Identification of protein targets for natural anti-diabetic active compounds

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vorgelegt von

Najlaa Bassalat

aus Hajaa, Qalqalia, Palestine

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Referent: Prof. Dr. Jörg Labahn

Korreferent: Prof. Dr. Oliver H. Weiergräber

Co-Betreuer/in: Prof. Hilal Zaid

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of Heinrich Heine University Düsseldorf

Dedication

This thesis is devoted to the soul of my beloved father, whose absence is felt in every moment of my life. His love, wisdom, and endless sacrifices shaped who I am and continue to light my path. Though he is no longer here to witness this achievement, every step of this journey carried his voice, his prayers, and his dreams for me. This accomplishment will always belong to him.

To my precious mother, my greatest source of strength, patience, and unconditional love, I owe more than words can express. Your prayers carried me through moments of weakness, and your unwavering faith in me gave me the courage to continue when the path felt impossible. Every page of this work reflects your sacrifices and your endless compassion.

To my sisters and brothers, thank you for being my shelter and my strength. Your love, understanding, and encouragement gave me comfort during the most difficult moments and reminded me that I was never alone.

To my supervisor, **Prof. Hilal Zaid,** I express my deepest gratitude for your guidance, patience, and unwavering support throughout this journey. Your belief in my abilities, your thoughtful advice, and your constant encouragement played a vital role in bringing this work to completion. Your mentorship extended beyond academic guidance and left a lasting impact on my growth as a researcher.

To my friends, who walked beside me through this long and demanding journey, thank you for your kindness, motivation, and belief in me. And especially to my best friend **Haneen**, who shared my fears, hopes, and perseverance—your presence transformed exhaustion into strength and challenges into moments of hope.

This work is not mine alone; it is a reflection of the love, guidance, prayers, and sacrifices of everyone who stood by me throughout this journey.

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Abbreviations

DM Diabetes mellitus

T1DM Diabetes Mellitus type 1

IDDM Insulin-dependent diabetes mellitus

DKA Diabetic ketoacidosis

IAAs Insulin autoantibodies

ZnT8 Zinc transporter isoform 8

GADAs Glutamic acid decarboxylase autoantibodies

ICAs Islet cell autoantibodies

ICA512 Islet cell antigen 512

IgG Immunoglobulin G

T2DM Diabetes Mellitus type 2

NIDDM non-insulin-dependent diabetes mellitus

SGLT2 Sodium-glucose co-transporter-2

HDLs High-density lipoproteins

GDM Gestational diabetes mellitus

GLUTs Glucose transporters

PIP_3 Phosphatidylinositol (3,4,5)-trisphosphate

Akt protein kinase B

WHO World Health Organization

A.E *Abelmoschus esculentus*

CAM Complementary and alternative medicine

FA Ferulic acid

CA Caffeic acid

ROS Reactive oxygen species

DN Diabetic nephropathy

DR Diabetic retinopathy

DPN Diabetic peripheral neuropathy

SA	Stearic acid
VA	Vanillic acid
PM	Plasma membrane
GLUT4	Glucose transporter-4
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffer saline
IRS1	Insulin Receptor Substrate 1
BSA	Bovin serum albumin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
PVDF	Polyvinylidene fluoride
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
AS160	Akt substrate of 160 kDa
ECL	Electrochemiluminescence
GAP	GTPase-activating protein
DEPC	Diethyl Pyrocarbonate-treated water
Q RT-PCR	q Real time Polymerase reaction
STZ	Streptozotocin
SE	Standard error
C. intybus	*Cichorium intybus*
GC-MS	Gas Chromatography-Mass Spectrometry
ISV	Isovanillic acid
SYA	Syringic acid
PA	Palmitic acid
DB	3,4-dihydroxy benzoic acid
BZ	Benzoic acid
HM	2-hydroxy4-methoxy benzoic acid
TB	3,4,5 trihydroxy benzoic acid

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1. Introduction

1.1 Diabetes mellitus

Diabetes mellitus (DM), also referred to as diabetes, is a hallmark of the chronic and varied condition hyperglycemia, a physiologically aberrant state typified by persistently high blood glucose levels. Hyperglycemia is caused by abnormalities in either insulin secretion, insulin action, or both. Diabetes has a complicated etiology, a progressive course, and a variety of manifestations [1, 2]. It presents as metabolic dysfunctions of the proteins, fats, and carbohydrates. The impact of hyperglycemia on several body organs extends to the metabolic dysfunctions of proteins, fats, and carbohydrates.

DM also affects blood vessel’s structure and function. The detrimental effects of hyperglycemia and the related metabolic abnormalities on the normal construction and function of the micro- and macro-vasculature develop gradually. Microvascular and macrovascular ailments result from structural and functional abnormalities of organ systems. These complications impact the body's organs, including the kidneys, heart, nerves, and eyes, and are characterized by organ damage, dysfunction, and, eventually, organ failure. Complications might include retinopathy, which may eventually lead to blindness; nephropathy and possible renal failure; coronary heart disease and hypertension; and neuropathy, which can be peripheral or autonomic. Autonomic neuropathy is characterized by cardiovascular, gastrointestinal, and genitourinary (including sexual) dysfunctions, while long-term peripheral neuropathy is frequently linked to foot infections, including ulcers that necessitate amputations, and Charcot joint (osteoarthropathy) [3-5]. One of the main causes of diabetes-related morbidity and death is atherosclerotic cardiovascular disease, which is a combination of coronary heart disease, peripheral arterial disease, and cerebrovascular illness [1, 4, 5].

Diabetes is one of the most significant and difficult health problems facing the world's population today, which is becoming increasingly common worldwide. In most parts of the world, the rise in prevalence of diabetes has coincided with fast

economic growth, which has resulted in urbanization and the adoption of contemporary lifestyles[6].

The physiological variables that exist at the time of assessment and diagnosis have an impact on the complex etiology and different presentations of diabetes mellitus. The current categorization is helpful in clinical evaluation of the disease and in determining the necessary treatment because it is based on both the etiology and the pathophysiology of the disease. This categorization separates diabetes into three categories: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus [1, 7].

1.1.1 Diabetes Mellitus type 1 (T1DM)

T1DM, referred to as juvenile-onset diabetes or insulin-dependent diabetes mellitus (IDDM), accounts for 5–10% of all diabetes cases. T cell-mediated death of pancreatic β-cells is a hallmark of this autoimmune disease, leading to insulin insufficiency and eventually, hyperglycemia[8, 9]. Although the exact pathophysiology of this autoimmunity is yet unknown, it has been discovered that both hereditary and environmental factors have crucial roles. The development of this pancreatic β-cell-specific autoimmunity can occur gradually in adults (late onset) or quickly in most cases, as in infants and children (juvenile onset) [10].

Variability in the rate of immune-mediated loss of pancreatic β cells frequently determines how the disease will ultimately progress. Some children and adolescents may experience abrupt β-cell loss and subsequent failure, which can result in diabetic ketoacidosis (DKA). DKA is frequently referred to as the disease's initial presentation. Others have very gradual disease progression with moderate elevations in fasting blood glucose levels, which only become severe hyperglycemic with or without ketoacidosis when there are physiological stressors such as severe infections. In some patient cases, in specific adults, β-cells may still be able to secrete just enough insulin to keep them from developing ketoacidosis for years. However, these patients develop severe hyperglycemia and ketoacidosis as a result of gradual insulin insufficiency, which makes them insulin dependent. Affected patients in the early, middle, or even late stages of their

lives become severely or completely insulin deficient. This group of patients require insulin treatment for survival, despite the unpredictable course of this kind of diabetes. Regardless of the onset of diabetes, this severe or complete insulin insufficiency shows up as low or undetectable plasma C-peptide levels [1, 8, 9].

Numerous immunological markers, particularly autoantibodies, are indicative of type 1 diabetes. These autoantibodies normally target the immune-mediated β-cell destruction. The autoantibodies include insulin autoantibodies (IAAs), islet-specific zinc transporter isoform 8 (ZnT8), tyrosine phosphatases IA-2 and IA-2α, glutamic acid decarboxylase autoantibodies (GADAs), such as GAD65, and islet cell autoantibodies (ICAs) to β-cell cytoplasmic proteins, such as autoantibodies to islet cell antigen 512 (ICA512). Although at least one of these autoantibodies can be used to clinically diagnose this condition, 85–90% of individuals with newly diagnosed type 1 diabetes typically have multiple immunological markers[1, 11]. The most significant of these autoantibodies is GAD65, which is present in roughly 80% of all T1DM patients at diagnosis. ICAs are present in 69–90% of patients, and IA-2α is present in 54–75% of patients at clinical presentation. The prevalence of IAAs, which are significant immunological markers found in newborns and early children at risk for diabetes, declines with increasing diabetes start age. One significant sign of developing type 1 diabetes in patients who have never received insulin treatment is the existence of IAAs [1, 11]. Approximately 70% of diabetic newborns and young children have IAAs at the time of diagnosis. In patients on insulin therapy, the IAAs also have significant inhibitory effects on insulin activity. This immunological response has been seen in at least 40% of patients receiving insulin treatment, albeit in various degrees of intensity; as a result, it exhibits distinct clinical symptoms even though it is not frequently clinically noteworthy[12]. These autoantibodies vary in their affinities and binding capabilities for insulin and are primarily composed of polyclonal immunoglobulin G (IgG) antibodies. Both low insulin affinity with high insulin binding capacity and high insulin affinity with low insulin binding capacity can occur together with the detection of IAAs. Clinical symptoms are caused by IAAs' high insulin-binding capability and poor insulin affinity. The characteristic hyperglycemia in the immediate postprandial period,

which results in markedly elevated insulin requirements and later unpredictable hypoglycemic episodes (postprandial hypoglycemia), is caused by these antibodies' binding to insulin at high titers, which inhibits or delays its action [13].

The rate at which beta cells are destroyed varies somewhat; it frequently happens quickly for some people and slowly for others[14]. The loss of the duct gland's ß-islet cells results in a severe lack or insufficiency of hormone output. Hormone injections are necessary for treatment [15].

When fasting hyperglycemia is first identified, the diagnosis of type 1 diabetes is supported by evidence of autoimmune β-cell destruction. This is typically demonstrated by the presence of islet-specific autoantibodies, including insulin autoantibodies (IAA), glutamic acid decarboxylase antibodies (GAD65A), insulinoma-associated antigen-2 antibodies (IA-2A), and zinc transporter 8 antibodies (ZnT8A). Collectively, these autoantibodies are detected in approximately 85–90% of individuals at the time of initial diagnosis [16].

1.1.2 Diabetes Mellitus type 2 (T2DM)

T2DM, referred to as adult-onset diabetes or non-insulin-dependent diabetes mellitus (NIDDM), accounts for 90–95% of all cases of diabetes. This kind of diabetes is distinguished by two primary abnormalities linked to insulin: Insulin resistance and malfunction of β-cells [17-19].

A reduced sensitivity, or responsiveness, of cells in peripheral organs, specifically the muscle, liver, and adipose tissue, to insulin is the result of disruption of multiple cellular pathways, which causes insulin resistance. When insulin sensitivity is reduced in the early stages of the disease, β-cell hyperfunction is triggered, which results in an increase in insulin secretion to maintain normoglycemia. Thus, hyperinsulinemia, or elevated amounts of circulating insulin, avoids hyperglycemia. But over time, the β-cells' increasing release of insulin is unable to adequately offset the decline in insulin sensitivity. Additionally, β-cell function starts to deteriorate, and eventually, β-cell malfunction results in insulin insufficiency. Hyperglycemia results from the inability to sustain normoglycemia. In most situations, insulin secretion is adequate to avoid DKA, even when insulin levels are

low [19]. However, DKA can occur in extremely stressful situations, e.g., at the time of infections or other pathological situations. Atypical antipsychotics (second-generation antipsychotic medications), corticosteroids, and sodium-glucose co-transporter-2 (SGLT2) inhibitors are among the medications that can induce DKA [20, 21]. When there are no significant physiological stressors present, patients with type 2 diabetes may not need insulin therapy both at the onset of the disease or even later on [17-19].

Since type 2 diabetes is a very slow-moving, asymptomatic disease, even mild hyperglycemia takes years to develop. As a result, it is often not diagnosed until the advanced stages of the disease, which show the classic symptoms of severe hyperglycemia, including blurred vision, weight loss, growth impairment, polyuria, and polydipsia. Several known and unknown variables contribute to the pathogenesis/etiology of this form of diabetes. It can be characterized as a combination of strong environmental factors and genetic (polygenic) predispositions. T2DM has been more often linked to aging, obesity, a family history of the disease, a lack of physical activity, and the adoption of contemporary lifestyles that include pathophysiological disorders such dyslipidemia and hypertension. Unlike type 1 diabetes, this condition has not been linked to immune response genes, such as autoimmunity, and as a result, there is no immune-mediated death of pancreatic β-cells [22, 23].

Since obesity induces insulin resistance by affecting tissue sensitivity to insulin, it plays a significant role in the homeostatic dysregulation of systemic glucose. As a result, the majority of T2DM patients, are overweight or obese [24]. Indeed, the elevated body fat content is a major risk factor for type 2 diabetes, and the distribution of body fat, as well as its overall quantity, are major indicators of the onset of insulin resistance and ultimately, hyperglycemia. This form of diabetes has often been linked to increased visceral obesity or belly fat, rather than the increased gluteal/subcutaneous fat seen in peripheral obesity[25]. Patients with type 2 diabetes frequently exhibit several cardiovascular risk factors, including hypertension and lipoprotein metabolic abnormalities, which are typified by

elevated triglycerides and low levels of high-density lipoproteins (HDLs), linked to increased body fat content or obesity. T2DM is often linked to the development of a number of microvascular and macrovascular ailments resulting from dietary and sedentary life style for the long run and the resulting varied metabolic abnormalities that lead to hyperglycemia, especially in the middle and later age decades [24, 25].

1.1.3 Gestational diabetes mellitus (GDM)

GDM is the form of diabetes or glucose intolerance identified at the beginning of pregnancy or over the course of the pregnancy, typically in the second or third trimester. This earlier definition also covered any undiagnosed type 2 diabetes that might start before or at the onset of pregnancy. The International Association of the Diabetes and Pregnancy Study Groups' most recent guidelines, however, do not include diabetes diagnosed at the beginning of pregnancy or later in high-risk women, such as those who are obese, where any level of glucose intolerance is referred to as previously undiagnosed overt diabetes rather than GDM. GDM often goes away shortly after childbirth or pregnancy termination and is distinct from any underlying diabetes in women who are pregnant [26].

Early in pregnancy, both fasting and postprandial blood glucose levels are typically lower than normal. However, during the third trimester of pregnancy, blood glucose levels rise, and when they approach diabetic levels, the disease is referred to as gestational diabetes mellitus (GDM). GDM is responsible for almost 90% of all cases of diabetes and related complications that arise during pregnancy. GDM affects between 1% and 14% of pregnancies, and the demographics being studied have a significant impact on its prevalence. The Middle Eastern nations, Pacific Islanders, Asian Indians, Aboriginal Australians, and women from China, Japan, Korea, and Mexico are the groups most likely to have GDM. The frequency is lower among Black women and is the lowest among non-Hispanic white women [27, 28]. The chance of developing GDM increases with age, obesity, prior pregnancies with large babies, and any prior history of poor glucose tolerance or GDM[29, 30]. Additionally, a higher lifetime risk of developing type 2 diabetes has been linked to GDM. Therefore, it is strongly advised that these females undergo routine and

lifetime screening for any form of glucose impairment to guarantee an early diagnosis of type 2 diabetes [31-33].

1.2 Glucose Metabolism

Glucose is the main source of energy and fuel for human cells and is the universal language for energy consumption. It is obtained from the consumed diet, treated within the gastrointestinal tract and by the liver tissue, and transferred from circulation to target cells. The regulation of glucose uptake and exocytosis through the plasma membranes is crucial for maintenance its bodily homeostasis [34]. Fat, proteins, and carbohydrates eventually decompose to form glucose, which acts as fuel for metabolic processes in the body. Glycogenesis, glycolysis, gluconeogenesis, and glycogen degradation are just a few of the multiple processes that constitute glucose metabolism.

Glycolysis is one major enzyme-induced metabolism mechanism that promotes glucose metabolism and conversion to pyruvate in body cells [35]. Occurring in ten consecutive cytosolic chemical reactions, this catabolic process leads to the net production of two ATP molecules per one glucose molecule. The total efficiency of ATP composition is only about 43%, with the remaining 57% get lost in heat form. Each resulting pyruvate is converted in the mitochondria into acetyl-Co-enzyme A, with the release of the NADH electron carrier, via pyruvate dehydrogenase enzyme. Acetyl CoA then enters the Krebs cycle, releasing two molecules of carbon dioxide, 2 molecules of GTP, and eight electrons that are received by four electrons carriers, namely three NADH molecules and one $FADH_2$ molecule. Consequent oxidative phosphorylation releases on average nine ATP molecules per cycle [36].

To maintain constant levels of sugar and glucose in the blood during fasting, cytosol, mitochondria, and the endoplasmic reticulum of hepatocytes undergo a series of metabolic activities that create glucose from non-carbohydrate precursors. These processes are regulated by insulin, glucagon, and cortisol [37].

On average, the level of blood sugar during fasting drops within the period of 3-4 hours of fasting, reaching 80-90 mg/dL. In cases of starvation, the glucose

formation process from glycogen, fat and eventually protein reserves provide the body cells with their glucose needs. Postprandially, blood sugar rises on average to 120-140 mg/dL and returns to normal levels in a two-hour period due to the body's feedback mechanisms. High blood glucose leads to insulin secretion including a massive glucose uptake by cells. Conversely, glucagon is released due to hypoglycemia, thereby raising blood glucose levels. Therefore, poor insulin secretion and inadequacy clinically lead to diabetes mellitus[37].

1.2.1 α-Amylase and α-Glucosidase: Primary Gatekeepers Before Glucose Transport

Before glucose reaches the intestinal transporters, carbohydrate-digesting enzymes such as α-amylase and α-glucosidase initiate the first step in maintaining glucose balance by beginning the breakdown of dietary polysaccharides. α-Amylase, secreted by the salivary glands and pancreas, hydrolyzes complex starches into shorter oligosaccharides and disaccharides [38]. Subsequently, α-glucosidase, an enzyme located on the small intestine's membrane, further breaks down these carbohydrates into free glucose molecules, which are then absorbed by the small intestine [39]. Therefore, the coordinated activity of these digestive enzymes before absorption plays a vital role in regulating the amount and rate of glucose entering the bloodstream, contributing to postprandial glucose homeostats.

These enzymes are key targets for anti-diabetic medications. By inhibiting α-amylase and α-glucosidase, pharmacological agents slow carbohydrate digestion, reducing the rapid appearance of glucose in the bloodstream and preventing sharp post-meal blood sugar spikes[38, 39]. To enhance the effectiveness of intestinal enzyme inhibitors in managing type 2 diabetes mellitus (T2DM), recent research has focused on developing dual-enzyme inhibitors that simultaneously target both α-amylase and α-glucosidase [40].

1.2.2 Glucose Transport and Defect in Transport Activity

Since glucose is a hydrophilic large molecule that cannot pass freely across the plasma membrane, its transport must be facilitated by a carrier system.

Hexokinase phosphorylates glucose inside the cell at a pace that is significantly faster than its uptake from the extracellular part in the majority of cell types. As a result, compared to the external concentration of glucose, which is typically 5 mM, the internal concentration is significant enough to keep the cell from death. Due to this notable concentration difference between the cell's inside and exterior, can facilitate the diffusion of glucose into the cell [41]. In recent years, advances in genetics have shed new light on the types and physiology of various glucose transporters, of which there are two main types: Sodium–glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs), both of which can be divided into many family members [42].

Transporters vary in terms of their specificity, distribution and regulatory mechanisms. Glucose transporters have received specific attention as therapeutic targets for different diseases. Sodium–glucose linked transporter-1 (SGLT1) was the first glucose-1 transporter to be widely detected and studied. It consists of fourteen transmembrane helices (TM0-TM13), of which both the COOH and NH_2 termini face the extracellular space. Members of the SGLT family have molecular weights ranging between 60 and 80 kDa and consisting of 580-718 amino acids. GLUT proteins consist of twelve transmembrane helices spanning the membrane, with intracellularly located amino and carboxyl termini The amino acid sequence of GLUT proteins has been found to show a 28-65% match with GLUT 1. GLUT major transporters are classified into GLUT1-5. Among the several transporters, GLUT2 is mainly expressed in beta cells in the pancreas, liver, and kidneys. GLUT2 acts as a glucose sensor in beta cells for marine organisms, but human beta cells essentially express GLUT1 [42].

In hepatocytes, GLUT1 is associated with the two-way transmission of glucose controlled by hormones, such as thyroid hormone. The GLUT2 on the hepatocellular membrane regulates the entry and exit of glucose to and from the cell, respectively, thereby controlling the metabolism of glucose in the liver. In contrast, GLUT2 in intestinal brush edge cells and kidney pipe cells is associated with glucose absorption and reabsorption, respectively [42]. GLUT3 is the main

GLUT isoform in the brain. It has a significant, high affinity for glucose, a property consistent with its function of transferring glucose to cells that have higher requirements of glucose. GLUT4 is the predominant glucose transporter in skeletal muscle and plays a critical role in regulating glucose uptake and metabolism. Numerous studies have established a strong positive correlation between GLUT4 protein levels and the efficiency of muscle glucose uptake. Research using mouse skeletal muscle models has shown that overexpression of GLUT4 significantly enhances glucose uptake under various conditions, increasing basal uptake by 20–300%, insulin-stimulated uptake by 60–200%, and contraction-induced uptake by 35% [43]. Conversely, the targeted loss of GLUT4 in skeletal muscle results in a 70–80% reduction in basal glucose uptake and a complete inhibition of glucose uptake induced by insulin or muscle contraction. These findings highlight the indispensable role of GLUT4 in facilitating glucose uptake in skeletal muscle during short-term stimuli [43]. Additionally, GLUT4 is the most abundant glucose transporter in skeletal muscle and is regarded as a rate-limiting factor for glucose uptake and metabolism, particularly in the resting state of the muscle. This underscores its pivotal role in maintaining glucose homeostasis and its potential as a therapeutic target for metabolic disorders [44].

Glucose is reabsorbed from glomerular filtrate by SGLT2, the primary glucose transporter in kidney tubules. High levels of glucose are excreted in urine by people who have a genetic abnormality in the SGLT2 gene, which impairs glucose reabsorption [45].In cardiac sarcolemma SGLT1 expression, mediated through leptin, was found to increase in T2DM and ischemia and to decrease in T1DM. These SGLT1 levels were found to be correlated with insulin levels. Increased levels of SGLT1 lead to increased uptake of glucose in cardiomyocytes of T2DM patients [46].

GLUT1, together with SGLT1 reabsorb the glucose found in bile in the intrahepatic bile ducts. Bile volume is decreased by the osmotic gradient created by this reabsorbed glucose, which facilitates the removal of water from the bile. Patients with diabetes have greater liver glucose concentrations as a result of their elevated

blood sugar levels. The reduced bile secretion seen in diabetic people is probably explained by the fact that an increase in glucose transport results in a decrease in bile flow and vice versa [47]. Mutations in the GLUT2 gene can cause severe Fanconi-Bickel Syndrome, which is characterized by dwarfism, proximal renal tubular failure, increased cholesterol, fasting hypoglycemia, postprandial hyperglycemia, and hepatorenal glycogen buildup that causes hepatomegaly [48].

T2DM patients with insulin resistance have a reduced capacity to use extracellular glucose by their skeletal muscle cells. The skeletal muscles of these patients have normal GLUT4 levels, suggesting that their translocation to the membrane is compromised, maybe as a result of an aberrant buildup of GLUT4 in the membrane compartments or a malfunction in the insulin signaling pathway [49].

1.2.3 Hormonal Regulation for Glucose Metabolism

The two main opposing hormones, insulin and glucagon, which are in charge of lowering and raising glucose levels, respectively, are secreted by the pancreas, which plays a major role in controlling glucose metabolism. Acinar or exocrine cells, which make up the majority of the pancreas, discharge digestive hormones into the duodenum through the pancreatic and accessory pancreatic ducts. The pancreas performs an endocrine function through the islets of Langerhans, which are located within the exocrine tissue and constitute 1-2% of the organ's mass [50].

1.3. Insulin Signaling Pathway

Whole-body glucose homeostasis following escalated serum glucose levels is maintained via insulin-induced glucose uptake in skeletal muscles and adipose tissue. Glucose transporter GLUT4 rapidly moves to the plasma membrane through the process of exocytosis. The deficiency in GLUT4 trafficking may induce the progression to insulin resistance and type 2 diabetes. Phosphoproteomics, the large-scale study of protein phosphorylation/dephosphorylation, is directly associated with insulin signaling, especially since several crucial protein’s phosphorylation and dephosphorylation processes govern the translocation of GLUT4 to the plasma membrane. Therein, IRS-1, Akt, TBC1D4/AS160, and

regulatory phosphatases such as PTEN are major contributors to this signaling cascade (figure1.3) [51-53].

Insulin binds to insulin receptor to induce the phosphorylation of insulin receptor substrate-1 (IRS-1) at the tyrosine residue, leading to its subsequent activation. This leads to the induction of the canonical PI3K–Akt pathway. Phosphorylated IRS-1 activates PI3K, which in turn generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). As a result, membrane localization and the activation of Akt follow. Mass-spectrometry-based studies have demonstrated that the fluctuation of IRS-1 phosphorylation levels, due to inhibitory serine phosphorylation, is well-correlated to insulin resistant states in human skeletal muscles [54]. A study by Stuart et al on human subjects shows that hyper-phosphorylation of serine residues at IRS-1 (most importantly Ser337 and Ser636) occurs in metabolic syndrome muscles, which reduces the propagation of the insulin signal, thereby lowering insulin-stimulated GLUT4 translocation[55]. Thus, the signal of IRS-1 phosphorylation is a multifaceted regulatory network, and not solely a binary “on/off” action.

PTEN is a lipid phosphatase that works downstream of IRS-1 and antagonizes the PI3K signaling, mainly by dephosphorylating PIP_3 to PIP_2 [56]. Studies have demonstrated PTEN as a potent negative regulator of Akt and GLUT4 trafficking. Overexpression of PTEN in adipocytes reduces Akt phosphorylation and consequently inhibits insulin-induced GLUT4 translocation. Conversely, knockouts of PTEN have increased PIP_3 levels, leading to promoted Akt phosphorylation, and the resulting augmented uptake of glucose by GLUT4 [57, 58]. Related phosphatases, such as inositol polyphosphate phosphatase (SKIP), produce similar effects: elevated PIP_3 dephosphorylation inhibits GLUT4 vesicle trafficking, indicating that the equilibrium between phosphatase-driven dephosphorylation and kinase-driven phosphorylation of PIP isoforms is a crucial cornerstone for insulin action [59]. Akt (protein kinase B) is the control center that links insulin signal transduction to GLUT4 vesicle translocation. Early mechanistic studies demonstrated that constitutively active Akt can initiate the process of GLUT4

exocytosis, without the need of insulin action. Conversely inhibition of Akt prevents the fusion of GLUT4 vesicles with the plasma membrane[60, 61]. More recent reviews highlight that Akt isoform specificity and substrate selectivity govern GLUT4 translocation [53].

TBC1D4, also known as AS160, is a fundamental Akt substrate in the Akt-AS160-GLUT4 axis. Akt, via the insulin signaling cascade, phosphorylates TBC1D4 and subsequently inhibits the activity of its Rab GTPase-activating protein (GAP). As a result, Rab10 and Rab8A remain in their active GTP-bound states. This activation is pivotal for GLUT4 vesicle release from intracellular compartments and their consequent fusion with the plasma membrane. AS160 mutants that are not subject to Akt phosphorylation inhibit GLUT4 translocation[62]. *In vivo*, knock-in mutation in mice of key Akt phosphorylation sites on TBC1D4 (e.g., AS160-Thr649) to alanine leads to diminished muscle glucose uptake and attenuated GLUT4 translocation[63]. Studies on human skeletal muscle demonstrate that insulin promotes TBC1D4 phosphorylation and its interaction with the 14-3-3 proteins; In obesity and type 2 diabetes, this cascade is disrupted, causing defective GLUT4 trafficking [64].

Our understanding of insulin resistance is reshaped based on the development of the concept of phospho-proteomics, where insulin effect is contingent upon the balance between phosphorylation and dephosphorylation of signaling proteins. Insulin-resistant muscle and adipose tissues exhibit selective dysregulation of phosphatase-mediated dephosphorylation, rather than a steady decline of kinase activity. This results in a subsequent effect on proteins involved in the upstream signaling and downstream trafficking. Consequently, the loss of pertinent dephosphorylation attenuates several nodes in the IRS-1 → PI3K → Akt → TBC1D4 axis [51].

Taken together, current findings suggest a clear model: moving GLUT4 to the cell surface relies on a delicate balance of phosphorylation events. The process begins with IRS-1, gets amplified as PI3K generates PIP_3, is driven forward by inducing

Akt, TBC1D4 and Rab-controlled vesicle movement. This process ultimately led to GLUT4 vesicle fusion with the PM.

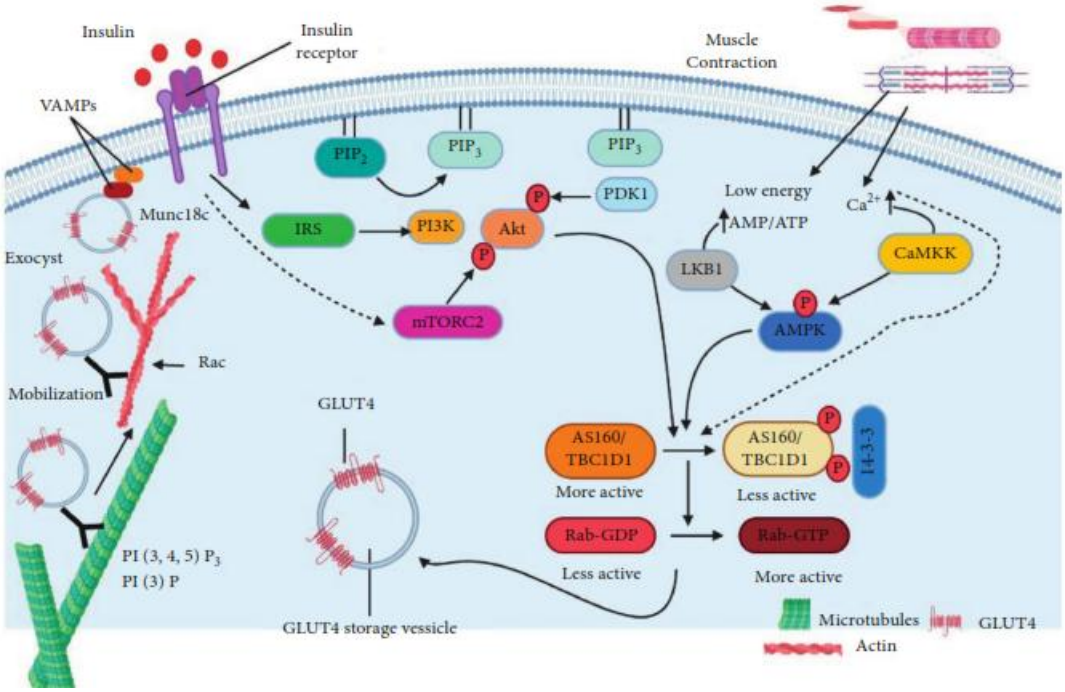


Figure 1.3 Simplified overview of insulin signaling in the skeletal muscle [65]

1.4. Drugs Targeting Key Nodes of Glucose Homeostasis in T2DM

Type 2 diabetes mellitus (T2DM) is characterized by disruptions in both the regulation of glucose before absorption and insulin signaling after absorption. Consequently, existing treatments and natural antidiabetic agents focus on two primary levels:

(1) Digestive enzymes α-amylase and α-glucosidase, which control the rate of dietary carbohydrate breakdown. (2) Intracellular insulin-signaling nodes, including IRS-1, PI3K, Akt, AS160/TBC1D4, PTEN, and GLUT4 translocation, which regulate glucose uptake in muscle and adipose tissue.

1.4.1. Drugs Targeting α-Glucosidase and α-Amylase

Pharmacological blockade of intestinal α-glucosidase and α-amylase enzymes diminishes the breakdown of starch. This intervention subsequently mitigates

postprandial glycemic spikes. Traditional pharmacotherapies, including acarbose, miglitol, and voglibose, exert their effects through this enzymatic inhibition[66].

Recent rigorous biochemical investigations establish that chlorogenic acid, a prominent dietary polyphenol, acts as an inhibitor of both α-amylase and α-glucosidase enzymes. This inhibition occurs via mixed or competitive mechanisms, resulting in reduced enzyme activity and subsequently reduced glucose liberation [67]. Structural validation of chlorogenic acid's inhibitory function has been achieved through docking simulations and spectroscopic analyses. These approaches substantiate chlorogenic acid capacity to interfere with enzyme function at the molecular level [68].

Hydroxycinnamic acids, specifically caffeic acid and ferulic acid, function as significant inhibitors of digestive enzymes. Caffeic acid and its derivatives demonstrate powerful antidiabetic activity *in vivo*, coupled with consistent enzyme inhibition across various experimental models[69]. **S**tructure–activity analyses verify that the hydroxylation configurations of caffeic and ferulic acids influence their binding affinity to α-glucosidase[70].Ferulic acid exhibits significant inhibitory activity against α-amylase and α-glucosidase enzymes. This indicates its potential in diabetes treatment inhibition [71].

Numerous aromatic carboxylic acids have been identified as natural α-glucosidase inhibitors in medicinal plants. These include vanillic acid, isovanillic acid, benzoic acid, and 3,4-dihydroxybenzoic acid. Additional compounds such as 3,4,5-trihydroxybenzoic acid (gallic acid), 2-hydroxy-4-methoxybenzoic acid, and syringic acid have also been documented for their inhibitory activity [72].

1.4.2. Drugs Targeting the IRS-1 → PI3K → Akt → AS160 → GLUT4 Axis

Insulin-facilitated glucose uptake depends on the activation of IRS-1 and subsequent PI3K signaling pathways. This cascade leads to Akt phosphorylation and the inactivation of AS160/TBC1D4, enabling GLUT4 translocation to the cell surface. Impairments in any of these processes can result in insulin resistance. Such impairments ultimately hinder efficient glucose clearance from the bloodstream, thereby exacerbating hyperglycemic conditions characteristic of

metabolic disorders [72]. Therefore, elucidating the molecular mechanisms underlying these signaling disruptions is essential for developing targeted therapeutic strategies aimed at restoring insulin sensitivity and ameliorating metabolic dysregulation [73].

The importance of particular nodes—namely IRS-1/2, PI3K, Akt, AS160/TBC1D4, PTEN, and GLUT4 was highlighted as essential targets for pharmacological intervention [51].

PTEN functions as the primary negative regulator of the PI3K/Akt signaling pathway. It decreases the availability of PIP_3, thereby attenuating insulin-mediated signal transduction. Overexpression or heightened activity of PTEN is associated with the development of peripheral insulin resistance [51, 74]. This dysregulation of PTEN expression can contribute to metabolic disturbances by impairing glucose uptake and lipid metabolism, ultimately exacerbating the pathophysiological processes underlying type 2 diabetes mellitus [75]. Consequently, dysregulation of PTEN expression can disrupt cellular homeostasis and contribute to the pathogenesis of metabolic disorders characterized by impaired glucose uptake and utilization [76].

1.5 Medicinal plants with Anti-diabetic activities

The growing interest in herbal medicine over the past decade can be attributed to concerns over the long-term side effects of synthetic drugs, particularly those used for managing diabetes. Various classes of synthetic medications, such as biguanides, sulfonylureas, thiazolidinediones, α-glucosidase inhibitors, GLP-1 agonists, dopamine-2 agonists, DPP-4 inhibitors, and SGLT-2 inhibitors, are commonly prescribed. However, prolonged use of these drugs can lead to adverse effects, including cancer, liver damage, and allergic reactions. As a result, many individuals are increasingly turning to natural remedies, which are perceived to be less toxic than synthetic alternatives. The adage "Let food be the medicine, and medicine be the food" reflects the growing recognition that natural treatments offer numerous benefits, particularly due to their safety and minimal side effects [77].

Natural plant extracts are gaining significant attention in the development of new therapeutic agents. Recent research highlights their effectiveness in treating or preventing a wide range of diseases, including cardiovascular conditions, atherosclerosis, diabetes, and cancer. Furthermore, herbal remedies have long been used globally for managing chronic illnesses [78]. Medicinal plants have emerged as a key area of interest, particularly for their potential in diabetes management. Over 410 medicinal plants have demonstrated experimentally proven anti-diabetic properties, offering therapeutic benefits with limited side effects often associated with conventional treatments[79].

These plant extracts contain bioactive compounds such as tannins, alkaloids, flavonoids, and phenolics, which are essential for enhancing pancreatic function by regulating glucose absorption in the intestines and promoting insulin secretion. As such, medicinal plants present a promising and cost-effective alternative for diabetes treatment, making them an important focus in the search for alternative therapies for the condition. In developing countries, approximately 70–95% of the population relies on herbal medicinal plants for basic healthcare. The World Health Organization (WHO) estimates that around 65–80% of the global population depends primarily on herbal plants to treat various illnesses. This reliance is often due to limited access to modern medical facilities or the perception that herbal plants are a safer and more accessible source of healthcare [80]. Palestine, known for its rich biological diversity, boasts a wide range of traditional herbs used in local medicine. The region is home to over 2,600 plant species, with more than 700 identified as medicinal plants or used as botanical pesticides. A study by Ali-Shtayeh et al. (2000) found that at least 63 plant species are still in active use for treating a variety of ailments, including skin conditions, urinary tract issues, gastric problems, prostate diseases, diabetes and cancer. Further ethnopharmacological studies have highlighted the use of herbal medicine in Palestine for addressing gastrointestinal disorders, urological conditions, scalp diseases, and cancer treatment [81].

1.5.1 *Abelmoschus Esculentus*

Abelmoschus esculentus (A.E), commonly known as okra shown in figure (1.4.1), is a member of the Malvaceae family, native to Africa but extensively cultivated and consumed in Asia, Southern Europe, and the Americas. Traditionally, okra has been used in folk medicine to enhance metabolic health, including in the management of T2DM [82, 83]. The okra fruit contains proteins, fiber, polysaccharides, vitamins, and unsaturated fatty acids, as well as phenolic compounds such as polyphenols, terpenoids, alkaloids, and flavonoids [84]. These phytochemicals contribute to various health benefits, including antioxidant, anti-inflammatory, cardioprotective, gastroprotective, hepatoprotective, and particularly hypoglycemic effects, making okra both nutritionally and functionally valuable [85].

Figure 1.5.1 ***Abelmoschus Esculentus***[86]

1.5.2. *Cichorium intybus*

Cichorium intybus (c. intybus) is a perennial herbaceous plant with a somewhat woody stem, belonging to the family Asteraceae. Typically, it produces vibrant blue flowers, though white or pink variants are infrequent. Common chicory is also referred to as blue daisy as shown in figure 1.4.2. It originates from regions in western Asia, North Africa, and Europe. Root chicory has historically been cultivated across Europe as an alternative to coffee. Its roots are subjected to baking, roasting, and grinding processes for use as an additive. This practice is particularly prevalent in the Mediterranean region, where the plant is indigenous[87]. Recent scientific investigations have elucidated the multifaceted

pharmacological effects of chicory, highlighting its potential as a complementary agent in managing various metabolic and inflammatory disorders[88]. Cichorium intybus demonstrates potential for the regeneration of pancreatic beta cells. It contains bioactive compounds such as antioxidants, phenolics, and flavonoids, which contribute to its antidiabetic properties by addressing hyperglycemia, oxidative stress, inflammation, and hyperlipidemia. The multifaceted effects of chicoric acid on glucose homeostasis are linked to its influence on multiple interconnected biological processes and pathways [89].

Figure 1.5.2 ***Cichorium intybus***[90]

1.5.3 Natural Plant-Based Medicine in Healthcare

Herbal medicine, a key component of complementary and alternative medicine (CAM), has been used for centuries to treat various illnesses and maintain health. CAM encompasses a diverse array of healthcare systems, practices, and products not typically considered part of conventional medicine (AlQathama, 2016). According to the US National Center for Complementary and Alternative Medicine, herbal medicine involves the use of plant-derived products applied or consumed for therapeutic purposes. This ancient practice dates back over 5,000 years, yet scientific validation for its efficacy remains limited in many areas. Herbal medicines are widely utilized across healthcare systems, particularly in developing regions. The World Health Organization (WHO) estimates that approximately 80% of the

population in developing countries, particularly in Africa and Asia, rely on herbal medicine for primary healthcare needs (WHO, 2019).

Furthermore, the popularity of herbal remedies has risen significantly in developed countries over the past two decades. For instance, 75% of the population in France and 42% in the United States report using herbal medicines, with similarly high usage observed in Middle Eastern nations such as Egypt (86%) and Saudi Arabia (88%) [91]. This widespread adoption of herbal medicine has sparked considerable academic, industrial, and economic interest. Notably, chronic conditions such as diabetes mellitus, which are associated with high morbidity and mortality rates, have become a focus for integrating herbal remedies into therapeutic strategies. The global demand for herbal medicines underscores their potential role in modern healthcare while highlighting the need for further research to substantiate their safety and effectiveness [92].

1.5.4 The Role of Medicinal Plants in Diabetes Management

Diabetes is a global health concern, with the World Health Organization (WHO) estimating that 347 million people worldwide were affected by the condition in 2013, a figure projected to double by 2030. More recently, WHO reported that 1.6 million deaths were directly attributable to diabetes, disproportionately affecting middle-income populations in Asia, Africa, and South America [91]. Alarmingly, diabetes claims one life every six seconds, with annual death tolls reaching 5 million surpassing fatalities from HIV (1.5 million), tuberculosis (1.5 million), and malaria (0.6 million) combined. If not properly managed, diabetes can result in severe complications, including damage to vital organs such as the kidneys, heart, eyes, blood vessels, and nerves. The use of herbal remedies by diabetic patients is often motivated by dissatisfaction with conventional treatments and concerns about their potential side effects. Many patients perceive herbal medicines as safer and more effective alternatives due to their natural origins [93]

Medicinal plants have been recognized for their significant role in managing diabetes mellitus, a serious metabolic disorder. These plants are reported to possess potent antidiabetic properties without harmful side effects. They contain

bioactive compounds such as flavonoids, alkaloids, phenolics, and tannins, which enhance pancreatic function by promoting insulin secretion or reducing intestinal glucose absorption. Scientific literature has identified approximately 410 medicinal plants with experimentally validated antidiabetic properties, though the mechanisms of action have been comprehensively studied for only 109 of these plants. Plant extracts have demonstrated the ability to modulate critical metabolic pathways, including glycolysis, gluconeogenesis, the Krebs cycle, glycogen synthesis and degradation, insulin synthesis and release, cholesterol metabolism, and carbohydrate absorption [94].

1.5.5 Phytochemicals

Phytochemicals are naturally occurring bioactive compounds found in plants that offer significant health benefits to humans. These compounds are categorized into primary metabolites, which include proteins, sugars, nucleic acids (pyrimidines and purines), amino acids, and chlorophylls, and secondary metabolites, such as flavonoids, alkaloids, saponins, phenolics, terpenes, curcumins, phytosterols, glucosides, lignans, and anthraquinones.

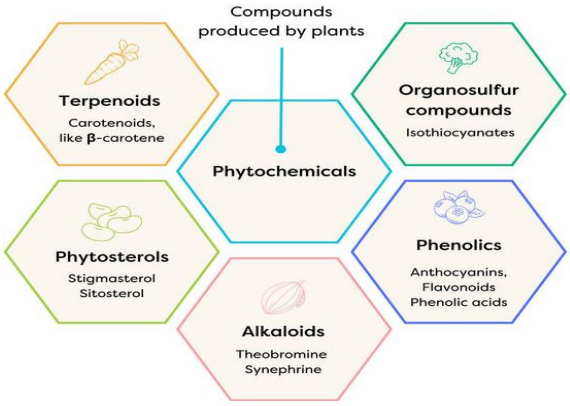


Figure 1.5.5 Phytochemicals in Plants Types [35]

Phytochemicals are found in a variety of plant-based sources, including fruits, vegetables, beverages, and cereals. They are primarily located in plant parts such as stems, roots, flowers, leaves, and seeds, often concentrated in the outer layers of these tissues. The concentration of phytochemicals varies depending on factors

such as the plant species, growing conditions, and methods of processing or cooking. Given their diverse therapeutic properties, medicinal plants containing phytochemicals are invaluable in both complementing and potentially substituting modern medical treatments [95].

1.5.6 Phenolic Compounds in Medicinal Plants

Medicinal plants play a vital role in healthcare worldwide, serving as both standalone remedies and supplements to conventional treatments [96]. For centuries, plants have been utilized for food, flavoring, and medicinal purposes [97]. Various plant parts, possess diverse bioactive properties such as antioxidant and anti-inflammatory activities [98]

Among the most prevalent bioactive components are phenolic compounds, which include phenolic monoterpenes (e.g., carvacrol, thymol), diterpenes (e.g., carnosol, carnosic acid), and a range of phenolic acids like hydroxybenzoic acids (e.g., p-hydroxybenzoic, gallic, vanillic), phenylpropanoic acids (e.g., caffeic, rosmarinic, ferulic, chlorogenic), and flavonoids (e.g., naringenin, quercetin, catechin) [99]. Plant phenolics have gained attention for their potential as effective antibiofilm and antifungal agents [100].

Research has also shown a strong association between the levels of phenolic and flavonoid compounds and the anti-inflammatory and antioxidant properties of medicinal plants. Furthermore, studies indicate that the phenolic content of plants is closely linked to their antioxidant activity [101], and are also considered precursors in the synthesis of pharmaceutical drugs used to treat a wide range of diseases, serving as crucial defense mechanisms to protect against harmful insects, pathogens, and adverse environmental conditions [95].

1.5.7 Anti-diabetic mechanism of natural phenolic compounds

Anti-diabetic mechanisms of natural phenolic compounds have gained significant attention due to their potential therapeutic effects. Phenolic compounds constitute a broad and heterogeneous class of secondary metabolites derived from plants, characterized by the presence of one or more hydroxylated aromatic rings. These compounds are extensively distributed within the plant kingdom and are prevalent

in human diets. This group includes hydroxycinnamic acids such as caffeic acid, ferulic acid, and chlorogenic acid, as well as hydroxybenzoic acids like syringic acid and various benzoic acid derivatives. All of these share a phenolic structural framework and significantly contribute to the antioxidant properties of foods and beverages. Phenolic acids are found abundantly in a variety of dietary sources, including fruits, vegetables, grains, coffee, tea, wine, and other plant-based foods consumed globally [102]. For example, chlorogenic acid and caffeic acid are among the principal polyphenols found in coffee, which epidemiological studies have linked to reduced risk of T2DM, potentially through effects on glucose metabolism, oxidative stress, and insulin sensitivity [103]. Ferulic acid is predominantly found in cereals and whole grains [104], and syringic acid and benzoic acid derivatives are present in berries, nuts, and fermented products [105]. Owing to their structural similarities and widespread dietary occurrence, these phenolic compounds have attracted considerable interest for their antioxidant, anti-inflammatory, and metabolic regulatory properties.

While some studies suggest that phenolics could help manage diabetes, further investigation, particularly with human trials, is necessary to substantiate these claims. More research is needed to understand the effects of pure phenolic compounds or phenolic-enriched diets in the context of diabetes management [106].

1.5.8 Ferulic Acid

Ferulic acid (FA), also known as (E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid, is a phenolic compound commonly found in medicinal herbs and everyday foods. It has been demonstrated to possess several pharmacological effects, including antihyperglycemic, antihyperlipidemic, and antioxidant properties, which contribute to the management of diabetes and its complications. FA exhibits free radical scavenging activities, offering potential benefits in controlling cancer, cardiovascular diseases, as well as in hepatic protection, antimicrobial action, and anti-inflammatory treatments. In particular, its role in preventing and managing diabetes and related complications is well-documented. The antidiabetic effects of

FA are linked to its ability to lower blood glucose levels, enhance antioxidant enzyme activity, and prevent lipid peroxidation in pancreatic tissues in diabetic rats. [107, 108].ferulic acid reverses palmitate-induced insulin resistance by restoring IRS-1 tyrosine phosphorylation, reactivating PI3K and Akt, and increasing GLUT4 translocation [109].

1.5.9 Caffeic Acid

Caffeic acid (CA), also known as 3,4-dihydroxycinnamic acid, is a nonflavonoid phenolic acid naturally present in various herbs, foods, and beverages. As a potent antioxidant, CA helps reduce the formation of reactive oxygen species (ROS). It exerts anti-diabetic effects through multiple mechanisms, including the activation of antioxidant enzymes, inhibition of the nuclear transcription factor-κB (NF-κB) signaling pathway, and stimulation of the nuclear erythroid 2-related factor 2 (NrF2) transcription factor [110]. Caffeic acid has been recognized for its dual role in regulating blood glucose levels, promoting insulin secretion from β cells while also helping to reduce insulin resistance, making it an effective agent in the management of diabetes mellitus [111]. It also inhibits α-glucosidase and α-amylase[69, 70], making it a dual-action antidiabetic agent.

1.5.10 Chlorogenic Acid

Chlorogenic acid, also known as 5-caffeoylquinic acid (5-CQA), is a phenolic compound commonly found in plant-based human diets. It belongs to the hydroxycinnamic acid family and is formed through the combination of caffeic acid and quinic acid. Recent research has highlighted its wide range of Pharmacological properties, including hypoglycemic, hypolipidemic, anti-inflammatory, and antioxidant effects[112]. Specifically, Chlorogenic acid has demonstrated the ability to alleviate and prevent diabetes mellitus (DM) and is beneficial in managing complications associated with DM, such as diabetic nephropathy (DN), diabetic retinopathy (DR), and diabetic peripheral neuropathy (DPN). Chlorogenic acid has also been shown to improve insulin resistance, a primary cause of DM. [113] Studies indicate that chlorogenic acid enhances IRS-1-PI3K-Akt activation and promotes GLUT4 translocation in L6 myotubes. Chronic

administration of Chlorogenic acid to Lepr (db/db) mice improved insulin sensitivity, glucose tolerance, and lipid profiles, while reducing hepatic steatosis and increasing glucose uptake in skeletal muscles. Additionally, Chlorogenic acid activates AMPK, contributing to beneficial metabolic effects such as reduced fatty acid synthesis and hepatic glucose production [114]. Also, as demonstrated [67, 68], chlorogenic acid inhibits both digestive enzymes and enhances glucose metabolism, thereby reducing postprandial glucose spikes.

1.5.11 Syringic Acid

Syringic acid (SA) is an endogenous phenolic compound derived from gallic acid. It is present in various plants, including olives, walnuts, and grapes. SA is recognized for its potent antioxidant, anti-inflammatory, and antimicrobial activities. These properties suggest its potential utility in managing metabolic disorders such as diabetes and cardiovascular diseases[115]. Additionally, SA exhibits chemotherapeutic potential by inhibiting cell proliferation and supporting bone health [116]. It also functions as a microbial metabolite and may serve as a biomarker for specific food sources. Also, improves glycemic control, corrects carbohydrate-metabolism enzymes, and shows potent antidiabetic effects *in vivo* [116].

1.5.12 Benzoic Acid Derivatives

Benzoic acid and its derivatives have garnered significant attention for their potential antioxidant and antidiabetic properties. These compounds exhibit remarkable antioxidant activity, effectively neutralizing free radicals and reducing oxidative stress. Evidence suggests that benzoic acid and its derivatives may provide therapeutic benefits in diabetes management by functioning as both antioxidants and enzyme inhibitors. Classified as a simple phenolic compound in plants, benzoic acid is characterized by its carboxyl group (-COOH) attached to a benzene ring, meeting the criteria for phenolic acids. Hydroxylated benzoic acids like 3,4-dihydroxybenzoic acid, gallic acid. Benzoic acid and its derivatives play vital roles in various physiological processes and act as precursors for more complex phenolic compounds. They are widely recognized for their antioxidant,

antimicrobial, and anti-inflammatory properties, contributing to the plant's defense systems. These attributes further underscore their potential health benefits, including their role in managing diabetes [117].

1.5.13 Vanillic acid

Vanillic acid (VA), a phenolic compound derived from vanillin, is commonly found in various fruits and vegetables. It is well-known for its potent antioxidant and antimicrobial properties, particularly its effectiveness against bacteria, molds, and yeasts. As an intermediate compound in vanillin biosynthesis, vanillic acid also exhibits pharmacological effects such as antimutagenic, antitumor, and anticlastogenic activities, positioning it as a promising nutraceutical agent. Its ability to address key aspects of diabetes pathogenesis—including oxidative stress, inflammation, and glucose metabolism—further underscores its therapeutic potential. Present in numerous antidiabetic plants, vanillic acid significantly contributes to their medicinal properties and stands out as a candidate for further research in diabetes treatment. Notably, studies suggest that vanillic acid may improve insulin signaling by enhancing the expression of GLUT4, a vital glucose transporter in muscle and fat tissues, thereby promoting efficient glucose uptake [118, 119].

1.5.14 D-Pinitol: A Natural Insulin-Sensitizer

D-Pinitol has shown direct activation of PI3K and Akt, enhancement of GLUT4 translocation, and improved glycemic outcomes *in vivo* [120].

1.5.15 Fatty Acids: Palmitic, Stearic, and Myristic Acid

Palmitic acid, stearic acid, and myristic acid are long-chain saturated fatty acids commonly found in both animal and plant foods. Palmitic acid (C16:0) is one of the most abundant saturated fatty acids in the human diet, occurring in high amounts in palm oil, dairy products, meat, and cocoa butter, and it also comprises a substantial proportion of total fatty acids in the body and human milk fat. Stearic acid (C18:0) and myristic acid (C14:0) similarly occur in foods such as milk fat, butter, cocoa butter, and coconut oil, albeit typically at lower relative levels than palmitic acid [121].

These saturated fatty acids are not antidiabetic agents; rather, palmitic acid is a key inducer of insulin resistance via IRS-1 dysregulation and inflammatory signaling. The metabolic effects of palmitate and its role in insulin resistance is well documented [122].

1.6 The aim of the study

The aim of this study was to evaluate the antidiabetic activity of fourteen selected natural compounds. The study investigates the potential effect of these compounds as hypoglycemic agents *in vitro* and *in vivo* and examines their mechanism of action. The compounds were selected based on their potential activity in preliminary tests on anti-diabetic medicinal plant crude extracts and based on published data elsewhere. The natural compounds are: Caffeic acid, ferulic acid, isovanillic acid, vanillic acid, chlorogenic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid.

Specific aims:

1. Testing the toxicity of the natural compounds using MTT assay, for identifying the safe concentration, for further testing.
2. To elucidate the mechanism underlying the response of skeletal muscle to natural anti-diabetic candidate compounds, and to examine the acute effects of these compounds on glucose uptake and on the potential mechanism for integration of insulin signaling. L6-GLUT4myc, the glucose transporter-4 (GLUT4) with a myc epitope at the exofacial loop, is used to monitor GLUT4 translocation to the PM in L6 skeletal muscle cells.
3. To identify the protein target(s) in the insulin signaling pathway for the active natural compound(s).
4. To evaluate the antihyperglycemic activity of the natural compounds *in vivo* in a diabetic mice model.
5. To evaluate the inhibitory potential of the natural compounds on the digestive enzymes α-amylase and α-glucosidase.

2. Materials and Methods

2.1 Instruments and Materials

Table 2.1.1 Instruments used

Instruments	**company**
Biosafety cabinet	Biobase
CO_2 incubator	Memmert
Centrifuge	Hettich
Inverted microscope	Olympus-CKX53
Elisa reader	Bio-Rad
q-PCR	Thermo fisher scientific
PCR	Analytik Jena
Heat block	Gni lab
ChemiDoc XRS gel system	Bio-Rad
Microscope DMi8	Leica
Spectrophotometer	Jenway
Nanodrop spectrophotometer	Thermo fisher scientific
Refrigerated centrifuge	Hettich
Analytical balance	ae ADAM
Orbital shaker	Bellco
pH meter	Hanna

Table 2.1.2 Materials used

Materials	**Company**
Acrylamide/bis-acrylamide	Sigma
Acarbose	Sigma
Anti-AKt	Sigma
Anti-β-Actin (ACTB) Antibody	Sigma
Anti-c-myc antibody	Sigma
Anti-rabbit IgG	Sigma
Amphotericin B solution	Sigma
AS160 Primer F 5`	Thermo fisher scientific
AS160 Primer R 5	Thermo fisher scientific
AS160	Cell signaling
Benzoic acid	Thermo Scientific
Citric acid	Sigma
Chlorogenic acid	Sigma
Caffeic acid	Alfa Aesar
Dapi	Sigma
Ethanol	Sigma

Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma
Ether	Sigma
Fetal Bovine Serum	Biological Industries
Ferulic acid	Sigma
Fluorescence mounting medium	Sigma
Glycine	Sigma
GLUT4 Primer F 5`	Thermo fisher scientific
GLUT4 Primer R 5`	Thermo fisher scientific
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	Thermo fisher scientific
Glycero	Sigma
Goat serum	Gibco
L-Glutamine solution Sigma	Sigma
HEPES, sodium salts	Sigma
High-capacity cDNA reverse transcription kit	Applied Biosystems™
House Keeping Gene F 5(18S rRNA)	Thermo fisher scientific
House Keeping Gene R 5(18S rRNA)	Thermo fisher scientific
IGEPAL	Sigma
Insulin solution from bovine pancreas	Sigma
Isopropanol	Sigma
Intestinal α-glucosidase enzyme	Sigma
β-Mercaptoethanol	Sigma
Methanol	Sigma
Minimum Essential Medium Eagle	Sigma
Myristic acid	Alfa Aesar
MEM- non-essential amino acid	Sigma
Non-fat dry milk	Cell signaling
3,5-dinitrosalicylic acid	Sigma
O-Phenylenediamine (OPD)	Sigma
Okadaic acid	Sigma
Palmitic acid	Thermo Scientific
Pancreatic α-amylase enzyme	Sigma
Paraformaldehyde	Sigma
Penicillin-streptomycin solution	Sigma
Phosphate buffer saline	Sigma
Phospho-AS160(Thr642) Polyclonal antibody	Thermo fisher scientific
Phospho-IRS1 (Tyr612) polyclonal antibody	Thermo fisher scientific
Phospho-PTEN (Ser380, Thr382, Ser385) Polyclonal Antibody	Thermo Scientific
Protease inhibitor	Sigma
D-pintol	Alfa Aesar

p-nitro-phenyl α-D-glucopyranoside	Sigma
PageRuler™ Plus Prestained Protein Ladder, (10 to 250) kDa	Thermo fisher scientific
PTEN Primer F 5(for 177bp product)	Thermo fisher scientific
PTEN Primer R 5(for 177bp product)	Thermo fisher scientific
PVDF membrane	GE Healthcare Life Science
Rabbit anti-rabbit IgG Secondary antibody, HRP	Thermo fisher scientific
IRS1 Polyclonal Antibody	Thermo fisher scientific
IRS1 Primer F 5`	Thermo fisher scientific
IRS1 Primer R 5	Thermo fisher scientific
Sodium Dodecyl Sulfate	Sigma
Sodium fluoride	Sigma
Sodium orthovanadate	Sigma
Sodium phosphate	Sigma
Sodium sulfate	Sigma
Na_2CO_3	Sigma
SYBR® Green master mixes	Thermo Fisher Scientific
Stearic acid	Alfa Aesar
Super Signal™ West Pico PLUS Chemiluminescent Substrate	Thermo fisher scientific
Syringic acid	Thermo Scientific
Streptozocin	Sigma
Tetramehylethylene diamine	AppliChem
Thiazolyl blue tetrazolium bromide MTT	Sigma
Triton x	Sigma
Trypsin	Sigma
Tween 20	Sigma
Tris-HCL	Sigma
Trizma® base - 2-Amino-2-(hydroxymethyl)-1,3-propanediol	Sigma
Trizol reagent	Thermo Scientific
Vanillic acid	Thermo fisher scientific
3,4-Dihydroxy benzoic acid	Alfa Aesar
3,4,5-Trihydroxybenzoic acid	Thermo fisher scientific
2-Hydroxy-4-methoxybenzoic acid	Thermo Scientific

2.2 Buffers

Table 2.2.1 Buffers for gel electrophoresis

Buffers	**Compositions**
SDS-Sample buffer (5X)	62 mM Tris, 2 % (w/v) SDS 5 % (v/v) β-Mercaptoethanol 20%(w/v) Glycerol 0.2 % (w/v) Bromophenol blue
SDS-PAGE running buffer	25 mM Tris 192 mM Glycine 0.1 % (w/v) SDS%
Transfer Buffer for Western blot	39 mM Glycine 48 mM Tris base 20% (v/v) Methanol
TBS buffer	20 mM Tris base pH 7.6 137 mM NaCl
TBS-T buffer	TBS buffer 0.1% (w/v) Tween 20
Blocking buffer	TBS-T buffer 5% (w/v) non-fat milk

Table 2.2.2 Sodium Dodecyl Sulphate–Polyacrylamide Gel

Components	**10 % resolving gel (20 ml)**	**5 % stacking gel (5 ml)**
H_2O	7.9 ml	3.4 ml
30% Acrylamide	6.7 ml	0.83 ml
1.5 M Tris, pH 8.8	5 ml	-
1 M Tris, pH 6.8	-	0.63 ml
10 % SDS	0.2 ml	0.05 ml
10 %Ammonium Persulfate	0.2 ml	0.05 ml
TEMED	0.008 ml	0.005 ml

2.3 Plant Extract Preparation

The methanolic extract of dried *Abelmoschus esculentus* fruit, aerial and the root parts of *Cichorium intybus* were prepared as follows: fifty grams of the dried part of each plant were powdered and incubated in Erlenmeyer flask with 100 mL methanol. The flasks were then sonicated for 2 h at 60 °C and further incubated in dark glass bottles for 24 h for complete extraction. The stock extracts were kept at −20°C in air-tight glass containers [123].

2.4 L6 Cell Culture and Treatment

Rat L6 muscle cell line that stably expressing myc-tagged GLUT4 (L6-GLUT4myc) obtained from Kerafast (Boston, MA, USA) and were cultured in a-MEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an atmosphere of 95% air and 5% CO2. For every experiment, L6 cells that were approximately 80% confluent were collected or subcultured [123].

2.5 MTT Assay

The colorimetric tetrazolium dye MTT technique depends on the process by which mitochondrial succinate dehydrogenase in living cells changes the yellow tetrazolium bromide into its purple formazan derivative. In 96-microtiter plate wells, 20,000 cells were seeded and incubated for 24 h. Subsequently the cells were incubated with increasing concentrations (0 mg/mL, 0.125mg/mL, 0.250 mg/mL, 0.500 mg/mL, 1 mg/mL) of plant extracts and the natural active compounds (0 mg/mL, 0.125mg/mL, 0.250 mg/mL, 0.500 mg/mL, 1 mg/mL): Caffeic acid, ferulic acid, isovanillic acid, vanillic acid, chlorogenic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid for 1 or 24 h at 37 °C. Then the cells were maintained in serum-free MEM media for 3 hours. Next, 100 µL contained 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were added to each well, and the plates were incubated for 4 hours at 37 °C in the dark. Following the removal of the media, the cells were treated with 100 µL of acidic isopropanol (0.08 N HCl) for 15 minutes in

order to dissolve the formazan crystals. Using a microtiterplate reader, the MTT formazan's absorbance was measured at 570 nm [124].

% Cell viability: $\frac{A570\ nm\ of\ treated\ sample}{A570\ nm\ of\ nontreated\ sample} \times 100$

2.6 GLUT4 Translocation to the PM

Surface myc-tagged GLUT4 was measured in intact cells as described previously [125]. Briefly, L6-GLUT4 myc cells were seeded in 24 well-plates and incubated for 24 h. A. esculentus, C. intybus extracts and active compounds: Caffeic acid, ferulic acid, isovanillic acid, vanillic acid, chlorogenic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid were added to the cells for 24 h for overnight experiments or 0.5 and 1 h for short exposure experiments. After serum starvation for 3 h the samples treated with or without 100 nM insulin for 20 min. The cells were washed twice with ice-cold PBS, fixed with 3% paraformaldehyde for 15 min, then blocked with 3% (v/v) goat serum for 10 min, incubated with polyclonal anti-myc antibody (1:200) for 1 h at 4 ◦C, washed ten times with PBS and incubated with goat-anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:1000) for 1 h at 4 ◦C, then washed ten times with PBS at room temperature. Half milliliter of o-phenylenediamine dihydrochloride reagent was added to each well. And the plates were incubated in the dark at room temperature for 20–30 min. The reaction was stopped by adding 0.25 mL of 3 M HCl. The absorbance was measured by using a spectrophotometer at 492 nm. Background absorbance obtained from 3 wells in each 24-well plate untreated with anti-myc antibody was subtracted from all values.

2.7 Immunostaining of Surface GLUT4

L6-GLUT4myc cells were grown on 18mm-diameter glass coverslips in 12 well plates and incubated for 24 h. Selected natural compounds were added to the cells either for 0.5 or 24 h and followed by serum starvation for 3 h and treated with or without 100 nM insulin for 20 min at 37 °C. The cells were washed twice with ice

cold phosphate buffer saline (PBS), then incubated with polyclonal anti-myc antibody (1:200) for 1h at 4◦C. Then the cells were washed twice with ice-cold PBS, and the antibody blocked with 5% (v/v) goat serum for 15 min. The cells were incubated with CY3-conjugated goat anti-rabbit IgG (1:750 dilution) with 1 µg/ml Dapi solution to stain also cell nuclei for 30-45 min, washed five times with PBS and mountd coverslip up site down on slides with a drop of mounting medium. Cells were examined with a DMi8 microscope.

2.8 IRS1, AKT, PETEN, and AS160 protein levels and phosphorylation in L6-GLUT4-myc cells detection

2.8.1 L6-myc GLUT4 cells lysis

Lysates were prepared from the incubated pretreated cells with natural compounds for 24 h or 0.5 h. Briefly, cells were rinsed with ice-cold PBS, the PBS was aspirated completely, and cells proceed quickly to cell lysis buffer. (1 % (v/v) Nonidet P-40, 10%Glycerol, 1 mM EDTA,87 mM NaCl, 50 mM NaF, 20 mM HEPES, pH 7.5, 1:500 dilution of Protease Inhibitor Cocktail, 10 nM okadaic acid, 1 mM sodium orthovanadate) then the cell monolayer scraped with a cell scraper and the suspension was transferred to a 1.5 mL microtube and place on ice for 10 min. Lysates were then centrifuged at 18,000 x *g* for 15 min and frozen at -20°C. Protein concentration in lysates were determined on the supernatants using the Bradford method.

2.8.2 Estimation of Protein concentration in lysates

Protein concentration was determined using the Bradford method. A series of Bovin serum albumin (BSA) protein solutions with known concentrations were prepared and added to separate cuvettes (0.2, 0.5, 1.0, 2.0 mg/mL). In parallel, 10, 20 and 50 □l of each lysate was added to other cuvettes. All tubes volumes were set to 0.5 mL with distilled water. Equal volume (500 µL) of Bradford reagent were added to each cuvette and incubated for 15min. The absorbance of each standard and sample was measured at 595 nm using a spectrophotometer. The absorbance values of the standard BSA solutions were plotted against their known concentrations to create a standard curve. linear regression was used to calculate

the concentration of protein in unknown Lysates based on their absorbance values. The equation of the line (y = mx + b) was used where y is the absorbance, m is the slope, x is the concentration, and b is the y-intercept [126].

2.8.3 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely employed technique for the separation of proteins based on their molecular weight [127]. To initiate the process, protein samples collected during the lysis were mixed with 4X SDS loading buffer and incubated at 70 °C For 10 minutes. This step ensures denaturation of the proteins and allows sodium dodecyl sulfate (SDS) to bind to the positively charged and hydrophobic residues, facilitating uniform migration. The prepared samples were loaded onto a 10% polyacrylamide gel and subjected to electrophoresis at 80V voltage for the stacking gel and 120V voltage for the resolving gel, until then front dye migrated out of the gel. To determine protein sizes, a prestained protein marker was included as a reference.

2.8.4 Western Blotting

Western blotting, a widely utilized analytical technique in protein analysis, enables the detection and characterization of specific proteins within complex biological samples [127]. Following the separation of proteins through SDS-PAGE, the transfer of proteins from the gel onto polyvinylidene fluoride (PVDF) membranes was accomplished using the western blotting system to minimize non-specific binding, the membranes were incubated in a blocking solution composed of 5% non-fat dry milk dissolved in TBS-T buffer at 4 °C overnight with gentle shaking. The membranes were subsequently washed two times with TBS-T buffer at room temperature and incubated with the first antibody to be tracked (Insulin Receptor Substrate 1 (IRS1), Protein Kinase B (AKT), phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN), and Akt substrate of 160 kDa (AS160)) or with anti-phosphorylation formula of the antibody for 1 h. Then the membranes were washed 10 times with TBS-T buffer, and incubated in Goat anti-rabbit IgG Secondary antibody, HRP for 1 h. The membranes were then rinsed 5 times in TBS-T. The bands were detected via Electrochemiluminescence (ECL), and the

resulting chemiluminescent signal was captured using the ChemiDoc MP Imaging System.

2.9 RNA Extraction

2.9.1 L6-GLUT4myc cells treatment

3000,000 L6-GLUT4 myc cells were seeded per each well of 6 well plates and incubated for 24 h until the cells were confluent. Next, 0.125 mM caffeic acid, ferulic acid, benzoic acid, 3,4 dihydroxy benzoic acid, 3,4,5 trihydroxy benzoic acid and 2-hydroxy4-methoxy benzoic acid were added to the cells for 24 h or 0.5 h followed by serum starvation for 3 h. Subsequent treatment with and without 100 nm insulin for 20 min. The cells were washed twice with ice-cold PBS, collected and stored at -80C [123].

2.9.2 Lyse samples and separate phases

After relocating the samples of treated cells under the hood, 500 µl of Trizol reagent was added to each sample, which was then vortexed and placed on ice for a period of 10 minutes. After a 5-minute centrifugation at 12,000 × g at 4°C, the clear supernatant was transferred to a new tube and the process was repeated. The formed supernatant layer was then removed, and 200 µl of chloroform was added to each tube, thoroughly mixed by shaking, and incubated for 5 minutes to allow the nucleoprotein complex to completely dissociate. In order to improve separation into a lower red phenol-chloroform layer, an interphase, and a colorless upper aqueous phase, the centrifugation period was extended to 15 min at 16,000 × g at 4◦C [128].

2.9.3 Isolation and precipitation of RNA

250 µl of precooled isopropanol was added to the aqueous phase. It was then incubated for 15 minutes at 4°C to ensure the RNA precipitates sufficiently, and centrifuged for 15 minutes at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The supernatant was discarded [128].

2.9.4 RNA Washing and Solubilizing

After resuspending the pellets in 500 µl of 75% ethanol for every 500 µl of Trizol lysis reagent, the sample underwent a brief vortex and was centrifuged for 5 minutes at 12,000 × g at 4°C. After using a pipettor to discard the supernatant, the RNA pellet was allowed to air dry for five to ten minutes. After adding 50 microliters of Diethyl Pyrocarbonate-treated water (DEPC) water to resuspend the pellet, the quality of each RNA extraction was verified using the Nanodrop Spectrophotometer, based on the ratios of absorbance between A260 nm and A280 nm. At -80°C, it was finally stored [128].

2.10 cDNA Synthesis

First-strand cDNA synthesis was performed via reverse transcription using the High-capacity cDNA reverse transcription kit (Applied Biosystems™, Life Technologies, Darmstadt, Germany). The amount of total RNA from each methanolic extract added to each cDNA reaction was always quantified using Nanodrop to reach 1000 ng. The RT master mix for each sample was prepared on ice as follow: 2 µl of 10× RT Buffer, 0.8 µl of 25× dNTP Mix (100Mm), 2 µl of 10x RT Random Primer, 1 µl Multi RT were added and completed to 10 µl with RNase Free Water then it was mixed gently. 10 µL of 2× RT master mix with10 µL of each RNA sample, by pipetting, tubes were then sealed and spin down. Samples were run with the PCR thermal cycling conditions as follows: 25°C for 10minute, 37°C for 120minute and 85°C for 5minute. The generated cDNA samples were diluted ten-fold using RNAse-free water and used as a template for RT-PCR analysis [129].

2.11 q Real time Polymerase reaction (q RT-PCR)

Syber Green Master Mix (Applied Biosystem, Thermo Fisher Scientific) was used to determine the mRNA expression for each cDNA sample, and the reactions contained 10µl of Syber Green Master Mix, 1µl of each primer (Biotech), 3µl of cDNA template, and RNase free water to a reaction volume of 20 µl. The cycling conditions were performed on a quantitative PCR machine (Bio Rad) as follow: a 10min initiation of Taq polymerase activity at 95°C, 35 cycles of PCR amplification

at 95°C for 15 s, and a 1 min annealing/elongation step at 60°C. Primers sequences used were as follows:

Housekeeping Gene: Rat 18S rRNA F: 5’ GTAACCCGTTGAACCCCATT

3’R: 5’ CCATCCAATCGGTAGTAGCG 3’

Rat AS160 F: 5’-AGAAGGGGTCCCTAAAAGTCGGC-3’

R: 5’-GTTGGGCAATCTGTGTCTCAGGC-3’

Rat GLUT4 F: 5`- GTGTGGTCAATACCGTCTTCACG 3`

R: 5`-CCATTTTGCCCCTCAGTCATTC 3`

Rat IRS1 F: 5`ATGGCGAGAGCCCTCCGGATACC 3`

R:5`CTCATAATACTCCAGGCGCGC 3`

Rat PTEN F: 5’-CAGAAGAAGCCCCGCCACCAG-3’

R; 5’-AGAGGAGCAGCCGCAGAAATG-3’

Rat AKT1 F: 5` GAGGGAAGAATGGACGAAAG 3`

R: 5` TTCCCAGGAGTTTGAGGTAT 3`

Rat AKT 2 F: 5` CATCCCTTTCTAACAACACTA 3`

R: 5` CTGTAAACAACATTGCGTGA 3`

Relative quantification results obtained for the target genes were normalized using the two to the delta-delta C-T (2-ΔΔCT) method [130].

2.12 Animals

C57BL/6 mice, often referred to as (C57 black) males, 4-6 weeks old, 25-30 g were used at the animal unit of Prof. Zaid at the Arab American University, Jenin. All mice were housed in (a 25×30×30 cm cage) under healthy conditions at 21-24°C with 40-60% humidity and a 12 h light/dark cycle. All mice were fed standard rodent chow and filtered water and acclimated for 2 weeks before the beginning of the

experiment. All procedures were approved under the principles of animal welfare according to Arab American University.

2.13 Induction of diabetes

Streptozotocin (STZ), a substance that exhibits a preference for toxicity toward pancreatic cells, is used to cause diabetes in mice. Streptozotocin preparation was done daily for immediate injection by freshly dissolving in sodium citrate buffer in the morning. Mice get an injection of the diabetic medication at a dose of 50 mg/Kg body weight for 5 days. During this time, the diabetic mice were fed on a (10%) sucrose diet that was added to their water [123].

For the experiment, mice were divided into four groups (n=8/group) for each treatment as shown below.

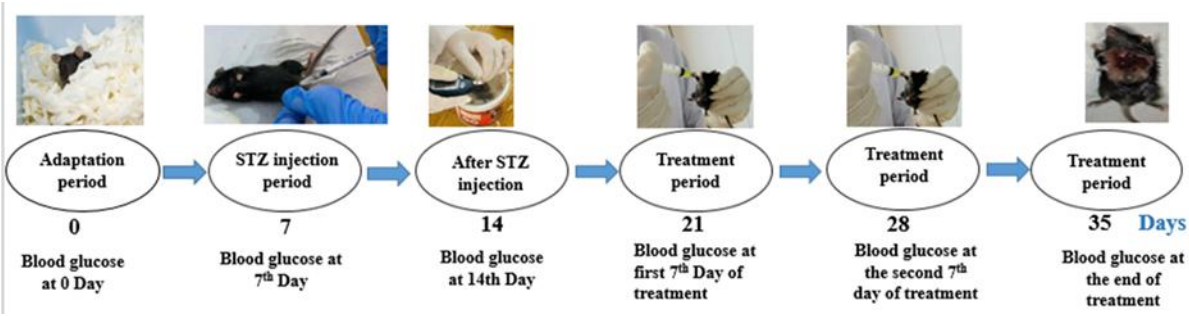


Figure 2.13 The timetable of the animal experiment.

Group I: Control mice kept under normal dietary conditions.

Group II: Control Mice fed (oral gavage) continuously once a day for 21 days with selected natural compounds dosage of (100mg/Kg).

Group III: Diabetic control mice

Group IV: Diabetic mice fed (gavage) with selected natural compound at the above-mentioned dosages for 21 days [123].

On the 9th-day post STZ injection blood glucose level was be monitored. Mice were conceded diabetic if their blood glucose level was above 200 mg/dL. Then

treatment of each natural compounds (caffeic acid, ferulic acid, isovanillic acid, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid and 2-hydroxy4-methoxy benzoic acid) was introduced to the desired group with a concentration of 100mg/Kg (Figure 2.13).

2.14 Blood glucose monitoring

The blood glucose levels of all groups were checked first after the STZ, a diabetes agent, had been injected into mice for 5 days (on the 9th day) as show in figure 2.13, then throughout the trial (10 days after the mice had been given the natural compounds), and lastly after 21 days of natural compounds administration (by gavage). Blood samples were taken from the tail vein using a digital glucometer and a glucose strip test[123] .

2.15 α-Amylase Inhibitory Method

Pancreatic α-amylase inhibition assay was performed according to Adisakwattana et al. [131]. Using acarbose as a positive control. Pancreatic α-amylase (4 units/mL) was dissolved in 0.1 M sodium phosphate buffer, pH 6.9. Ferulic acid, isovanillic acid, vanillic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid, or acarbose as a positive control of different dilutions (0.5mM, 1mM, 8mM, 10mM) was preincubated with 250 µL of the enzyme solution at 37°C for 10 min. The reaction was initiated by adding 500 µL of the substrate solution (1%starch in 0.1 M sodium phosphate buffer, pH 6.9). After 5 min of incubation, the reaction was stopped by adding 1 mL of 96 mM 3,5-dinitrosalicylic acid solution to the reaction mixture. The mixtures were heated at 100 °C for10 min to stop the reaction and then cooled to room temperature in a cold-water bath. Subsequently, the reaction mixtures were diluted 10 times with distilled water. The absorbance was recorded at 540 nm using a spectrophotometer. The α-amylase inhibitory potential was calculated utilizing the following equation:

I (%) = (ABS blank – ABS test) / ABS blank ∗ 100%

where I (%) is the α-amylase inhibitory percentage.

2.16 Intestinal α-Glucosidase Inhibitory Method

The assay of intestinal α-glucosidase inhibitory activity was performed according to Kim et al [132] with a slight modification. The reaction mixture consisting of natural active compound, or acarbose as a positive control at varying concentrations was premixed with 100 µL of 0.1 M sodium phosphate buffer, pH 6.9. 15 µL of α-glucosidase (0.1 unit/µL) was added and preincubated at 37 ° C for 10 min. The reaction mixture was set to 750 µL with distilled water. The reaction was initiated by adding 250 µL of 20 mM p-nitrophenyl α-D-glucopyranoside and further incubated for10 min. The reaction was terminated by the addition of100 µL of 0.1 M Na_2CO_3. The amount of released product (p-nitrophenol) was measured at 405 nm using a spectrometer. Intestinal α-glucosidase enzyme inhibitory potential was measured utilizing the following equation:

I (%) = (ABS blank – ABS test) / ABS blank ∗ 100%

where I (%) is the α-glucosidase inhibitory percentage.

2.17 Data analysis

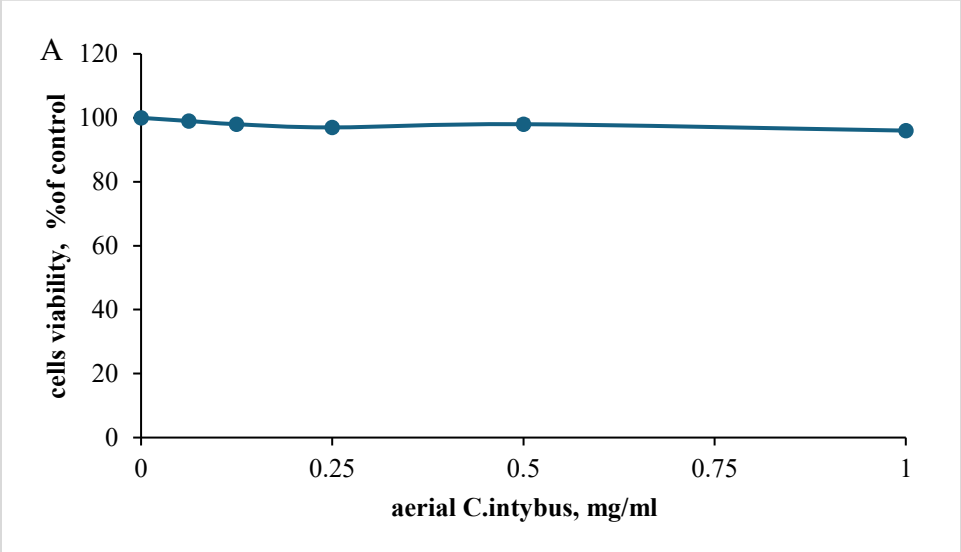
The data was expressed as mean ± standard error (SE), the t-test was used for the statistical analysis and significance measures ($P<0.05$) were considered to be statistically significant. SPSS Program version 23 was used to analyze the results.

Curves connecting data points in figures are spline functions if not indicated otherwise.

3. Results

3.1 MTT cell Viability Test for *Abelmoschus esculentus* and *Cichorium intybus* extracts

To evaluate the potential cytotoxic effects of A. esculentus and C. intybus extracts on L6- GLUT4-myc cell lines the MTT assay was utilized. This colorimetric assay measures cell metabolic activity by converting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble formazan crystals via mitochondrial enzymes in viable cells. L6-GLUT4-myc cells were treated with methanolic extracts of A. esculentus fruits and C. intybus aerial and root parts.



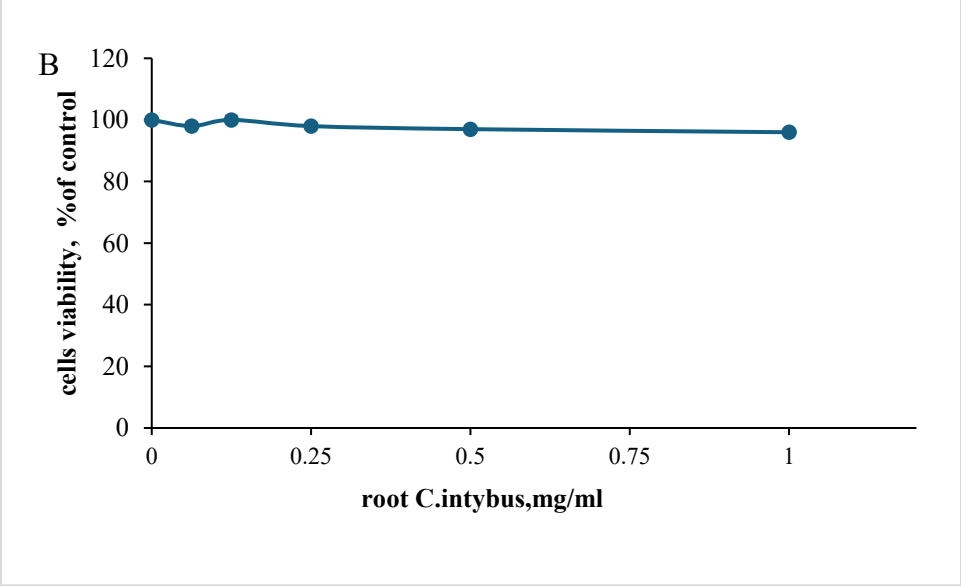


Figure 3.1.1 Effect of methanol C. intybus extracts on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mg/ml) for 24 h. (A) C. intybus aerial part, (B) C. intybus root part. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

The cells were treated with A. esculentus and C. intybus extracts up to 1 mg/ml for 24 hours. Following the incubation, the optical density of the dissolved formazan crystals was measured at 570 nm using a microplate reader The concentration of these plants extracts that led to less than 10% of cell death were consider safe, non- toxic concentration [102].

C. intybus both aerial and root part extracts were non-toxic up to 0.125 mg/ml while A. esculents fruit extract was non- toxic up to 0.250 mg/ml (figures 3.1.1 and 3.1.2).

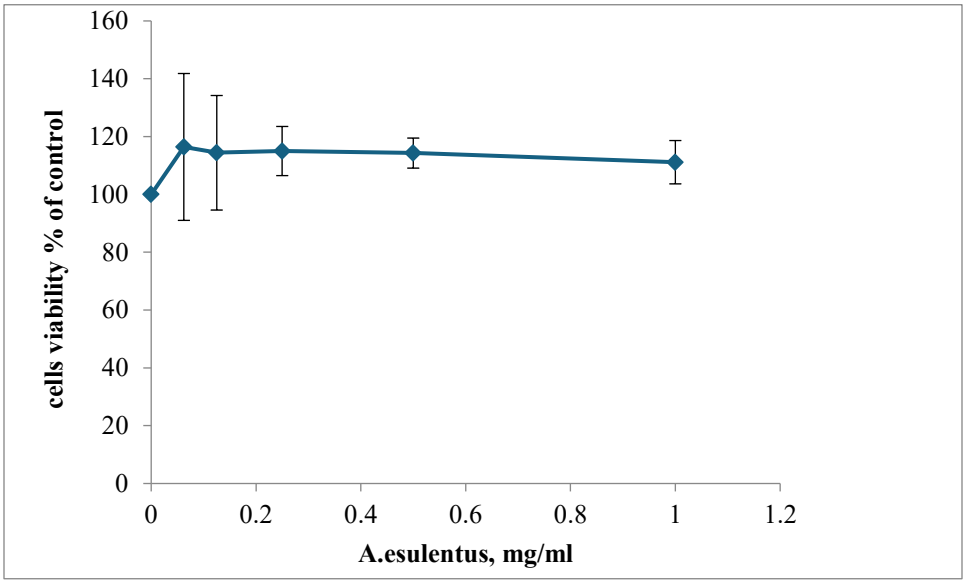


Figure 3.1.2 Effect of methanol A. esculentus fruit extract on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mg/ml) for 24 h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

Following laboratory screening tests on methanol extracts of *Abelmoschus esculentus* (A. esculentus) and *Cichorium intybus* (C. intybus), a Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted to examine the chemical composition of these two plants. Consequently, 14 phytochemicals were chosen for this study as they possess potential anti-diabetic activity according to the literature

3.2 MTT Cell Viability Test for Natural pure compounds

To assess the cytotoxicity of potential natural anti-diabetic compounds on L6-GLUT4-cells, the MTT assay was utilized. This colorimetric assay measures cell metabolic activity by converting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble formazan crystals via mitochondrial enzymes in viable cells. L6-GLUT4-myc cells were treated with the following active compounds: Caffeic acid, ferulic acid, isovanillic acid, vanillic acid, chlorogenic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid.

The L6 muscle cells were treated with pure compound at 0.0625 mM, 0.125 mM, 0.250 mM, 0.5 mM, and 1 mM for either 1 or 24 hours. Following the incubation, the optical density of the dissolved formazan crystals was measured at 570 nm using a microplate reader. Natural compounds concentrations that led to less than 10% of cell death were considered safe, non-toxic concentrations (Table 3.2.1 and Figures 3.2.1-3.2.14).

Caffeic acid, ferulic acid and palmitic acid were non-toxic up to 0.125 mM at 1 h exposure (figures 3.2.1, 3.2.2 and 3.2.8). While isovanillic acid, stearic acid, benzoic acid, 3,4-dihydroxy benzoic acid and 2-hydroxy4-methoxy benzoic acid were non-toxic up to 0.250 mM at 1 h exposure (figures 3.2.3, 3.2.9, 3.2.11, 3.2.12 and 3.2.14). However, Chlorogenic acid, D-pinitol, myristic acid and syringic acid were non-toxic up to 0.5 mM at 1 h exposure (figures 3.2.5, 3.2.7, 3.2.10 and

3.2.6). Vanillic acid and 3,4,5 trihydroxy benzoic acid were non-toxic up to 0.0625 mM at 1h exposure (figures 3.2.4 and 3.2.13).

Caffeic acid, chlorogenic acid, stearic acid, benzoic acid, 3,4dihydroxy benzoic acid and 2-hydroxy4-methoxy benzoic acid were safe up to 0.250 mM at 24 h exposure (figure 3.2.1, 3.2.5, 3.2.9, 3.2.11, 3.2.12 and 3.2.14). Whilst ferulic acid, isovanillic acid, palmitic acid, myristic acid and 3,4,5 trihydroxy benzoic acid were safe up to 0.125 mM at 24 h exposure (figure 3.2.2, 3.2.3, 3.2.8, 3.2.10 and 3.2.13). Vanillic acid and D-pintol were safe up to 0.5 mM at 24 h exposure (figure 3.2.4 and 3.2.7).

Table 3.2.1 Safe (non-toxic) maximal concentrations of the tested pure phytochemicals in L6-GLUT4myc cells after 1- and 24-h exposure.

Compounds name	Maximal safe concentration	
	at 1 h exposure	at 24 h exposure
Caffeic acid	0.125 mM	0.250 mM
Ferulic acid	0.125 mM	0.125 mM
Isovanillic acid	0.250 mM	0.125 mM
Vanillic acid	0.0625 mM	0.5 mM
Chlorogenic acid	0.5 mM	0.250 mM
D-pintol	0.5 mM	0.5 mM
Palmitic acid	0.125 mM	0.125 mM
Stearic acid	0.250 mM	0.250 mM
Myristic acid	0.5 mM	0.125 mM
Benzoic acid	0.250 mM	0.250 mM
3,4 dihydroxy benzoic acid	0.250 mM	0.250 mM
3,4,5 trihydroxy benzoic acid	0.0625 mM	0.125 mM
2-hydroxy 4-methoxy benzoic acid	0.250 mM	0.250 mM
Syringic acid	0.5 mM	0.125 mM

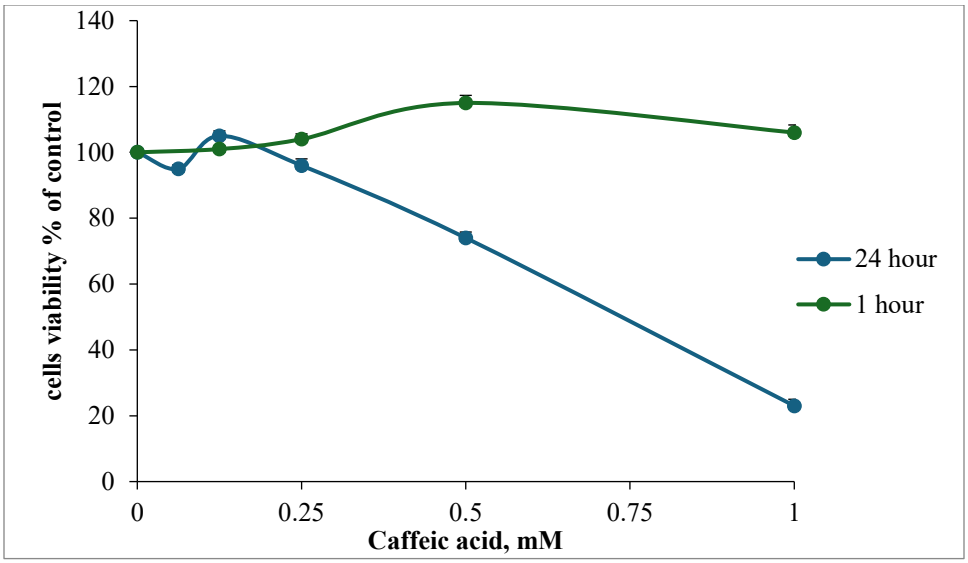


Figure3.2.1 Effect of caffeic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

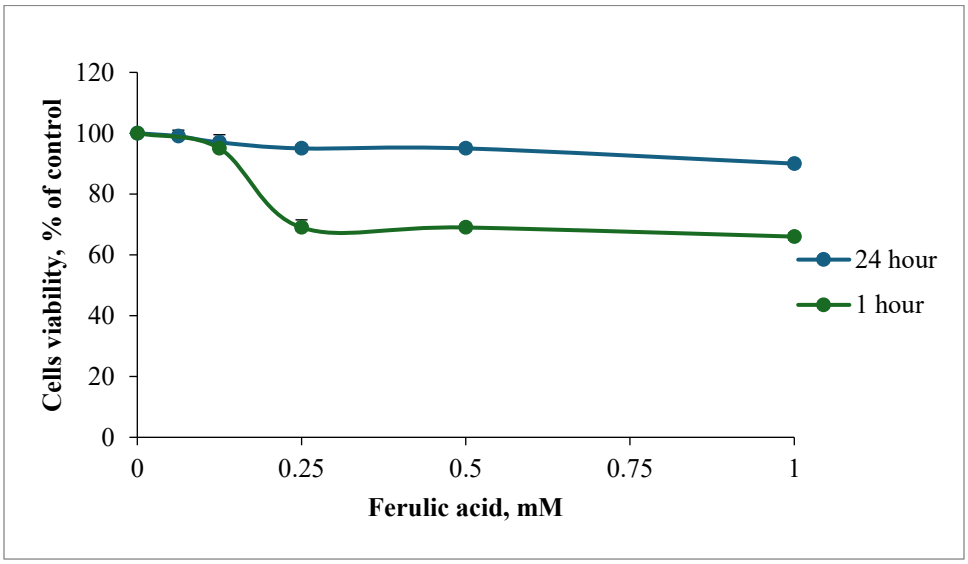


Figure 3.2.2 Effect of ferulic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

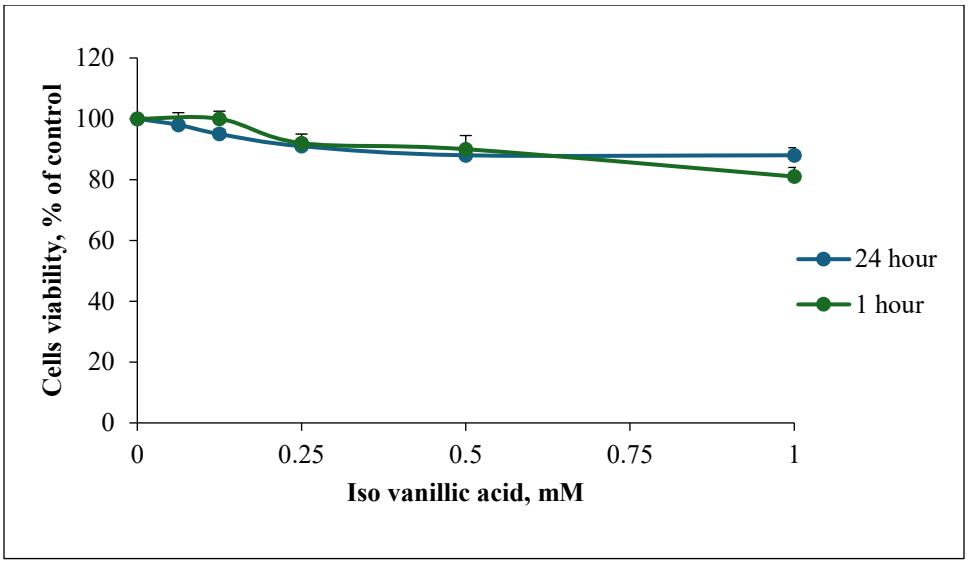


Figure 3.2.3 Effect of Iso vanillic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

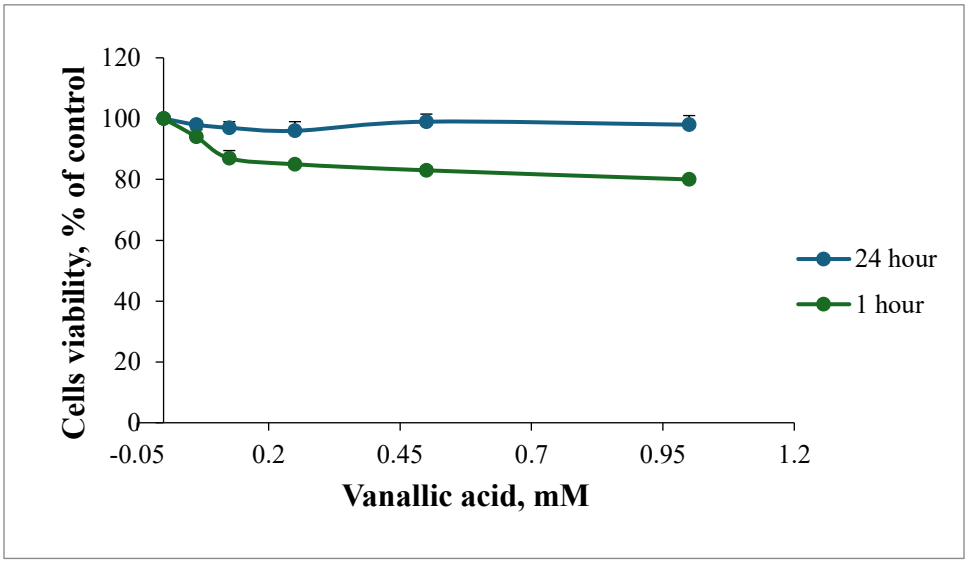


Figure 3.2.4 Effect of Vanillic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

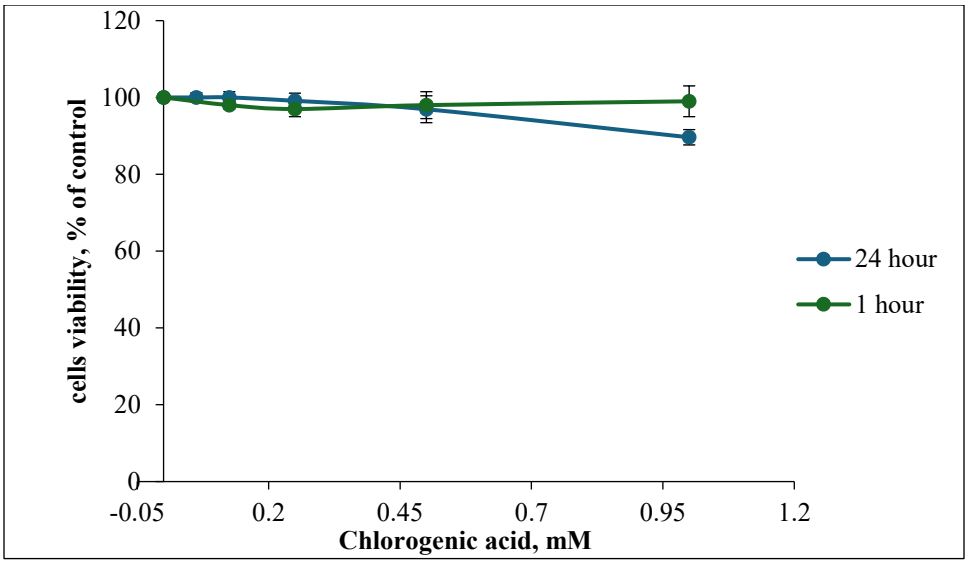


Figure 3.2.5 Effect of Chlorogenic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

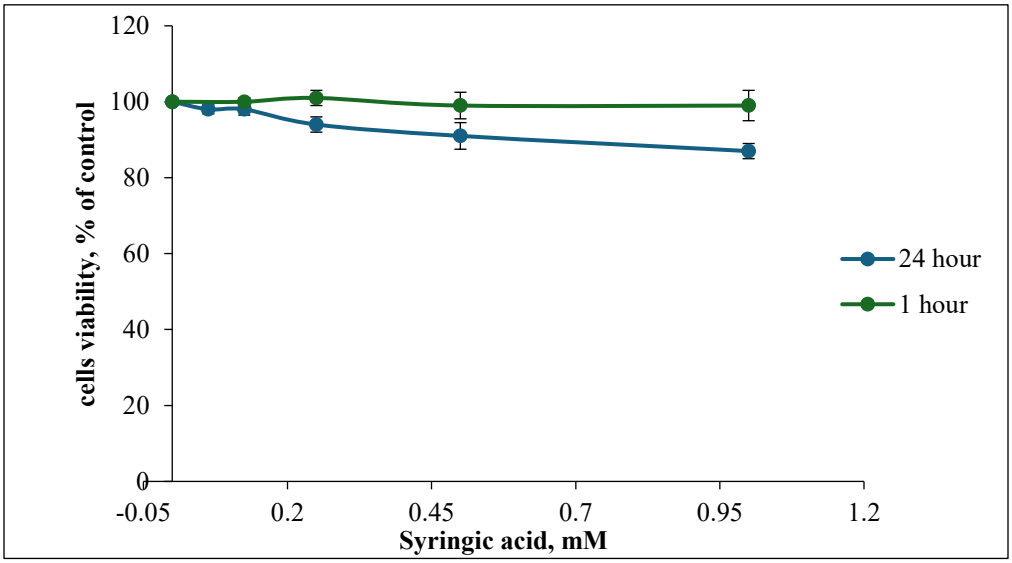


Figure 3.2.6 Effect of Syringic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

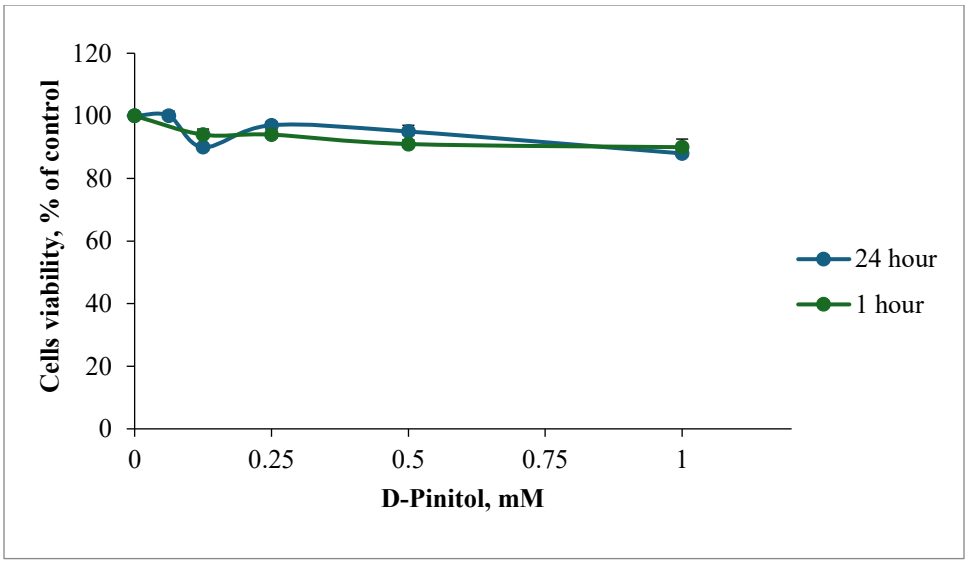


Figure 3.2.7 Effect of D-Pinitol on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

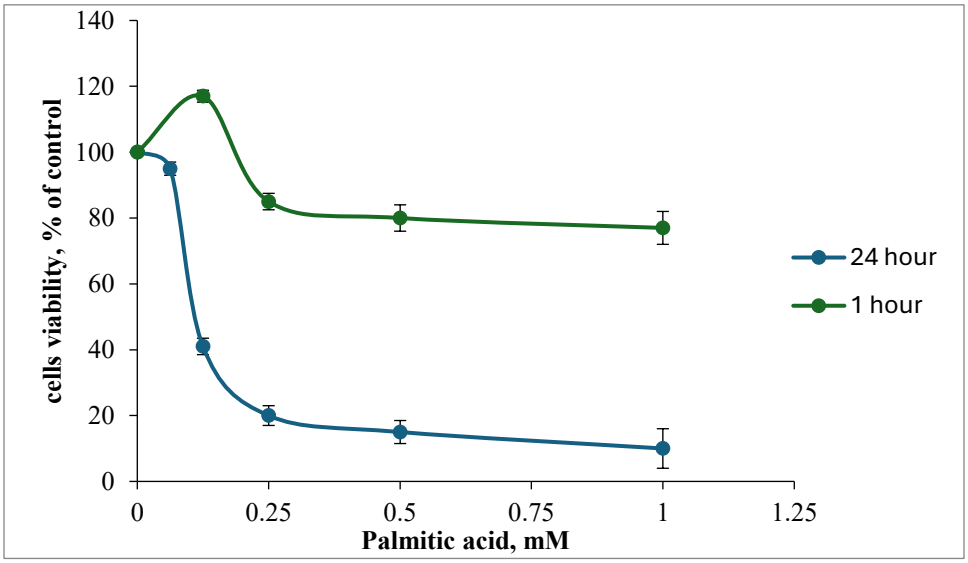


***Figure 3.2.8 Effect* of Palmitic acid on cell viability examined by MTT assay.** L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

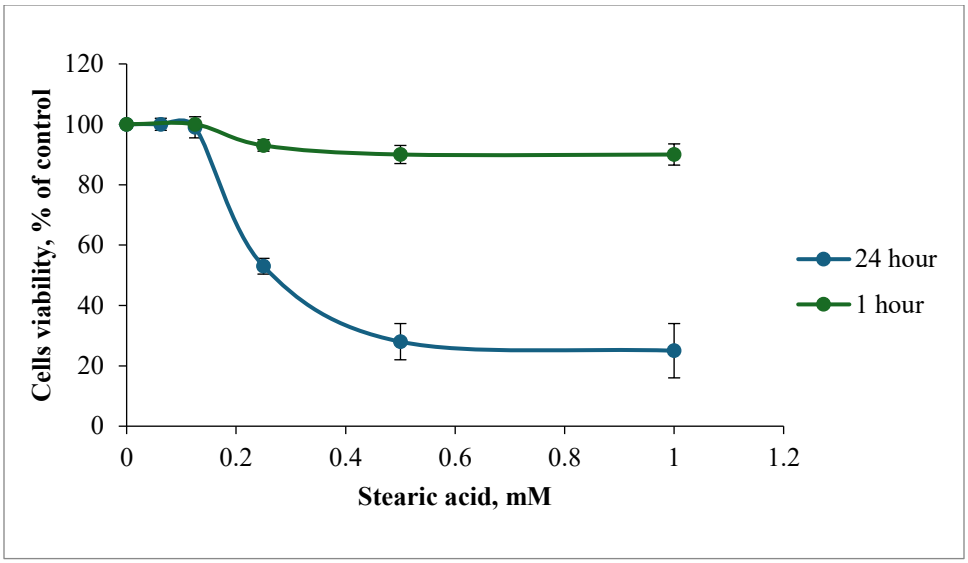


Figure 3.2.9 Effect of Stearic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

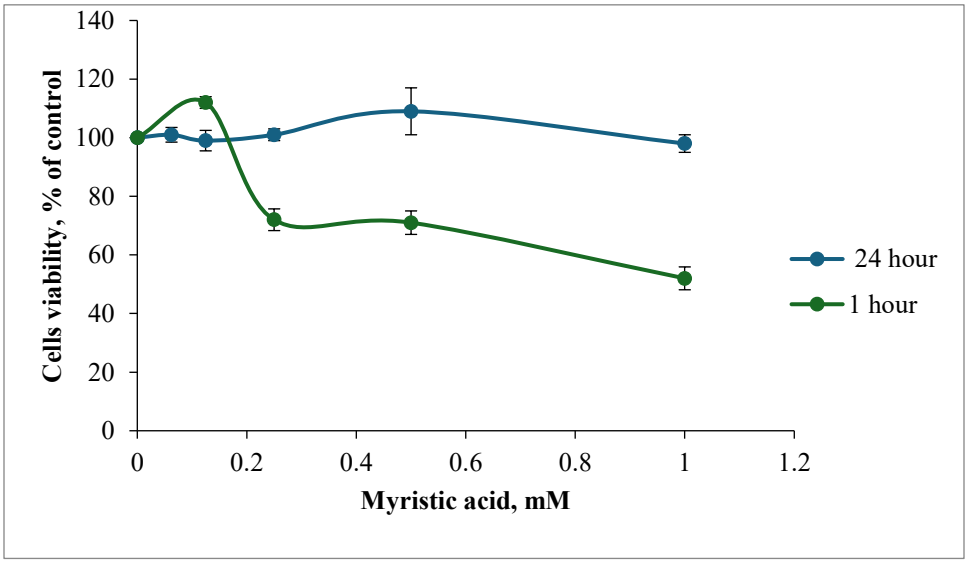


Figure 3.2.10 Effect of Myristic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

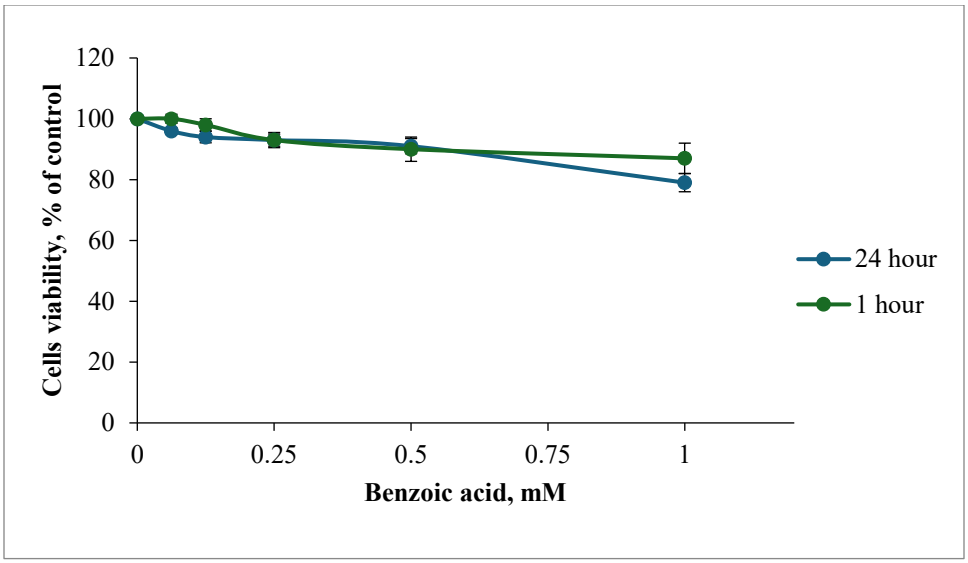


Figure 3.2.11 Effect of Benzoic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

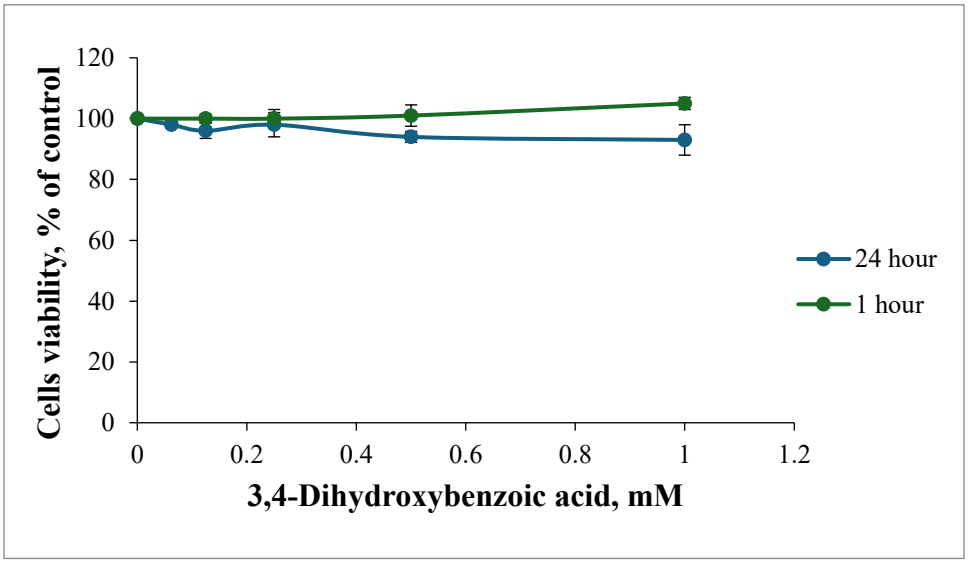


Figure 3.2.12 Effect of 3,4-Dihydroxybenzoic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

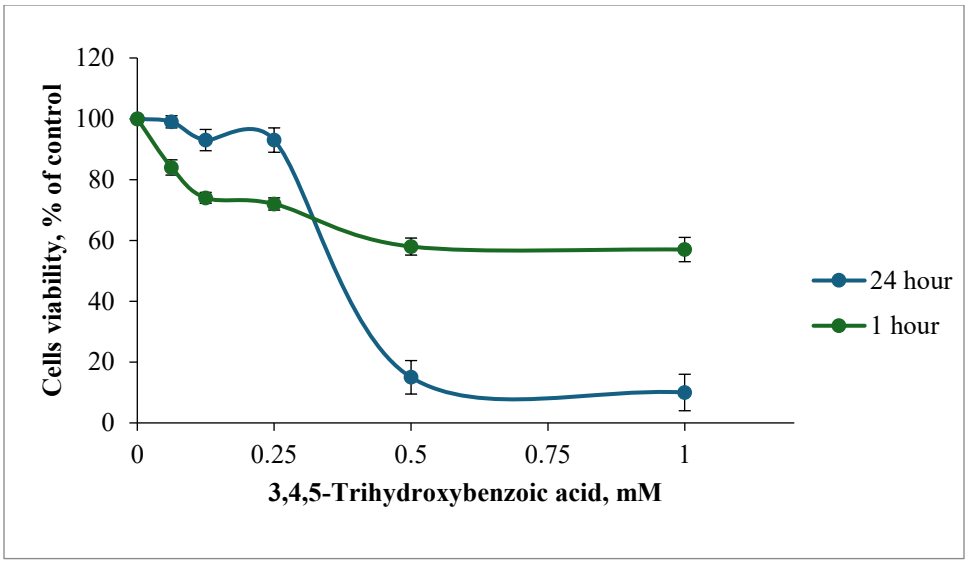


Figure 3.2.13 Effect of 3,4,5-Trihydroxybenzoic acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

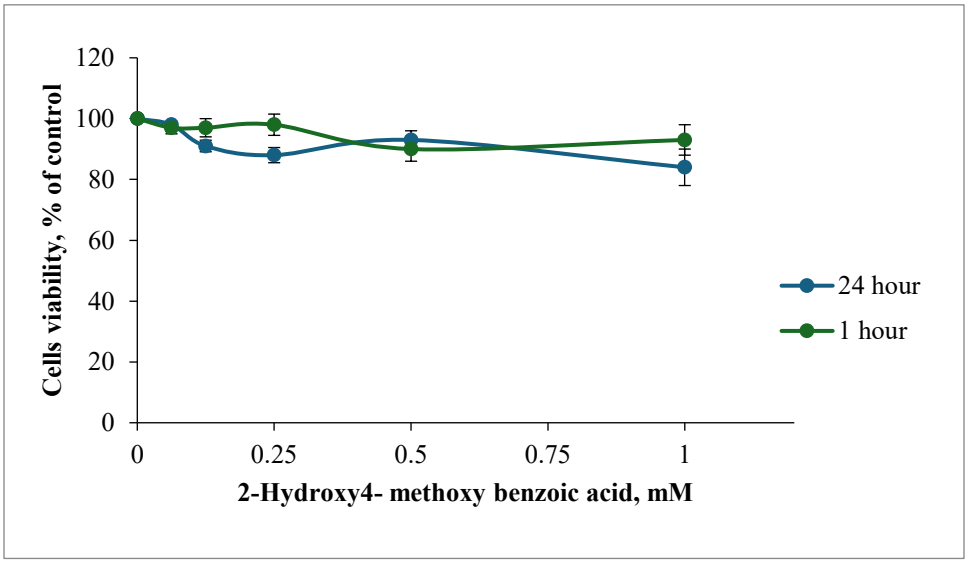


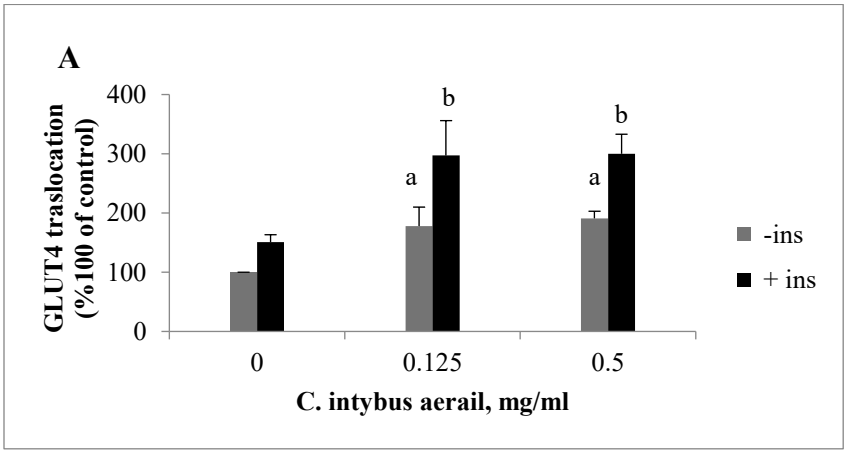
Figure 3.2.14 Effect of 2-Hydroxy4-methoxy acid on cell viability examined by MTT assay. L6-GLUT4myc cells (20,000 cell/well) were seeded in 96-well plate and were exposed to increasing concentrations (0−1 mM) for either 24 h or 1h. Values given represent means ± SEM (% of untreated control cells) of three independent experiments carried out in triplicates.

3.3 Effects of natural compounds on GLUT4 Translocation to the PM

In type 2 diabetes, GLUT4 translocation to the PM is impaired. Insulin and some other ingredients can increase the translocation of GLUT4 to the PM [1]. Therefore, the effects of methanol extracts of A. esculentus fruit and C. intybus from both aerial and root sections on the translocation of GLUT4 to the plasma membrane was measured as described in the methods in the presence and absence of insulin. Cells were exposed to selected safe nontoxic concentrations of A. esculentus fruit and C. intybus aerial and root methanol extracts.

C. intybus aerial extract increased GLUT4 translocation at 0.125 mM by 96.9±59% and 78±32% respectively in present and absence of insulin. Also, the translocation increased at 0.5 mM exposure by 98.6±33% and 91±12% in present and absence of insulin respectively after 24 h exposure (figure 3.3.1A). Moreover, GLUT4 translocation increased in cells treated with the C. intybus root part extract at 0.125 mM by 104.4±9% and 222±9 % in presence and absence of insulin respectively.

The translocation increased in L6-GLUT4-myc cells when the cells treated with 0.5 mM by 112.5±34% and 245±18 % in present and absence of insulin respectively after 24 h exposure (figure 3.3.1B).



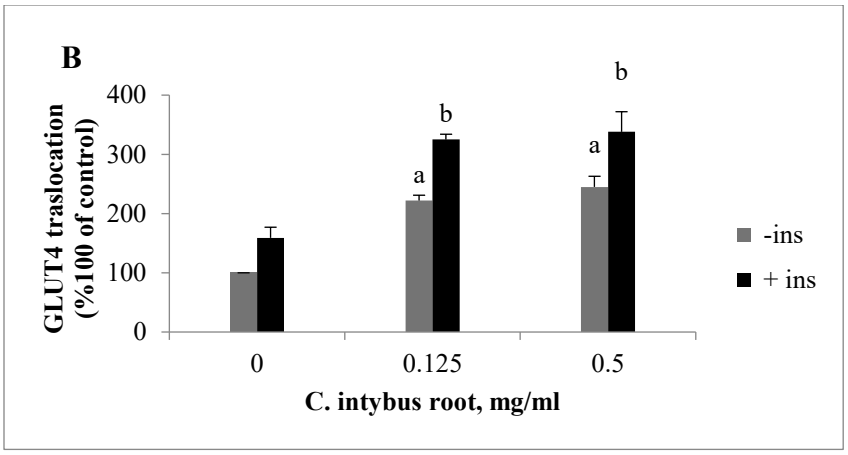


Figure 3.3.1 GLUT4 translocation to the plasma membrane.

L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to C. intybus at 24 h treatment. (A) C. intybus aerial part extract. (B) C. intybus root part extract without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

The translocation of GLUT4 was markedly enhanced in L6-GLUT4-myc cells following treatment with A. esculentus extract at a concentration of 0.250 mM. The increase was 85.6±30% in the presence of insulin and 216±40% in its absence. Similarly, GLUT4 translocation was augmented in L6-GLUT4- cells exposed to 0.5 mM of the extract. The observed increases were 96.2±88% with insulin and 240±81% without insulin after 24 h of treatment. These results are depicted in Figure 3.3.2.

Based on the results obtained from extracts of A. esculentus and C. intybus, and GC/MS tests, fourteen natural compounds with anti-diabetic properties were selected for further investigation. The selected compounds are: Caffeic acid, ferulic acid, isovanillic acid, vanillic acid, chlorogenic acid, D-pinitol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, 2-hydroxy-4-methoxybenzoic acid, and syringic acid.

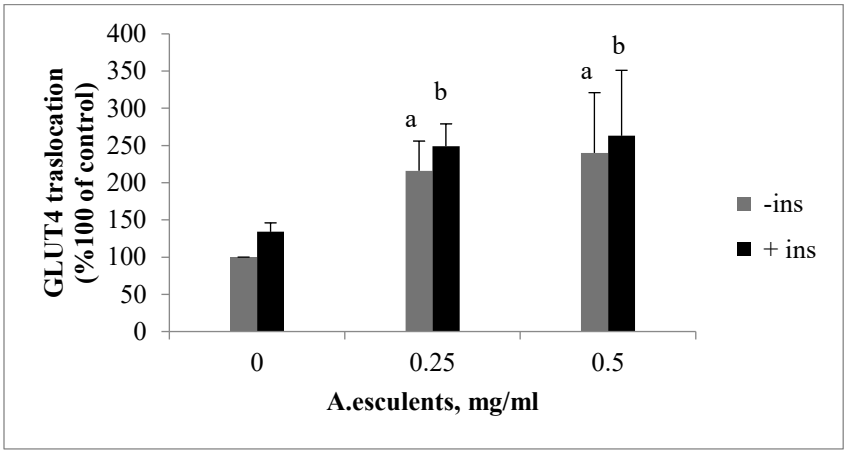


Figure 3.3.2 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to A. esculents extract at 24 h treatment. without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

The impact of these compounds on GLUT4 translocation to the plasma membrane was assessed under both insulin-stimulated and unstimulated conditions. Treatments were conducted over 24 h, as well as for 0.5 and 1 h durations, following the procedures outlined in the methods section.

Caffeic acid (CA) enhanced GLUT4 translocation by 31 ± 17% at a concentration of 0.125 mM in the absence of insulin, and by 24.5 ± 27% in the presence of insulin after 24 h of treatment (Figure 3.3.3A). In cells exposed to 0.250 mM CA for 0.5 h, GLUT4 translocation increased by 27.8 ± 7% when insulin was present. No significant effect on GLUT4 translocation was observed following 1 hour of CA treatment (Figure 3.3.3B).

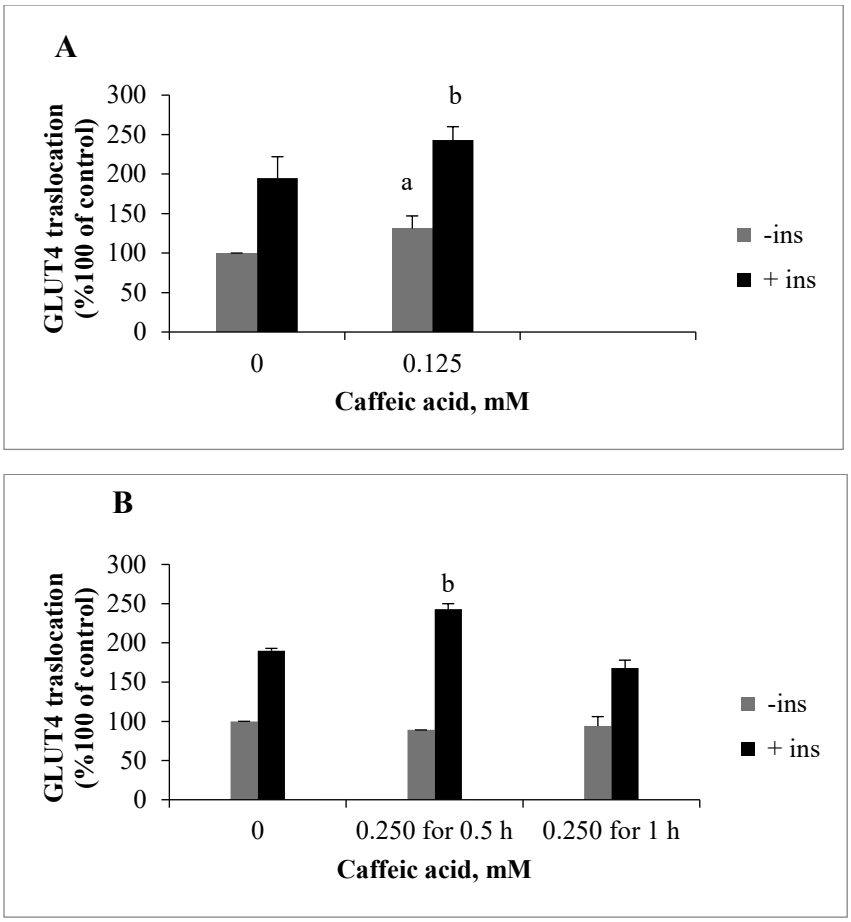


Figure 3.3.3 GLUT4 translocation to the plasma membrane.

L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to CA for 24 h treatment (A) and 0.5, 1h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

In cells treated with 0.125 mM Ferulic acid (FA) for 24 h, GLUT4 translocation was observed to be 18 ± 10% in the presence of insulin and 19 ± 12% without insulin (Figure3.3.4A). Short-term exposure to 0.125 mM FA for 0.5 h resulted in a greater increase in GLUT4 translocation, reaching 27 ± 7% in the presence of insulin (Figure3.3.4B).

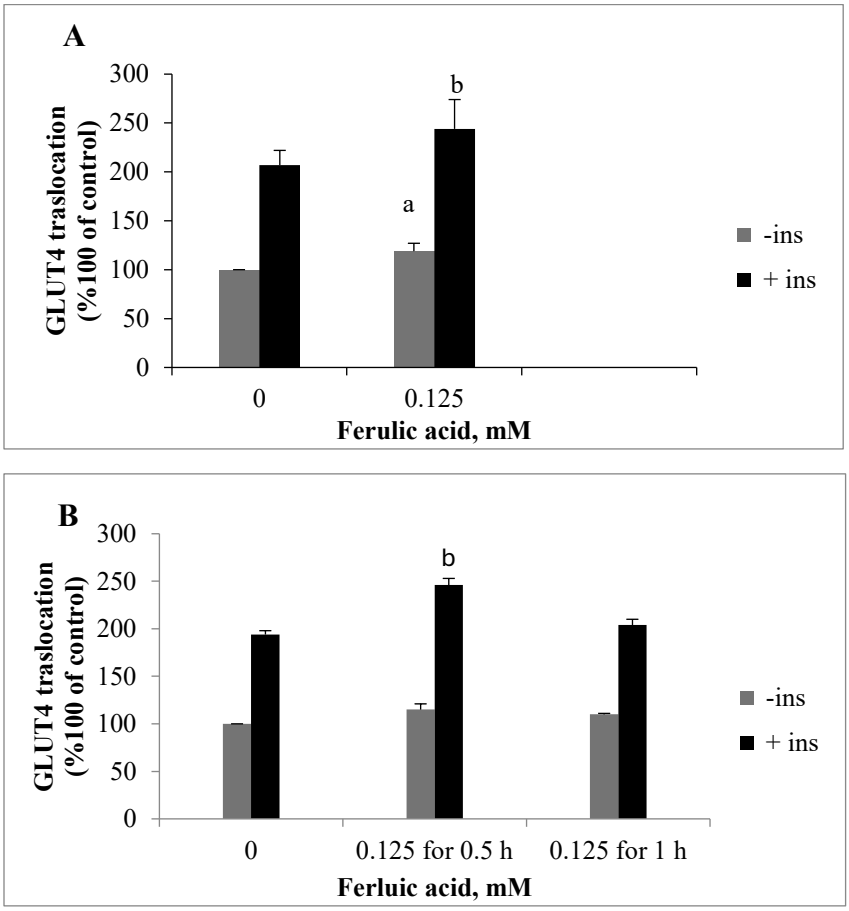


Figure 3.3.4 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to FA for 24 h treatment (A), and 0.5, 1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

Isovanillic acid (ISV) treatment did not influence GLUT4 translocation to the plasma membrane, irrespective of exposure duration (figure 3.3.5). In contrast, exposure to vanillic acid (VA) at concentrations of 0.250 mM and 0.5 mM for 24 h resulted in increases of 23.8% ± 20% and 12% ± 25%, respectively, in the presence of insulin. Similarly, in the absence of insulin, VA treatment at these

concentrations led to increases of 36% ± 9% and 30% ± 9%, respectively, after 24 h. A shorter exposure of 1 hour to 0.0625 mM VA in the presence of insulin produced a 27.9% ± 24% increase in GLUT4 translocation (figure 3.3.6).

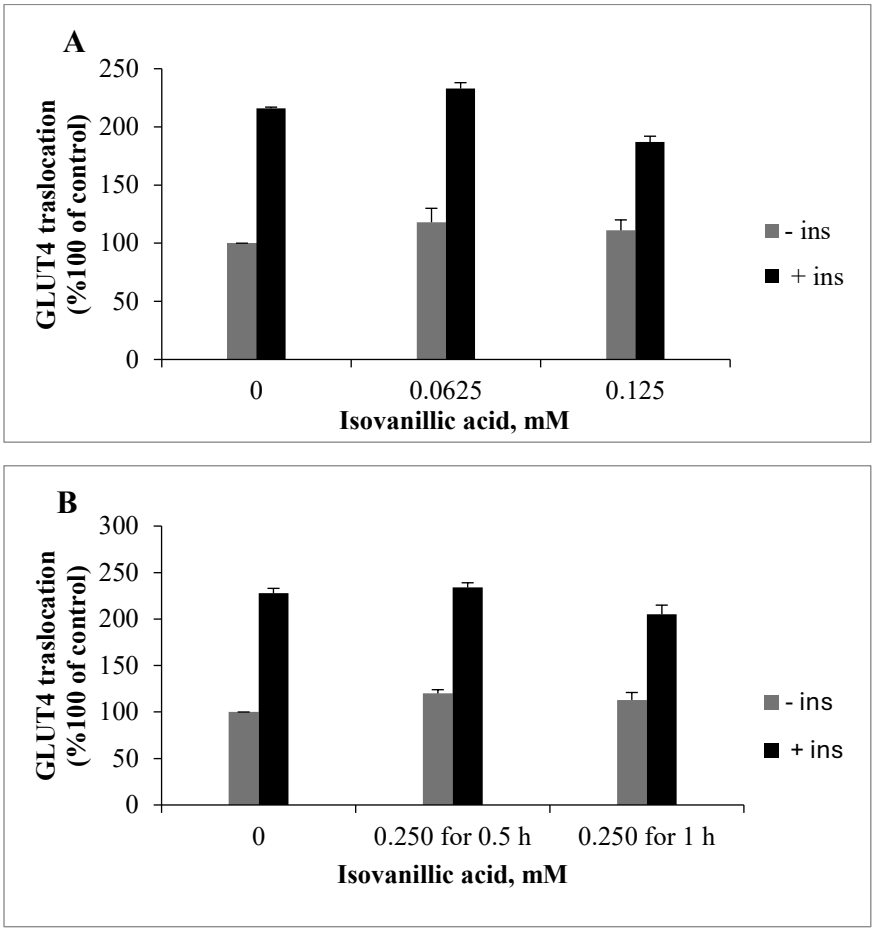


Figure 3.3.5 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to ISV for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates.

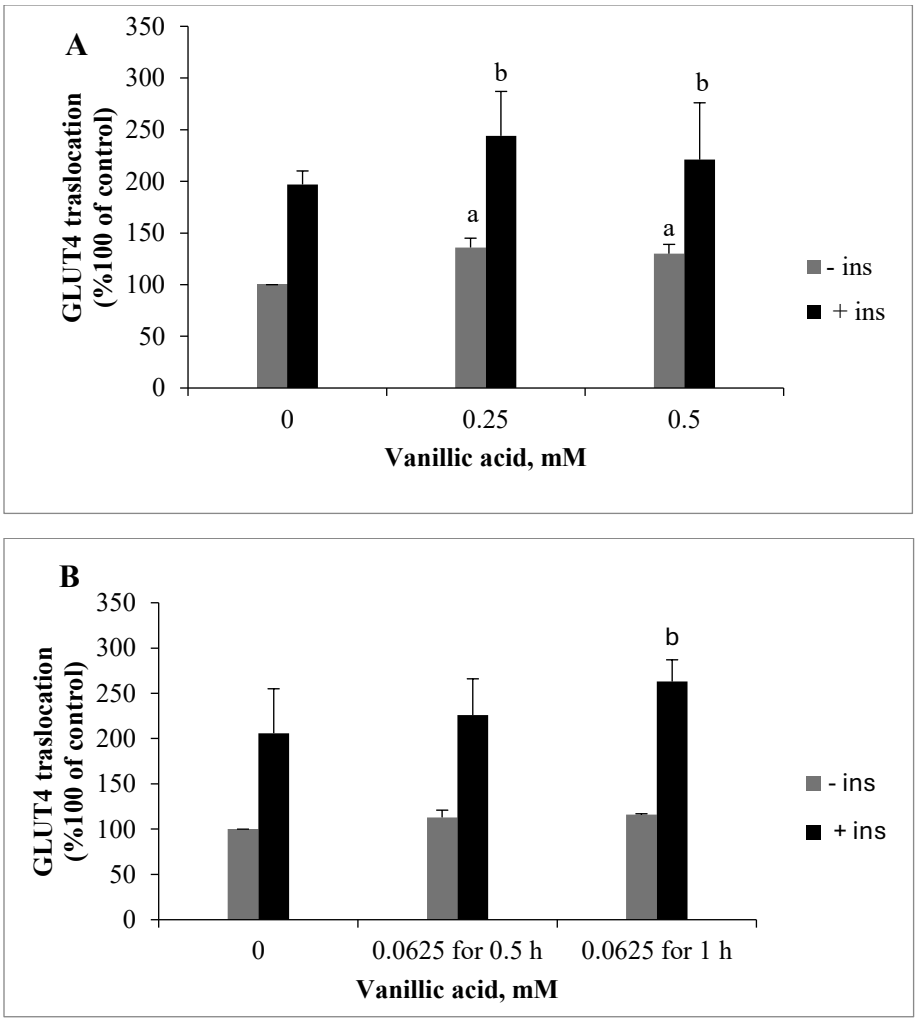


Figure 3.3.6 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to VA for 24 h treatment (A), and 0.5, 1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

Exposure to chlorogenic acid resulted in an increase in GLUT4 translocation by 32.9±20% and 44.3±30% at concentrations of 0.125 mM and 0.250 mM, respectively, following 24 h of treatment in the presence of insulin. In the absence of insulin, the translocation increased by 28±11% and 38±11% at the same

concentrations after 24 h of exposure. Additionally, treatment with chlorogenic acid at 0.5 mM for durations of 0.5 and 1 h enhanced GLUT4 translocation in the presence of insulin by 36±27% and 32.8±20%, respectively (Figure 3.3.7).

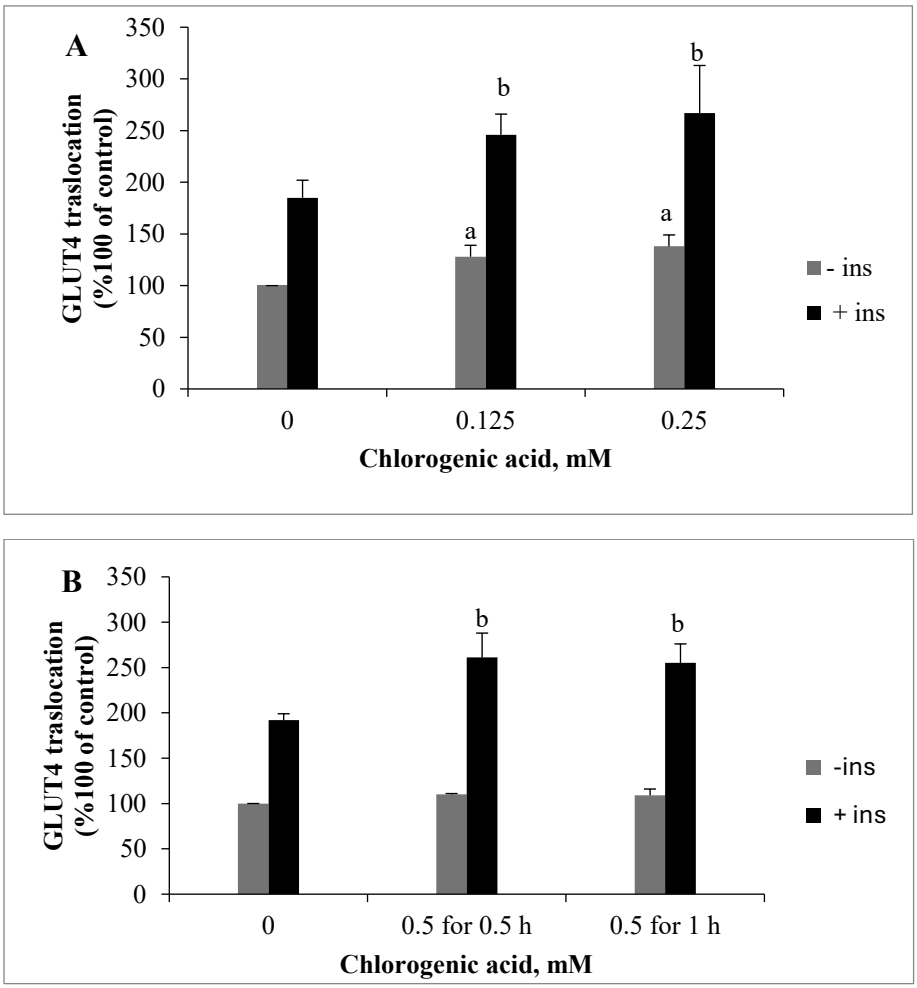


Figure 3.3.7 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to chlorogenic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

Syringic acid (SYA) markedly enhanced GLUT4 translocation, with increases of 39±20% and 52.7±20% following 24 h of treatment at concentrations of 0.0625 mM and 0.125 mM, respectively, in the presence of insulin. Similar elevations in GLUT4 translocation were observed when cells were exposed to the same concentrations of SYA for 24 h in the absence of insulin, with increases of 54±11% and 46±11%.

Additionally, treatment with syringic acid at a concentration of 0.5 mM for durations of 0.5 and 1 hour resulted in more pronounced increases, reaching levels of 68±15% and 76.6±16%, respectively (Figure 3.3.8).

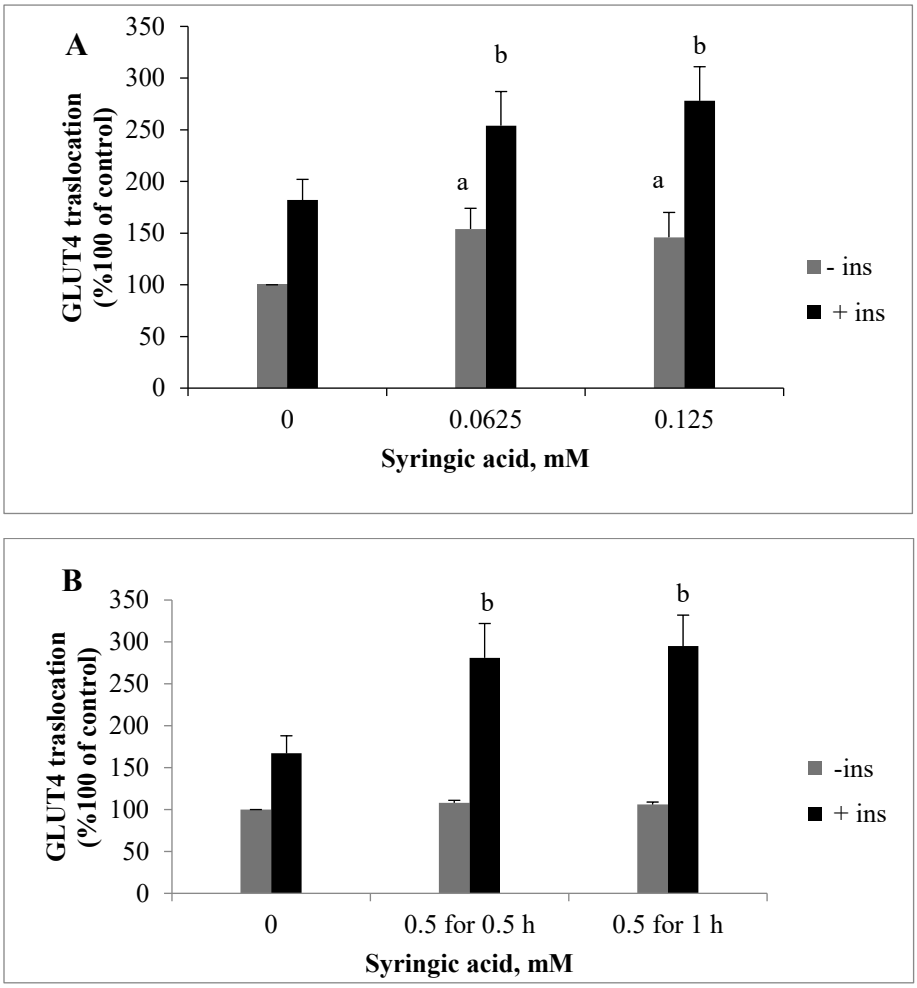


Figure 3.3.8 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to syringic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described

in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

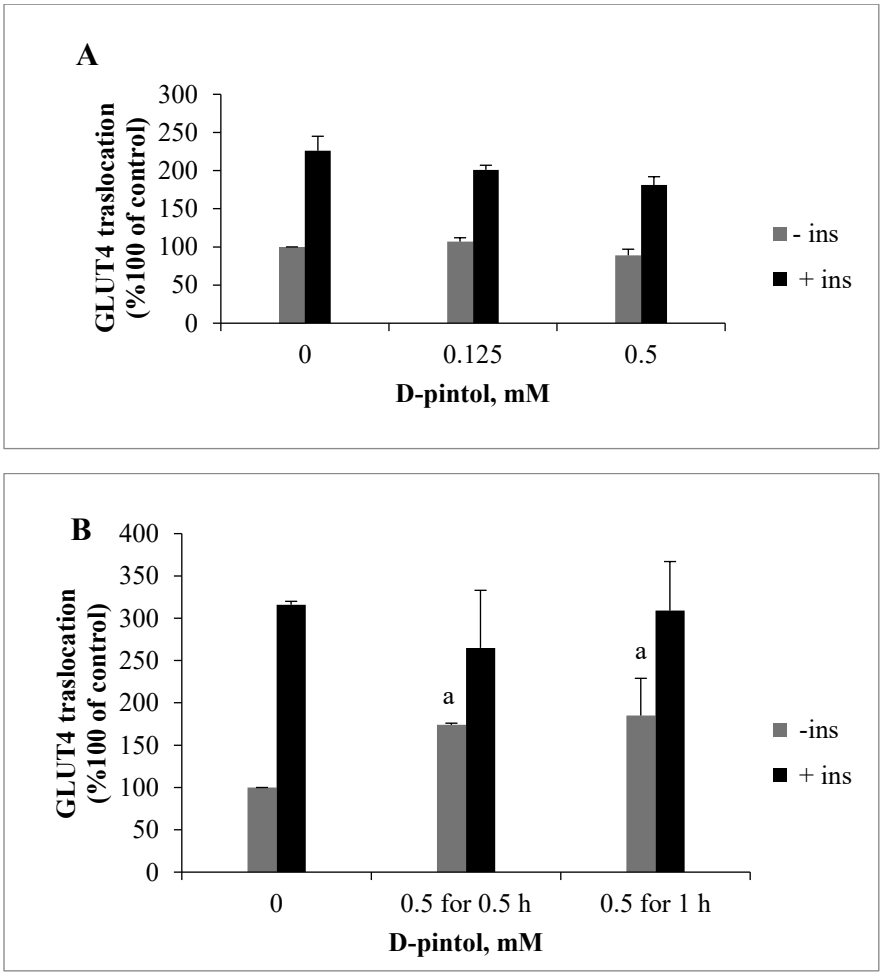


Figure 3.3.9 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to D-pintol for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group.

Treatment with D-pintol or palmitic acid (PA) at 24 hours did not influence GLUT4 translocation. However, exposure to 0.5 mM D-pintol resulted in increased GLUT4 translocation at 0.5 and 1 hour in the absence of insulin, with elevations of 74±2% and 85±20%, respectively (Figure 3.3.9). PA augmented GLUT4 translocation by 25±10% and 31±11% when administered at 0.125 mM for 0.5 and 1 hour, respectively, in the presence of insulin (Figure 3.3.10).

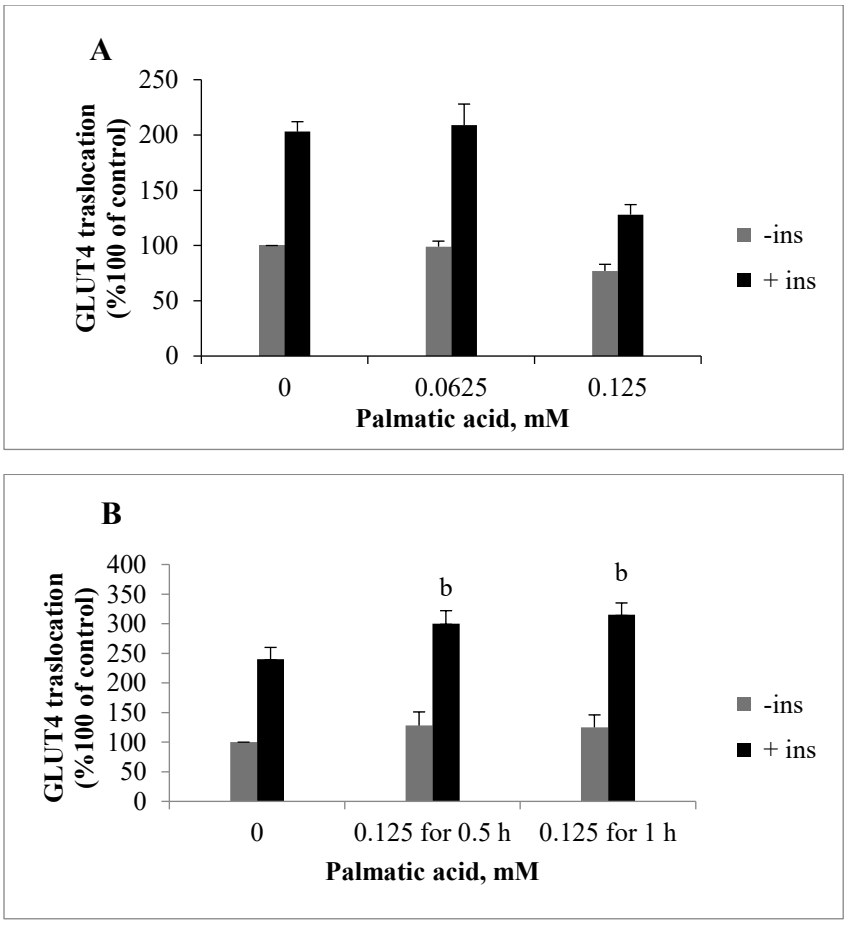


Figure 3.3.10 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to palmitic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (b) compared with (+ins) control group.

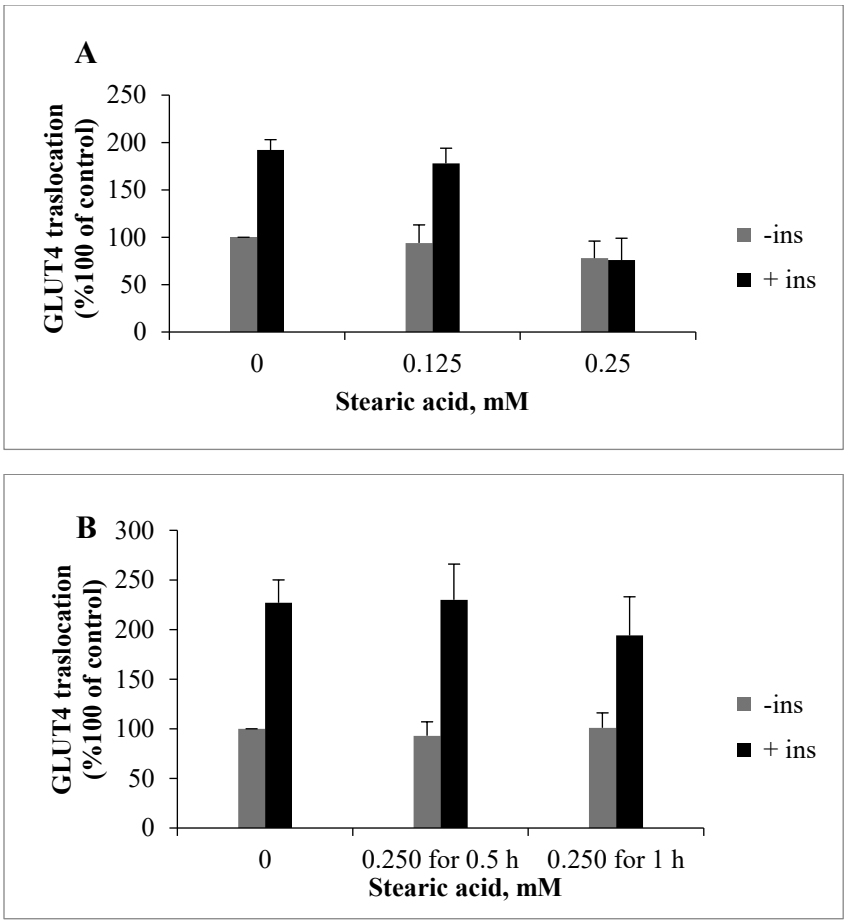


Figure 3.3.11 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to stearic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates.

The administration of Myristic acid and Stearic acid did not significantly influence the GLUT4 content in the PM Figures 3.3.11 and 3.3.12.

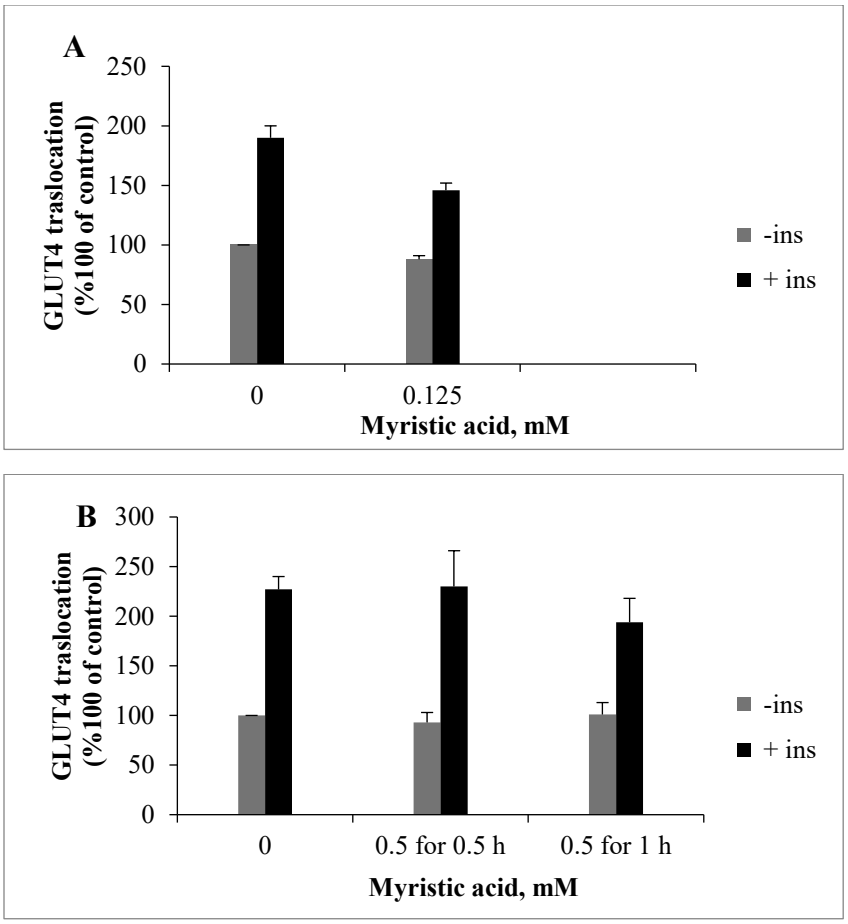


Figure 3.3.12 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to Myristic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates.

Benzoic acid at a concentration of 0.125 mM augmented GLUT4 translocation by 17.5±6% in the presence of insulin. In the absence of insulin, exposure to 0.125 mM and 0.250 mM benzoic acid for 24 h resulted in increases of 26±9% and 33±5%, respectively. A 0.250 mM dose of benzoic acid for 0.5 h in the presence of insulin produced a 15±20% enhancement in GLUT4 translocation (Figure 3.3.13).

Similarly, treatment with 3,4-dihydroxybenzoic acid at 0.125 mM and 0.250 mM in the presence of insulin elevated GLUT4 translocation by 21±10% and 34.2±16%, respectively. In the absence of insulin, 24 h exposure to these concentrations of

3,4-dihydroxybenzoic acid resulted in increases of 44±20% and 21±9%. Additionally, a 1-hour exposure to 0.250 mM 3,4-dihydroxybenzoic acid without insulin led to a 23±11% increase in GLUT4 translocation (Figure 3.3.14).

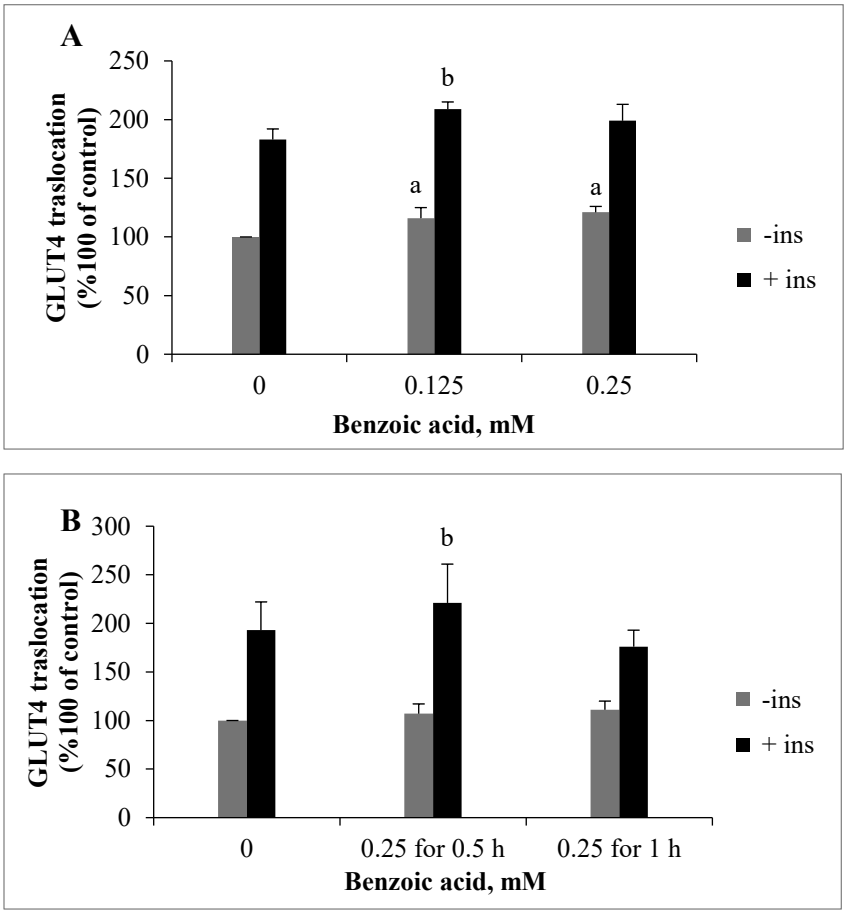


Figure 3.3.13 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to benzoic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

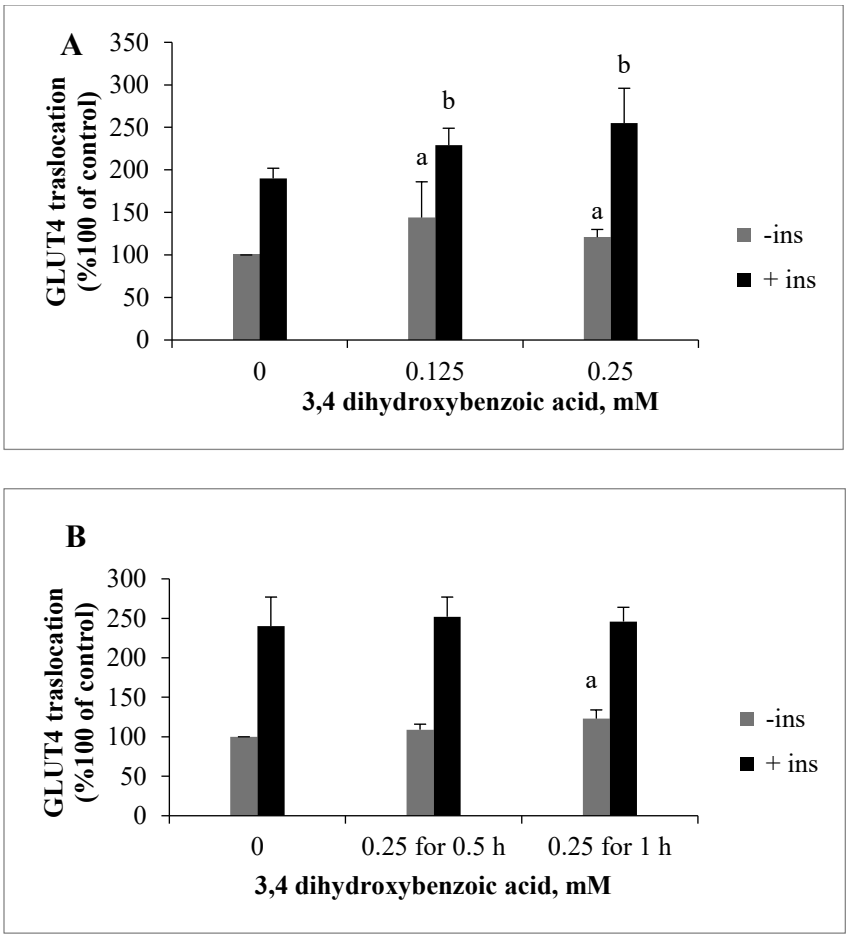
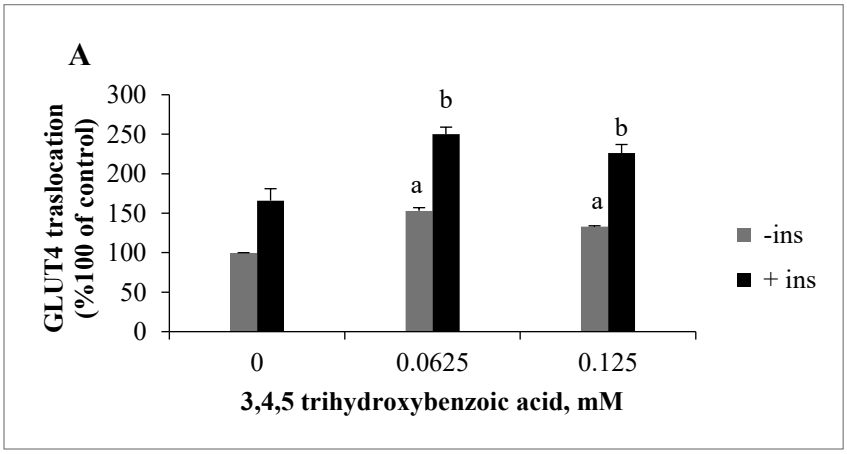


Figure 3.3.14 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to 3,4dihydroxybenzoic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

Exposure of cells to 3,4,5-trihydroxybenzoic acid at concentrations of 0.0625 mM and 0.125 mM resulted in an increase in GLUT4 translocation by 53 ± 6% and 33 ± 1%, respectively, after 24 h in the absence of insulin. In the presence of insulin, the same concentrations led to increases of 50.6 ± 9% and 36 ± 11% under identical conditions. Brief exposure durations of the compound (0.5 and 1 hour at

0.0625 mM) enhanced GLUT4 translocation by 18 ± 9% and 20 ± 11% in the absence of insulin. At 0.0625 mM for 0.5 h, translocation increased by 25 ± 16% in the presence of insulin (Figure 3.3.15).

Treatment with 2-hydroxy-4-methoxybenzoic acid for 24 h increased GLUT4 translocation by 34 ± 17% and 28 ± 14% at concentrations of 0.125 mM and 0.250 mM, respectively, in the absence of insulin. In the presence of insulin, the translocation was enhanced by 27.3 ± 15% and 11 ± 14% at the same concentrations. Short-term exposure (0.5 and 1 hour at 0.250 mM) resulted in increases of 22 ± 17% in the absence of insulin and 25.3 ± 11% and 12 ± 9% in the presence of insulin at 0.5 and 1 hour, respectively (Figure 3.3.16).



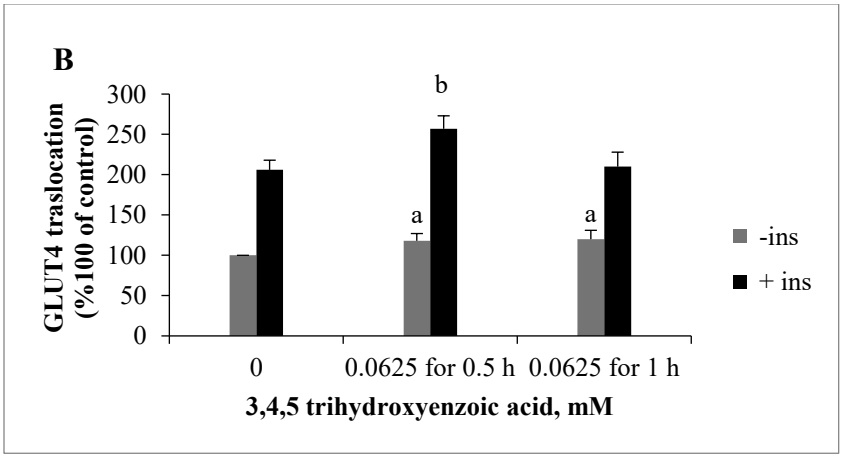


Figure 3.3.15 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to 3,4,5 trihydroxybenzoic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was

quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

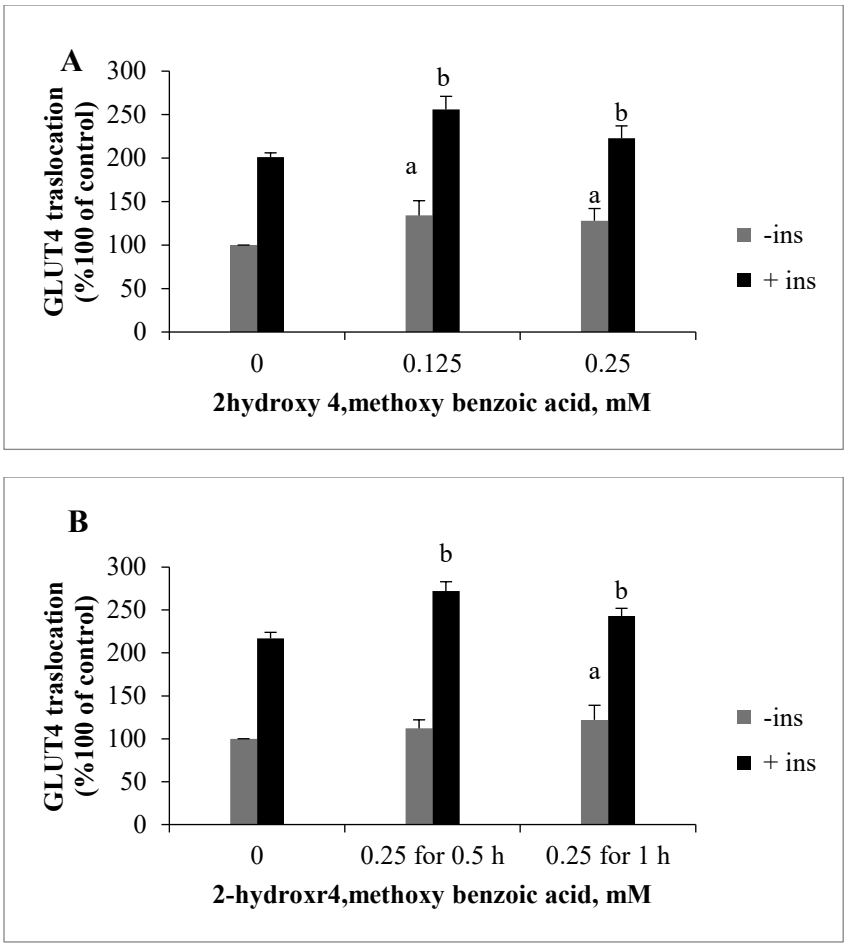
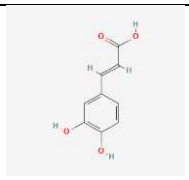
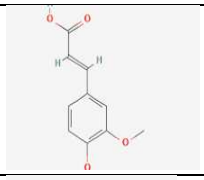
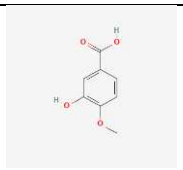
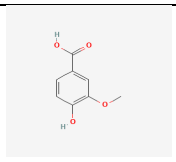
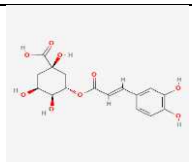
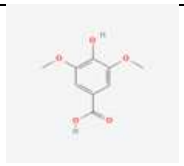
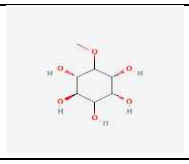
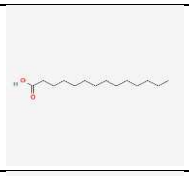
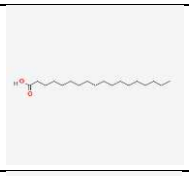
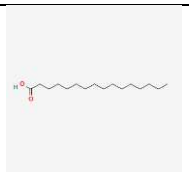
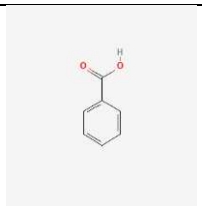
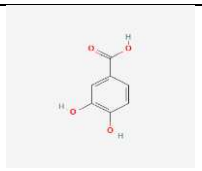
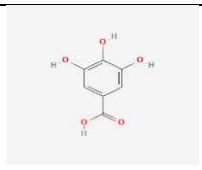
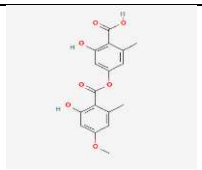


Figure 3.3.16 GLUT4 translocation to the plasma membrane. L6-GLUT4myc cells (150,000 cell/well) were seeded in a 24-well plate and were exposed to 2-hydroxy4, methoxy benzoic acid for 24 h treatment (A), and 0.5,1 h treatment (B); without (−) or with (+) insulin as described in the methods. Surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. $p < 0.05$ Statistical significance: (a) compared with (-ins) control group, (b) compared with (+ins) control group.

Table 3.3 Effect of the tested natural compounds on GLUT4 translocation after 0.5- and 24-hour exposure.

Compound		% of GLUT4 translocations Concentration of the compound in brackets (mM)			
		without insulin, 0.5 h	with insulin, 0.5 h	without insulin, 24 h	with insulin, 24 h
Caffeic acid		89±0% (0.250)*	243±7% (0.250)	131±16% (0.125)	243±17% (0.125)
Ferulic acid		115±6% (0.125)	246±7% (0.125)	119±10% (0.125)	244±12% (0.125)
Isovanillic acid		120±5% (0.250)	234±5% (0.250)	118±12% (0.0625)	233±5% (0.0625)
Vanillic acid		113±8% (0.0625)	226±24% (0.0625)	136±6% (0.250)	244±25% (0.250)
Chlorogenic acid		110±1% (0.5)	261±27% (0.5)	138±11% (0.250)	267±30% (0.250)
Syringic acid		108±3% (0.5)	281±30% (0.5)	146±24% (0.125)	278±20% (0.125)
D-pintol		174±2% (0.5)	265±30% (0.5)	107±5% (0.125)	201±6% (0.125)

Myristic acid		93±10% (0.5)	230±36% (0.5)	88±6% (0.125)	146±10% (0.125)
Stearic acid		93±14% (0.250)	230±30% (0.250)	94±10% (0.125)	178±16% (0.125)
Palmitic acid		128±20% (0.125)	300±10% (0.125)	99±5% (0.0625)	209±19% (0.0625)
Benzoic acid		107±10% (0.250)	221±20% (0.250)	116±9% (0.125)	209±6% (0.125)
3,4-dihydroxy-benzoic acid		109±7% (0.250)	252±25% (0.250)	144±10% (0.125)	229±20% (0.125)
3,4,5-trihydroxy-benzoic acid		118±9% (0.0625)	257±16% (0.0625)	153±4% (0.0625)	250±9% (0.0625)
2-hydroxy,4-methoxy- benzoic acid		112±10% (0.250)	272±11% (0.250)	134±17% (0.125)	256±15% (0.125)

3.4 Surface GLUT4 detection by Immunostaining

To test the effect of the natural compounds on GLUT4 translocation at the level of single cells, immunostaining assay was applied and cells were recorded by microscopy as detailed in the methods. L6-GLUT4myc cells were seeded on cover slips and treated with an active compound for a specific time and then the non-permeabilized cells were reacted with anti-myc polyclonal antibody which was subsequently detected with Cy3-conjugated goat anti-mouse IgG. DAPI was used to stain the nucleus.

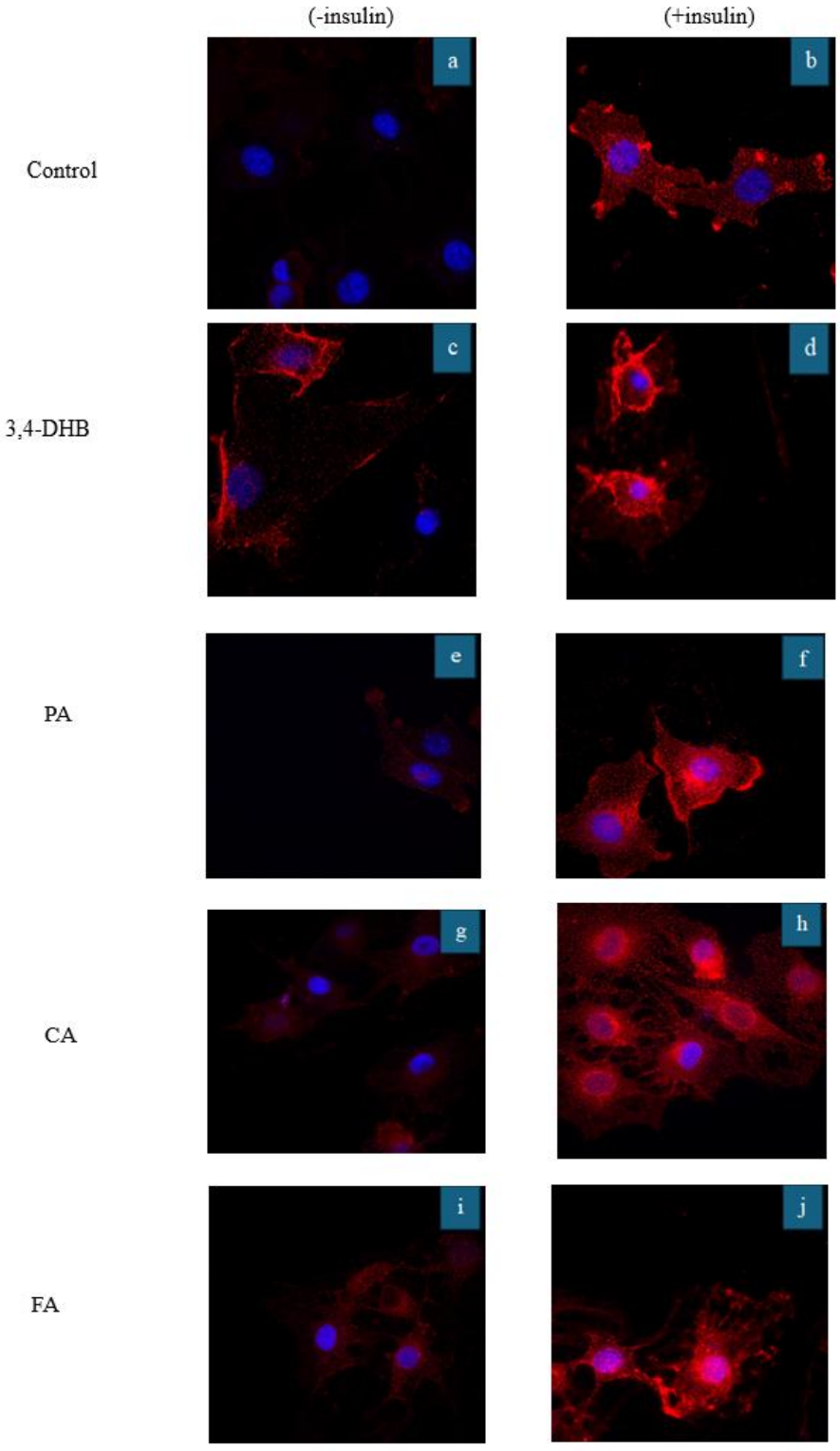

(-insulin)
(+insulin)
Control
3,4-DHB
PA
CA
FA
a
b
c
d
e
f
g
h
i
j

Figure 3.4.1 Immunostaining of GLUT4 in non-permeabilized L6-myc cells re-attached onto coverslips. L6-myc cells were stained with anti-myc polyclonal antibody (C3956), and subsequently with Cy3 goat anti-mouse IgG to detect surface GLUT4. L6-GLUT4-myc cells were treated without or with insulin(100nM) for 20 min at 37℃. Shown are representative LEICA DMi8 microscopy images(100X). A: L6-myc cells without insulin, B: L6-myc cells stimulated with insulin, C: L6-myc cells treated with 0.125 mM 3,4-dihydroxy benzoic acid (3,4DHB) for 24 h without insulin, D: : L6-myc cells treated with 0.125 mM 3,4-dihydroxy benzoic acid for 24 h with insulin, E:L6-myc cells treated with 0.3 mM palmitic acid (PA) for 1 h without insulin, F: L6-myc cells treated with 0.3mM palmitic acid for 1 h with insulin, G: L6-myc cells treated with 0.125 mM caffeic acid (CA) for 24 h without insulin, H: L6-myc cells treated with 0.125 mM caffeic acid treated for 24 h with insulin, I: L6-myc cells treated with 0.125 mM ferulic acid (FA) for 24 h without insulin and J: L6-myc cells treated with 0.125 mM ferulic acid for 24 h with insulin.

In the basal state, a low level of surface GLUT4 was detected (Fig.3.4.1a), and the intensity of the staining increased upon insulin stimulation (Fig.3.4.1b). Moreover, using this detection system it was demonstrated that insulin increases the amount of GLUT4myc at the cell surface when L6-myc cells were pre-treated with 0.3 mM palmitic acid for 1 h (Fig.3.4.1f) and 0.125mM of 3,4-dihydroxy benzoic acid for 24 h exposure (Fig.3.4.1d). Also, the level of GLUT4 enhanced when the insulin stimulated cells treated with 0.125mM of caffeic acid and ferulic acid for 24 h exposure (Fig.3.4.1h&j). In contrast, a low amount of GLUT4 at the PM was detected at non-insulin stimulated cells treated with 0.3mM palmitic acid for 1 h exposure comparing with basal cells (Fig.3.4.1e) while the levels of PM GLUT4 enhanced at non-insulin stimulated cells treated with 0.125mM of 3,4-dihydroxy benzoic acid, caffeic acid and ferulic acid at 24 h exposure comparing with basal cells (Fig.3.4.1c, g, i).

3.5. Effects of natural compounds on IRS1, AKT, PETEN, and AS160 protein levels and phosphorylation in L6-GLUT4-myc cells

Western blot analysis of Insulin Receptor Substrate 1 (IRS1) and Protein Kinase B (AKT), phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN), and Akt substrate of 160 kDa (AS160) protein revealed notable variations in their expression and phosphorylation states following treatment with a selected set of natural compounds.

According to the above results for the fourteen selected natural anti-diabetic compounds and their effect on enhancing GLUT4 translocation in L6-GLUT4-myc cells, six compounds were chosen: caffeic acid, ferulic acid, benzoic acid, 3,4 dihydroxy benzoic acid, 3,4,5 trihydroxy benzoic acid and 2-hydroxy4-methoxy benzoic acid. These compounds were evaluated for their impact on the levels and phosphorylation states of IRS1, AKT, PTEN, and AS160 proteins in L6-GLUT4-myc cells.

The Western blot analysis demonstrated a notable increase in IRS1 protein expression in L6-myc cells treated with 0.125 mM 2-hydroxy-4-methoxybenzoic acid for both 24 h and 0.5 h, relative to untreated control cells maintained without insulin. This suggests a potential modulatory role of the compound on insulin signaling pathways (Figure 3.5.1).

Conversely, exposure to 0.125 mM benzoic acid over 0.5 h did not induce a significant alteration in IRS1 expression relative to control cells, irrespective of insulin treatment. However, after 24 h of incubation with 0.125 mM benzoic acid, a substantial reduction in IRS1 expression was detected (Figure 3.5.1).

AKT expression was examined at 0.5 h and 24 h post-treatment with 0.125 mM benzoic acid, both with and without insulin. At 0.5 h, there was no significant change in AKT expression relative to control cells. Conversely, at 24 h, treatment with 0.125 mM benzoic acid and 0.125 mM 2-hydroxy-4-methoxybenzoic acid led to a marked decrease in AKT expression (Figure 3.5.1).

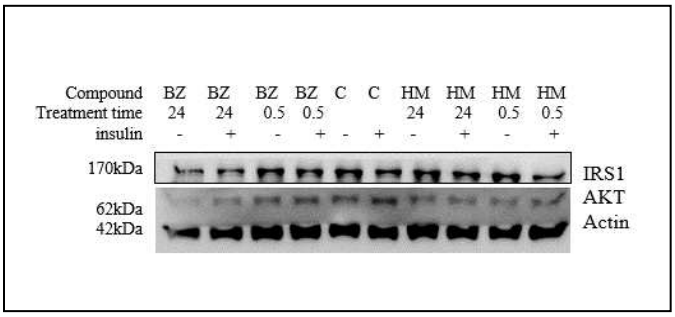


Figure 3.5.1 Effect of BZ and HM on IRS1 and AKT protein expression. This western blot analysis illustrates the impact of benzoic acid (BZ) and 2-hydroxy4-methoxy benzoic acid (HM) treatment on the protein levels of AKT and IRS1 in L6-GLUT4-myc cells treated with 0.125mM of benzoic acid or 2-hydroxy4-methoxy benzoic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against AKT and IRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

L6-myc cells were exposed to benzoic acid and 2-hydroxy-4-methoxybenzoic acid for durations of 0.5 h and 24 h, both in the presence and absence of insulin. Benzoic acid appears to influence the insulin signaling pathway either directly or indirectly. This compound promotes the phosphorylation of AKT, as evidenced by increased levels of phosphorylated AKT relative to control cells. In the absence of insulin, phosphorylated AKT was not detectable in any of the treatment groups. This observation emphasizes the requirement of insulin for AKT activation and underscores the pathway's specificity under the experimental conditions (Figure 3.5.2).

Notably, the levels of phosphorylated IRS1 remained unchanged in the treated cells relative to control samples. Although 0.125 mM benzoic acid and 0.125 mM 2-hydroxy-4-methoxybenzoic acid promote AKT phosphorylation, they do not seem to influence the phosphorylation status of IRS1, an upstream signaling component. This observation suggests that these compounds may exert their

effects downstream of IRS1 or via alternative pathways that bypass IRS1 regulation (Figure 3.5.2).

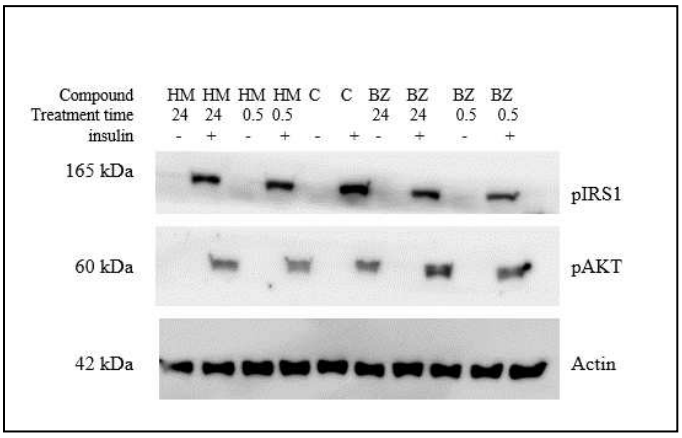


Figure 3.5.2 Effect of BZ and HM on pIRS1 and AKT protein expression. This western blot analysis illustrates the impact of benzoic acid (BZ) and 2-hydroxy4-methoxy benzoic acid (HM) treatment on the protein levels of phosphorylated AKT (pAKT) and phosphorylated IRS1(pIRS1) in L6-GLUT4-myc cells treated with 0.125mM of benzoic acid or 2-hydroxy4-methoxy benzoic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against pAKT and pIRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The study examined the impact of benzoic acid and 2-hydroxy-4-methoxybenzoic acid on the expression of AS160 and PTEN in L6-myc cells under both insulin-free and insulin-present conditions. No significant alterations in the expression levels of AS160 and PTEN were detected following treatment with either 0.125 mM of benzoic acid or 0.125 mM of 2-hydroxy-4-methoxybenzoic acid (Figure 3.5.3).

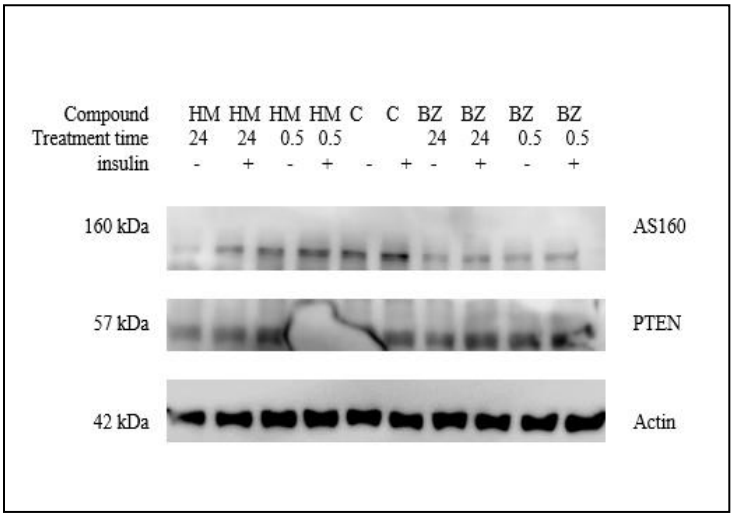


Figure 3.5.3 Effect of BZ and HM on AS160 and PTEN protein expression. This western blot analysis illustrates the impact of benzoic acid (BZ) and 2-hydroxy4-methoxy benzoic acid (HM) treatment on the protein levels of AS160 and PTEN in L6-GLUT4-myc cells treated with 0.125mM of benzoic acid or 2-hydroxy4-methoxy benzoic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against As160 and PTEN, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The treatment of L6 myocytes with 0.125 mM 3,4,5-trihydroxybenzoic acid and 0.125 mM 3,4-dihydroxybenzoic acid for 24 h elicited a notable upregulation of AKT expression in both insulin-treated and untreated cells. Conversely, the expression levels of IRS1 were unaffected in either basal or insulin-stimulated conditions following exposure to these compounds at the specified concentrations (Figure 3.5.4).

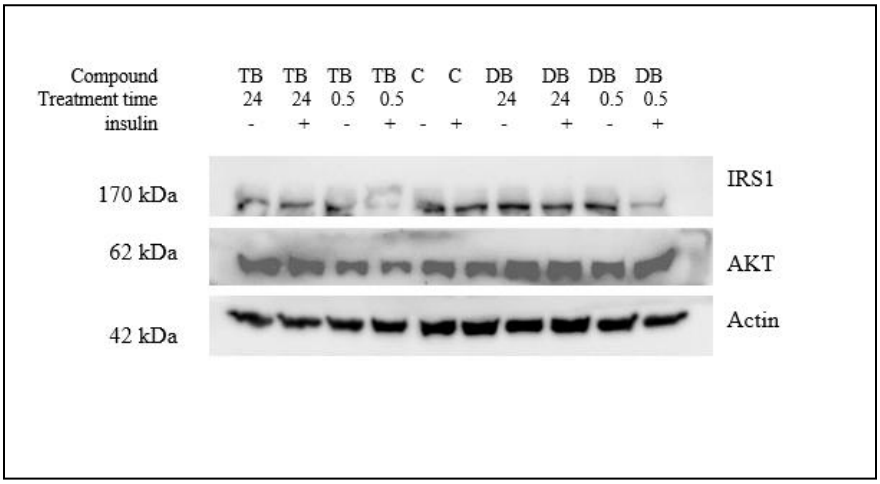


Figure 3.5.4 Effect of TB and DB on IRS1 and AKT protein expression. This western blot analysis illustrates the impact of 3,4,5 trihydroxy benzoic acid (TB) and 3,4 dihydroxy benzoic acid (DB) treatment on the protein levels of AKT and IRS1 in L6-GLUT4-myc cells treated with 0.125mM of 3,4,5 trihydroxy benzoic acid or 3,4 dihydroxy benzoic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against AKT and IRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The effect of 3,4,5-trihydroxybenzoic acid and 3,4-dihydroxybenzoic acid on the phosphorylation levels of AKT (pAKT) and insulin receptor substrate 1 (pIRS1) in L6-myc cells was examined. Cells were exposed to each compound for both 0.5 h and 24 h, with treatments conducted in the presence and absence of insulin. In insulin-stimulated conditions, treatment with 0.125 mM 3,4-dihydroxybenzoic acid for 24 h significantly elevated pAKT levels relative to controls. This indicates a potential enhancement of insulin signaling via AKT activation by 3,4-dihydroxybenzoic acid. However, pIRS1 expression did not exhibit changes following treatment with either 3,4,5-trihydroxybenzoic acid or 3,4-dihydroxybenzoic acid, suggesting these compounds do not modulate IRS1 phosphorylation under the tested conditions. In the absence of insulin, neither

pAKT nor pIRS1 signals were detectable in L6 myocytes treated with either compound, as shown in figure 3.5.5.

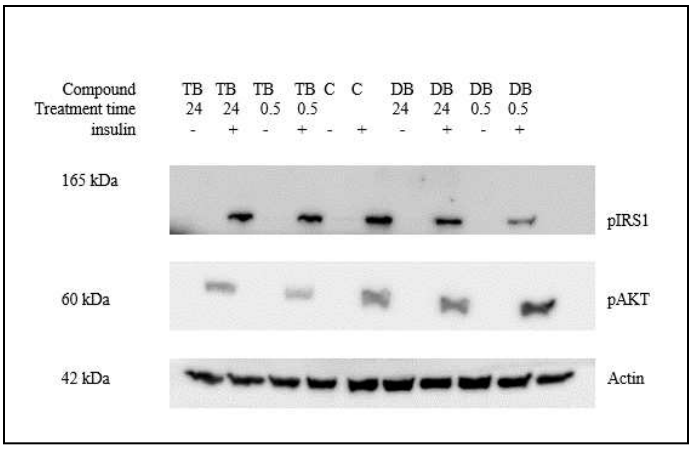


Figure 3.5.5 Effect of TB and DB on pIRS1 and pAKT protein expression. This western blot analysis illustrates the impact of 3,4,5 trihydroxy benzoic acid (TB) and 3,4 dihydroxy benzoic acid (DB) treatment on the protein levels of phosphorylated AKT (pAKT) and phosphorylated IRS1(pIRS1) in L6-GLUT4-myc cells treated with 0.125mM of 3,4,5 trihydroxy benzoic acid or 3,4 dihydroxy benzoic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against pAKT and pIRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The effects of ferulic acid on the expression levels of IRS1 and AKT proteins were analyzed at 0.5 h and 24 h following treatment. A notable increase in AKT protein levels was detected in L6-myc cells exposed to 0.125 mM ferulic acid, independent of insulin administration, suggesting a possible enhancement of AKT activation. This activation is significant due to AKT's involvement in critical cellular functions such as metabolism and growth. Caffeic acid treatment for 0.5 h resulted in elevated IRS1 levels both in the presence and absence of insulin, indicating a potential upregulation of IRS1 expression within insulin signaling pathways. Ferulic acid prompted an increase in IRS1 levels after 0.5 h of treatment in the absence

of insulin. However, it did not alter IRS1 expression when insulin was present. These results suggest that ferulic acid's effect on IRS1 expression is limited, particularly in the context of insulin presence, when compared to caffeic acid. (Refer to figure 3.5.6 for detailed data.

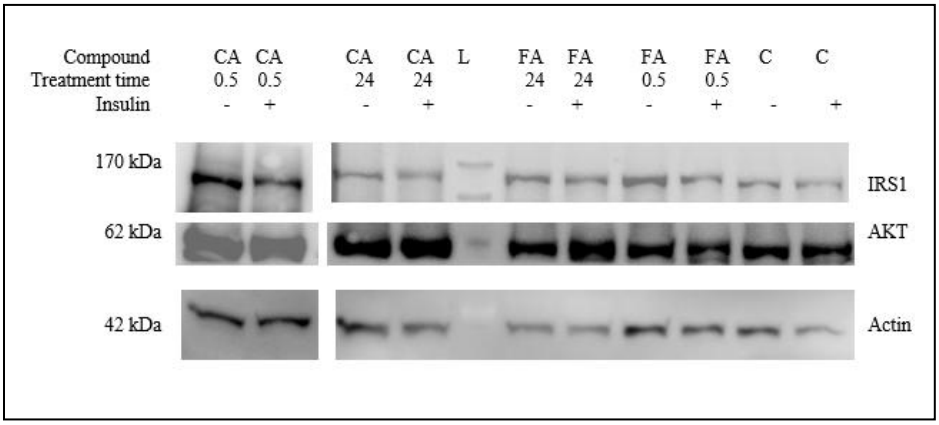


Figure 3.5.6 Effect of CA and FA on IRS1 and AKT protein expression. This western blot analysis illustrates the impact of caffeic acid (CA) and ferulic acid (FA) treatment on the protein levels of AKT and IRS1 in L6-GLUT4-myc cells treated with 0.125mM of caffeic acid or ferulic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against AKT and IRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The effects of caffeic acid treatment at 0.5 h and 24 h were examined on the expression levels of pAKT and pIRS1 in L6-myc cells, under both insulin-stimulated and unstimulated conditions. A notable increase in pIRS1 levels was detected after a 0.5 h exposure to 0.125 mM caffeic acid when insulin was present (Figure 3.5.7).

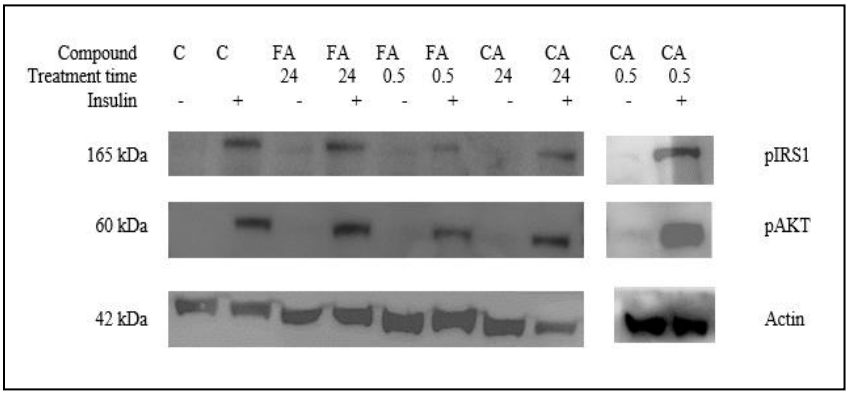


Figure 3.5.7 Effect of CA and FA on pIRS1 and pAKT protein expression. This western blot analysis illustrates the impact of caffeic acid (CA) and ferulic acid (FA) treatment on the protein levels of phosphorylated AKT (pAKT) and phosphorylated IRS1(pIRS1) in L6-GLUT4-myc cells treated with 0.125mM of caffeic acid or ferulic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against pAKT and pIRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The impact of ferulic acid on the expression of AS160 and PTEN proteins in L6-myc cells was examined. After 24 h of exposure to 0.125 mM ferulic acid, a significant increase in AS160 protein levels was observed under both insulin-present and insulin-absent conditions compared to control samples. This suggests that ferulic acid may enhance the expression or stability of AS160, potentially strengthening AS160-mediated signaling within the insulin pathway. In contrast, at the 0.5 h time point, no significant changes in AS160 levels were detected, indicating that the effects of ferulic acid are dependent on prolonged exposure. Additionally, PTEN protein levels also showed a marked increase after 24 h of treatment with 0.125 mM ferulic acid, regardless of insulin presence, as illustrated in figure 3.5.8.

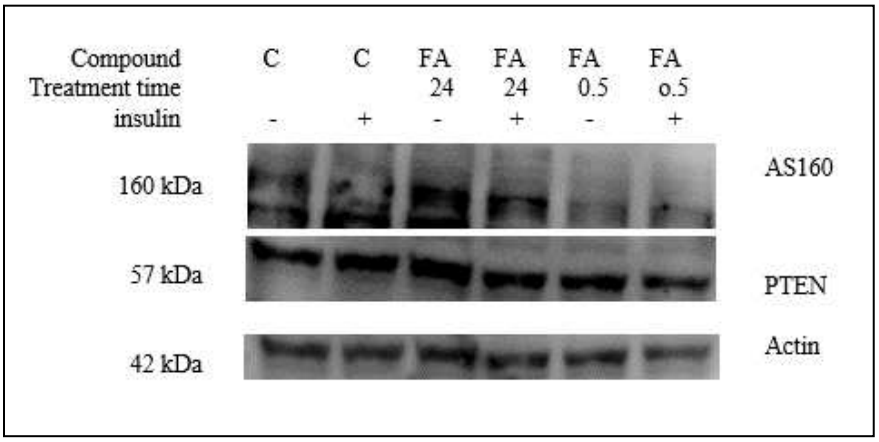


Figure 3.5.8 Effect of FA on As160 and PTEN protein expression. This western blot analysis illustrates the impact of ferulic acid (FA) treatment on the protein levels of AS160 and PTEN in L6-GLUT4-myc cells treated with 0.125mM of ferulic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against As160 and PTEN, followed by appropriate secondary antibodies. β-actin was used as a loading control.

The effect of ferulic acid and caffeic acid on pAS160 and pPTEN was also examined. Treatment with 0.125 mM ferulic acid for 0.5 h in the presence of insulin resulted in a notable elevation of pAS160 levels relative to control cells. This finding implies that ferulic acid may potentiate insulin signaling or associated pathways in the short term (0.5h). Similarly, exposure to 0.125 mM caffeic acid for 24 h in the presence of insulin also significantly increased pAS160 levels compared to controls. This suggests that caffeic acid exerts a sustained influence on insulin signaling pathways. In both cases, no significant alterations in pPTEN levels were observed compared to control cells, irrespective of the treatment duration. This indicates that the modulation of PAS160 by FA and CA does not involve changes in PTEN phosphorylation status (Figure 3.5.9).

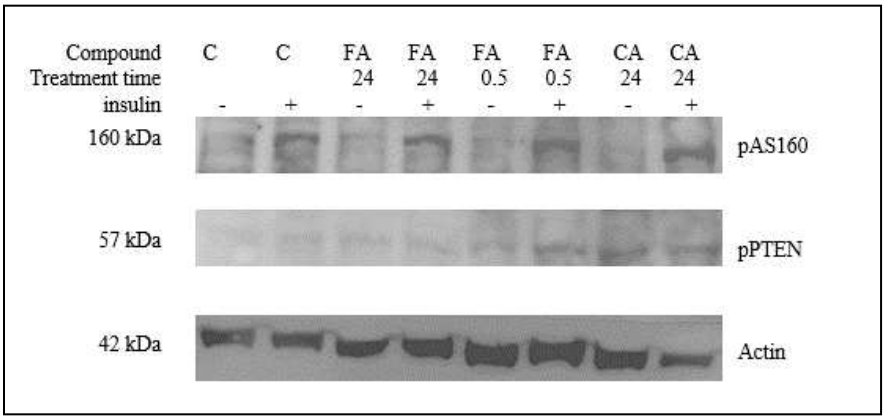


Figure 3.5.9 Effect of FA and CA on pAS160 and pPTEN protein expression. This western blot analysis illustrates the impact of ferulic acid (FA) and caffeic acid (CA) treatment on the protein levels of phosphorylated AS160 (pAS160) and phosphorylated PTEN (pPTEN) in L6-GLUT4-myc cells treated with 0.125mM of ferulic acid or caffeic acid for either 24 h or 0.5 h in presence and absence of insulin. Equal amounts of cell lysate (80 µg protein) were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against pAKT and pIRS1, followed by appropriate secondary antibodies. β-actin was used as a loading control.

3.6 Effects of natural compounds on GLUT4, IRS1, PETEN, and AS160 Expression in Glucose Metabolism Pathways

In this study, the effects of selected natural compounds on the mRNA expression levels of GLUT4, PTEN, AS160, and IRS1 were examined in L6-GLUT4myc cells using reverse transcription–polymerase chain reaction (RT-PCR). The natural compounds evaluated included caffeic acid, ferulic acid, benzoic acid, 3,4-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, and 2-hydroxy-4-methoxybenzoic acid. These compounds were selected based on their previously observed ability to enhance GLUT4 translocation in serum-starved and insulin-stimulated L6-GLUT4myc cells. Relative mRNA expression levels were normalized to 18S ribosomal RNA (18-srRNA), which was used as the internal reference gene

due to its stability and widespread use as a housekeeping gene in qRT-PCR analyses of skeletal muscle cells.

3.6.1 GLUT4 mRNA Expression Analysis

The influence of caffeic acid and ferulic acid on GLUT4 mRNA expression in L6 myoblast cells was examined under conditions both with and without insulin treatment. The insulin-free control condition served as the baseline (100% relative expression) for normalization across all samples. Insulin administration resulted in an increase in GLUT4 expression to 138±0.2%, indicating that insulin upregulates GLUT4 gene expression. In the presence of insulin, the natural compounds exhibited distinct effects on GLUT4 expression levels.

The study examined the impact of caffeic acid and ferulic acid on GLUT4 mRNA expression in L6 myoblast cells, both with and without insulin treatment. The control condition without insulin served as the baseline, representing 100% relative expression for normalization purposes. Insulin administration resulted in an increase in GLUT4 expression to 138±0.2%, confirming its role in upregulating GLUT4.

The effects of the natural compounds on GLUT4 expression varied when combined with insulin. The most pronounced enhancement was observed with caffeic acid at a concentration of 0.125 mM for 24 h in the presence of insulin, reaching 365 ± 0.018%. A shorter treatment duration of 0.5 h with 0.125 mM caffeic acid also significantly increased GLUT4 expression by 254±0.43%, demonstrating a statistically significant difference compared to the control condition (Figure 3.6.1.1).

Treatment with ferulic acid at a concentration of 0.125 mM for 24 hours resulted in a 243±0.1% increase in GLUT4 expression. Conversely, exposure to 0.125 mM ferulic acid for 0.5 hours led to a 128±0.25% increase in GLUT4 expression. This suggests that there was no statistically significant difference from the control in the short-term treatment.

The effect of the benzoic acid derivates namely, 3,4-dihydroxybenzoic acid (DB), benzoic acid (BZ), 3,4,5-trihydroxybenzoic acid (TB), and 2-hydroxy-4-methoxybenzoic acid (HM) on GLUT4 mRNA levels was examined. The Cells were treated with each compound for 0.5 hours and 24 hours at 0.125 mM, with and without insulin stimulation. The insulin dependence of these compounds was then established by comparing +insulin to -insulin for each compound.

Treatment with 0.125 mM of DB significantly elevated GLUT4 expression relative to the control, with increases of 245±0.01% at 24 h and 192±0.25% at 0.5 h in the presence of insulin. The enhancement was more substantial at 24 hours, particularly following insulin stimulation, implying a synergistic interaction between DB and insulin on GLUT4 expression. Similarly, 0.125 mM of BZ increased GLUT4 expression at both time points, by 221±0.02% at 24 h and 291±0.43% at 0.5 h, with a notable augmentation after insulin stimulation at 0.5 h (Figure 3.6.1.2).

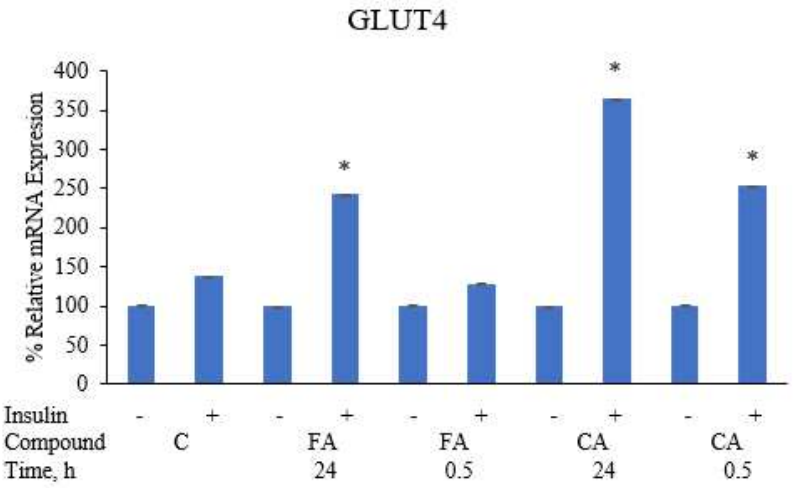


Figure 3.6.1.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on GLUT4 gene expression before and after insulin stimulation compared with control (C) cells. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *$p < 0.05$ Statistical significance.

TB did not induce a significant change in GLUT4 levels at 0.5 h; however, at 24 h, 0.125 mM of TB in the presence of insulin resulted in a 182±0.3% increase in GLUT4 expression. In contrast, 0.125 mM of HM did not produce a significant effect on GLUT4 expression at either 24 h or 0.5 h (Figure 3.6.1.2).

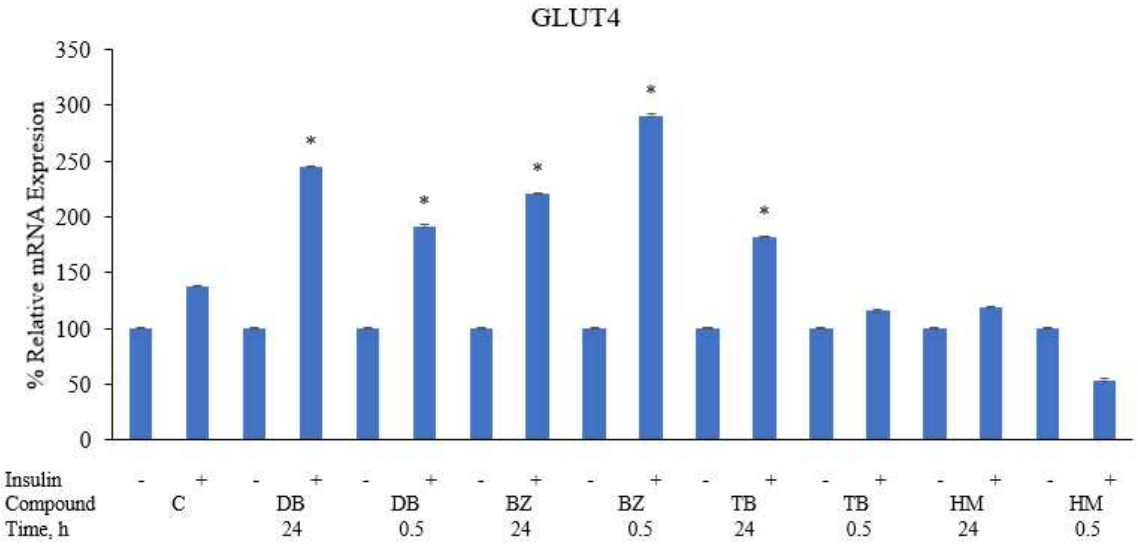


Figure 3.6.1.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on GLUT4 gene expression before and after insulin stimulation and compared with control (C) cell. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.6.2 PTEN mRNA Expression Analysis

The effect of the above six compounds on PTEN mRNA expression in L6 muscle cells was also investigated in the presence and absence of insulin administration. To normalize all samples and establish a common reference, the baseline (100% relative expression) was the insulin-free control condition (-insulin control). The

insulin-dependent PTEN levels altered by CA and FA at 0.125 mM were then calculated by comparing +insulin to -insulin for each compound.

The expression of PTEN significantly decreased to 46±0.25% following insulin administration without plant extract (control + insulin), relative to the control group. This indicates that insulin alone suppresses PTEN expression, which functions as a negative regulator of insulin signaling. The observed reduction in PTEN expression was altered when combined with CA and FA.

PTEN expression was diminished in the presence of 0.125 mM FA combined with insulin, decreasing to 52±0.9% at 24 h and 75±4.5% at 0.5 h. This reduction suggests a notable downregulation that could potentially enhance insulin signaling pathways. Additionally, CA at the same concentration (0.125 mM) with insulin significantly suppressed PTEN levels, reducing them to 48±0.8% at 24 h and 87±0.2% at 0.5 h (Figure 3.6.2.1).

PTEN expression was significantly diminished following treatment with 0.125 mM of DB, reaching 38±0.76% at 24 h and 42±0.35% at 0.5 h. Similarly, 0.125 mM of BZ reduced PTEN levels by 59±3.7% at 24 h and 62±3.1% at 0.5 h in the presence of insulin. Treatment with 0.125 mM of TB and HM for 24 h also resulted in decreased PTEN expression, reaching 60±3.8% and 61±5%, respectively, under insulin conditions (Figure 3.6.2.2).

When the cells were treated with 0.125mM of TB and HM at 0.5 h, PTEN expression was reduced to 80% but there was no statistically significant difference from the control for both TB and HM in the presence of insulin (Figure 3.6.2.2). None of the compounds had any significant effect on PTEN mRNA expression in the absence of insulin.

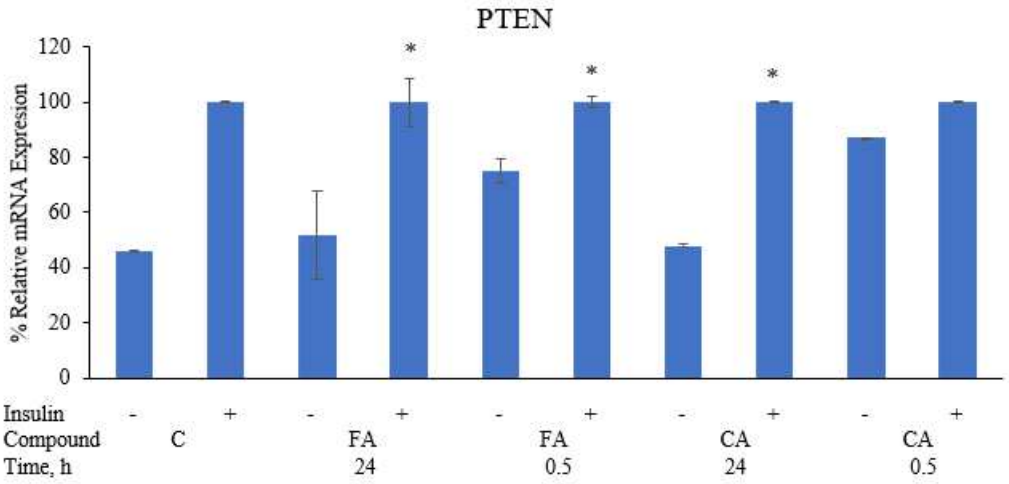


Figure 3.6.2.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on PTEN gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

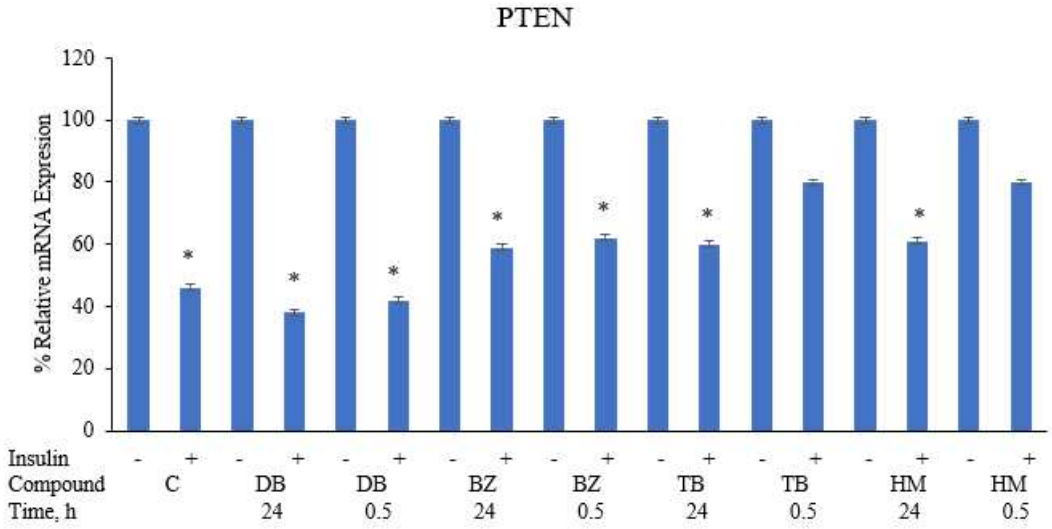


Figure 3.6.2.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on PTEN gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.6.3 AS160 mRNA Expression Analysis

The expression levels of AS160 in L6 muscle cells were examined under conditions with and without the six natural compounds, both in the presence and absence of insulin. The results indicate that insulin markedly reduces AS160 expression, achieving only 23±4% of the baseline levels observed in untreated cells (Figure 3.6.3.1).

AS160 mRNA expression was significantly restored in insulin-treated cells following exposure to CA at a concentration of 0.125 mM. Expression levels increased to 200 ± 0.45% after 24 h and to 144 ± 0.23% after 0.5 h of treatment. In comparison, FA treatment resulted in a moderate but significant upregulation of AS160 expression. Cells treated with 0.125 mM FA exhibited AS160 expression levels of 167 ± 0.2% after 24 h and 133 ± 2.2% after 0.5 h in the presence of insulin (Figure 3.6.3.1).

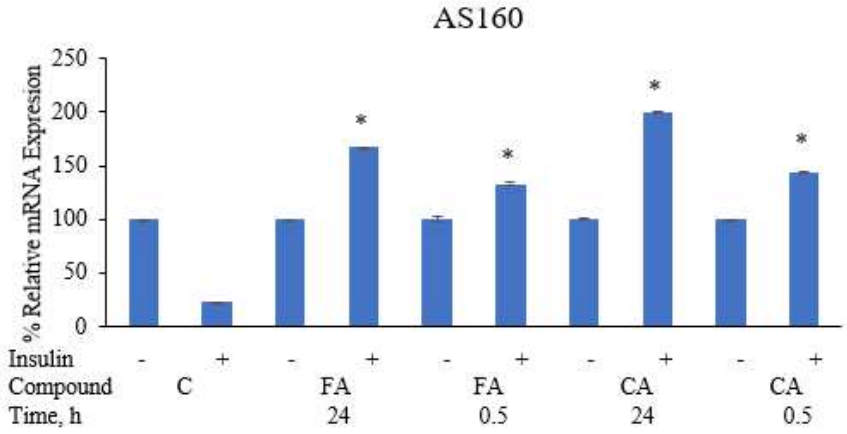


Figure 3.6.3.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on AS160 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

AS160 expression was elevated following treatment with 0.125 mM DB, reaching 210±1.1% at 24 h and 151±0.9% at 0.5 h in the presence of insulin. Similarly, exposure to 0.125 mM BZ for 24 h increased AS160 expression to 139%, with a

modest rise to 108% at 0.5 h under insulin conditions. Conversely, treatment with 0.125 mM TB for 24 h resulted in an increase to 182±0.3%, while a slight elevation to 116±0.5% was observed at 0.5 h in the presence of insulin. In contrast, administration of 0.125 mM HM for either 24 h or 0.5 h led to a reduction in AS160 expression to 98±0.8% and 79±0.4%, respectively (Figure 3.6.3.2).

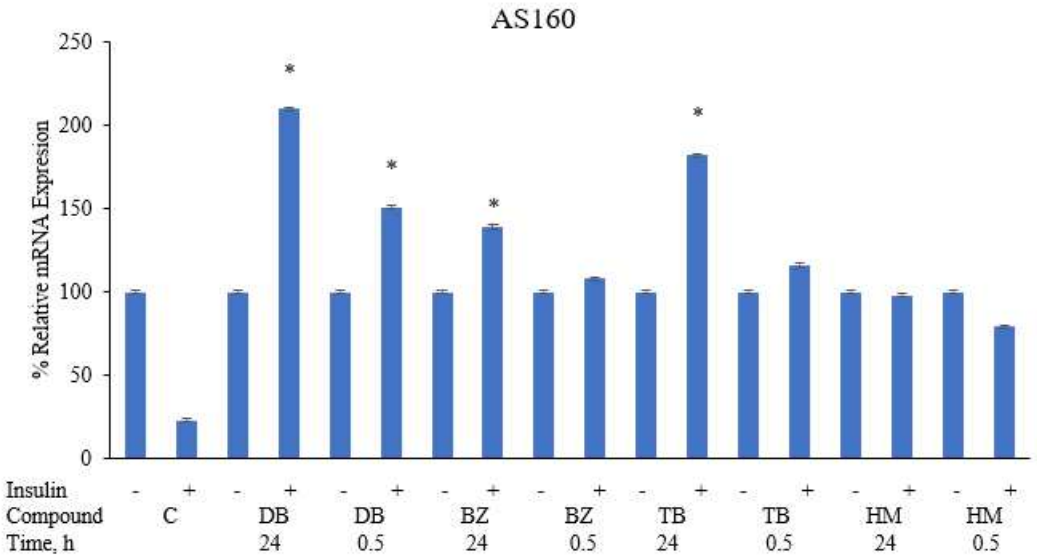


Figure 3.6.3.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on AS160 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.6.4 IRS1 mRNA Expression Analysis

Expression for IRS1 in the L6 muscle cells challenged with the six selected natural compounds was tested without and with insulin. The control data suggest that insulin increased IRS1 expression significantly to 170%.

A significant increase in IRS1 mRNA expression was detected in cells stimulated with insulin and treated with CA and FA. Treatment with CA at 0.125 mM resulted

in a notable elevation of IRS1 levels, reaching 261 ± 0.01% after 24 h and remaining elevated at 210 ± 0.4% after 0.5 h. Similarly, FA at 0.0125 mM markedly enhanced IRS1 expression, attaining 228 ± 0.32% at 24 h and 180 ± 0.25% at 0.5 h under insulin-stimulated conditions (Figure 3.6.4.1).

IRS1 expression was elevated in cells exposed to 0.125 mM DB at 24 h, reaching 241±0.8%, and increased to 162±0.5% at 0.5 h in the presence of insulin. Similarly, treatment with 0.125 mM BZ resulted in increased IRS1 levels, attaining 208±0.9% at 24 h and 239±0.2% at 0.5 h in insulin-treated cells. Additionally, 0.125 mM TB treatment enhanced IRS1 expression to 189% at 24 h and 109±0.5% at 0.5 h under insulin stimulation. Conversely, 0.125 mM HM treatment reduced IRS1 levels to 88±0.84% at 24 h and 56±0.9% at 0.5 h, both in the presence of insulin. None of the tested compounds significantly affected IRS1 expression in the absence of insulin treatment (Figure 3.6.4.2).

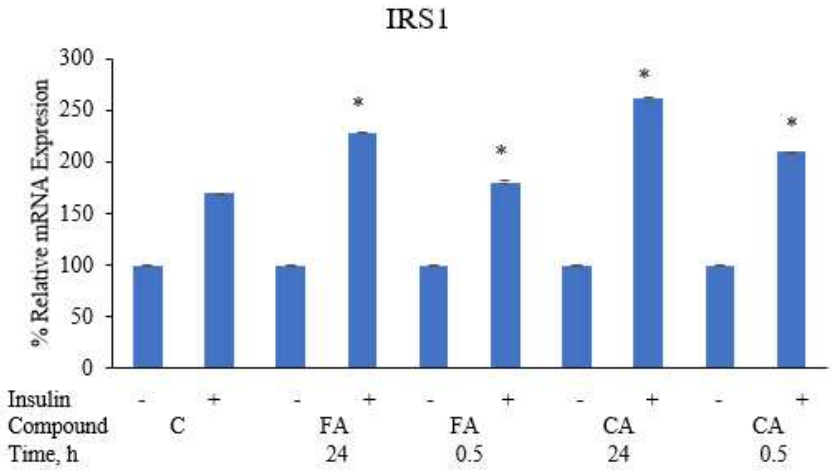


Figure 3.6.4.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on IRS1 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

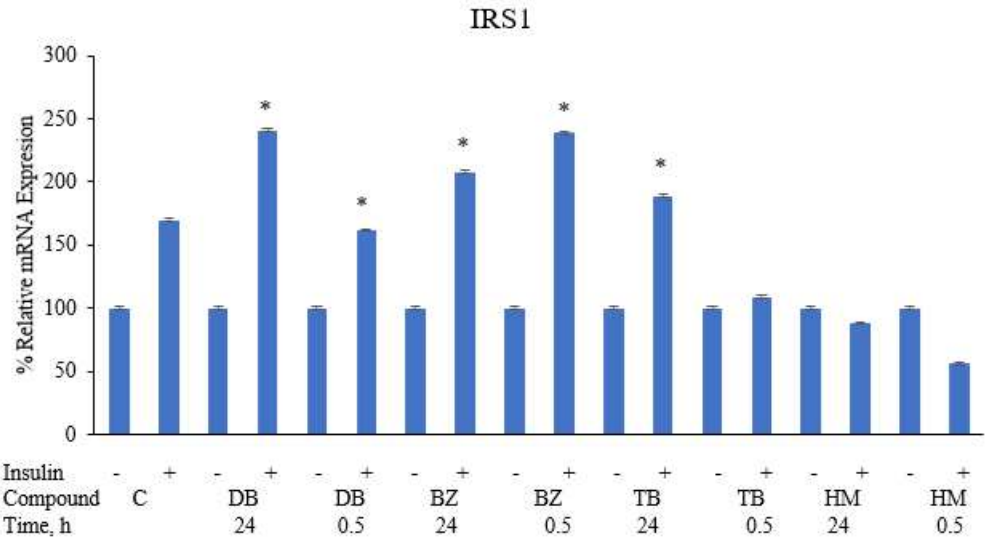


Figure 3.6.4.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on IRS1 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.6.5 AKT1 mRNA Expression Analysis

The impact of six specific compounds on AKT1 mRNA expression in L6 muscle cells was examined both in the presence and absence of insulin treatment. All samples were normalized against a baseline, which was set as the insulin-free control condition, representing 100% relative expression. The changes in insulin-dependent AKT1 levels induced by CA and FA at a concentration of 0.125 mM were determined by comparing the expression levels with and without insulin for each compound. When insulin was administered without the presence of the active compounds, AKT1 expression significantly increased to 120±0.003%, indicating an upregulation due to insulin alone.

Treating the cells with CA and FA led to increase in AKT1 expression. It was increased in cells treated with 0.125mM of FA (+insulin) and reached 110±2% and 120±1.5% at 24 h and 0.5 h respectively, indicating a significant regulation that

may improve insulin signaling. Under insulin-stimulated conditions, AKT1 mRNA expression responded differently to treatment with the six tested compounds.

CA at 0.125 mM exerted a significant inhibitory effect, reducing AKT1 expression to 65 ± 2% at 24 h and to 90 ± 3.2% at 0.5 h (Figure 3.6.5.1). Conversely, DB and BZ treatments resulted in a consistent upregulation of AKT1 expression, with peak levels observed after 24 h of exposure.

TB and HM also increased AKT1 expression following 24 h of treatment; however, their short-term effects were less pronounced, as AKT1 levels at 0.5 h did not differ significantly from the insulin-treated control (Figure 3.6.6.2). Notably, the absence of insulin abolished these modulatory effects, as none of the compounds significantly influenced AKT1 mRNA expression in absence of insulin.

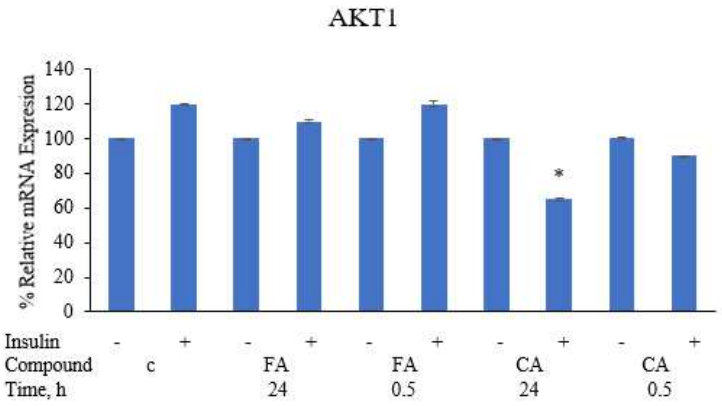


Figure 3.6.5.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on AKT1 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

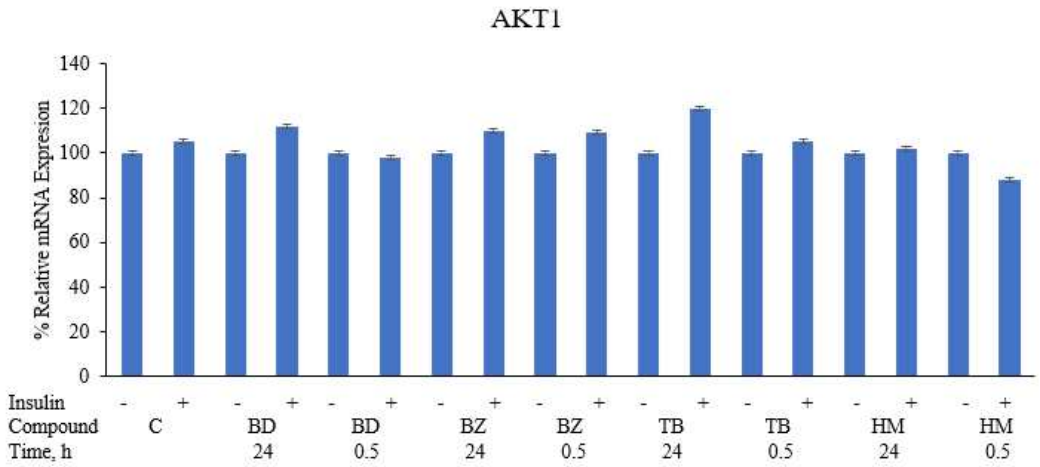


Figure 3.6.5.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on AKT1 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.6.6 AKT2 mRNA Expression Analysis

The influence of the six selected compounds on AKT2 mRNA expression in L6 muscle cells was evaluated under both insulin-stimulated and insulin-free conditions. For standardization purposes, the insulin-free control condition was defined as the baseline and assigned a relative expression value of 100%. Insulin-dependent changes in AKT2 expression following treatment with CA and FA at a concentration of 0.125 mM were determined by comparing expression levels in the presence and absence of insulin for each compound.

In the absence of plant extracts, insulin treatment alone resulted in a marked upregulation of AKT2 mRNA expression, reaching 109 ± 0.6% relative to the insulin-free control. This finding confirms that insulin stimulation significantly enhances AKT2 expression in L6 muscle cells.

Different findings were observed, when paired with CA and FA. AKT2 expression was increased in 0.125mM of FA (+insulin) and reached 135±1.2% and 112±1.6% at 24 h and 0.5 h respectively. AKT2 was also statistically significantly suppressed by CA at 0.125mM (+insulin) which decreased AKT2 levels to 55±0.95% and 85±0.3% at 24 h and 0.5 h respectively (Figure 3.6.6.1).

AKT2 expression was markedly elevated in cells exposed to 0.125 mM of DB, reaching 109±0.6% at 24 hours and 92±0.35% at 0.5 hours. Similarly, 0.125 mM of BZ increased AKT2 expression to 124±3.7% at 24 hours and 119±3.1% at 0.5 hours in the presence of insulin. In addition, treatment with 0.125 mM of TB and HM for 24 hours resulted in increased AKT2 expression, reaching 102±0.8% and 112±2.5% respectively, in the presence of insulin. When cells were treated with 0.125 mM of TB and HM for 0.5 hours, AKT expression also increased; however, these changes were not statistically significant compared to controls.

None of the compounds significantly affected AKT2 mRNA expression in the absence of insulin (Figure 3.6.6.2).

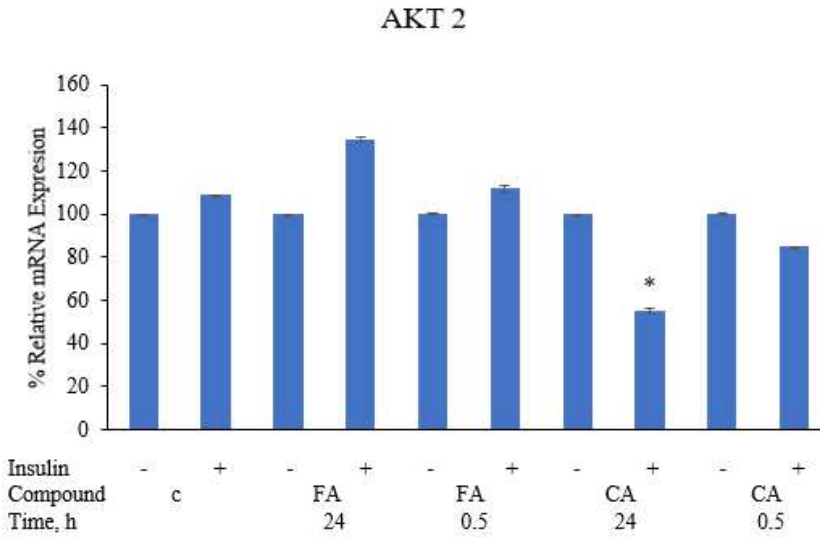


Figure 3.6.6.1 Effects of 0.125mM caffeic acid (CA) and ferulic acid (FA) at 0.5 h treatment and 24 h treatment on AKT2 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

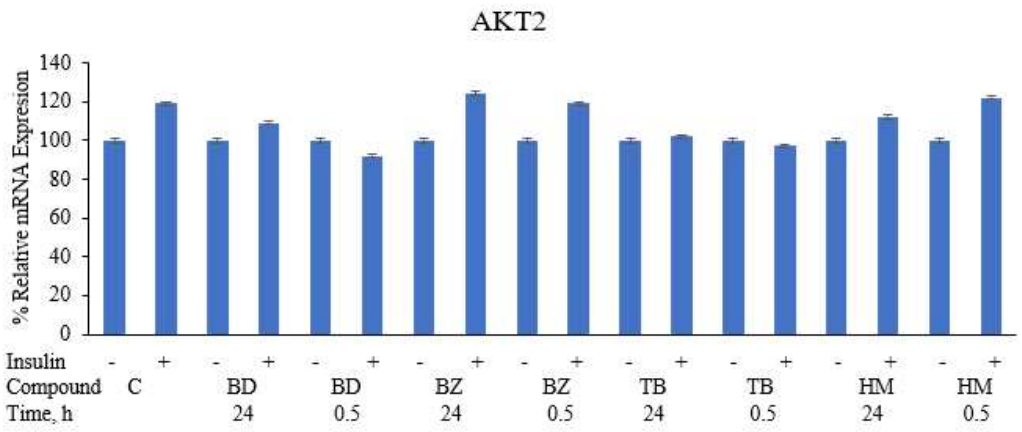


Figure 3.6.6.2 Effects of 0.125mM of 3,4 dihydroxy benzoic acid (DB), benzoic acid (BZ), 3,4,5 trihydroxy benzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) at 0.5 h and 24 h treatments on AKT2 gene expression before and after insulin stimulation. Values given represent means ± SEM (relative to untreated control cells) of three independent experiments carried out in triplicates. *p < 0.05 Statistical significance.

3.7 Effects of Natural Compounds on Streptozotocin induced diabetic Mice Blood Glucose levels

The effects of selected natural compounds caffeic acid, ferulic acid, isovanillic acid, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid and 2-hydroxy4-methoxy benzoic acid on blood glucose levels in diabetic mice induced by streptozotocin (STZ) was evaluated after 3 weeks of 100mg/Kg treatment of each compound.

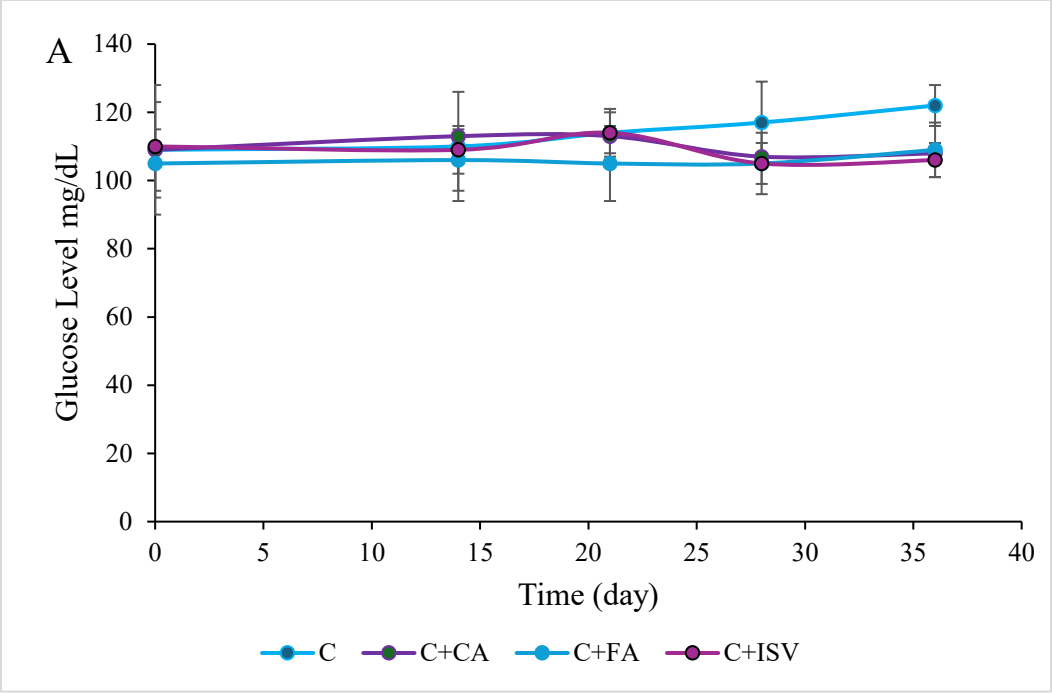
A clear reduction in blood glucose levels was observed in diabetic mice following a three-week treatment with CA, FA, and ISV at a dose of 100 mg/Kg. When compared with the diabetic control group, all treated groups showed a marked improvement in glycemic status, with the effect becoming evident as early as the first week of treatment. By day 36, blood glucose levels reached 487 ± 8 mg/dL in diabetic control mice, while substantially lower values were recorded in the CA, FA, and ISV treated groups (336 ± 10 mg/dL, 320 ± 8 mg/dL, and 321 ± 9 mg/dL, respectively) (Figure 3.7.1B).

In non-diabetic mice, blood glucose levels remained within the normal range throughout the experimental period. The control group exhibited a glucose level of 122 mg/dL, whereas mice treated with CA, FA, and ISV maintained comparable levels of 108 mg/dL, 109 mg/dL, and 106 mg/dL, respectively, with no statistically significant differences observed. (Figure 3.7.1A).

Blood glucose levels were reduced in mice with induced diabetes following treatment with 100 mg/Kg of BZ, TB, DB, and HM for three weeks. Among the tested compounds, HM demonstrated the most pronounced hypoglycemic effect. On day 36, diabetic control mice exhibited a blood glucose level of 493 ± 6 mg/dL, whereas treated groups showed significantly lower levels of 364 ± 9 mg/dL (BZ), 382 ± 12 mg/dL (TB), 347 ± 9 mg/dL (DB), and 277 ± 8 mg/dL (HM) (Figure 3.6.2B).

In contrast, treatment with these compounds did not induce statistically significant changes in blood glucose levels in non-diabetic mice. Control mice maintained a glucose level of 127 ± 6 mg/dL, while mice treated with 100 mg/Kg of BZ, TB, DB, and HM exhibited levels of 114 ± 15 mg/dL, 120 ± 13 mg/dL, 122 ± 4 mg/dL, and 124 ± 4 mg/dL, respectively, throughout the experimental period (Figure 3.7.2A).

Mice blood glucose decreased from 451±7mg/dL for diabetic mice to 311±3 mg/dL, 252±7 mg/dL, 310±5 mg/dL for mice treated with 100mg/Kg of PA, MA and SA respectively (figure 3.7.3B). No significant changes in the blood glucose levels in the mice treated with the vehicle Bovin serum albumin (BSA) compared with control diabetic mice was observed. Moreover, the blood glucose level in non-diabetic mice was 114±10 mg/dL and no statically significant in glucose blood levels in non-diabetic mice groups treated with 100mg/Kg of PA, MA and SA (Figure 3.7.3A).



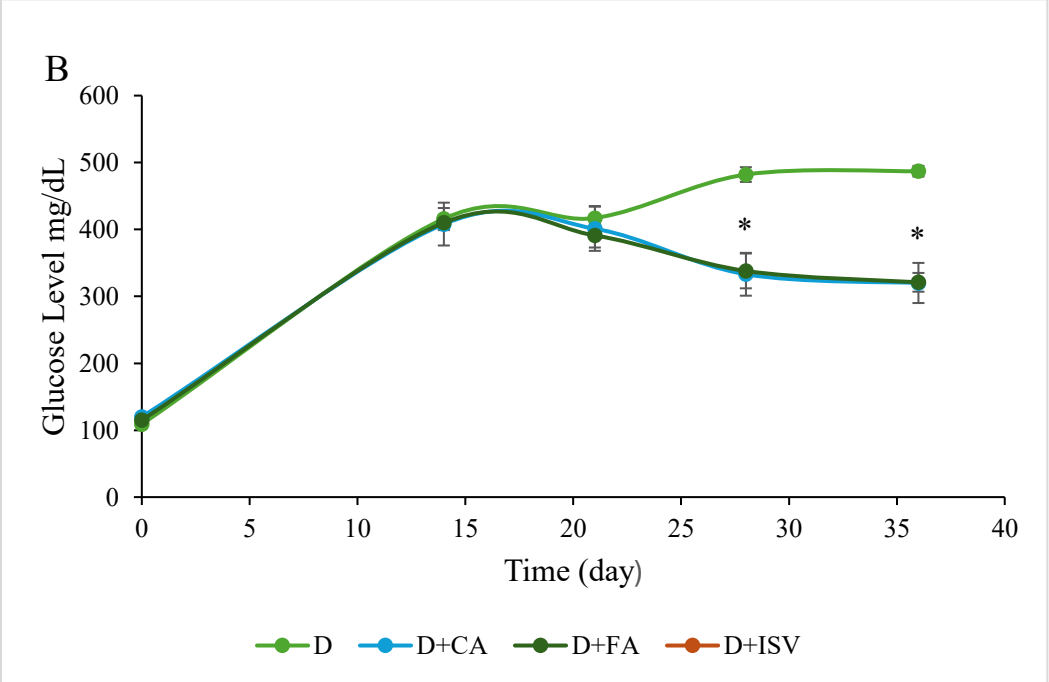


Figure 3.7.1 Blood glucose levels (mg/dL); A: for control mice (C) treated with 100mg /kg of caffeic acid (CA), ferulic acid (FA) and isovanillic acid (ISV) for 3 weeks. B: STZ-induced diabetic mice (D) treated with 100mg/Kg of CA, FA and ISV for 3 weeks. Values are expressed as mean ± SEM. * $p < 0.05$, significant as compared with diabetic group.

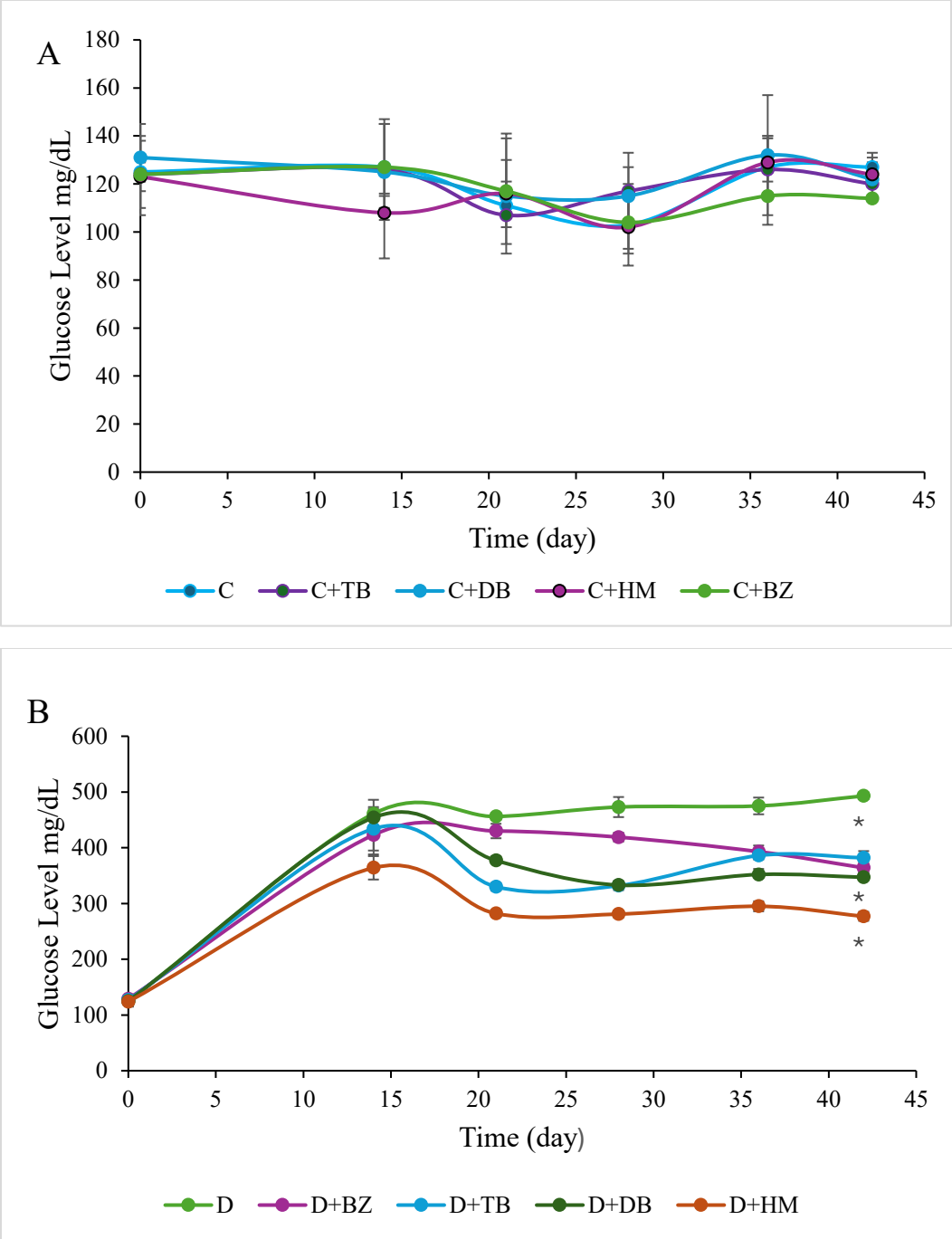
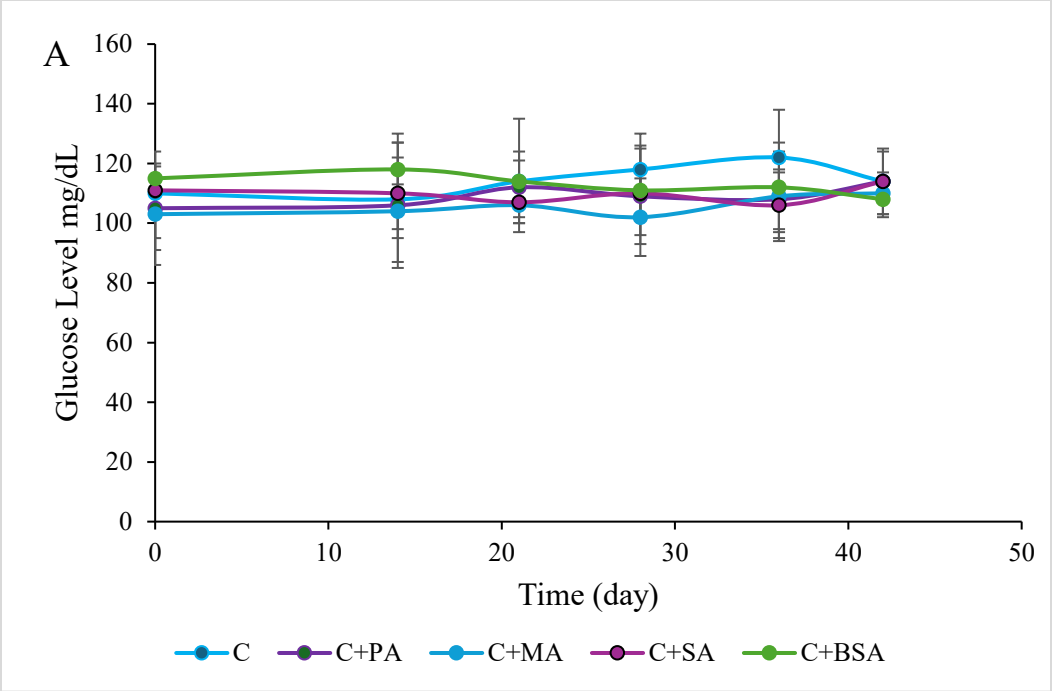


Figure 3.7.2 Blood glucose levels (mg/dL); A: for control mice(C) treated with 100mg /kg of benzoic acid (BZ), 3,4-dihydroxy benzoic acid (DB), 3,4,5 trihydroxybenzoic acid (TB) and 2-hydroxy4-methoxy benzoic acid (HM) 3 weeks. B: STZ- induced diabetic mice (D) treated with 100mg/Kg of BZ, DB, TB and HM for 3 weeks. Values are expressed as mean ± SEM. * $p < 0.05$, significant as compared with diabetic group.



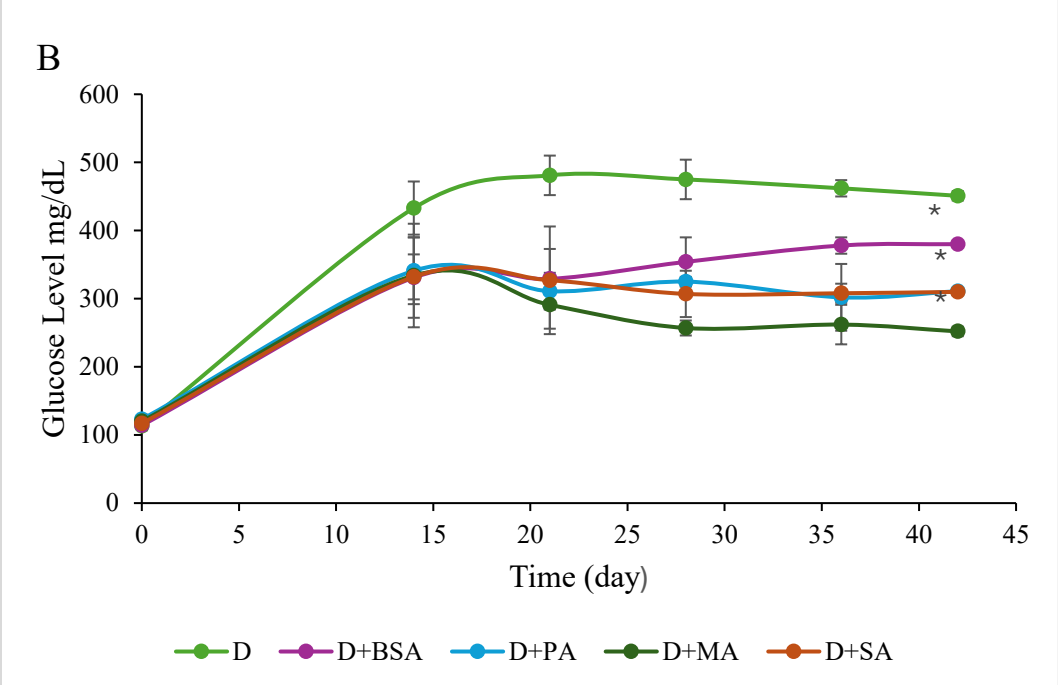


Figure 3.7.3 Blood glucose levels (mg/dL); A: for control mice(C) treated with 100mg /kg of palmitic acid (PA), stearic acid (SA), myristic acid (MA) and Bovin serum albumin (BSA) for 3 weeks. B: STZ- induced diabetic mice (D) treated with 100mg/Kg of PA, SA and MA for 3 weeks. Values are expressed as mean ± SEM. * $p < 0.05$, significant as compared with diabetic group.

3.8 Effects of natural compounds on pancreatic α-amylase and intestinal α-Glucosidase enzymes

The potential inhibitory effect of the natural compounds caffeic acid, ferulic acid, isovanillic acid, vanillic acid, D-pintol, palmitic acid, stearic acid, myristic acid, benzoic acid, 3,4-dihydroxy benzoic acid, 3,4,5 trihydroxybenzoic acid, 2-hydroxy4-methoxy benzoic acid and syringic acid

The inhibitory effects on α-amylase and α-glucosidase were evaluated according to the procedures outlined in the materials and methods section. Acarbose at a concentration of 0.5 mM served as the reference inhibitor, achieving 93% inhibition.

The data demonstrated that these compounds suppressed enzyme activity in a concentration-dependent manner. Consequently, the IC_{50} values were determined (refer to Figure 3.8.1-4 and Table 3.8).

The IC_{50} values for α-amylase inhibition by FA, ISV, CA, and VA were 3.9 ±3 mM, 6.8 ±0.4 mM, 1.05 ±0.25 mM, and 5.8 ±4 mM, respectively. Similarly, for DP, PA, SA, and MA, the IC_{50} values were 5.2 ±9 mM, 6.8 ±2 mM, 9.5 ±6 mM, and 2.3 ±7 mM, respectively. The IC_{50} values for BA, DB, TB, HM, and SYA were 9.4 ±8 mM, 3.7 ±7 mM, 5.7 ±0.9 mM, 2.7 ±7 mM, and 5.8 ±3 mM, respectively.

These natural compounds exhibited a dose-dependent inhibitory effect on α-glucosidase activity, and the corresponding IC_{50} values were determined. The IC_{50} values for ISV and CA were found to be below 1 mM, while those for FA, VA, BZ, DB, TB, and HM were 3.6±4 mM, 1.4±2.5 mM, 7±3 mM, 2.1±3 mM, 1.7±2.5 mM, 6.3±1 mM, and 4.8±3 mM, respectively.

Acarbose at a concentration of 0.5 mM served as a reference standard, achieving 53% inhibition of α-glucosidase activity.

Table 8.3 IC_{50} values of natural compounds against intestinal α-glucosidase and α-amylase

Compounds name	For α-Amylase (mM)	For α-Glucosidase (mM)
Acarbose	<0.5	<0.5
Ferulic acid	3.9 ±3	3.6±4
Isovanillic acid	6.8 ±0.4	>1
Caffeic acid	1.05 ±0.25	> 1
Vanillic acid	5.8 ±4	1.4±2.5
D-pintol	5.2 ±9	*
Palmitic acid	6.8 ±2	*
Stearic acid	9.5 ±6	7±3
Myristic acid	2.3 ±7	*
Benzoic acid	9.4 ±8	2.1±3
3,4-dihydroxy benzoic acid	3.7 ±7	1.7±2.5
3,4,5 trihydroxybenzoic acid	5.7 ±0.9	6.3±1
2-hydroxy4-methoxy benzoic	2.7 ±7	4.8±3
Syringic acid	5.8 ±3	*

* **Not tested.** The IC_{50} for α-glucosidase could not be determined for some compounds due to limited solubility under the assay condition, which lacks a heating step required to enhance solubility, unlike the amylase assay.

In summary, the tested natural compounds exhibited varying inhibitory activities against both pancreatic α-amylase and intestinal α-glucosidase. For most compounds, inhibition of α-glucosidase was moderate, whereas a more pronounced inhibitory effect was observed against α-amylase. This pattern suggests that these compounds may predominantly modulate pancreatic starch digestion rather than directly limiting intestinal glucose release.

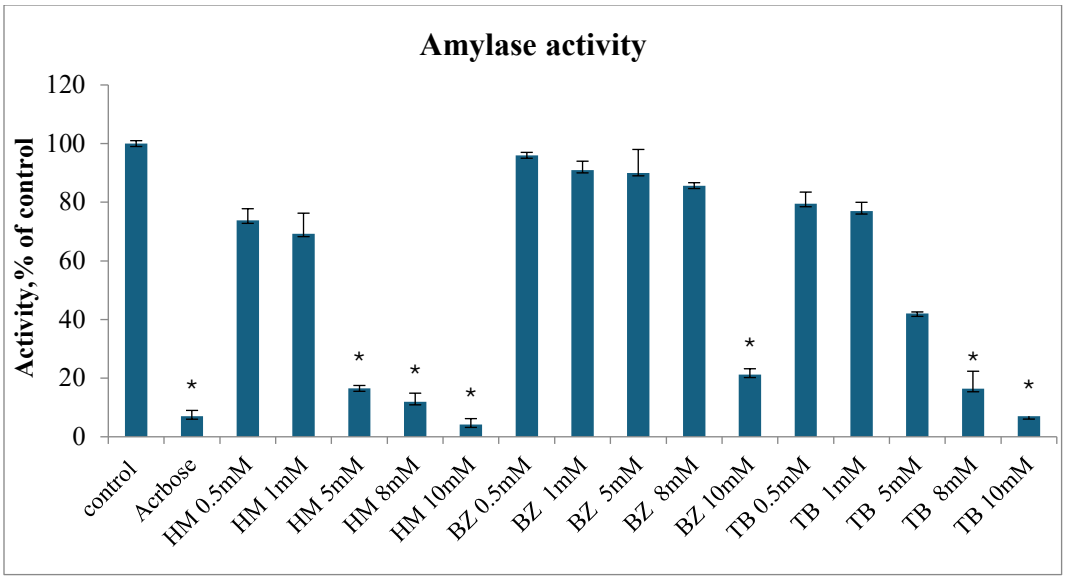


Figure 3.8.1 Effects of 2-hydroxy4-methoxy benzoic (HM), benzoic acid (BZ) and 3,4,5 trihydroxybenzoic acid (TB). Concentrations are 0.5mM, 1mM, 5mM, 8mM and 10mM for α-amylase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. $*p < 0.05$, which is considered significant as compared with controls.

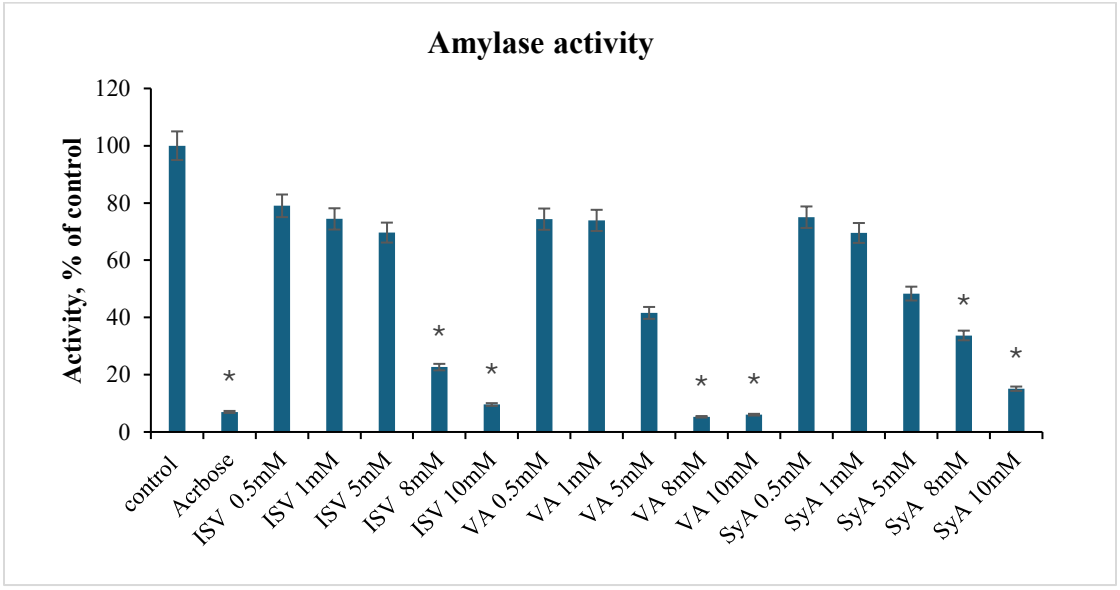


Figure 3.8.2 Effects of isovanillic acid (ISV), vanillic acid (VA) and syringic acid (SY). Concentrations ar 0.5mM, 1mM, 5mM, 8mM and 10mM on α-amylase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. $*p < 0.05$, which is considered significant as compared with controls.

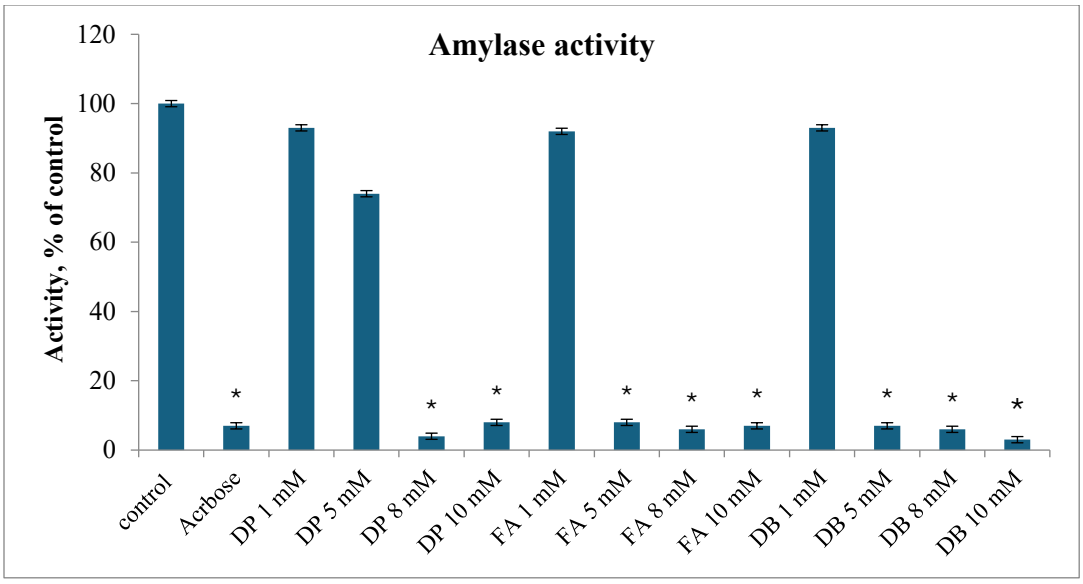


Figure 3.8.3 Effects of D-pintol (DP), ferulic acid (FA) and 3,4-dihydroxy benzoic (DB) acid. Concentrations are 1mM, 5mM, 8mM and 10mMon α-amylase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. ∗p < 0.05, which is considered significant as compared with controls.

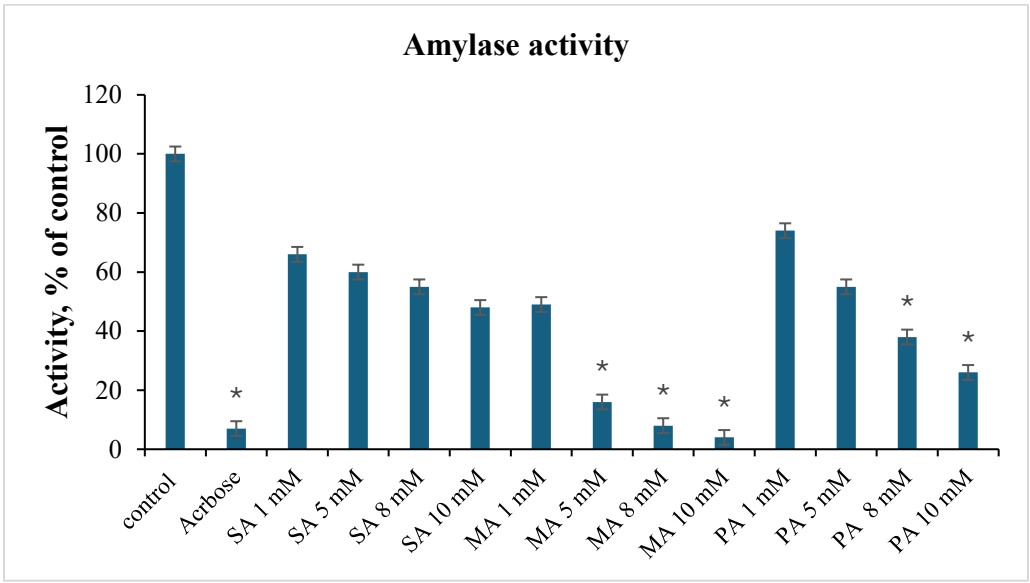


Figure 3.8.4 Effects of palmitic acid (PA), stearic acid (SA) and myristic acid (MA). Concentrations are 1mM, 5mM, 8mM and 10mM on α-amylase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. ∗p < 0.05, which is considered significant as compared with controls.

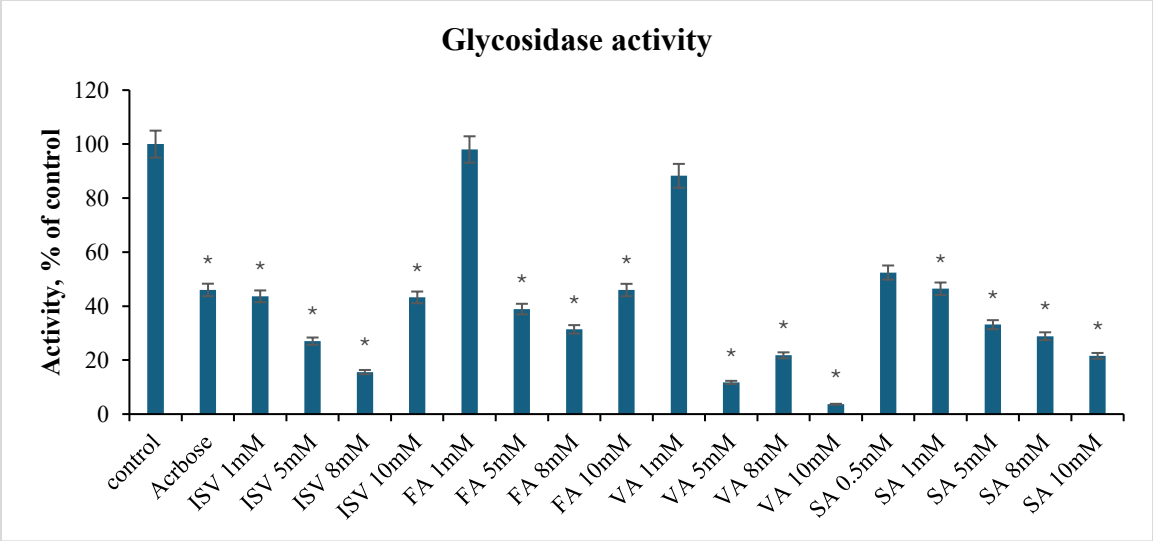


Figure 3.8.5 Effects of isovanillic acid (ISV), vanillic acid (VA), ferulic acid (FA) and stearic acid (SA). Concentrations are1mM, 5mM, 8mM and 10mM on α-glycosidase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. ∗p < 0.05, which is considered significant as compared with controls.

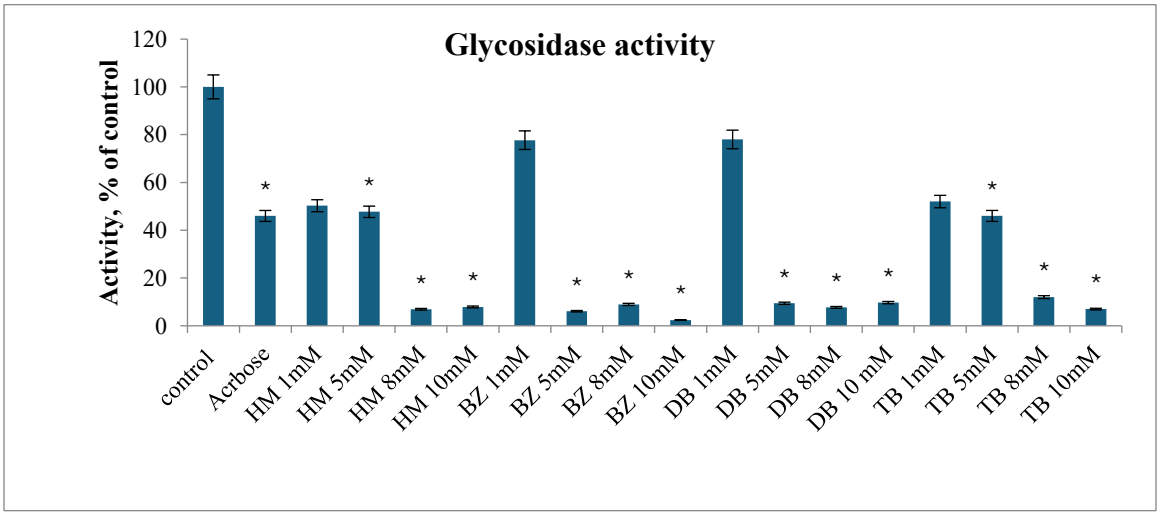


Figure 3.8.6 Effects of 3,4-dihydroxy benzoic acid (DB), 3,4,5 trihydroxybenzoic acid (TB), benzoic acid (BZ) and 2-hydroxy4-methoxy benzoic acid (HM). Compound concentrations are 1mM, 5mM, 8mM and 10mM on α-glycosidase activity. Values represent the mean ± SEM of three experiments. The t-test was conducted using SPSS, version 23.0. ∗p < 0.05, which is considered significant as compared with controls.

4.Discussion

4.1 Overview of Study Rationale and Principal Findings

Type 2 diabetes mellitus is distinguished by compromised insulin signaling pathways. This results in impaired glucose absorption in peripheral tissues, notably skeletal muscle. A key pathological characteristic is the diminished translocation of glucose transporter-4 (GLUT4) to the cell membrane [133]. Bioactive compounds extracted from medicinal plants have garnered growing interest as prospective antidiabetic agents. This interest is primarily due to their structural heterogeneity, favorable safety profiles, and ability to act through multiple biological targets [134, 135]. Recent investigations have elucidated that these phytochemicals may modulate key signaling pathways involved in insulin sensitivity, thereby offering a promising avenue for the development of adjunctive therapies aimed at restoring metabolic homeostasis in individuals with type 2 diabetes mellitus [136, 137]. Nonetheless, for numerous such compounds, the specific molecular targets and signaling pathways mediating their antidiabetic actions are not yet comprehensively characterized [138].

The present study aimed to systematically assess the antidiabetic efficacy of specific natural compounds through an integrated experimental framework. This approach incorporated cellular toxicity assessments, GLUT4 translocation measurements, protein signaling pathway analysis, gene expression profiling, and *in vivo* validation using a streptozotocin-induced diabetic murine model. This multi-tiered methodology facilitated the identification of compounds that promote glucose uptake mechanisms in skeletal muscle cells. Moreover, it revealed their capacity to influence critical elements within the insulin signaling pathway.

The principal findings of this work demonstrate that several phenolic and benzoic acid derivatives significantly enhance GLUT4 translocation to the plasma membrane in L6-GLUT4myc skeletal muscle cells. These effects were both insulin-dependent and insulin-independent, varied according to exposure duration, and were compound-specific. Importantly, the enhancement of GLUT4 translocation was supported by complementary immunofluorescence data, strengthening the reliability of the observed effects.

The existing literature underscores the significant role of phenolic and benzoic acid derivatives in modulating GLUT4 translocation within skeletal muscle cells, which is pivotal for glucose uptake and insulin sensitivity [125]. Similarly, extracts rich in phenolic compounds, including benzoic acids and their derivatives, have demonstrated capacity to stimulate GLUT4 expression and translocation in skeletal muscle cells [139]. Abelmoschus esculentus (okra) extract contains diverse phenolic compounds with antioxidant and antidiabetic activities, which may influence glucose metabolism. These bioactive constituents potentially modulate key enzymatic pathways and enhance insulin sensitivity, thereby contributing to the amelioration of hyperglycemic conditions [140]. Similarly, Cichorium intybus (chicory) extract is rich in phenolic constituents that have been shown to improve insulin sensitivity and facilitate GLUT4 translocation [141]. The presence of these phenolic compounds in both extracts suggests a possible mechanism for their antidiabetic effects, involving the activation of pathways that increase GLUT4 presence on muscle cell membranes. This process enhances glucose uptake and contributes to the regulation of blood glucose levels in hypoglycemic conditions.

At the molecular level, the benzoic acid derivates tested in this study modulated the expression and phosphorylation status of critical insulin signaling intermediates, particularly AKT and AS160, while exerting limited or no effects on IRS1 phosphorylation in most cases. Transcriptional analyses further revealed insulin-dependent regulation of GLUT4, PTEN, AS160, and IRS1 mRNA levels, indicating that these compounds influence glucose metabolism at both post-translational and gene expression levels. Finally, a subset of the compounds significantly reduced blood glucose levels in diabetic mice, providing physiological relevance to the cellular findings.

Further supporting this, Lee and his colleagues investigated hydroxybenzoic acid derivatives and found that their anti-diabetic effects involve down-regulation of microRNA-1271, which subsequently modulates the IRS1/PI3K/AKT/FOXO1 pathway—an essential signaling cascade in insulin action. This finding is in line with my study and indicates that phenolic acids may exert their beneficial effects by targeting molecular pathways directly involved in insulin signaling [142].

In addition, Naz *et al* provided a comprehensive overview of food polyphenols, including phenolic acids, emphasizing their capacity to protect pancreatic β-cells, promote proliferation, and inhibit enzymes like α-glucosidases and α-amylases, thereby contributing to improved glucose regulation. These mechanisms collectively suggest that phenolic acids can influence multiple facets of glucose metabolism, from insulin secretion to peripheral glucose uptake [143]. Overall, these studies collectively suggest that phenolic acids can improve insulin sensitivity and glucose metabolism through various mechanisms, including modulation of AMPK activity, regulation of insulin signaling pathways, protection of β-cell function, and anti-inflammatory effects. Such multifaceted actions position phenolic acids as promising candidates for managing metabolic disorders like T2DM.

4.2 Safety Profiling and Experimental Validity

A critical prerequisite for evaluating the biological activity of any pharmacologically active compound is the establishment of non-toxic concentration ranges. In this study, the MTT cell viability assay was employed to assess the cytotoxic effects of both plant extracts and purified natural compounds on L6-GLUT4myc skeletal muscle cells. The use of mitochondrial metabolic activity as a readout ensured sensitive detection of early cytotoxic responses.

The results clearly demonstrated that the methanolic extracts of Abelmoschus esculentus and Cichorium intybus exhibited low cytotoxicity within defined concentration ranges, enabling their use in downstream functional assays. C. intybus both aerial and root part extracts were non-toxic up to 0.125 mg/ml *Eray et al* reported IC_{50} for C. intybus methanol extract at 0.69 mg/ml for HepG2 cell line as tested by XTT (a very close assay for the MTT used in my study) [144]. While A. esculents fruit extract according to my study was non- toxic up to 0.250 mg/ml. This is supported by numerous studies that have proven Abelmoschus esculentus extract to be safe up to concentrations of 1 mg/ml. Hafeez et al. have demonstrated that cell viability remains around 50% upon exposure to methanolic leaf extract at a concentration of 2.38 mg/ml [145].

Purified compounds displayed markedly different toxicity profiles, with maximal safe concentrations varying according to chemical structure, exposure duration, and compound class. Short-term exposure generally allowed higher concentrations to be tolerated compared to 24-hour treatment, underscoring the importance of time-dependent toxicity assessment. Most studied active compounds in this project were safe up to 0.5 mM at short time exposure (one hour) while at 24 h exposure they safe up to 0.250 mM. For instance, the exposure of the muscle cells in this study to ferulic acid and caffeic acid for 24 h was non-toxic up to 0.250 mM. The same finding was observed by Caparica *et al* who reported that FA and CA compounds were safe up to 0.25mM on 786-O human cell lines [146]. Others reported higher safe concentrations for benzoic acid when exposed to Phoenix cell line. In their study, he IC_{50} values following 48- and 72-hour exposures to benzoic acid in the Phoenix cells were 410.54±32.29 and 231.16±25.25 µg/ml, which are equivalent to 3.33 and 1.9 mM respectively [147].

This rigorous safety profiling strengthens the experimental validity of subsequent findings in several ways. First, it ensured that observed increases in GLUT4 translocation or signaling protein modulation were not secondary to cellular stress or compromised viability. Second, it enabled the use of physiologically relevant concentrations that are more likely to reflect pharmacologically achievable levels. Finally, the identification of compound-specific toxicity thresholds highlights the necessity of individualized dosing strategies when evaluating natural products, rather than assuming class-wide safety.

By carefully restricting all mechanistic and functional analyses to concentrations that resulted in less than 10% cell death, the study minimizes confounding effects and supports the interpretation that the observed metabolic effects arise from specific biological actions rather than non-specific cytotoxicity.

4.3 Natural Compounds as Regulators of GLUT4 Trafficking

The translocation of GLUT4 from intracellular storage vesicles to the plasma membrane represents a pivotal step in insulin-stimulated glucose uptake in skeletal muscle. Impairment of this process is a hallmark of insulin resistance and

type 2 diabetes. Therefore, the ability of natural compounds to enhance GLUT4 translocation constitutes a strong indicator of potential antidiabetic efficacy.

In the present study, both plant extracts and purified natural compounds significantly increased GLUT4 translocation in L6-GLUT4myc cells, albeit with substantial variability in magnitude, insulin dependence, and temporal dynamics. Notably, extracts from Cichorium intybus and Abelmoschus esculentus enhanced GLUT4 translocation even in the absence of insulin, suggesting the presence of bioactive constituents capable of bypassing or supplementing canonical insulin signaling pathways. The reviewed literature underscores the significant role of Cichorium intybus and Abelmoschus esculentus in modulating insulin signaling pathways, particularly through the regulation of GLUT4 translocation. Multiple studies highlight that Cichorium intybus exerts beneficial effects on glucose metabolism by promoting GLUT4 translocation, thereby enhancing cellular glucose uptake and insulin sensitivity [140, 148, 149]. Specifically, Cichorium intybus leaf extract has been shown to increase insulin secretion and improve glucose uptake, which are critical in managing hypoglycemic conditions [150, 151]. Additionally, the plant's bioactive compounds appear to influence insulin-producing cells, further contributing to its antidiabetic potential [149, 152].

Similarly, Abelmoschus esculentus (okra) has demonstrated promising effects on blood glucose regulation, partly through its impact on GLUT4 expression and translocation. Evidence indicates that okra improves blood glucose and lipid profiles, with mechanisms involving the upregulation of insulin-responsive glucose transporter GLUT4 mRNA expression [140, 148, 153]. The plant's effects are also associated with modulation of key signaling molecules such as IRS-1 and PI3K, which are integral to insulin signaling pathways[154-156].

Furthermore, phytochemicals such as quercetin, a flavonoid present in Cichorium intybus, are recognized for their capacity to influence insulin signaling. Quercetin has been identified as having substantial pharmacological capacity, potentially contributing to the enhancement of GLUT4 translocation and insulin sensitivity [153, 157, 158]. The collective findings suggest that both plants contain bioactive

compounds capable of activating the PI3K/Akt pathway, a central route in insulin signaling that facilitates GLUT4 translocation to the cell membrane [156].

Among the purified compounds, phenolic acids such as caffeic acid, ferulic acid, chlorogenic acid, syringic acid, and several benzoic acid derivatives emerged as potent enhancers of GLUT4 translocation. These compounds are classified as carboxylic acids. They are subdivided into hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids include gallic acid, p-hydroxybenzoic acid, syringic acid, and vanillic acid [159]. Hydroxycinnamic acids encompass caffeic acid, ferulic acid, and chlorogenic acid [160]. Caffeic acid appears to enhance insulin-dependent glucose transport in skeletal muscle under conditions of insulin resistance. The cellular mechanisms underlying this process remain unclear. However, tumor necrosis factor α (TNFα), a cytokine belonging to the TNF ligand superfamily, has been implicated in the pathogenesis of insulin resistance [161] .Activation of the TNF receptor triggers stimulation of the nuclear factor-κB (NF-κB) signaling pathway through inhibitor κB kinase (IKK). IKK functions as the primary regulator of NF-κB activation in response to inflammatory stimuli, and the IKK/NF-κB pathway is considered central to the development of insulin resistance in peripheral tissues, including skeletal muscle[162]. *Egawa et al* demonstrate that, under insulin-stimulated conditions, caffeine-induced insulin resistance—characterized by activation of IKK/NF-κB signaling and suppression of Akt Ser473 phosphorylation—was mitigated by treatment with caffeic acid in rat skeletal muscle [163]. A study demonstrated that ferulic acid enhances glucose transport in L6 skeletal muscle cells independently of insulin. This effect is dependent on both the concentration of ferulic acid, specifically below 50 μM, and the duration of exposure, within five hours [164] which is consistent with our findings of increased glucose transport.

The stimulatory influence of chlorogenic acid on glucose transport in skeletal muscle was initially documented by Prabhakar and Doble. They observed that incubation with chlorogenic acid (25 μM) induced insulin-independent glucose uptake within three hours in differentiated L6 skeletal muscle cells. Subsequently, *Ong et al* [165]demonstrated that exposure of isolated skeletal muscle from db/db

mice and L6 cells to chlorogenic acid (1–10 mM) for durations ranging from one to twenty-four hours enhanced insulin-independent glucose transport. They further identified that this effect was inhibited by pretreatment with compound C, an AMPK inhibitor, but remained unaffected by wortmannin, a PI3K inhibitor [164].These results imply that chlorogenic acid promotes glucose transport in skeletal muscle through mechanisms that are both insulin-independent and reliant on AMPK activation. Also a study reported that treatment with gallic acid (10 μM) for 30 min induces GLUT4 translocation and insulin-independent glucose transport in 3T3-L1 adipocytes [166].

In my study, the tested phenolic derivates demonstrated both acute (0.5–1 h) and sustained (24 h) effects, indicating that they may act through multiple mechanisms, including rapid signaling events and longer-term cellular adaptations. In contrast, certain fatty acids showed minimal or no effect on GLUT4 trafficking, highlighting a degree of structural specificity in the regulation of glucose transporter dynamics. Moreover, this project results indicates that GLUT4 translocation to the PM was augmented in L6 myc cells exposed to 0.125 mM palmitic acid for a duration of up to 1 h. This finding aligns with previous experiments conducted in our laboratory, which demonstrated that brief exposure to palmitic acid promotes the translocation of GLUT4 [125]. Furthermore, Liu *et al.* observed an increase in glucose uptake in skeletal muscle subjected to 300 μM palmitic acid for the same time period [167]. Similarly, short-term fatty acid treatment of adipocytes with palmitic acid enhances basal glucose uptake [168, 169]. In contrast, prolonged exposure to palmitic acid, ranging from 16 to 24 hours, resulted in the development of insulin resistance in myocytes [169].

A recent study explored the impact of fatty acids on glucose metabolism and inflammation, revealing that obesity-induced palmitic acid promotes inflammation and glucose dysregulation via GPRs/NF-κB/KLF7 signaling pathways. While not directly focused on phenolic acids, this study highlights the importance of inflammatory pathways in glucose metabolism disorders, which phenolic acids may modulate given their known anti-inflammatory properties [170].

Importantly, several compounds increased GLUT4 translocation in the absence of insulin, suggesting insulin-mimetic or insulin-sensitizing properties. This finding is particularly relevant in the context of insulin resistance, where endogenous insulin signaling is impaired. The observation that insulin further amplified the effects of many compounds indicates potential synergistic interactions, whereby natural compounds enhance insulin responsiveness rather than simply replacing insulin action.

The consistency between the quantitative colorimetric assay and qualitative immunofluorescence imaging provides strong validation of these findings. The visualization of increased surface GLUT4 at the single-cell level confirms that the observed effects reflect genuine transporter redistribution rather than assay artifacts.

Overall, these data demonstrate that selected natural compounds act as functional regulators of GLUT4 trafficking, positioning them as promising candidates for further investigation as modulators of skeletal muscle glucose uptake.

4.4 Targeting the Insulin Signaling Cascade: AKT, AS160, PTEN, and IRS1

To elucidate the molecular mechanisms underlying the observed enhancement of GLUT4 translocation, this study examined the effects of selected natural compounds on key nodes of the insulin signaling pathway, namely IRS1, AKT, AS160, and PTEN. These signaling intermediates form a tightly regulated cascade that governs insulin-stimulated glucose uptake in skeletal muscle, as these proteins are critical effectors of GLUT4 trafficking.

One of the most consistent findings across the tested compounds was the activation of AKT, particularly in the presence of insulin. Several compounds, including caffeic acid, ferulic acid, benzoic acid, 3,4-dihydroxybenzoic acid, and 3,4,5-trihydroxybenzoic acid, increased AKT protein expression and/or phosphorylation. The absence of phosphorylated AKT in insulin-deprived conditions, regardless of compound treatment, confirms that AKT activation in this

system remains insulin-dependent and that these compounds function primarily as insulin sensitizers rather than insulin mimetics at the level of AKT phosphorylation.

Interestingly, modulation of AKT activity occurred largely without parallel changes in IRS1 phosphorylation. While certain compounds increased IRS1 protein or mRNA expression, phosphorylation of IRS1 was generally unaffected. This dissociation suggests that the compounds may enhance insulin signaling downstream of IRS1, possibly by facilitating signal propagation from the insulin receptor to AKT or by modulating intermediary regulatory mechanisms. Such selective pathway engagement is biologically significant, as impaired IRS1 signaling is a common defect in insulin-resistant states, and bypassing this bottleneck may restore downstream signaling efficiency.

AS160, a direct substrate of AKT and a key regulator of GLUT4 vesicle trafficking, emerged as a major functional target of several compounds. Both transcriptional and protein-level analyses revealed that caffeic acid, ferulic acid, and specific benzoic acid derivatives restored or enhanced AS160 expression under insulin-stimulated conditions. Moreover, increased phosphorylation of AS160 was observed following short-term ferulic acid treatment and prolonged caffeic acid exposure, providing a mechanistic link between AKT activation and enhanced GLUT4 translocation.

PTEN, a negative regulator of the phosphoinositide 3-kinase (PI3K)/AKT pathway, was also modulated by the tested compounds, particularly at the transcriptional level. Insulin alone suppressed PTEN expression, and this effect was further amplified by several natural compounds. The absence of significant changes in PTEN phosphorylation suggests that these compounds primarily influence PTEN abundance rather than its acute enzymatic activity. Suppression of PTEN expression would be expected to prolong or intensify AKT signaling, thereby facilitating sustained GLUT4 translocation.

Taken together, these findings indicate that the natural compounds examined in this study selectively enhance insulin signaling by reinforcing downstream signaling components (AKT and AS160) and relieving inhibitory constraints

(PTEN), rather than uniformly activating the entire insulin receptor cascade. This mode of action may be particularly advantageous in insulin-resistant conditions, where proximal signaling defects limit insulin responsiveness.

4.5 Integration of Transcriptional and Protein-Level Regulation of Glucose Metabolism

A notable strength of this study lies in the parallel assessment of gene expression and protein-level regulation for key components of glucose metabolism. The combined analysis provides important insights into the temporal and mechanistic layers through which natural compounds modulate insulin sensitivity and GLUT4 trafficking.

The transcriptional upregulation of GLUT4 observed for several compounds occurred predominantly in the presence of insulin, highlighting a strong insulin-dependent synergism. Compounds such as caffeic acid and 3,4-dihydroxybenzoic acid markedly increased GLUT4 mRNA expression, particularly following prolonged exposure. This suggests that, in addition to acutely mobilizing existing GLUT4 pools, these compounds may enhance the cellular capacity for glucose uptake by increasing GLUT4 synthesis.

Similarly, IRS1 mRNA expression was elevated by multiple compounds under insulin-stimulated conditions, despite limited effects on IRS1 phosphorylation. This transcriptional upregulation may serve a compensatory role, replenishing IRS1 protein levels and supporting sustained insulin responsiveness over longer time scales.

The regulation of AS160 expression further reinforces the coordinated nature of these responses. While insulin alone suppressed AS160 transcription, several compounds effectively restored or enhanced AS160 mRNA levels. This reversal aligns with the observed increases in AS160 protein and phosphorylation, indicating that transcriptional regulation contributes to the functional restoration of GLUT4 trafficking machinery.

Consistent with this project results, Ottana *et al* reported in an *invitro* and *in silico* study that benzoic acid derivates suppressed protein tyrosine phosphatase 1B

(PTP1B), an inhibitor of Akt and IRS1, thereby leading to the enhancement of Akt and IRS1 phosphorylation and eventually to augmenting GLUT4 translocation in the PM [171].

In contrast, PTEN expression was consistently downregulated by insulin and further suppressed by several compounds. This coordinated suppression at the transcriptional level provides a plausible mechanism for prolonged enhancement of PI3K/AKT signaling, complementing the acute phosphorylation events detected at the protein level.

In insulin-free conditions, control cells exhibited basal expression levels of AKT1 and AKT2, reflecting constitutive transcription required for cellular homeostasis [172]. Upon insulin stimulation, AKT2 mRNA expression increased, whereas AKT1 expression remained largely unchanged, consistent with the established role of AKT2 as the principal mediator of insulin signaling [173]. In the presence of insulin, the inhibitory effect of caffeic acid on AKT1 expression persisted, indicating that caffeic acid can override insulin-mediated transcriptional regulation of AKT1. A less pronounced reduction in AKT2 expression was observed, suggesting partial interference with insulin-responsive signaling. Previous studies have reported that caffeic acid can impair insulin-stimulated PI3K/AKT signaling, particularly in cancer and hyperproliferative models [174].

While caffeic acid continued to suppress AKT2 expression, albeit to a lesser extent, ferulic acid showed attenuation of this inhibitory effect, suggesting partial preservation of insulin-responsive AKT2 signaling. This finding aligns with previous reports indicating that ferulic acid can improve insulin sensitivity and support glucose homeostasis by maintaining AKT-dependent signaling pathways [175].

Preservation of AKT2 expression under insulin-stimulated conditions may therefore contribute to improved glucose uptake and metabolic regulation.

In contrast, changes in AKT1 expression appeared to have a limited direct impact on glucose metabolism. AKT1 is primarily associated with cell growth and survival, and its transcriptional regulation is less closely linked to insulin-mediated metabolic

effects [172].Thus, the stronger modulation of AKT2 relative to AKT1 observed in this study provides mechanistic support for the metabolic relevance of the findings.

Overall, these results suggest that phenolic acid–induced modulation of AKT2 gene expression directly influences insulin-dependent glucose metabolism. Caffeic acid may impair glucose metabolic signaling by suppressing AKT2 expression, whereas ferulic acid appears to exert a more balanced effect, potentially supporting insulin sensitivity under stimulated conditions. These findings highlight the importance of AKT2-specific transcriptional regulation as a key determinant of glucose metabolic outcomes.

Treatment of L6 myc skeletal muscle cells with benzoic acid resulted in minimal changes in AKT1 and AKT2 mRNA expression under both insulin-free and insulin-stimulated conditions, as determined by quantitative real-time PCR. In the absence of insulin, a slight reduction in AKT1 expression was observed, while AKT2 mRNA levels remained comparable to those of control cells. In the presence of insulin, benzoic acid treatment did not significantly alter the expression of AKT1 or AKT2 compared with insulin-stimulated controls.

The concordance between gene expression and protein signaling data supports the conclusion that these natural compounds exert multi-layered regulatory effects, acting both acutely through phosphorylation-dependent signaling and chronically through transcriptional reprogramming. Such dual-level regulation is likely to be critical for sustained metabolic improvements in insulin-resistant states.

4.6 *In Vivo* Antihyperglycemic Effects and Physiological Relevance

While *in vitro* studies provide valuable mechanistic insights, the physiological relevance of any proposed antidiabetic intervention ultimately depends on its efficacy in whole-animal models. In this study, the antidiabetic potential of selected natural compounds was evaluated using a streptozotocin-induced diabetic mouse model, which is widely employed to assess glucose-lowering effects.

Treatment with several compounds, including caffeic acid, ferulic acid, isovanillic acid, benzoic acids derivatives and fatty acids resulted in significant reductions in blood glucose levels compared to untreated diabetic controls. Notably, these

effects became evident within the first week of administration and were sustained throughout the treatment period, indicating both rapid and persistent antihyperglycemic activity.

Numerous investigations have demonstrated the hypoglycemic properties of caffeic acid. Intravenous administration of caffeic acid in STZ-induced diabetic rats at doses ranging from 0.5 to 5 mg/Kg resulted in a rapid reduction in plasma glucose levels [176]. Also, Ferulic acid incorporated at 0.01% and 0.1% of the basal diet markedly reduced blood glucose levels in STZ-induced diabetic mice and in KK-Ay mice, a concentration of 0.05% effectively suppressed hyperglycemia [177].

The benzoic acid derivatives tested here, namely 3,4-dihydroxybenzoic acid (DB), benzoic acid (BZ), 3,4,5-trihydroxybenzoic acid (TB), and 2-hydroxy-4-methoxybenzoic acid (HM) have garnered attention for their potential roles in metabolic regulation, particularly in the context of glucose homeostasis and insulin sensitivity. Benzoic acid derivatives are a class of organic compounds that are characterized by the presence of a benzoic acid moiety, which consists of a benzene ring attached to a carboxylic acid group. These compounds are widely studied for their diverse biological activities and potential therapeutic applications. Variations in the hydroxyl and methoxy substituents on the benzene ring can significantly influence the pharmacological properties of these derivatives. For instance, hydroxyl groups can enhance antioxidant activity, while methoxy groups may improve solubility and bioavailability. Chronic administration of TB at doses ranging from 25 to 100 mg/Kg/day over a period of 4 to 16 weeks led to a significant decrease in elevated fasting serum glucose levels across various diabetic models, including STZ-induced diabetic rats [178], high-fat diet-induced diabetic mice [179], and rats with combined high-fat diet and STZ-induced diabetes [180, 181]. In another study, TB (20 mg/Kg for 30 days) decreased body weight gain, fasting blood glucose and insulin levels in diabetic rats. It also significantly improved the level of PPAR-γ expression, and enhanced glucose uptake through translocation and activation of GLUT4 in the PI3K/Akt signaling pathway [180]. Furthermore, 2-Hydroxy-4-methoxybenzoic acid, extracted from the roots of

Homidium indicus, was orally administered at a dose of 500 µg/Kg to both diabetic and non-diabetic rats. Post-treatment, diabetic rats exhibited normalization of glycosylated hemoglobin, total cholesterol, triglycerides, and LDL-cholesterol levels, alongside restored plasma insulin levels, glycosylated hemoglobin, and liver glycogen [182]. These results are consistent with my findings that benzoic acids derivatives may exert hypoglycemic effects, thereby aiding in the treatment of diabetes mellitus.

Fatty acids such as palmitic acid, myristic acid, and stearic acid are saturated fatty acids commonly found in dietary fats and have significant effects on metabolic processes. Recent research suggests that these fatty acids can influence glucose metabolism and insulin sensitivity, which are critical factors in the development and management of hypoglycemia.

Experimental studies have begun to explore the direct effects of these fatty acids on diabetic conditions. For instance, stearic acid supplementation could prevent alcohol-induced liver damage by modulating gut microbiota, which is increasingly recognized as a factor in glucose metabolism and insulin sensitivity [183]. Although that study focused on liver injury, it suggests a broader role for stearic acid in metabolic regulation. Chronic oral administration of myristic acid ameliorated glucose tolerance (24–28% decrease in blood glucose levels during glucose tolerance tests) and reduced insulin-responsive blood glucose levels (~20% decrease) in male NSY mice. Also, in the same study, administration of palmitic acid on NSY mice slightly reduced blood glucose concentration in a glucose tolerance test [184]. However, the effects were weaker and appeared later compared with myristic acid. Palmitoleic acid has also been reported to be effective on hyperglycemia in a diabetic KK-Ay mice [185] . Therefore, these saturated fatty acids may contribute to the regulation of glucose homeostasis. They might also provide a protective effect against diabetes-related complications. This hypothesis is consistent with the results obtained from the in *vivo* experiments in the current project.

The in vivo findings align closely with the cellular mechanisms identified in L6 skeletal muscle cells. Compounds that enhanced GLUT4 translocation, activated AKT signaling, and suppressed PTEN expression *in vitro* were also those that exhibited the most pronounced glucose-lowering effects in diabetic mice. This concordance strengthens the interpretation that improved skeletal muscle glucose uptake contributes, at least in part, to the observed reduction in blood glucose levels.

However, it is important to acknowledge the limitations of the streptozotocin model, which primarily reflects β-cell dysfunction and hyperglycemia rather than the full complexity of type 2 diabetes–associated insulin resistance. Despite this limitation, the ability of the tested compounds to lower blood glucose in this model underscores their therapeutic potential and justifies further investigation in models that more closely mimic insulin resistance.

Overall, the *in vivo* data provide crucial validation of the cellular findings and support the conclusion that selected natural compounds exert biologically meaningful antidiabetic effects, reinforcing their relevance as candidates for further preclinical and translational studies.

4.7 Study Limitations and Methodological Considerations

Despite the strength and breadth of the experimental design, several limitations should be acknowledged when interpreting the findings of this study. Recognizing these limitations is essential for contextualizing the results and guiding future research.

First, although the L6-GLUT4myc skeletal muscle cell line is a well-established and highly informative model for studying GLUT4 trafficking and insulin signaling, it cannot fully recapitulate the complexity of glucose metabolism *in vivo*. Skeletal muscle glucose uptake in whole organisms is influenced by systemic factors such as circulating hormones, inflammatory mediators, neural input, and inter-organ crosstalk, which are not represented in an isolated cell culture system. Therefore, while the cellular findings provide robust mechanistic insights, they should be interpreted as part of a broader physiological framework.

Second, the study focused primarily on the PI3K/AKT signaling axis and its downstream targets. Although this pathway is central to insulin-mediated glucose uptake, alternative signaling routes—such as AMP-activated protein kinase (AMPK), mitogen-activated protein kinases (MAPKs), and calcium-dependent pathways—may also contribute to the insulin-independent effects observed for some compounds. The absence of data on these pathways limits the ability to fully delineate the spectrum of molecular mechanisms involved.

Third, the concentrations of natural compounds used *in vitro* were selected based on cytotoxicity thresholds and experimental feasibility rather than pharmacokinetic data. While this approach ensures cellular safety and biological relevance, it does not directly address issues of bioavailability, metabolism, or tissue distribution *in vivo*. These factors are critical for translational application and warrant further investigation.

Regarding the *in vivo* component, the streptozotocin-induced diabetic mouse model primarily reflects hyperglycemia resulting from pancreatic β-cell damage. Although this model is widely used and valuable for assessing glucose-lowering effects, it does not fully mimic the insulin resistance that characterizes T2DM. Consequently, the glucose-lowering effects observed in this study may reflect both insulin-independent and insulin-sensitizing mechanisms, and their relative contributions cannot be definitively resolved within this model alone.

Finally, while the study examined multiple natural compounds, it did not explore potential synergistic or antagonistic interactions between them. Given that many of these compounds coexist in medicinal plants and dietary sources, combination effects may be biologically relevant and merit future exploration.

4.8 Future Directions

The findings of this study open several avenues for future research. One important direction is the evaluation of the most promising compounds—particularly caffeic acid, ferulic acid, and selected benzoic acid derivatives—in additional *in vivo* models that more closely resemble type 2 diabetes and insulin resistance, such as

high-fat diet–induced diabetic models. These studies would help clarify the insulin-sensitizing versus insulin-independent actions of the compounds.

Further mechanistic studies are also warranted to investigate alternative signaling pathways that may contribute to GLUT4 translocation, particularly in insulin-independent conditions. Examination of AMPK activation, mitochondrial function, and cellular redox status could provide a more comprehensive understanding of how these compounds influence glucose metabolism.

From a molecular perspective, structure–activity relationship analyses could be undertaken to identify the functional groups responsible for the observed biological effects. Such studies may facilitate the rational design or selection of more potent analogues with improved efficacy and safety profiles.

In addition, pharmacokinetic and bioavailability studies are essential to determine whether effective concentrations observed *in vitro* can be achieved in target tissues *in vivo*. These investigations would represent a critical step toward clinical translation.

Finally, given the transcriptional effects observed for GLUT4, IRS1, AS160, and PTEN, long-term studies examining epigenetic regulation and sustained metabolic adaptation may provide insight into whether these compounds can induce durable improvements in insulin sensitivity.

4.9 Concluding Remarks

In conclusion, this study provides a comprehensive evaluation of the antidiabetic potential of selected natural compounds using an integrated *in vitro* and *in vivo* approach. The results demonstrate that several phenolic and benzoic acid derivatives enhance GLUT4 translocation in skeletal muscle cells through selective modulation of insulin signaling pathways, particularly by activating AKT, promoting AS160 function, and suppressing PTEN expression.

Importantly, these compounds act primarily as insulin sensitizers, amplifying insulin-dependent signaling rather than indiscriminately activating upstream components such as IRS1 phosphorylation. At the transcriptional level, the

coordinated regulation of GLUT4, IRS1, AS160, and PTEN further supports their role in improving glucose handling capacity in skeletal muscle.

The ability of selected compounds to significantly reduce blood glucose levels in diabetic mice provides physiological relevance to the cellular findings and underscores their therapeutic promise. While further studies are required to address translational challenges, the present work advances our understanding of how natural compounds can modulate glucose metabolism and identifies promising candidates for future antidiabetic intervention.

Overall, this dissertation contributes novel mechanistic insights into the role of natural compounds in regulating insulin signaling and GLUT4 trafficking and lays a strong foundation for subsequent preclinical and translational research.

5. Summary

Natural bioactive compounds have gained increasing attention for their potential to modulate metabolic disorders such as diabetes; however, their effects on key insulin-signaling mediators in skeletal muscle cells remain insufficiently characterized. Accordingly, this study evaluated the antidiabetic potential of selected natural compounds using an integrated experimental approach that included cytotoxicity assessment, GLUT4 translocation analysis, insulin-signaling and gene expression profiling, and *in vivo* validation in a streptozotocin-induced diabetic mouse model.

The results demonstrated that both crude plant extracts and isolated natural compounds significantly stimulated GLUT4 translocation in L6-GLUT4myc skeletal muscle cells, although the magnitude, insulin dependence, and temporal responses varied among treatments. Notably, extracts from Cichorium intybus and Abelmoschus esculentus enhanced GLUT4 translocation even under insulin-free conditions, suggesting the involvement of alternative or complementary regulatory mechanisms beyond canonical insulin signaling.

Furthermore, phenolic compounds and benzoic acid derivatives exhibited a clear capacity to enhance GLUT4 translocation, with several phenolic acids inducing pronounced increases under insulin-stimulated conditions, while selected benzoic acid derivatives produced moderate yet consistent effects. Importantly, some phenolic compounds, including ferulic acid and chlorogenic acid, promoted GLUT4 translocation independently of insulin, highlighting their potential to modulate glucose uptake through insulin-independent pathways. In contrast, most tested fatty acids showed minimal or no effect on GLUT4 trafficking, underscoring a degree of structural specificity in the regulation of glucose transporter dynamics. However, palmitic acid transiently augmented GLUT4 translocation to the plasma membrane in L6-GLUT4myc cells following short-term exposure of up to 1 hour.

To elucidate the molecular basis of enhanced GLUT4 translocation, this study evaluated the effects of selected natural compounds on key insulin-signaling

mediators, including IRS1, AKT, AS160, and PTEN, using integrated transcriptional and protein-level analyses. The results demonstrated a consistent potentiation of AKT activation under insulin-stimulated conditions, with the compounds caffeic acid, ferulic acid, and selected benzoic acid derivatives increasing AKT expression and phosphorylation. However, these compounds fails to induce AKT activation in the absence of insulin, thereby indicating an insulin-sensitizing rather than insulin-mimetic mode of action. This enhanced AKT signaling was mechanistically linked to increased AS160 expression and phosphorylation, counteracting insulin-associated transcriptional suppression and reinforcing downstream signaling required for GLUT4 vesicle mobilization. In parallel, several compounds promoted insulin-dependent upregulation of GLUT4 and IRS1 gene expression, suggesting a synergistic interaction with insulin that supports sustained glucose uptake capacity.

The strong concordance between gene expression and protein signaling data indicates that these natural compounds exert multilayered regulatory effects, acting acutely through phosphorylation-dependent signaling events and chronically through transcriptional modulation. This dual mode of regulation is likely essential for achieving sustained metabolic improvements, particularly under insulin-resistant conditions. Collectively, these coordinated transcriptional and post-translational actions demonstrate that natural compounds enhance insulin signaling efficiency through an integrated regulatory mechanism, ultimately promoting effective GLUT4 trafficking in skeletal muscle cells.

While *in vitro* experiments provided mechanistic insight into the ability of selected natural compounds to enhance GLUT4 translocation and insulin signaling in skeletal muscle cells, the physiological relevance of these effects was confirmed using a streptozotocin-induced diabetic mouse model. Compounds that promoted GLUT4 trafficking in L6-GLUT4myc cells, including caffeic acid, ferulic acid, isovanillic acid, benzoic acid derivatives, and selected fatty acids, also led to significant reductions in blood glucose levels in diabetic mice compared with untreated controls. These antihyperglycemic effects became evident within the first

week of administration and were sustained throughout the treatment period, indicating a close concordance between the cellular enhancement of glucose uptake mechanisms and improved systemic glycemic control *in vivo*.

Overall, the *in vivo* findings validate the cellular observations and demonstrate that selected natural compounds exert biologically relevant antidiabetic effects, supporting their potential for further preclinical investigation and translational development.

6. References

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Zusammenfassung

Natürliche bioaktive Verbindungen rücken aufgrund ihres Potenzials zur Modulation von Stoffwechselstörungen wie Diabetes zunehmend in den Fokus der Forschung. Ihre Wirkung auf wichtige Mediatoren der Insulin-Signalübertragung in Skelettmuskelzellen ist jedoch noch unzureichend charakterisiert. Daher untersuchte diese Studie das antidiabetische Potenzial ausgewählter Naturstoffe mittels eines integrierten experimentellen Ansatzes. Dieser umfasste Zytotoxizitätsbestimmungen, GLUT4-Translokationsanalysen, Insulin-Signal- und Genexpressionsprofilierung sowie eine In-vivo-Validierung in einem Streptozotocin-induzierten diabetischen Mausmodell.

Die Ergebnisse zeigten, dass sowohl rohe Pflanzenextrakte als auch isolierte Naturstoffe die GLUT4-Translokation in L6-GLUT4myc-Skelettmuskelzellen signifikant stimulierten. Ausmaß, Insulinabhängigkeit und zeitliche Reaktionen variierten jedoch zwischen den Behandlungen. Extrakte aus Cichorium intybus und Abelmoschus esculentus verstärkten die GLUT4-Translokation sogar unter insulinfreien Bedingungen, was auf die Beteiligung alternativer oder komplementärer Regulationsmechanismen jenseits der kanonischen Insulin-Signalübertragung hindeutet.

Phenolverbindungen und Benzoesäurederivate zeigten zudem eine deutliche Fähigkeit zur Steigerung der GLUT4-Translokation. Mehrere Phenolsäuren induzierten unter insulininduzierten Bedingungen einen ausgeprägten Anstieg, während ausgewählte Benzoesäurederivate moderate, aber konsistente Effekte hervorriefen. Bemerkenswerterweise förderten einige Phenolverbindungen, darunter Ferulasäure und Chlorogensäure, die GLUT4-Translokation unabhängig von Insulin, was ihr Potenzial zur Modulation der Glukoseaufnahme über insulinunabhängige Wege unterstreicht. Im Gegensatz dazu zeigten die meisten getesteten Fettsäuren nur einen minimalen oder gar keinen Effekt auf den GLUT4-Transport, was auf eine gewisse strukturelle Spezifität bei der Regulation der Glukosetransporterdynamik hinweist. Palmitinsäure steigerte jedoch nach einer

kurzzeitigen Exposition von bis zu einer Stunde vorübergehend die GLUT4-Translokation zur Plasmamembran in L6-GLUT4myc-Zellen.

Um die molekularen Grundlagen der verstärkten GLUT4-Translokation aufzuklären, untersuchte diese Studie die Wirkung ausgewählter Naturstoffe auf wichtige Mediatoren der Insulin-Signalübertragung, darunter IRS1, AKT, AS160 und PTEN, mittels integrierter Transkriptions- und Proteinanalysen. Die Ergebnisse zeigten eine konsistente Potenzierung der AKT-Aktivierung unter insulininduzierten Bedingungen. Kaffeesäure, Ferulasäure und ausgewählte Benzoesäurederivate erhöhten die AKT-Expression und -Phosphorylierung. Diese Verbindungen induzierten jedoch keine AKT-Aktivierung in Abwesenheit von Insulin, was auf einen insulin-sensibilisierenden und nicht auf einen insulin-mimetischen Wirkmechanismus hindeutet. Diese verstärkte AKT-Signalübertragung war mechanistisch mit einer erhöhten AS160-Expression und -Phosphorylierung verknüpft, wodurch die insulinbedingte Transkriptionshemmung aufgehoben und die für die GLUT4-Vesikelmobilisierung notwendige nachgeschaltete Signalübertragung verstärkt wurde. Parallel dazu förderten mehrere Verbindungen die insulinabhängige Hochregulierung der GLUT4- und IRS1-Genexpression, was auf eine synergistische Wechselwirkung mit Insulin hindeutet, die eine anhaltende Glukoseaufnahmekapazität unterstützt.

Die starke Übereinstimmung zwischen Genexpressions- und Proteinsignaldaten deutet darauf hin, dass diese Naturstoffe vielschichtige regulatorische Effekte ausüben, die akut durch phosphorylierungsabhängige Signalereignisse und chronisch durch transkriptionelle Modulation wirken. Dieser duale Regulationsmechanismus ist wahrscheinlich essenziell für nachhaltige metabolische Verbesserungen, insbesondere unter insulinresistenten Bedingungen. Zusammengenommen zeigen diese koordinierten transkriptionellen und posttranslationalen Wirkungen, dass Naturstoffe die Effizienz der Insulinsignalisierung durch einen integrierten Regulationsmechanismus steigern und letztendlich den effektiven GLUT4-Transport in Skelettmuskelzellen fördern.

Während In-vitro-Experimente mechanistische Einblicke in die Fähigkeit ausgewählter Naturstoffe zur Steigerung der GLUT4-Translokation und Insulin-

Signalgebung in Skelettmuskelzellen lieferten, wurde die physiologische Relevanz dieser Effekte mithilfe eines Streptozotocin-induzierten diabetischen Mausmodells bestätigt. Verbindungen, die den GLUT4-Transport in L6-GLUT4myc-Zellen förderten, darunter Kaffeesäure, Ferulasäure, Isovanillinsäure, Benzoesäurederivate und ausgewählte Fettsäuren, führten im Vergleich zu unbehandelten Kontrolltieren auch zu einer signifikanten Senkung des Blutzuckerspiegels bei diabetischen Mäusen. Diese blutzuckersenkenden Effekte zeigten sich innerhalb der ersten Behandlungswoche und hielten während des gesamten Behandlungszeitraums an. Dies deutet auf eine enge Korrelation zwischen der zellulären Steigerung der Glukoseaufnahme und der verbesserten systemischen Blutzuckerkontrolle in vivo hin.

Insgesamt bestätigen die In-vivo-Befunde die zellulären Beobachtungen und zeigen, dass ausgewählte Naturstoffe biologisch relevante antidiabetische Wirkungen entfalten. Dies unterstreicht ihr Potenzial für weitere präklinische Untersuchungen und die translationale Entwicklung.

Erklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe verfasst habe.
Die vorliegende Arbeit wurde weder in der jetzigen noch in ähnlicher Form bei einer anderen Institution eingereicht.
Es wurden zuvor keine Promotionsversuche unternommen.

Ort, Datum 

Najlaa Bassalat

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