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Molecular Approach to Identify Anti-inflammatory Potential of Stevioside in HFD-induced Type 2 Diabetic Rats: Evidence From *in Vivo* Study

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Abstract

Stevioside is a natural sweetener derived from the leaves of the Stevia rebaudiana plant. It has gained popularity as a sugar substitute due to its intense sweetness without adding calories or affecting blood sugar levels, making it a suitable option for people with diabetes or those looking to reduce their sugar intake. Studies have shown that stevioside has glucose lowering effects. Previous studies have shown that it has significant role in skeletal muscle but its role on expression of inflammatory signaling molecules in adipose tissue against high diet and sucrose-induced type-2 diabetes in experimental rats is yet to be done. The current research was undertaken to investigate if stevioside could also exert its antidiabetic effects by circumventing adipocyte induced inflammation, a key driving factor for insulin resistance in obese individuals. Effective dose of stevioside (20 mg/kg b.wt) was administered orally for 45 days to high fat diet and sucrose induced type-2 diabetic rats. Interestingly, stevioside treatment restores the elevated serum levels of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and sterol regulatory element binding protein-1c (SREBP-1c) and enhances Peroxisome Proliferator–activated receptor-γ (PPAR-γ) in adipocytes of diabetic rats. The gene expression of IR, GLUT4 and PPAR-γ mRNA were also significantly activated in stevioside treated groups but reduced IL-1 beta, IlL-6, IKKB, TNF-alpha and NFkB mRNA expression in diabetic adipose tissue. More importantly, stevioside acts very effectively as metformin to circumvent inflammation and insulin resistance in diabetic rats. Our results clearly show that stevioside inhibits obesity induced insulin resistance by ameliorating the inflammatory events and upregulating insulin signalling molecules.

Keywords: *Stevioside, HFD-T2DM, pro inflammation, insulin signalling; adipose tissue, obesity; signaling pathways; Therapautics.*

Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by chronic hyperglycaemia, insulin resistance, and impaired insulin secretion. It is a global health concern with an increasing prevalence, largely attributed to sedentary lifestyles and poor dietary choices [1-5]. A significant complication of T2DM is the chronic low-grade inflammation that accompanies the disease, leading to the development of various comorbidities, including cardiovascular diseases, neuropathy, and nephropathy. One of the key factors contributing to T2DM is low grade inflammation, which plays a crucial role in the pathogenesis of diabetes mellitus. Inflammation in T2DM is multifaceted, involving the activation of pro-inflammatory pathways and the release of inflammatory mediators, such as cytokines and chemokines. These inflammatory processes lead to systemic insulin resistance and impaired glucose

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homeostasis, exacerbating the diabetic condition [6,7]. Hyperglycaemia itself can further perpetuate inflammation, creating a vicious cycle that contributes to the progression of T2DM and its associated complications. The inflammatory response in T2DM is primarily driven by elevated levels of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which result from macrophage activation in adipose tissues and insulin-resistant states [8-10].

High-fat diet (HFD)-induced obesity and insulin resistance in animal models have been widely used to study the pathophysiology of T2DM, as they mimic the diet-induced metabolic disturbances seen in humans [11]. HFD consumption leads to excess calorie intake, obesity, and the accumulation of lipid deposits, particularly in adipose tissues and liver, exacerbating insulin resistance and inflammation [12, 13]. These models display chronic inflammation, characterized by an upregulation of inflammatory markers, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP), among others. The presence of chronic inflammation in these models closely parallels the systemic inflammation observed in individuals with T2DM. Traditional treatments for T2DM mainly involve lifestyle modifications and pharmaceutical interventions, such as insulin sensitizers and anti-diabetic medications. However, these approaches may have limitations, side effects, and variable efficacy, prompting the search for alternative therapeutic options. Natural products and plant-derived compounds have gained attention due to their potential as adjunctive therapies in managing T2DM and its associated complications [14-18].

Stevioside, a natural sweetener extracted from the leaves of Stevia rebaudiana, has drawn increasing interest in recent years due to its potential anti-diabetic and anti-inflammatory properties. Stevioside has been shown to improve insulin sensitivity, reduce blood glucose levels, and inhibit inflammation in various in vitro and in vivo models [19,20]. However, the precise molecular mechanisms underlying its anti-inflammatory effects, especially in the context of T2DM induced by HFD, remain incompletely understood. This study aims to investigate the anti-inflammatory potential of Stevioside in the context of HFD-induced T2DM using in vivo experiments. By elucidating the specific pathways and molecular targets through which stevioside exerts its anti-inflammatory effects, we hope to provide valuable insights into its therapeutic potential in managing T2DM-associated inflammation [21,22].

To address this hypothesis, we will employ a well-established rat model of HFD-induced T2DM and administer Stevioside as an intervention. We will evaluate the impact of Stevioside on key parameters, including glucose homeostasis, insulin resistance, inflammatory markers, and relevant molecular pathways, to gain insights into its potential as a therapeutic agent for T2DM-associated inflammation. The findings of this study could contribute to our understanding of the molecular mechanisms involved in the anti-inflammatory effects of Stevioside in T2DM and offer a promising avenue for the development of novel therapeutic strategies that may complement existing approaches for the management of T2DM and its associated inflammatory complications.

Materials and Methods

Chemicals

The entire chemicals and reagents used in this research were of the molecular and analytical grade acquired from Sigma Chemical Company (St. Louis, MO, USA); MP Biomedicals (Santa Ana, CA 92,707 USA) and Sisco Research Laboratories (Mumbai, India). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Ray Biotech (3607 Parkway Lane, Suite 100 Norcross, GA 30,092 Illinois, USA). Rat specific primers were

obtained from Eurofins Scientific, Bangalore, India.

Animals

As stated by the national guiding principle and procedure approved by the institutional animal ethics committee (IAEC No: BRULAC/SDCH/SIMATS/IAEC/04-2022/107 dated 25th April 2022). 150–180 days old (180–200g) healthy adult male Wistar albino rats were kept in hygienic polypropylene cages in specific humidity (65% ± 5%) and temperature (21 ± 2◦C) with stable 12 h light and 12h dark schedule at the Central Animal House Facility, BRULAC, Saveetha Dental College and Hospitals, Chennai-600 077, India.

Induction of Type-2 Diabetes in Animals

Rats were fed with HFD comprising of 66% standard rat feed, 3% cholesterol, 1% cholic acid and 30% coconut oil for 60 days. Through drinking water 30% sucrose was given [23]. After the treatment period, rats with fasting blood glucose >120 mg/dL were selected as type-2 diabetic rats. Feeding with HFD and sucrose water was done until the end of the study.

Experimental Design

Animals were arbitrarily divided into five groups with each group consisting of 6 animals. Group I- normal rats; Group II- type-2 diabetic rats; Group III-type-2 diabetic rat treated with stevioside (20 mg/kg b.wt/day), orally for 30 days; Group IV—type-2 diabetic rats treated with metformin (50 mg/kg, b.wt/day), orally for 30 days. Oral glucose tolerance test (OGT) and insulin tolerance test (ITT) were done in control and experimental rats two days before sacrifice. After the treatment period, the animals were anesthetized with sodium thiopentone (40 mg/kg body weight) through cardiac puncture, blood was collected, and sera were separated and stored at −80 °C. After clearing the blood from the organs by perfusing 20 mL of isotonic sodium chloride solution through the left ventricle, organs were immediately dissected out and used for further studies.

Fasting Blood Glucose (FBG)

Blood glucose levels were assessed following an overnight fasting period using On-Call Plus blood glucose test strips from ACON Laboratories Inc., USA. Blood samples were obtained by pricking the tip of the rat's tail, and the outcomes were quantified & presented in units of mg/dL.

Estimation of Serum TNF-α, SREBP-1c and PPARγ

Serum TNF-α, SREB-1c and PPARγ levels were assayed using rat insulin ELISA kit obtained from Ray Biotech (3607 Parkway Lane, Suite 100 Norcross, GA 30,092 Illinois, USA) as per the manufacturer's instruction. Intra-assay coefficient of variation was <10.0% and inter-assay coefficient of variation was <12.0%. Results for TNF-α, and SREBP-1c were expressed as npg/mL while the PPARγ level was expressed as ng/mL.

mRNA Expression Analysis Real Time-RT-PCR

To perform RT-PCR analysis 2µg of RNA was used and it was processed by RT-PCR kit (PrimeScript 1st strand cDNA Synthesis Kit, catalog no. 6110B, TAKARA BIO INDIA PVT LTD. New Delhi-110044, India). Firstly, using mRNA template along with oligo DT, dNTPs, and reverse transcriptase enzyme cDNA was synthesized. These constituents were blended with a DNA primer in a reverse transcriptase buffer and allowed to incubate at 37°C for one hour to facilitate cDNA conversion. Followed by conversion RT-PCR was performed using gene specific oligonucleotide primers. The initial PCR activation was set at 95°C for 5 minutes. The ensuing three-step PCR cycles encompassed denaturation at 95°C for 2 minutes, annealing at 60°C for 30 seconds, and extension at 73°C for 30 seconds. This PCR amplification procedure was iterated for 30 cycles, and for comprehensive product

extension, a final extension step at 73°C C for 5 minutes was executed (TB Green® Premix Ex Taq™ II, Catalog no. RR820A, TAKARA BIO INDIA PVT LTD. New Delhi-110044, India). Furthermore, gene-specific oligonucleotide primers for the housekeeping gene β-actin were incorporated into the same PCR reaction vial and co-amplified together with the target genes (Bio Rad C1000 Touch Thermal Cycler System, USA). A list of primers used in this study are given in Table 1.

Table 1. List of Gene Specific Primers Used

Name of the gene	Primer Sequence	Reference
Rat IR	Sense primer: 5'- GCC ATC CCG AAA GCG AAG ATC-3' Anti-sense primer: 5'- TCT GGG TCC TGA TTG CAT-3'	[24]
Rat GLUT4	Sense primer: 5'- GGG CTG TGA GTG AGT GCT TTC - 3' Anti-sense primer: 5'- CAG CGA GGC AAG GCT AGA - 3'	[24]
Rat TNF-α	Sense primer: 5′-GTCGTAGCAAACCACCAAGC-3' Anti-sense primer: 5′-CTCCTGGTATGAA ATGGCAAA-3'	[24]
Rat IL1-β	Sense primer: 5'-GGGATGATGACGACCTGC-3' Anti-sense primer: 5'- CCACTTGTTGGCTTATGTT-3'	[25]
Rat IL-6	Sense primer: 5′-GTGAGAAGTATGAGAAGTGTGA-3' Anti-sense primer: 5′-GCAGGATGAGAATGATCTTTG-3'	[24]
Rat IKKB	Sense Primer: 5′-TGGCATGGAAACGGATAACTGA-3' Anti-sense primer: 5′-CTGGAACTCTGTGCCTGTGGAA-3'	[24]
Rat β-actin	Sense primer: 5'- AAG TCC CTC ACC CTC CCA AAA G-3' Anti-sense primer: 5'- AAG CAA TGC TGT CAC CTT CCC-3'	[24]

Results

Effects of Stevioside on Fasting Blood Glucose in HFD-induced Type-2 Diabetic Rats

To assess the potential anti-diabetic effects of Stevioside, FBG was measured in the control and experimental animal groups. As depicted in Figure 1, the administration of Stevioside exhibited a notable reduction ($p < 0.05$) in elevated FBG levels in diabetic rats, comparable to the effects of metformin (Figure 1).

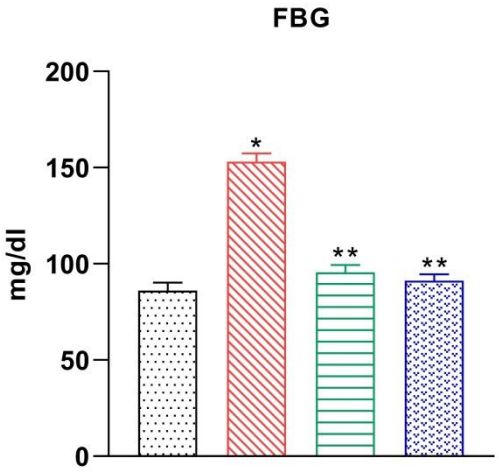

Figure 1. Effect of Stevioside on FBG in Type-2 Diabetic Rats

Effects of Stevioside on TNF-α, SREB-1c and PPARγ in HFD-induced Type-2 Diabetic Rats

Adiponectin, leptin, resistin, TNF-α is considered as possible serum markers of metabolic syndrome and their expression levels are majorly regulated by SREP-1c and PPAR-γ. Hence, their levels were analyzed in the serum of control and experimental rats. The acquired data showed a significant increase ($p < 0.05$) in, TNF-α, and SREP-1c but PPAR-γ concentration was considerably decreased in diabetic rats. However, treatment with stevioside restored the altered levels these markers in type-2 diabetic rats as effectively as metformin (Figure 2A-C).

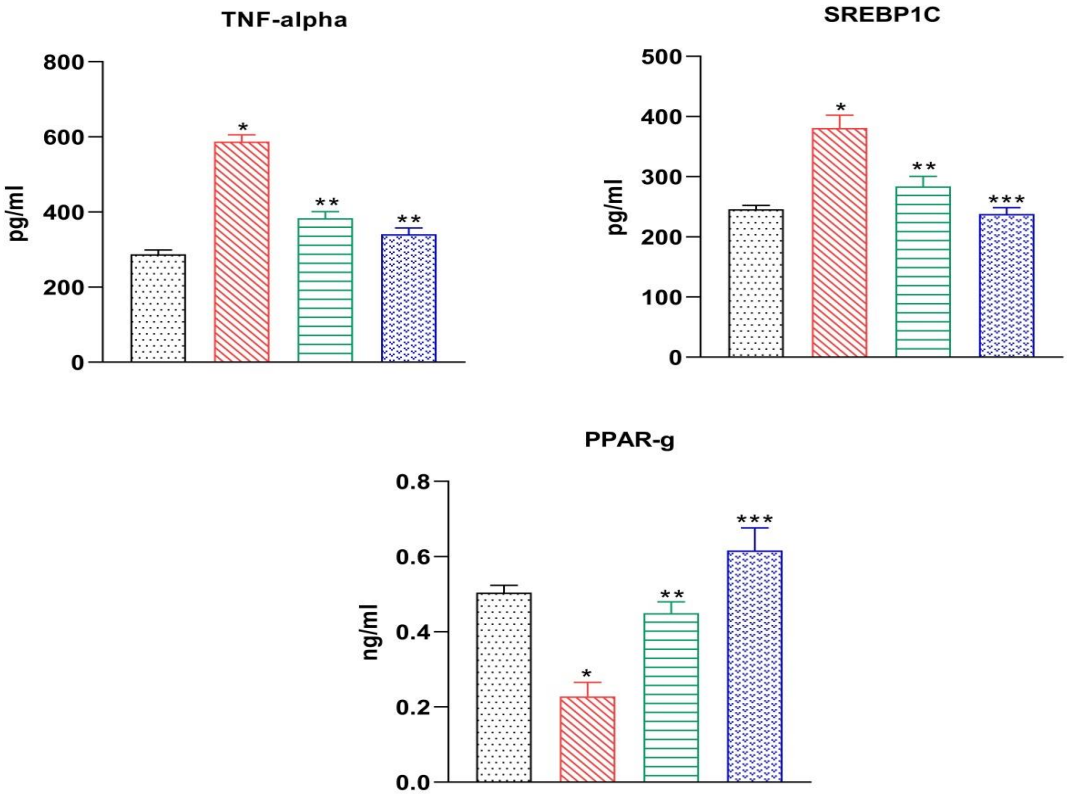

Figure 2A-C. Effect of Stevioside on TNF-α, SREB-1c and PPARγ in Type-2 Diabetic Rats

Effects of Stevioside on Insulin Receptor and GLUT 4 mRNA Expression Adipose Tissue of HFD-induced Type-2 Diabetic Rats

The insulin receptor is a protein found on the surface of cells, particularly in muscle, fat, and liver cells. It plays a critical role in regulating blood sugar levels. When insulin binds to its receptor, it initiates a signaling cascade that leads to the translocation of GLUT4 transporters to the cell membrane. GLUT4 is responsible for transporting glucose from the bloodstream into the cell. In the present study insulin receptor (IR) and GLUT4 mRNA expression was found to be significantly reduced in HFD-induced type-2 diabetic rats compared to control. Stevioside treatment fascinatingly ($p<0.05$) improved the expression of both IR and GLUT4 mRNA suggesting that stevioside has significant role in mediating insulin signalling in adipose tissue (Figure 3 A & B).

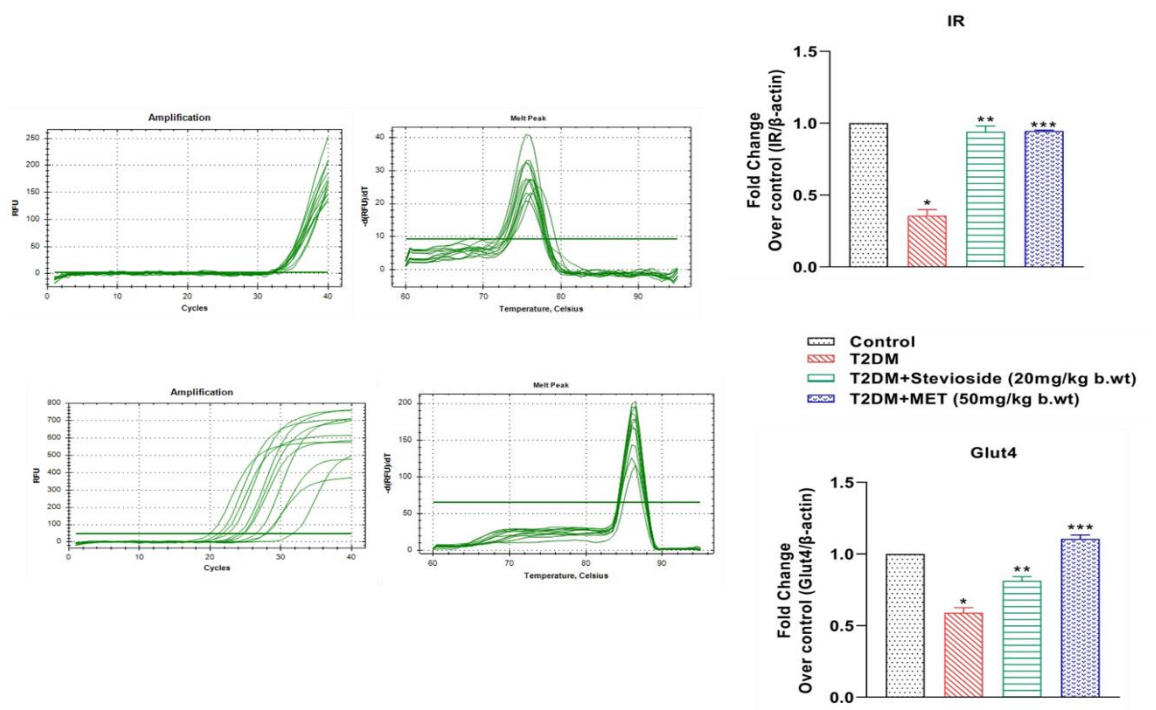

Figure 3 A & B. Effect of Stevioside on mRNA Expression of Insulin Receptor and GLUT 4 mRNA in Adipose Tissue of Type-2 Diabetic Rats

Effects of Stevioside on IL-1β and IL-6 mRNA Expression Adipose Tissue of HFD-induced Type-2 Diabetic Rats

Interleukin-1 beta (IL-1β) and interleukin-6 are pro-inflammatory cytokines that play a role in the pathogenesis of diabetes mellitus and can impact insulin signaling. In diabetes, chronic low-grade inflammation can lead to increased production of these cytokines. Both IL-1β and TNF-α can interfere with insulin signaling pathways in various ways. Elevated levels of these cytokines are associated with the development of insulin resistance in type 2 diabetes. In HFD-induced rats, both IL-1β and IL-6 mRNA were significantly elevated suggesting that pro-inflammation induced insulin resistance whereas stevioside reduced the same (Figure 4 A & B).

Effects of Stevioside on TNF-α and IKKB mRNA Expression Adipose Tissue of HFD-induced Type-2 Diabetic Rats

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that has been implicated in the development of insulin resistance. One of the key pathways through which TNF-α influences insulin resistance is by activating IκB kinase beta (IKKβ). IKKβ is an enzyme that triggers the degradation of IκB, a protein that normally inhibits nuclear factor-kappa B (NF-κB). When TNF-α stimulates IKKβ, it leads to the activation of NF-κB, which then induces the expression of various genes involved in inflammation. This chronic low-grade inflammation can disrupt insulin signaling pathways and impair the response of insulin-sensitive cells to glucose uptake, contributing to insulin resistance. Therefore, the TNF-α/IKKβ/NF-κB pathway is a critical mechanism linking inflammation to insulin resistance in conditions like obesity and type 2 diabetes. Hence, in the present study, we mesured TNF-α and IKKB mRNA level in adipose tissue. Both the mRNA was upregulated in type-2 diabetic rats. Stevioside at a dose of 20mg significantly reduced the mRNA whose effects were found to be near to that of the standard drug metformin (Figure 5 A & B).

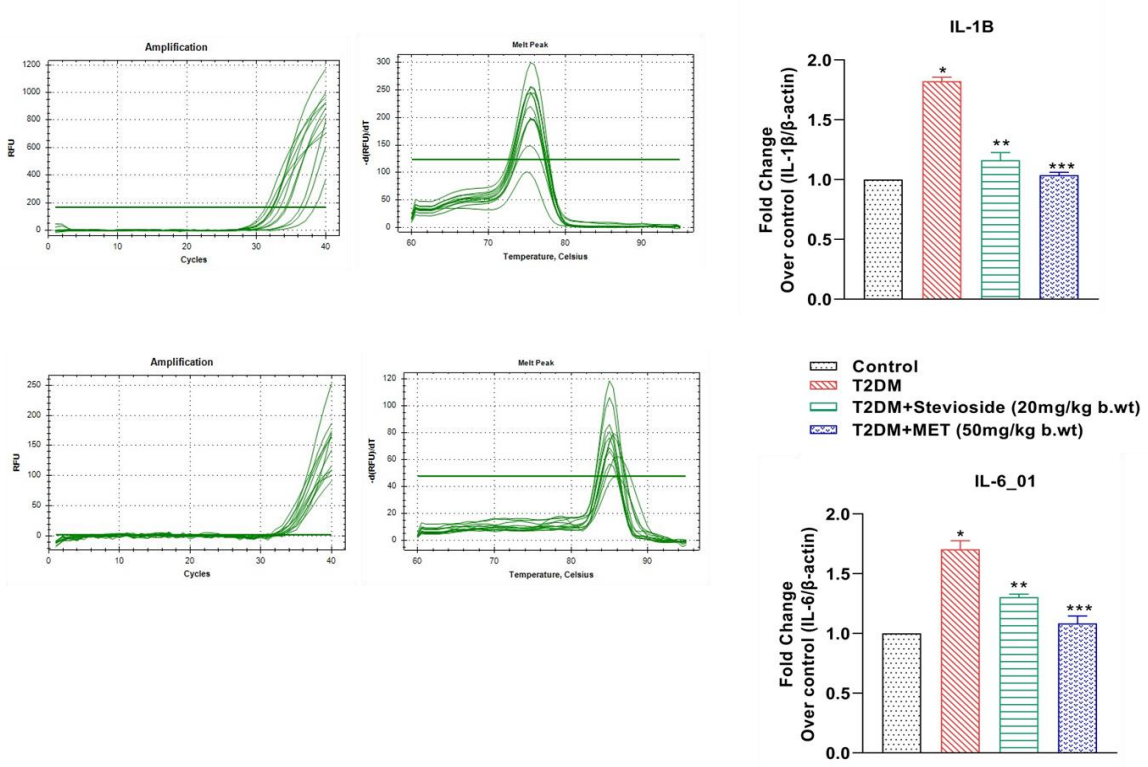

Figure 4 A & B. Effect of Stevioside on mRNA Expression of IL-1β and IL-6 mRNA in Adipose Tissue of Type-2 Diabetic Rats

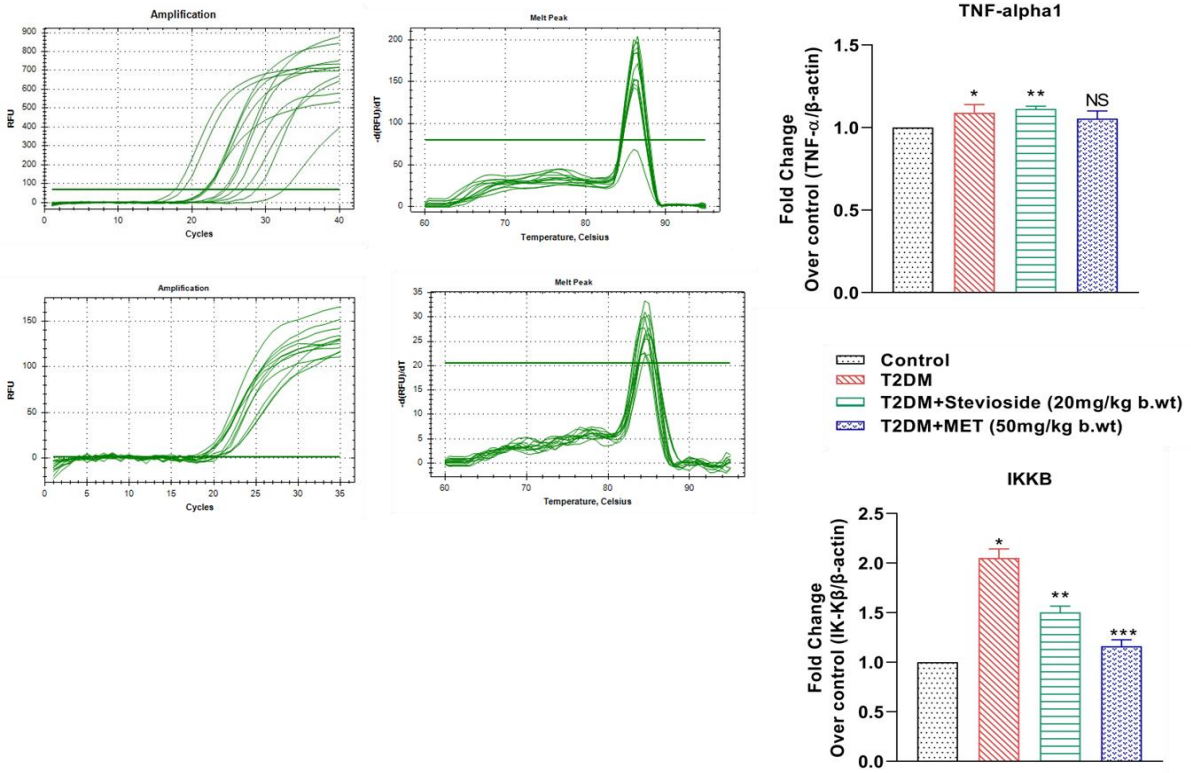

Figure 5 A& B. Effect of Stevioside on mRNA Expression of TNF-α and IKKB mRNA in Adipose Tissue of Type-2 Diabetic Rats

Effects of Stevioside on NFkB and PPARγ mRNA Expression Adipose Tissue of HFD-induced Type-2 Diabetic Rats

Nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ) are two key transcription factors that play opposing roles in insulin resistance. NF-κB is involved in the promotion of inflammation and is often activated in response to pro-inflammatory signals, like TNF-α. When NF-κB is overactive, it can lead to increased inflammation and insulin resistance by interfering with insulin signaling pathways. PPARγ, on the other hand, is a nuclear receptor that plays a role in regulating glucose and lipid metabolism. It is a key target for certain diabetes medications, like thiazolidinediones (TZDs), which enhance insulin sensitivity. Activation of PPARγ promotes glucose uptake and adipocyte differentiation, reducing insulin resistance. In the present study, NFkB mRNA expression was upregulated ($p<0.05$) while PPARγ expression was significantly reduced in HFD-induced T2DM rats. Stevioside treatment, normalized both the mRNA level (Figure 6A & B).

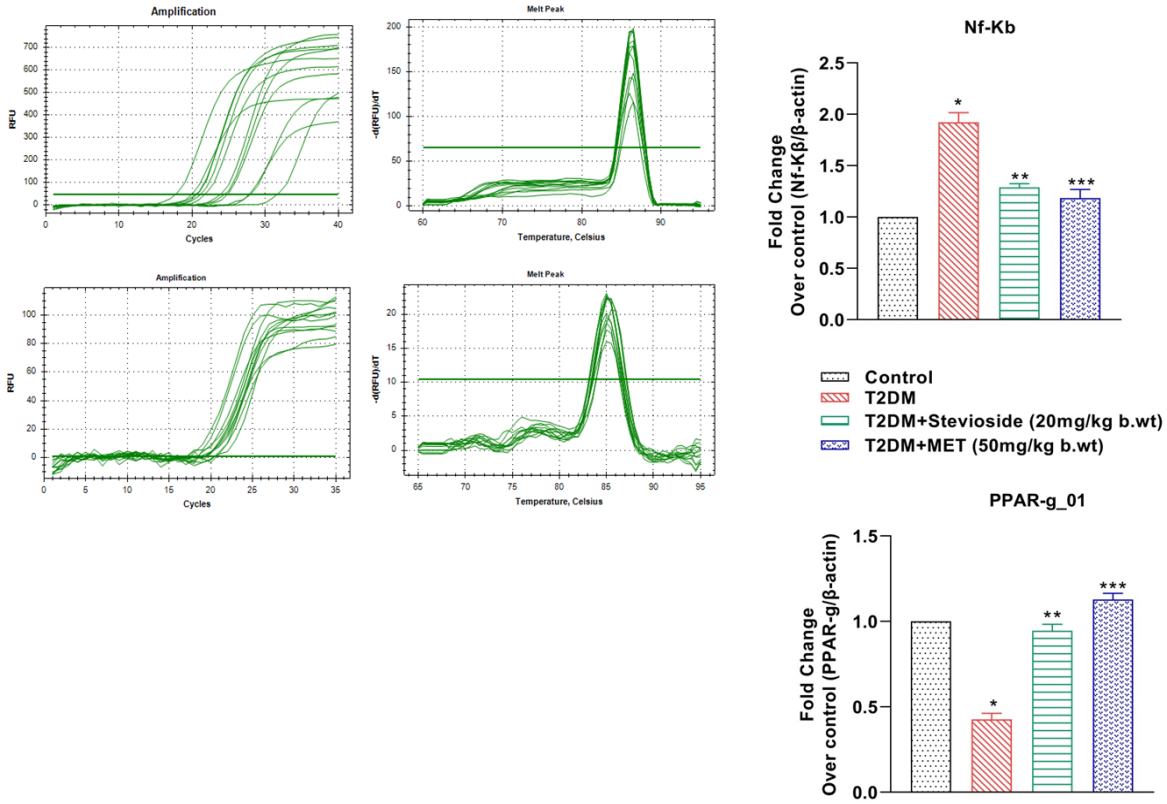

Figure 6A& B. Effect of Stevioside on mRNA Expression of NFkB and PPARγ mRNA in Adipose Tissue of Type-2 Diabetic Rats

Discussion

Type 2 diabetes is a multifaceted, diverse, and genetically influenced condition that is progressively becoming a major contributor to illness and death. Insulin resistance within the body plays a pivotal role in driving elevated blood sugar levels in Type 2 diabetes. This disease is a primary factor in the development of severe health issues, including vision impairment, the necessity for limb amputations, as well as the onset of other critical conditions such as kidney failure and cardiovascular diseases like strokes and heart attacks [26]. The global prevalence of diabetes currently affects around 425 million individuals, and this figure

is projected to rise to 629 million by the year 2045. Type 2 diabetes, which accounts for approximately 90% of all diabetes cases worldwide, is considerably more widespread than Type 1 diabetes. Traditional pharmacological therapies come with numerous unwanted side effects and a substantial risk of subsequent ineffectiveness. As a result, there is a growing desire for natural products that possess antidiabetic properties while causing fewer adverse effects [27]. Numerous experimental investigations have indicated that rats, when subjected to a high-fat diet, exhibit insulin resistance, closely resembling the clinical characteristics and development of Type 2 diabetes mellitus in humans. Additionally, the elevated levels of free fatty acids generated during high-fat diet regimens impede the expression of various insulin signalling molecules. We assessed the mRNA expression level of insulin receptor and GLUT4 (glucose transporter) molecules which are key molecules in insulin dependent glucose homeostasis. The levels were reduced in the diabetes group compared with the control. Upon treatment with Stevioside there is a significant increase in their expression levels which are nearly normalized to healthy. Stevioside potentially influences the expression of these signalling molecules as compared to that of the standard antidiabetic drug.

Numerous research investigations have demonstrated that TNF-alpha and IL-6 impede the uptake of glucose induced by insulin by affecting the insulin signalling pathway, which includes the insulin receptor, IRS, and Glut-4 [28, 29]. This interference contributes to the advancement and emergence of insulin resistance. Inflammation is believed to be a significant factor in the onset of Type 2 diabetes mellitus, leading to a decrease in insulin sensitivity, as indicated by research by Duncan et al. in 2003. Circulating markers of low-grade inflammation and glucose homeostasis such as TNF-α, IL-1β, IL-6, IKκB, NFκB, SREBP-1C have been identified as robust indicators of an elevated risk for developing Type 2 diabetes mellitus. We assessed the serum levels of inflammatory cytokines and a few essential signalling effector molecules which are responsible for glucose homeostasis through insulin dependent signalling events. SREBP-1C is one of the crucial insulin signalling molecule which negatively regulates glucose homeostasis, and its levels were elevated during diabetes mellitus. Medicament of Stevioside has a potential influence on these signalling molecules which results in the reduction in their expression levels. Inflammatory cytokines such as TNF-α, IL-1β, IL-6, IKκB, NFκB levels were elevated during diabetes which is one of the pathogenesis of inflammation mediated diabetes mellitus. And the mRNA expression levels of these signalling molecules were altered and significantly reduced upon stevioside treatment. PPARs are transcription factors that rely on specific molecules to function and play a pivotal role in maintaining energy balance by overseeing the regulation of both carbohydrate and lipid metabolism [30-32].

Among these, PPAR-γ, a member of the nuclear receptor superfamily of ligand-dependent transcription factors, plays a critical role in the development of fat cells and the maintenance of glucose equilibrium [33]. It is recognized for its ability to influence insulin sensitivity by controlling various hormones, cytokines, and proteins involved in insulin resistance [34]. PPAR-γ ligands are effective in suppressing the expression of numerous pro-inflammatory genes in macrophages [35]. Changes in the expression of PPAR-γ have been observed in rats with Type 2 diabetes [36]. The present study also confirmed the alterations in the level of PPAR-γ which is reduced. Stevioside treatment alters the expression level of PPAR-γ which is significantly increased compared with the control group. And these results suggest that Stevioside can act as a potential therapeutic approach for diabetes mellitus.

Numerous antidiabetic medications are currently being excessively utilized, yet their adverse effects render them unsuitable and hazardous for use. This urgent situation calls for the exploration of antihyperglycemic drugs with minimal side effects and maximal effectiveness, prompting our investigation into natural peptides with a strong affinity for receptors involved in glucose regulation in our latest study. Based on the latest research findings, Stevioside has demonstrated promising anti-inflammatory and anti-hyperglycaemic properties. It appears to achieve this by regulating various inflammatory molecules, including IL-1β, IL-6, TNF-α, IKKβ, NFKβ, and SREBP-1c, while also restoring the normal levels insulin receptor and GLUT4 as well as PPAR-γ.

Furthermore, the study conducted in high-fat diet-induced type-2 diabetic rats indicates that Stevioside effectively modulates gene expression related to these processes. Consequently, the findings strongly suggest that Stevioside merits further investigation in clinical studies aimed at developing effective and safe treatments for type 2 diabetes.

Conclusion

The study reveals that Stevioside shows significant potential in mitigating inflammation in type 2 diabetic rats induced by a high-fat diet. This research offers valuable understanding of the molecular mechanisms that make Stevioside a potential therapeutic option for addressing inflammation in type 2 diabetes. To gain a more comprehensive understanding of how Stevioside achieves its anti-inflammatory effects, additional investigations are necessary to clarify the exact signalling pathways and specific cellular targets involved.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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