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Molecular and Epigenetic Studies on the Effect of Hesperidin on IRS-1/Akt/GLUT4 Signaling Molecules in the Gastrocnemius Muscle of Streptozotocin-induced Type-2 Diabetic Rats

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Abstract

Diabetes mellitus is a significant global health issue, affecting 425 million people worldwide, with projections estimating an increase to 629 million by 2045. The need for potent pharmacological agents is urgent, as current oral hypoglycemic drugs have adverse side effects. Hesperidin, a bioflavonoid with anti-hyperglycemic and anti-hyperlipidemic properties, offers promise as a natural therapeutic option. This study aimed to evaluate hesperidin's molecular and epigenetic effects on insulin signal transduction in the gastrocnemius muscle of STZ-induced type 2 diabetic rats. Methods involved dividing fully-grown male Wistar rats into five groups: Healthy control, STZ-induced diabetic, Diabetes+Hesperidin (100mg/kg), Diabetes+Metformin (50mg/kg), and Control+Hesperidin. At the experiment's conclusion, blood samples and gastrocnemius muscle tissues were collected to measure fasting blood glucose, serum insulin, antioxidant enzymes, oxidative stress markers, and histopathological and mRNA expression of insulin signalling molecules. Data were analyzed using one-way ANOVA, with significance set at $p<0.05$. Results indicated that STZ-induced diabetic rats exhibited significant increases in hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress markers, along with reduced superoxide dismutase (SOD) activity. Histopathological analysis revealed reduced muscle fibres and disrupted skeletal fibres. Additionally, mRNA expression of IRS-1, Akt, and GLUT 4 was significantly reduced. Remarkably, hesperidin treatment normalized these altered parameters. In conclusion, hesperidin effectively regulates insulin signalling in skeletal muscle, reducing diabetic risk. Thus, hesperidin shows potential as a therapeutic candidate for treating type 2 diabetes and its associated complications.

Keywords: *Akt, Bioflavonoid, Diabetes, Glut4, Health and Well-being, Hesperidin, IRS-1, Novel Method, Streptozotocin.*

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Introduction

The widespread illness, diabetes is a primary cause of cardiovascular disease (CVD), a major worldwide health concern in the modern period, and it develops as a result of oxidative stress brought on by obesity and hyperglycemia in skeletal muscles. It has spread to epidemic proportions throughout the world and had a significant negative influence on human life and health economics [1]. Therefore, cutting-edge innovative medications that mitigate these pathogenic occurrences should be prioritized as a key component.

According to statistics from around the world, approximately 74% of people are unable to purchase allopathic medicine products. As a result, they must rely on traditional remedies, which are mostly made from plants [2]. Hesperidin is a bioflavonoid (flavanone glycoside) with hypolipidemic and hypoglycemic hypoglycemic properties that are abundant in oranges and lemons. Hesperidin, one of several biophenols, is important as a naturally occurring medicinal substance [3]. The majority of citrus plants contain hesperidin, a powerful vitamin that animals consume through diet. As per the European Food Safety Authority (EFSA), consuming hesperidin in conjunction with diosmin, troxerutin, and other substances is properly described. Maintaining normal venous-capillary permeability is the purported result, and it has positive physiological effects [4].

Alongside genetic changes, epigenetic modifications brought on by an inactive lifestyle and ecological stressors in response to imbalances in energy consumption and expenditure cause metabolic diseases like diabetes and obesity to develop and worsen. Insulin resistance can be produced by reducing the transcriptional function of important beta-cell genes by methylation of DNA, histone changes, and increased production of non-coding RNAs. Together with inflammation associated with obesity, increased Reactive Oxygen Species (ROS), & impairment to DNA in multiple body parts, epigenetics have some significance in the expression of the underlying gene networks responsible for insulin resistance and insufficiency [5]. A few other investigations have shown that hesperidin modifies the action of enzymes governing glucose and normalizes blood glucose levels. Loss of body weight persisted even after serum lipid levels decreased in STZ-administered diabetic mice & the modulatory action of biotransformation enzymes was the main 3cause. Trained immunity is based on epigenetic regulation of histone changes that cause chromatin to open permanently [6]. The objective of this experimental investigation is to identify and evaluate the therapeutic properties of hesperidin, a bioflavonoid derived from orange peel (a citrus fruit), on the molecules that regulate insulin signal in the gastrocnemius (skeletal) muscle of obese & hyperglycemia-instigated type 2 diabetes (T2DM) rats.

Materials and Methods

Animals

The 150–180-day-old adult male Wistar albino rats were used in this study and they were kept in conventional settings, which included a standard temperature of 21 ± 2 ◦C, a continuous 12-hour light/dark cycle, and specific humidity as per the standard guidelines (Animal Ethical Approval no: BRULAC / SDCH / SIMATS / IAEC / 04-2022 /101).

Induction of T2DM

To induce Diabetes, Streptozotocin (STZ) (40 mg/kg BW) mixed in cold citrate buffer (0.1 M, pH 4.5) that was freshly prepared was injected intraperitoneally once to develop diabetes for fasted (overnight) experimental rats. For a whole day, animals administered with streptozotocin were given a 20% glucose

solution in order to avert the first hypoglycemia mortality caused by the treatment. Animals given streptozotocin injections showed signs of hyperglycemia a few days later. Rats with diabetes were identified by detecting their high plasma glucose levels (using the glucose oxidase method) 72 hours after receiving a streptozotocin injection.

Experimental Design

Five groups of mature male Wistar albino rats were raised to vigorous adulthood. Group-I: Healthy control; Group-II: STZ-induced type-2 diabetic rats; Group-III: Diabetes+Hesperidin (100mg/kg.bwt); Group-IV: Diabetes+Metformin (50mg/kg.bwt); Group-V: Control+Hesperidin. All the animals were anaesthetized, blood and sera were separated & stored at -80˚C. Gastrocnemius muscles were separated out and utilized for epigenetic studies.

Fasting Blood Glucose (FBG)

After an overnight fast, FBG measurements were taken using test strips (On Call Plus) from ACON Lab. Inc., USA. The tail tip was used to draw blood and the results were measured as mg/dL.

Oral Glucose Tolerance Test (OGTT)

While fasting through the night, the rats were administered glucose orally (10 mL kg; 50% w/v), and the blood glucose levels were measured five times (0, 30, 60, 120, and 180 minutes) by On-Call Plus test strips. The FBG value were reading to measure the zero-minute reading, & the outcomes shown as mg/dL.

Hemoglobin and Glycosylated Hemoglobin Measurement

The cyanmethemoglobin method was used to measure haemoglobin (Hb) levels [7] and Bannon's approach [8] was used to measure glycosylated haemoglobin (HbA1C) levels. Grammes per deciliter (g/dL) were used to express haemoglobin content, while milligrammes per gramme of haemoglobin (mg/g) were used to express glycosylated haemoglobin value.

Histopathological Examination

Hematoxylin and eosin dye are applied to stain gastrocnemius muscle after they had been histopathologically examined using formalin (10% neutral buffered) preserved in paraffin [9,10]. The 'Olympus' light microscope equipped with a 'Nikon' digi-cam was used to identify the semi-thin sections, which had a magnification of x200, that had been generated using the LKB ultra-microtome.

Glucose-6-Phosphatase Assay

A one-hour incubation period at 37 °C was required for 0.5 mL of substrate, 0.1 mL of homogenate tissue, and 0.3 mL of citrate buffer. After that, 10% TCA was administered to halt the reaction using Fiske and Subbarow's technique [11]. The value was determined at 640 nm and glucose-6-phosphatase was measured.

Fructose-1,6 Bisphosphatase Assay

The fructose-1,6 bisphosphatase assay was conducted using the Gancedo JM and Gancedo C technique [12]. The final mixture (2.3 mL) was incubated with the rest of the substances for 15 minutes at 37°C: Tris-HCl buffer, substrate, $MgCl_2$, KCl, EDTA, and tissue homogenate. 10% TCA then stopped the reaction.

Analysis of mRNA Expression

Real-time PCR, cDNA conversion, and total RNA separation

The total RNA was isolated using a TRIR kit. The reverse transcriptase (RT) kit came from Eurogentec (Seraing, Belgium). To make cDNA, 2μg of total RNA were utilized. The genes in a Real-Time PCR system were amplified using the following conditions: a 5-minute initial denaturation at 95 ◦C, 40 cycles

of 95 ℃ (30 sec), 59 to 60 ℃ (30 sec), and 72 ℃ (30 sec). Relative quantification was established through an examination of the melt and amplification curves.

Statistical analysis

The statistics is presented as mean ± S.E.M (standard error of mean). ANOVA was used with the Windows version of the SPSS software package to evaluate group differences. For inter-group comparisons, post hoc analysis was carried out based on the least significance difference (LSD); Significant changes were defined as p values less than 0.05.

Results

Hesperidin's Impact on Variations in Weight (b.wt) of Rats

Figure 1 shows the variations in the body weight of the experimental and control rats. Experimental rats with streptozotocin-induced diabetes showed a sharp decrease in body weight. Rats given hesperidin alone and normal controls showed no apparent alterations. After taking hesperidin and metformin orally, all of the alterations seen in rats given streptozotocin considerably improved.

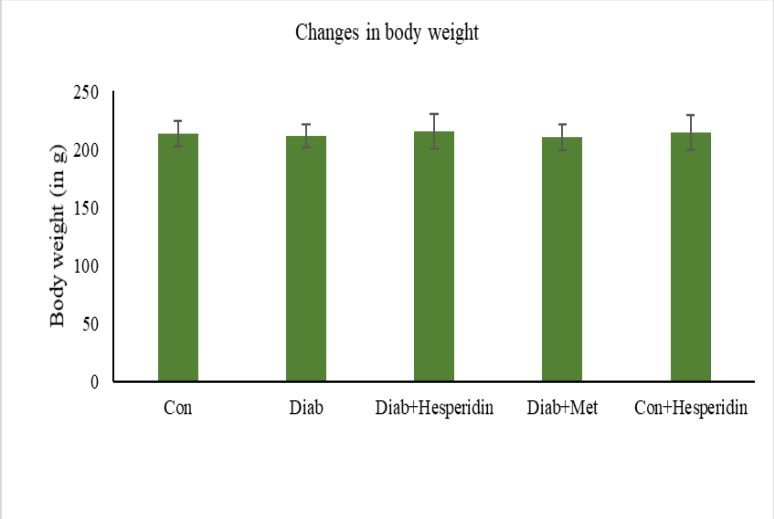

Figure 1. Hesperidin's Effect on Experimental and Control Rats' Body Weight.

Hesperidin's Effect on Plasma Glucose and Serum Insulin Concentrations

This study investigated the effects of hesperdin on FBG, serum insulin, and testosterone levels (Table1). The results showed that hesperidin supplementation significantly ($p < 0.05$) reduced fasting blood glucose levels, decreased serum insulin levels and these findings suggest that hesperidin has a positive impact on these metabolic parameters

Table 1. Effects of Hesperidin on Fasting Blood Glucose and Serum Insulin Level.

Parameter	**Control**	**Diabetes Induced**	**Diabetic + Hesperidin (100 mg/kg b.wt)**	**Diabetic + metformin (50 mg/kg b.wt)**	**Control + Hesperidin (100 mg/kg b.wt)**
Glucose (mg/dL)	88.54± 6.50	260.66± 14.01^{a}	128.30± 6.70 ^{ab}	120.73± 6.60^{ab}	88.45± 5.77^{bcd}
Serum insulin (µIU/mL)	17.85 ± 1.40	7.45± 0.66 ^{a}	16.80± 1.09 ^{b}	18.20± 1.55^{b}	17.50 ± 1.42^{b}

Hesperidin's Impact on OGTT

The results of the OGT showed that rats fed STZ-induction had higher FBG levels than the control group (Figure 2). They also had significantly higher blood glucose levels at 60 and 120 minutes after the oral glucose load. Rats treated with 100 mg/kg body weight of hesperidin had significantly lower fasting blood glucose levels than the diabetes group. The control group and the metformin-treated group had normal blood glucose levels after the oral glucose load. These findings suggest that hesperidin may improve OGT in STZ-induced diabetic rats by reducing fasting blood glucose levels.

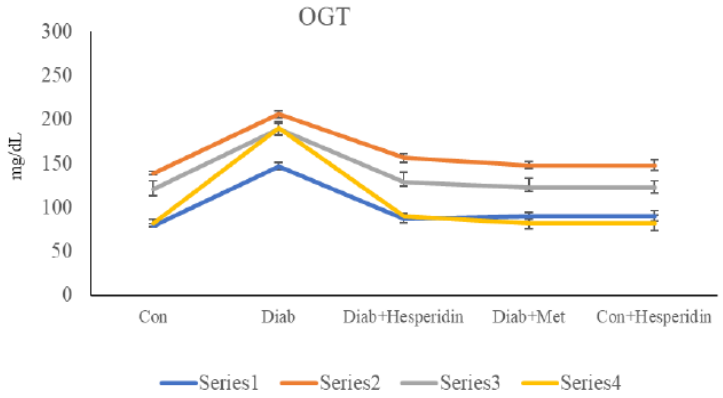

Figure 2. Effects of Hesperidin on Fasting Blood Glucose and Serum Insulin Level.

Hesperidin's Influence on Hb and HbA1c Levels

Table 2 displays total Hb and HbA1c levels in experimental and control animals. Comparing the diab. mice to normal contrl. rats, the diab. mice had considerably lower Hb levels and higher HbA1c levels. Hesperidin plus metformin treatment dramatically reduced Hb and HbA1c values. Apparently Hb and HbA1c levels in control animals adminstering with 100 mg per kg BW of hesperidin didn't notbly alter. By which we are able to see that treatment of Hesperidin clearly controls the Hb and HA1c levels.

Table 2. Effects of Hesperidin on Haemoglobin and Glycosylated Haemoglobin.

Groups	**Hb (g per dl)**	**HbA_{1c} (% Hb)**
Control	13.50± 1.25	5.30 ± 0.35
Diabetes	8.01 ± 0.65^{a}	09.95 ± 1.05^{a}
Diab + Hesperidin (100 mg)	11.30±1.22a^{b}	7.87± 0.62^{ab}
Diab + Metformin (50mg)	11.80 ± 1.09^{ab}	6.34± 0.50^{abc}
Control + Hesperidin (100 mg)	14.40 ± 1.20^{abc}	4.95 ± 0.38^{abcd}

Hesperidin's Influence on the Histopathological Modifications in Gastrocnemius Muscle

The onset of diabetes in the group with diabetes caused a break in the muscle fibers of the gastrocnemius and a reduction in the total number of muscle fibers. In the diabetic group, the connective tissue space was comparatively greater than in the control group. Focal atrophy is noted Chronic inflammation in the intermuscular connective tissue was noted (Figure 3a-e).

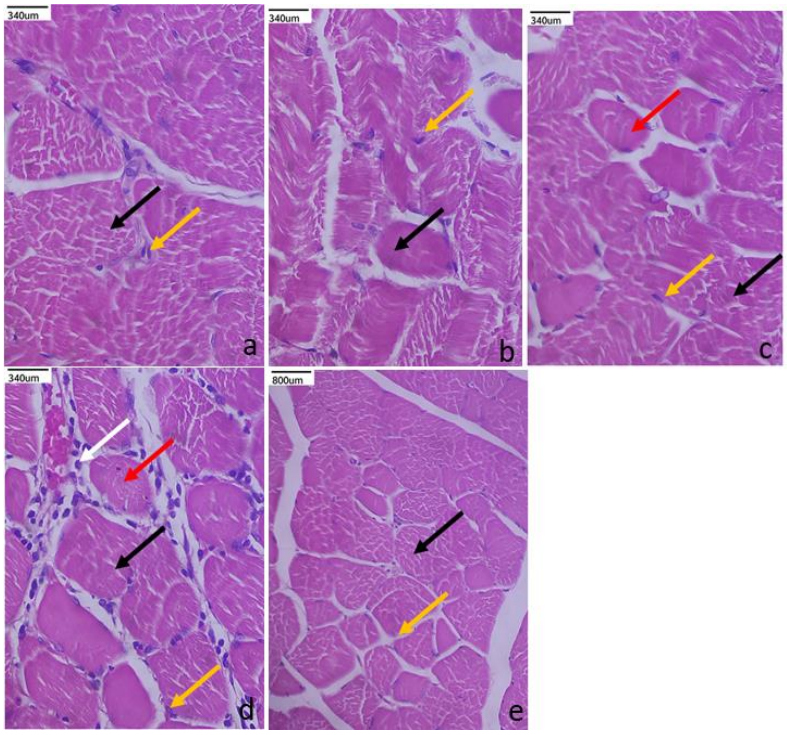

Figure 3a-e. Hesperidin Action on Skeletal Muscle on STZ Induced Rats.

Effects on Gluconeogenic Enzyme Determination

The activity of four carbohydrate metabolic enzymes (HK, G6P, and FBP) was measured in the gastrocnemius muscle of control and diet-induced diabetic rats. The diabetic rats had significantly lower activity of HK than the control rats. Conversely, the diabetic rats had significantly higher activity of G6P and FBP than the control rats. These results suggest that diabetes may disrupt carbohydrate metabolism in the gastrocnemius muscle. Hesperidin treatment normalized the altered levels of enzymes to that of the control levels (Table 3).

Table 3. Effects of Hesperidin on Haemoglobin and Glycosylated Haemoglobin.

Groups	**Hexokinase (units/g protein)**	**Glucose-6-phosphatase (Units/min/mg protein)**	**Fructose 1, 6 bisphosphatase units/h/mg protein)**
Control	157.60± 6.10	0.159±0.10	4.22±1.80
Diabetes	118.36 ± 8.20^{a}	0.49 ±0.07^{a}	11.13 ±2.25^{b}
Diab + Hesperidin (100 mg)	150.62± 12.1^{ab}	0.269±0.12^{ab}	6.90 ± 1.20^{c}
Diab + Metformin (50mg)	154.60 ±10.11^{b}	0.260±0.12^{ab}	6.20±1.10^{ab}
Nor. + Hesperidin (100 mg)	158.38 ± 7.20^{b}	0.180±0.20^{bcd}	4.30±1.60^{bcd}

Hesperidin's Influence on Lipid Peroxidation (LPO) and H_2O_2 Production

We observed the influence of Hesperidin on LPO and H_2O_2 production in skeletal muscle to note that after the administration of Hesperidin for the Diabetic rats, the values were significantly reduced the details were illustrated in Figure 4 & 5.

LPO

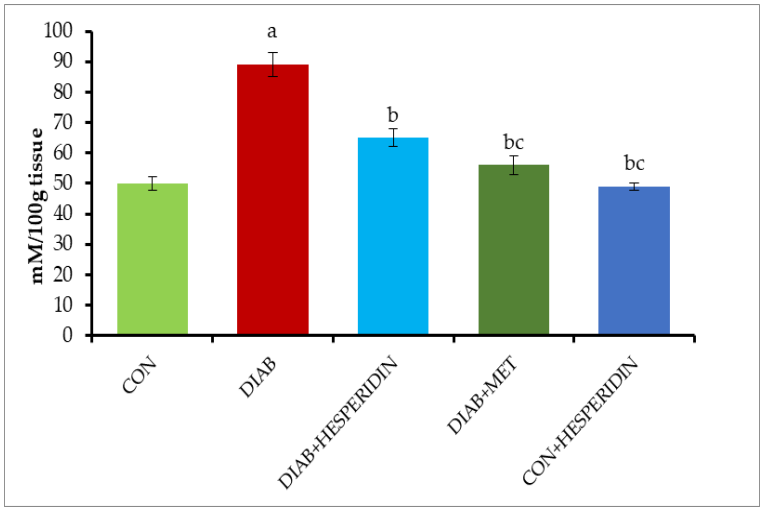

Figure 4. Effects of Hesperidin on LPO Activity in the Gastrocnemius Muscle.

H_2O_2

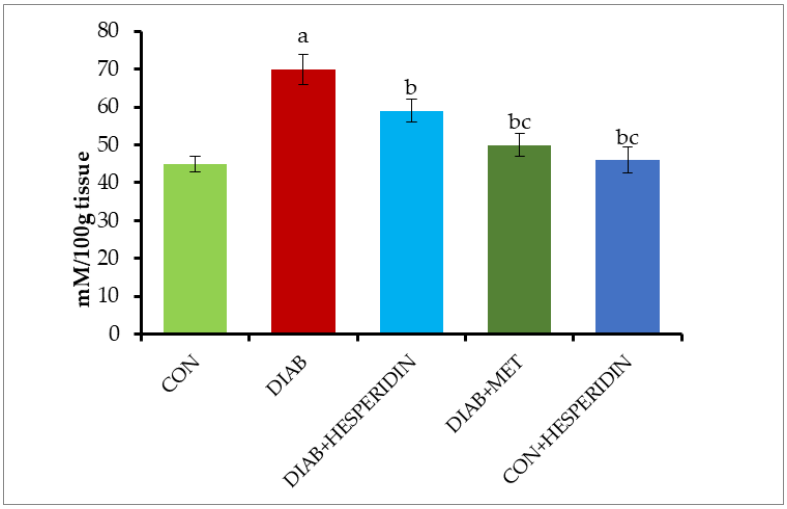

Figure 5. Effects of Hesperidin on H_2O_2 Activity in the Gastrocnemius Muscle.

Hesperidin's Influence on Antioxidant Enzymes (SOD and CAT) in the Skeletal Muscle

In the present study, both SOD and CAT activity were found to be significantly reduced in STZ-induced rats compared to control (Figure 6 & 7). However, hesperidin treatment, increased the same and the levels of the same were found to be equal to that of the control level.

SOD

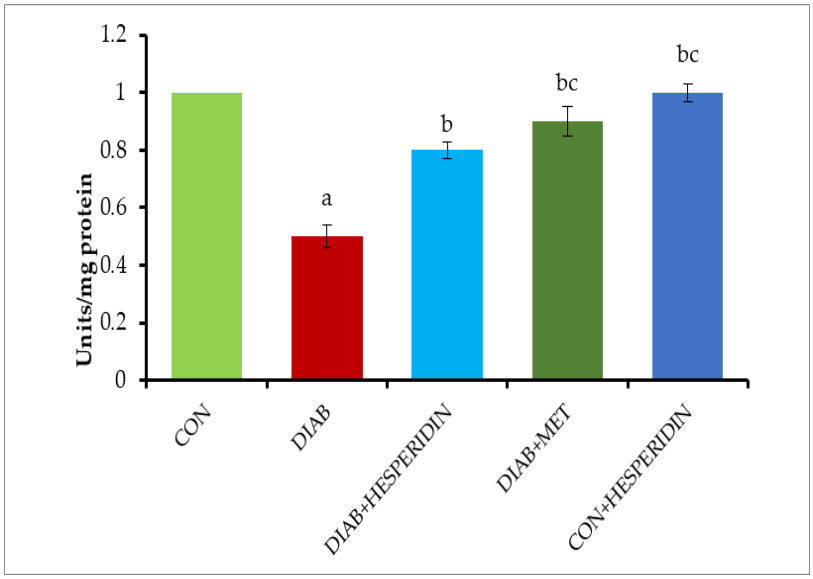

Figure 6. Effects of Hesperidin on SOD Activity in the Gastrocnemius Muscle.

CAT

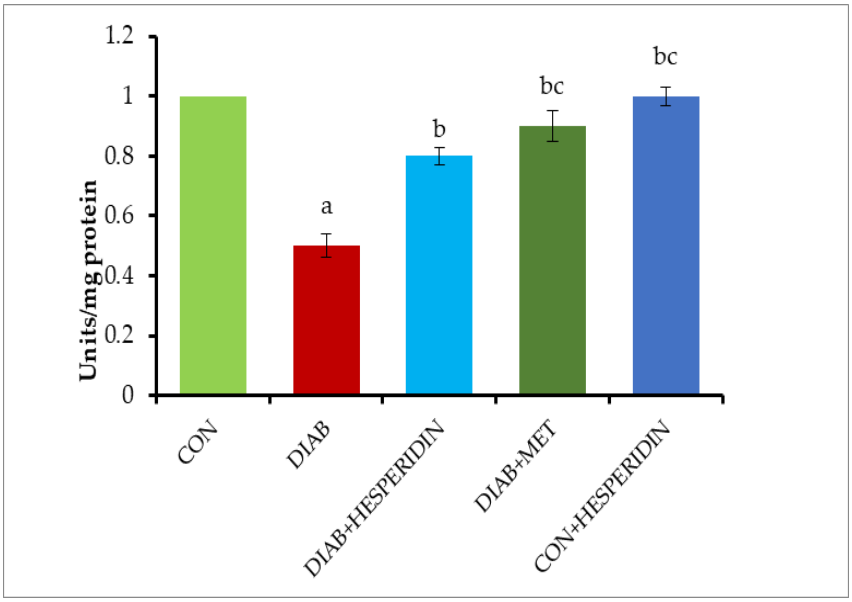

Figure 7. Effects of Hesperidin on CAT Activity in the Gastrocnemius Muscle.

Hesperidin's Influence on IRS-1, Akt, and GLUT4 mRNA Expression in the Skeletal Muscle of STZ-Induced Rats

We observed that hesperidin treatment to the STZ-induced type-2 diabetic rats upregulated the gene expression of IRS-1, Akt and GLUT 4 in the gastrocnemius muscle of type-2 diabetic rats compared to control rats (Figure 8-10).

IRS-1 mRNA

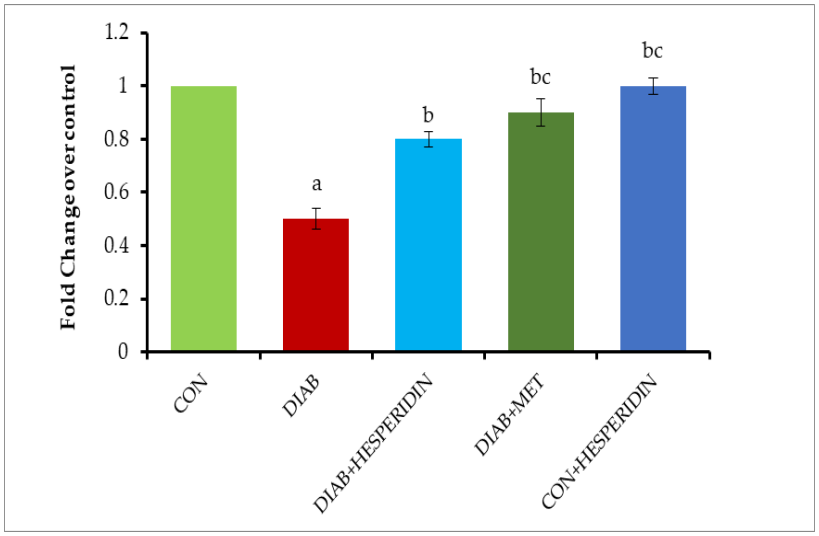

Figure 8. Effects of Hesperidin on IRS-1 mRNA Expression in the Gastrocnemius Muscle

Akt mRNA

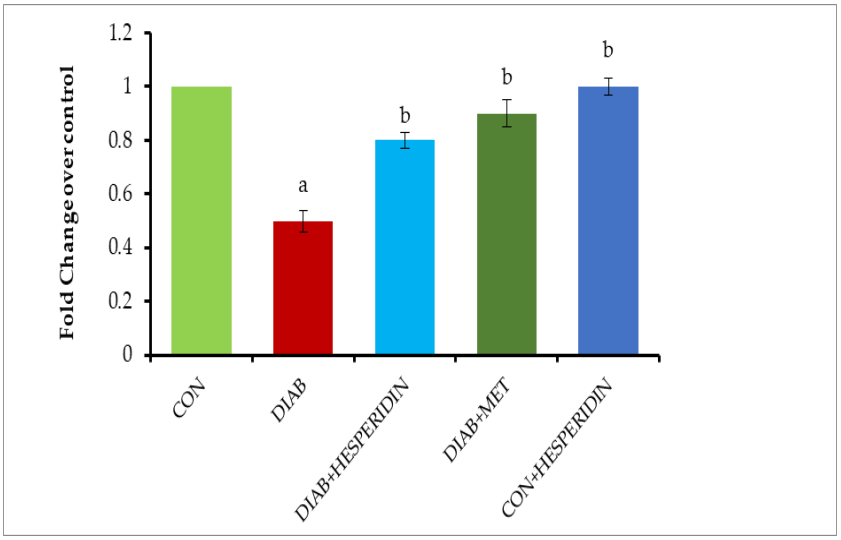

Figure 9. Effects of Hesperidin on Akt mRNA Expression in the Gastrocnemius Muscle

GLUT4 mRNA

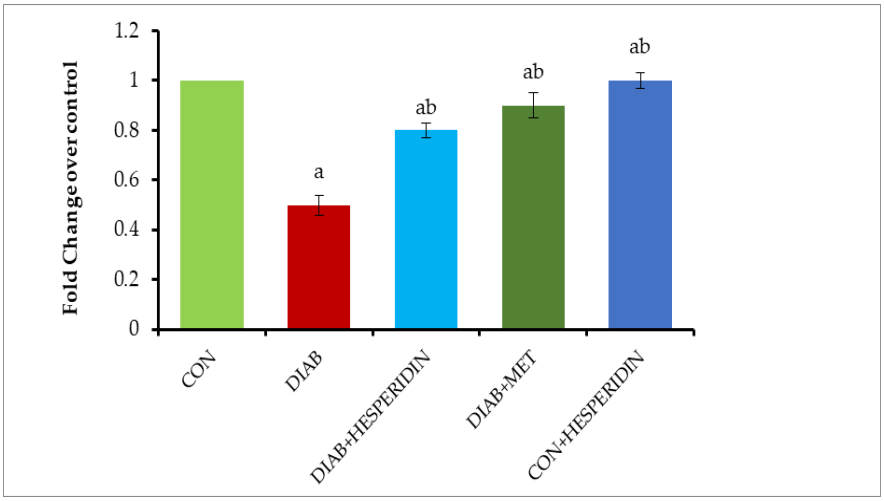

Figure 10. Effects of Hesperidin on GLUT 4 mRNA Expression in the Gastrocnemius Muscle.

Discussion

The prevalence of diabetes has increased and is on the rise in recent decades, and it will continue to change in the levels and disturbances in the generation and uptake of glucose, insulin secretion, insulin action, and insulin resistance may lead to illness [13]. It appears that hormonal interactions and various stressors may negatively affect this condition. One of the main causes of T2DM and the main issue related to obesity and a metabolic condition is peripheral insulin resistance. Reduced insulin-induced glucose uptake in skeletal muscle, a variety of post-receptor intracellular flaws, including lessened glucose transport, glucose phosphorylation, and decreased glucose oxidation and glycogen production, can all contribute to insulin resistance. Diabetes is a significant medical disorder that has many causes. Due to its numerous causes, diabetes is a serious medical condition [14].

Hyperinsulinemia and dysregulated insulin production are intimately linked to persistently high blood insulin levels in type 2 diabetes [15]. Streptozotocin induction alters beta cell activity and leads to an aberrant structure of insulin that ultimately prevents it from binding to insulin receptors in target organ cells. This may open the door to lessening the insulin receptor's ability to bind to insulin receptor substrates, which in turn may lessen the activity of downstream signaling pathways like PI3K, Akt, and AS 160. Reduced GLUT4 transporter translocation ultimately results in decreased glucose absorption and decreased glucose metabolism [16].

To keep blood glucose levels normal, insulin usually controls the processes of glycolysis and gluconeogenesis that promoting oxidation and absorption of glucose including muscle and adipose cells [17]. Alterations to oxidation of glucose in metabolic organs under diabetes produce regular swings in the enzymic activity linked to these pathways, which in turn results in insulin resistance in these organs [18]. The two main enzymes involved in gluconeogenesis are fructose-1,6 bisphosphatase and glucose-6-phosphatase. Since insulin normally acts as a inhibitor of gluconeogenic enzymes, the state of insulin shortage is the cause of the activation of these enzymes. Higher blood glucose levels result from increased gluconeogenic enzyme activity in type 2 diabetes (T2DM) since it creates H_2 & mixes with NADP+ to form NADPH and enhance lipogenesis [19]. In our investigation, we observed that the gastrocnemius muscles of diabetic mice had considerably higher amounts of fructose-1,6 bisphosphonates and glucose-6-phosphatase. In this paper, we found that giving hesperidin to experimental rats with high-fat diets and streptozotocin-administered T2DM increased their insulin sensitivity in the gastrocnemius muscle. Hesperidin has been reported to normalize glycolysis and gluconeogenesis by raising the glucokinase activity, phosphorylating IR and phosphoinositide-dependent kinase 1 (PDK1), and lowering the activity of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in liver. Hesperidin boosted glucose absorption in primary rat adipocytes in a cell-based experiment. By stimulating the IR/PDK1 pathway, Peng et al. shown the powerful preventative impact of hesperidin against HFD-induced insulin resistance [20]. Glycolysis generally affects insulin secretion as well as several cell metabolic activities. Important glycolysis enzymes HK and PK deficiencies may result in decreased glucose absorption and glycolysis, which can lead to insulin resistance [20].

As a result of defective insulin signaling, HK and PK were reduced in diabetic rats produced with streptozotocin and a HFD in the present study. The skeletal muscle of diab. mice treated with hesperidin had higher concentrations of these glycolytic enzymes than the group treated with metformin. As stated by Pari et al. [21], the phytocompound coumarin likewise exhibited a comparable

characteristic, raising the amounts of glycolytic enzymes in diab. mice relative to normal control. Similarly, increased levels of glycolytic enzymes in the skeletal muscle of diabetics due to flavonoids [22].

Insulin signaling, mostly through its effects on metabolic organs, is essential for regulating several physiologic processes, including glucolipid homeostasis. β-subunit tyrosine autophosphorylation, which is brought on by insulin binding to IR, phosphorylates more substrates and starts a signaling cascade that oxidizes glucose to produce energy. Various signaling pathways can become disrupted and results in insulin resistance. A HFD disorders glucose and lipid homeostasis, which impacts insulin signaling and changes how insulin signaling molecules normally operate, ultimately resulting in insulin resistance. In particular, insulin signaling molecule change may be more pronounced in skeletal muscle—where majority of (80%) glucose oxidation occurs—than the other organs including the liver and adipose tissue.

Insulin signaling pathways heavily rely on IRS-1, that is exclusive to bordering tissues like skeletal fibres and adipose tissue. Ineffective insulin binding to the insulin receptor leads to diabetes, which causes IRS-1 to be phosphorylated on the serine rather than the tyrosine kinase, which reduces IRS-1 activation and reduces the activity of lessening insulin signaling molecules like PI3 kinase, Akt, and AS160 [23]. The activity of Hesperidin on IRS-1, in the skeletal muscle of rats with HFD and STZ-administered rat shows while comparing with controls, the diabetic group had lessened levels of IRS-1 mRNA. Reduced activity of IRS-1 could result from changes in gluconeogenic and glycolytic processes.

Reduced binding of the insulin receptor to IRS-1 may result from modified gluconeogenic and glycolytic enzyme activity, which may have led to diminished IRS-1 activity. As a result, there was less Akt activation. In diabetic skeletal muscle, Zhang et al.'s study [24] revealed that fucoxanthin treatment increased IRS-1 mRNA levels. In T2DM skeletal muscle, Folium Mori led to increased levels of IRS-1 gene expression, according to a different study by Cai et al. [25]. When compared to metformin, the Hesperidin therapy increased IRS-1 levels and demonstrated the mechanism behind its antidiabetic effects.

Immunohistochemical studies support hesperidin’s antidiabetic properties and its increase of the IRS-1 and Akt. According to our research, hesperidin therapy improved skeletal muscle's insulin sensitivity by activating these protein targets that are part of the insulin signaling cascade, much like metformin does. While compared with control rats, the effect of these molecules’ staining was diminished in the skeletal muscle of diabetic rats.

Amplified IRS-1 deprivation was shown in the lipid tissue of a T2DM mouse model by Wang et al. [26], who also proposed that impaired glucose uptake was caused by malfunctioning GLUT4. According to Li et al. [27], dioscin therapy controlled IRS-1 and Akt levels in a manner similar to that of the normal group, which in turn activated insulin signalling [28-30]. Our study's immunohistochemistry staining may help to explain how the ethanolic extract of C. papaya regulates the gluconeogenic and glycolytic enzymes and increases the mRNA composition of IRS-1 and Akt.

Conclusion

Our current research suggests that hesperidin, a naturally occurring bioflavonoid, may lower the risk of developing diabetes via controlling insulin signaling in skeletal muscle. Hesperidin could therefore be evaluated as a likely plant based therapeutic medication for the management of type 2 diabetes and its consequences.

Conflict of Interest

The authors hereby declare that there is no conflict of interest.

Acknowledgement

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References

[1] Manna, P., & Jain, S. K., 2015, Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies. *Metabolic Syndrome and Related Disorders*, 13(10), 423–444. https://doi.org/10.1089/met.2015.0095.

[2] Bindu Jacob, & Narendhirakannan, R.T., 2019, Role of medicinal plants in the management of diabetes mellitus: a review. *3 Biotech*, 9(1), 4. https://doi.org/10.1007/s13205-018-1528-0.

[3] Kim, J., Wie, M. B., Ahn, M., Tanaka, A., Matsuda, H., & Shin, T., 2019, Benefits of hesperidin in central nervous system disorders: a review. Anatomy & cell biology, 52(4), 369–377. https://doi.org/10.5115/acb.19.119.

[4] Giannini, I., Amato, A., Basso, L., Tricomi, N., Marranci, M., Pecorella, G., Tafuri, S., Pennisi, D., & Altomare, D. F., 2015, Flavonoids mixture (diosmin, troxerutin, hesperidin) in the treatment of acute hemorrhoidal disease: a prospective, randomized, triple-blind, controlled trial. *Techniques in Coloproctology*, 19(6), 339–345. https://doi.org/10.1007/s10151-015-1302-9.

[5] Prasad, M., Rajagopal, P., Devarajan, N., Veeraraghavan, V. P., Palanisamy, C. P., Cui, B., Patil, S., & Jayaraman, S., 2022, A comprehensive review on high -fat diet-induced diabetes mellitus: an epigenetic view. *The Journal of Nutritional Biochemistry*, 107, 109037. https://doi.org/10.1016/j.jnutbio.2022.109037.

[6] Georgel, P. T., & Georgel, P. (2021). Where Epigenetics Meets Food Intake: Their Interaction in the Development/Severity of Gout and Therapeutic Perspectives. *Frontiers in Immunology*, 12, 752359. https://doi.org/10.3389/fimmu.2021.752359.

[7] Drabkin, D.L., Austin, J.H., 1932, Spectrophotometric Studies: I. Spectrophotometric Constants for Common Hemoglobin Derivatives in Human, Dog, and Rabbit Blood. *Journal of Biological Chemistry*, 1932, 98, 719-733. 10.4236/oalib.1100648.

[8] Bannon P., 1982, Effect of pH on the elimination of the labile fraction of glycosylated hemoglobin. *Clinical Chemistry*, 28(10), 2183.

[9] Slaoui, M., & Fiette, L., 2011, Histopathology procedures: from tissue sampling to histopathological evaluation. Methods in molecular biology (Clifton, N.J.), 691, 69–82. https://doi.org/10.1007/978-1-60761-849-2_4.

[10] Gabe, M., 1968, Techniques Histologiques, 6th ed.; Massie e Cie: Paris, France, 1113p.

[11] Slaoui, M., & Fiette, L., 2011, Histopathology procedures: from tissue sampling to histopathological evaluation. *Methods in Molecular Biology (Clifton, N.J.)*, 691, 69–82. https://doi.org/10.1007/978-1-60761-849-2_4.

[12] Gancedo, J. M., & Gancedo, C., 1971, Fructose-1,6-diphosphatase, phosphofructokinase and glucose-6-phosphate dehydrogenase from fermenting and non fermenting yeasts. *Archiv fur Mikrobiologie*, 76(2), 132–138. https://doi.org/10.1007/BF00411787.

[13] Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., Ostolaza, H., & Martín, C., 2020, Pathophysiology of Type 2 Diabetes Mellitus. *International Journal of Molecular Sciences*, 21(17), 6275. https://doi.org/10.3390/ijms21176275.

[14] Merz, K. E., & Thurmond, D. C., 2020, Role of Skeletal Muscle in Insulin Resistance and

Glucose Uptake. *Comprehensive Physiology*, 10(3), 785–809. https://doi.org/10.1002/cphy.c190029.

[15] Thomas, D. D., Corkey, B. E., Istfan, N. W., & Apovian, C. M., 2019, Hyperinsulinemia: An Early Indicator of Metabolic Dysfunction. *Journal of the Endocrine Society*, 3(9), 1727–1747. https://doi.org/10.1210/js.2019-00065.

[16] Wilcox G., 2005, Insulin and insulin resistance. *The Clinical Biochemist. Reviews*, 26(2), 19–39.

[17] Zhao, C., Yang, C., Wai, S. T. C., Zhang, Y., P Portillo, M., Paoli, P., Wu, Y., San Cheang, W., Liu, B., Carpéné, C., Xiao, J., & Cao, H., 2019, Regulation of glucose metabolism by bioactive phytochemicals for the management of type 2 diabetes mellitus. *Critical Reviews in Food Science and Nutrition*, 59(6), 830–847. https://doi.org/10.1080/10408398.2018.1501658.

[18] Stanley Mainzen Prince, P., & Kamalakkannan, N., 2006, Rutin improves glucose homeostasis in streptozotocin diabetic tissues by altering glycolytic and gluconeogenic enzymes. *Journal of Biochemical and Molecular Toxicology*, 20(2), 96–102. https://doi.org/10.1002/jbt.20117.

[19] Latha, M., & Pari, L., 2003, Antihyperglycaemic effect of Cassia auriculata in experimental diabetes and its effects on key metabolic enzymes involved in carbohydrate metabolism. *Clinical and Experimental Pharmacology & Physiology*, 30(1-2), 38–43. https://doi.org/10.1046/j.1440-1681.2003.03785.x.

[20] Peng, P., Jin, J., Zou, G., Sui, Y., Han, Y., Zhao, D., & Liu, L., 2021, Hesperidin prevents hyperglycemia in diabetic rats by activating the insulin receptor pathway. *Experimental and Therapeutic Medicine*, 21(1), 53. https://doi.org/10.3892/etm.2020.9485.

[21] Pari, L., & Rajarajeswari, N., 2009, Efficacy of coumarin on hepatic key enzymes of glucose metabolism in chemical induced type 2 diabetic rats. *Chemico-biological Interactions*, 181(3), 292–296. https://doi.org/10.1016/j.cbi.2009.07.018.

[22] Gothandam, K., Ganesan, V. S., Ayyasamy, T., & Ramalingam, S., 2019, Antioxidant potential of theaflavin ameliorates the activities of key enzymes of glucose metabolism in high fat diet and streptozotocin - induced diabetic rats. Redox report : Communications in free radical research, 24(1), 41–50. https://doi.org/10.1080/13510002.2019.1624085.

[23] Boucher, J., Kleinridders, A., & Kahn, C. R., 2014, Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor Perspectives in Biology*, 6(1), a009191. https://doi.org/10.1101/cshperspect.a009191.

[24] Zhang Y, Xu W, Huang X et al., 2018, Fucoxanthin ameliorates hyperglycemia, hyperlipidemia and insulin resistance in diabetic mice partially through IRS-1/PI3K/Akt and AMPK pathways. *Journal of Functional Foods*, 48:515–524. 10.1016/j.jff.2018.07.048.

[25] Cai, S., Sun, W., Fan, Y., Guo, X., Xu, G., Xu, T., Hou, Y., Zhao, B., Feng, X., & Liu, T., 2016, Effect of mulberry leaf (Folium Mori) on insulin resistance via IRS-1/PI3K/Glut-4 signalling pathway in type 2 diabetes mellitus rats. *Pharmaceutical Biology*, 54(11), 2685–2691. https://doi.org/10.1080/13880209.2016.1178779.

[26] Wang, Y., Nishina, P. M., & Naggert, J. K., 2009, Degradation of IRS1 leads to impaired glucose uptake in adipose tissue of the type 2 diabetes mouse model TALLYHO/Jng. *The Journal of Endocrinology*, 203(1), 65–74. https://doi.org/10.1677/JOE-09-0026.

[27] Li, H., Yu, L., & Zhao, C., 2019, Dioscin attenuates high-fat diet-induced insulin resistance of adipose tissue through the IRS-1/PI3K/Akt signaling pathway. *Molecular Medicine Reports*, 19(2), 1230–1237. https://doi.org/10.3892/mmr.2018.9700.

[28] Ramalingam, K., Yadalam, P. K., Ramani, P., Krishna, M., Hafedh, S., Badnjević, A., Cervino, G., & Minervini, G., 2024, Light gradient boosting-based prediction of quality of life among oral cancer-treated patients. *BMC Oral Health*, 24(1), 349. https://doi.org/10.1186/s12903-024-04050-x.

[29] Neralla, M., M, H., Preethi, A., Selvakumar, S. C., & Sekar, D., 2024, Expression levels of microRNA-7110 in oral squamous cell carcinoma. *Minerva Dental and Oral Science*, 73(3), 155–160. https://doi.org/10.23736/S2724-6329.23.04801-5.

[30] Ramsundar, K., Jain, R. K., Balakrishnan, N., & Vikramsimha, B., 2023, Comparative evaluation of bracket bond failure rates of a novel non-primer adhesive with a conventional primer-based orthodontic adhesive - a pilot study. *Journal of Dental Research, Dental Clinics, Dental Prospects*, 17(1), 35–39. https://doi.org/10.34172/joddd.2023.36953.