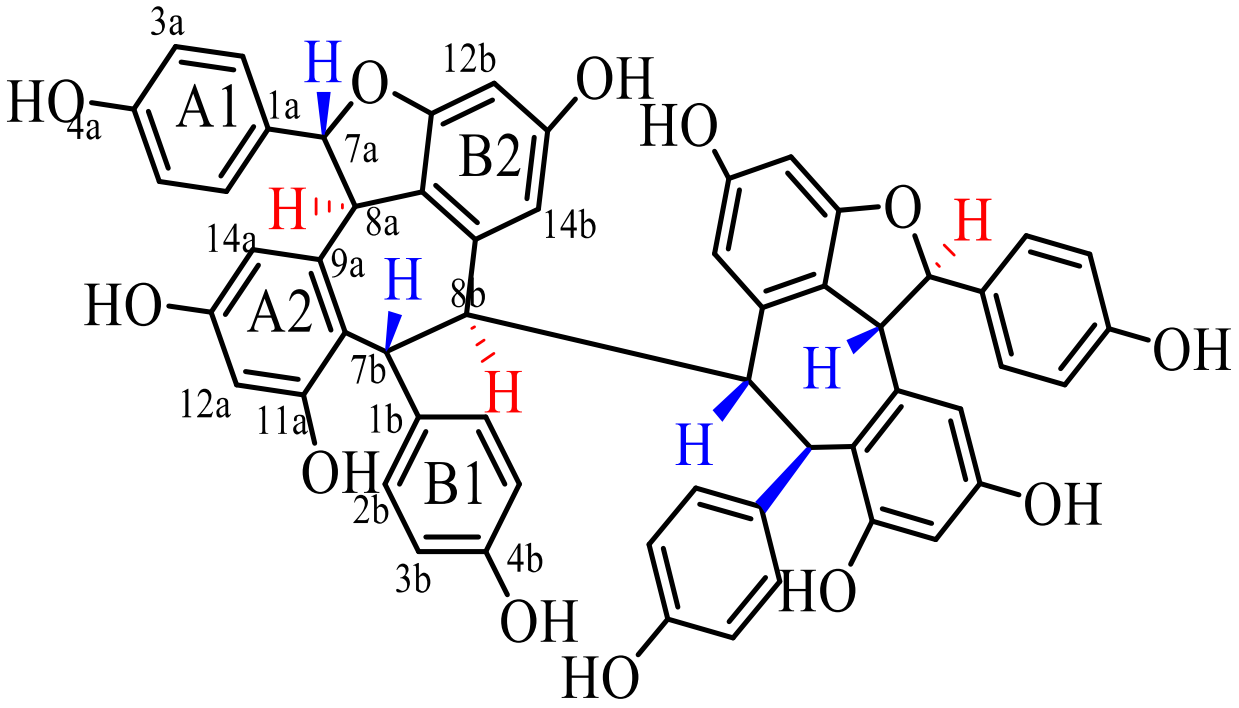


Fig 2.5. Structure of (-) Hopeaphenol

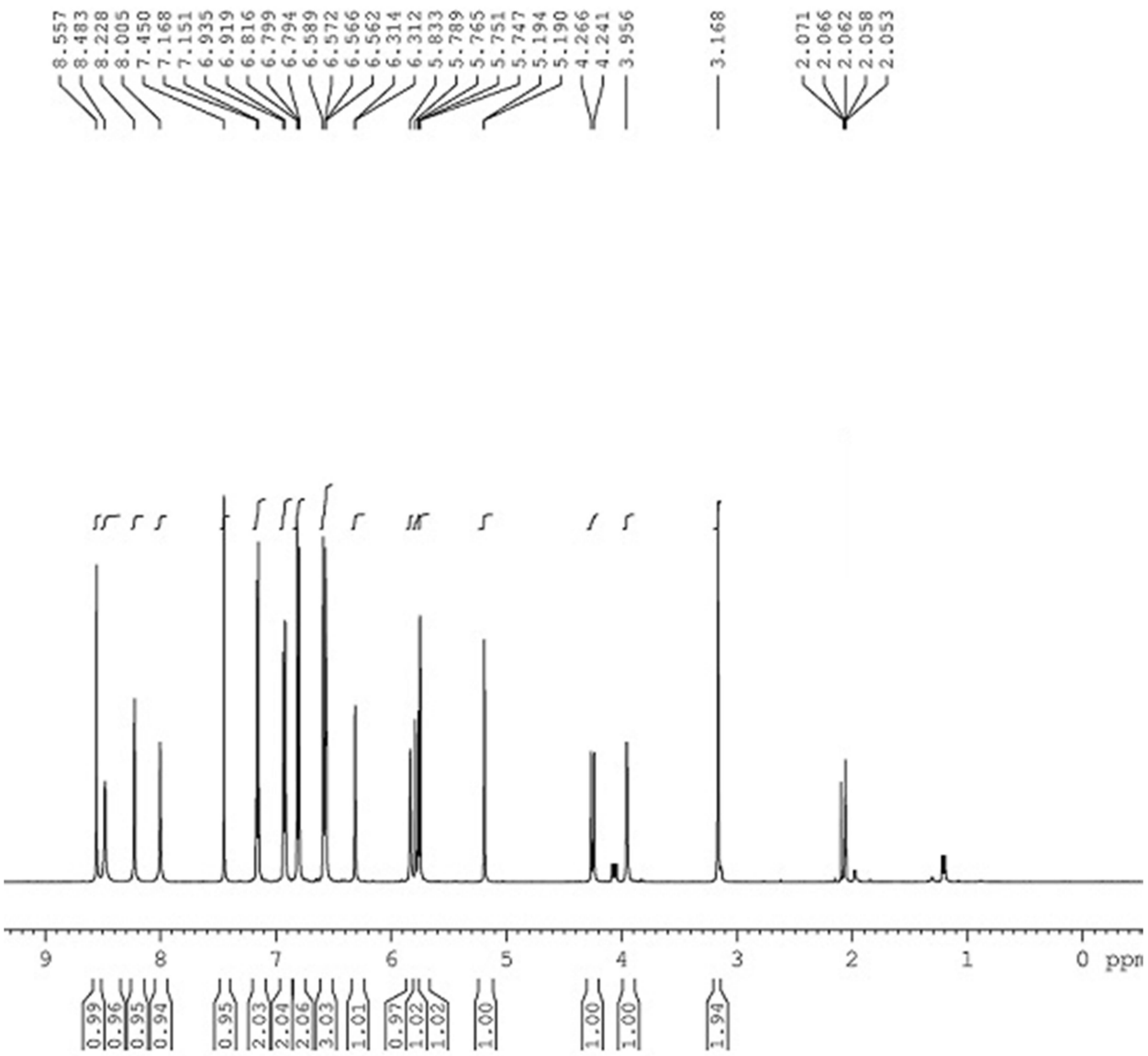


Fig 2.6. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of (-) Hopeaphenol

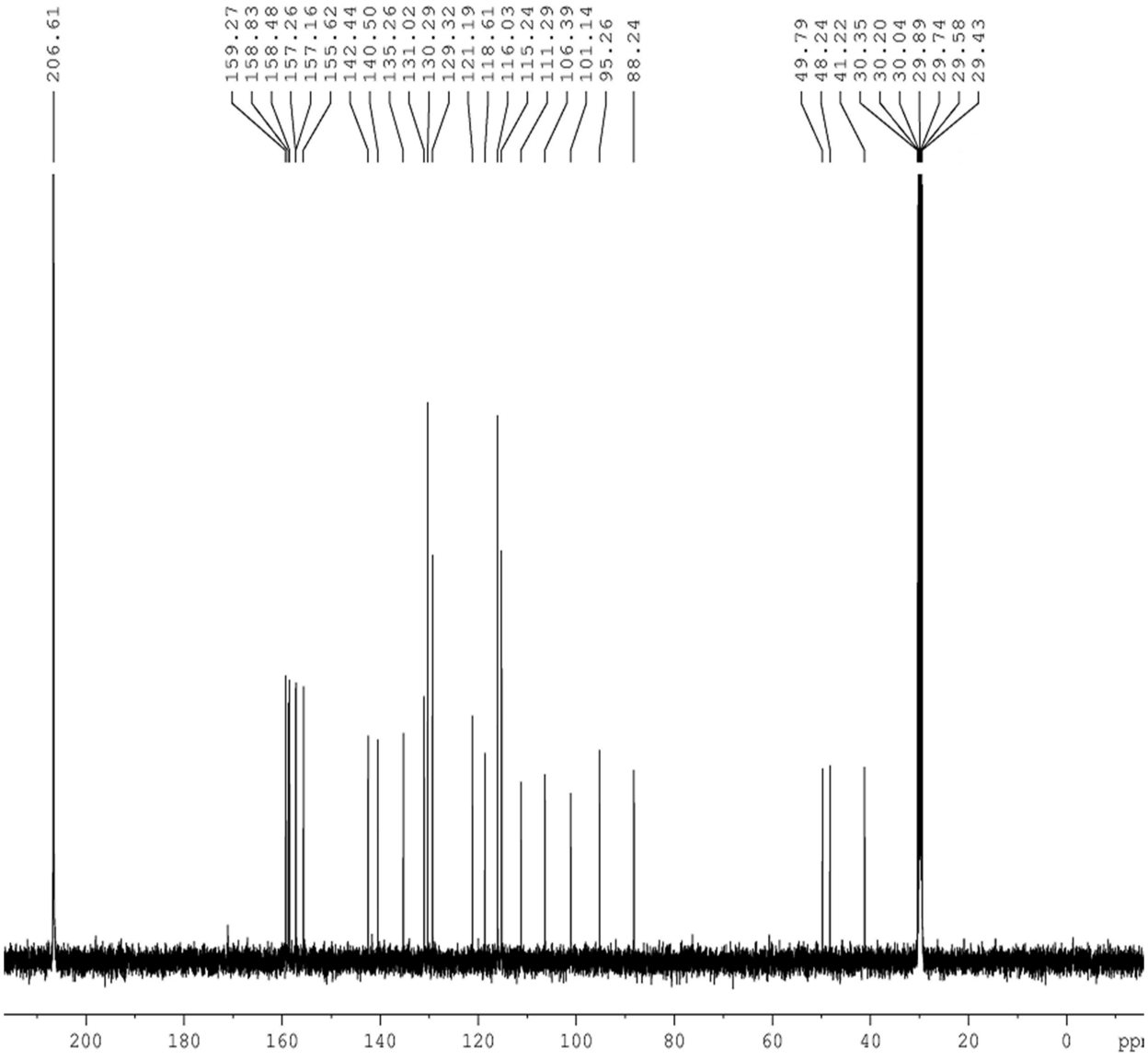


Fig 2.7. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of (-) Hopeaphenol

2.3.3. Vaticaphenol A

Vaticaphenol A is resveratrol tetramer (Fig 2.8) isolated from the stem bark of *Vatica chinensis* L. According to Keylor (2015), the oxidation of ε-Viniferin followed by radical dimerization results in the generation of Vaticaphenol A. This resveratrol tetramer is reported for its anti-microbial activity (Zgoda-Pols et al., 2002). The NMR spectra of Vaticaphenol A is depicted in Fig 2.9 and Fig 2.10.

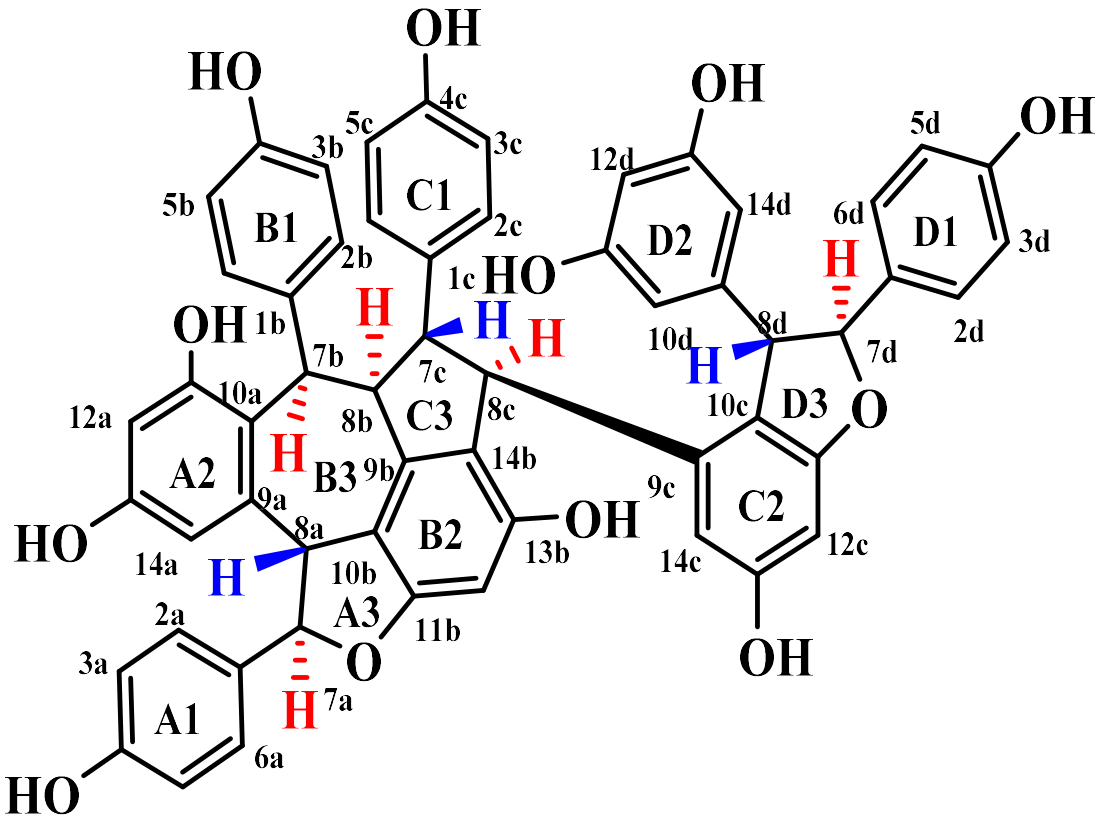


Fig 2.8. Structure of Vaticaphenol A

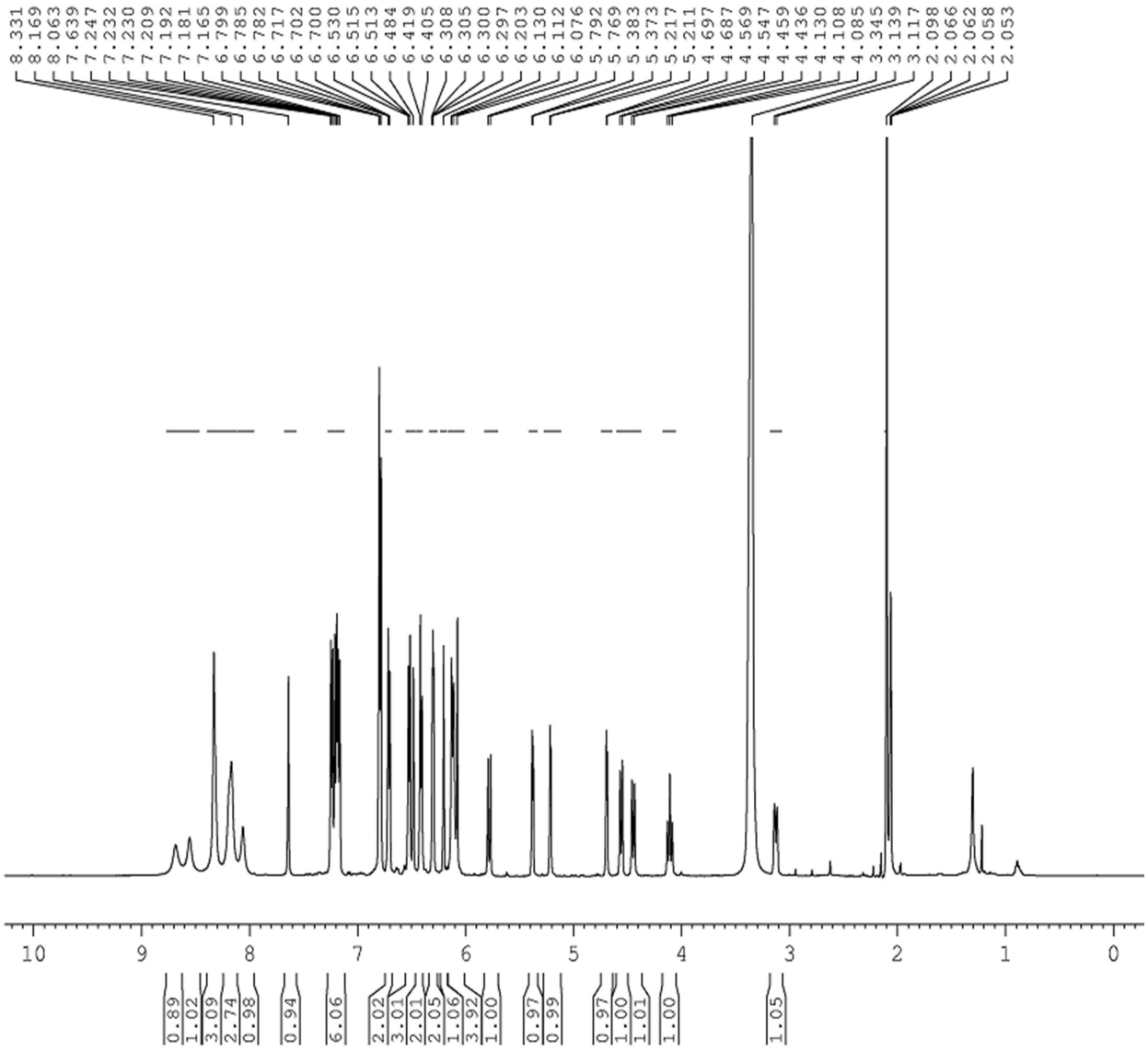


Fig 2.9. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Vaticaphenol A

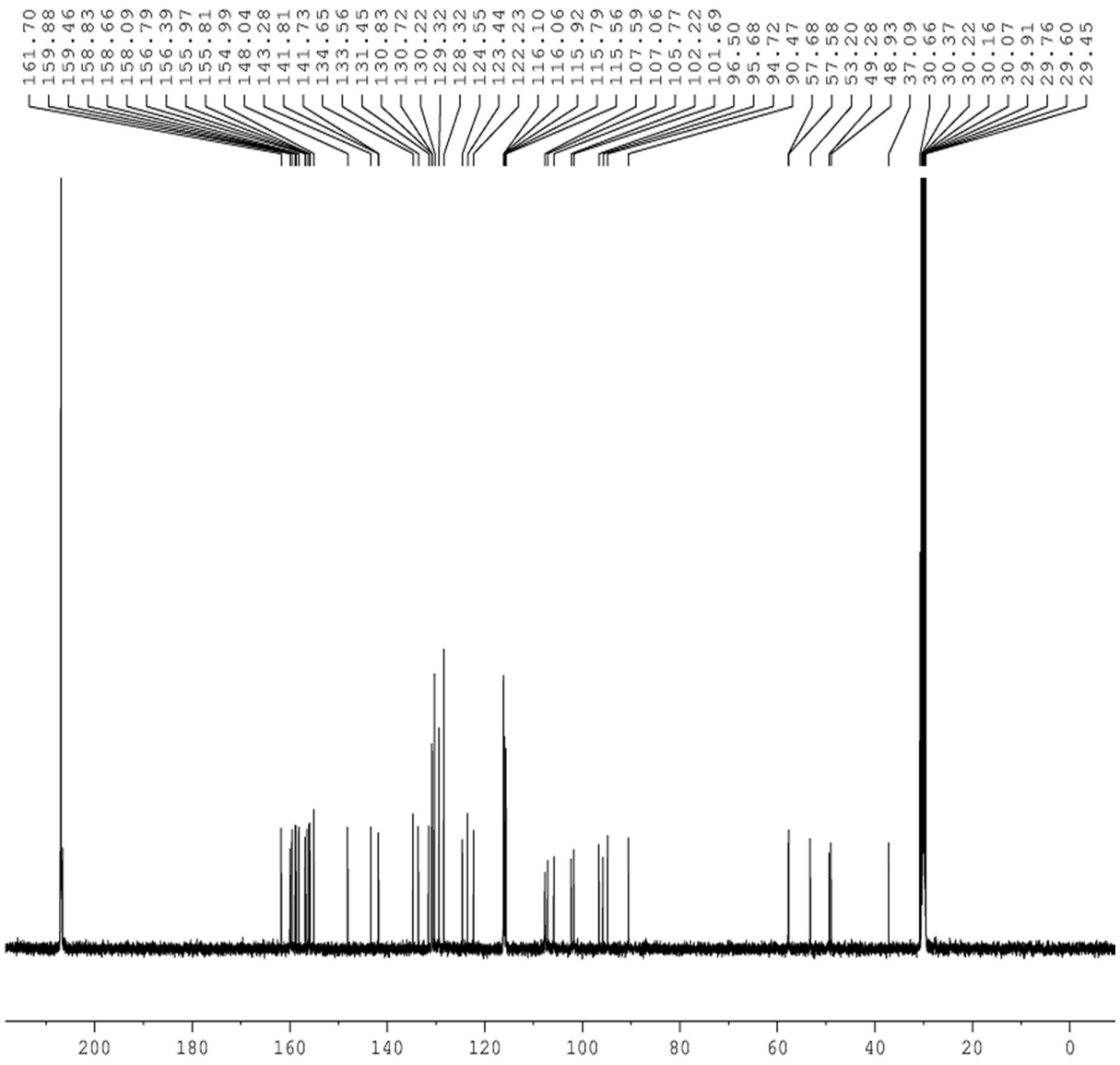


Fig 2.10. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Vaticaphenol A

2.3.4. α-Viniferin

α-Viniferin is a resveratrol trimer (Fig 2.11) isolated from the stem bark of *Hopea ponga* L. This oligostilbenoid is widely reported for various biological activities like anti-inflammatory activity, anti-oxidant activity, anti-arthritis activity, and anti-tumor activity. α-Viniferin was also reported for its potent inhibitory effect on cytochrome P450 in human liver microsomes (HLM) (Sim et al., 2014). A recent study established its role in suppressing angiogenesis via blocking the VEGFR-2/p70 pathway (Cho et al.,2020).The NMR spectra of α-Viniferin is depicted in Fig 2.12 and Fig 2.13.

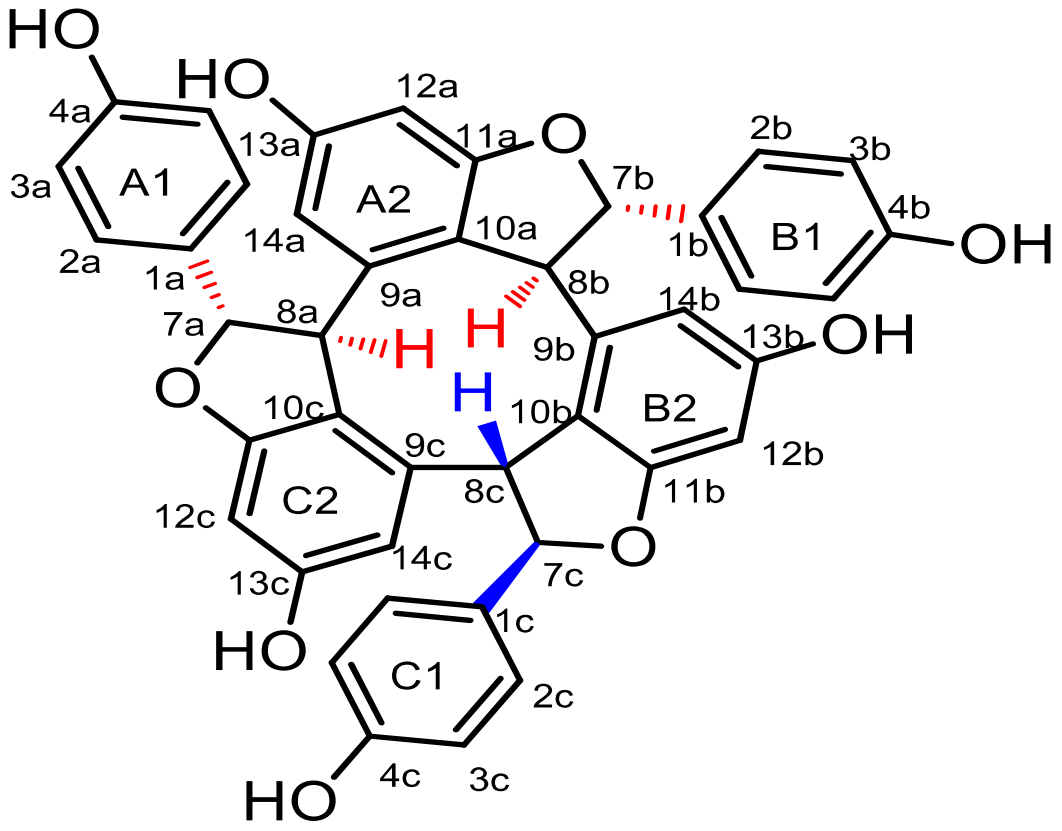


Fig 2.11. Structure of α-Viniferin

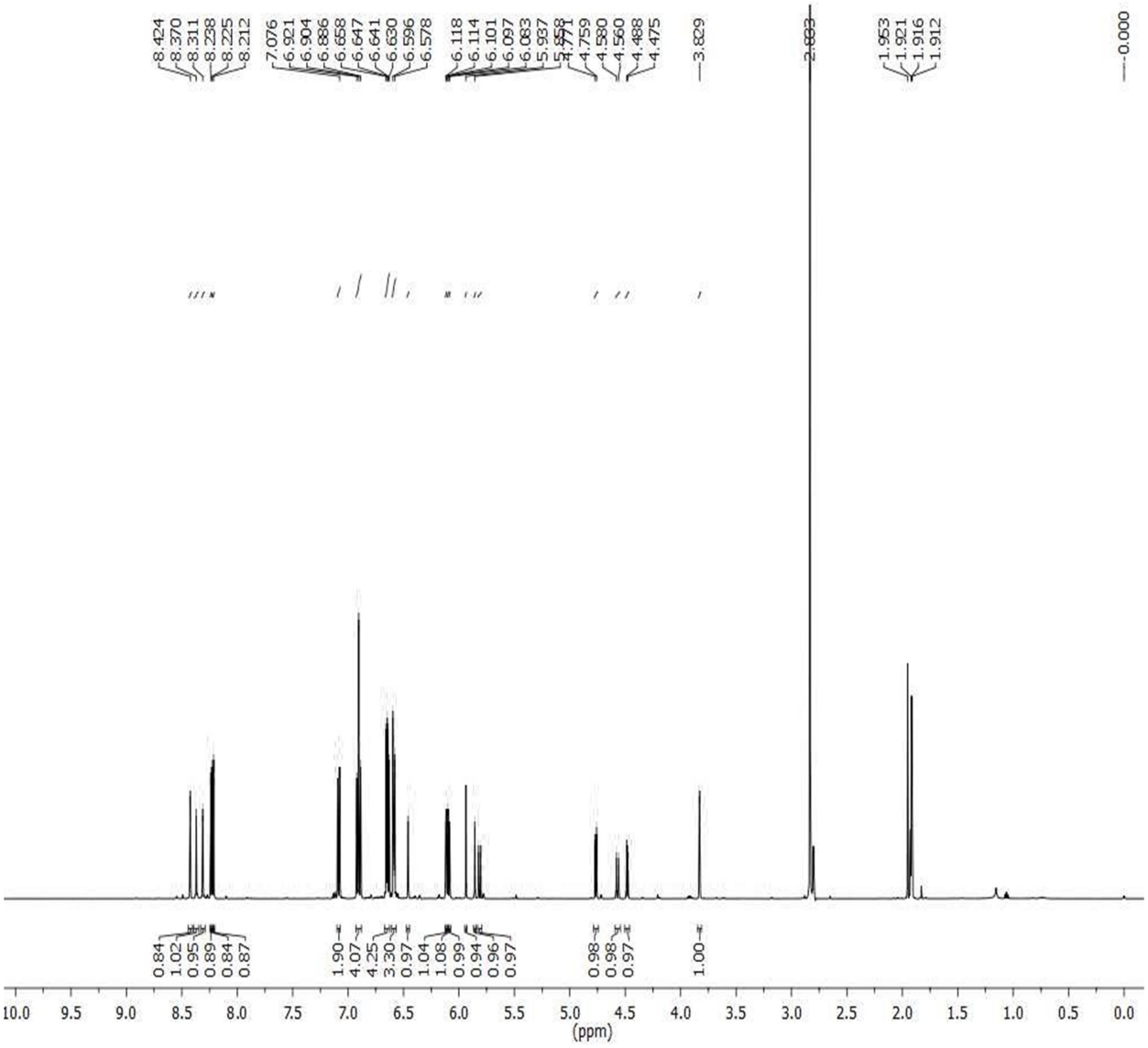


Fig 2.12. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of α-Viniferin

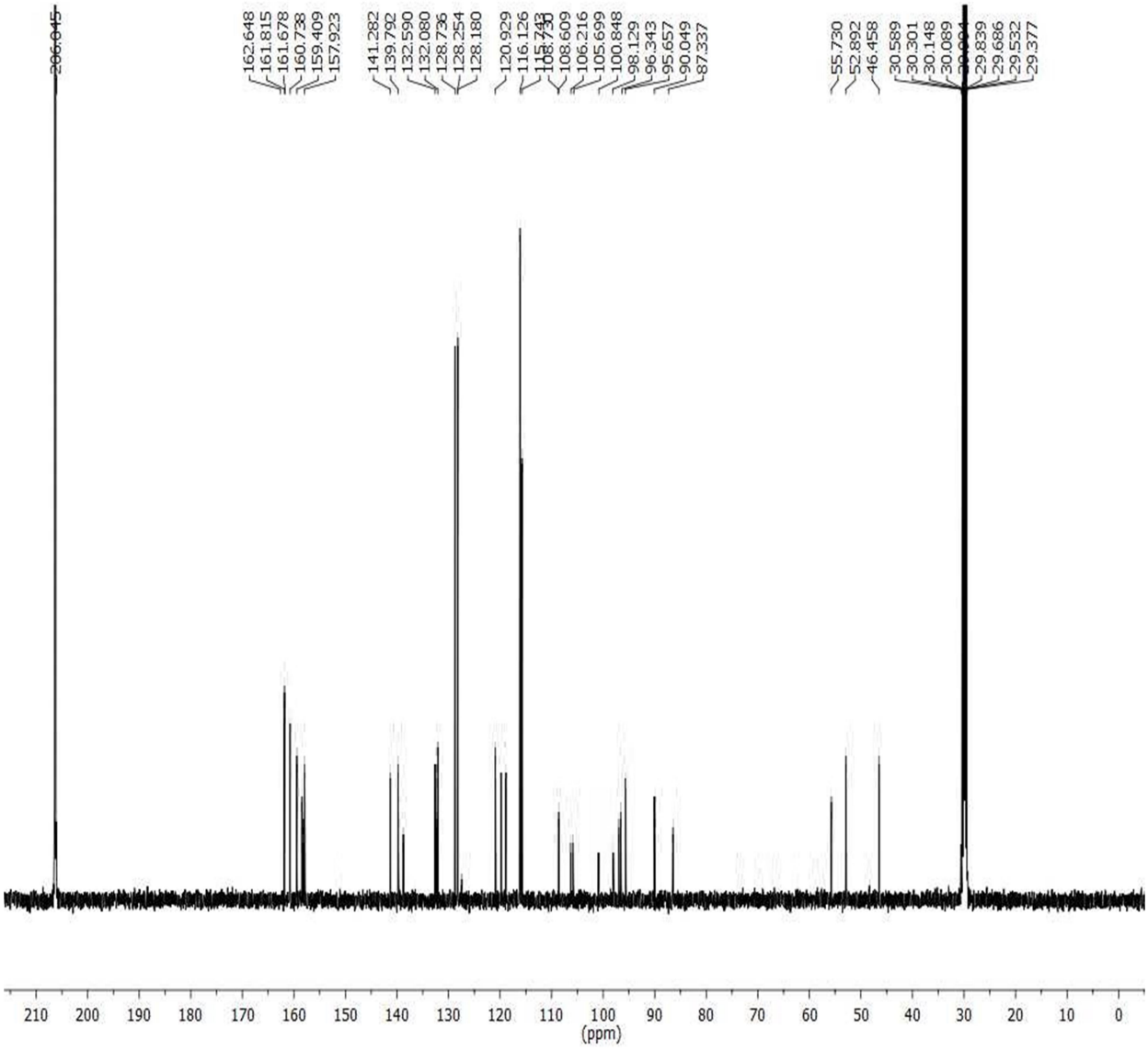


Fig 2.13. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of α-Viniferin

2.3.5. NIIST C1 (Novel compound)

NIIST C1 is a novel compound (Fig 2.14) isolated from the stem bark of *Vatica chinensis* L. This compound is a non-heterocyclic resveratrol tetramer and this compound is reported for the first time from natural sources. The NMR spectra of NIIST C1 is depicted in Fig 2.15 and Fig 2.16.

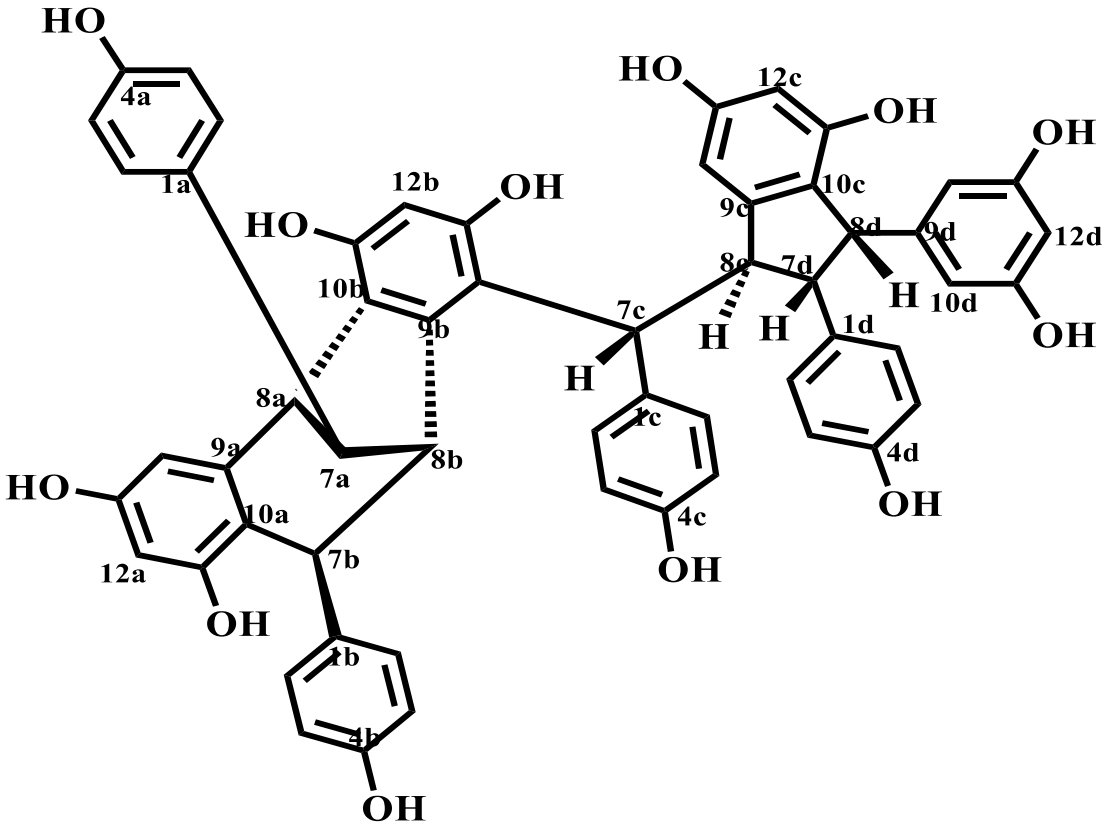


Fig 2.14. Structure of NIIST C1

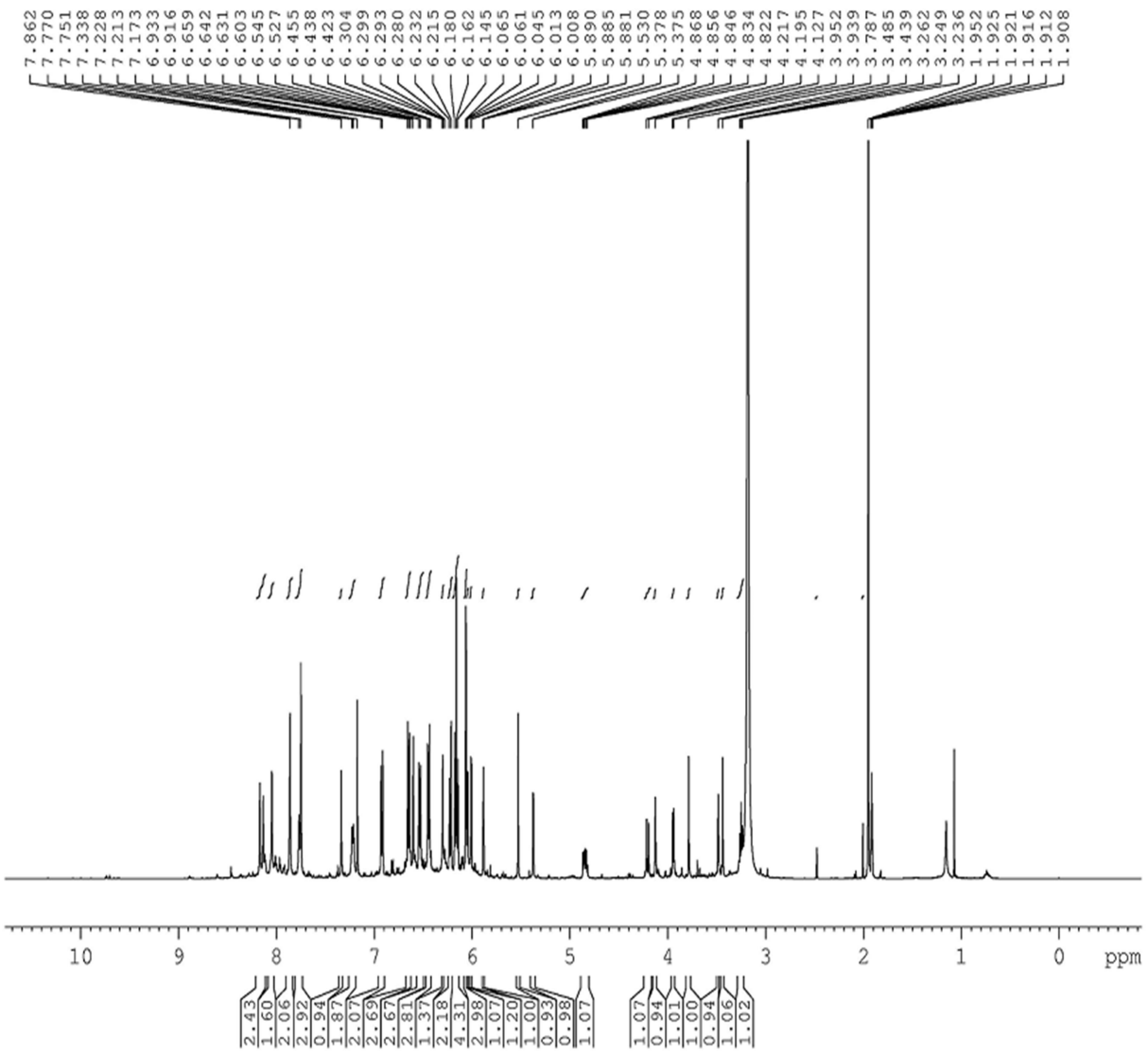


Fig 2.15. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of NIIST C1

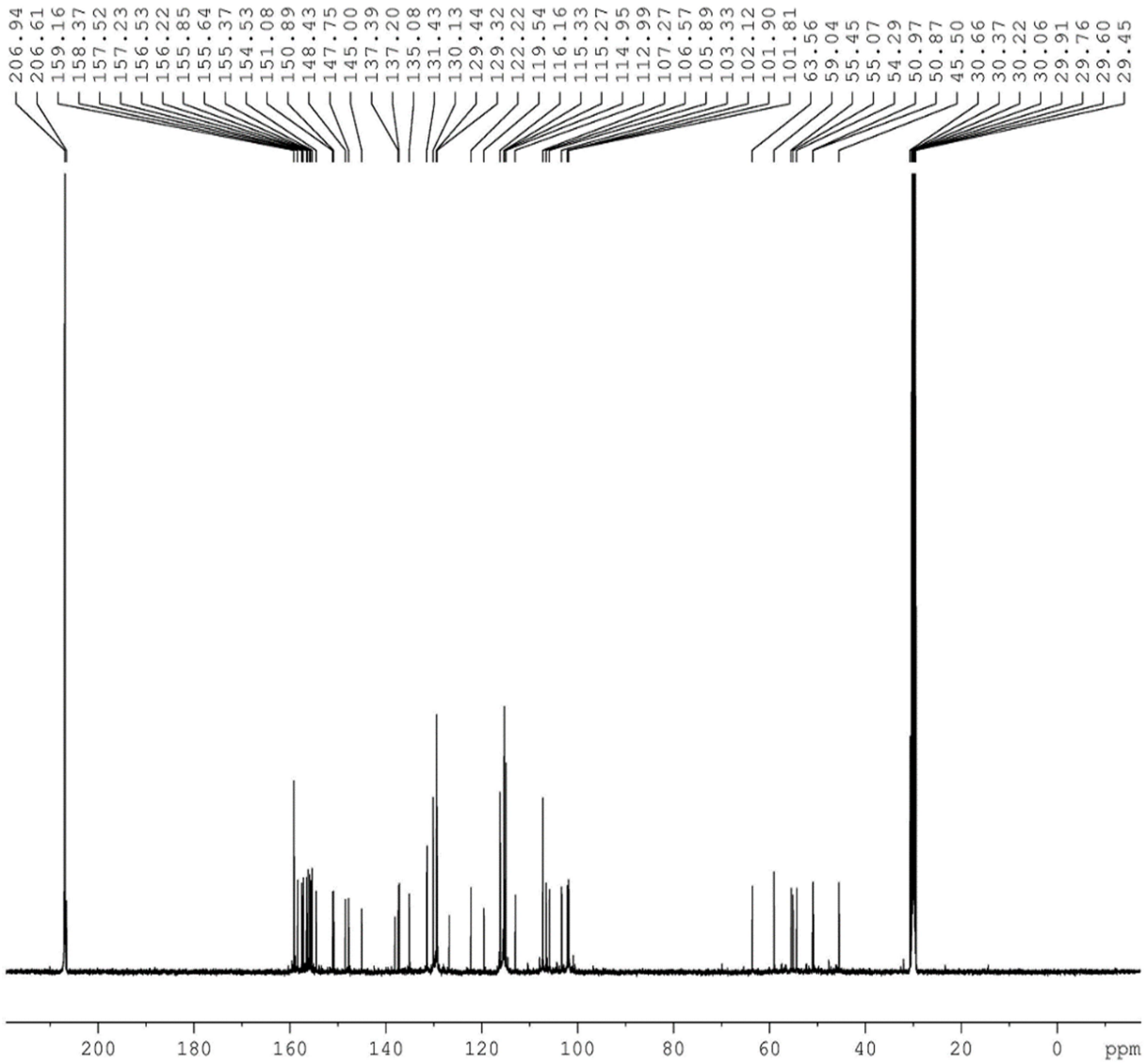


Fig 2.16. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of NIIST C1

2.3.6. 2, 4, 8 - trihydroxyphenanthrene-2-*O*-glucoside (THPG)

2, 4, 8 - trihydroxyphenanthrene-2-*O*-glucoside is a resveratrol oligomer (Fig 2.17) isolated from the stem bark of *Hopea ponga* L. There were no reports with respect to its biological activity. The NMR spectra of THPG is depicted in Fig 2.18 and Fig 2.19.

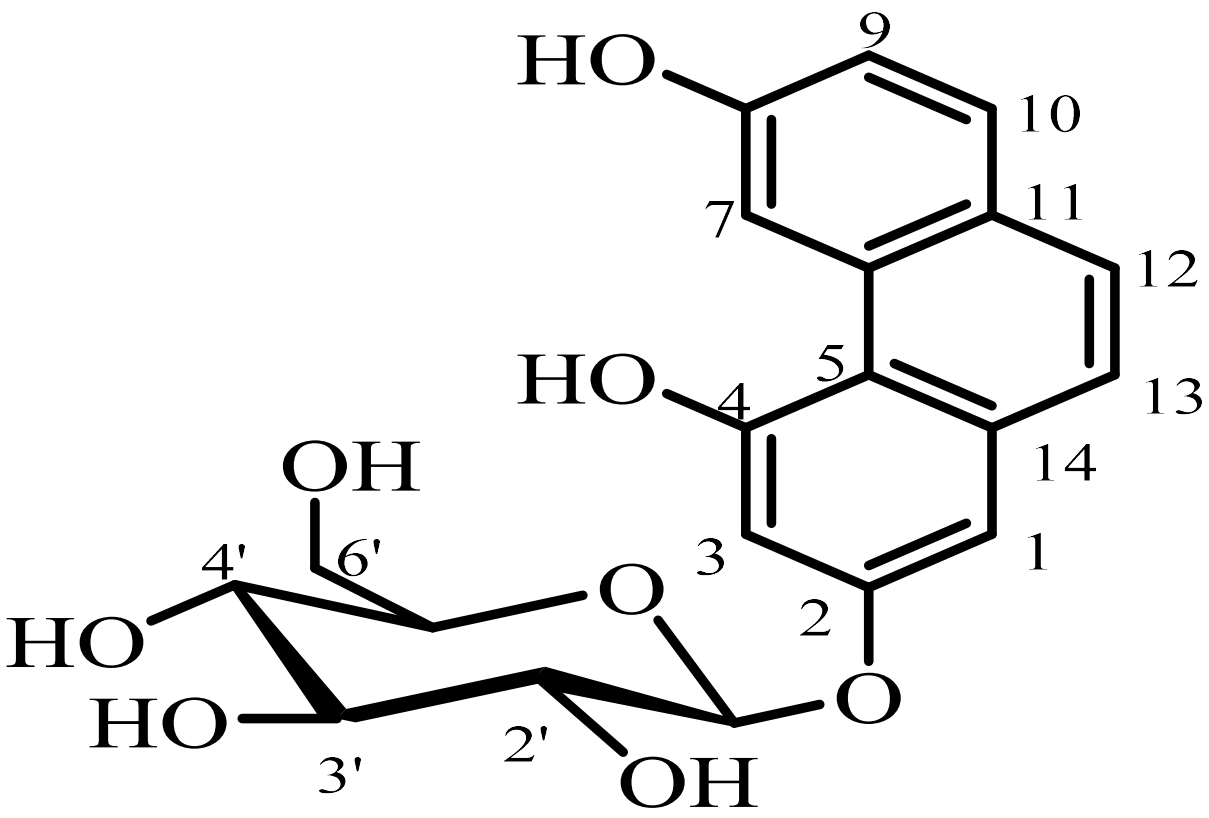


Fig 2.17. Structure of 2, 4, 8 - trihydroxyphenanthrene-2-*O*-glucoside

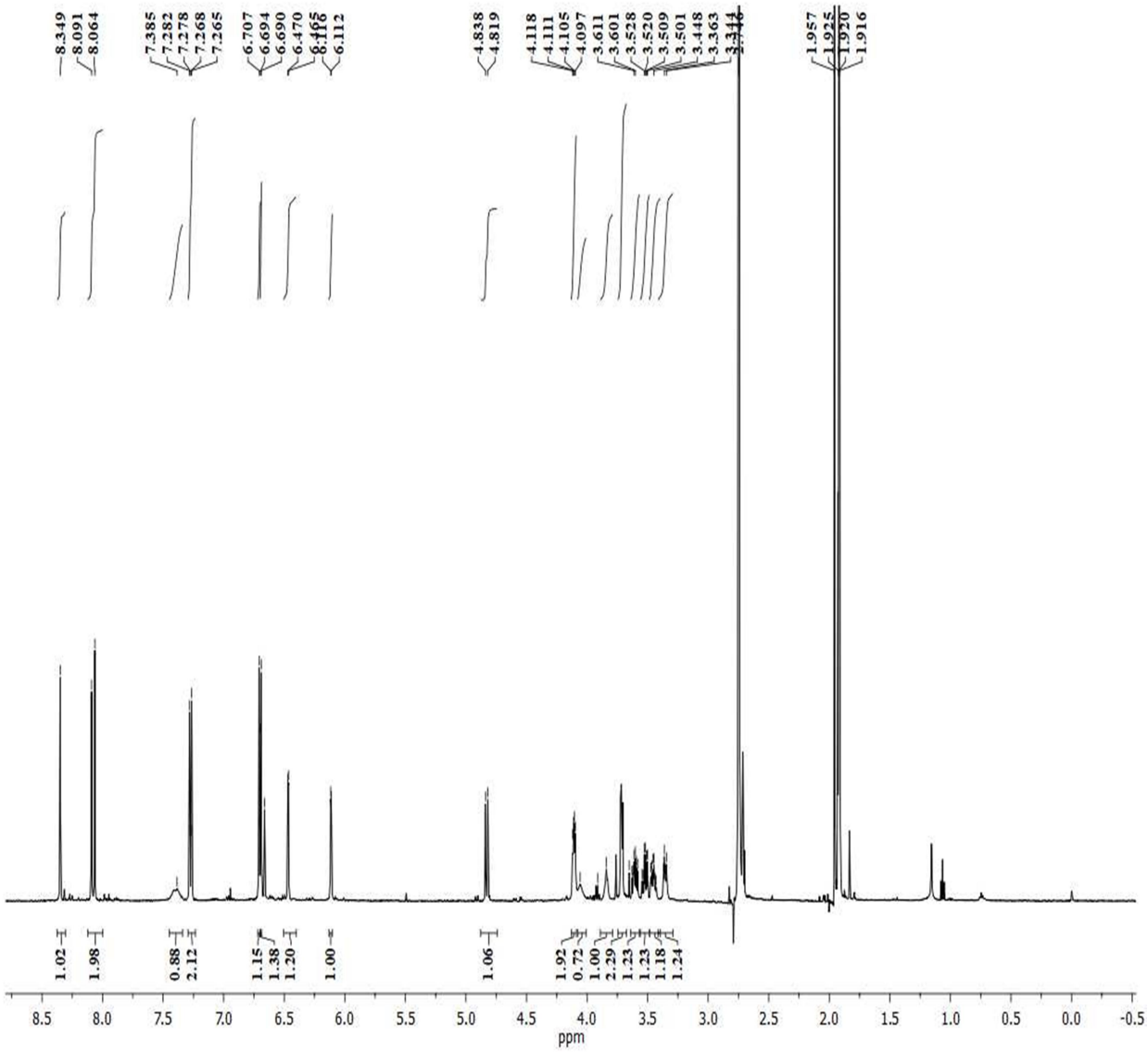


Fig 2.18. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of 2, 4, 8-trihydroxyphenanthrene-2-*O*-glucoside

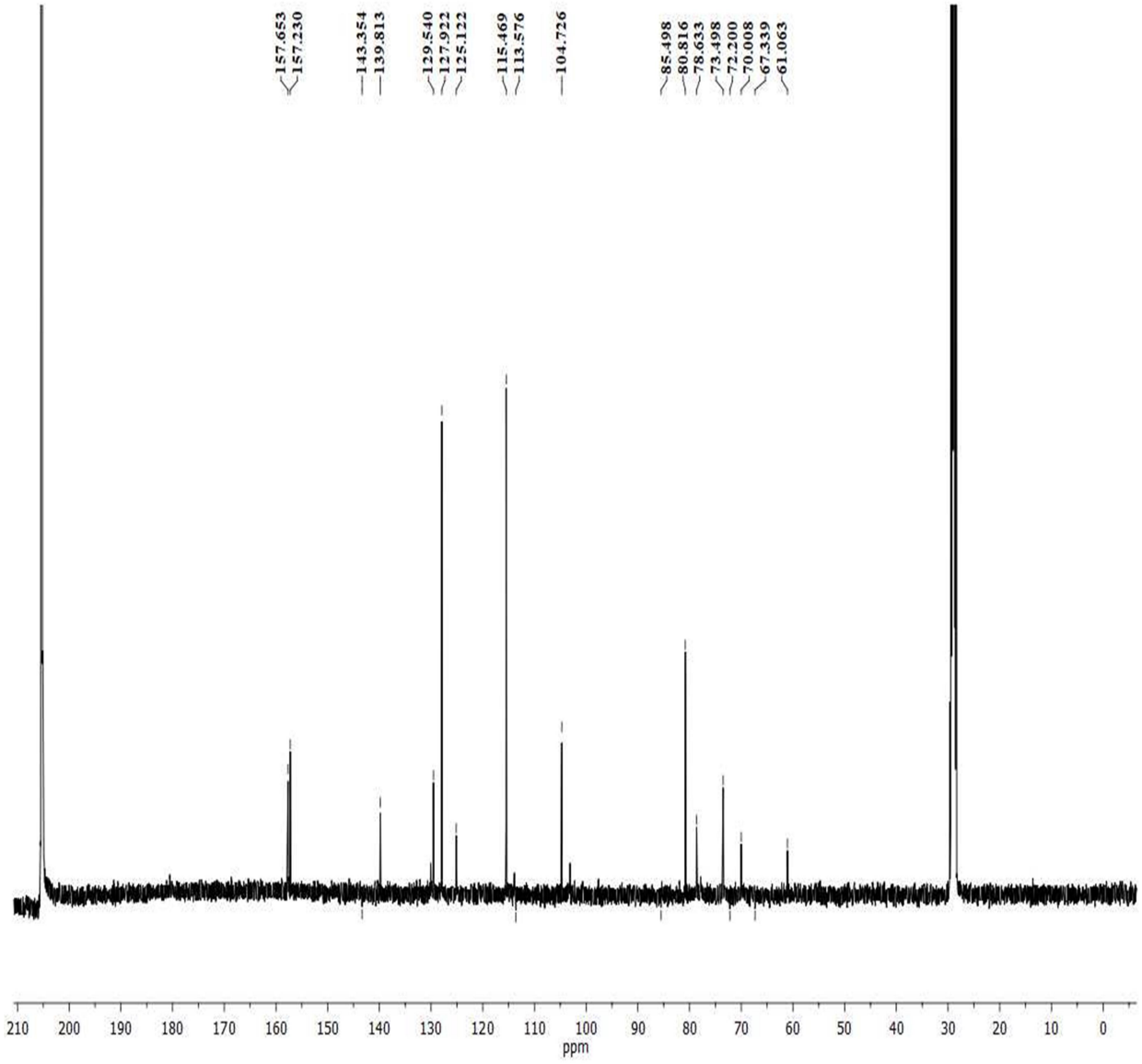


Fig 2.19. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of 2, 4, 8-trihydroxyphenanthrene-2-*O*-glucoside

2.3.7. Resveratrol hexamer (Vaticanol M)

Vaticanol M is a resveratrol hexamer (Fig 2.20) isolated from the stem bark of *Vatica chinensis* L. This is a complex molecule belonging to oligostilbene group. No activity has been reported so far for this complex molecule. The NMR spectra of Resveratrol hexamer is depicted in Fig 2.21 and Fig 2.22.

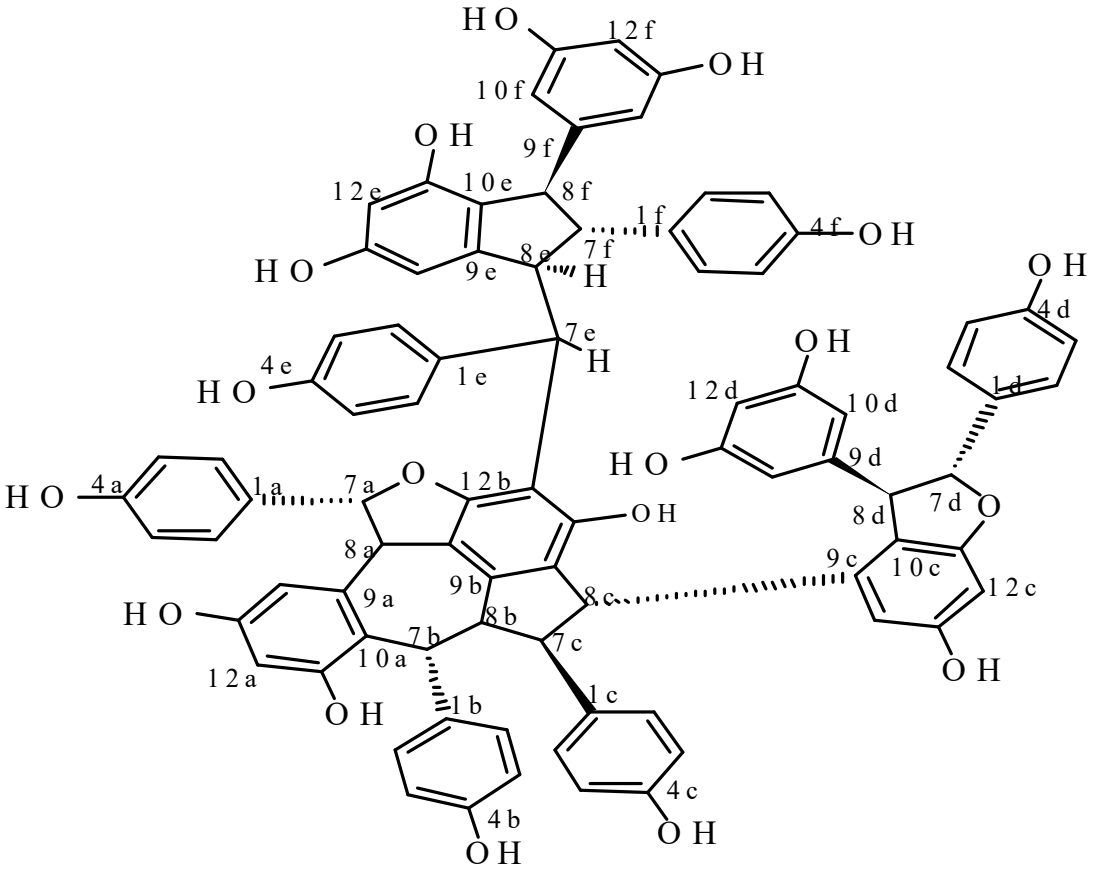


Fig 2.20. Structure of Resveratrol hexamer

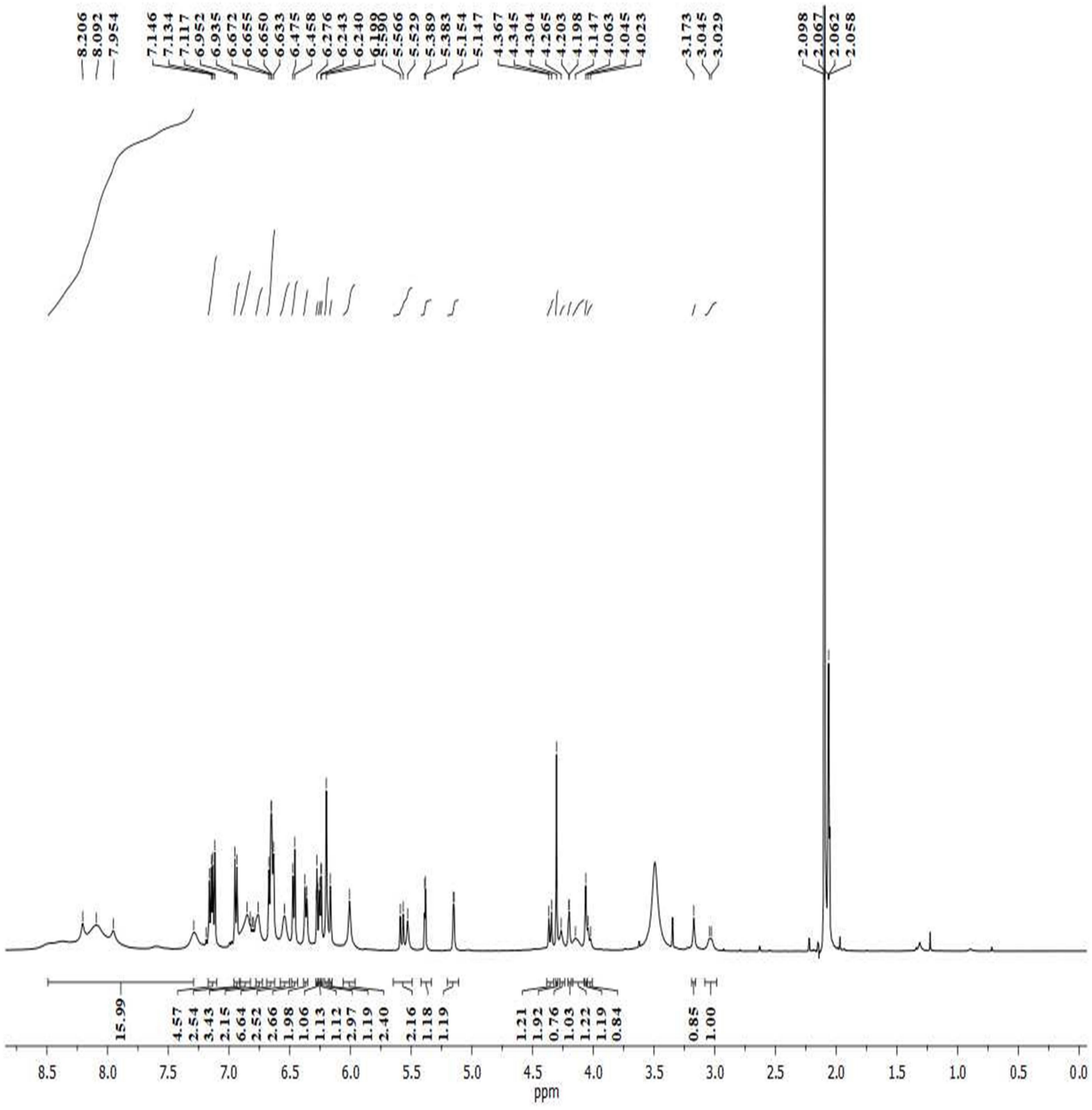


Fig 2.21. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of Resveratrol hexamer

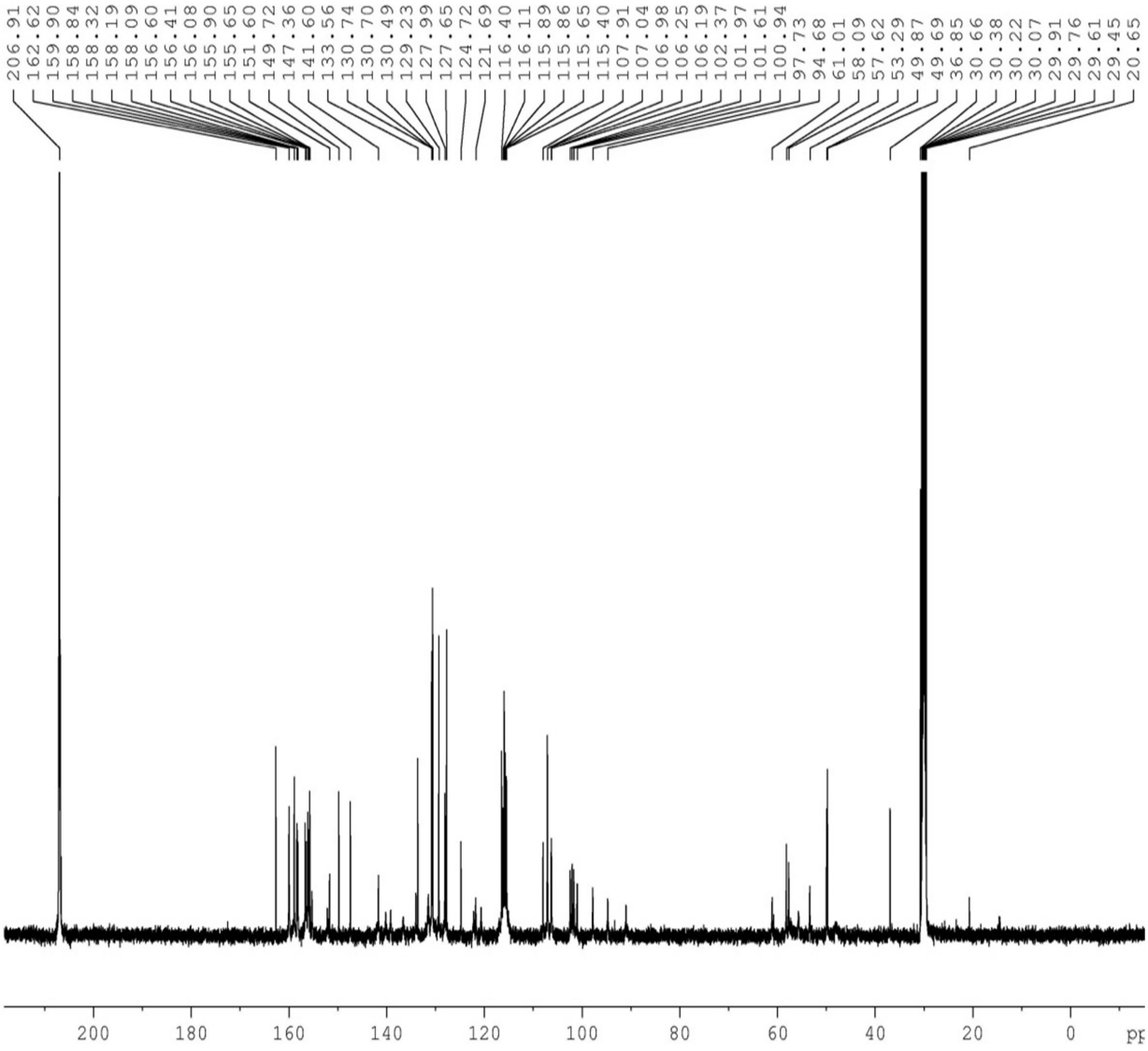


Fig 2.22. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of Resveratrol hexamer

2.3.8. ε-Viniferin

ε-Viniferin is a resveratrol dimer (Fig 2.23) isolated from the stem bark of *Vatica chinensis* L. Various biological activities were reported with this resveratrol oligomer such as anti-microbial activity (Catinella et al., 2020; Yadav et al., 2019), anti-diabetic activity in terms of activation of PPARs in *in vitro* and *in vivo* models (Tsukamoto et al., 2010). The NMR spectra of ε-Viniferin is depicted in Fig 2.24 and Fig 2.25.

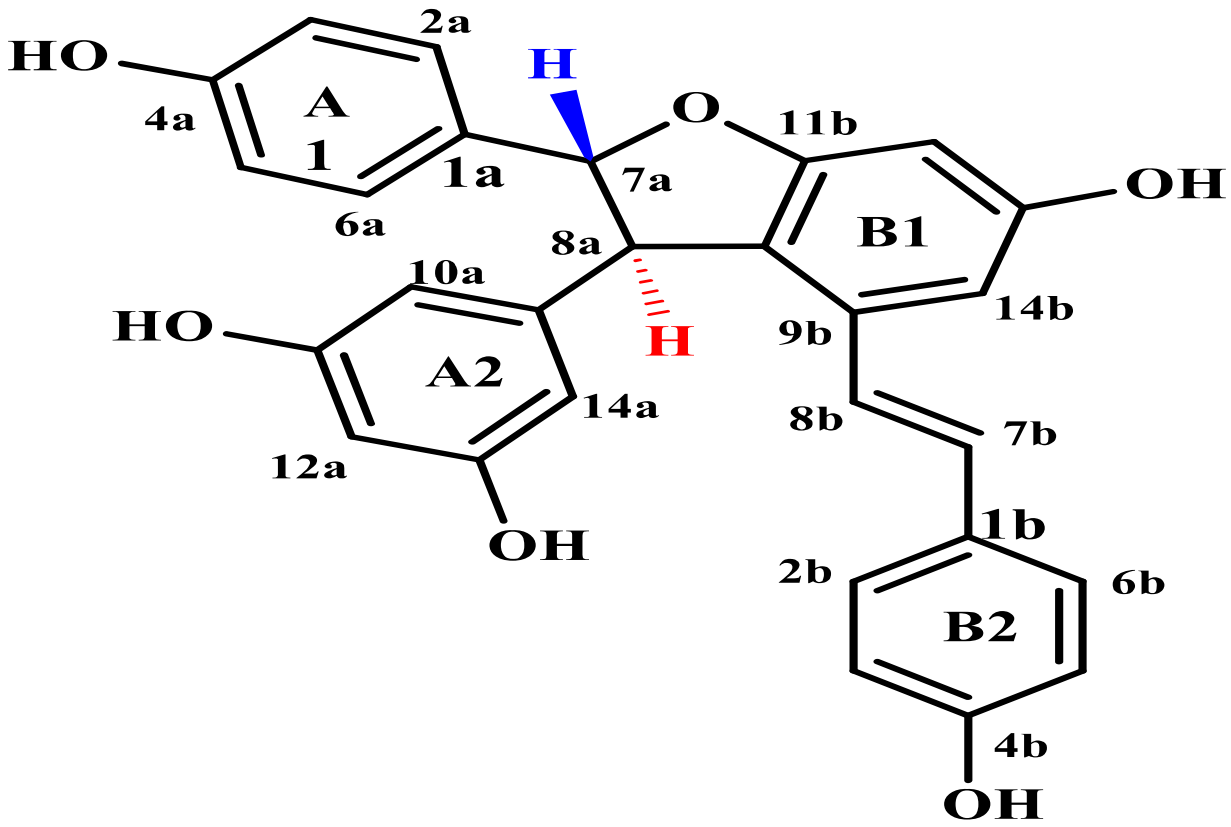


Fig 2.23. Structure of ε-Viniferin

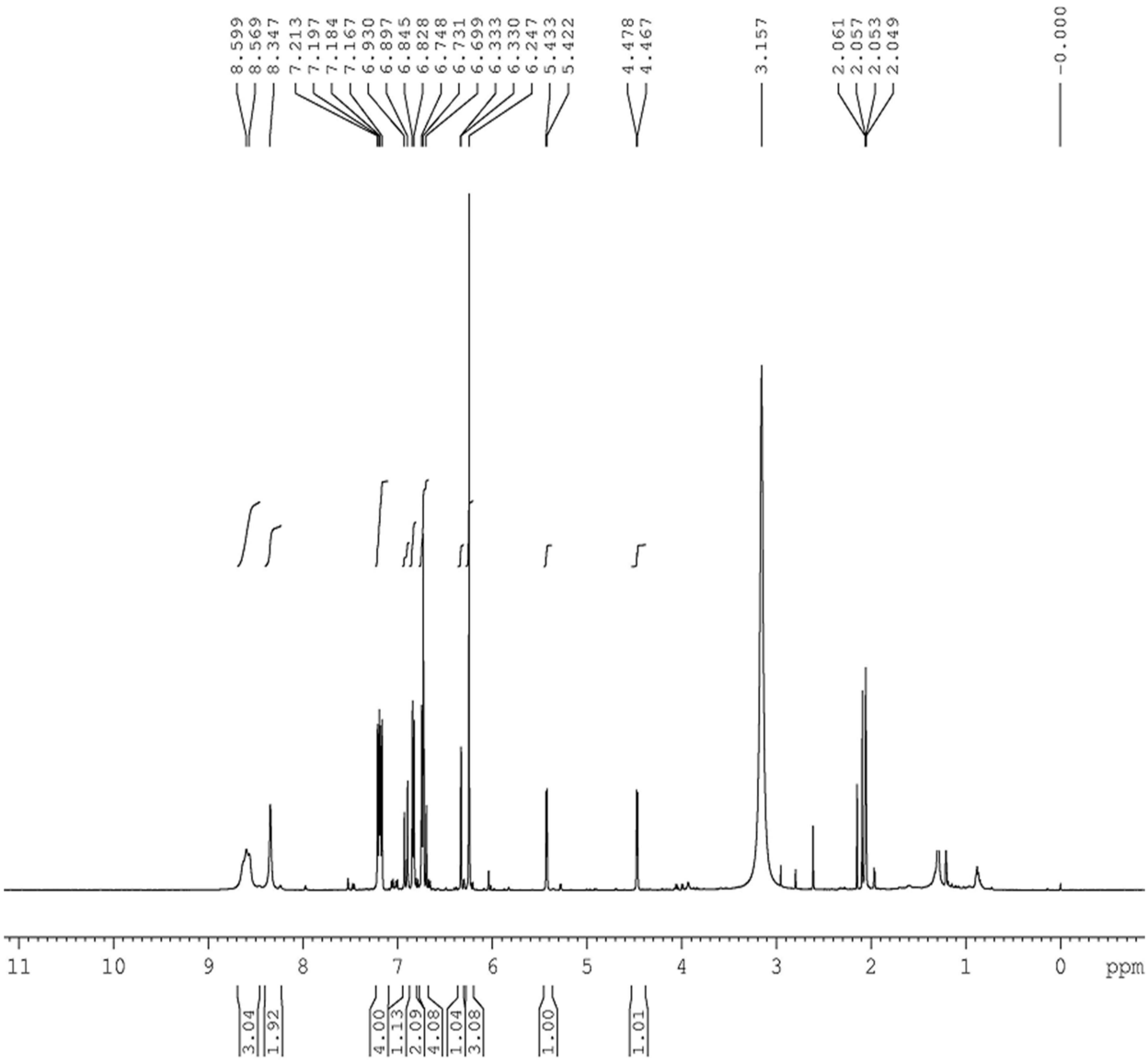


Fig 2.24. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of ε-Viniferin.

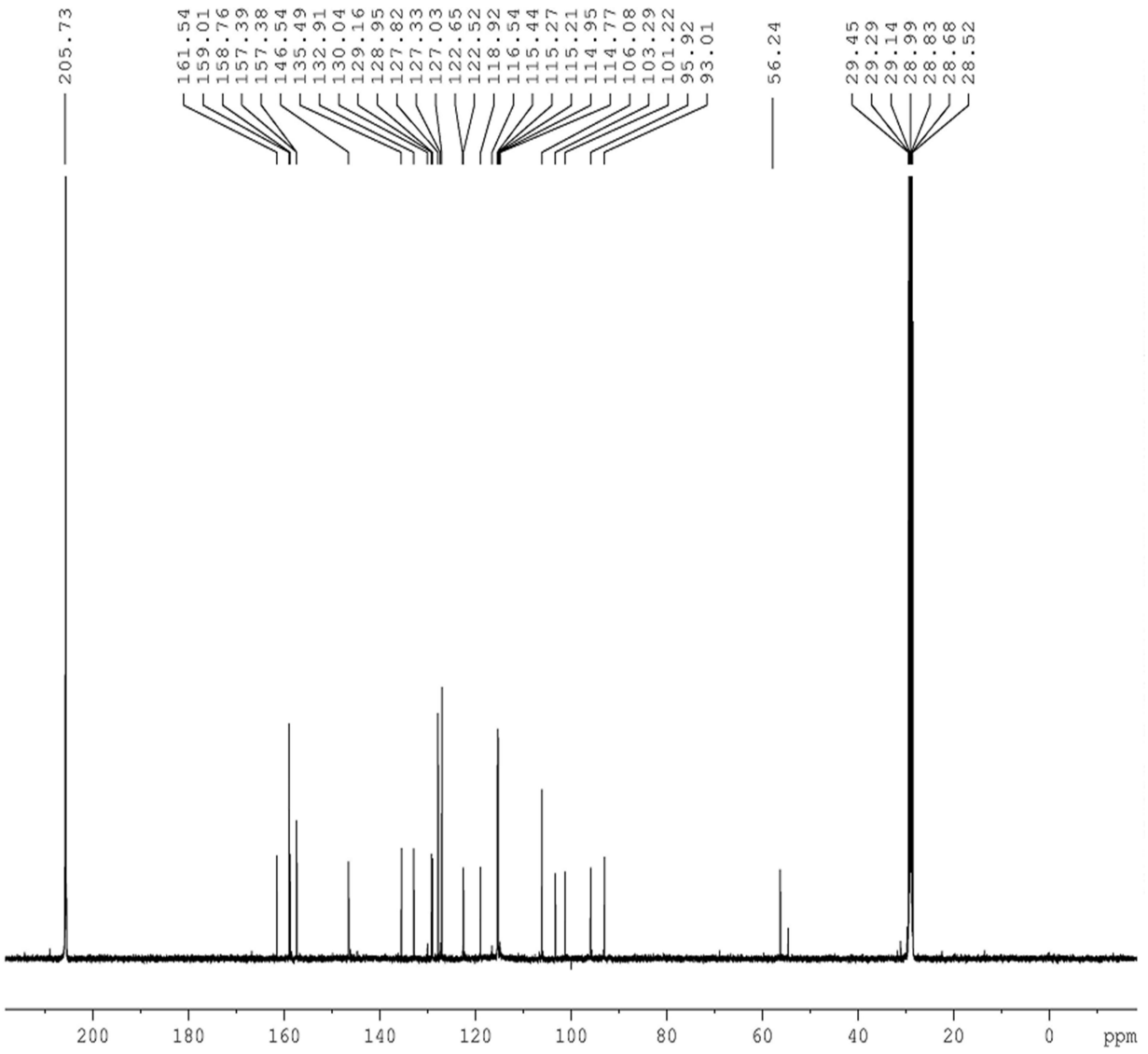


Fig 2.25. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of ε-Viniferin.

We primarily focused on screening the anti-diabetic potential of these eight resveratrol oligomers isolated from the stem bark of different plants belonging to Dipterocarpaceae family in terms of glucose uptake and insulin secretion for insulin signaling and insulin secretion studies respectively.

The insulin signaling studies were carried out in skeletal muscle cell lines, L6 to investigate the role of compounds in enhancing glucose uptake and the translocation of GLUT4 which are the utmost hallmarks in the treatment of diabetes (Zhao et al., 2019). Skeletal muscle is a main tissue for the consumption of blood glucose and principal target cells for the action of insulin. Insulin stimulates the uptake of glucose in skeletal muscle tissue by increasing the translocation of functional glucose transport molecules GLUT4 onto the plasma membrane (Das et al., 2015).

The insulin secretion studies were carried out in pancreatic beta cell lines, Beta-TC-6 cell lines. It is one among the few cell lines possessing characteristics of pancreatic beta cells. This mouse pancreatic insulinoma cell line secretes insulin upon glucose stimulation. This cell line expresses abundant insulin and trace amounts of glucagon and somatostatin owing to the corresponding genes present in the cell line.

2.4. Objectives

- To evaluate glucose uptake potential of the resveratrol oligomers using L6 myotubes.
- To evaluate the insulin secretion potential of the resveratrol oligomers using Beta-TC-6 cell lines.

The experimental design for screening the Resveratrol Oligomers for their anti-diabetic potential in L6 skeletal muscle cell lines and pancreatic Beta-TC-6 cell lines are depicted below in Fig 2.26.

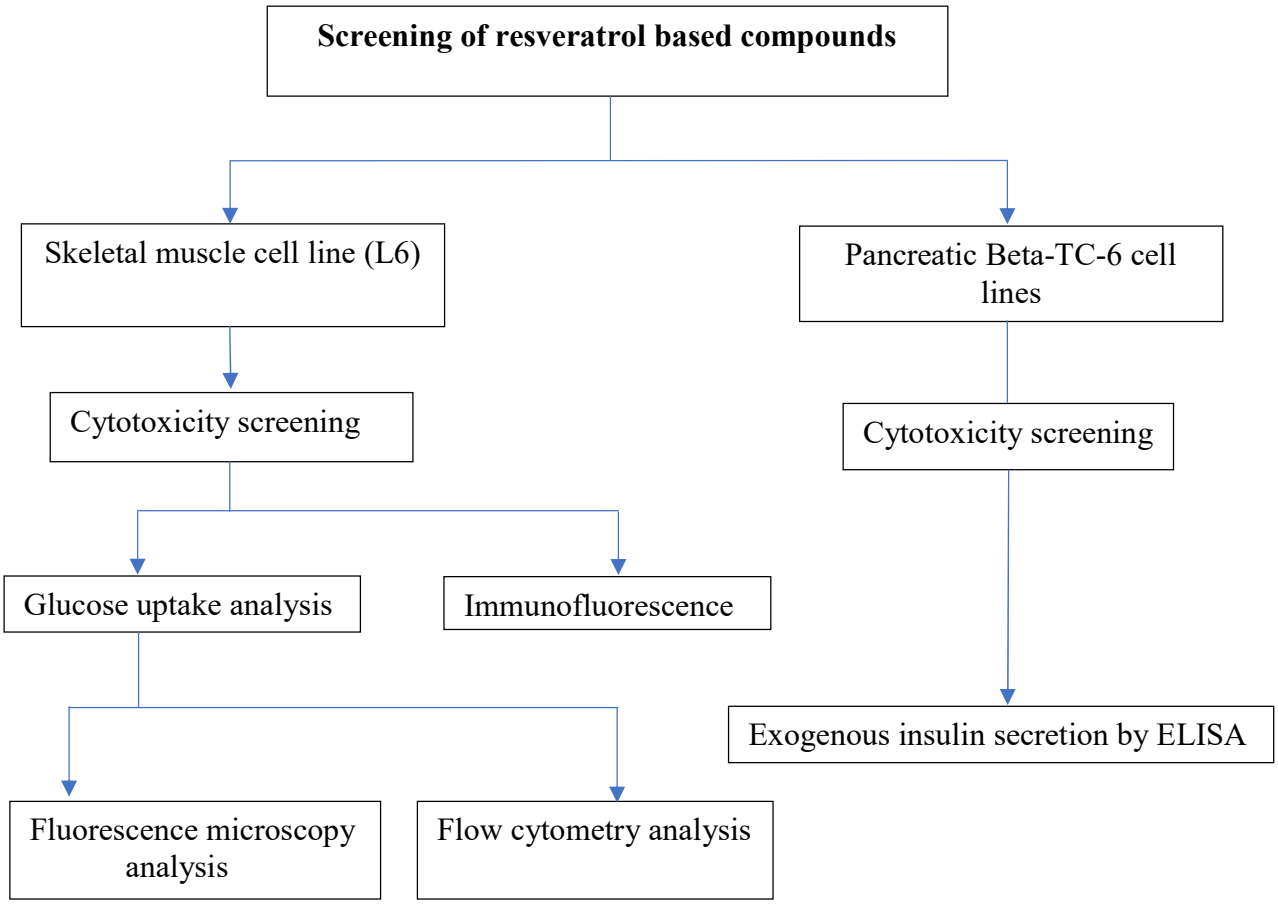


Fig 2.26. Flow chart showing the experimental design for screening of resveratrol oligomers in L6 myoblasts and Beta-TC-6 cell lines.

2.5. Materials and methods

2.5.1. Materials

Dulbecco's modified Eagles's media (DMEM), Fetal Bovine Serum (FBS), Horse Serum (HS) Bovine Serum Albumin (BSA), 10X Trypsin, 100X Anti-biotic antimycotic mix, phosphate buffer saline (PBS) were purchased from Himedia Laboratories, India; Metformin, insulin (Bovine), MTT (Thiazolyl blue) were obtained from Millipore Sigma (United States); 2-NBDG [2- (7- Nitrobenz-2-oxa-1,3- diazol- 4-yl) amino- 2-deoxy- D- glucose] was acquired from Invitrogen Life Technologies, United States; primary and secondary antibodies were purchased from Cell Signaling Technology (Massachusetts, USA), Rat/Mouse ELISA kit to measure insulin were acquired from Merck Millipore (Burlington, US). L6 myoblast and Beta-TC-6 cell lines were procured from National Centre for Cell Sciences, Pune, India. Rest of the chemicals expended were of standard analytical grade.

2.5.2. Cell culture and treatment

Rattus skeletal myocytes, L6 myoblasts were cultured in high-glucose DMEM (25mM glucose) added with 10% Fetal Bovine Serum, 1% 1X antibiotic- antimycotic mix in a humidified atmosphere of 95% air and 5% CO_2 at 37^{o}C. For differentiation into myotubes, L6 cells were cultured in DMEM with 2% horse serum on attaining 70% confluency for 4-5 days.

Mouse derived pancreatic Beta-TC-6 insulinoma cell lines procured from NCCS were cultured in DMEM containing 25mM glucose supplemented with 10% FBS, 1% 1X antibiotic-antimycotic mix under similar conditions as L6 myoblasts. The cell cultures were trypsinized within 7-10 days after attaining 2:3 confluency.

2.5.3. Cytotoxicity studies by means of MTT

The L6 myoblasts were trypsinized and seeded in 24 well plates at a density of $5x10^4$ cells/ well. When a monolayer of 70 % cells were formed, flicked off the medium and pretreated the cells in the presence and absence of resveratrol based compounds in different concentrations (25, 50, 75 and 100 μM) for a period of 24 hours. After incubation, drained the solution from the wells and MTT dissolved in DMEM plain media (0.5 g/L) was added to respective wells and

incubated for 4h at 37^{o} C in a CO_2 incubator. 300 µL of 10 % SDS in DMSO was added to each well and was gently shaken for 45 minutes to dissolve the formed blue formazan product. The absorbance was measured colorimetrically (570 nm) by using a microplate reader (BIOTEK-USA) as a sign of metabolically active cells (Palanivel, 2005).

Percentage cytotoxicity was analyzed by the following calculation-

$$\text{Percentage of Toxicity} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right] \times 100$$

Similarly, the Beta-TC-6 cell lines were pretreated with resveratrol oligomers for a period of 24 hours and percentage cytotoxicity was determined.

2.5.4. Glucose (2-NBDG) uptake in L6 myotubes

L6 skeletal muscle cell lines grown in 96 well black clear bottom plates (BD Biosciences, New Jersey) were allowed to differentiate by maintaining in DMEM with 2 % horse serum for 4-5 days and the extent of differentiation was evaluated by examining multinucleation by means of forming myotubes. After incubation with resveratrol oligomers with the least cytotoxic concentration in the presence of insulin (100 nM) for 24 hours, culture medium was substituted with fresh DMEM medium in the absence and presence of 100 µM fluorescent tagged glucose moiety, 2-NBDG (Invitrogen), and incubated at 37^{o} C for 30 minutes. After incubation, the cells were washed twice using phosphate-buffered saline (PBS) and the fluorescence gained by the cells were acquired by a Fluorescent Microscope (Pathway 855, BD Bioscience, USA) equipped with filters in the FITC range (excitation, 490 nm and emission, 525 nm). The average fluorescence intensity in cells of different groups were evaluated by BD Image Data Explorer software for 2 dissimilar experiments.

2.5.5. 2-NBDG uptake analysis by flow cytometry

The enhancement in 2-NBDG uptake induced by the resveratrol based compounds were validated by flow cytometry analysis. Following treatment with the resveratrol oligomers for a period of 24 hours, the medium was decanted from each well and replenished with new plain

medium in the presence and absence of 100 μM fluorescent 2-NBDG and underwent thirty minutes incubation. The cells washed in PBS were subsequently trypsinized and suspended again in ice cold saline buffer. The cells were exposed to flow cytometry analysis using Becton Dickinson fluorescence-activated cell sorting (BD FACS) Aria II (BD Biosciences) at FITC range (excitation 490 nm, emission 525 nm band pass filter). The unlabeled cells were corrected for auto fluorescence and the fluorescence intensities were analyzed by BD FACS DIVA software.

2.5.6. Immunofluorescence assay

The measurement of surface GLUT4 levels under various pretreatment conditions was accomplished through immunofluorescence assay. The cells were seeded in a black well clear bottomed plate followed by differentiation for 4-5 days and were then treated with the compounds (Ampelopsin F, (-) Hopeaphenol, NIIST C1 and THPG) which were found to be most active in the 2-NBDG uptake assay along with metformin for a period of 24 hours and then stimulated with insulin (100 nM) for 10–30 minutes. The pretreated cells were then washed with saline buffer and fixed with 4% formaldehyde for five minutes. Followed by 3X wash with PBS, cell permeabilization were accomplished with 0.2 % triton X for ten minutes. Subsequently the cells were blocked using 5% BSA for one hour and incubated with monoclonal GLUT4 antibody solution (1:250 dilution in blocking buffer) at 4^{o} C overnight. Followed by 3X wash, 1 hour incubation was given with Alexa flour 488-conjugated goat anti-mouse IgG secondary antibody (1:400 dilution in blocking buffer). Images were acquired by using a Fluorescent Microscope (Pathway 855, BD Bioscience, United States) equipped with filters in the Alexa fluor range (excitation, 490 nm and emission, 525 nm).

2.5.7. Insulin Secretion Assay

Pancreatic Beta-TC-6 cell lines were seeded in a 24 well plate in the growth medium at 37^{o} C in an atmosphere of 5% CO_2. Insulin concentrations were quantified by means of an ELISA kit exclusive for rat/mouse insulin (Millipore Corporation, United States). The beta cells were pretreated with compounds in low glucose media (1g/ L) at safer concentration obtained from MTT assay for a period of 24 hours. After the period of incubation, the culture medium was collected and the insulin secreted into the media was utilized for insulin ELISA assay for

quantification of insulin levels according to the manufacturer's instructions. Glyburide was used as the positive control. The exogenous insulin secreted are measured colorimetrically at 450 nm. The results were expressed in ng/mL of insulin produced and secreted onto the medium.

2.5.8. Statistical analysis

All the experiments were executed in triplicates. The data were presented as mean ± standard deviations and significance of differences determined by one-way ANOVA followed by Tukey's test. P value at $p \leq 0.05$ was regarded as statistically significant.

2.6. Results and Discussion

2.6.1. MTT assay

The cytotoxicity of the eight resveratrol based compounds was determined by means of MTT assay as described by Mosmann (1983). MTT dye was applied to assess the cell viability based on which the dead cells do not reduce the tetrazolium salts. The metabolically active cells reduce this MTT upon entering the mitochondria by NAD(P)H-dependent oxidoreductase enzymes into a dark purple colored formazan product. Hence the amount of formazan produced could be related to the number of active cells. The cytotoxicity of the resveratrol oligomers in L6 myoblasts for a period of 24 hours is depicted below in Table 2.2.

Table 2.2. MTT based cytotoxicity assay of resveratrol oligomers (expressed in percentage) in L6 myoblasts

Compounds	Concentration of compounds (in µM)			
	25 µM	**50 µM**	**75 µM**	**100 µM**
Ampelopsin F	17.94 ± 0.006	19.09 ± 0.0007	22.47 ± 0.009	30.98 ± 0.0127
(-)Hopeaphenol	0.58 ± 0.002	6.77 ± 0.004	9.08 ± 0.009	10.88 ± 0.0007
Vaticaphenol A	2.09 ± 0.007	9.87 ± 0.0007	17.51 ± 0.002	20.32 ± 0.018
α-Viniferin	1.73 ± 0.008	2.45 ± 0.001	4.03 ± 0.004	7.49 ± 0.031
NIIST C1	1.22 ± 0.007	2.81 ± 0.004	13.11 ± 0.057	19.02 ± 0.002
THPG	19.09 ± 0.007	30.8 ± 0.015	34.29 ± 0.011	40.27 ± 0.017
Resveratrol Hexamer	24.21 ± 0.011	28.53 ± 0.001	33.21 ± 0.009	39.05 ± 0.024
ε- Viniferin	9.37 ± 0.004	11.82 ± 0.007	14.8 ± 0.0007	15.78 ± 0.002

The compound α-Viniferin showed less than 10% cytotoxicity up to 100 µM concentration and hence did not confer any significant lethality to L6 cell lines. (-) Hopeaphenol, NIIST C1 and ε-Viniferin revealed the safety of the compounds in L6 cell lines by showing more than 80% cell viability up to 100 µM concentration. Vaticaphenol A and Ampelopsin F were found to increase their toxicity by more than 20 % in 75 and 100 µM concentrations.. Reduction of cell

viability was observed with high concentrations of Trihydroxy phenanthrene glucoside & Resveratrol hexamer (Fig 2.27).

Hence, based on the results obtained from MTT cytotoxicity assay, 50 µM concentration of Ampelopsin F, (-) Hopeaphenol, NIIST C1, α-Viniferin, ε-Viniferin and Vaticaphenol A were used for further studies while THPG and resveratrol hexamer were fixed at 25 µM concentration for further studies.

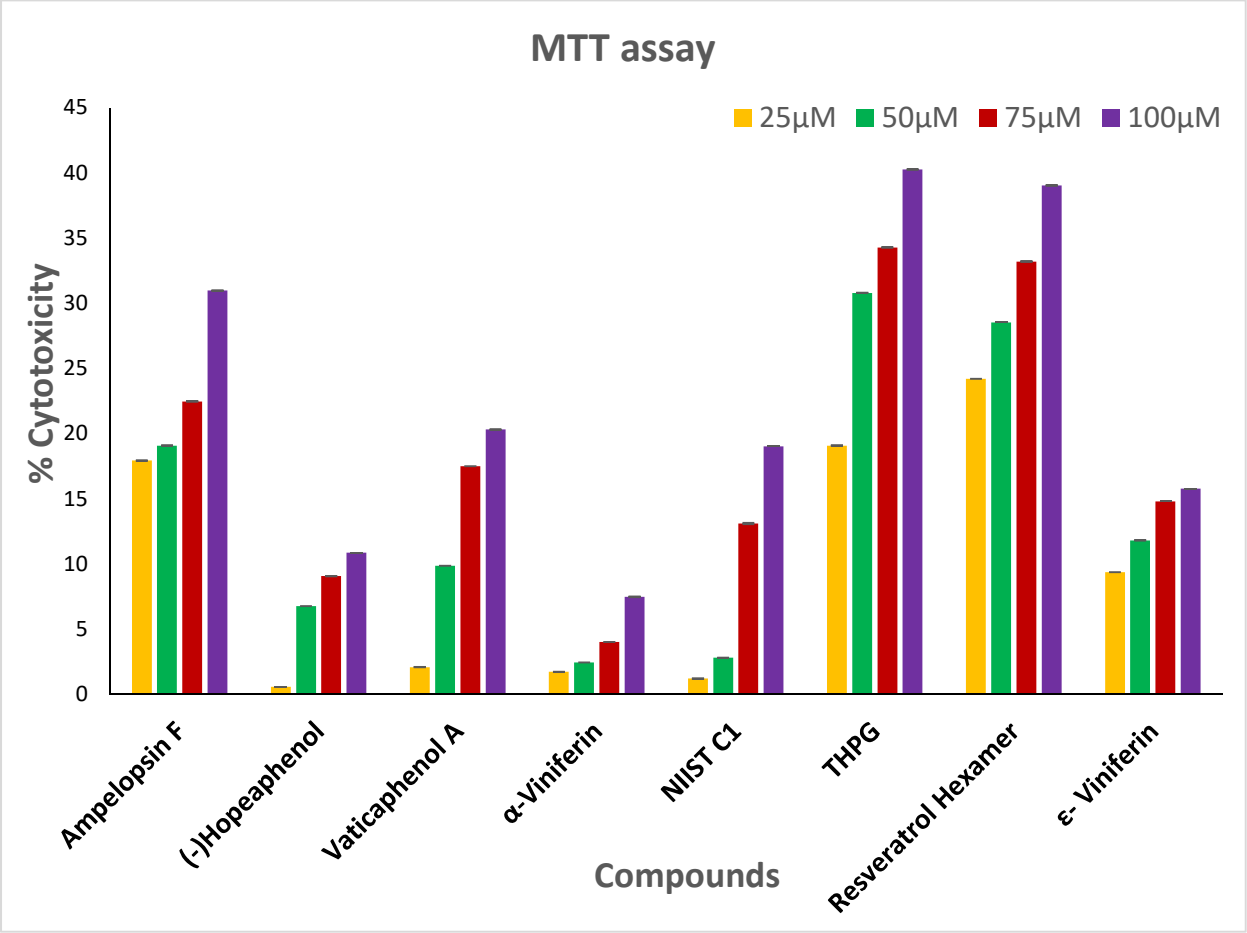
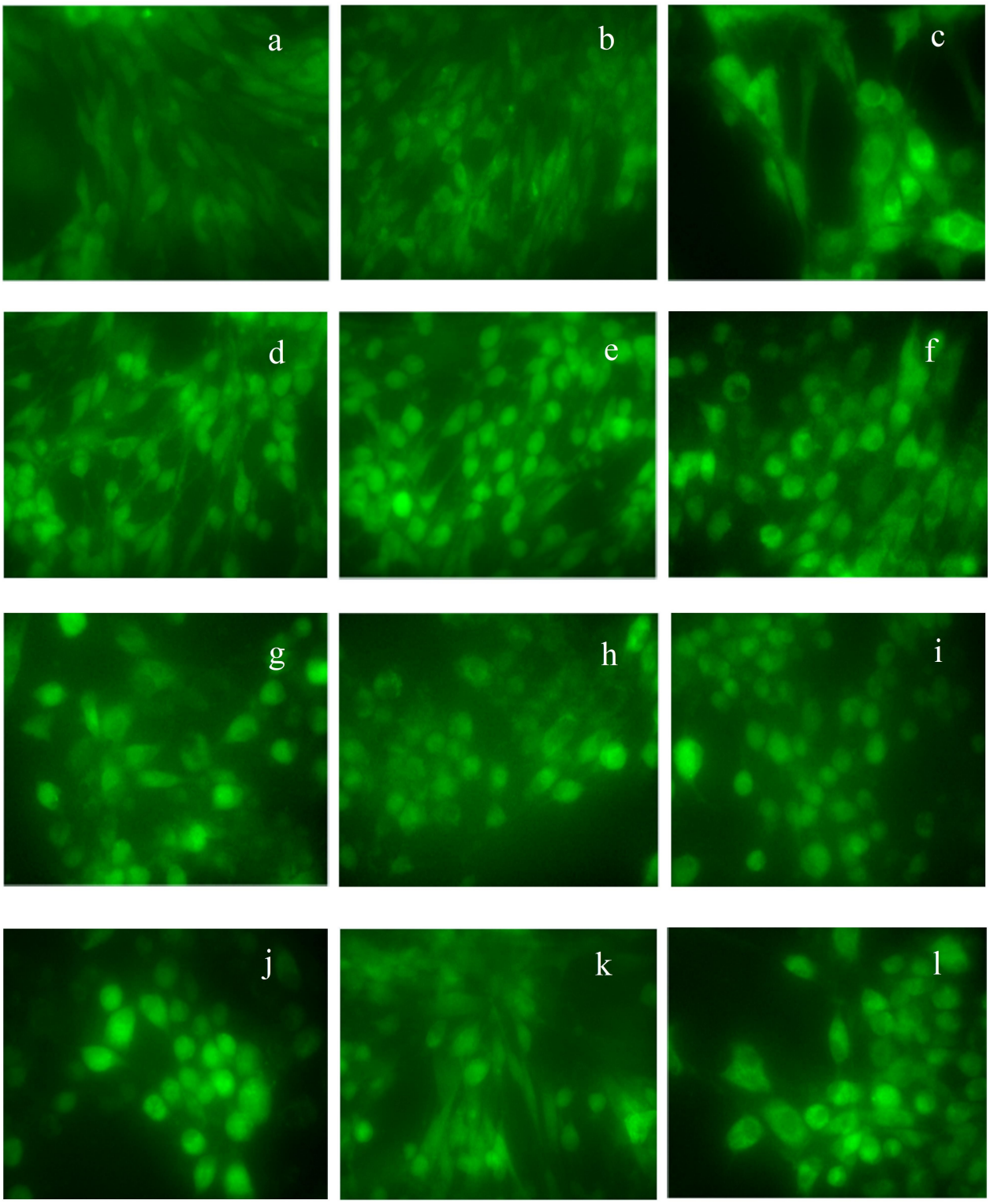


Fig 2.27. MTT cytotoxicity assay of resveratrol based compounds. L6 myoblasts were treated with various concentrations of these compounds (25 µM, 50 µM, 75 µM and 100 µM) for a period of 24 hours and the cytotoxicity of resveratrol oligomers was determined by MTT assay. All values were represented as means ± SD (n=3).

2.6.2. 2-NBDG uptake analysis by confocal microscopy

The anti-diabetic potential of the resveratrol oligomers in L6 myoblast was analyzed via 2-NBDG uptake pretreated for 24 hours. In glucose uptake assay, cells are directly incubated with 2-NBDG, a fluorescent analog of D-glucose. Investigation of the anti-diabetic potential of resveratrol oligomers was primarily evaluated by monitoring the 2-NBDG uptake in L6 skeletal myoblasts. The endocytosis of 2-NBDG in rat L6 myoblasts stimulated by the compounds were monitored in the presence and absence of insulin as shown in the Fig 2.28. Insulin treated cells showed a slight increase in the uptake of glucose than untreated cells. Metformin (100 μM), a potent anti-diabetic drug was used as the positive control for the comparative evaluation with the resveratrol oligomers. Metformin treated cells demonstrated a significant uptake of 2-NBDG than untreated cells as well as with insulin treated cells. Most of compounds showed enhanced glucose uptake in L6 myotubes except for Vaticaphenol A, Resveratrol Hexamer and ε-Viniferin. L6 myotubes pretreated with these compounds showed lesser uptake than untreated cells. Ampelopsin F, (-) Hopeaphenol, NIIST C1, THPG and α-Viniferin treated cells showed enhanced uptake of 2-NBDG when compared to untreated cells and insulin treated cells. The cells treated with Ampelopsin F, (-) Hopeaphenol, NIIST C1 and α-Viniferin demonstrated increased glucose uptake in L6 myotubes which was at par with the positive control metformin (100 μM). Cells pretreated with Ampelopsin F and THPG showed an increase in the uptake of glucose in the presence of insulin (100 μM) relatively better than in the absence of insulin. (-) Hopeaphenol, NIIST C1 and α-Viniferin treated cells didn't show significant difference in 2-NBDG uptake in the presence and absence of insulin.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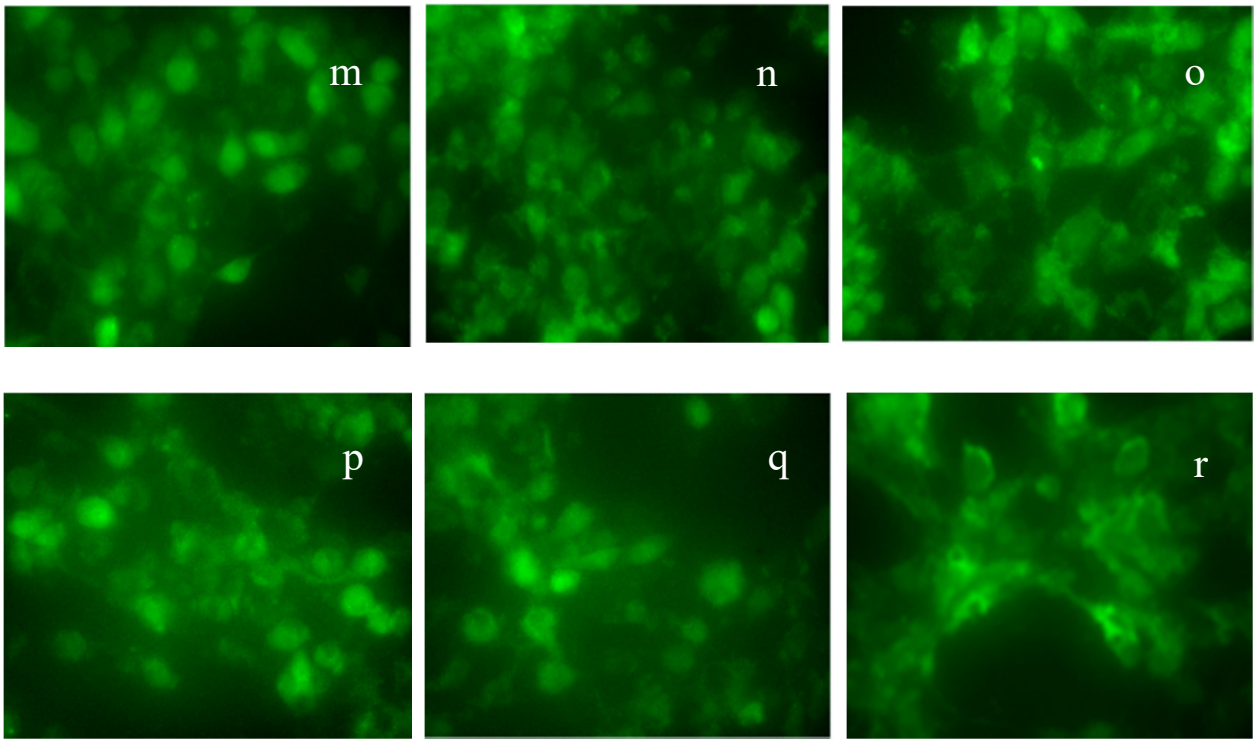


Fig. 2.28. 2-NBDG uptake assay by fluorescent microscopy in L6 rat myotubes: Fluorescent images of resveratrol oligomers pretreated for 24 hours at 40X magnification (a) control cells, (b) insulin treated (c) metformin (100 µM) (d) Ampelopsin F (50 µM) (e) Ampelopsin F + Insulin (f) (-) Hopeaphenol (50 µM) (g) (-) Hopeaphenol + Insulin (h) Vaticaphenol A (50 µM) (i)Vaticaphenol A + Insulin (j)) α-Viniferin (50 µM) (k) α-viniferin + Insulin (l) NIIST C1 (50 µM) (m) NIIST C1 + Insulin (n) Trihydroxy phenanthrene glucoside (25 µM) (o) Trihydroxy phenanthrene glucoside + Insulin (p) Resveratrol hexamer (25 µM) (q) Resveratrol hexamer + Insulin (r) ε-Viniferin (50 µM). Magnification 40 X. Scale bar corresponds to 100 µM.

The relative fluorescent intensity analysis by BD Image Data Explorer software revealed a quantitative analysis of the glucose uptake upon pretreatment with the compounds as shown in the Fig 2.29. Relative fluorescence intensity showed a two fold increase in the uptake of glucose in L6 myotubes upon pretreatment with Ampelopsin F, (-) Hopeaphenol, NIIST C1 and α-Viniferin than that of untreated cells. THPG was observed with 1.3 fold increase than that of control cells. Vaticaphenol A & ε-Viniferin were shown with a slight decrease in glucose uptake when comparing to untreated cells.

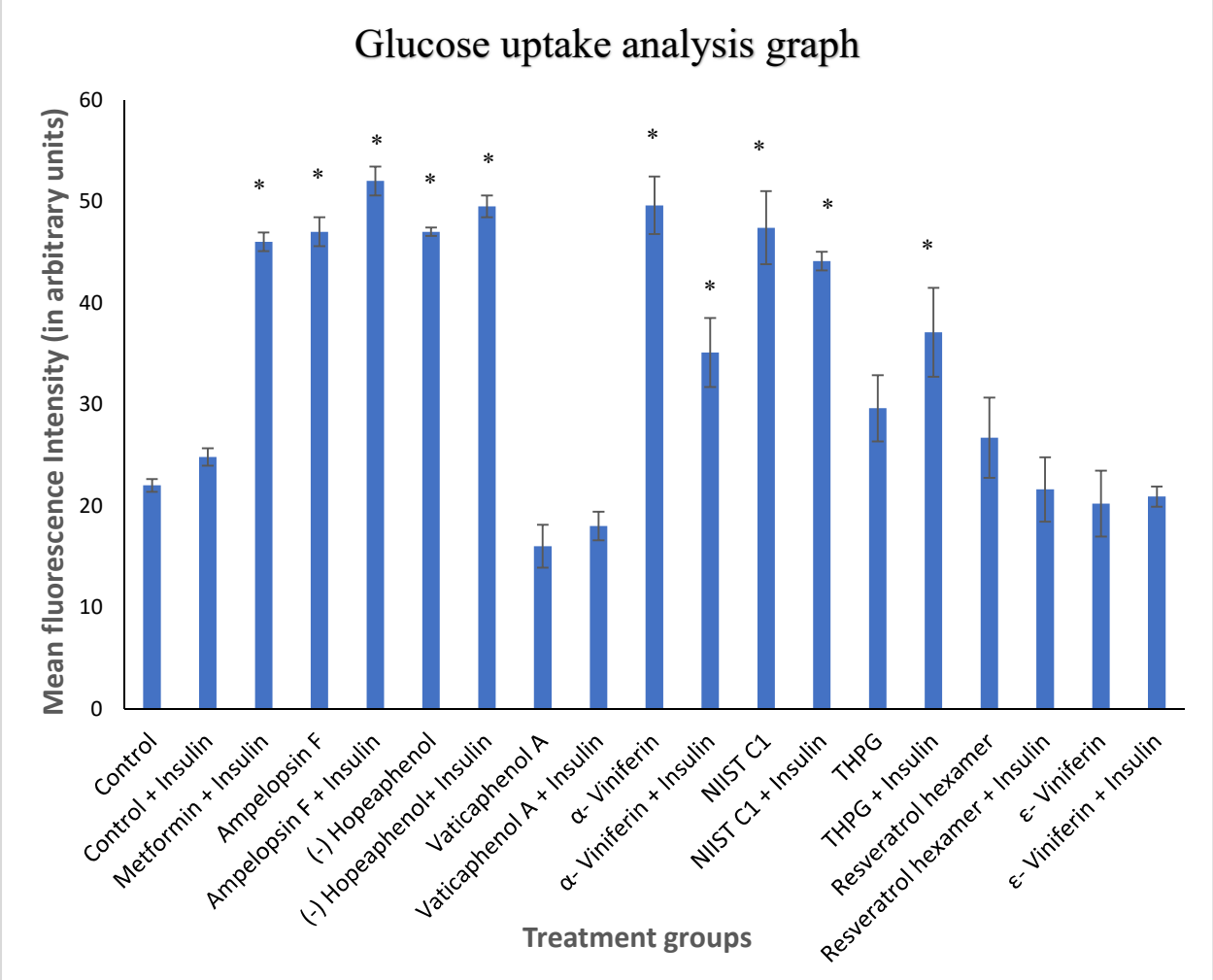


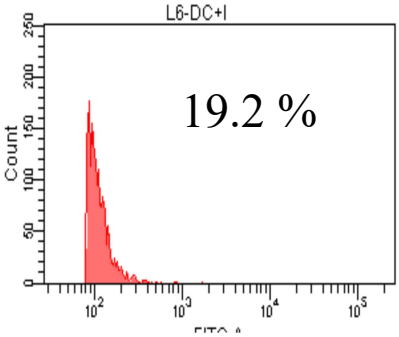
Fig 2.29. Fluorescence intensity analysis graph. L6 myoblast pretreated with the resveratrol based compounds Ampelopsin F (50 µM), (-) Hopeaphenol (50 µM), VaticaphenolA (50 µM), NIIST C1 (50 µM), THPG (25 µM), α-Viniferin (50 µM), Resveratrol hexamer (25 µM) and ε-Viniferin (50 µM) in the presence and absence of insulin. All data were represented as means ± SD (n=3). *p* values were determined by one way ANOVA followed by Tukey's test.*$p \leq 0.05$ versus control.

2.6.3. Flow cytometry analysis

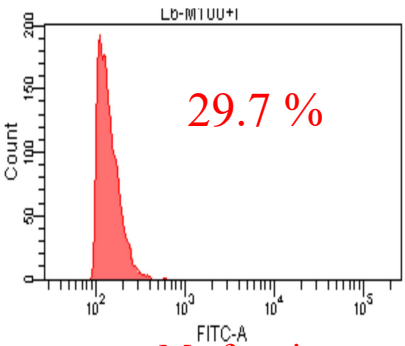
To confirm the 2-NBDG uptake of the compounds in L6 myotubes, flow cytometry analysis was performed to detect the fluorescence within the cells following the pretreatment with resveratrol oligomers at the active concentration for a period of 24 hours. Here also the cellular

glucose uptake was monitored using the fluorescent tagged glucose moiety 2-NBDG in L6 myotubes after 24 hours pretreatment with the resveratrol oligomers. Flow cytometry results were also found to be analogous to the qualitative results obtained from fluorescence microscopy. Flow cytometry analysis revealed a 2-NBDG uptake of 19.2 % in insulin treated cells. A remarkable increase in the uptake of glucose was found in metformin treated cells when compared to insulin treated cells. The positive control, metformin showed 29.7 % uptake of glucose. Ampelopsin F, (-) Hopeaphenol and NIIST C1 treated cells showed increased uptake of glucose similar to the positive control metformin. Ampelopsin F showed 31.7 % and 28.7 % uptake in the presence and absence of insulin respectively. A slight increase in the fluorescence was observed in Ampelopsin F treated cells along with insulin. No significant difference in the uptake of glucose was observed in (-) Hopeaphenol treated cells in the presence and absence of insulin. However, a significant increase in glucose uptake was observed when compared to insulin treated cells. (-) Hopeaphenol treated cells demonstrated 27.8 % uptake of glucose in the absence of insulin & showed 28.8 % uptake in the presence of insulin (Fig 2.30).

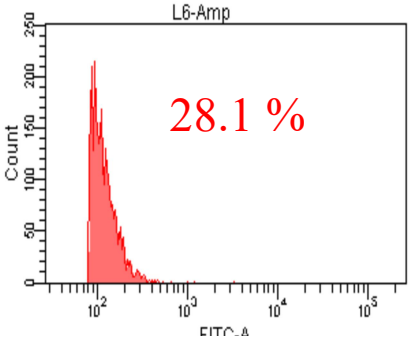
Vaticaphenol A and ε-Viniferin didn't show significant increase in the uptake of glucose in L6 myoblasts upon pretreatment under identical conditions. These compounds showed some kind of inhibition for the uptake of glucose in L6 myotubes. Vaticaphenol A showed 12.6 % and 11.4 % uptake of glucose in the absence and presence of insulin. ε-Viniferin showed 13.4 % and 15.5 % 2-NBDG uptake in the absence and presence of insulin. NIIST C1 treated cells showed 23.1 % glucose uptake without insulin and 27.7 % in the presence of insulin. Similarly THPG & Resveratrol hexamer treated cells demonstrated a slight increase in glucose uptake in the presence of insulin. THPG & Resveratrol hexamer pretreatment increased the count of cells possessing 2- NBDG from 23.9 % and 21.1 % to 32.2 % & 28.1 %, respectively, in the presence of insulin.



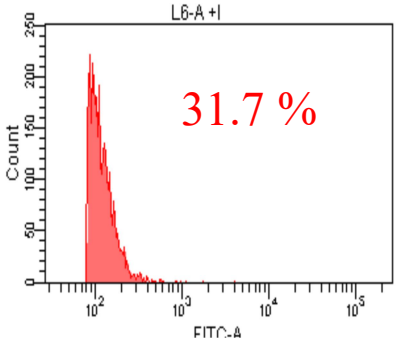
Control + Insulin



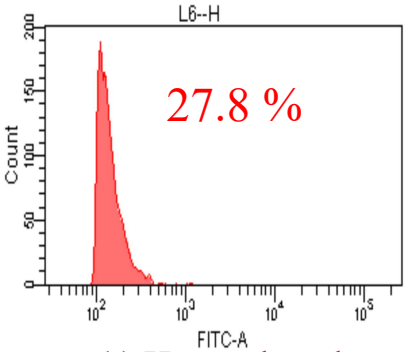
Metformin



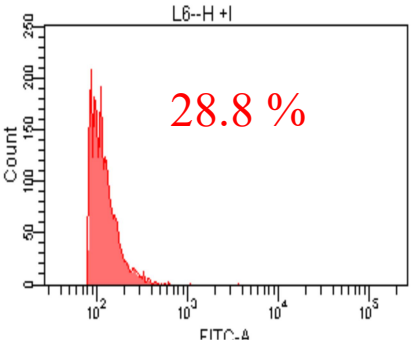
Ampelopsin F



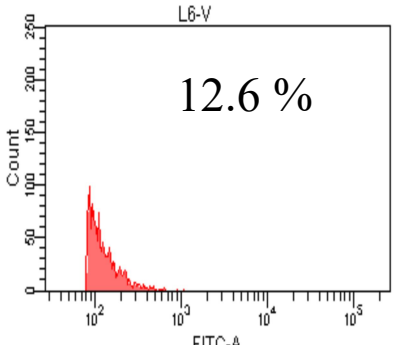
Ampelopsin F +Insulin



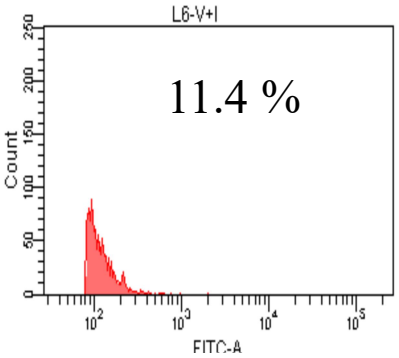
(-) Hopeaphenol



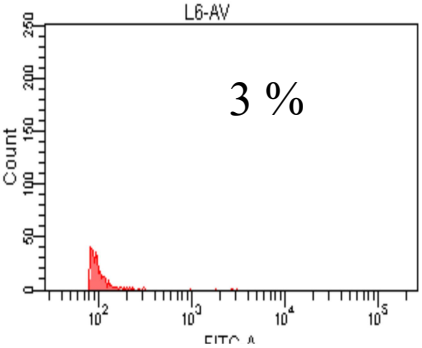
(-) Hopeaphenol +Insulin



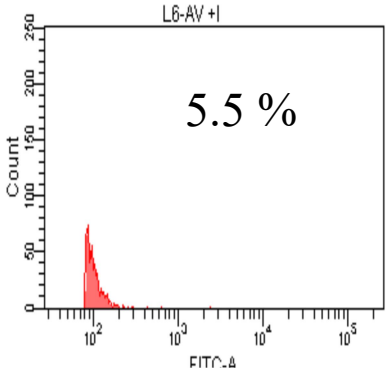
Vaticaphenol A



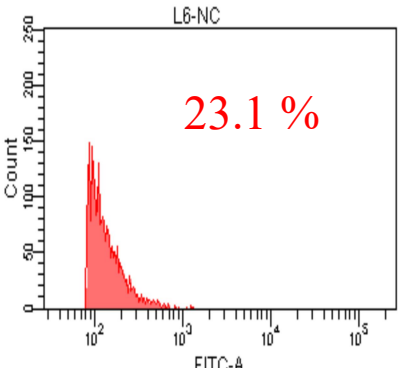
Vaticaphenol A + Insulin



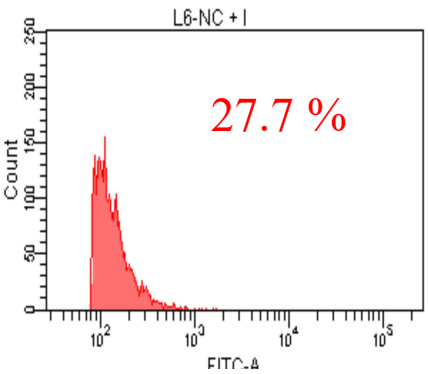
α-Viniferin



α-Viniferin + Insulin



NIIST C1



NIIST C1 + Insulin

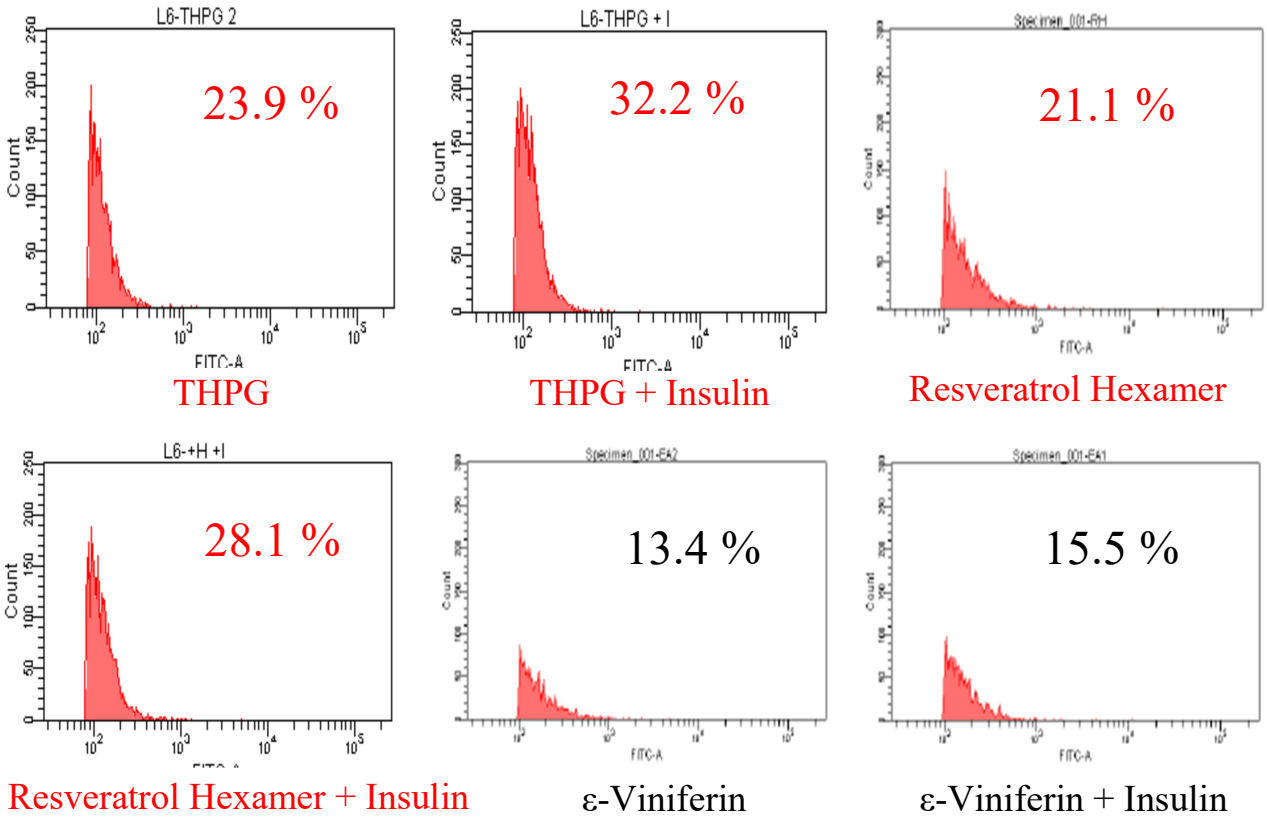


Fig 2.30. Flow cytometry analysis of fluorescent labelled 2-NBDG uptake in differentiated L6 skeletal muscle cell lines: The groups represent insulin treated cells, cells treated with positive control metformin (100 µM) and the compounds, Ampelopsin F (50 µM), (-) Hopeaphenol (50 µM), Vaticaphenol A (50 µM), α-Viniferin (50 µM), NIIST C1 (50 µM), THPG (25 µM), Resveratrol Hexamer (25 µM) and ε-Viniferin (50 µM) in the presence and absence of insulin (100 nM).

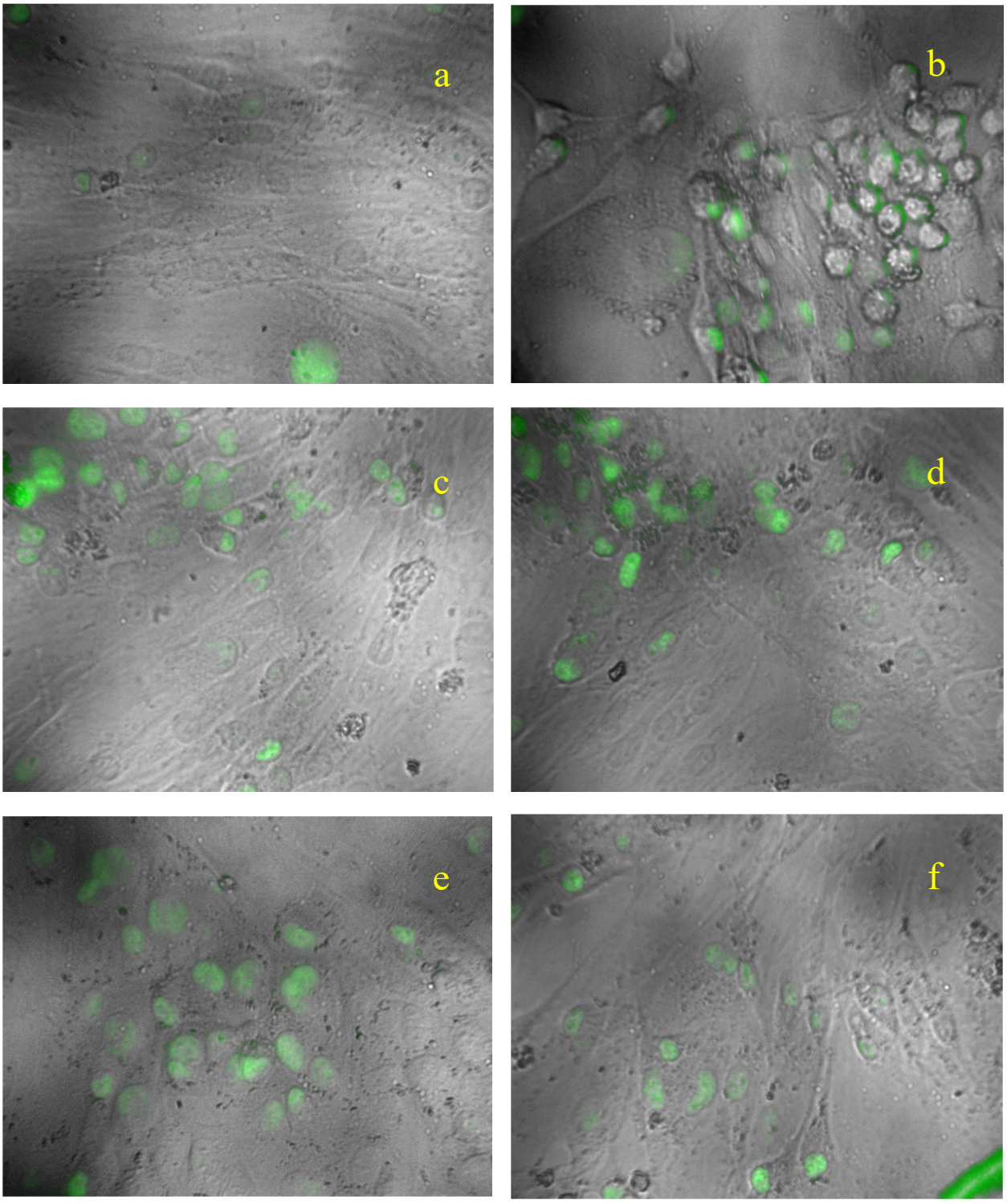
Skeletal muscle is a major tissue associated with insulin stimulation as well as insulin independent glucose uptake (DeFronzo and Tripathy, 2009). Insulin enhances glucose uptake in skeletal muscle by GLUT4 transporters, shifted towards the plasma membrane from the cytoplasm (Hajiaghaalipur et al., 2015). GLUT4 is the major transporter expressed in skeletal muscle cells and insulin dependent uptake of glucose primarily occurred in the skeletal muscles (Richter and Hargreaves, 2013). Since the defects in insulin stimulated glucose uptake result in the progression of diabetes mellitus, L6 skeletal muscle cell lines have been extensively used to elucidate the mechanistic roles of natural compounds for the uptake of glucose (Peterson and

Shulman, 2018). Some citrus flavonoids like quercetin, rutin, naringin and naringenin were reported to possess significant anti-diabetic activity via increased 2-NBDG uptake in L6 cell lines (Dhanya et al., 2017; Dhanya et al., 2015). Other natural compounds such as, Promalabaricone B, isolated from *Myristica fatua* has been reported for its anti-diabetic potential in L6 myotubes (Prabha et al., 2018). Resveratrol was reported with increased 2-NBDG uptake by ameliorating the high fat diet induced abnormalities in mice (Lu et al., 2021). α-Viniferin was reported to bear anti-diabetic activity *in vivo* 100 mg/kg acute pretreatment in mice (Morikawa et al., 2012). One of the initial study published by our group revealed the anti-diabetic potential of α-Viniferin and Trihydroxy phenanthrene glucoside in modulating digestive enzymes, protein glycation and enhancing glucose uptake in L6 myotubes (Sasikumar and Lekshmy et al., 2019). (-) Hopeaphenol isolated from *Vateria indica* along with its enantiomer (+) Hopeaphenol isolated from *Ampelocissus indica* was reported to exhibit anti-diabetic activity in terms of glucose uptake in L6 myotubes and inhibition of digestive enzymes (Sasikumar et al., 2016). In the present study, Ampelopsin F, NIIST C1, THPG were reported for the first time possessing anti-diabetic potential in L6 myoblasts. Ampelopsin F, (-) Hopeaphenol, NIIST C1 and THPG were found to be the most active compounds in enhancing the uptake of glucose in L6 myotubes. The qualitative results obtained from fluorescence microscopy strongly supported the data acquired from flow cytometry analysis. Therefore, the glucose uptake capability of the resveratrol oligomers plays a substantial role in understanding its efficacy in the management of diabetes mellitus.

2.6.4. Immunofluorescence assay

GLUT4 translocation was observed by immunofluorescence assay using a fluorescent-labelled secondary antibody after compound pretreatment for a period of 24 hours. Those resveratrol oligomers which showed potential in the uptake of glucose were further analyzed for the translocation of GLUT4, the glucose transporter. Ampelopsin F, (-) Hopeaphenol, NIIST C1 and THPG in the presence and absence of insulin were selected to perform this experiment owing to its increased activity in 2-NBDG uptake. The compounds, Ampelopsin F and (-) Hopeaphenol were found to be effective in up-regulating GLUT 4 translocation on pre-treatment that was far better than that of untreated cells and at par with the positive control group treated

with Metformin (Fig 2.31). NIIST C1 and THPG treated cells also exhibited the translocation of GLUT4 better than that of untreated cells.



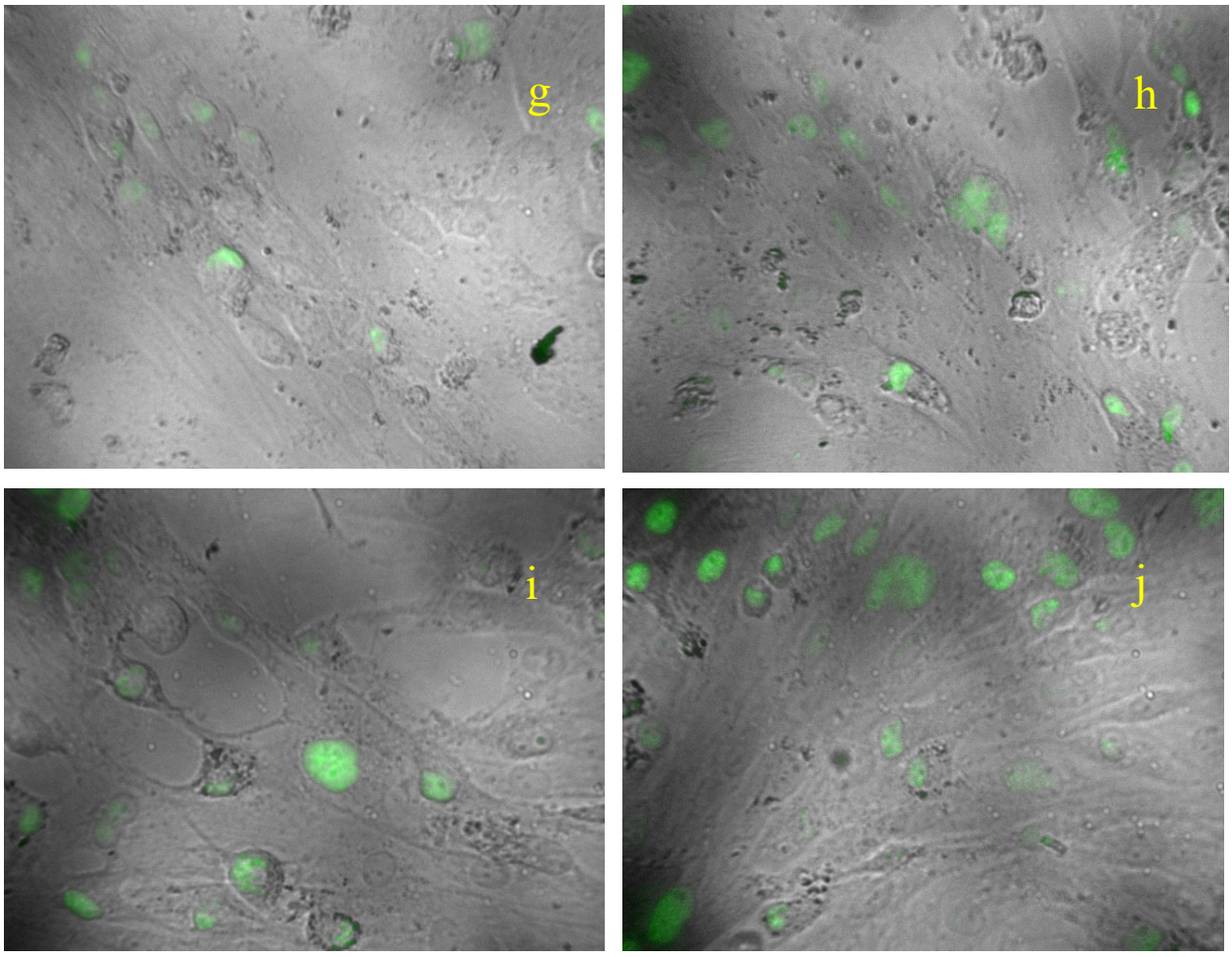


Fig 2.31. Modulation of GLUT4 expression. The increase in GLUT4 expression was monitored by means of immunofluorescence assay using a fluorescent labelled secondary antibody visualized in fluorescence microscope at 40X magnification. L6 myotubes pretreated with resveratrol oligomers in the absence and presence of insulin along with the positive control metformin (100 µM). (a) control cells, (b) metformin (100 µM) (c) Ampelopsin F (50 µM) (d) Ampelopsin F + Insulin (e) (-) Hopeaphenol (50 µM) (f) (-) Hopeaphenol + Insulin (g) NIIST C1 (50 µM) (h) NIIST C1 + Insulin (i) Trihydroxy phenanthrene glucoside (25 µM) (j) Trihydroxy phenanthrene glucoside + Insulin. Magnification 40 X. Scale bar corresponds to 100 µM.

The upregulation and modulation of GLUT4, the major glucose transporter present in skeletal muscles appears very crucial to decrease the onset of diabetes mellitus (Olson, 2012). The treatment with resveratrol *in vivo*, results in the increased uptake of glucose facilitated by the translocation of GLUT4 to the plasma membrane (Penumathsa et al., 2008). The insulin

sensitizing drug, troglitazone pretreated for a period of 24 hours in L6 myotubes was found to induce the translocation of GLUT4 (Yonemitsu et al., 2001). The effect of insulin in inducing the translocation of GLUT4 onto the plasma membrane had been detected and reported using direct immunofluorescence assay (Wang et al., 1998). Neferine, a dibenzyl isoquinoline alkaloid obtained from dietary lotus seeds were found to enhance the expression of GLUT4; its translocation and fusion with plasma membrane resulted in the increased glucose uptake in L6 cell lines (Zhao et al., 2019). The stimulation with insulin attributes to the increased GLUT4 translocation which is the most downstream event in the insulin signaling cascade (Funaki et al., 2004). This immunofluorescence assay could generate large clusters of stain with tiny spots dispersed throughout the interior of cells (Bradley et al., 2014). The results obtained after immunologically labeled GLUT4 transporters at the cell surface could be related to glucose uptake obtained in Section 2.6.2 and Section 2.6.3 (Zeigerer et al., 2002), signifying the stimulation of translocation of GLUT4 upon pretreatment with compounds is likely accountable for better 2-NBDG uptake demonstrated by L6 myotubes. This is the first report showing the enhanced translocation of GLUT4 by the resveratrol oligomers Ampelopsin F, (-) Hopeaphenol, NIIST C1 and THPG.

2.6.5. Cytotoxicity in Beta-TC-6 Cell lines

The cytotoxicity of the resveratrol oligomers at varying concentrations (25, 50, 75 and 100 μM) were measured in Beta-TC-6 cell lines (Table 2.3) for a period of 24 hours by means of MTT assay as described for the first time by Mosmann (1983).

Table 2.3. MTT based cytotoxicity assay of resveratrol oligomers (expressed in percentage) in Beta-TC-6 pancreatic beta cell lines.

Compounds	Concentration of compounds (µM)			
	25 µM	**50 µM**	**75 µM**	**100 µM**
Ampelopsin F	7.87 ± 0.107	14.20 ± 0.158	21.97 ± 0.038	39.89 ± 0.038
(-) Hopeaphenol	9.13 ± 0.311	13.70 ± 0.155	15.92 ± 0.010	21.54 ± 0.111
Vaticaphenol A	3.45 ± 0.138	7.16 ± 0.018	14.86 ± 0.102	24.87 ± 0.030
α-Viniferin	1.85 ± 0.105	8.43 ± 0.004	11.71 ± 0.033	16.81 ± 0.024
NIIST C1	6.20 ± 0.043	15.84 ± 0.014	25.18 ± 0.030	27.28 ± 0.022
THPG	2.92 ± 0.004	5.84 ± 0.136	12.30 ± 0.016	18.27 ± 0.002
Resveratrol Hexamer	1.63 ± 0.041	15.45 ± 0.046	17.10 ± 0.037	19.44 ± 0.109
ε-Viniferin	2.81 ± 0.044	4.44 ± 0.043	7.49 ± 0.053	20.18 ± 0.001

Cytotoxicity of the eight resveratrol oligomers were observed in a dose dependent manner in pancreatic beta cell lines. The concentration at 50 µM was found to be less than 20 % toxic to the cells treated with resveratrol oligomers (Fig 2.32). α-Viniferin, THPG and resveratrol hexamer

were found to be least toxic among the others. These oligostilbenoids showed less than 20 % cytotoxicity in Beta-TC-6 cell lines up to 100 µM concentration. (-) Hopeaphenol, Vaticaphenol A and ε-Viniferin were observed with 15.92 %, 14.86 % and 7.49 % cytotoxicity on 75 µM concentration. Ampelopsin F and the novel compound NIIST C1 were found with more than 80% viable cells in 50 µM concentration. Since the compounds did not show detectable cytotoxicity at 50 µM concentration, this concentration was fixed as safe dose for further studies *in vitro*.

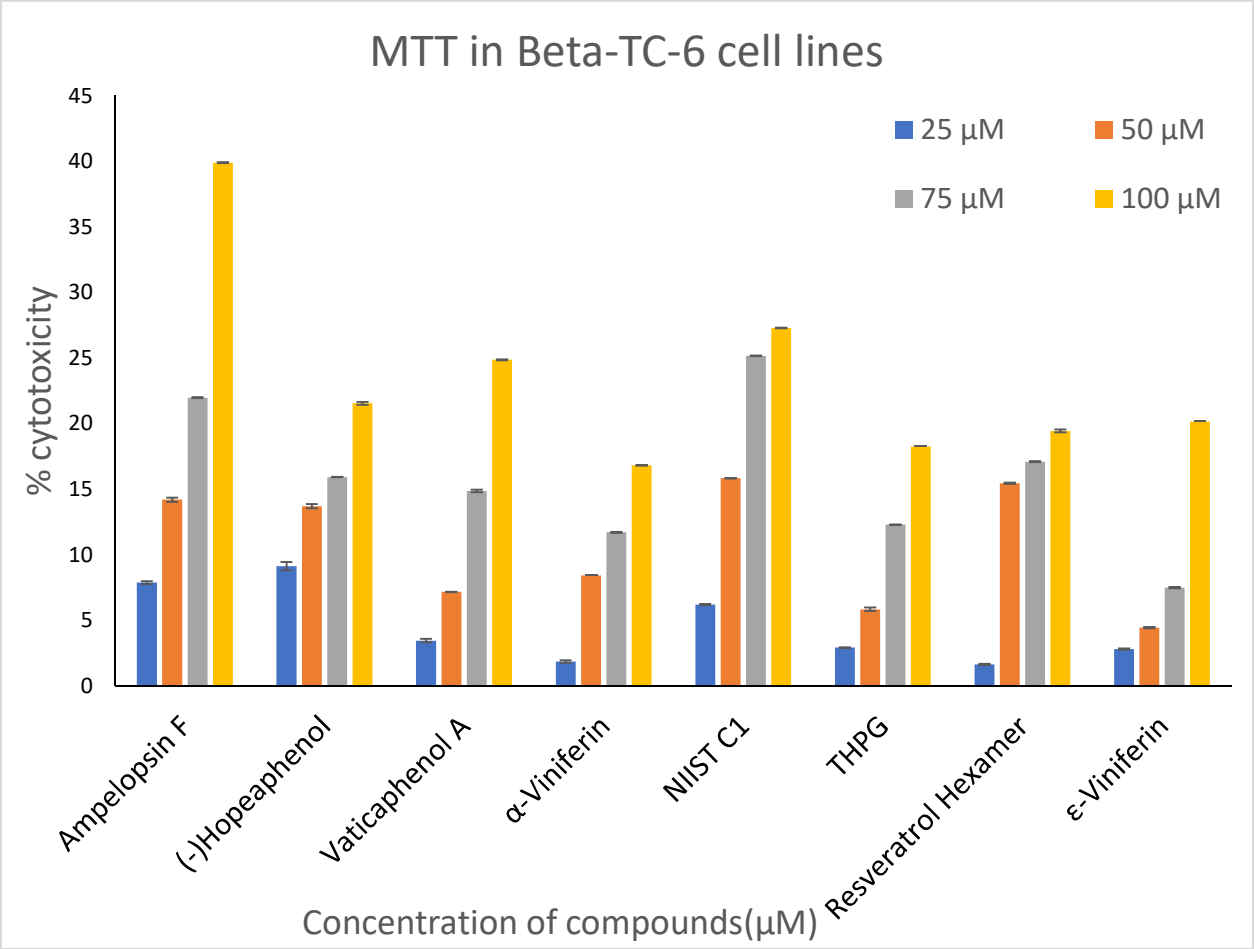


Fig 2.32. MTT cytotoxicity assay of resveratrol based compounds in Beta-TC-6 cell lines. Pancreatic beta cell lines were treated with various concentrations of these compounds (25 µM, 50 µM, 75 µM and 100 µM) for 24 hours and the cytotoxicity of resveratrol based compounds were resolved by MTT assay. All data are represented as means ± SD (n=3).

2.6.6. Insulin secretion studies

Insulin secretion in response to compound pretreatment in Beta-TC-6 cell lines (Table 2.4) was monitored by means of ELISA using insulin ELISA kit according to manufacturer's instructions.

Table 2.4. Insulin secretion assay using Insulin ELISA in Beta-TC-6 cell lines

Compounds	Insulin (ng/mL)
Control	725 ± 19.80
Glyburide	3112 ± 21.21
Ampelopsin F	2600 ± 22.62
(-)Hopeaphenol	2690 ± 16.98
Vaticaphenol A	1220 ± 26.87
α-Viniferin	982.5 ± 14.8
NIIST C1	1637.5 ± 13.43
THPG	530 ± 31.11
Resveratrol Hexamer	577.5 ± 17.68
ε-Viniferin	532.5 ± 19.1

The positive control, Glyburide (100 nM) showed a 4 times increase in the secretion of insulin than untreated cells. An increase in insulin secretion was observed with Ampelopsin F and (-) Hopeaphenol treated cells in which both showed a 3.5 fold increase in insulin secretion compared to the unstimulated control and found to be similar as compared to the positive control Glyburide (100 nM) treated cells (Fig 2.33). The novel compound, NIIST C1 treated cells was found to have a 2 fold increment in the secretion of insulin when compared to untreated cells.

Vaticaphenol A and α-Viniferin treated cells showed 1.6 times and 1.3 times insulin secretion, respectively, than that of control cells (Table 2.4).

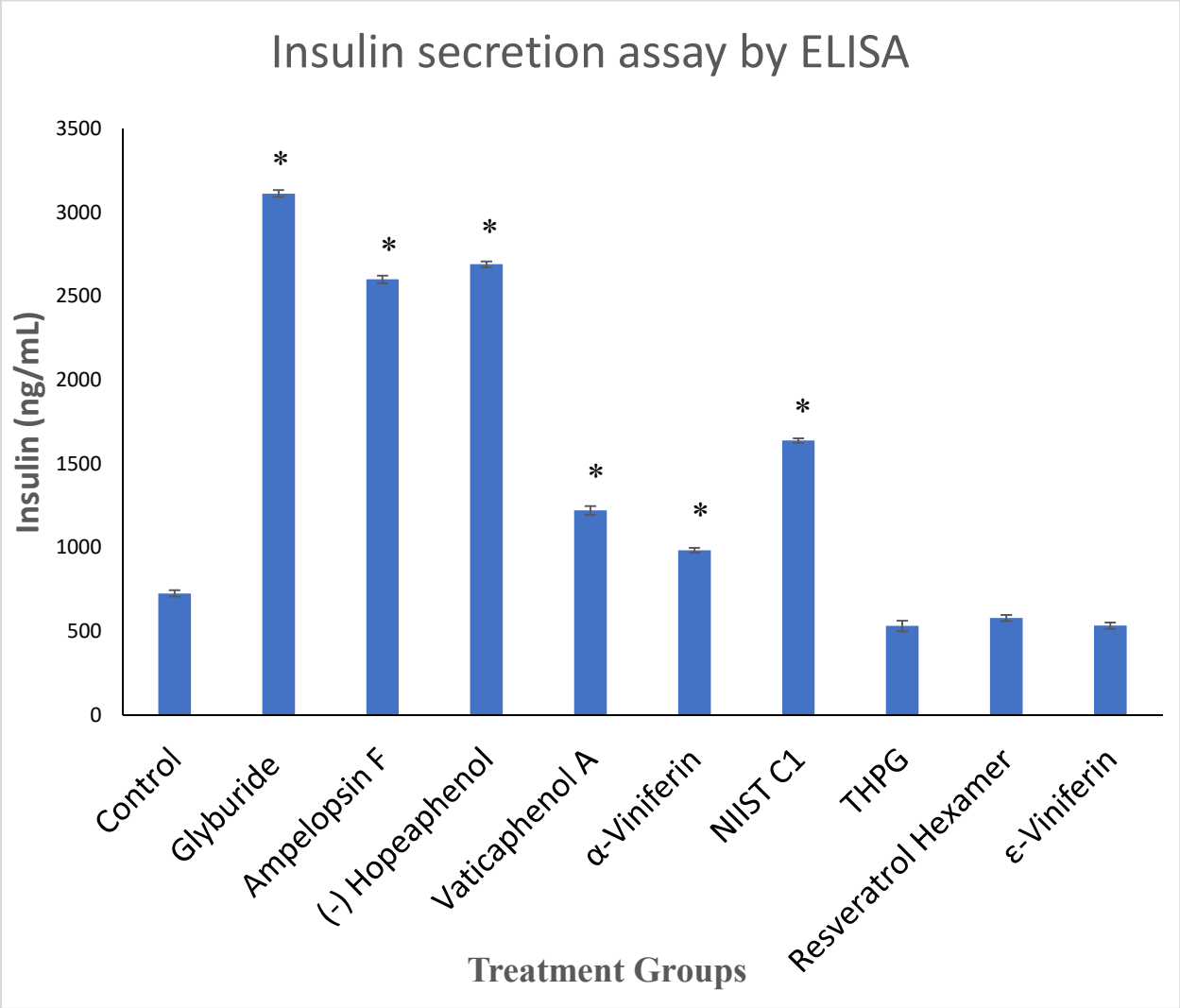


Fig 2.33. The insulin secretion potential of the compounds determined by ELISA. After 24 hours pretreatment, the exogenous insulin secreted by the pancreatic beta cells was measured by means of ELISA. The cells were pretreated with the positive control Glyburide (100 nM) along with the resveratrol oligomers Ampelopsin F (50 µM), (-) Hopeaphenol (50 µM), Vaticaphenol A (50 µM), α-Viniferin (50 µM), NIIST C1 (50 µM), THPG (50 µM), Resveratrol hexamer (50 µM) and ε-Viniferin (50 µM)

Natural compounds, such as, Berberine, Conophylline, Curcumin, Epigallocatechin-3-Gallate, Genistein, Kinsenoside, Quercetin, Resveratrol, Silymarin etc are a few that have been reported to have insulin secretion potential and possess positive effects on the pancreatic beta cell function (Oh, 2015). Verticinone, a steroidal alkaloid found in *Fritillaria imperialis* has been reported with significant anti-diabetic activity in β-TC-6 cell lines by enhancing the insulin secretion at low concentrations (Boojar et al., 2020). Resveratrol and curcumin have been reported to enhance the function of pancreatic beta cells in MIN6 cell lines (Rouse et al., 2014). Resveratrol has been reported to trigger a rise in insulin secretion in Sprague Dawley rats (Ku et al., 2012). Resveratrol was reported for its insulin secretion potential upon glucose stimulation *in vitro* via activation of SIRT1 (Vetterli et al., 2011). Resveratrol aids in the attenuation of amyloid formation and increased the secretion of insulin in INS1 cell lines (Lv et al., 2018). In the present study, Ampelopsin F and (-) Hopeaphenol exhibited significant insulin secretion potential in beta cell lines similar to the positive control Glyburide. In addition to the glucose uptake ability of Ampelopsin F and (-) Hopeaphenol in L6 myoblasts as mentioned in Section 2.6.2 and 2.6.3, the insulin secretion potential of these resveratrol oligomers, thus demonstrating a multi-targeted approach in the management of diabetes mellitus. The observation of anti-diabetic potential by enhancing the secretion of insulin in beta cells by the resveratrol oligomers is being reported for the first time through this study.

2.7. Summary

The results from this chapter suggested the anti-diabetic potential of the resveratrol oligomers isolated from the stem bark of different plants belonging to Dipterocarpaceae family. About eight resveratrol based compounds were used in this study. Ampelopsin F, Vaticaphenol A, a novel compound NIIST C1, resveratrol hexamer (Vaticanol M), and ε-Viniferin isolated from *Vatica chinensis* L., (-) Hopeaphenol isolated from *Vateria indica* L. and α-Viniferin and Trihydroxy phenanthrene glucoside (THPG) isolated from *Hopea ponga* L. were the compounds used in the studies. The experimental data of the present chapter signifies the anti-diabetic potential of the resveratrol based compounds in terms of insulin signaling and insulin secretion in L6 myoblast and Beta-TC-6 cell lines respectively.

In skeletal muscle cell lines

- The resveratrol oligomers were standardized for its concentration for a period of 24 hours in skeletal muscle cell lines (L6).
- The modulation in glucose uptake was monitored by means of fluorescence microscopy and flow cytometry
- GLUT4, the glucose transporter, translocation was monitored in L6 myotubes
- The resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family showed the enhanced glucose uptake and translocation of GLUT4.
- The most active compounds in enhancing glucose uptake- Ampelopsin F, (-) Hopeaphenol, NIIST C1, α-Viniferin and THPG

In Beta-TC-6 cell lines

- The resveratrol oligomers were standardized for its concentration for a period of 24 hours in pancreatic beta cell lines (Beta-TC-6).
- The increase in insulin secretion exogenously was quantified by means of ELISA after compounds pretreatment.
- The resveratrol oligomers isolated from different plants belonging to Dipterocarpaceae family showed the enhanced insulin secretion.
- The most active ones in increasing insulin secretion were- Ampelopsin F and (-) Hopeaphenol.

CHAPTER 3

Elucidation of molecular mechanism of the resveratrol oligomers in insulin secretion and insulin signaling pathway

3.1. Introduction

The beta cells present in the pancreas aids in maintaining glucose homeostasis via the secretion of insulin. Glucose, the chief stimulator for insulin secretion upon entering the beta cells results in an increase in the ATP to ADP ratio. This results in the closure of K_{ATP} channels and opening of voltage dependent calcium channels (VDCC). This rise in intracellular Ca^{2+} concentration initiates the exocytosis of insulin, hence results in increased insulin secretion. Though effective anti-hyperglycemic drugs are available in the market, the side effects including vascular complications are reported in many patients suffering from diabetes. The preservation of beta cell function showed better advantages over insulin replacement. Several studies were reported showing the positive effects of polyphenols in the management of diabetes. Their beneficial effects needed clinical intervention for a better conclusion regarding beta cell function.

The decreased responsiveness of insulin on target tissue is not only the major pathophysiological condition of non-insulin dependent diabetes mellitus but also occurs in insulin dependent diabetes mellitus (Fonseca; 2009). Skeletal muscle accounts for around 80% glucose disposal of whole body under insulin stimulated conditions (DeFronzo and Devjit; 2009). The primary rate limiting step in glucose metabolism is glucose transport and its impairment disturbs whole body glucose homeostasis which ultimately leads to the pathogenesis of diabetes mellitus (Solis-Herrera et al., 2021). GLUT4 isoform is highly expressed with maximal insulin stimulated glucose uptake in skeletal muscle cells (Richter and Hargreaves; 2013)). Glucose uptake has been stimulated in skeletal myocytes via 2 diverse pathways. One is IRS-1/PI3-kinase/AKT signaling pathway stimulated by insulin through binding to the insulin receptor which ultimately leads to the translocation of glucose transporter GLUT4 from the cytoplasm to the cell membrane for the uptake of glucose whereas AMPK is the other intermediate for transporting the GLUT4 towards the cell membrane (Schultze et al., 2012). Hypoxia, muscle contraction and similar metabolic stresses result in a decline in ATP levels leading to an increment in cellular AMP/ATP ratio which eventually activates the AMPK pathway and upon activation, AMPK triggers an increase in glucose uptake (Richter and Ruderman, 2009).

3.2. Objectives

The two resveratrol based compounds, Ampelopsin F and (-) Hopeaphenol showed anti-diabetic activity in Beta-TC-6 cell lines and L6 myoblasts and hence these two compounds were selected for exploring the molecular mechanism in these cell lines. Therefore, in the present study we chose to understand the potential of the resveratrol dimer Ampelopsin F isolated from *Vatica chinensis* and resveratrol tetramer (-) Hopeaphenol from *Vateria indica* in the management of targets linked to diabetes mellitus. The major objectives of the chapter are-

- To explore the possible mechanism of action of the resveratrol oligomers in insulin secretion in Beta-TC-6 cell lines
- To explore the possible mechanism of action of the resveratrol oligomers in insulin signaling pathway in L6 cell lines.

Based on the objectives listed above, this chapter has been divided into two sub-chapters

a) Elucidation of molecular mechanism of the resveratrol oligomers in insulin secretion (Section 3.3).
b) Elucidation of molecular mechanism of the resveratrol oligomers in insulin signaling (Section 3.4).

3.3) Elucidation of molecular mechanism of the resveratrol oligomers in insulin secretion

The experimental design for investigating the molecular mechanism of resveratrol oligomers Ampelopsin F and (-) Hopeaphenol in insulin secretion is depicted below in Fig 3.1.

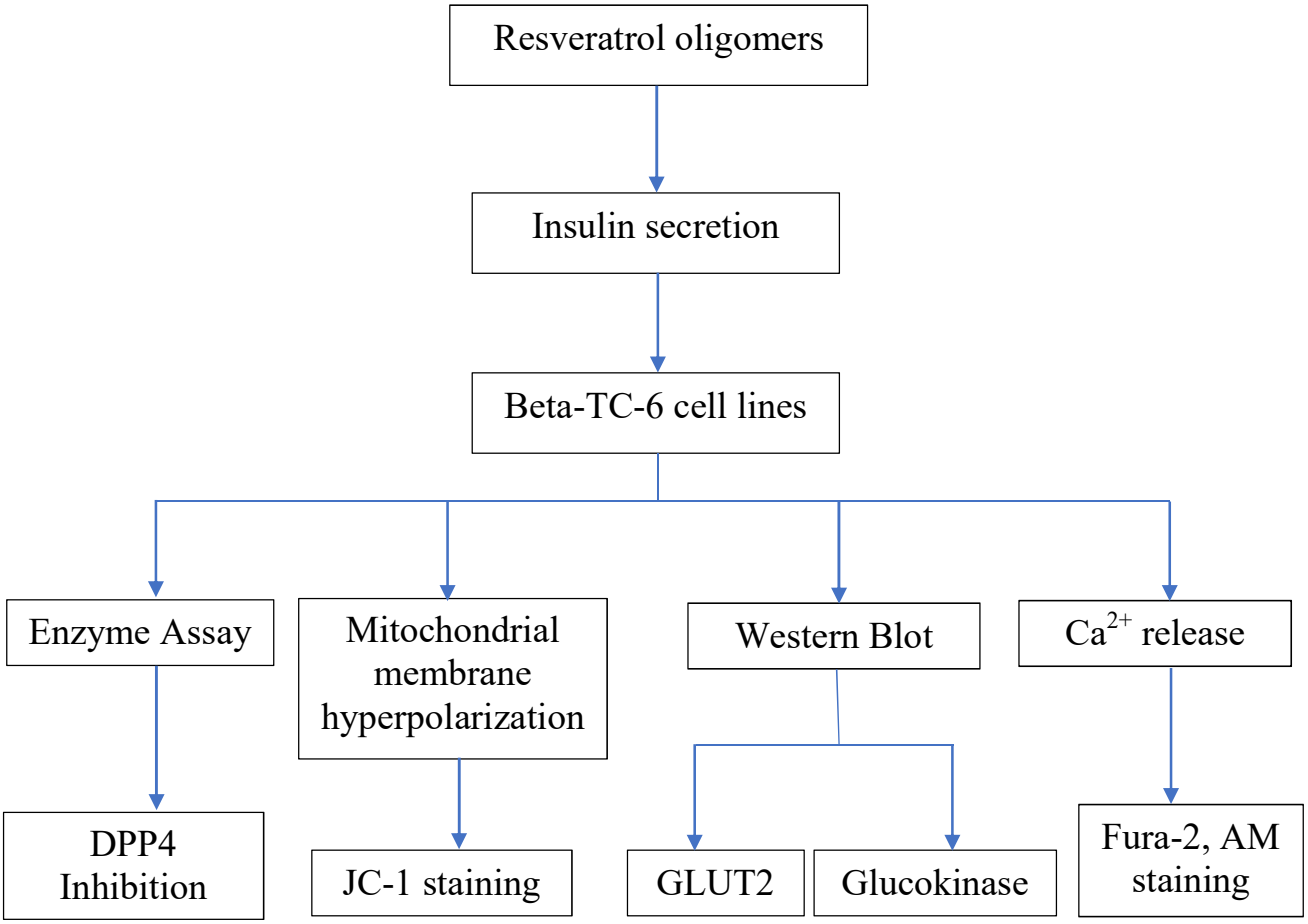


Fig 3.1. Schematic representation of experimental design in Beta-TC-6 cell lines to elucidate the mechanism of action.

3.3.1. Material and methods

Bovine serum albumin, streptomycin ampicillin-amphotericin B mix, Glyburide, Fura-2, AM and JC-1 kit were purchased from Sigma (USA); phospho-specific or pan-specific antibodies against GLUT2 and glucokinase from Cell Signaling Technology; Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 Ham (DMEM/F12, 1:1 Mixture), DMSO were obtained from Himedia laboratories. Fetal bovine serum was purchased from Gibco (United States). Beta-TC-6 pancreatic beta cell lines were procured from NCCS, Pune, India. The additional chemicals expended were of standard analytical grade.

3.3.1.1. Cell culture and treatment

Beta-TC-6, pancreatic beta cell lines were maintained in DMEM/F12 (1:1 Mixture) supplemented with 10% fetal bovine serum and 10% antibiotic- antimycotic mix at 37^{o}C under 5% CO_2 atmosphere. Cells were seeded at 1:3 ratio after trypsinization. The cells were trypsinized within 7-10 days after attaining 2:3 confluency.

3.3.1.2. Determination of mitochondrial membrane potential in Beta-TC-6 cell lines

Glucose induced insulin secretion is characterized by hyperpolarization of the mitochondrial membrane followed by the production of ATP. The hyperpolarization of the mitochondrial membrane was determined by staining the cells with the cationic lipophilic dye, JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'-tetraethy1 benzimidazolocarbocyanine iodide). In normal cells under physiological condition, owing to the electrochemical potential gradient, JC-1 forms aggregates in the mitochondrial matrix, emitting red fluorescence. Alteration in mitochondrial membrane potential obstructs the deposition of JC-1 dye in the mitochondrial membrane, and hence the dye diffuses through the mitochondria resulting in the formation of JC-1 monomers, generating a shift from red to green fluorescence (Sivandzade et al., 2019). The cells after pretreatment with resveratrol oligomers and positive control were incubated with JC-1 staining solution for 20 minutes at 37^{o} C. The staining solution was discarded and washed with plain media. The mitochondrial membrane potential (MMP) stimulated by the resveratrol oligomers in Beta-TC-6 cell lines were determined by fluorescence microscopy. The hindrance of JC-1 dye accumulating in the mitochondria is due to the disturbance in the mitochondrial membrane potential and hence,

the dye is scattered over the cell resulting in a shift from red fluorescence (which implies the presence of JC-1 aggregates) to green fluorescence (which implies the presence of JC-1 monomers). Images were acquired (IX83 inverted microscope, Olympus Life Science, Japan) and fluorescence intensity was measured using the software, Cellsens (Olympus Life Science, Japan). The green fluorescence emitted by JC-1 monomers, was imaged using 490nm excitation and 530 nm emission filters, and the red fluorescence for JC-1 aggregates was imaged using 525nm excitation and 590 nm emission filters. Valinomycin (1µg/ mL) was used as positive control to determine the depolarization of mitochondrial membrane.

3.3.1.3. Determination of calcium efflux using Fura-2, AM

The intracellular Ca^{2+} level was determined according to the method described by Chandra et al., (1989). Fura-2, AM was loaded from a 2 mM stock dissolved in dimethyl sulfoxide (DMSO) to the culture medium and incubated the cells in the presence and absence of dye for 30 minutes at 37^{o} C in a 5% CO_2 incubator. The stain was discarded and washed with plain media and then visualized under a fluorescence microscope (Pathway 855, BD Bioscience, USA) at an excitation-emission wavelength of 350 and 510 nm, respectively.

3.3.1.4. Western blotting

The Beta-TC-6 cell lines were cultured in T25 flasks and after attaining 80% confluency, the cells were treated with Ampelopsin F and (-) Hopeaphenol at 50 µM concentration and Glyburide at 100 nM concentration. The untreated cells were used as control. Following 24 hours incubation, the cells were washed off with plain media and extracted in lysis buffer (containing RIPA, protease inhibitor cocktail and triton X). The cells in buffer were kept on ice for 30 minutes with intermittent vortexing followed by freeze thaw up to 5 cycles. The samples were then centrifuged at 12000 rpm for 20 minutes and the supernatants collected were subjected to protein estimation using a standard BCA kit (Pierce Rockford, IL, USA). The lysates were normalized to get an equal concentration. The proteins separated in 10% SDS polyacrylamide gel were transferred onto PVDF membranes. After one hour blocking in 5% skimmed milk, the transferred membranes were incubated overnight with primary antibodies for GLUT2 (1:1000), Glucokinase (1:1000) and β-actin (1:1000) followed by incubation with analogous secondary antibody conjugated with horse-radish peroxidase which was used at 1:2000 dilution. The

development of bands was detected by exposing to ECL substrate solution (chemiluminescence substrate) (Biorad, USA) and quantified by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

3.3.1.5. DPP4 activity assay

Dipeptidyl peptidase 4 or adenosine deaminase complexing protein or CD26 is a major protein involved in the metabolism of glucose. The inhibition of DPP4 enhances the active forms of incretins which allows the prolonged secretion of insulin from the beta cells of pancreas (Deacon, 2019). The activity of this dipeptidyl peptidase 4 was evaluated by means of a DPP4 activity assay kit (Abcam, USA) according to the instructions from the manufacturer. Results were expressed as the amount of 7-amino-4-methyl coumarin (AMC) produced by the hydrolysis of DPP4 substrate by DPP4 enzyme per minute at 37^{o} C.

3.3.1.6. Statistical analysis

All experiments were done in triplicates and the data were expressed as mean ± standard deviations and significance of differences were determined by one-way ANOVA followed by Tukey's test. P value at $p \leq 0.05$ was regarded as statistically significant.

3.3.2. Results and discussion

3.3.2.1. Determination of change in mitochondrial membrane potential

To determine the mitochondrial membrane potential, here we used the dye JC-1, which emit green fluorescence under depolarized conditions and emit red fluorescence under hyperpolarized condition. About five groups were included to determine the mitochondrial membrane potential. Two resveratrol oligomers Ampelopsin F and (-) Hopeaphenol were treated for a period of 24 hours in Beta-TC-6 cell lines besides valinomycin treated, Glyburide treated and control groups. The control cells were observed with well-polarized mitochondria, as reflected by the elevated fluorescence of JC-1 aggregates emitting red fluorescence. The deeply depolarized cells emitting green fluorescence was observed in cells treated with Valinomycin (1 µg/ mL). A significant increase in red fluorescence was observed in Glyburide, Ampelopsin F and (-) Hopeaphenol treated cells through fluorescent microscopy (Fig 3.2).

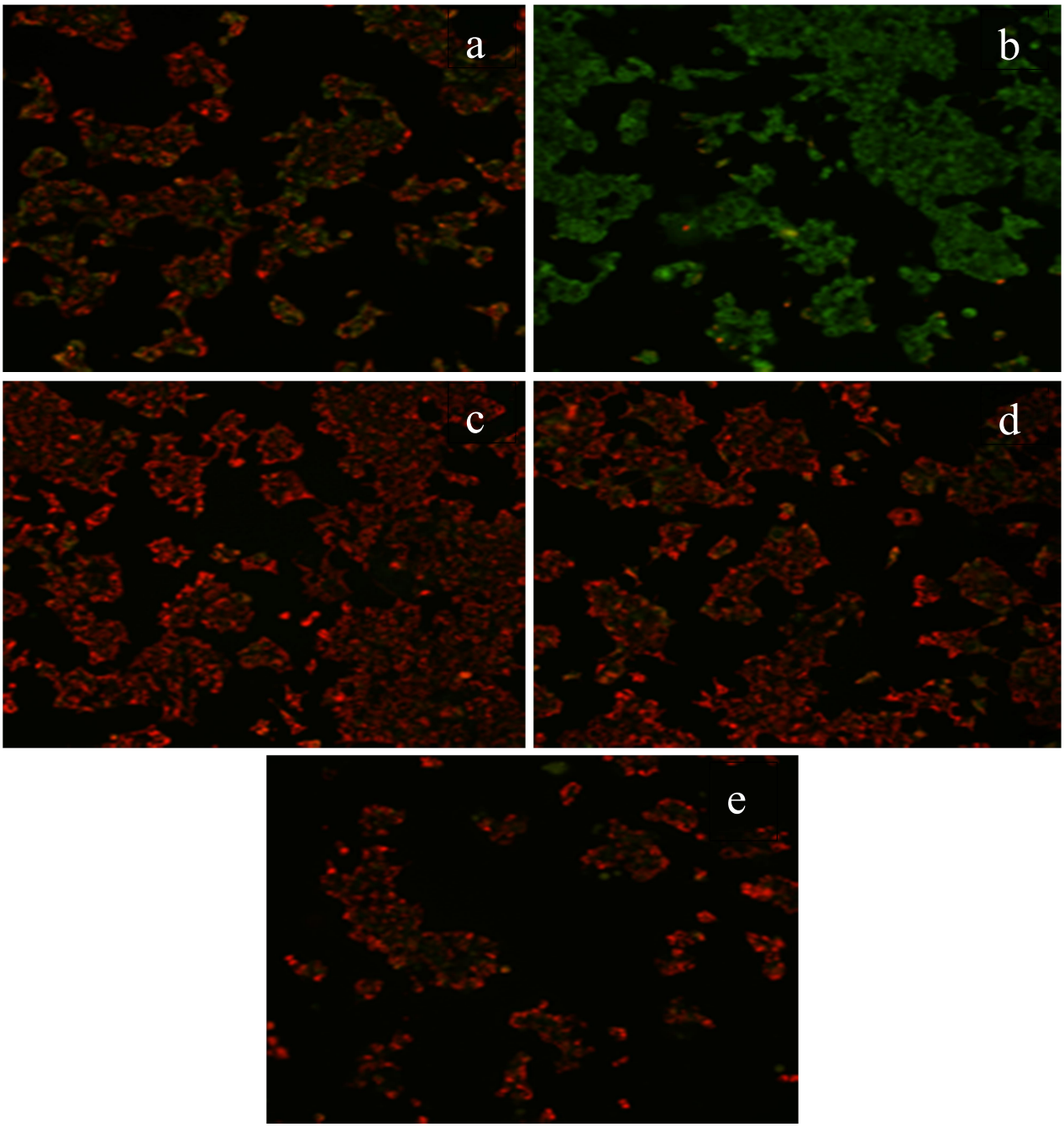


Fig 3.2. Determination of mitochondrial membrane hyperpolarization in Beta-TC-6 cell lines: Fluorescent images of cells stained with the fluorescent dye JC-1 followed by the pretreatment with the resveratrol oligomers. a) control cells, b) valinomycin treated cells (1 µg/ mL), c) Glyburide treated cells (100 nM), d) Ampelopsin F treated cells (50 µM) and e) (-) Hopeaphenol treated cells (50 µM). Magnification 20X. Scale bar corresponds to 50 µM.

The change in mitochondrial membrane potential observed after the fluorescence microscopy was quantified and the ratio of red to green fluorescence was determined. From the analysis, (Fig 3.3), a significant increase ($p \leq 0.05$) in red fluorescence was observed in Glyburide treated cells and Ampelopsin F treated cells as compared to control group. There was slight decrease in the red and green fluorescence in (-) Hopeaphenol treated cells when compared to control.

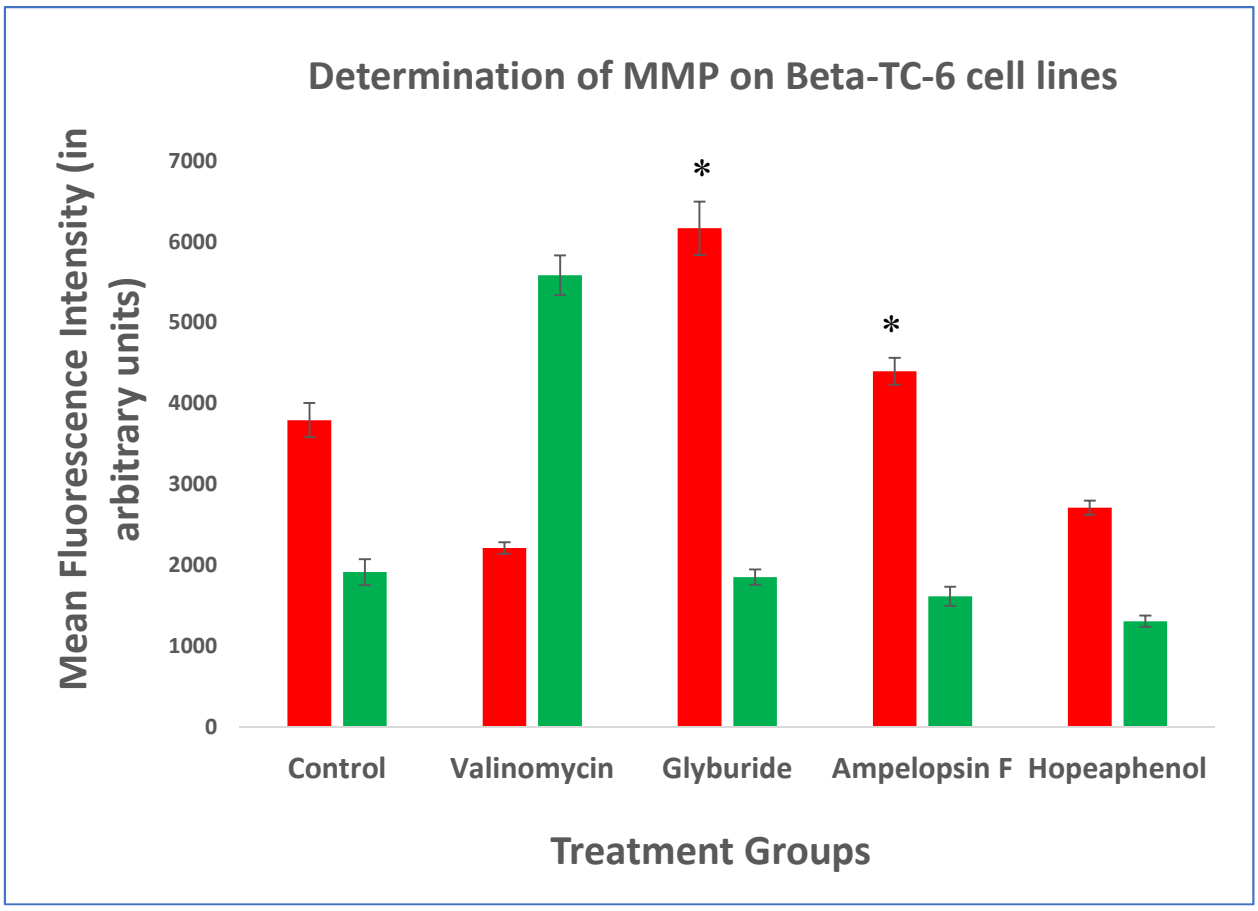


Fig. 3.3. Fluorescence intensity analysis of JC-1 aggregates (red) and JC-1 monomers (green). Relative fluorescent intensity measured and observed with a slight hyperpolarization of mitochondrial membrane in resveratrol oligomers treated cells. Untreated cells: control, Positive control: Valinomycin (1 µg/ mL) treated and Glyburide (100 nM) treated cells. Resveratrol oligomers Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM). The results were expressed in terms of mean ± SD of three determinations using one-way ANOVA. The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ versus control.

The ratio of red to green fluorescence was also generated (Fig 3.4) from the above data. This data signifies the potential of compounds in changing the mitochondrial membrane potential. The Glyburide treated and Ampelopsin F treated cells were observed with a slight hyperpolarization of the mitochondrial membrane. As this hyperpolarization of the mitochondrial membrane marked as a sign of increased insulin secretion, the data showed here supported the results obtained from the section 2.6.6 with respect to increased insulin secretion.

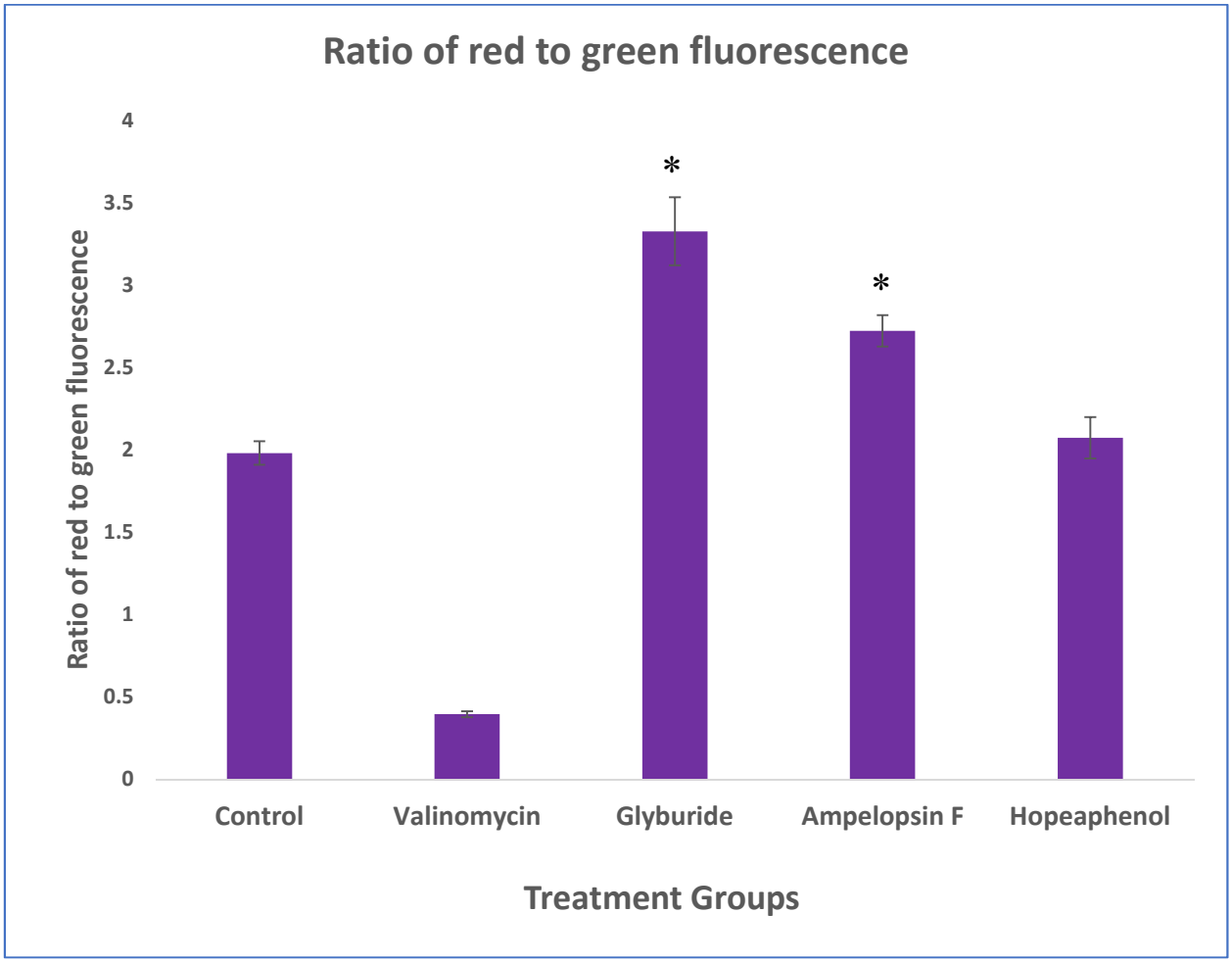


Fig 3.4. Determination of ratio of red to green fluorescence in Beta-TC-6 cell lines. Bar diagram shows the ratio of JC-1 aggregates (red) to JC-1 monomers (green). Untreated cells: control, Positive control: Valinomycin (1 µg/ mL) treated and Glyburide (100 nM) treated cells. Resveratrol oligomers Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM). The results were expressed in terms of mean ± SD of three determinations using one-way ANOVA. The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ versus control.

Valinomycin is a K+ ionophore which depolarizes the mitochondria without altering the morphology of the cell (Inai et al., 1997). The change in mitochondrial membrane potential upon glucose stimulation results in the hyperpolarization of the mitochondrial membrane and aids in the secretion of insulin (Gerencser, 2018). Previous reports have shown a concentration dependent increase in insulin secretion as a result of glucose-induced mitochondrial hyperpolarization (Wikstrom et al, 2007) and thus mitochondrial membrane hyperpolarization was emphasized as a suitable predictor of insulin secretion. Gerencser (2018) had reported that the amount of insulin secreted upon glucose stimulation was directly proportional to the magnitude of the mitochondrial membrane hyperpolarization. Glucolipotoxicity inhibits the production of NADPH and subsequently prevents mitochondrial membrane hyperpolarization (Schultheis et al., 2019). The activity of mitochondria stimulates the exocytosis of insulin by the rise in mitochondrial calcium concentration ($[Ca^{2+}]_m$) (Maechler et al., 1997). In pancreatic beta insulinoma cell line MIN6, the loss of mitochondrial membrane integrity caused by simvastatin induced toxicity was retained by restoring the mitochondrial membrane potential by Sesamol, a known antioxidant found in sesame (Ghadge et al., 2020). Our data showed an increase in red fluorescence in beta cells pretreated with Glyburide, Ampelopsin F and (-) Hopeaphenol implying the hyperpolarization of mitochondrial membrane. This change in mitochondrial membrane potential sequentially leads to increased ATP production, plasma membrane hyperpolarization and ultimately resulted in enhanced insulin secretion. Section 2.6.6 showed the enhanced insulin secretion in Glyburide treated as well as in Ampelopsin F treated cells correlated the mitochondrial membrane hyperpolarization as the predictor of insulin secretion.

3.3.2.2. Intracellular calcium efflux

As the secretion of insulin is dependent on calcium release, the exocytosis of insulin requires a significant rise in the concentration of calcium in order to elicit the fusion of insulin granules with the plasma membrane. To observe the effect of resveratrol oligomers in the release of calcium ions in Beta-TC-6 cell lines, the dye Fura-2, AM was used to determine intracellular calcium concentrations. The control cells showed a normal physiological intracellular calcium concentration and the increased intracellular calcium efflux was demonstrated in the positive control, Glyburide treated cells. Ampelopsin F pretreatment demonstrated a significant rise in the concentration of calcium twice as that of control cells and was at par with the positive control,

Glyburide (100 nM). (-) Hopeaphenol pretreatment induced an increase in the intracellular calcium level when compared to control cells but was not as intensified as the positive control, Glyburide (Fig 3.5).

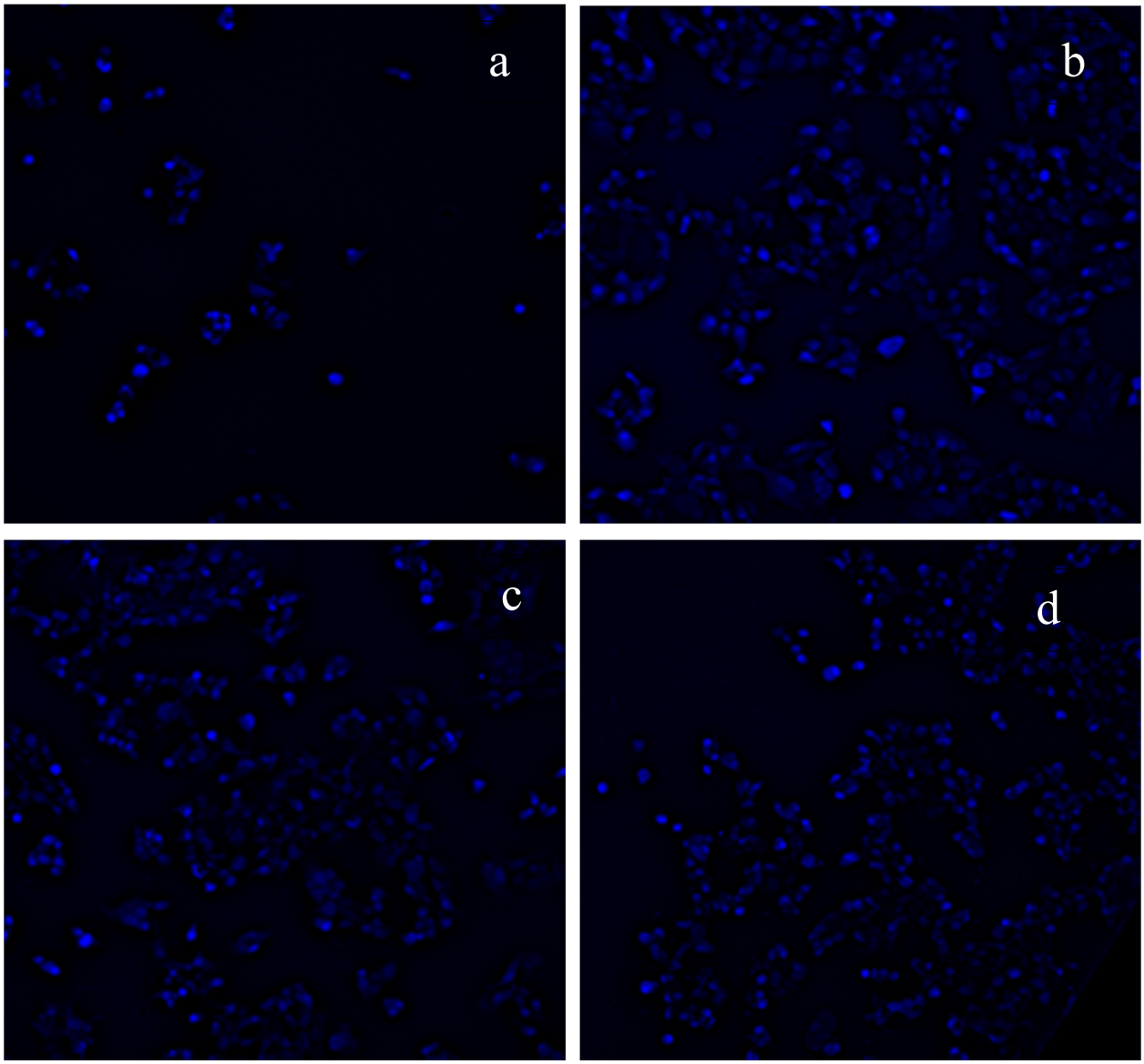


Fig 3.5. Determination of intracellular calcium efflux. Fluorescent images of the cells stained with Fura-2, AM to determine the concentration of calcium in Beta-TC-6 cell lines. a) control cells, b) Glyburide treated cells (100 nM), c) Ampelopsin F treated cells (50 µM) and d) (-) Hopeaphenol treated cells (50 µM). Magnification 20X. Scale bar corresponds to 50 µM.

The effect of calcium efflux upon pretreatment with resveratrol oligomers in Beta-TC-6 cell lines observed via the fluorescence microscopy was quantified. From the graph (Fig 3.6), a significant increase in cytoplasmic calcium concentration was observed in Glyburide treated cells and Ampelopsin F treated cells as compared to control group. (-) Hopeaphenol treated cells showed a slight decrease in fluorescence than Glyburide treated cells but significantly increased the intracellular calcium concentration than control cells.

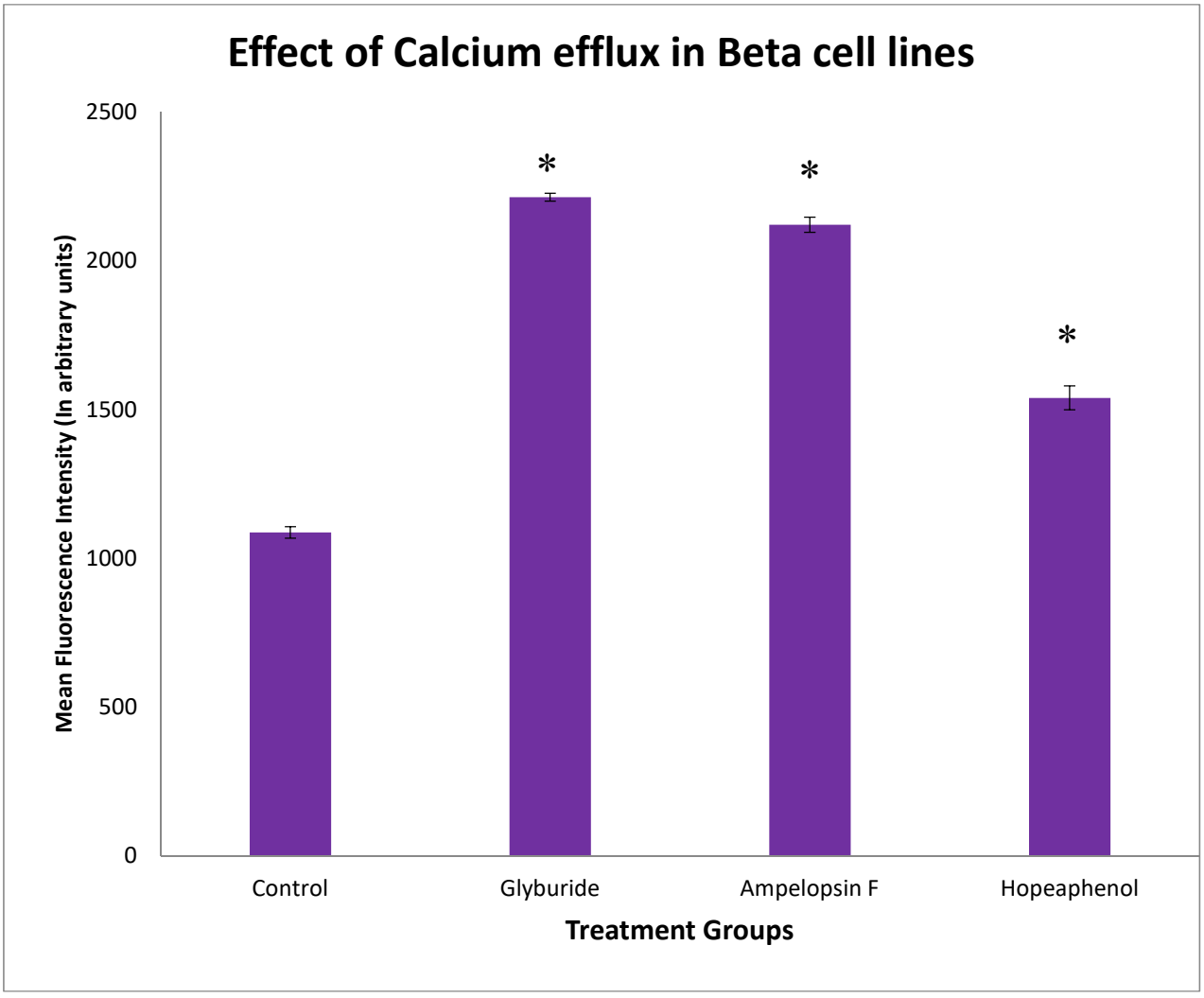
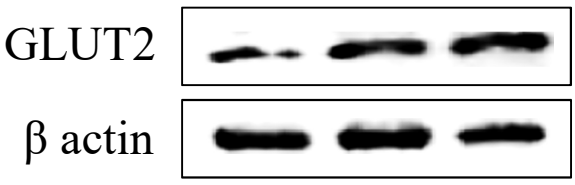


Fig 3.6. Intracellular calcium levels in Beta-TC-6 cell lines. Control represents untreated cells, Positive control: Glyburide treated cells (100 nM) and resveratrol oligomers Ampelopsin F treated cells (50 µM) and (-) Hopeaphenol treated cells (50 µM). The results were expressed in terms of mean ± SD of three determinations using one-way ANOVA. The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ versus control.

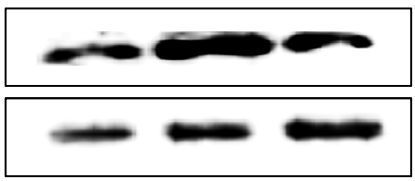
Intracellular calcium concentration acted as a proximal marker (Fu et al, 2013) and has been reported to play a substantial role in the physiological release of insulin from pancreatic beta cells upon glucose stimulation (Klec et al., 2019). Resveratrol stimulates insulin secretion via an increase in the concentration of calcium upon glucose stimulation in a concentration dependent manner (Pezzolla et al., 2015). Natural compounds, such as, Quercetin was reported to stimulate the secretion of insulin in INS1 cell lines upon glucose or KCl stimulation (Youl et al., 2010) and an increase in calcium concentration was noticed following pretreatment with quercetin in isolated islets (Hii and Howell, 1985). With respect to quinoline compounds, the calcium efflux reportedly occurred on pretreatment with higher concentrations of quinoline compounds in RIN-5AH beta cells and in rat pancreatic islets (Orfi et al., 2017). Our results demonstrated an increase in the release of calcium in beta cells, which trigger the exocytosis of insulin granules ultimately leading to the release of insulin. The rise in intracellular calcium concentration is concordant with our observations in the insulin secretion potential of the compounds in beta cell lines as mentioned in section 2.6.6.

3.3.2.3. Western blot analysis

GLUT2, the glucose transporter is highly expressed in pancreatic beta cells of rodents upon glucose stimulation. The results from the western blot analysis showed an increased expression of the glucose transporter GLUT2 in Beta-TC-6 cell lines pretreated with resveratrol oligomers. Ampelopsin F induced a significant increment in the protein expression of GLUT2 when compared to the control (1.5 times) and a similar expression profile relatable to that of the positive control Glyburide (100 nM) (Fig 3.7). (-) Hopeaphenol also stimulated an upregulation in the expression of GLUT2 better than control cells (1.25 times), but not as good as the expression of Glyburide treated cells.



Lane 1- Control, 2- Glyburide, 3- Ampelopsin F



Lane 1- Control, 2- Glyburide, 3- (-) Hopeaphenol

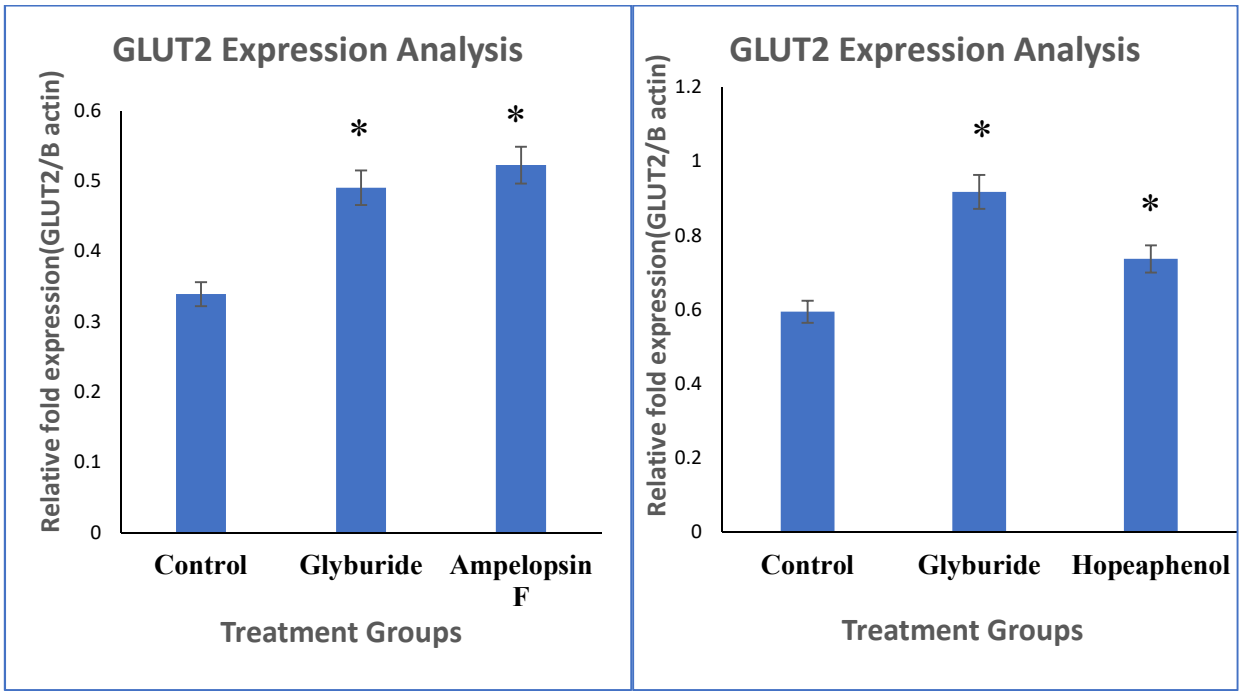
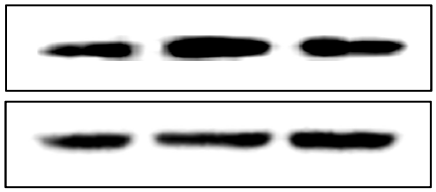


Fig 3.7. GLUT2 protein expression in Beta-TC-6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol: Western blot analysis for GLUT2 expression: Beta-TC-6 cell lines were treated with Glyburide (100 nM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours. Cell lysates were examined for protein expression of GLUT2 by western blot analysis with β -actin as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. $*p \leq 0.05$ versus control.

Glucokinase is the key enzyme involved in the glycolytic pathway for the phosphorylation of glucose to glucose-6-phosphate. The positive control Glyburide showed a significant increase in the expression of glucokinase in beta cell lines when compared to control cells (2 times). The expression of glucokinase in Ampelopsin F treated cells revealed a significant increase than control cells (2.5 times) and was at par with the positive control Glyburide (Fig 3.8) while (-) Hopeaphenol treated cells showed a slight increase in the expression of glucokinase when compared to control group (1.3 times), but was not significant when compared to the positive control Glyburide.

Lane 1- Control, 2- Glyburide, 3- Ampelopsin F



Lane 1- Control, 2- Glyburide, 3- (-) Hopeaphenol

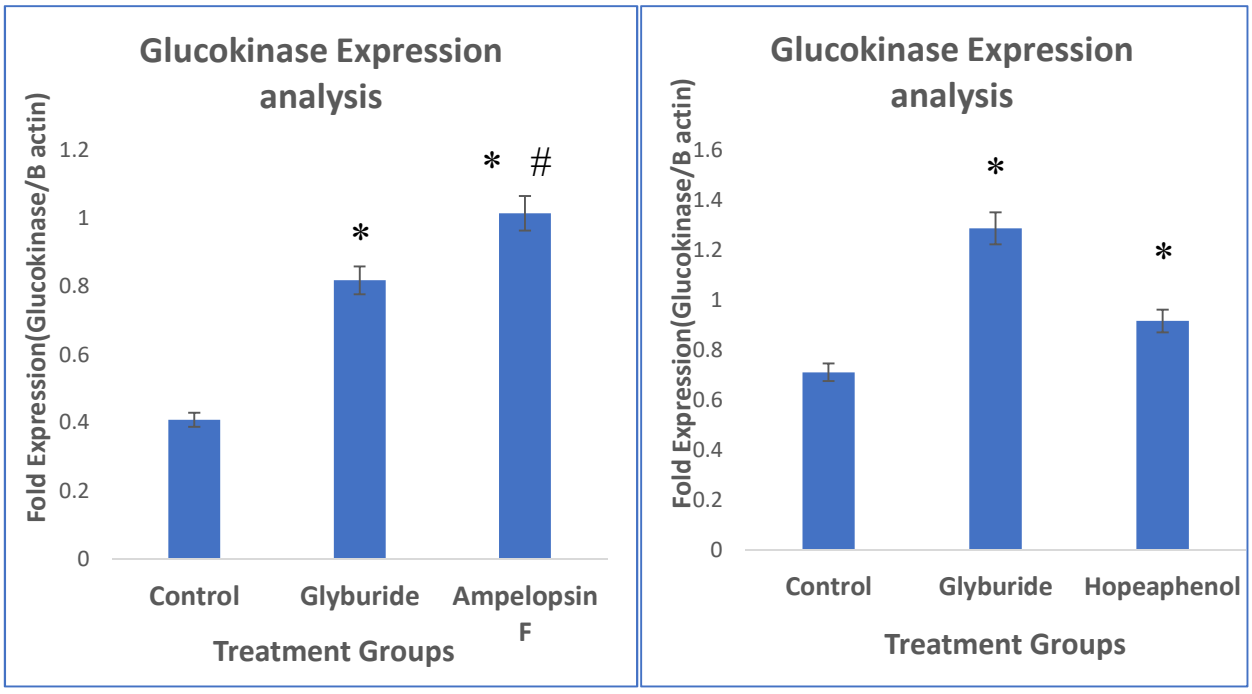


Fig 3.8. Glucokinase protein expression in Beta-TC-6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol: Western blot analysis for Glucokinase expression: Beta-TC-6 cell lines were treated with Glyburide (100 nM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours. Cell lysates were examined for protein expression of Glucokinase by western blot analysis with β- actin as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. $*p \leq 0.05$ versus control, $\#p \leq 0.05$ versus Glyburide.

Low et al (2021) stated that the decreased expression of GLUT2 was found to be associated with decreased ATP production and hence stipulated to defects in glucose stimulated insulin secretion in Maturity Onset Diabetes of the Young (MODY3) patients. Hyperglycemia results in

the degradation of cell surface GLUT2, adversely affecting the function of beta cells (Hou et al., 2009). Hence GLUT2 is a target molecule in diabetes upon stimulation with low doses of streptozotocin (Wang and Gleichmann, 1998). Palmitic acid downregulates the expression of GLUT2 and glucokinase and negatively regulates the function of pancreatic islets (Gremlich et al., 1997). Vitexin, a C-glycosyl flavonoid improved glucose induced insulin secretion via upregulation of GLUT2 (Wickramasinghe et al., 2021). Estrogen 2 has been reported to improve GLUT2 protein content and upregulate glucokinase enzyme and thereby the secretion of insulin in INS 1 cell lines and in Sprague-Dawley rats (Bian et al., 2019). Exposure to high glucose results in the functional impairment of glucokinase and its reduced interaction with mitochondria leading to the downregulation of glucokinase (Kim et al., 2005). Several lab studies demonstrated the association of glucokinase present in beta cells with the insulin granules (Rizzo et al., 2002). In our study, western blot analysis in pancreatic beta cell lines pretreated with resveratrol oligomers and positive control, Glyburide, demonstrated an improved expression of GLUT2 and glucokinase. These two proteins are considered to be glucose sensors and catalyzes the metabolism of glucose. The increased expression of GLUT2 and glucokinase in resveratrol oligomers treated cells and Glyburide treated cells demonstrated the mechanism similar to glucose induced insulin secretion. These data obtained from western blot analysis in Beta-TC-6 cell lines correlated with the increased insulin secretion induced by the compounds as observed in Section 2.6.6.

3.3.2.4. DPP4 activity assay

As DPP4 is a negative regulator of incretins, GLP-1 and GIP, the inhibition of DPP4 allows the sensitization of incretins which ultimately leads to the prolonged secretion of insulin. Aimed at muti-targeted drug approach, we studied the potential of resveratrol oligomers Ampelopsin F and (-) Hopeaphenol in inhibiting the enzyme DPP4 by means of DPP4 activity assay. A positive control enhancing the activity of DPP4 was used according to manufacturer's instruction. The incubation with positive control demonstrated 2.5 times increase in the activity of DPP4 when compared to control. Ampelopsin F reduced the activity of DPP4 by 2.5 times and 6.5 times as compared to control and positive control, respectively. (-) Hopeaphenol incubated groups showed 9.5 times decrease in activity as compared to control and showed 19 times decrease in activity when relating to positive control of DPP4 activity (Fig 3.9).

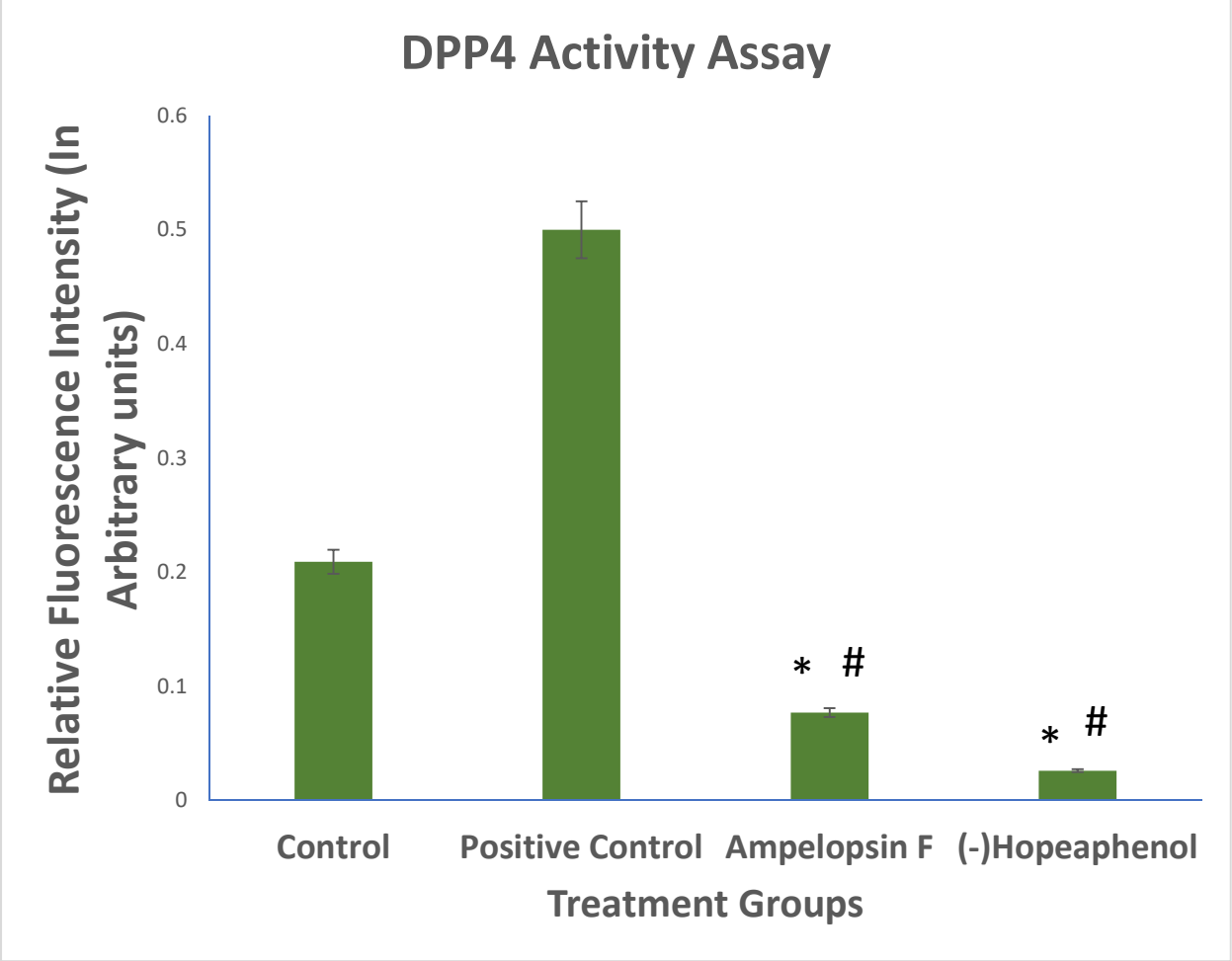


Fig 3.9. Determination of decrease in DPP4 activity: The inhibitory potential of resveratrol oligomers in DPP4 enzyme was measured. Control represents untreated groups, positive control represents the positive control (2 µL/100mL) catalyzing DPP4 activity which was present in the kit; Resveratrol oligomers Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM). The results were expressed in terms of mean ± SD of three determinations using one-way ANOVA. The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ versus control, $\#p \leq 0.05$ versus positive control.

Sitagliptin is the most widely used DPP4 inhibitor used for the treatment of diabetes mellitus. A combinatorial drug consisting of sitagliptin along with metformin is also extensively used for the management of diabetes mellitus (St. Onge et al., 2012). Resveratrol, luteolin, apigenin has been reported as the potent natural DPP4 inhibitors in *in vitro* studies and molecular docking simulation studies (Singla et al., 2019). The huge affinity towards DPP4 enzyme was reported with resveratrol, curcumin and syringic acid. Curcumin was reported to show the inhibition of

DPP4 in Caco-2 cell lines (Huang et al., 2019). DPP4 has been a potential drug target for treating diabetes mellitus. Our compounds, Ampelopsin F and (-) Hopeaphenol demonstrated the inhibition of DPP4 which can result in the prolonged secretion of insulin that could be an added advantage for supporting the insulin secretion potential by the beta cells.

In our study, the resveratrol oligomers, Ampelopsin F and (-) Hopeaphenol, induced the mitochondrial membrane hyperpolarization, increased intracellular calcium concentration and induced the expression of GLUT2 and glucokinase in pancreatic beta cell lines which ultimately resulted in the increased insulin secretion. This enhancement in the secretion of insulin was found to be similar to glucose stimulated insulin secretion (GSIS). In addition to insulin secretion, our studies also demonstrated inhibitory potential of DPP4 thus highlighting the multi-targeted approach of these two compounds in the management of diabetes mellitus.

3.3.3. Summary

In this chapter, two compounds, resveratrol dimer- Ampelopsin F and resveratrol tetramer- (-) Hopeaphenol, were selected to explore the mechanistic role in stimulating the secretion of insulin in Beta-TC-6 cell lines. Glyburide at 100 nM concentration was used as the positive control. The standardized concentration 50 μM was used for the compounds in pancreatic beta cells. The study revealed the potential of these two resveratrol oligomers in stimulating insulin secretion analogous to glucose stimulated insulin secretion (GSIS) in Beta-TC-6 cell lines. These two resveratrol based compounds also showed their potency in the inhibition of DPP4, which is another extensively used drug target in the treatment of diabetes mellitus.

The key findings of this chapter is summarized here below:

- A slight hyperpolarization of the mitochondrial membrane was observed upon pretreatment with Ampelopsin F (50 μM) and (-) Hopeaphenol (50 μM) which was at par with the positive control Glyburide (100 nM)
- An increase in Ca^{2+} concentration was observed upon pretreatment with Glyburide (100 nM), Ampelopsin F (50 μM) and (-) Hopeaphenol (50 μM) complementing the increment in insulin secretion in beta cell lines.

- The increased protein expression of GLUT2, the glucose transporter and the enzyme glucokinase suggested the contribution of the resveratrol oligomers in insulin secretion analogous to the glucose stimulated insulin secretion (GSIS).
- Both the compounds also showed its antidiabetic potential by demonstrating the inhibition of DPP4, an enzyme responsible for the degradation of incretins, thus showing the potency for prolonged insulin secretion.

3.4) Elucidation of molecular mechanism of the resveratrol oligomers in insulin signaling

The experimental design for investigating the molecular mechanism of resveratrol oligomers in insulin signaling pathway is depicted below in Fig 3.10.

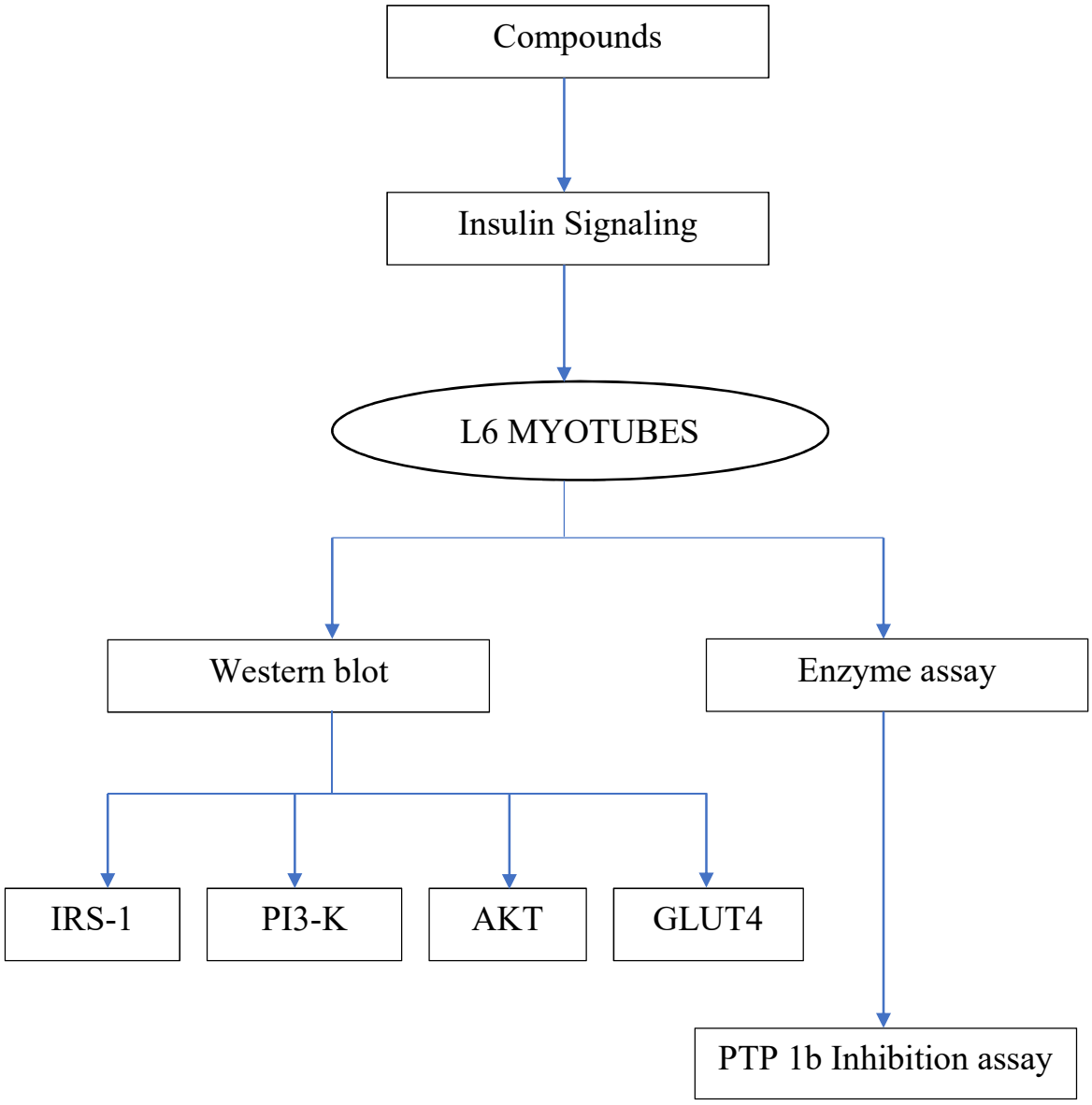


Fig 3.10. Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action of resveratrol oligomers.

3.4.1. Materials and methods

DMEM, BSA, streptomycin ampicillin-amphotericin B mix, insulin, metformin, HEPES, dithiothreitol, EDTA (Ethylene diamine tetra acetic acid) para nitropheny1 phosphate (PNPP) and Sodium orthovanadate were obtained from Sigma-Aldrich (USA); phospho-specific or pan-specific antibodies against GLUT 4, AKT, IRS-1, PI3-K and the corresponding anti-rabbit HRP-conjugated secondary antibodies were purchased from Cell Signaling Technologies. DMSO was obtained from Himedia laboratories. Fetal bovine serum was purchased from Gibco (United States). L6 myoblast cell lines were procured from National Centre for Cell Sciences, Pune, India. Rest all chemicals expended were of standard analytical grade.

3.4.1.1. Cell culture and treatment

Skeletal myocytes, L6 cell lines were maintained in Dulbecco's modified Eagle's media complemented with 10% antibiotic-antimycotic mix at 37^0 C under 5% CO_2 atmosphere. For cellular differentiation to generate myotubes, the cells were maintained in differentiation medium having 2% horse serum for 5-7 days.

3.4.1.2. Western blotting

Differentiated L6 skeletal myotubes (5-7 days) seeded in T25 flasks/plates were pretreated with resveratrol oligomers in the presence of insulin at its standardized concentrations and metformin at 100 μM concentration for 24 hours. The untreated cells were used as control. Following 24 hours incubation, the cells were washed off with plain media and extracted in lysis buffer (containing RIPA, protease inhibitor cocktail (Toche, Mannheim, Germany) and triton X). The cells in buffer were kept on ice for 30 minutes with intermittent vortexing followed by freeze thaw up to 5 cycles. The samples were then centrifuged at 12000 rpm for 20 minutes and the supernatants collected were subjected to protein estimation using a standard BCA kit (Pierce Rockford, IL, USA). Supernatants were warehoused at -80^o C until western blot analysis. The lysates were normalized to get an equal concentration. The samples were heated for ten minutes at 75^oC in reducing sample buffer (62.5 mM Tris-HC1 pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol and 0.01% bromophenol blue) just before the separation. The proteins were separated in 10% SDS polyacrylamide gel and were transferred onto PVDF membranes. After one hour blocking in 5% skimmed milk, the membranes were incubated overnight with primary

antibodies for GLUT 4, AKT, IRS-1, PI3-K and β-actin (1:1000) followed by incubation with analogous horse-radish peroxidase conjugated secondary antibody at 1:2000 dilution. The development of bands was detected by exposing to ECL substrate solution (chemiluminescence substrate) (Biorad, USA) and quantified by densitometry using a Chemi Doc XRS digital imaging system and the Multi Analyst software from Bio-Rad Laboratories (USA).

3.4.1.3. PTP 1b assay

PTP 1b is a phosphatase enzyme which negatively regulates insulin signaling pathway and hence involved in glucose homeostasis. This phosphatase enzyme catalyzes the dephosphorylation of IR and IRS subsequently leading to the downregulation of insulin signaling pathway (Abdelsalam, 2019). The activity of this phosphatase enzyme on cells was analyzed expending 100 µL p-nitropheny1 phosphate (PNPP) buffer (25 mM HEPES, pH 7.2, 50 mM NaC1, 5 mM dithiothreitol, 2.5 mM EDTA). This assay catalyzed by the phosphatase enzyme was based on the hydrolysis of PNPP (substrate of PTP 1b enzyme) to p-nitrophenol, an yellow-colored product. The potency of the chromogen formed was quantified at 410 nm on a microplate reader (BIOTEK, USA). Results were demonstrated as percentage inhibition comparative to control.

$$\text{Percentage inhibition} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right] \times 100$$

3.4.1.4. Statistical analysis

All experiments were conducted in triplicates and the data were expressed as mean ± standard deviations and significance of differences were determined by one-way ANOVA followed by Tukey's test. P value at $p \leq 0.05$ was regarded as statistically significant.

3.4.2. Results and discussion

3.4.2.1. Western blot analysis

3.4.2.1.1. IRS -1 expression analysis

To investigate the mechanism of resveratrol oligomers in insulin signaling pathway, the protein expression analysis were carried out. The studies were conducted on the key proteins involved in insulin signaling pathway which comprises IRS-1, PI3-K, AKT and GLUT4. The cells grown in T25 flasks underwent differentiation and were pretreated with resveratrol oligomers, Ampelopsin F and (-) Hopeaphenol, in the presence of insulin and the positive control, metformin. The expression studies were conducted by western blot analysis on phosphorylated proteins with the corresponding pan specific proteins. An additional group, insulin control, was used for carrying out the western blot analysis to understand the effect of compounds in insulin signaling pathway. The insulin control demonstrated an increased expression of p-IRS-1 in L6 myotubes. Though metformin treated cells didn't show a marked increase in the expression of p-IRS-1, a significant increase in its expression was observed when compared to control cells. An increase in the expression of phosphorylated IRS-1 was observed in Ampelopsin F treated cells and (-) Hopeaphenol treated cells which was at par with the insulin treated control cells (Fig 3.11).

Lane 1-Control, 2- Insulin Control, 3-Metformin, 4- Ampelopsin F

Lane 1-Control, 2- Insulin Control, 3-Metformin, 4- (-) Hopeaphenol

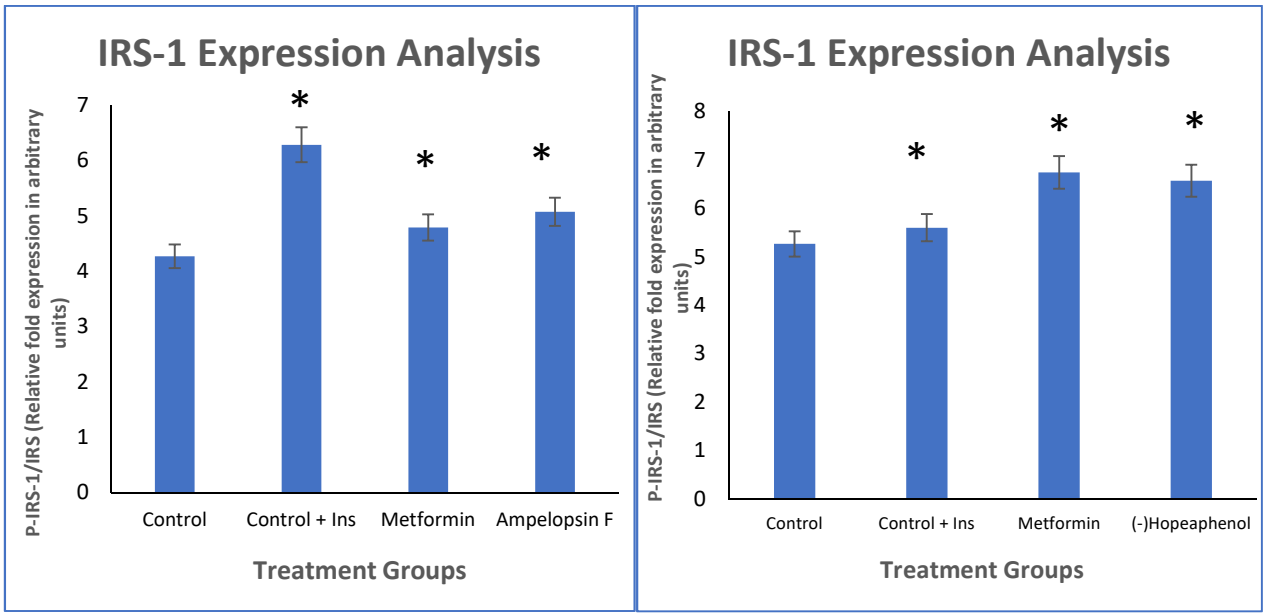


Fig 3.11. a) IRS-1 protein expression in L6 cell lines on pretreatment with Ampelopsin F and b) (-) Hopeaphenol: Western blot analysis for IRS-1 expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) along with insulin (100 nm) for a period of 24 hours. Cell lysates isolated were studied for protein expression of p-IRS-1 by western blot analysis with pan specific IRS-1 as loading control. All data were represented as means ± SD (n=3), the significance was accepted at $p \leq 0.05$. *$p \leq 0.05$ versus control

3.4.2.1.2. PI3-K expression analysis

The western blot analysis were extended to determine the expression of phosphorylated PI3-K, which is the key protein involved in insulin transduction signaling pathway. A weaker expression of PI3-K was observed in metformin treated cells. Insulin treated cells showed an increase in the expression of PI3-K than control cells. Similarly, a significant increment in the expression of PI3-K was observed in Ampelopsin F treated cells when compared to control cells. Ampelopsin F treated cells showed 5.2 times and 5.8 times increase in the expression of phosphorylated PI3-K than control cells and metformin treated cells respectively. But there was a feeble expression of PI3-K in (-) Hopeaphenol treated groups (Fig 3.12) than control and insulin control.

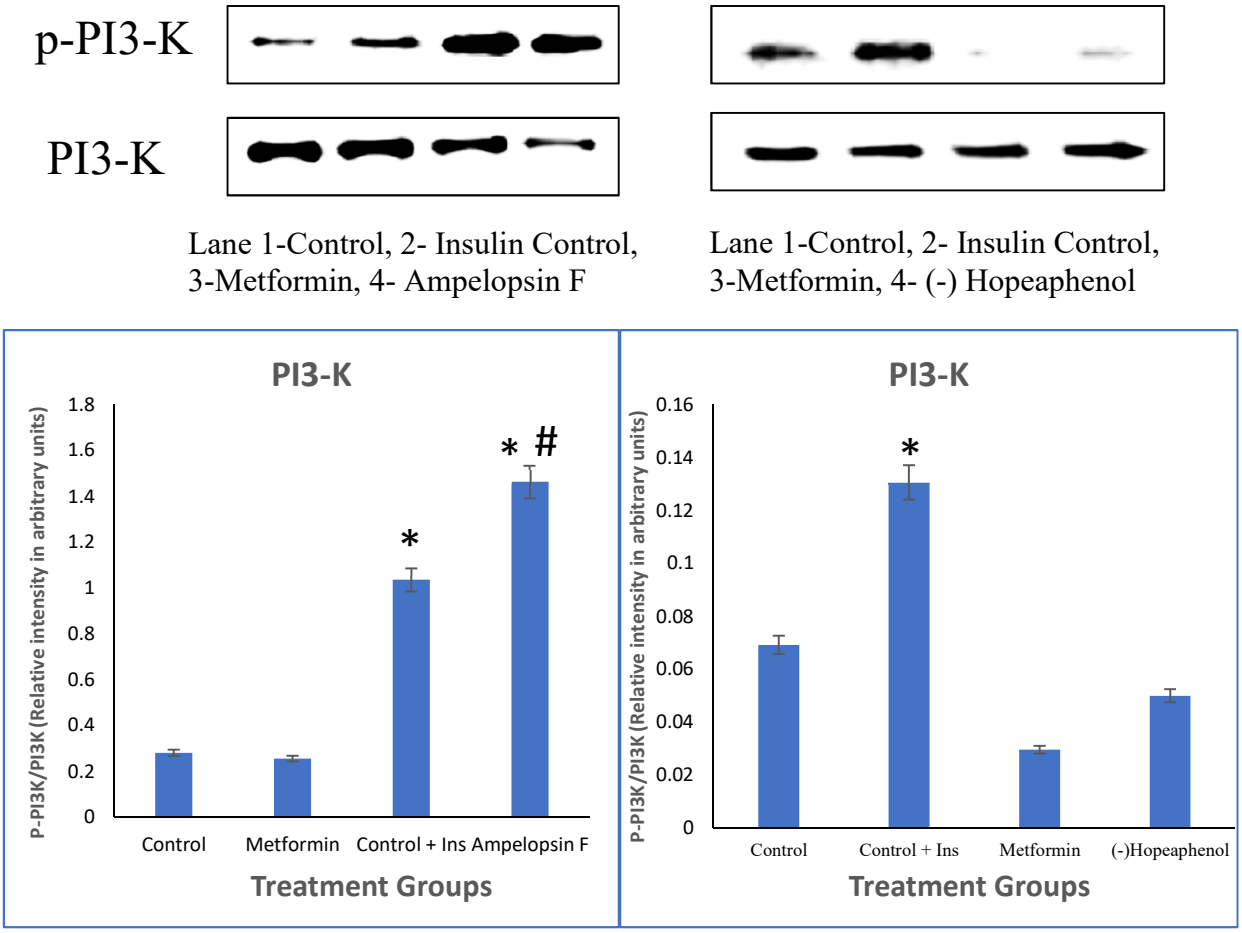
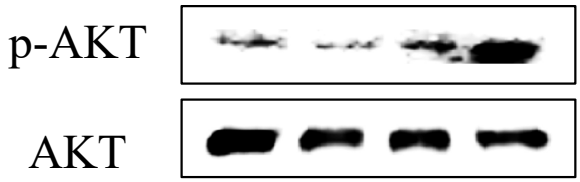


Fig 3.12. PI3-K protein expression in L6 cell lines on pretreatment with Ampelopsin F and b) (-) Hopeaphenol: Western blot analysis for PI3-K expression:**.** L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) along with insulin (100 nm) for a period of 24 hours. Cell lysates prepared were studied for protein expression of p-PI3-K by western blot analysis with pan specific PI3-K as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus metformin.

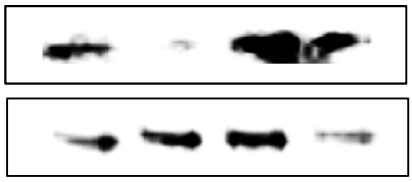
3.4.2.1.3. AKT expression analysis

AKT is a most essential protein involved in insulin dependent signaling pathway. The activation of AKT aids in stimulating the translocation of GLUT4 catalyzing via the phosphorylation of AS160 substrate protein. In our study, insulin and metformin treated cells showed an increased expression of p-AKT in L6 myotubes when compared to control cells. An increase in the phosphorylation of AKT was observed in Ampelopsin F treated cells and (-)

Hopeaphenol treated cells. Ampelopsin F treated groups showed 3.5 times increased expression than control cells, 2.5 times than insulin treated cells and 1.9 times than metformin treated groups. There was an increase of 1.8 times expression of phosphorylated AKT in (-) Hopeaphenol treated cells when compared to control cells and 1.4 times than that of metformin treated cells (Fig 3.13).



Lane 1- Control, 2- Insulin Control, 3- Metformin, 4- Ampelopsin F



Lane 1- Control, 2- Insulin Control, 3- Metformin, 4- (-) Hopeaphenol

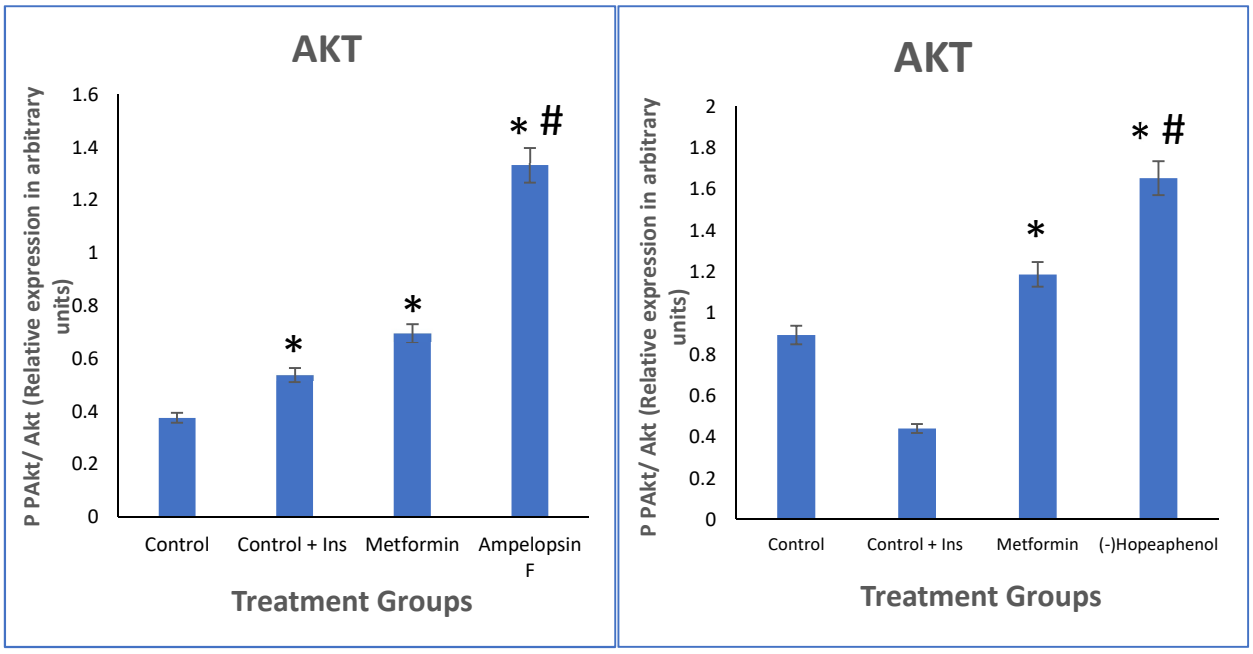
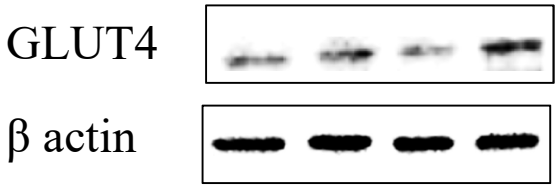


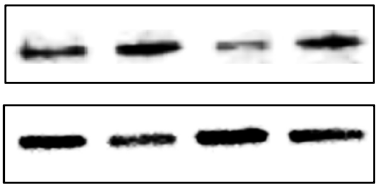
Fig 3.13. AKT protein expression in L6 cell lines on pretreatment with Ampelopsin F and b) (-) Hopeaphenol: Western blot analysis for AKT expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) along with insulin (100 nm) for a period of 24 hours. Cell lysates prepared were studied for protein expression of p- AKT by western blot analysis with pan specific AKT as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus metformin.

3.4.2.1.4. GLUT4 expression analysis

GLUT4 is the major glucose transporter expressed in skeletal muscle cells for the facilitated transport of glucose. The increased expression of GLUT4 demonstrated the anti-diabetic efficacy of compounds in insulin stimulating and insulin independent pathway. Metformin treated cells demonstrated an increment in the expression of GLUT4 in L6 myotubes. There was an increase of 1.4 times in the expression of GLUT4 in metformin treated cells when compared to control cells (Fig 3.14). Ampelopsin F treated cells showed a similar kind of expression of GLUT4 as noticed in metformin treated cells. Ampelopsin F treated cells demonstrated 1.5 times increase in the expression of GLUT4 than control cells and was at par with the positive control metformin. The treatment with (-) Hopeaphenol in L6 myotubes showed 2.3 times increase in the expression of GLUT4 when compared to control cells. The increased expression of GLUT4 in (-) Hopeaphenol treated cells than metformin treated cells implied the anti-diabetic potential of the resveratrol tetramer in L6 myotubes.



Lane 1- Control, 2- Metformin, 3- Insulin Control, 4- Ampelopsin F



Lane 1- Control, 2- Metformin, 3- Insulin Control, 4- (-) Hopeaphenol

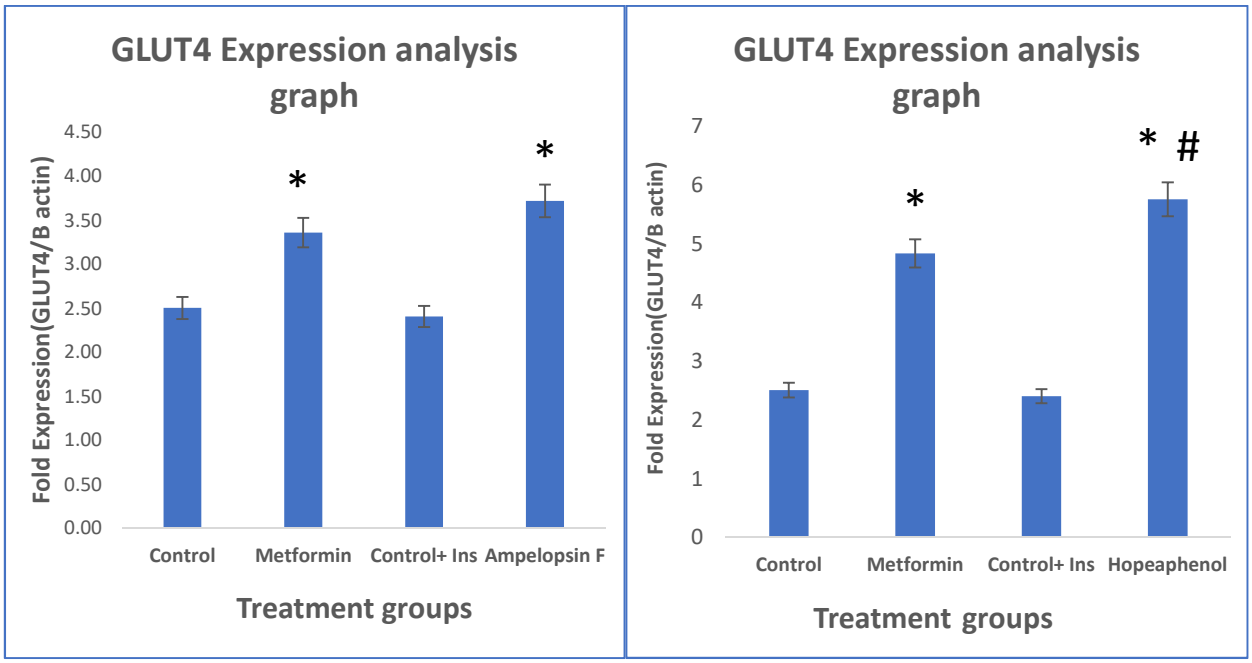


Fig 3.14. GLUT4 protein expression modulation in L6 myotubes on pretreatment with Ampelopsin F and b) (-) Hopeaphenol: Western blot analysis for GLUT4 expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) along with insulin (100 nm) for a period of 24 hours. Cell lysates prepared were studied for protein expression of GLUT4 by western blot analysis with β actin as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. $*p \leq 0.05$ versus control.

Insulin stimulated glucose uptake is majorly facilitated by phosphatidylinositol 3-kinase (PI3-K) dependent signal transduction pathway. The activation of proteins majorly depends on its phosphorylation on the corresponding amino acid residues. The binding of insulin induces the dimerization and autophosphorylation of the insulin receptor which in turn phosphorylates the tyrosine residues of insulin receptor substrate IRS-1 (Hubbard, 2013). These phosphorylated IRS proteins activate PI3-K, generating a lipid product phosphatidylinositol 3,4,5-triphosphate (PIP_3). The increment in PIP_3 concentration leads to the activation of AKT (Guo, 2014). The increased expression of AKT results in the translocation of GLUT4 from the cytosol to the plasma membrane and thereby modulating the uptake of glucose (Saltiel and Pessin, 2003).

Metformin, is a well-known anti-diabetic drug used in the treatment of diabetes mellitus. Metformin was reported to activate IRS-1 in human hepatocytes (Gunton et al., 2004) and sensitize insulin signaling in insulin resistant C2C12 myotubes (Kumar and Dey 2002). Turban and co-workers (2012) reported the non-participation of metformin in PI3-K dependent uptake of glucose in L6 myotubes. Though metformin was reported to downregulate PI3-K and AKT in anaplastic thyroid cancer cells (Nozhat et al., 2018), metformin activated AKT in the presence of insulin and stimulated the GLUT4 translocation in human granulosa cell lines (Rice et al., 2010). Metformin was shown to increase the expression of GLUT4 as it was reported for the acute increase in GLUT4 translocation in L6 myotubes (Sajan et al., 2010).

D-Pinitol, a naturally derived inositol, has been reported to activate PI3-K/ AKT dependent insulin signaling pathway in the hypothalamus of Wistar rats (Medina-Vera et al., 2021). Hong and the co-workers (2014) reported that the insulin resistance induced by high fat diet could be reverted by administering resveratrol by restoring the phosphorylation of PI3-K and AKT. Quercetin was reported to activate PI3-K/AKT signaling in L6 myotubes by promoting GLUT4 translocation and expression (Dhanya et al., 2017). The activation of AKT via its phosphorylation could be insulin dependent or insulin independent (Huang et al., 2018).

Recent study reported by our research group showed that the resveratrol oligomers, α-Viniferin and Trihydroxy phenanthrene glucoside (THPG), isolated from *Hopea ponga* were able to modulate the expression of GLUT4 in L6 myotubes (Sasikumar and Lekshmy et al., 2019). In the present study, we observed the increased expression of IRS-1, AKT and GLUT4 in metformin treated cells, whereas, there was no change in the expression of phosphorylated PI3-K in L6 myotubes. Ampelopsin F treated cells showed the increased expression of all key proteins involved in insulin signaling pathway, phosphorylated IRS-1, PI3-K, AKT and GLUT4 and thus implied its role in insulin dependent signaling transduction pathway. There was an increased expression of phosphorylated IRS-1, AKT and GLUT4 proteins in L6 myotubes pretreated with (-) Hopeaphenol. Though PI3-K is the major protein involved in insulin dependent signaling pathway, the down regulation of PI3-K in (-) Hopeaphenol treated cells pointed out the disengagement of (-) Hopeaphenol in insulin dependent signaling pathway. The increased expression of GLUT4 in L6 myotubes treated with metformin, Ampelopsin F and (-)

Hopeaphenol correlated with the translocation of GLUT4 observed from immunofluorescence assay cited in Section 2.6.4.

From the results obtained from our study, we hypothesize that the activation of AKT in Ampelopsin F treated cells were through PI3-K/ AKT signaling pathway whereas the increased expression of phosphorylated AKT in (-) Hopeaphenol treated cells implied that it wasn't via PI3-K/ AKT signaling pathway.

3.4.2.2. PTP 1b Inhibition assay

The inhibition of PTP 1b allows the sensitization of insulin receptor which ultimately leads to the uninterrupted uptake of glucose. Sodium orthovanadate was used as the positive control as this strongly inhibits the activity of this phosphatase enzyme (Ghonime et al., 2012). Here, sodium orthovanadate demonstrated 59 % inhibition of PTP 1b enzyme and metformin showed 44 % inhibition of PTP 1b enzyme in L6 myotubes. The pretreatment with Ampelopsin F showed 54 % inhibition which is at par with the positive control sodium orthovanadate. (-) Hopeaphenol treated cells showed 42.5 % inhibition and was at par with the activity demonstrated by metformin treated cells (Fig 3.15).

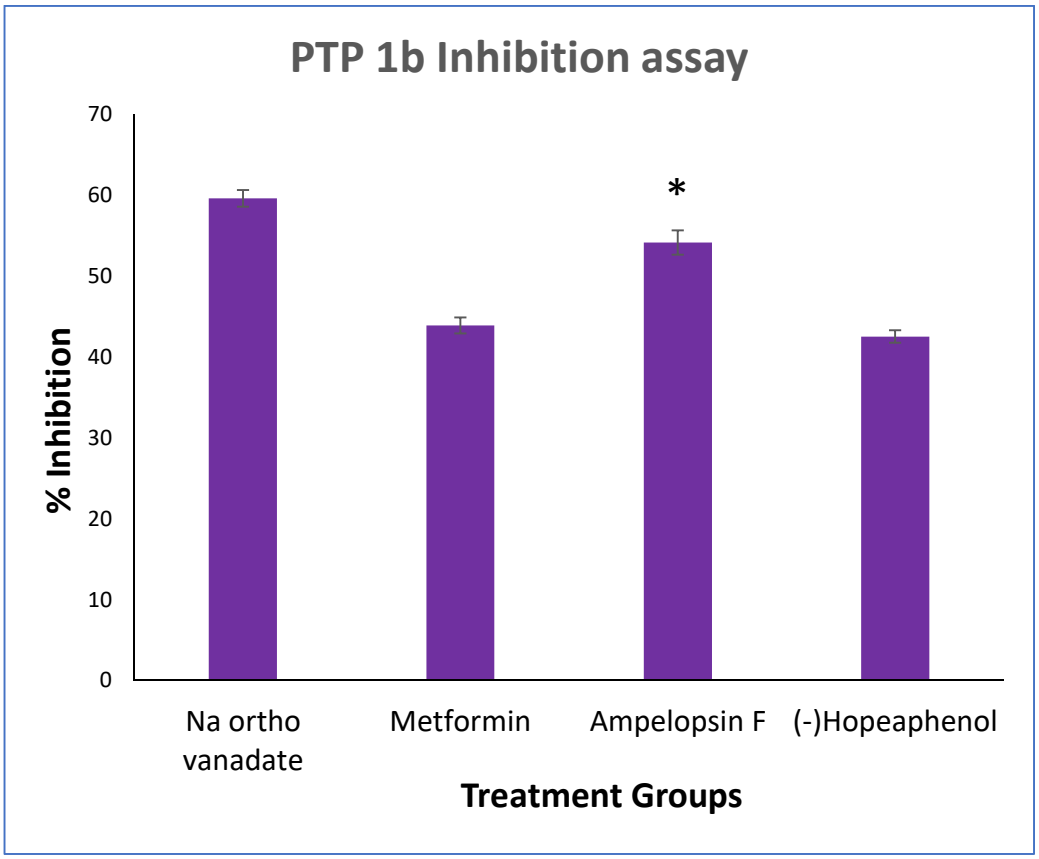


Fig 3.15. PTP 1b inhibition assay on L6 myotubes: The inhibitory potential of resveratrol oligomers in PTP 1b phosphatase enzyme was measured. Positive control represents sodium ortho vanadate (100 nM) and metformin (100 µM) along with Resveratrol oligomers Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM). The results were expressed in terms of mean ± SD of three determinations, the significance was accepted at $p \leq 0.05$. $*p \leq 0.05$ versus metformin.

Several PTP 1b inhibiting drugs have been disapproved on reaching different phases of clinical trials owing to their adverse side effects. Several investigators are trying to explore the identification of compounds from natural resources for the inhibition of PTP 1b in the management of diabetes mellitus (Jiang et al., 2012; Zhao et al., 2018). Berberine (Bustanji et al., 2006) and papaverine (Bustanji et al., 2009) have been reported as two potent natural PTP 1b inhibitors. Shibata and co-workers (2013) reported activation of PI3-K/AKT pathway of saturated and unsaturated fatty acids along with the inhibition of PTP 1b. Saturated fatty acids like palmitic acid, stearic acid, nonadecyclic acid, arachidic acid have been reported as potent inhibitors of PTP 1b (Steinmann et al., 2012). Flavonoids like apigenin and luteolin have been

reported with better PTP 1b inhibitory potential (Zhao et al., 2018). Our findings suggested the potential role of two resveratrol-based compounds in the inhibition of PTP 1b in L6 myotubes. Ampelopsin F is involved in stimulating PI3-K dependent insulin signaling pathway and also showed its potency in the inhibition of the phosphatase enzyme, PTP 1b. These data suggested the surplus characteristic of the resveratrol dimer Ampelopsin F to overcome insulin resistance. Though (-) Hopeaphenol was not found to be engaged in stimulating PI3-K dependent insulin signaling pathway, the potent inhibition of PTP 1b enzyme exhibited by the compound is independent of insulin transduction signaling pathway. In addition to the insulin signaling pathway, the inhibitory potential of PTP 1b by the resveratrol oligomers suggested the multi-targeted approach of the resveratrol oligomers in the management of diabetes mellitus.

3.4.3. Summary

The resveratrol dimer, Ampelopsin F and resveratrol tetramer (-) Hopeaphenol, were investigated to understand the mechanistic role in insulin signaling transduction pathway in L6 skeletal muscle cell lines. Metformin at 100 μM concentration was used as the positive control. The standardized concentration 50 μM was used for the compounds in skeletal muscle cell lines. The study revealed the potential of the resveratrol dimer Ampelopsin F in stimulating insulin signaling pathway whereas the downregulation of PI3-K by resveratrol tetramer, (-) Hopeaphenol, indicated the role in insulin independent pathways. These two resveratrol based compounds also showed their potential in the inhibition of PTP1b, which is a drug target in the treatment of diabetes mellitus.

The key findings of this chapter is summarized as follows

- Expression analysis of IRS-1, PI3-K and AKT revealed the role of resveratrol dimer Ampelopsin F in insulin dependent signaling
- The decreased expression of PI3-K in (-) Hopeaphenol treated cells implied that its mechanism of action in enhancing the uptake of glucose is insulin independent.
- There was an increased protein expression of GLUT4, which is the most downstream event in insulin signaling pathway in both the compound treated cells suggesting the

upregulation and translocation of GLUT4 transporter, which ultimately leads to increased glucose uptake

- Both the compounds were involved in the inhibition of phosphatase enzyme PTP 1b comparable to the positive control orthovanadate.

CHAPTER 4

Elucidation of molecular mechanism of the resveratrol oligomers in AMPK signaling pathway

4.1. Introduction

A target pathway of pharmacological importance in the treatment of diabetes mellitus is 5'-adenosine monophosphate-activated protein kinase (AMPK) signaling pathway (Joshi et al., 2019). AMPK is a conserved serine/threonine kinase in which its activation stimulates insulin sensitization and henceforth a perfect therapeutic target for the management of glucose homeostasis (Musi et al., 2001). A well-known drug currently used in the management of diabetes mellitus is metformin (Kim et al., 2016). This drug is reported to act via regulation of AMPK pathway (Min et al., 2018). Therefore, drugs which are involved in the activation and regulation of AMPK pathway could be the potential leads for the management of diabetes mellitus. AMPK is a major nutrient sensor and hence would be activated under low cellular energy levels and therefore a master regulator of metabolic homeostasis (Zhang et al., 2009). Upon activation, this serine/threonine kinase stimulates the uptake of glucose in skeletal muscle as well as fatty acid oxidation in adipose tissue. It also decreases the production of hepatic glucose and thereby maintaining glucose homeostasis (Hardie., 2013). The activation of AMPK is mediated majorly via two signals, AMP dependent signal facilitated by an upstream kinase tumor-suppressor liver kinase B1 (LKB1) and Ca^{2+} dependent one facilitated by calcium/calmodulin-dependent protein kinase β (CaMKKβ).

There are many polyphenols from natural sources reported to possess significant potential in regulating glucose homeostasis. Most of them were reported with the activation and regulation of AMPK pathway. Those include resveratrol, curcumin, berberine, quercetin, epigallocatechin gallate, hispidulin, theaflavin, apigenin, genistein (Zang et al., 2006) etc.

In the previous chapter, the resveratrol oligomers Ampelopsin F and (-) Hopeaphenol showed its anti-diabetic potential in insulin secretion pathway analogous to glucose stimulated insulin secretion (GSIS). While exploring the mechanistic role of resveratrol based compounds in insulin signaling pathway, it was observed that the Ampelopsin F treated cells had a positive effect in insulin signaling pathway. The downregulation of PI3-K in (-) Hopeaphenol treated groups implied the anti-diabetic potential of the compound is insulin independent.

Hence, this chapter explores the anti-diabetic potential of resveratrol oligomers in L6 myotubes in terms of modulating AMPK pathway.

4.2. Objectives

- To explore the possible mechanism of action of the resveratrol oligomers in AMPK pathway
- To explore the alterations in mitochondria followed by AMPK activation.

The experimental design for investigating the molecular mechanism of resveratrol oligomers in AMPK signaling pathway is depicted below in Fig 4.1

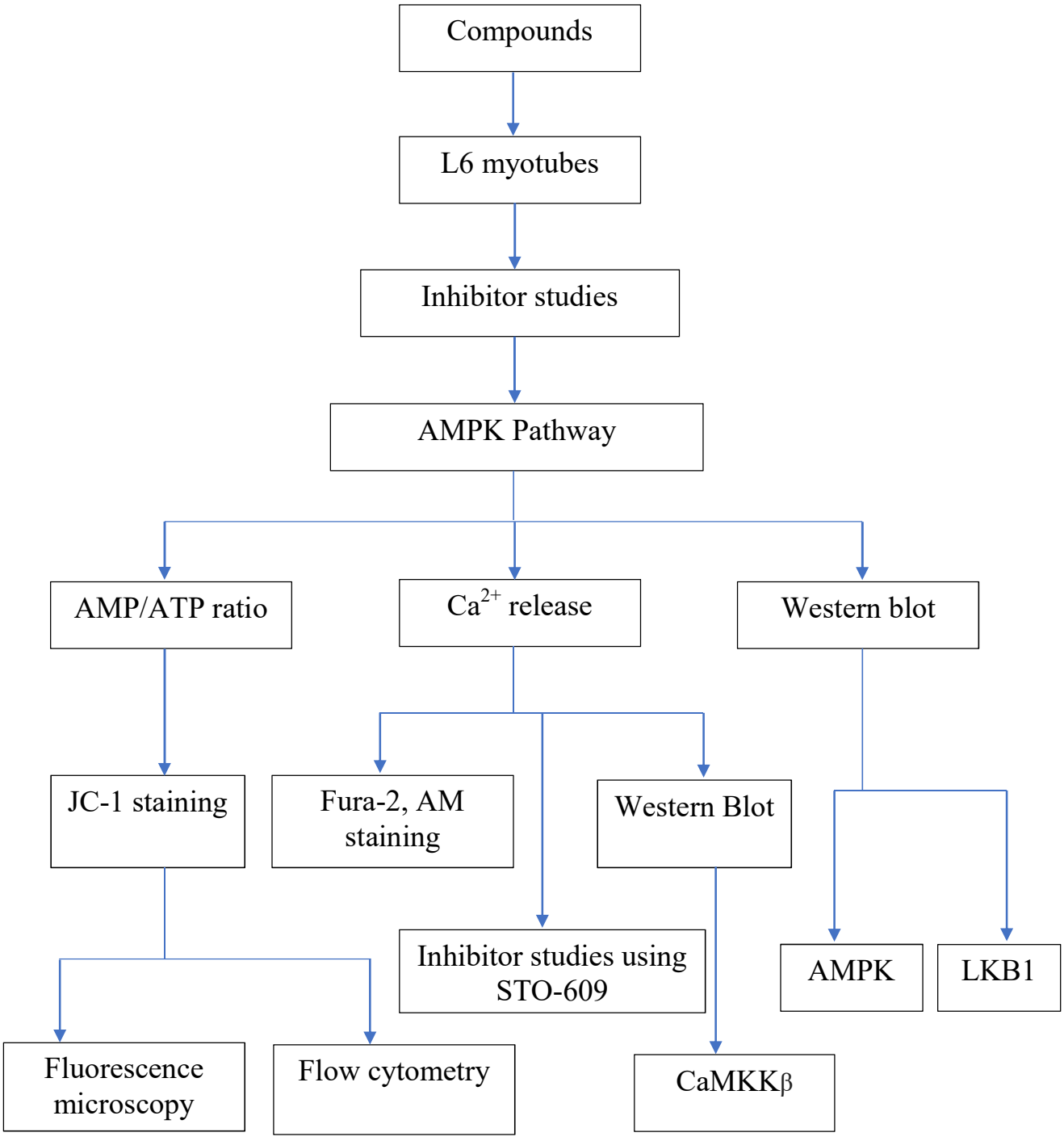


Fig 4.1. Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action in AMPK signaling

The experimental design for investigating the molecular mechanism of resveratrol oligomers in AMPK signaling pathway downstream to AMPK activation is depicted below in Fig 4.2.

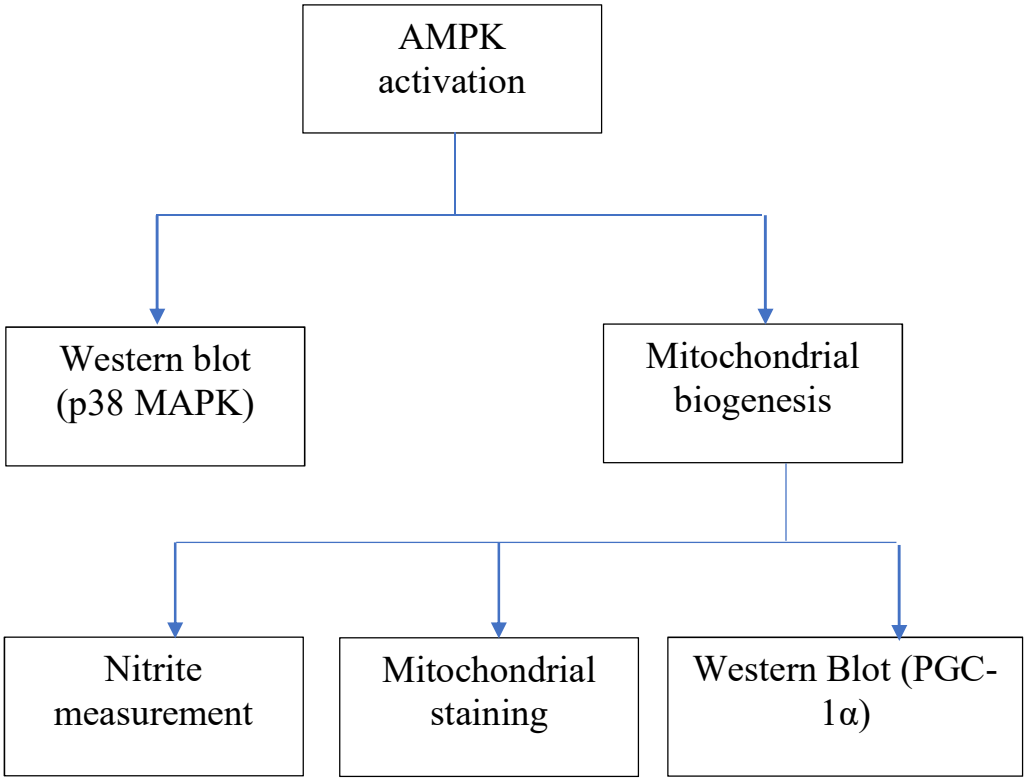


Fig 4.2. Schematic representation of experimental design in L6 skeletal muscle cell lines to elucidate the mechanism of action downstream to activation of AMPK.

4.3. Materials and methods

DMEM, BSA, ampicillin-amphotericin B mix, insulin, metformin, wortmannin, dorsomorphin, Fura-2,AM, STO-609, Griess reagent, mitochondria isolation kit and JC-1 mitochondrial staining kit were obtained from Sigma (MO, United States); phospho-specific or pan-specific antibodies against LKB1, AMPK, p38MAPK, CaMKKβ and PGC-1α and HRP-conjugated anti-rabbit were procured from Cell Signaling Technologies. DMSO was obtained from Himedia laboratories. Mitotracker deep red was purchased from Invitrogen (USA). Fetal bovine serum was purchased from Gibco (United States). L6 skeletal muscle cell lines for *in vitro* studies were obtained from NCCS, Pune, India. Rest all chemicals expended were of standard analytical grade.

4.3.1. Cell culture

L6 skeletal myocytes at low passages were grown in Dulbecco's modified Eagle's media supplemented with 10% antibiotic – antimycotic mix at 37°C under 5% CO_2 atmosphere. For cellular differentiation to generate myotubes, the seeded cells were provided with differentiation medium having 2% horse serum for 5-7 days.

4.3.2. Inhibitor analysis

Inhibitor studies were conducted to evaluate the role of resveratrol based compounds in insulin signaling pathway and AMPK pathway. To assess the outcome of kinase inhibitors, wortmannin (PI3K inhibitor) and dorsomorphin (AMPK inhibitor), entirely differentiated L6 myotubes were initially starved in serum free (SF) medium for a period of 1.5 hours. Subsequently, wortmannin (100 nM) and dorsomorphin (20 μM) were added on to the culture wells and cells were incubated for 30 minutes in the CO_2 incubator. Following this, cells were then treated with Ampelopsin F and (-) Hopeaphenol at a concentration of 50 μM for a period of 24 hours on serum free medium in the presence and absence of corresponding inhibitors. 2-NBDG uptake assay was conducted to analyze the effect of inhibitors in the glucose uptake potential of compounds in L6 cell lines. The pretreated cells were trypsinized, centrifuged, washed twice with saline buffer and then the suspended pellets were subjected to flow cytometry to analyze the fluorescence in cells.

4.3.3. Determination of Intracellular Calcium Levels

Analysis of intracellular calcium concentration was carried out to determine the role of resveratrol oligomers in stimulating CaMKKβ for the activation of AMPK. L6 myoblasts cultured in 96 black well plates were differentiated into myotubes after 5-7 days differentiation. The cells were treated with resveratrol oligomers for a period of 24 hours at 50 μM concentration. Intracellular calcium levels were determined using Fura-2,AM as described previously in section 3.3.1.3

4.3.4. Inhibitor analysis with STO-609

An increase in intracellular calcium concentration results in the activation of AMPK. This was confirmed by conducting inhibitor studies. STO-609 was used as a CaMKK inhibitor. The cells were pretreated with a 20 μM STO-609, in serum free media for one hour. Then the cells were treated with Ampelopsin F (50 μM) and (-) Hopeaphenol (50 μM) for 24 hours in the presence and absence of CaMKK inhibitor STO-609. 2-NBDG uptake assay was conducted to analyze the effect of STO-609 in the glucose uptake potential of compounds in L6 cell lines. As mentioned in Section 4.3.2, the cells after trypsinization were subjected to flow cytometry to analyze the fluorescence in cells.

4.3.5. Western Blotting

Differentiated L6 myoblast (5-7 day) cultured in T25 flasks/plates treated with resveratrol oligomers in the presence of insulin at 50 μM concentration and metformin at 100 μM concentration for 24 hours. The control cells were used as control and insulin-treated cells were used as insulin control. Western blot analysis was carried out for the cellular proteins LKB1, AMPK, CaMKKβ, p38 MAPK, and mitochondrial protein PGC-1α as described previously in section 3.3.1.4 using the cell lysates. Mitochondrial protein was determined via isolating the proteins using a mitochondrial isolation kit followed by the protocol mentioned previously for the western blot. The cellular/mitochondrial proteins transferred onto membranes were incubated with primary antibodies for AMPK, CaMKKβ, LKB1, PGC-1α, and β-actin (1:1000) followed by incubation with analogous horse-radish peroxidase conjugated secondary antibody at 1:2000 dilution.

4.3.6. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was determined to figure out the depolarization of the membrane using the dye JC-1. L6 cells were stained with the cationic lipophilic fluorochrome, JC-1 as previously described in section 3.3.1.2. A potent antibiotic, valinomycin (1 µg/mL), was treated as the positive control to determine the change in mitochondrial membrane potential.

For the quantitative estimation of mitochondrial membrane potential, flow cytometry analysis was performed. The cells followed by compounds pretreatment and 30 minutes incubation with JC-1 dye, were trypsinized, and the pellets were collected. These pellets were washed twice with PBS, strained, and subjected to flow cytometry analysis to determine the mitochondrial membrane potential.

4.3.7. Determination of mitochondrial content

Mitochondrial content was analyzed by means of mitotracker red (Mitotracker Deep Red FM, Invitrogen). Following pretreatment with resveratrol oligomers for 24 hours, the cells were incubated with 5 µM mitotracker red in plain media for 30 minutes at 37°C in CO_2 incubator. The media was then removed and washed twice with saline buffer and examined under fluorescence microscope. Fluorescence was quantified at excitation and emission wavelengths of 581 nm and 644 nm, respectively.

4.3.8. Measurement of nitrite content

A slight increase in the concentration of nitrite in cells denotes the increased mitobiogenesis. The amount of nitric oxide produced was assayed by measuring the nitrite concentration using Griess reagent in L6 myotubes. Following 24 hours of incubation with resveratrol oligomers Ampelopsin F and (-) Hopeaphenol, 50 µL of the pre-treated medium was transferred to a 96 well plate containing 50 µL of Griess reagent. The mixture was mixed gently and incubated for nearly ten minutes in room temperature. Then the absorbance was measured at 540 nm via multimode microplate reader. Sodium nitrite was used to plot the standard curve for this assay.

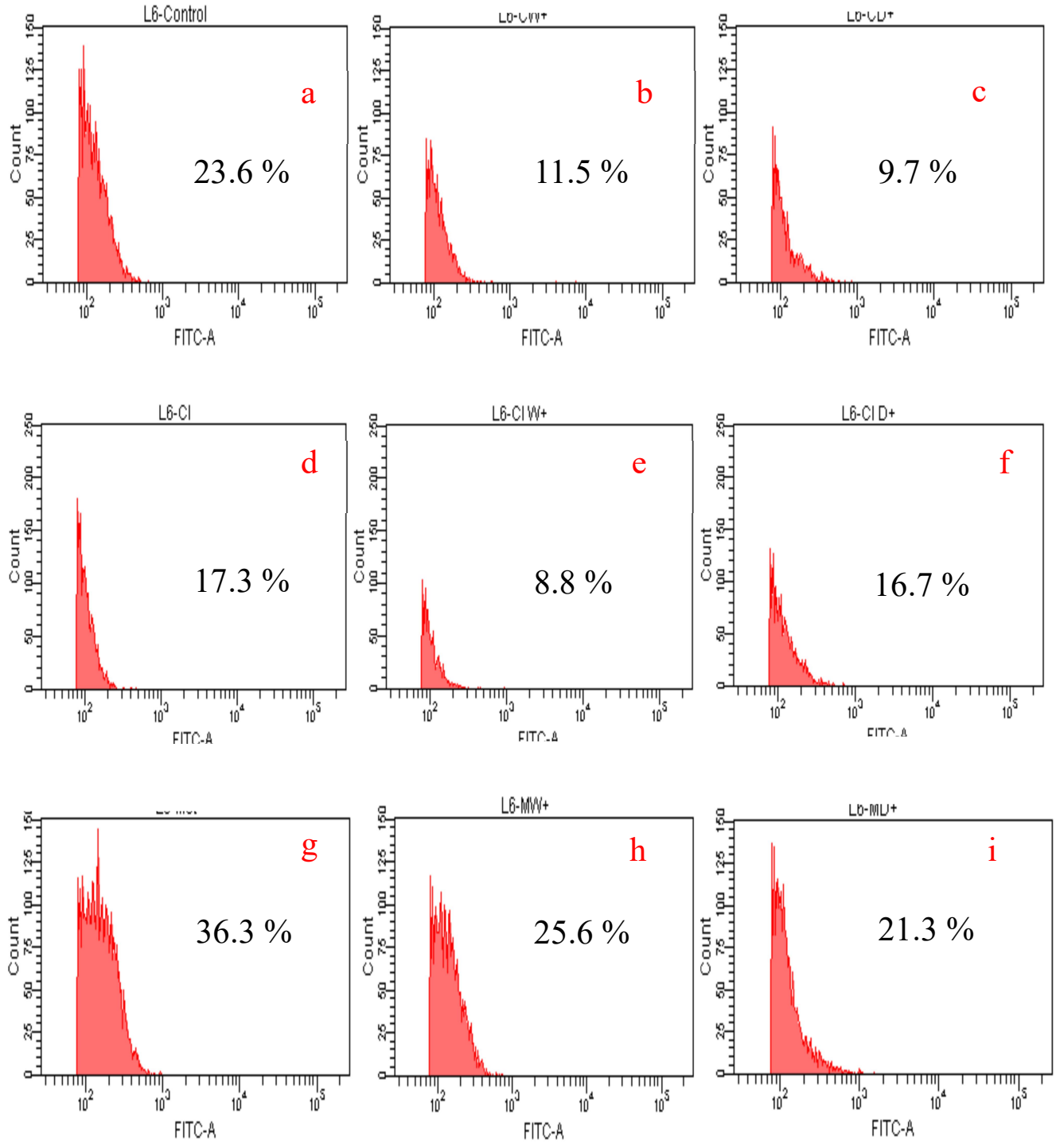
4.3.9. Statistical analysis

All the experiments were executed in triplicates. The data were expressed as mean ± standard deviations and significance of differences determined by one-way ANOVA followed by Tukey's test. P value at $p \leq 0.05$ was regarded as statistically significant.

4.4. Results and discussion

4.4.1. Inhibitor studies

The data from the previous chapters confirmed the anti-diabetic potential of Ampelopsin F and (-) Hopeaphenol, in stimulating uptake of glucose in L6 myotubes via the translocation of GLUT4. To examine whether the increased glucose uptake in L6 myotubes stimulated by resveratrol oligomers were intermediated via PI3K activation, we studied the role of selective inhibitors of PI3K (wortmannin) and AMPK (dorsomorphin) on the uptake of glucose. 2-NBDG uptake analysis in the presence and absence of inhibitors by flow cytometry demonstrated a reduction in the uptake of glucose in control cells in the presence of both the inhibitors, wortmannin and dorsomorphin. Control cells showed 23.6 % uptake of glucose, which was reduced to 11.5 % and 9.7 % in the presence of wortmannin and dorsomorphin, respectively. Insulin treated cells demonstrated 17.3 % 2-NBDG uptake that was reduced to 8.8 % when co-treated with wortmannin and no significant difference was observed on treating with dorsomorphin. Metformin treated cells resulted in 36.3 % 2-NBDG uptake which was significantly reduced to 25.6 % when co-treated with wortmannin. The presence of dorsomorphin in metformin treated cells showed a greater reduction in the uptake of glucose (21.3 %). Ampelopsin F treated cells showed 38.2 % glucose uptake which was significantly reduced to 29 % and 21.8 % in the presence of wortmannin and dorsomorphin, respectively (Fig 4.3). The pretreatment with (-) Hopeaphenol showed 37.5 % glucose uptake which was significantly reduced to 27.2 % in the presence of dorsomorphin in L6 myotubes. No significant difference in 2-NBDG uptake was noticed in (-) Hopeaphenol treated cells co-treated with wortmannin (36.6 %).


L6-Control
a
23.6 %
b
11.5 %
c
9.7 %
L6-CI
d
17.3 %
L6-CI W+
e
8.8 %
L6-CI D+
f
16.7 %
L6-MW+
h
25.6 %
L6-MD+
i
21.3 %
Count
FITC-A

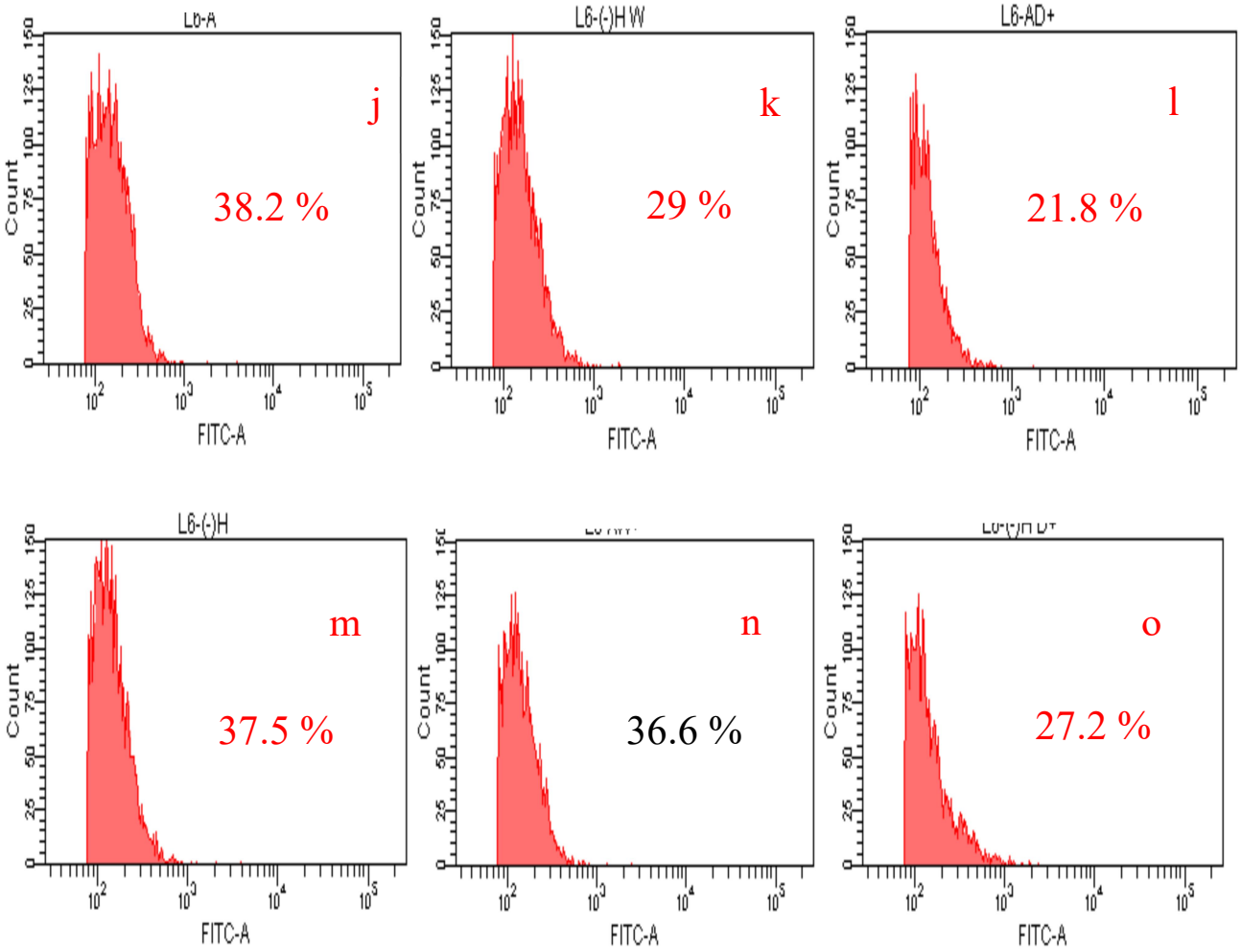


Fig 4.3. Inhibitor analysis in L6 myotubes by flow cytometry: Inhibitor studies in the presence and absence of inhibitors wortmannin and dorsomorphin. a) control, b) control cells co-treated with wortmannin (100 nM), c) control cells co-treated with dorsomorphin (20 µM), d) Insulin control (100 nM), e) Insulin control cells co-treated with wortmannin (100 nM), f) insulin control cells co-treated with dorsomorphin (20 µM), g) Metformin treated cells (100 µM) h) Metformin treated cells co-treated with wortmannin (100 nM), i) Metformin treated cells co-treated with dorsomorphin (20 µM), j) Ampelopsin F treated cells (50 µM), k) Ampelopsin F treated cells co-treated with wortmannin (100 nM), l) Ampelopsin F treated cells co-treated with dorsomorphin (20 µM), m) (-) Hopeaphenol treated cells (50 µM), n) (-) Hopeaphenol treated cells co-treated with wortmannin (100 nM), o) (-) Hopeaphenol treated cells co-treated with dorsomorphin (20 µM).

The effect of inhibitors, wortmannin and dorsomorphin on 2-NBDG uptake co-incubated with resveratrol oligomers were depicted graphically in Fig 4.4. The uptake of glucose in L6 myotubes was reduced partially in insulin treated cells along with wortmannin. Metformin treated cells showed decreased uptake of glucose (1.4 times and 1.7 times) when co-treated with wortmannin and dorsomorphin, respectively. Similarly, 1.3 times and 1.7 times decrease in 2-NBDG uptake was noticed in Ampelopsin F treated cells in presence of wortmannin and dorsomorphin, respectively. No significant difference was noticed in (-) Hopeaphenol treated cells in the presence and absence of wortmannin. But a significant reduction (1.4 times) in the uptake of glucose was noticed in (-) Hopeaphenol treated cells on co-incubation with dorsomorphin.

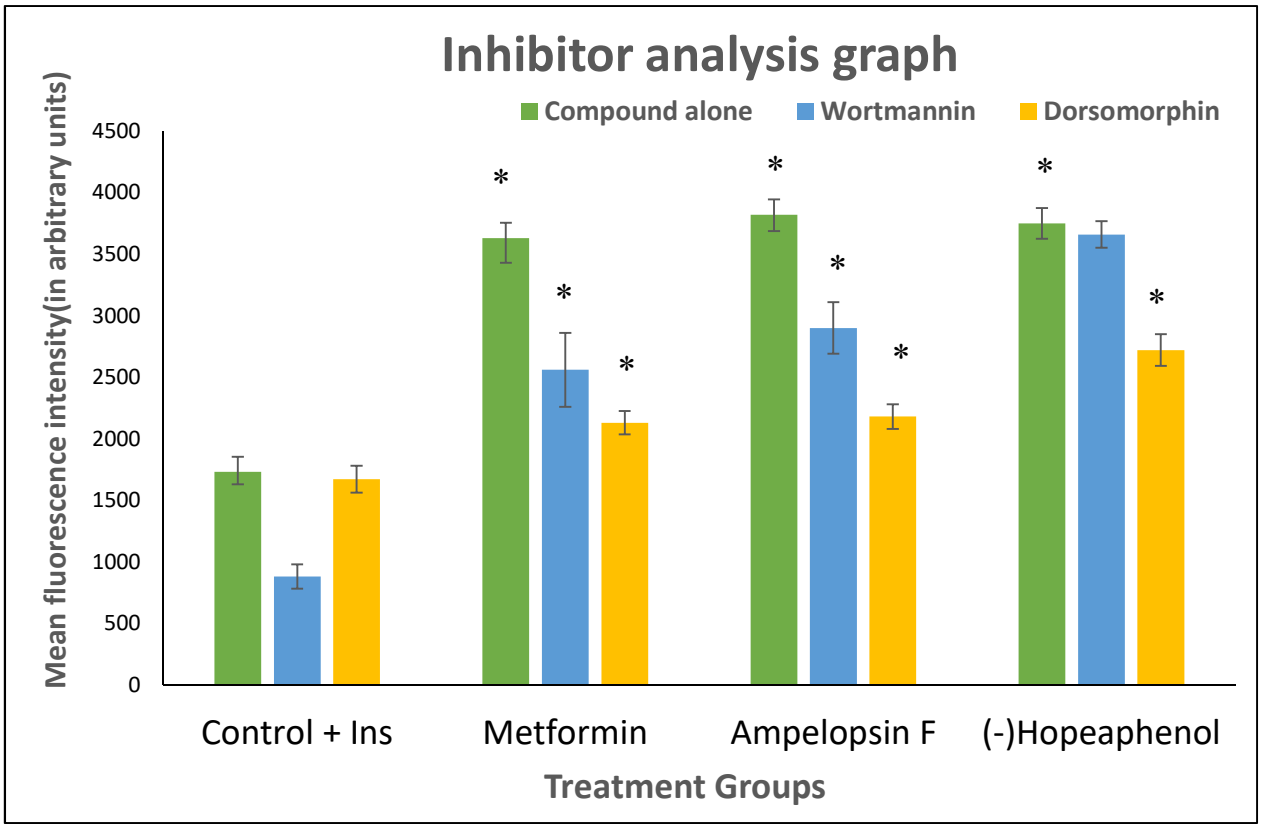


Fig. 4.4. Inhibitor studies in the presence and absence of inhibitors wortmannin and dorsomorphin. This study exposed the involvement of Ampelopsin F in both insulin signaling and AMPK signaling pathway while (-) Hopeaphenol in AMPK pathway alone. The results expressed in terms of mean ± SD of three determinations. The significance accepted at $p \leq 0.05$. $*p \leq 0.05$ versus control.

The involvement of PI3-K/AKT pathway and AMPK pathway was determined by using specific inhibitors (wortmannin and dorsomorphin) to demonstrate the accurate molecular pathway resulting in the increased glucose uptake induced by compounds. Phadannok and his colleagues (2020) reported that the extracts of *Astraeus asiaticus* reduced the uptake of glucose in the presence of the inhibitor wortmannin in L6 myotubes. α -lipoic acid has been reported to trigger the uptake of glucose via PI3-K/AKT dependent insulin signaling pathway and Konrad and his co-workers (2001) reported the reduced uptake of glucose in α -lipoic acid treated cells co-treated with wortmannin in L6 myotubes. The fruit juice of *Momordica charantia* treated along with wortmannin attenuated the uptake of glucose in L6 myotubes (Cummings et al., 2004). Compound C or dorsomorphin was reported as the inhibitor of AMP kinase (Liu et al.,2014), and hence the cells upon co-treatment with compounds and inhibitors aided in revealing the role of compounds in stimulating AMPK pathway. L6 myotubes co-treated with quercetin and dorsomorphin markedly reduced the uptake of glucose signifying the role of quercetin in AMPK signaling pathway (Dhanya et al., 2017). Abscisic acid (ABA) was reported to enhance the activation of AMPK in L6 myotubes and the presence of dorsomorphin abolished the effects induced by ABA in AMPK phosphorylation (Magnone et al., 2020).

Metformin has been reported to induce the translocation of GLUT4 and subsequently the uptake of glucose via AMPK dependent mechanism. Kumar and Dey (2002) reported the involvement of metformin in restoring the uptake of glucose in insulin resistant C2C12 muscle cells via insulin dependent and insulin independent pathways. Conversely, in skeletal muscle L6 cell lines, metformin regulated the glucose uptake independent of PI3-K/AKT signaling pathway (Turban et al., 2012). Several polyphenols, including dietary stilbenes, have been shown to possess anti-diabetic potential in animal models and human studies (Pandey and Rizwi, 2009). Resveratrol, the dietary stilbene, has been reported to promote GLUT4 translocation via activation of AMPK pathway (Penumathsa et al., 2008) and insulin signaling pathway via upregulation of IRS-1 (Chen et al., 2012) and AKT (Burgess et al., 2011).

The results obtained from the last chapter showed the stimulation of PI3-K/AKT dependent pathway by the resveratrol dimer Ampelopsin F, but not by (-) Hopeaphenol which was confirmed through results of the inhibitor studies in the current chapter. Metformin treated cells in presence of wortmannin showed a significant reduction in the 2-NBDG uptake and in the

presence of dorsomorphin strongly inhibited the uptake of glucose indicating the predominant role of metformin in stimulating the AMPK pathway.

The effect of Ampelopsin F on 2-NBDG uptake was wortmannin as well as dorsomorphin sensitive. Glucose uptake analysis by flow cytometry in skeletal muscle cells in the presence and absence of wortmannin (100 nM) and dorsomorphin (10 μM) in Ampelopsin F treated cells showed a significant decline in the glucose uptake which implied the involvement of this resveratrol dimer in stimulating insulin signaling pathway and AMPK signaling pathway.

The glucose uptake analysis in the presence and absence of inhibitors followed by (-) Hopeaphenol pretreatment pointing the effect of (-) Hopeaphenol on 2-NBDG uptake was wortmannin insensitive. The results showing the downregulation of PI3-K in (-) Hopeaphenol treated cells in the last chapter coincided with this chapters data where the co-incubation with wortmannin didn't show any significant difference in the uptake of glucose. However, a significant reduction of glucose uptake in (-) Hopeaphenol treated cells was observed in the presence of dorsomorphin which implies that this resveratrol tetramer was involved in stimulating AMPK pathway alone for inducing glucose uptake.

4.4.2. Calcium dependent AMPK signaling pathway

Intracellular Calcium Levels

In order to evaluate the modulation of intracellular calcium levels by resveratrol oligomers in L6 cells, Fura-2, AM, a fluorescent indicator of calcium was used. The fluorescent microscopic images for L6 myotubes followed by pretreatment with Ampelopsin F, (-) Hopeaphenol and the positive control metformin stained with Fura-2, AM were shown below in Fig 4.5. The control cells were observed with least signals of Fura-2, AM, indicating the normal physiological calcium concentration in L6 myotubes. The cells pretreated with the positive control, metformin, showed a noticeable increase in the cytosolic calcium levels as indicated by increased fluorescence when compared to control cells. The pretreatment of (-) Hopeaphenol to cells significantly altered calcium homeostasis in L6 cell lines by considerably raising the cytosolic calcium levels. The pretreatment of L6 myotubes with Ampelopsin F did not elicit any significant rise in cytosolic calcium levels when compared to control.

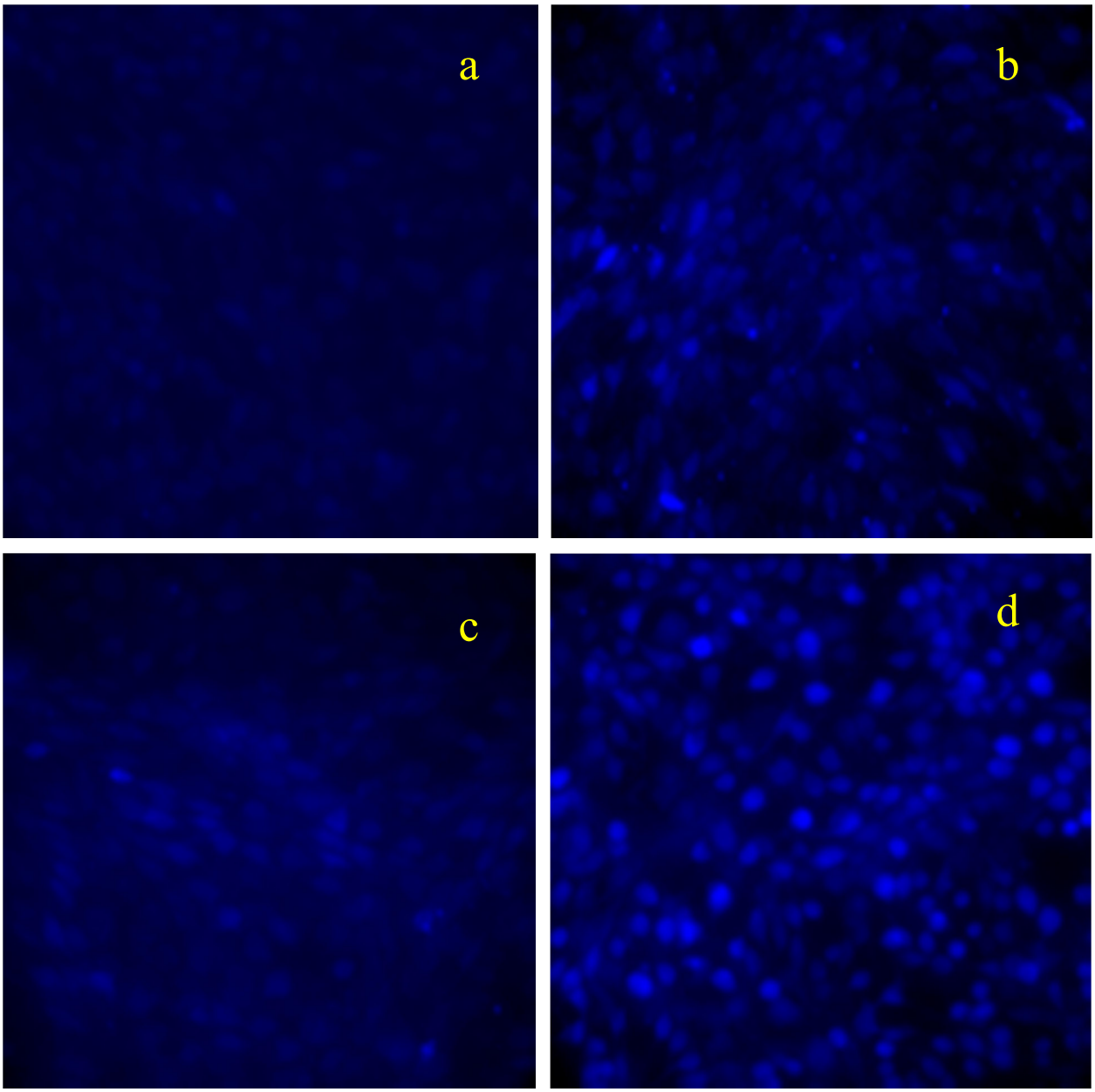


Fig 4.5. Determination of cytosolic calcium levels in L6 myotubes pretreated with resveratrol oligomers. Fluorescent images of cytosolic Ca^{2+} in L6 cell lines were investigated using Fura-2, AM. a) Control group; b) Metformin (100 µM); c) Ampelopsin F (50 µM) and d) (-) Hopeaphenol (50 µM). Magnification 20X. Scale bar corresponds to 50 µM.

The intracellular calcium concentration obtained from the fluorescence microscopy was quantified and plotted graphically (Fig 4.6) to analyze the intracellular calcium concentrations in untreated cells, metformin and resveratrol oligomers treated cells. Metformin treated cells

demonstrated 1.6 times increase in cytosolic calcium concentration when compared to control cells. (-) Hopeaphenol treated cells showed 2.4 times and 1.5 times increment in intracellular calcium concentration when compared with control cells and metformin treated cells, respectively. No significant difference in calcium concentration was noticed in Ampelopsin F treated cells.

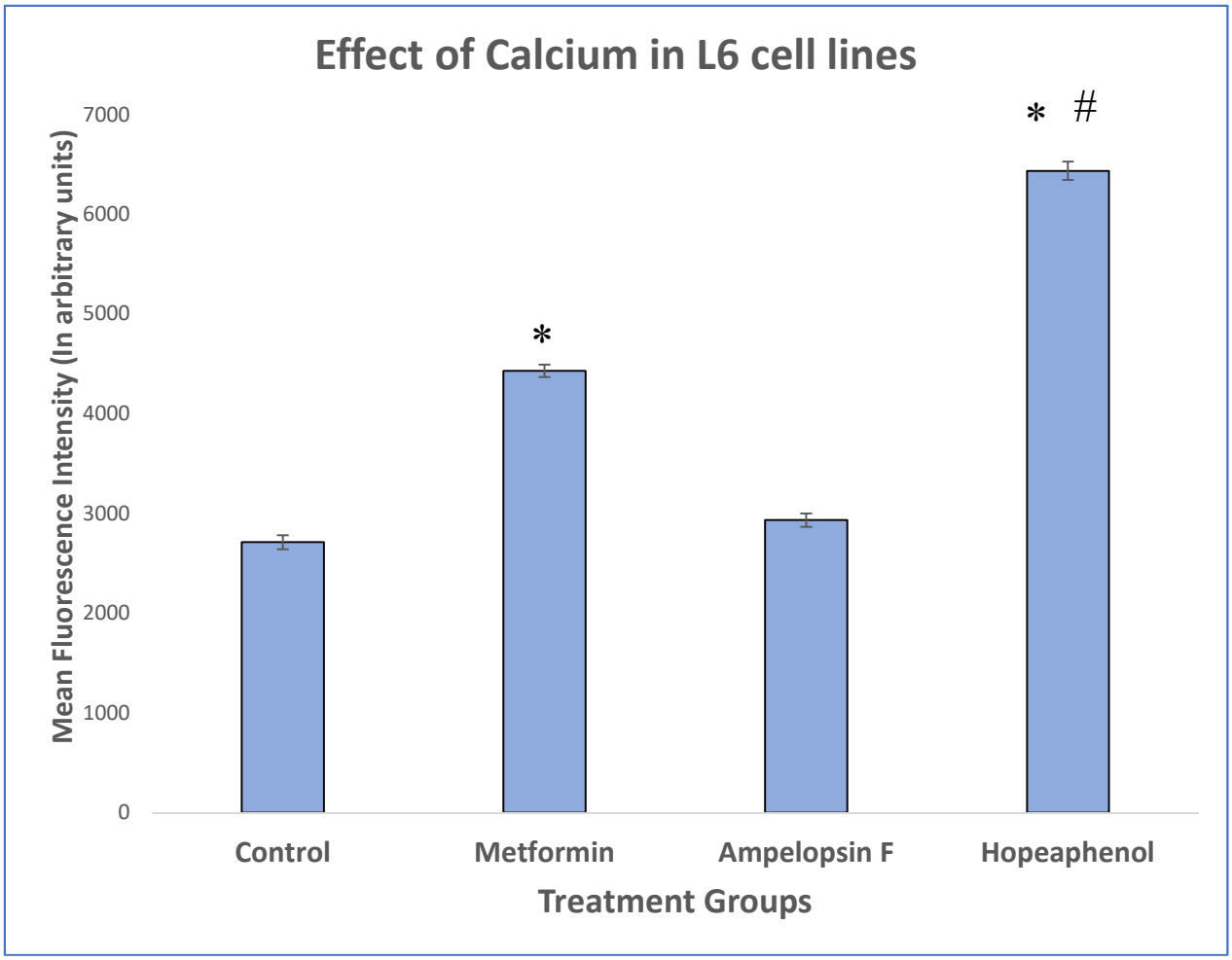
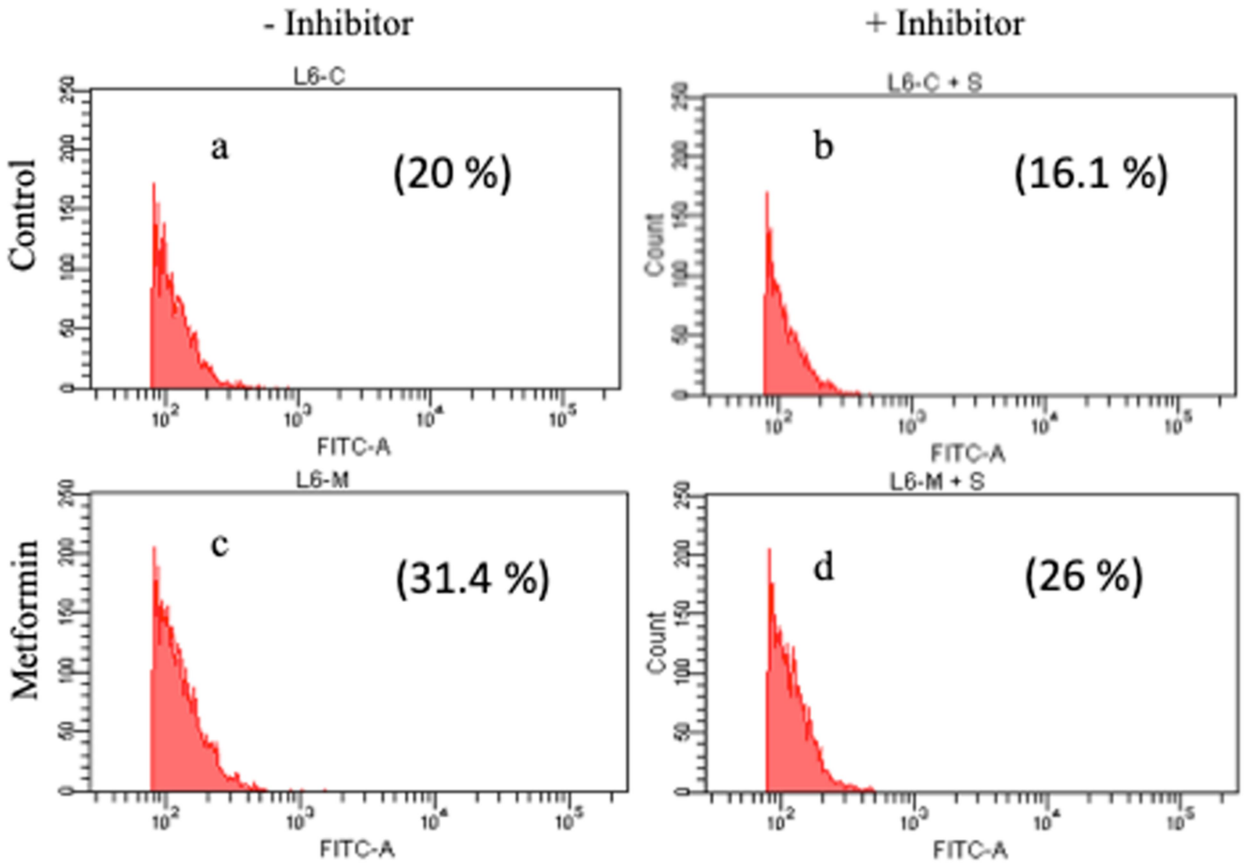


Fig 4.6. Fluorescence intensity analysis of cytosolic calcium levels in L6 myotubes. A significant increase in cytosolic calcium concentration observed in metformin treated and (-) Hopeaphenol treated cells when compared to control cells. No significant changes in intracellular calcium concentration in Ampelopsin F treated cells. The results were expressed in terms of mean ± SD of three determinations. The significance was established at $p \leq 0.05$. $*p \leq 0.05$ versus control, $\#p \leq 0.05$ versus metformin.

Inhibitor analysis with STO-609

To understand the role of the resveratrol tetramer, (-) Hopeaphenol in the stimulation of calcium dependent AMPK pathway, inhibitor studies were performed using STO-609, a specific and potent pharmacological inhibitor of CaMKK. A slight change in the uptake of glucose was found in L6 myotubes in control group with 16.1 % and 20 % uptake of glucose in the presence and absence of CaMKK inhibitor, STO-609 respectively. A similar trend was observed upon treatment with metformin in the presence and absence of inhibitor. Metformin treated cells showed 31.4 % 2-NBDG uptake in the absence of inhibitor whereas about 5 % decrease in the uptake of glucose was observed in L6 myotubes in the presence of STO-609 (26 %). 2-NBDG analysis followed by pretreatment with Ampelopsin F in the presence and absence of CaMKK inhibitor, STO-609, didn't show any marked difference with 32 % and 33.7 % uptake of glucose, respectively. As shown in Fig 4.7, STO-609 significantly attenuated (-) Hopeaphenol induced glucose uptake to 20.1 % from 30.3 %.



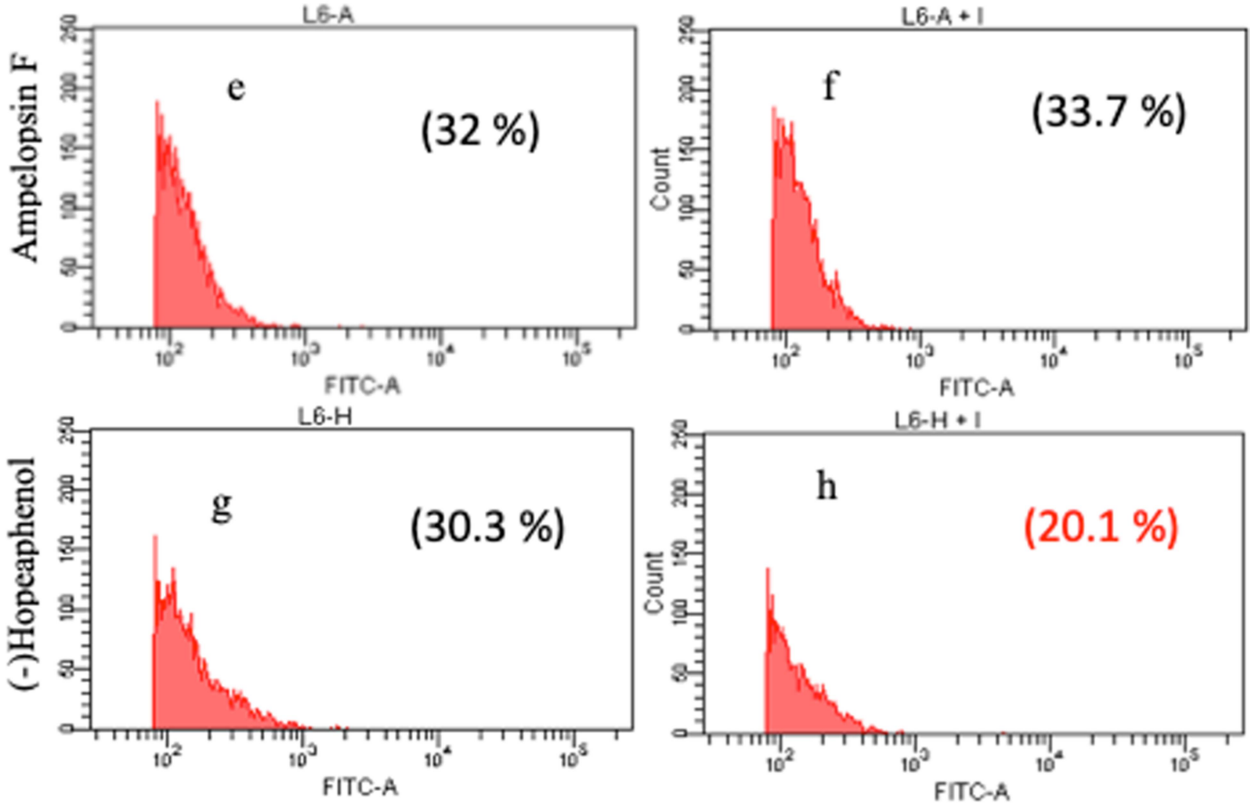


Fig 4.7. Inhibitor studies using a specific CaMKK inhibitor, STO-609. L6 myotubes followed by compounds pretreatment co-treated with STO-609 (20 μM) were subjected to flow cytometry analysis in order to study the involvement of resveratrol oligomers in triggering Ca^{2+}-dependent AMPK activation. a) and b) corresponds to control cells in the absence and presence of inhibitor respectively, c) and d) corresponds to metformin treated cells in the absence and presence of inhibitor respectively, e) and f) corresponds to Ampelopsin F treated cells in the absence and presence of inhibitor respectively, f) and g) corresponds to (-) Hopeaphenol treated cells in the absence and presence of inhibitor respectively.

Western blot for CaMKKβ expression analysis

To assess the potential role of calmodulin kinase in the activation of AMPK, western blot analysis was performed. The expression of CaMKKβ in resveratrol oligomers treated cells was determined in L6 myotubes. Control group and insulin treated cells didn't show any significant difference in the expression of CaMKKβ in L6 myotubes. Ampelopsin F treated cells didn't show a significant expression of CaMKKβ in L6 myocytes. However, the resveratrol tetramer,

(-) Hopeaphenol, induced a 1.5 fold increase in the expression of CaMKKβ than control cells (Fig 4.8).

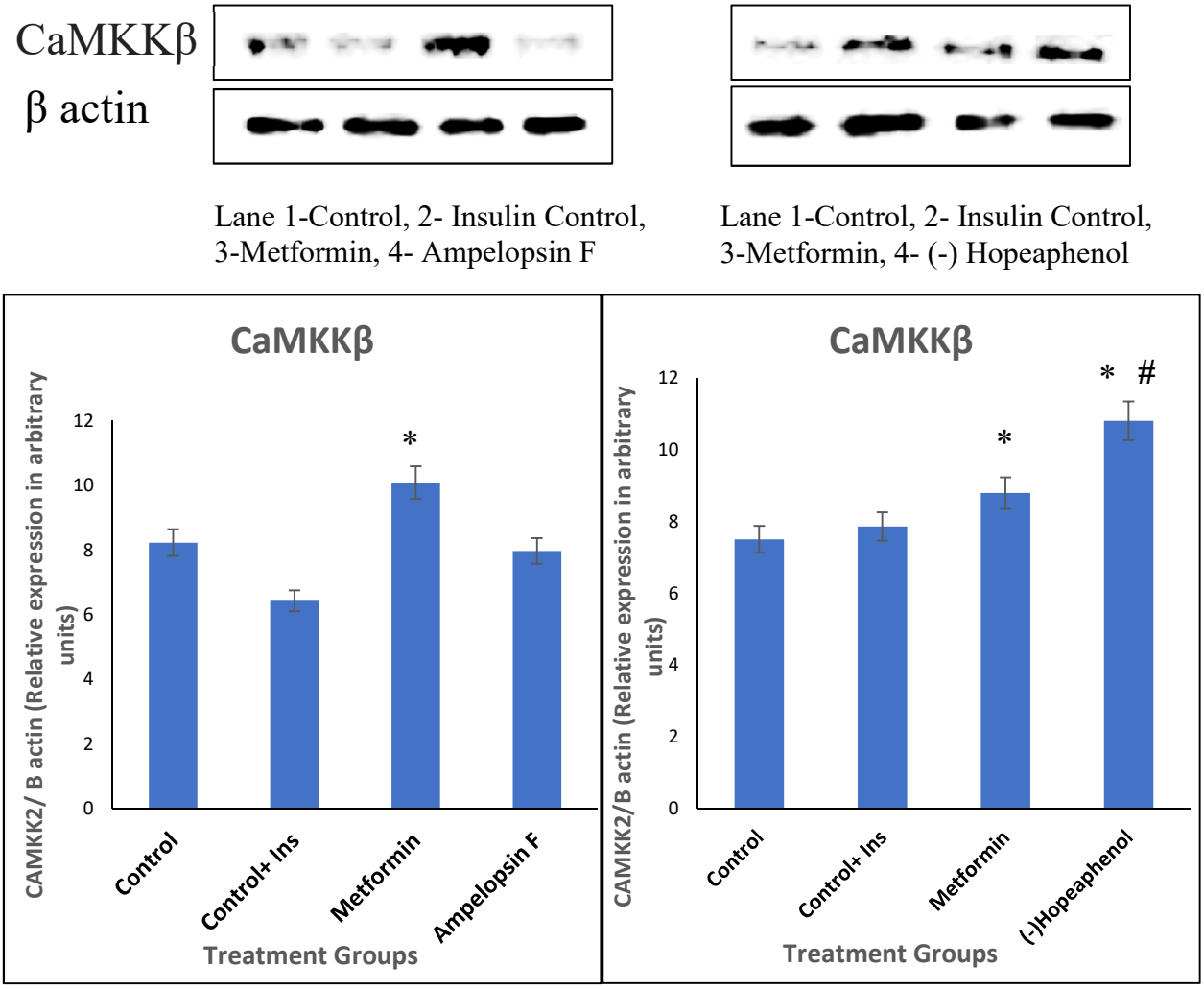


Fig 4.8. CaMKKβ protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol: Western blot analysis for CaMKKβ expression:**.** L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours in the presence of insulin (100 nm). Cell lysates were examined for protein expression of CaMKKβ by western blot analysis with β-actin as loading control. All data are represented as means ± SD (n=3), The significance accepted at $p \leq 0.05$. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus metformin.

Exercise, depletion of energy, contraction (Abott et al., 2009) and oxytocin (Lee et al., 2008) could activate AMP kinase through the upstream kinases Ca^{2+}/calmodulin-dependent kinase

(CaMKKβ) and liver kinase B1 (LKB1) (Hawley et al., 2005). This activation leads to prolonged uptake of glucose in the cells and hence is a major signaling molecule for the activation of AMPK pathway (Long and Zierath., 2006). CaMKKβ stimulates an increase in intracellular calcium and plays a significant role in stimulating AMPK in skeletal muscle cells. Calcium (Ca^{2+}) acts as a ubiquitous and acute second messenger which could aid in regulating diverse downstream cellular signaling events. The release of calcium occurs upon response to a stimulus either from cytosolic vesicles or inflow from the extracellular void via ion channels, ultimately resulting in attaching to the target proteins following metabolic modulation (Newton et al., 2016). CaMKKβ triggers the activation of AMPKα subunit upon rise in intracellular cytosolic Ca^{2+} concentration in an AMP independent manner (Hawley et al., 2005), subsequently followed by the formation of complex encompassing CaMKKβ, AMPKα and β subunits and Ca^{2+}/Calmodulin (Marcelo et al., 2016).

Resveratrol was reported to phosphorylate AMPK by the upstream kinase CaMKKβ (Park et al., 2012). Resveratrol was reported to show the AMPK activation mediated by the upstream kinase LKB1 which was dependent on SIRT1 proteins (Lan et al., 2017). The mechanistic role of resveratrol oligomers was found to be independent from the stilbene resveratrol. Baicalin, a major flavonoid isolated from *Scutellaria baicalensis* was reported to activate AMPK via Ca^{2+}/ CaMKKβ/ AMPK-dependent pathway (Ma et al., 2012). Other polyphenols like quercetin (Dhanya et al., 2017), resveratrol (Szkudelski and Szkudelska, 2014) were reported to activate AMPK via both CaMKKβ/AMPK-dependent and -independent mechanism of action. The inhibitor analysis using STO-609, a specific CaMKK inhibitor, revealed the role of compounds in stimulating CaMKKβ dependent AMPK activation (Ma and Yang et al., 2012).

In order to establish the involvement of resveratrol oligomers in Ca^{2+}/CaMKKβ/AMPK-dependent pathway, we performed staining with Fura-2, AM, inhibitor studies using CAMKK inhibitor STO-609 and expression studies on the protein CaMKKβ. Resveratrol dimer, Ampelopsin F, didn't impart significant changes in the calcium concentration in L6 myotubes. This data was supported by the inhibitor analysis using STO-609, a specific CaMKK inhibitor. No significant difference in the uptake of glucose implied the disengagement of Ampelopsin F in CaMKKβ mediated activation of AMPK. In addition, the insignificant expression of CaMKKβ in

Ampelopsin F treated cells obtained from western blot analysis stated the confirmation on the non-participation of Ampelopsin F in CaMKKβ/ AMPK pathway. Contrarily, the activation of AMPK stimulated by the resveratrol tetramer, (-) Hopeaphenol, was dependent on CaMKKβ. The increased intracellular calcium content in L6 myotubes pretreated with (-) Hopeaphenol was complementing the inhibitor based studies using STO-609 where there was a reduced glucose uptake compared to non-inhibitor group. In addition, the western blot analysis demonstrated an increased expression of CaMKKβ on pretreatment of (-) Hopeaphenol. These results clearly indicated that the activation of AMPK pathway by the resveratrol oligomer, (-) Hopeaphenol was CaMKKβ dependent.

Therefore, through calcium based studies, we have proved the disengagement of Ampelopsin F in stimulating Ca^{2+}/ CaMKKβ dependent AMPK pathway, while the resveratrol tetramer (-) Hopeaphenol was engaged in stimulating Ca^{2+}/ CaMKKβ/ AMPK-dependent pathway for glucose uptake in skeletal muscle cells.

4.4.3. Change in mitochondrial membrane potential in AMPK signaling pathway

Transient Mitochondrial Depolarization

Mitochondrial membrane potential was determined by means of staining with lipophilic dye, JC-1. The control cells showed normal mitochondrial membrane potential as indicated by the increased red fluorescence in (a) in Fig 4.9. A slight depolarization of the mitochondrial membrane was showed by the cells on metformin, Ampelopsin F and (-) Hopeaphenol pretreatment as there was a shift from red to green fluorescence in b), c), and d) images as indicated in Fig 4.9.

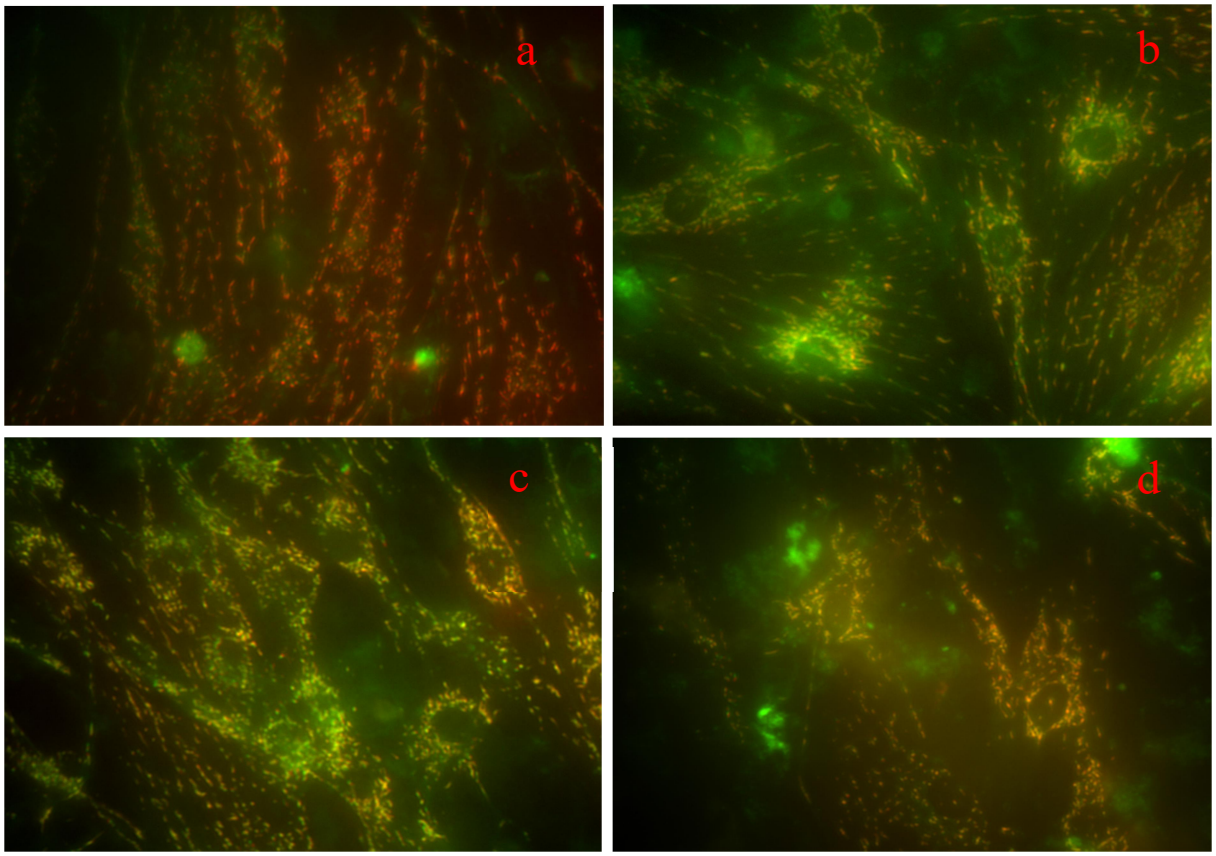
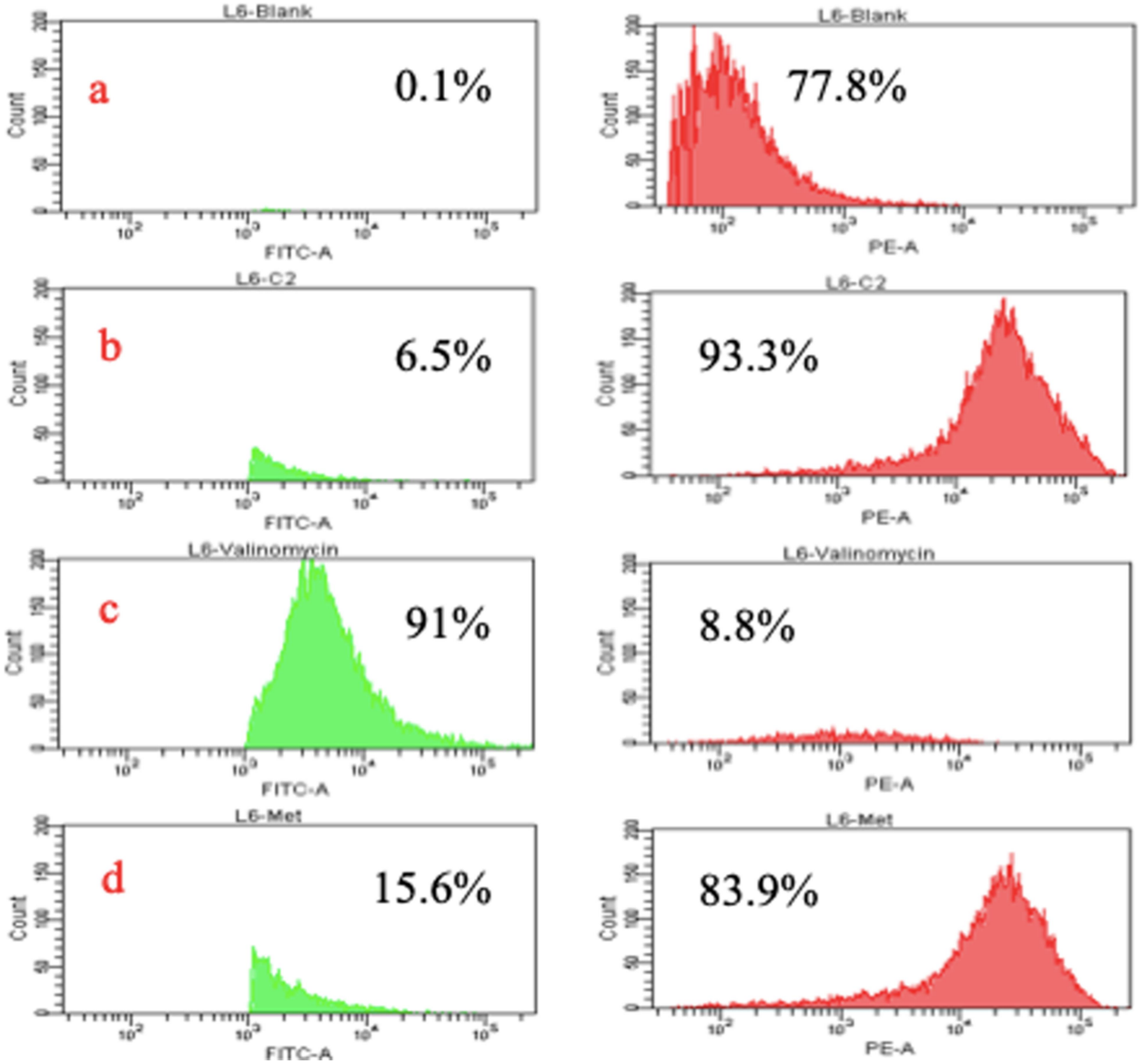


Fig 4.9. Determination of mitochondrial membrane potential using fluorescence microscopy. Change in mitochondrial membrane potential upon pretreatment with resveratrol based compounds was analyzed in L6 myotubes. The slight increment in green fluorescence when comparing to control cells showed a transient depolarization of mitochondrial membrane. a) Control cells, b) Metformin treated cells (100 µM), c) Ampelopsin F treated cells (50 µM) and d) (-) Hopeaphenol treated cells (50 µM). Magnification 40X. Scale bar corresponds to 20 µM.

Flow cytometry analysis for determination of mitochondrial membrane potential.

The results obtained from fluorescence microscopy on the mitochondrial membrane potential were further confirmed by flow cytometry analysis as shown in the Fig 4.10. The change in mitochondrial membrane potential was examined using the fluorescent dye JC-1. The control cells showed 93.3 % red fluorescence and 6.5 % green fluorescence. Valinomycin (1µg/mL) was used as the positive control. A highly depolarized mitochondrial membrane was observed in valinomycin treated cells (red fluorescence- 8.8 % and green fluorescence- 91 %). The anti-

diabetic drug, metformin, pretreatment showed 83.9 % of red fluorescence and 15.6 % of green fluorescence indicating a slight depolarization of mitochondrial membrane. A similar trend of membrane depolarization was demonstrated by the cells pretreated with Ampelopsin F and (-) Hopeaphenol. Ampelopsin F showed 77 % red fluorescence and 22.9 % green fluorescence while (-) Hopeaphenol showed 79.1 % red fluorescence and 19.6 % green fluorescence.



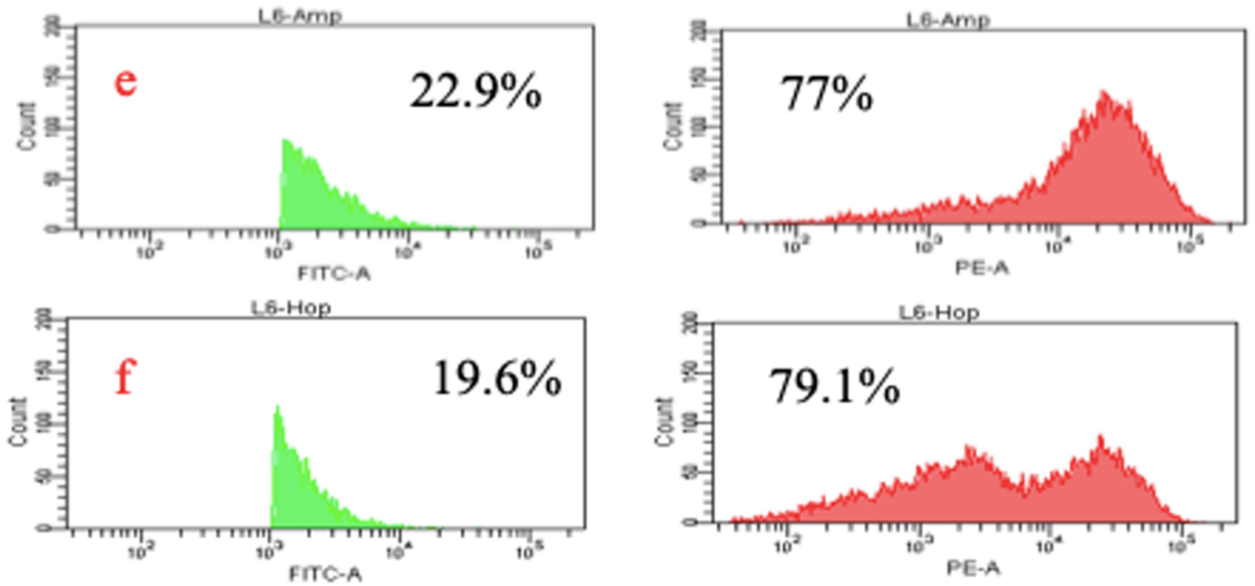


Fig 4.10. Determination of Mitochondrial membrane depolarization using JC-1 dye by means of flow cytometry analysis. A transient membrane depolarization was observed in Metformin (100 µM) treated (d), Ampelopsin F (50 µM) treated (e) and (-) Hopeaphenol (50 µM) (f) treated cells. Valinomycin (1 µg/mL) (e) was the positive control used for determining mitochondrial membrane potential. a) and b) represents blank and control respectively.

The fluorescent intensity obtained from flow cytometry is represented graphically as shown in Fig 4.11 (a). The transient depolarization of the mitochondrial membrane in L6 myotubes pretreated with resveratrol oligomers was observed by a shift from red to green fluorescence. The ratio of red to green fluorescence is also represented in Fig 4.11 (b) which shows the extent of depolarization of the mitochondrial membrane upon pretreatment with resveratrol oligomers.

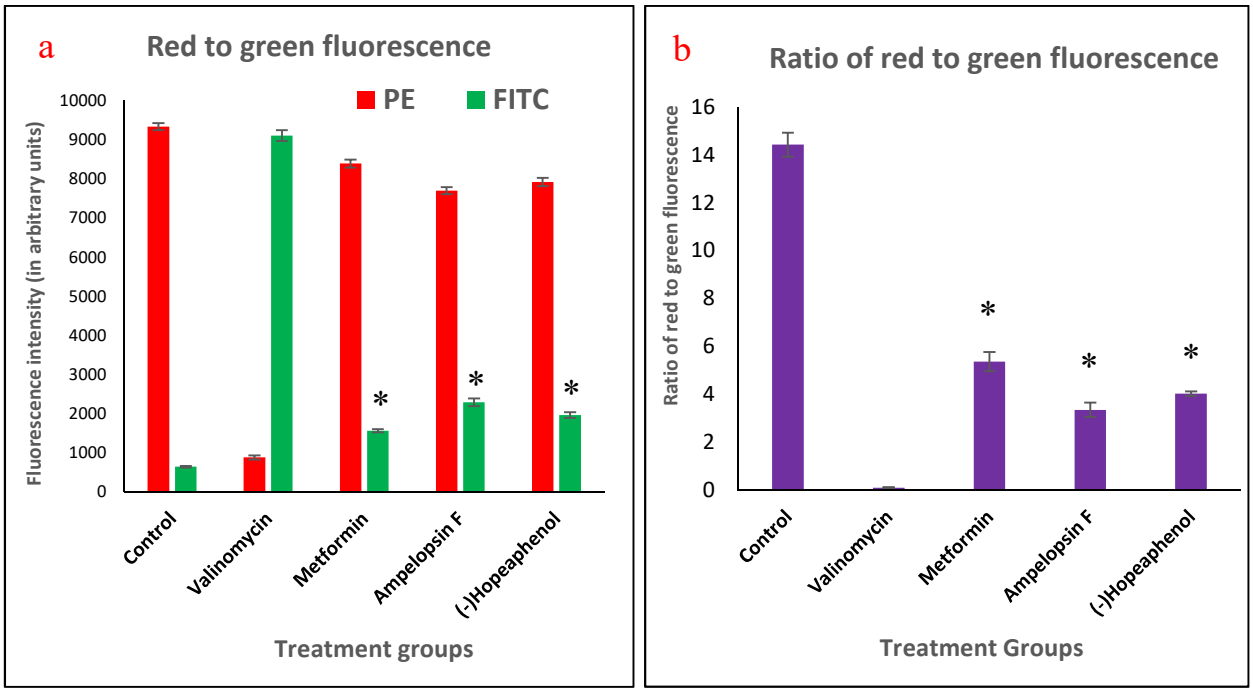


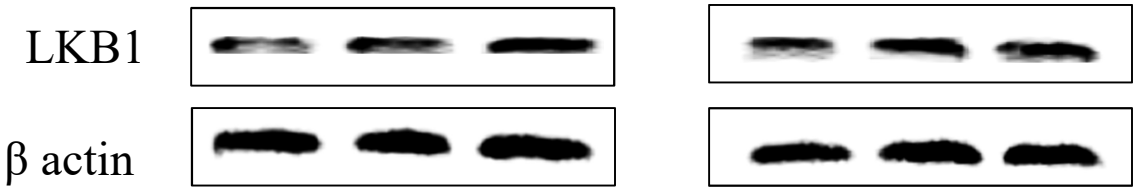
Fig 4.11. a) Determination of red to green fluorescence intensity in L6 myotubes. A slight decrease in red fluorescence complementing a slight increment in green fluorescence correlated the transient membrane depolarization in Metformin (100 µM) treated, Ampelopsin F (50 µM) treated and (-) Hopeaphenol (50 µM) treated cells. The highly depolarized valinomycin (1 µg/mL) treated cells were used as the positive control. **b) Determination of ratio of red to green fluorescence.** Ratio of red to green fluorescence was analyzed from the above mentioned graph The results were expressed in terms of mean ± SD of three determinations. The significance was accepted at $p \leq 0.05$. $*p \leq 0.05$ versus control

Change in mitochondrial membrane potential is an essential parameter for the production of ATP and a marker of mitochondrial function. The mild depolarized mitochondrial membrane has been reported to indicate the activation of AMPK via an increment in cellular AMP: ATP ratio resulting in enhanced glucose uptake in L6 myotubes (Qiu et al., 2010). Binding of AMP or ADP to the γ subunit of AMP kinase results in the phosphorylation of AMPK on Thr-172 (Willows et al., 2017). AMP can mediate the allosteric modification of AMPK by phosphorylating the α-subunit (Thr-172) (Sanders et al., 2007). Troglitazone, a thiazolidinedione class of anti-diabetic drug has been reported to induce a slight mitochondrial membrane depolarization but was not linked with insulin stimulation in L6 myotubes (Konrad et al., 2005). Quercetin, the

citrus flavonoid was reported to induce a transient mitochondrial depolarization in L6 myotubes (Dhanya et al., 2017). Similarly, our studies demonstrated a transient depolarization of the mitochondrial membrane upon pretreatment with resveratrol oligomers, Ampelopsin F and (-) Hopeaphenol in L6 myotubes. This change in mitochondrial membrane potential would lead to the activation of AMPK by resveratrol oligomers via increment in AMP/ATP ratio. In addition, these resveratrol based compounds showed anti-diabetic activity similar to the widely used anti-diabetic drug metformin.

4.4.4. LKB1 Expression analysis

To determine additional evidence regarding factors upstream of AMPK, we studied the involvement of Liver kinase B1 (LKB1). Herein, we examined the protein expression of LKB1 in L6 myotubes followed by pretreatment with resveratrol oligomers. The western blot analysis demonstrated an increment in the expression of LKB1 in metformin treated cells (1.4 fold) as compared to control group. Ampelopsin F treated cells and (-) Hopeaphenol treated cells resulted in a 1.5 and 1.3 fold increase in expression of LKB1 (Fig 4.12), respectively, compared to control cells. Compared to positive control, metformin, both the compounds demonstrated a slight increase in expression of LKB1.



Lane 1-Control, 2- Metformin, 3- Ampelopsin F

Lane 1-Control, 2- Metformin, 3-(-) Hopeaphenol

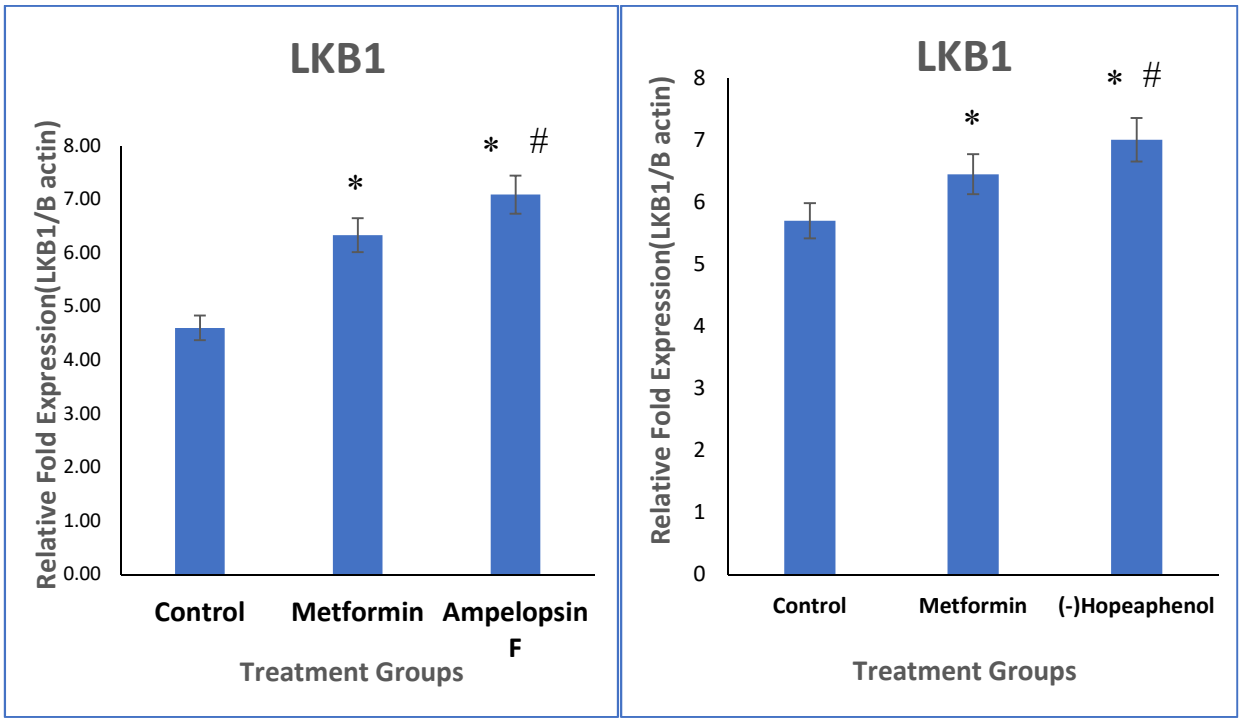


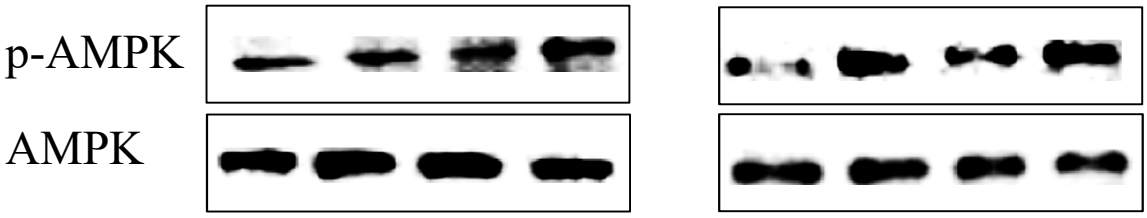
Fig 4.12. LKB1 protein expression in L6 cell lines on pretreatment with a) Ampelopsin F and b) (-) Hopeaphenol: Western blot analysis for LKB1 expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours. Cell lysates were examined for protein expression of LKB1 by western blot analysis with β-actin as loading control. All data were represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus metformin.

The kinase activity of LKB1 is not regulated by AMP according to Lizcano et al., (2004) and Sakamoto et al., (2004). Conversely some studies had demonstrated the stimulation of LKB1-dependent phosphorylation of Thr-172 upon the binding of AMP to AMPK. This results in the allosteric activation of AMPK (Sanders et al., 2007) and hence increased AMPK phosphorylation (Yamada et al. , 2010). Few studies have reported the activation of LKB1/AMPK signaling via the activation of SIRT1 (Zheng et al., 2012). Resveratrol is a potent compound known to activate SIRT1/LKB1/AMPK signaling pathway (Oyenihi et al., 2016). Quercetin was reported to enhance the expression of LKB1 in broilers (Wang et al., 2021). Metformin treated cells showed a significant increase in the expression of LKB1 in L6 myotubes. Both the resveratrol oligomers Ampelopsin F and (-) Hopeaphenol induced a significant upregulation of the protein LKB1 in L6 myotubes compared to control cells and

metformin treated cells. In agreement with the above cited reports suggesting the activation of AMPK associated with the increased expression of LKB1, our results too indicated the role of LKB1 mediated activation of AMPK that was adopted by the resveratrol oligomers Ampelopsin F and (-) Hopeaphenol for stimulating glucose uptake.

4.4.5. AMPK expression analysis

The protein expression studies were conducted to determine the phosphorylation of AMPK in L6 myotubes followed by compound pretreatment. In this study, AMPK was found to be upregulated in metformin treated cells. There was a significant upregulation of AMPK expression in L6 myotubes pretreated with the compounds. Around 1.6 times increase in expression was found in Ampelopsin F treated cells when compared to control cells (Fig 4.13). Compared to metformin treated cells, Ampelopsin F pretreatment showed a feeble increase in expression of phosphorylated AMPK. (-) Hopeaphenol treated cells showed about 6 times increase in the expression of AMPK than control cells. The expression profile of AMPK in (-) Hopeaphenol treated cells was at par with the expression of the positive control, metformin pretreated cells.



Lane 1-Control, 2- Insulin Control, 3-Metformin, 4- Ampelopsin F

Lane 1-Control, 2- Metformin, 3- Insulin Control, 4- (-) Hopeaphenol

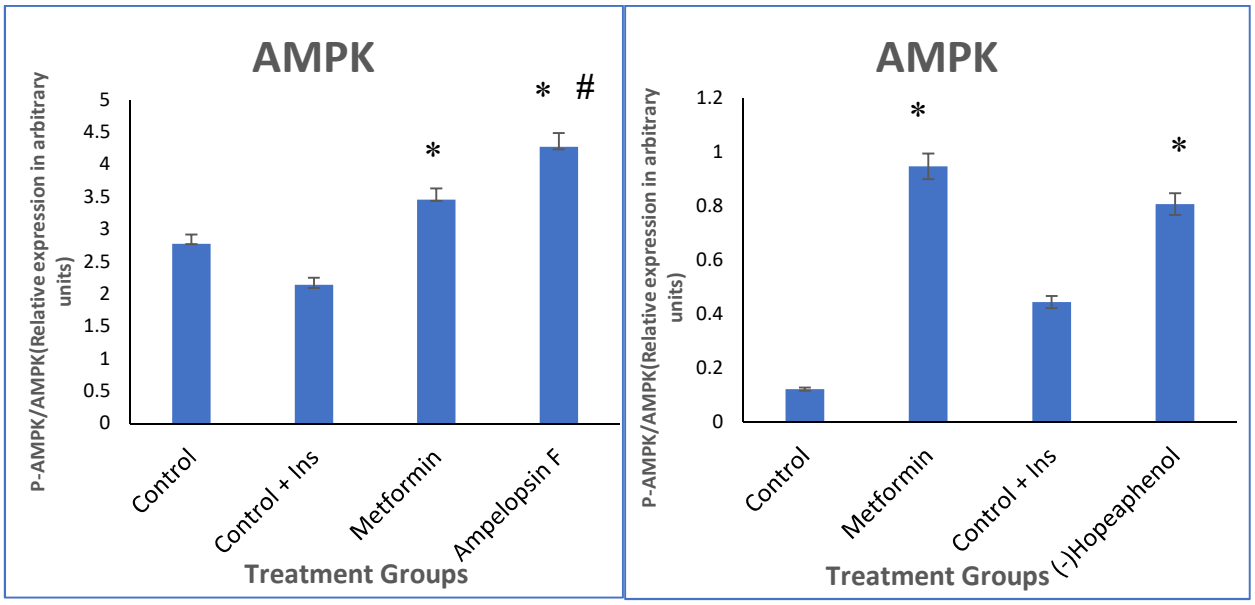


Fig 4.13. AMPK protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol: Western blot analysis for AMPK expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for a period of 24 hours in the presence of insulin (100 nm). Cell lysates were studied for the protein modulation of phosphorylated AMPK by western blot analysis with total AMPK as loading control. All data were represented as means ± SD (n=3), The significance was established at $p \leq 0.05$. *$p \leq 0.05$ versus control, #$p \leq 0.05$ versus metformin

The positive control, metformin which is the widely used anti-diabetic drug has been reported to trigger the uptake of glucose in skeletal muscles via activation of AMPK pathway (Rena et al., 2017). This drug was known to alter AMP/ATP ratio resulting in triggering the AMPK enzyme activation (Hawley et al., 2002). Most of the polyphenols screened for the anti-diabetic activity so far were found to activate AMPK pathway either alone or in combination with activating insulin signaling pathway. Quercetin (Dhanya et al.,2017), catechins (Thielecke and Boschmann; 2009), hydroxy cinnamic acids (Jung et al., 2007), procyanidins (Lu et al., 2011), tannins (Liu et al.,2005) etc were found to activate both insulin signaling pathway and AMPK pathway. The flavonoid, Kaempferide was reported to activate AMPK pathway in 3T3L1 adipocytes (Yong et al., 2015). Resveratrol was reported to activate AMPK in SIRT1 dependent manner (Oyenihi et al., 2016).

In the previous chapters, we had established the role of resveratrol oligomers (Ampelopsin F and (-) Hopeaphenol) in enhancing glucose uptake in skeletal cells (Section 2.6.2 and 2.6.3), which was mediated by the increased GLUT4 expression and translocation through western blot (Section 3.4.2.1.4) and immunofluorescence assays (Section 2.6.4), respectively. Through inhibitor studies (Section 4.4.1), we were successful in establishing the role of these resveratrol oligomers, in the activation of AMPK. The key requirements needed for the activation of AMPK includes the CaMKK mediated calcium influx and/or increase in AMP levels via alterations in mitochondrial membrane. We were able to demonstrate the non-participation of CaMKKβ signaling by Ampelopsin F for activating AMPK. Conversely, (-) Hopeaphenol triggered AMPK activation via the stimulation of CaMKKβ. Subsequently, using these compounds, the role of another key factor, i.e mitochondrial membrane potential, was investigated in the activation of AMPK. This revealed the involvement of both the resveratrol based compounds in altering the mitochondrial membrane potential. In addition, western blot studies were conducted to understand the expression profile of phosphorylated AMPK (active). The results revealed the increment in the expression of phosphorylated AMPK in both the resveratrol oligomers pretreated cells which was correlated to the parameters studied for stimulating AMPK activation. This enzyme activation could be leading to the modulation of GLUT4 expression and its translocation to the membrane in L6 myotubes ultimately resulting in the increased glucose uptake (as discussed in previous chapters).

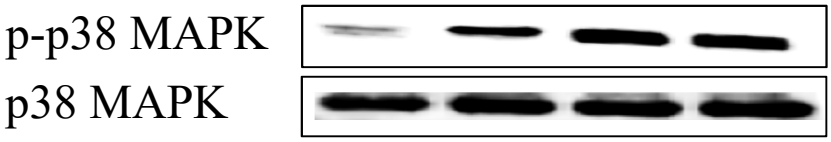
Therefore, through this chapter we were able to establish that the resveratrol dimer Ampelopsin F triggered the AMPK pathway via inducing alterations in the mitochondrial membrane and activation of LKB1. The resveratrol tetramer (-) Hopeaphenol mediated the activation of AMPK via AMP dependent stimulation of AMPK, Ca^{2+}/ CaMKKβ/ AMPK-dependent pathway and via the activation of LKB1.

4.4.6. Downstream events followed by post AMPK activation

4.4.6.1. p38 MAPK protein expression after AMPK activation

One of the downstream target proteins following the AMPK phosphorylation is p38 MAPK. In order to determine the role of resveratrol oligomers in AMPK/ p38 MAPK pathway, western

blot analysis was carried out to analyze the expression profile of p38 MAPK in L6 myotubes. Metformin showed 1.7 times increase in the expression of p38 MAPK in L6 myotubes. Ampelopsin F treated cells showed around 1.6 times increased expression of phosphorylated p38 MAPK in L6 myotubes than control cells and was at par with the positive control, metformin. (-) Hopeaphenol treated cells showed a two-fold increase in expression of phosphorylated p38 MAPK in L6 myotubes than control group and the expression of p38 MAPK was found to be significant than metformin treated cells (Fig 4.14).



Lane 1- Control, 2- Metformin, 3- Ampelopsin F, 4- (-) Hopeaphenol

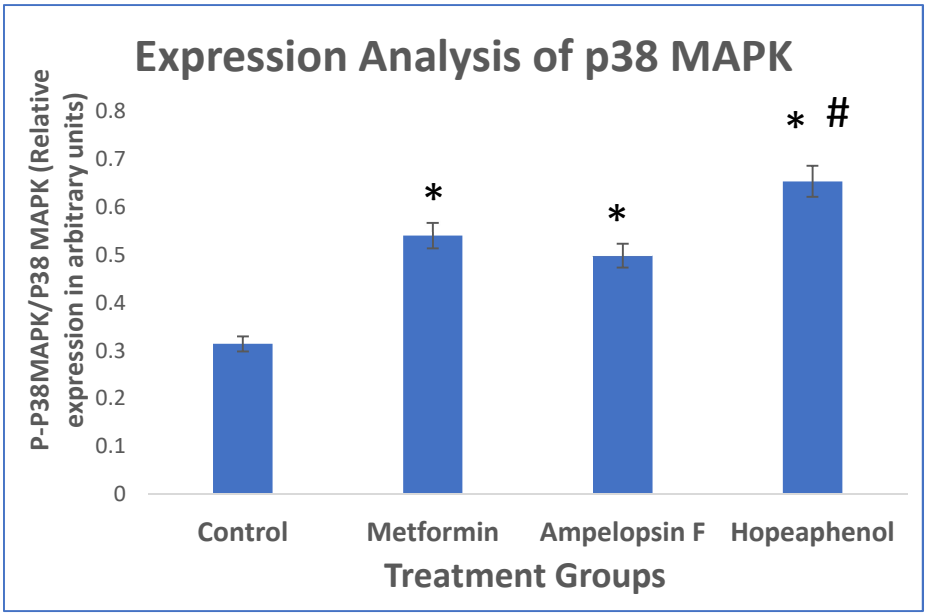


Fig 4.14. p38 MAPK protein expression in L6 cell lines on pretreatment with Ampelopsin F and (-) Hopeaphenol: Western blot analysis for p38 MAPK expression: L6 myotubes were treated with metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours. Cell lysates were examined for protein expression of phosphorylated p38 MAPK by western blot analysis with total p38 MAPK as loading control. All data are represented as means ± SD (n=3). The significance was established at $p \leq 0.05$. *$p \leq 0.05$ versus control. #$p \leq 0.05$ versus metformin.

p38 MAPK is the downstream kinase of AMPK and the inhibitory studies using p38 inhibitor had reported an impaired glucose uptake in skeletal muscles (Somwar et al., 2000). AICAR, one of the prominent AMPK activator, could activate p38 MAPK via its phosphorylation (Li et al., 2005). Curcumin was reported to induce glucose uptake via AMPK- p38 MAPK pathway and the inhibition of either of these proteins resulted in the decline of glucose uptake (Kim et al., 2010). The citrus flavonoid, quercetin was reported to initiate its anti-diabetic effect primarily through AMPK-p38 MAPK pathway in L6 myotubes (Dhanya et al., 2017). As discussed in the previous section (Section 4.4.5), we had discussed the role of resveratrol oligomers in stimulating/ upregulating upstream kinases of AMPK and as a result the activation of AMPK itself. p38 MAPK is the direct molecular target protein of AMPK and hence the upregulation of the active form of this protein reconfirmed the activation of AMPK induced by resveratrol based compounds. The increased expression of p38 MAPK pretreated with resveratrol based compounds falls in line with the AMPK- p38 MAPK signaling cascade involved in the uptake of glucose in L6 myotubes.

4.4.6.2. Post mitochondrial alterations after AMPK activation

Effect of resveratrol oligomers in mitochondrial content

To understand whether the activation of AMPK resulted in regulating energy homeostasis by triggering mitobiogenesis, mitochondrial content was measured using mitotracker red. The cells followed by metformin and resveratrol oligomers pretreatment were subjected to mitostaining. The mitochondrial content was quantified using the red fluorescence. Metformin treated cells exhibited an increment in red fluorescence than control cells as shown in Fig 4.15. Similarly, Ampelopsin F and (-) Hopeaphenol was observed with increased red fluorescence when compared to the control cells.

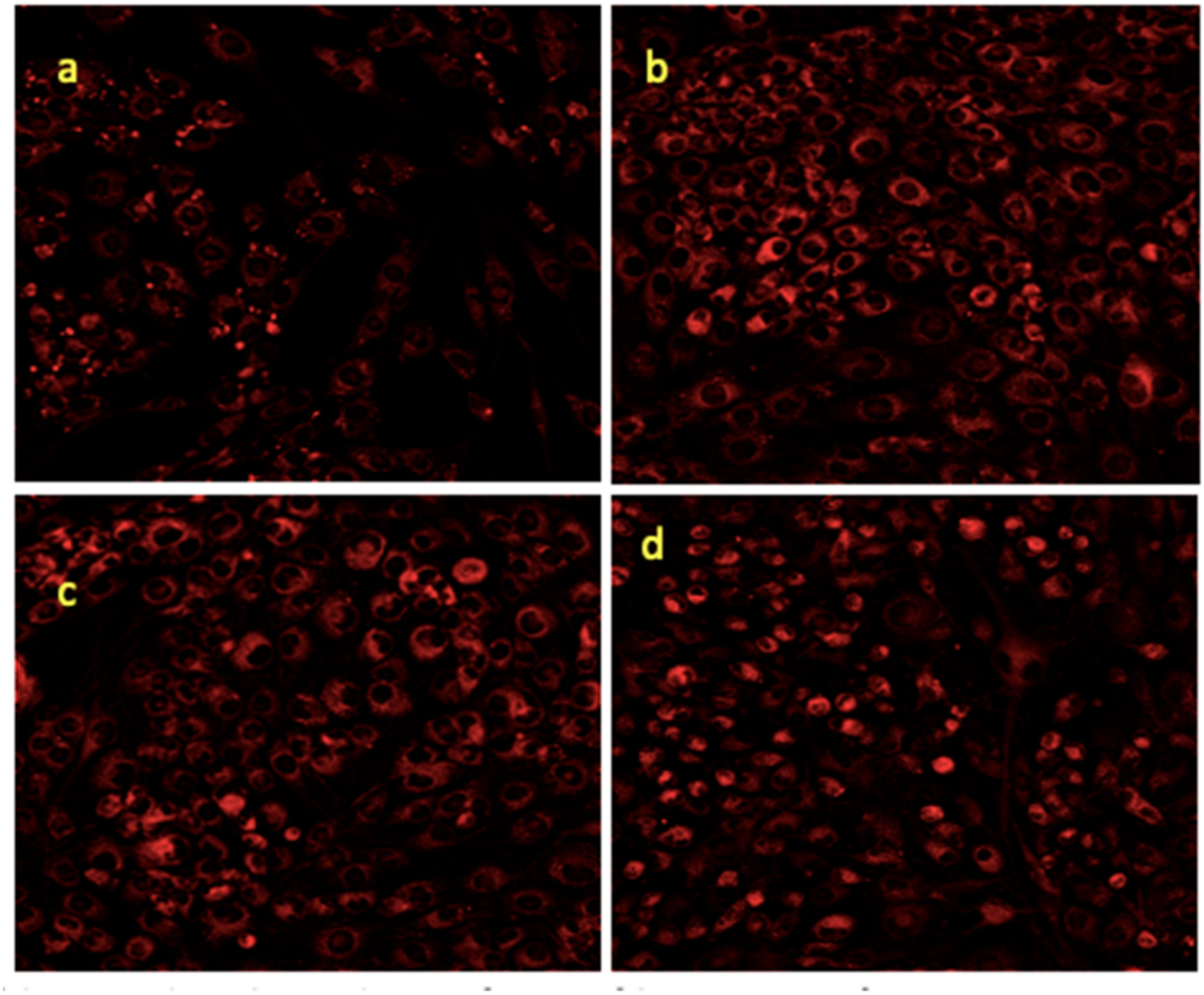


Fig 4.15. Determination of mitobiogenesis using Mitotracker Red in L6 skeletal myocytes. Fluorescent images of live mitochondria in L6 cell lines were resolved using mitotracker red. a) Control cells; b) Metformin (100 µM); c) Ampelopsin F (50 µM) and d) (-) Hopeaphenol (50 µM). Magnification 20X. Scale bar corresponds to 100 µM.

The increased mitobiogenesis obtained from the fluorescence microscopy was quantified and plotted (Fig 4.16) to statistically represent the rate of mitobiogenesis in control cells, metformin treated cells and resveratrol oligomers treated cells. A significant increase in the mitochondrial content was observed in metformin and resveratrol oligomers treated cells. As compared to control cells, 1.3 times, 1.4 times and 1.2 times increase in mitochondrial content was shown in metformin treated cells, Ampelopsin F treated cells and (-) Hopeaphenol treated cells, respectively.

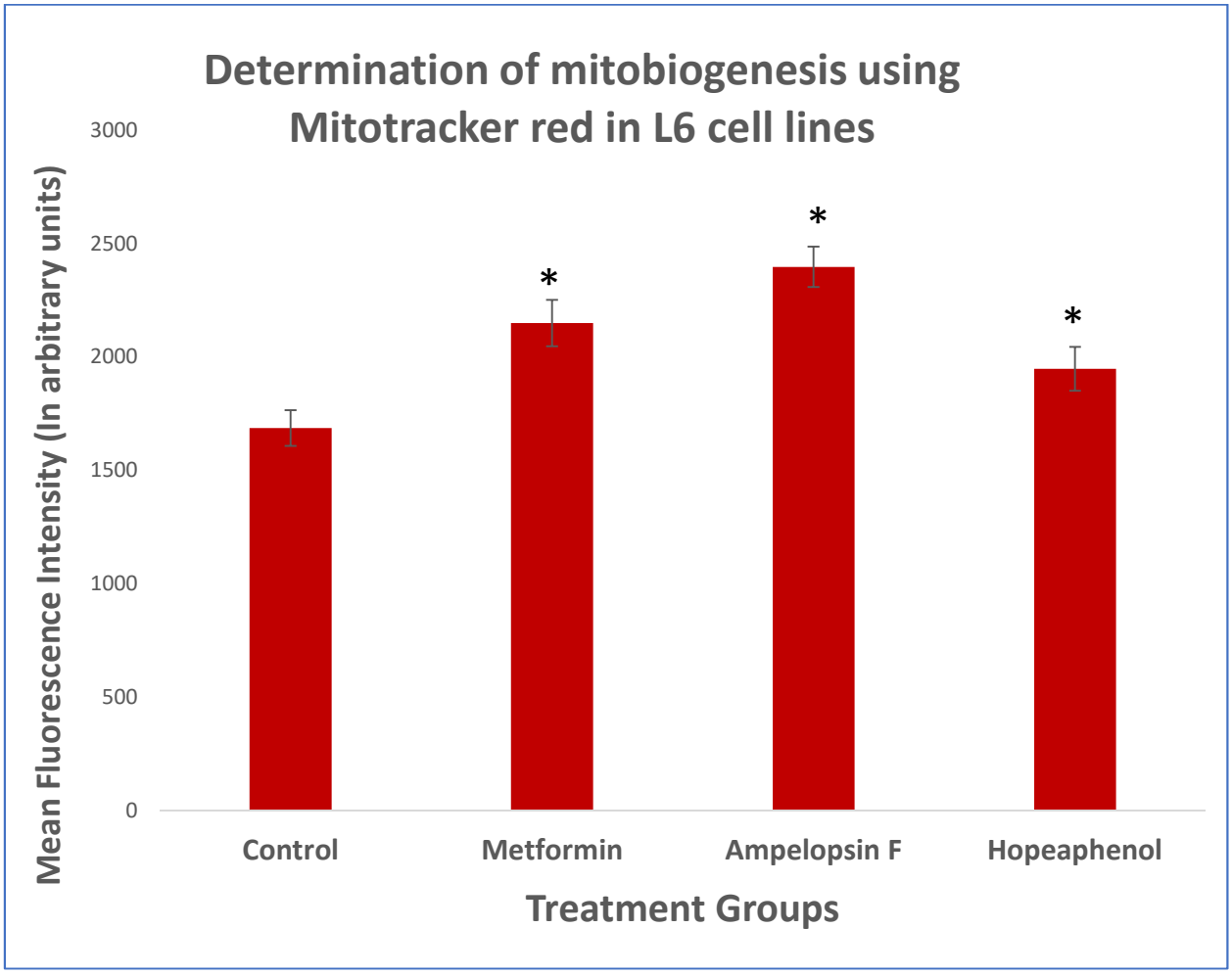


Fig 4.16. Fluorescence intensity analysis of mitobiogenesis in L6 myotubes. A significant increase in the rate of mitobiogenesis was observed in metformin treated (100 µM), Ampelopsin F treated (50 µM) and (-) Hopeaphenol (50 µM) treated cells when compared to control cells. The results expressed in terms of mean ± SD of three determinations. The significance was established at $p \leq 0.05$. $*p \leq 0.05$ versus control.

Measurement of nitrite in L6 cell lines

To determine the factors associated with mitobiogenesis, nitrite content in cells were measured using Griess reagent. Supernatants collected after incubation with resveratrol oligomers and the positive control metformin significantly increased the nitrite levels when compared to control cells. The positive control, metformin treated cells showed 1.2 times increase in the concentration of nitrite than control cells. Ampelopsin F (50 µM) and (-)

Hopeaphenol (50 µM) treated cells showed 1.3 times and 1.4 times increase in nitrite levels, respectively, than control cells (Fig 4.17).

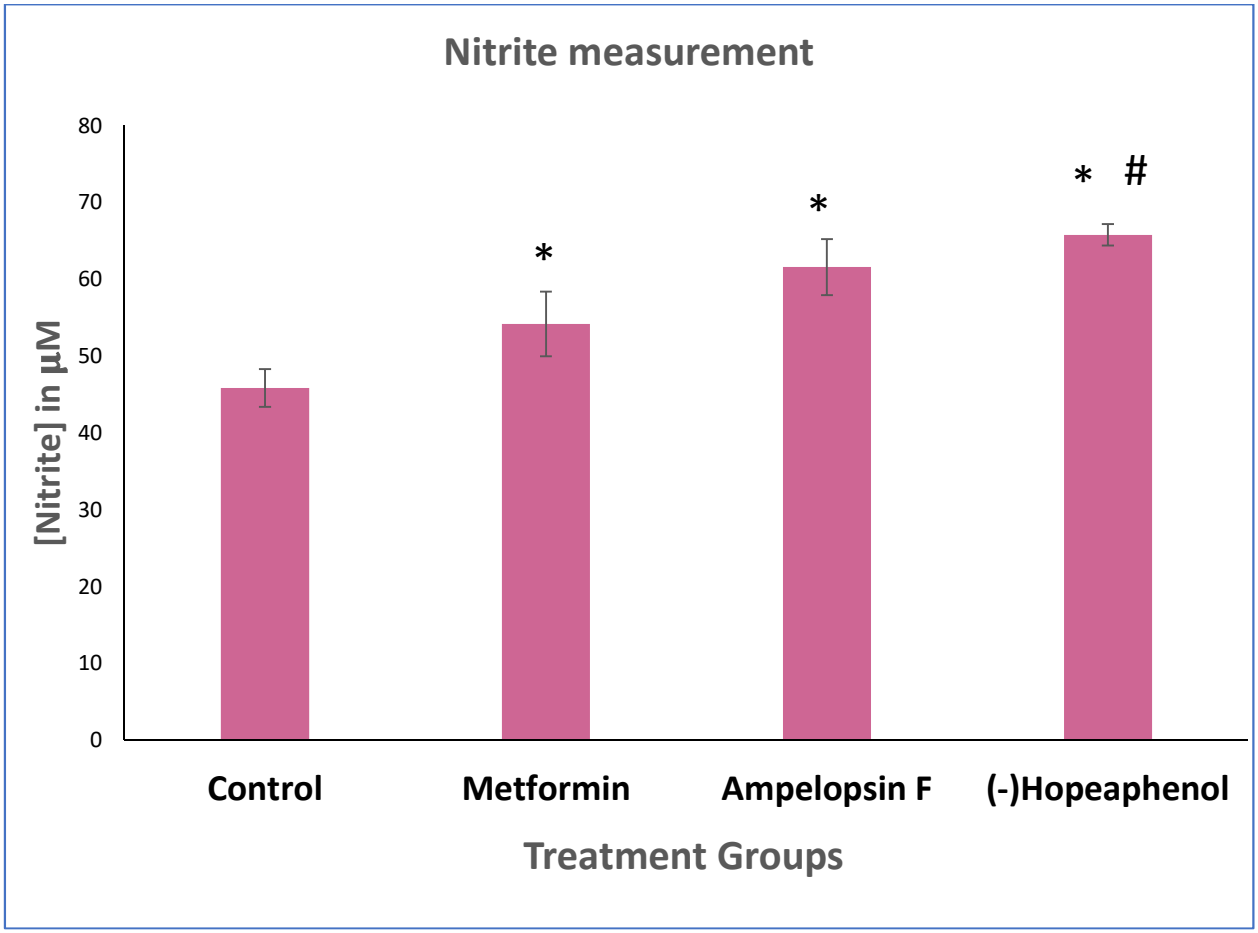
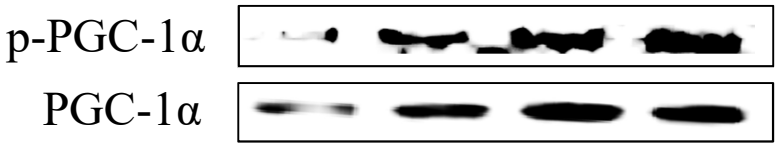


Fig 4.17. Determination of nitrite using Griess Reagent in L6 myotubes. A significant increase in nitrite concentration indicating the role of Nitric oxide in mitobiogenesis in Metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) treated cells as a result of AMPK activation. The results expressed in terms of mean ± SD of three determinations. The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ versus control, $\#p \leq 0.05$ versus metformin.

Determination of mitobiogenesis by increased protein expression of PGC-1α

To determine whether the mitochondrial biogenesis in L6 myotubes was mediated by the upregulation of PGC-1α, the protein expression studies were conducted to evaluate the effect of resveratrol oligomers in the expression of PGC-1α. The mitochondrial protein fraction isolated

after pretreatment with resveratrol oligomers, was subjected to western blot analysis. The positive control metformin treated cells showed 1.7 fold increase in expression of PGC-1α when compared to control cells. There was a significant increase in the expression of PGC-1α in Ampelopsin F treated as well as (-) Hopeaphenol treated cells. The expression of PGC-1α in Ampelopsin F treated cells and (-) Hopeaphenol treated cells showed 1.9 fold and 1.4 fold increment, respectively, than control cells. (Fig 4.18).



Lane 1- Control, 2- Metformin, 3- Ampelopsin F, 4- (-) Hopeaphenol

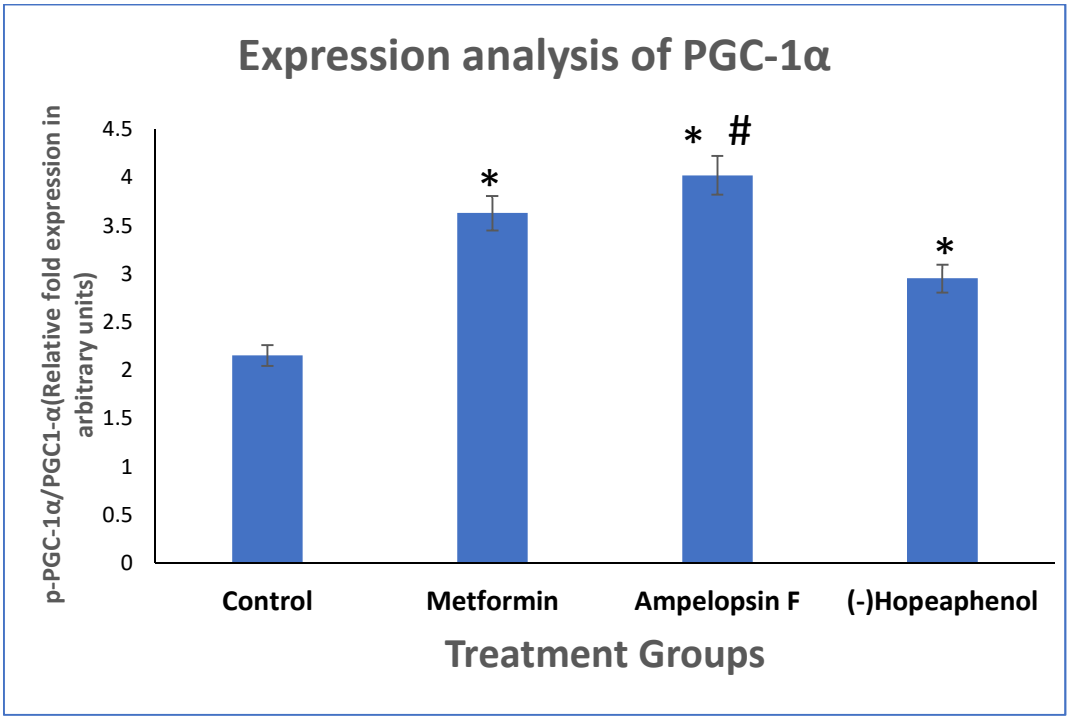


Fig 4.18. PGC-1α protein expression in L6 myotubes: L6 myotubes were treated with Metformin (100 µM), Ampelopsin F (50 µM) and (-) Hopeaphenol (50 µM) for 24 hours. Mitochondrial protein lysates were examined for protein expression of p-PGC-1α by western blot analysis with total PGC-1α as loading control. All data are represented as means ± SD (n=3), The significance was accepted at $p \leq 0.05$. $^{*}p \leq 0.05$ against control, $\#p \leq 0.05$ against metformin.

Nitric oxide was reported to have a positive effect on mitochondrial biogenesis (Knott and Wetzel, 2010). The expression of iNOS depends on inflammation and nNOS as well as eNOS were reported to be constitutively expressed in skeletal muscles (Tengan et al.,2012). Nisoli (2003) reported that eNOS produce nitric oxide which results in the activation of guanylate cyclase. As an outcome there is an increment in cyclic GMP (cGMP). This constitutively regulate the gene expression of PGC-1α and subsequently the increased rate of mitobiogenesis.
A substantial rise in the mitochondrial content was reported upon incubation with NO donor *S*-nitroso-*N*-acetyl penicillamine (SNAP) in skeletal muscle primary culture (Tengan et al., 2007). Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α) was found to be associated in NO dependent mitochondrial biogenesis (Lira et al., 2010). PGC-1α was unveiled as the primary central regulator in the initiation of mitobiogenesis. This co-transcriptional regulation factor was stimulating mitobiogenesis via the activation of nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2) and mitochondrial transcription factor A (mtTFA). An in vivo study reported an association between energy deprivation and mitochondrial biogenesis using a pharmacological AMPK activator β-guanidinopropionic acid (β-GPA), resulting in the AMPK activation and subsequently enhancing mitochondrial biogenesis via PGC-1α and the NRFs (Bergeron et al.,2001). Citrus flavonoids like naringenin and quercetin were found to modulate GLUT4 translocation via the phosphorylation of AMPK and also increase the expression of SIRT1 and PGC-1α which were found to be down-regulated in insulin resistant cells (Krishnamoorthy and Venkatraman, 2017). Resveratrol has also been known to improve the function of mitochondria via the activation of SIRT1 and PGC-1α under metabolic disease state (Lagouge et al., 2006).

Following AMPK activation, the necessity for energy expenditure results in increased mitochondrial biogenesis. This was accomplished by a transient increase in nitric oxide levels in L6 myotubes. This nitric oxide (NO) regulates the expression of PGC-1α and hence resulted in increased mitochondrial content. From the results of our study, metformin was found to enhance the mitobiogenesis in L6 myotubes as a result of AMPK activation. Similarly, both the resveratrol oligomers Ampelopsin F and (-) Hopeaphenol induced an increment in mitochondrial content which signifies the increased mitobiogenesis in L6 myotubes. This was confirmed with a

transient increase in the nitrite content in L6 cell lines upon compound pretreatment. In addition, the increased expression of PGC-1α after compound pretreatment in L6 myotubes was relatable to the above data which implied the increased mitobiogenesis. The pretreatment with resveratrol based compounds in L6 myotubes revealed the effect of compounds in triggering the mitochondrial biogenesis owing to post AMPK activation via the activation of AMPK- PGC-1α pathway.

To conclude, both the resveratrol oligomers, Ampelopsin F and (-) Hopeaphenol demonstrated anti-diabetic potential in L6 myotubes. The resveratrol dimer, Ampelopsin F, demonstrated its anti-diabetic potential by enhancing glucose uptake via stimulating PI3-K/AKT dependent pathway and the AMPK pathway. AMPK activation was found to be stimulated by modulating mitochondrial membrane potential and increasing the expression of LKB1. The resveratrol tetramer, (-) Hopeaphenol, imparted its anti-diabetic effect via stimulating the AMPK pathway alone. The activation of AMPK in L6 myotubes was triggered via Ca^{2+}/ CaMKKβ dependent AMPK signaling pathway, inducing a mild change in mitochondrial membrane potential and modulation in the expression of LKB1.

4.5. Summary

The results obtained from this chapter indicated the activation of AMPK by the resveratrol oligomers. Ampelopsin F stimulated AMPK signaling pathway via alterations in mitochondria and upregulation of LKB1. (-) Hopeaphenol, the resveratrol tetramer stimulated AMPK pathway via the activation of upstream kinases LKB1, CaMKKβ and alterations in mitochondria.

The key findings were summarized below:

- Inhibitor studies performed in L6 myotubes resulted in determining the role of Ampelopsin F in stimulating PI3-K/ AKT pathway and AMPK pathway, and the involvement of (-) Hopeaphenol in stimulating AMPK pathway alone.
- The transient depolarization in mitochondrial membrane was observed illustrating the role of resveratrol oligomers in the activation of AMPK via alterations in mitochondrial membrane.

- The efflux of Ca^{2+} was observed in (-) Hopeaphenol treated cells but not in Ampelopsin F treated cells.
- The expression analysis of CaMKKβ performed in L6 myotubes confirmed the role of (-) Hopeaphenol alone in stimulating calcium dependent AMPK signaling
- Inhibitor studies using STO-609, established the role of (-) Hopeaphenol in CaMKKβ dependent AMPK activation.
- The modulation of LKB1 in resveratrol oligomers treated cells resulted in determining its role in LKB1 mediated AMPK stimulation.
- The increased expression of AMP Kinase and p38 MAP kinase was observed which further confirmed activation of AMPK-p38 MAPK pathway for inducing glucose uptake.
- Mitobiogenesis, the downstream event of AMPK activation was increased upon pretreatment with resveratrol oligomers in L6 myotubes.

CHAPTER 5

Summary and Conclusion

Summary and Conclusion

This thesis primarily focused on investigating the anti-diabetic potential of resveratrol-based compounds isolated from different plants belonging to the Dipterocarpaceae family and explores the possible mechanism of action on various targets of diabetes. About eight resveratrol-based compounds isolated from different plants belonging to the Dipterocarpaceae family were screened for their anti-diabetic potential in L6 myoblasts and Beta-TC-6 cell lines. Initially, the cells were screened for their cytotoxicity for a period of 24 hours in both the cell lines by means of MTT assay. Based on the initial results, we investigated the molecular mechanism of action of two resveratrol oligomers, Ampelopsin F, a resveratrol dimer, and (-) Hopeaphenol, a resveratrol tetramer in Beta-TC-6 cell lines and L6 skeletal muscle cell lines in insulin secretion potential and insulin signaling potential, respectively. In addition, exploring the role of compounds in inhibiting DPP4, an enzyme involved in the inhibition of incretins, and PTP1b, a phosphatase enzyme involved in the negative regulation of insulin signaling pathway.

5.1. Screening of the resveratrol based compounds for their anti-diabetic potential

The anti-diabetic potential of compounds in insulin signaling was analyzed via 2-NBDG uptake pretreated with resveratrol-based compounds along with metformin (100 μM) as the positive control for a period of 24 hours in L6 myotubes. Most of the compounds showed increased glucose uptake which was at par with the positive control, metformin, which was further confirmed with flow cytometry analysis. The most active compounds in enhancing glucose uptake were found to be Ampelopsin F, (-) Hopeaphenol, α-Viniferin, NIIST C1, and THPG. These compounds were subjected to immunofluorescence assay to determine the modulation in GLUT4, the glucose transporter, and showed an increase in the expression of GLUT4.

The resveratrol oligomers were screened for the exogenous insulin secretion potential by means of ELISA using Beta-TC-6 cells. A four times increase in insulin secretion was observed in the positive control, Glyburide (100 nM) pretreated cells as compared to the untreated cells. (-) Hopeaphenol and Ampelopsin F treated cells showed a 3.5 times increase in insulin secretion than control and no significant increase in insulin secretion was observed in α-Viniferin and THPG treated cells. The most active compounds in insulin secretion were found to be Ampelopsin F and (-) Hopeaphenol.

5.2. Anti-diabetic potential of Ampelopsin F and (-) Hopeaphenol in insulin secretory pathway

Based on the above validations, the exploration of molecular mechanism for the resveratrol oligomers was reduced to 2 compounds, namely, Ampelopsin F and (-) Hopeaphenol, owing to their anti-diabetic potential in the initial studies. The exploration of the role of resveratrol oligomers in the insulin secretory pathway was carried out via the determination of hyperpolarization of mitochondrial membrane, the intracellular Ca2+ efflux, and the expression analysis of GLUT2 and glucokinase. The mitochondrial membrane potential in different pretreated groups of cells was determined (resveratrol oligomers (50 μM), positive control glyburide (100 nM), and valinomycin (1 μg/mL). The JC-1 aggregates in Glyburide, Ampelopsin F and (-) Hopeaphenol treated cells indicated the mitochondrial hyperpolarization which could lead to closure of ATP dependent potassium channels as mitochondrial hyperpolarization has been linked to the increased ATP levels. Intracellular Ca^{2+} efflux (that result in the exocytosis of insulin) in the Beta- TC-6 cell lines was determined in different group of cells. An increase in Ca^{2+} concentration was observed upon pretreatment with resveratrol oligomers complementing with the increment in insulin secretion in beta cell lines. Protein expression studies were carried out in cell lysates of different groups of cells. The protein expression of glucokinase and GLUT2 was examined by western blot analysis with β -actin as the loading control. The increased expression of GLUT2, the glucose transporter, and the enzyme glucokinase observed via western blot analysis suggested the contribution of the resveratrol oligomers in insulin secretion analogous to the glucose-stimulated insulin secretion (GSIS) (Fig 5.1).

The inhibition of DPP4 facilitated the better availability of the incretins and hence increasing the possibility to extend the release of insulin. Hence the inhibition of this particular protein has been extensively used as a drug target for the treatment of diabetes mellitus. Both the compounds, Ampelopsin F and (-) Hopeaphenol showed their anti-diabetic potential by demonstrating the inhibition of DPP4, an enzyme responsible for the degradation of incretins, thus showing the potency for prolonged insulin secretion.

The proposed molecular mechanism of the anti-diabetic potential of Ampelopsin F and (-)Hopeaphenol in Beta-TC-6 cell lines

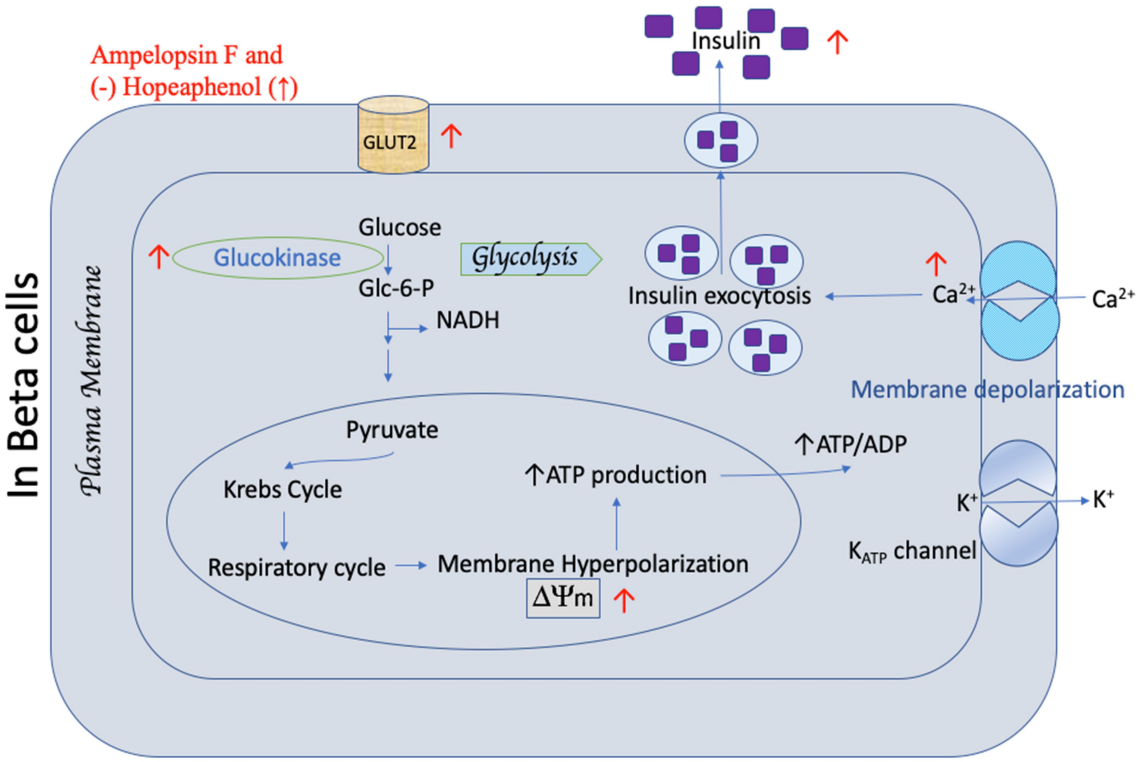


Fig 5.1. The proposed molecular mechanism of action of Ampelopsin F and (-) Hopeaphenol in Beta-TC-6 cell lines. An upregulation in the expression of GLUT2 and Glucokinase triggers glycolysis and subsequently the KREB's cycle leading to the hyperpolarization of the mitochondrial membrane. This enhances the production of ATP resulting in plasma membrane depolarization. The entry of Ca^{2+} into the cells to maintain the beta cell membrane potential ultimately leads to the exocytosis of insulin.

5.3. Anti-diabetic potential of Ampelopsin F and (-) Hopeaphenol in PI3-K/AKT dependent insulin signaling pathway

Under physiological conditions, the altered concentration of glucose stimulates the binding of insulin to the insulin receptor and hence the insulin signaling transduction pathway is activated. Following incubation with compounds in L6 cell lines, cell lysates were collected and examined for protein expression of key proteins involved in insulin signaling by western blot analysis. Expression analysis of IRS-1, PI3K, Akt revealed the role of resveratrol dimer Ampelopsin F in insulin-dependent signaling. The decreased protein

expression of PI3-K in (-) Hopeaphenol treated cells implied that its mechanism of action is insulin-independent. There was an increased protein expression of GLUT4, which is the most downstream event in the insulin signaling pathway in both the compound treated cells suggesting the upregulation and translocation of GLUT4 transporter, which ultimately leads to increased glucose uptake.

The Protein Tyrosine Phosphatase (PTP 1b) is the key enzyme involved in the desensitization of insulin receptors and hence affords a major role in the negative regulation of insulin signaling. The inhibition of this enzyme offers an assuring molecular-level therapeutic strategy in the management of type 2 diabetes mellitus. Both the compounds, Ampelopsin F and (-) Hopeaphenol induced the inhibition of PTP1b enzyme that was comparable to the positive control, orthovanadate.

5.4. Anti-diabetic potential of Ampelopsin F in AMPK signaling pathway

The role of compounds in insulin-dependent pathways and insulin-independent pathways was confirmed by deploying inhibitor studies (in the presence and absence of inhibitors Wortmannin and Dorsomorphin). This study revealed the action of Ampelopsin F, which activated both insulin signaling and AMPK signaling pathway. The activation of AMPK has been related to the increment in AMP/ATP ratio or via activation of LKB1 or via activation of CaMKKβ leading to the efflux of Ca^{2+}. The cells after the pretreatment with Ampelopsin F was analyzed for determining the mitochondrial membrane potential via fluorescence microscopy. A slight depolarization of the mitochondrial membrane was observed which indicated a rise in AMP/ATP ratio. This was confirmed with flow cytometry analysis.

CaMKKβ is a major protein involved in the activation of AMPK in response to a rise in intracellular calcium concentration. The increment in Ca^{2+} could act as a marker for the increased expression of CaMKK. No significant Ca^{2+} release was observed with Ampelopsin F treated cells upon staining with Fura-2, AM and hence the activation of the AMPK pathway by Ampelopsin F was via the change in AMP to ATP ratio alone. To confirm this, CaMKKβ protein expression studies in L6 myotubes were performed. A feeble expression of CaMKKβ was observed with Ampelopsin F treated cells. To confirm the same, STO-609, a selective inhibitor of CaMKK was used to perform the inhibitory analysis. No significant decrease in uptake was observed with Ampelopsin F treated cells co-treated with the inhibitor indicating that the activation of AMPK is Ca^{2+} independent. The role of LKB1 in the

activation of AMPK was determined by western blot analysis and an increase in expression of LKB1 was observed with Ampelopsin F treated cells. Expression analysis of AMPK confirmed the role of Ampelopsin F in insulin-independent signaling. With regards to an acute energy crisis, AMPK plays a precarious role in the regulation of intracellular energy metabolism. The activation of AMPK results in various physiological processes and therefore resulted in inducing mitochondrial biogenesis to escape from energy depletion. The expression analysis of p38- MAPK and PGC-1α entails the downstream activation of the AMPK pathway induced by Ampelopsin F and its role in mitobiogenesis (Fig 5.2).

The proposed molecular mechanism of the anti-diabetic potential of Ampelopsin F in L6 cell lines

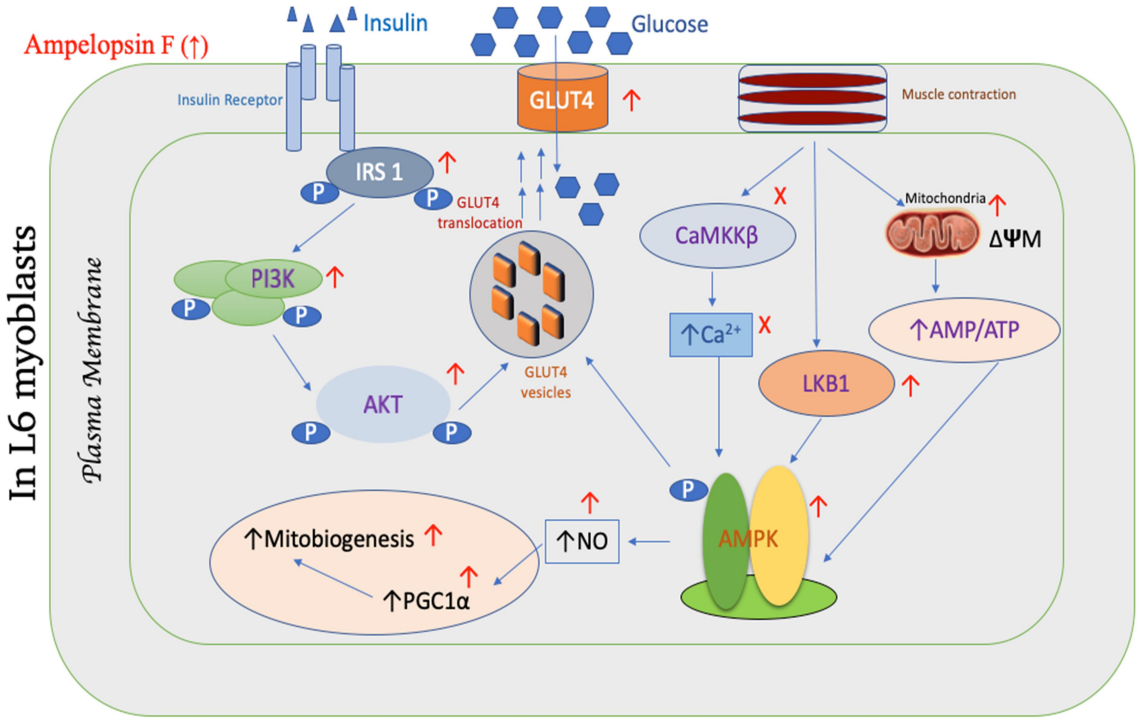


Fig 5.2. The proposed molecular mechanism of action of Ampelopsin F in L6 cell lines. Ampelopsin F stimulates both PI3-K/AKT pathway and AMPK pathway. Ampelopsin F along with insulin upregulates the expression of phosphorylated IRS-1, PI3-K, AKT and ultimately the glucose transporter GLUT4. Ampelopsin F stimulates the activation of AMPK via the upregulation of LKB1 and alterations in mitochondrial membrane. The activation of AMPK leads to increased mitobiogenesis via the upregulation of PGC-1α and a transient increase in nitrite levels in the cells.

5.5. Anti-diabetic potential of (-) Hopeaphenol in AMPK signaling pathway

The resveratrol tetramer (-) Hopeaphenol, downregulated the expression of PI3-K in insulin signaling pathway and the inhibitor studies with wortmannin co-treated with (-) Hopeaphenol in L6 myoblasts confirmed the same. The activation of AMPK pathway in (-) Hopeaphenol treated cells was confirmed using the inhibitor studies co-treated with dorsomorphin in L6 myotubes. The different parameters involved in the activation of AMPK when treated with (-) Hopeaphenol was studied and a slight depolarization of the mitochondrial membrane similar to Ampelopsin F was observed indicating an increment in AMP/ATP ratio and this was confirmed with flow cytometry analysis. The involvement of Ca^{2+} in the activation of AMPK was conducted. A significant release of Ca^{2+} in (-) Hopeaphenol treated cells was observed via fluorescence microscopy upon staining with Fura-2, AM. In addition, an increase in the expression of CaMKKβ in (-) Hopeaphenol treated cells was observed via western blot analysis. In the CaMKK inhibitor studies, the decreased uptake of glucose was observed in (-) Hopeaphenol treated cells co-treated with the inhibitor, STO-609 thus indicating the role of (-) Hopeaphenol in stimulating Ca^{2+} dependent activation of AMPK (Fig 5.3). The role of LKB1 in the activation of AMPK was determined by western blot analysis and an increase in expression of LKB1 was observed with (-) Hopeaphenol treated cells. Expression analysis of AMPK revealed its upregulation, which confirmed the role of (-) Hopeaphenol in AMPK signaling pathway. The increased expression of p38 MAPK and PGC-1α implies the downstream activation of the AMPK pathway induced by (-) Hopeaphenol and its potential role in mitochondrial biogenesis.

The proposed molecular mechanism of the anti-diabetic potential of (-) Hopeaphenol in L6 cell lines

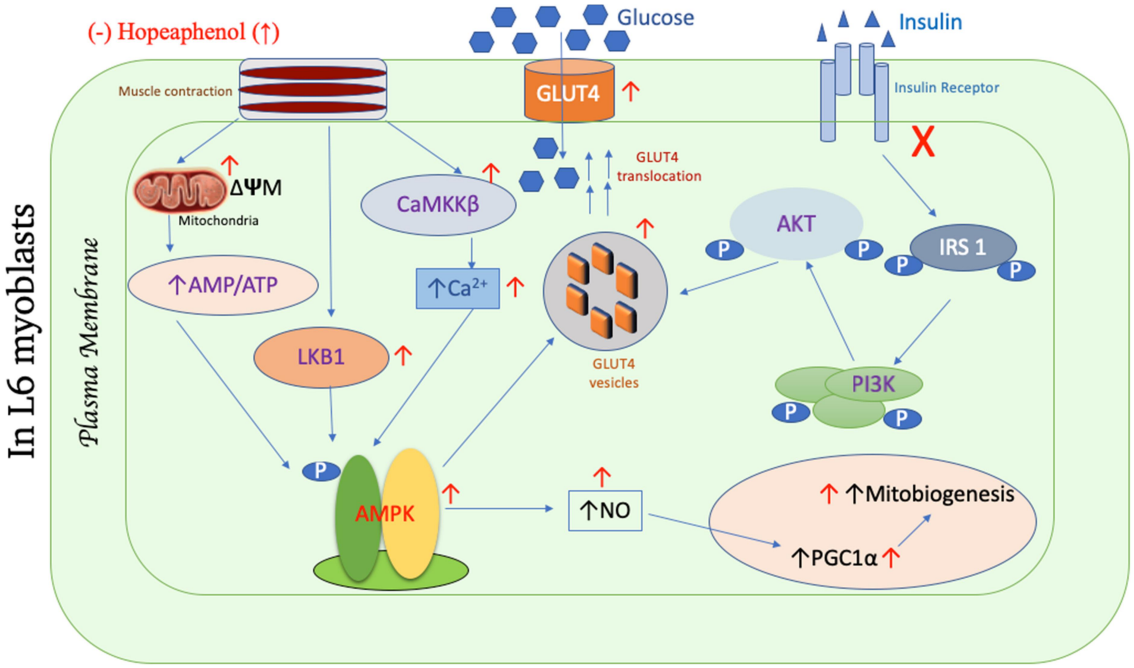


Fig 5.3. The proposed molecular mechanism of action of (-) Hopeaphenol in L6 cell lines. (-) Hopeaphenol stimulates AMPK pathway alone via the upregulation of LKB1, alterations in mitochondrial membrane and an increase in intracellular calcium levels. The activation of AMPK leads to increased mitobiogenesis via the upregulation of PGC-1α and a transient increase in nitrite levels in the cells.

Conclusion

Eight resveratrol-based compounds from different plants belonging to Dipterocarpaceae family were screened for their anti-diabetic potential. Based on the initial activity profile, two compounds (Ampelopsin F and (-) Hopeaphenol) were selected for investigating their molecular mechanism of action. Both the resveratrol-based compounds stimulated the insulin secretory pathway analogous to the GSIS. Ampelopsin F, the resveratrol dimer from *Vatica chinensis* activated both insulin dependent signaling and insulin independent signaling. Ampelopsin F stimulated the AMPK pathway via alterations in mitochondria and upregulation of LKB1. (-) Hopeaphenol, the resveratrol tetramer from *Vateria indica* stimulated insulin independent signaling pathway alone and the activation of AMPK via alterations in mitochondria, activation of LKB1 and CaMKKβ. Hence, the findings revealed for the first time that these two resveratrol oligomers are novel potential multi-targeted anti-diabetic leads targeting insulin secretion and insulin signaling pathways, besides the inhibition of PTP 1b and DPP4.

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ABBREVIATIONS

2- NBDG	2- (7- Nitrobenz-2-oxa-1,3- Diazol- 4-yl) Amino- 2-Deoxy- D- Glucose
ABA	Abscisic Acid
ADP	Adenosine Diphosphate
AGC kinase	Protein kinase A, G, and C
AMC	7-Amino-4-Methyl Coumarin
AMP	Adenosine Monophosphate
AMPK	5' Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of Variance
AS160	Akt Substrate of 160 kDa
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BCA	Bicinchoninic Acid
BCE	Before the Common Era
BD	Becton Dickinson
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ions
CaMKKβ	Ca^{2+}/Calmodulin-Dependent Protein Kinase β
cAMP	Cyclic Adenosine Monophosphate.
CBS	Cystathionine Beta Synthase
cGMP	Cyclic Guanosine Monophosphate
CD26	Cluster of Differentiation 26
CO_2	Carbon Dioxide
DKA	Diabetic Ketoacidosis
DMEM	Dulbecco's Modified Eagles's Media
DMSO	Dimethyl Sulfoxide
DPP4	Dipeptidyl Peptidase 4
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase

FACS	Fluorescence-Activated Single Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GCK	Glucokinase
GI	Gastro Intestine
GIP	Gastric Inhibitory Polypeptide
Glc-6-P	Glucose 6-phosphate
GLP-1	Glucagon-Like Peptide 1
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GSIS	Glucose Stimulated Insulin Secretion
GTP	Guanosine Triphosphate
HbA1c	Glycated Hemoglobin
HCl	Hydrochloric Acid.
HEPES	N-(2-Hydroxyethyl) Piperazine-N′-(2-ethanesulfonic acid)
HHEX	Hematopoietically-Expressed Homeobox Protein
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HLA-DRB	Human Leukocyte Antigen – DR isotype B
HLM	Human Liver Microsomes
HNF1A	Hepatocyte Nuclear Factor 1 Homeobox A
HNF 1B	Hepatocyte Nuclear Factor 1 Homeobox B
HNF4A	Hepatocyte Nuclear Factor 4 alpha
HRP	Horseradish Peroxidase
HS	Horse Serum
IDDM1	Insulin-dependent Diabetes Mellitus 1
IDF	International Diabetes Federation
IGF2BNP2	Insulin like Growth Factor 2 mRNA Binding Protein 2
IKK	IkB Kinase
IL-4	Interleukin 4
iNOS	Inducible Nitric Oxide Synthase
IR	Insulin Receptor

IR-A	Insulin Receptor Isoform A
IR-B	Insulin Receptor Isoform B
IRS	Insulin Receptor Substrate
IRS-1	Insulin Receptor Substrate 1
JC-1	5,5',6,6'-Tetrachloro- 1,1'3,3'-Tetraethy1Benzimidazolocarbocyanine Iodide
K^+ - ATP	ATP-sensitive Potassium Channels
KCl	Potassium Chloride
KCNJ1	Potassium Inwardly-Rectifying Channel, Subfamily J, Member 1
KCNJ11	Potassium Inwardly- Rectifying Channel Subfamily J, Member 11
K_M	Michaelis constant
LDH	Lactate Dehydrogenase
LDLc	Low-Density Lipoprotein Cholesterol
LKB1	Liver Kinase B1
Maf	Musculoaponeurotic Fibrosarcoma
Malonyl CoA	Malonyl Co-enzyme A
Mdm2	Murine Double Minute 2
MHC	Major Histocompatibility Complex
Mmol	Milli moles
MMP	Mitochondrial Membrane Potential
MODY	Maturity Onset Diabetes of the Young
MTNR1B	Melatonin Receptor 1B
mTORC2	Mammalian Target of Rapamycin Protein Complex 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mtTFA	Mitochondrial Transcription Factor A
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCCS	National Centre for Cell Sciences
NF-κB	Nuclear Factor Kappa B
NMR	Nuclear Magnetic Resonance
nNOS	Neuronal Nitric Oxide Synthase.
NO	Nitric Oxide
NRF	Nuclear Respiratory Factor

p38MAPK	p38 Mitogen-Activated Protein Kinases
PBS	Phosphate Buffer Saline
PC	Pyruvate Carboxylase
PDE3B	Phosphodiesterase 3B
PDH	Pyruvate Dehydrogenase
PDK-1	3-Phosphoinositide-Dependent Protein Kinase 1
Pdx	Pancreatic and Duodenal Homeobox
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1
PI3-K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-Triphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PNPP	Para Nitrophenyl Phosphate
PP	Pancreatic Polypeptide
PPARG2	Peroxisome Proliferator- Activated Receptor Gamma
PTP 1b	Protein-Tyrosine Phosphatase 1B
PVDF	Polyvinylidene Difluoride
RBC	Red Blood Cells
RIPA	Radioimmunoprecipitation Assay Buffer
RTI	Renal Tubule Infection
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SGK	Serum- and Glucocorticoid-Induced Protein Family of Kinases
SGLT1	Sodium/Glucose Cotransporter 1
SGLT2	Sodium/Glucose Cotransporter 2
SH2	Src-Homology 2
SIRT1	Silent Mating Type Information Regulation 2 Homolog1
SLC2A2	Solute Carrier Family 2, Facilitated Glucose Transporter Member 2
SNAP	*S*-nitroso-*N*-acetyl Penicillamine
SRP	Signal Recognition Particle

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAK1	TGF Beta Activated Kinase 1
TBC1D1	TBC1 Domain Family Member 1
TBC1D4	TBC1 Domain Family Member 4
TCA cycle	Tricarboxylic Acid Cycle
TCF7L2	Transcription Factor 7 Like 2
TG	Triglyceride
THPG	2, 4, 8 - Trihydroxyphenanthrene-2-*O*-Glucoside
TZD	Thiazolidinedione
VDCC	Voltage Dependent Calcium Channels
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
WHO	World Health Organization
XRS	Extensible Radio Specification
ΔΨm	Mitochondrial Membrane Potential

Publications

1. P. Sasikumar ‡, **K. Lekshmy** ‡ ,, S. Sini, B. Prabha, N. Anil Kumar, V. V. Sivan, M. M. Jithin, P. Jayamurthy, I.G. Shibi and K. V. Radhakrishnan; Isolation and characterization of resveratrol oligomers from the stem bark of *Hopea ponga* (Dennst.) Mabb. and their antidiabetic effect by modulation of digestive enzymes, protein glycation and glucose uptake in L6 myocytes; Journal of Etnopharmacology; 2019 May 23;236:196-204. (‡ contributed equally)

2. H.P. Syama, T. Sithara, **S. Lekshmy Krishnan**, P. Jayamurthy; Syzygium cumini seed attenuates LPS induced inflammatory response in murine macrophage cell line RAW264.7 through NF-κB translocation; Journal of Functional Foods ; 44 (2018) 218–226.

3. B. Prabha, S. Neethu, S. **Lekshmy Krishnan**, D.R. Sherin, M. Madhukrishnan, R. Ananthakrishnan, K.B. Rameshkumar, T.K. Manojkumar, P. Jayamurthy, K.V. Radhakrishnan; Antidiabetic potential of phytochemicals isolated from the stem bark of Myristica fatua Houtt. var. magnifica (Bedd.) Sinclair; Bioorganic & Medicinal Chemistry 26 (2018) 3461–3467.

Conferences

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- International Conference on Biotechnology and Biological Sciences, Biospectrum 2020, Department of Biotechnology, University of Engineering & Management, Kolkata, 19th to 21st of November, 2020 (Oral presentation).

- National Virtual Conference on Recent Breakthroughs in Biotechnology (NCRBB-2021) and Annual meet of Society for Biotechnologists, 22nd to 23rd January 2021 (Oral Presentation)

- **Best Poster Award,** 33rd Kerala Science Congress, January 25-30, 2021