https://doi.org/10.33472/AFJBS.6.5.2024.4120-4129

African Journal of Biological Sciences

Modulating Effect of Phenolic Compounds on The Insulin Secretion in RIN 5F Cells and Streptozotocin Induced Diabetes Rats

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Abstract

This study represents an initial investigation into the protective potential of phenolic compounds hesperetin, genistein, and gingerol against toxicity triggered by elevated glucose concentration in pancreatic β cells in vitro. Pancreatic β cells (RIN-5F) were grown under normal (5mM) and high (25mM) glucose concentrations, and the cell viability were assessed. Glucose stimulated insulin secretion was also measured after treatment of cells for 24 hr under normal and high glucose concentration and then treatment with phenolic compounds. Enhanced insulin secretion was observed in glucose-stimulated cells. Additionally, in streptozotocin (90 mg/kg, i.p) -induced diabetic rats, animals given phenolic compounds treatment for 8 weeks exhibited a significant drop in blood sugar levels accompanied by elevated insulin release. To summarize, hesperetin, genistein, and gingerol exhibited protective properties against toxicity induced by high glucose levels in RIN-5F cells, concurrently enhancing insulin secretion. Furthermore, these phenolic compounds also promote insulin secretion, decreased blood glucose and restored hepatic glycogen in diabetic rats indicating their modulating effects on insulin secretion and glycogen levels.

Keywords: Bioflavonoids, RIN5F cell line, streptozotocin, hesperetin, genistein, gingerol

Introduction

Diabetes mellitus is a widespread endocrine disorder distinguished by heightened levels of blood glucose resulting from insufficient insulin production or effectiveness within the pancreas. This disorder may manifest with or without accompanying complications that impact insulin function. (Subash-Babu et al., 2015; Tang et al., 2018).

Extensive clinical trials and epidemiological studies consistently demonstrate that a lack of insulin leads to high blood sugar levels, which in turn are associated with various serious health issues. These include coronary artery disease, stroke, kidney failure, vision loss, limb amputation, neurological problems, and premature mortality (Maida et al., 2022). In cases where oral hypoglycemic medications like sulfonylureas, biguanides, thiazolidinediones, and α-glucosidase

inhibitors failed towards effectively managing type II diabetes, insulin therapy becomes an alternative treatment option (Blahova et al., 2021). In conditions of high blood sugar levels, pancreatic β cells experience heightened metabolic activity and consequent cellular stress, which hampers their function and viability. This phenomenon, termed glucotoxicity, contributes to the deterioration of β-cell performance and survival (Böni-Schnetzler and Meier, 2019).

Contemporary medications for diabetes management, like rosiglitazone, a thiazolidinedione, are frequently prescribed by physicians to control elevated blood sugar levels and improve overall insulin sensitivity throughout the body. However, despite its efficacy in addressing insulin resistance, rosiglitazone use has been associated with increased cardiovascular risks, osteoporosis, and evidence suggests it may lead to weight gain and fluid retention (Vallon et al., 2009). Thus, there is a pressing need for a drug that can enhance insulin sensitivity without adverse side effects. Flavonoids derived from medicinal plants exhibit antioxidant properties and are pivotal in balancing cellular pro- and antioxidant processes without adverse effects. These dietary flavonoids are acknowledged as essential biological compounds (Park et al., 2020).

The hypoglycemic effects are linked to the distinct chemical components found in these plants, including flavonoids, polyphenolic compounds, among others. These contents may also be linked to elevated insulin and glucose metabolism. A comprehensive in vitro investigation was conducted to examine the impact of hesperetin, genistein and gingerol combination on insulin secretion, insulin activity. Moreover in vivo studies were also carried out in type II diabetic rats to evaluate the blood glucose lowering and insulin enhancing effects of these phenolic compounds after 8 weeks of oral administration to rats.

Material and method

1. Invitro studies

1.1. Cell line and chemicals

The Rat RIN-5F cells utilized in this in vitro study were procured from the "National Centre for Cell Science (NCCS)" in Pune. Chemicals such as glucose, 5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide trypsin, L-Glutamine-Penicillin-Streptomycin Solution and streptozotocin (STZ) were procured from Himedia Laboratories Private Limited, Maharashtra, India. Fetal bovine serum (FBS) and RPMI 1640 were obtained from Gibco, US. The experiment was conducted in duplicate, with each trial independently repeated at least three times.

1.2. Conditions of Cell culture

RIN-5F cells were cultivated in polystyrene flasks using RPMI 1640 medium (pH 7.4) supplemented with 10% fetal bovine serum (FBS). Subculturing of cells were done every three days until they reached confluence. Upon confluence, the cells detached from the flask using trypsin (0.25%)-EDTA (0.02%). Further cells were maintained in incubator at 5% CO_2 and 37°C.

1.3. MTT assay and STZ induced toxicity

RIN-5F cells were cultured in 96-well plates and exposed to various combinations of phenolic compounds for treatment (hesperetin: genistein: gingerol) at concentrations ranging from (1:1:1, 1:2:1, 1:3:1, 1:1:2, 1:1:3, 2:1:1, 3:1:1, 1:2:2, 1:2:3, 1:3:2, 1:3:3, 2:2:1, 2:2:3, 3:2:3, 3:2:2 µg/ml) for twenty four hours of incubation. Viability of cells were determined employing the MTT assay.

To optimize the dose and duration of streptozotocin treatment, RIN-5F cells were seeded at a density of 5×10^4 cells / well in 96 well cell culture plates. Streptozotocin (STZ) was dissolved in the cell

culture medium, and after 48 hours of cell attachment, various concentrations of STZ ranging from 1 mM to 40 mM were administered to the cells. The cells were then remained for 24 hours, following which cell viability was assessed using the MTT assay.

1.4. Insulin secretion assay

RIN-5F cells were plated at a density of 1.0 × 10^5 cells per well in a 24-well plate using RPMI 1640 medium. After a 72-hour incubation at 37°C, the medium was replaced with 0.5 mL of Krebs Ringer bicarbonate (KRB) buffer containing either 5 mM or 25 mM glucose for 40 minutes. Then, the buffer was replaced with 0.5 mL of KRB buffer treated with the phenolic compounds, while untreated cells served as the control. After a 3-hour incubation at 37°C, samples were collected from all wells and centrifuged. Insulin concentration in the media was measured using ELISA kits (Abbkine, China) according to the manufacturer's instructions, and the optical density was read at 450 nm using a BioRad multimode reader.

2. In-Vivo studies

2.1. Animal

Wistar albino rats of both sexes were employed in the current study. They were kept in 12 h light-dark cycles with 50–60% humidity and 20 ± 2°C. The Institutional Animal Ethics Committee (IAEC), gave its approval to the study (IAEC/2020/II-R02) recommendations, all experimental protocols were carried out as per CPSCEA guidelines.

Wistar albino adult rats (3 females: 1 male) were housed in same cage for mating. The vaginal smear was periodically checked for pregnancy. The female rats whose smears showed the presence of sperms were identified as pregnant and transferred in separate cage. After gestation period of 21-23 days, the animals delivered pups. Pups that were just five days old were given intraperitoneal injections of STZ (90 mg/kg, i.p) together with freshly made citrate buffer (pH – 4.5) to induce diabetes. Pups served as normal were given citrate buffer only. After 6 weeks of STZ injection, blood glucose was checked. Animals with fasting glucose level exceeding 160 mg/dL were considered diabetic and further divided into three groups viz. diabetic, and bioflavonoid treated group.

Eight animals (n = 8) were employed in each of the study groups. Bioflavonoid treatment group animals were given orally a combination of genistein (300 mg/kg), 6-gingerol (75 mg/kg) and hesperetin (100 mg/kg) till the experimental period of 8 weeks.

2.2. Biochemical parameters

Random blood glucose levels were assessed using a strip-based glucose monitoring device (Gluco One, Dr. Morepen, South Korea). Serum insulin levels were estimated utilizing an ELISA kit (Abbkine, China), adhering to the manufacturer's instructions. Insulin resistance was evaluated employing the homeostasis model evaluation method.

2.3. Oral glucose tolerance test (OGTT)

Before the OGTT, rats underwent a 16-hour fasting period. On the day of the experiment, fasting blood glucose levels were measured as described previously and considered as the baseline (0 min). A dose of 2.0 g/kg of body weight of glucose was orally administered to the rats immediately after the baseline measurement. Blood glucose levels were subsequently assessed at 30, 60, and 120 minutes after administering the glucose dose.

2.4. Measurement of total hemoglobin

Total hemoglobin was measured using the cyanomethemoglobin technique (Mahoney et al., 1993), while glycosylated hemoglobin (HbA1c) levels were determined using Bannon's modified method (Sabater et al., 1991).

2.5. Estimation of hepatic glycogen

Liver tissues weighing 0.5 grams were homogenized in 10 volumes of ice-cold buffer containing 30% of KOH. Afterward, glycogen content was precipitated using ethanol, pelleted, washed, and re-dissolved in distilled water. The glycogen content was then assessed by treating with anthrone reagent and measuring the absorbance at 625 nm (Mojibi and Rasouli, 2017).

2.6. Statistical analysis

Statistical analysis was performed utilizing one-way analysis of variance (ANOVA) followed by Tukey's post doc test with GraphPad Prism version 8.0 software. Significance was considered at $P < 0.05$.

3. Result

3.1. Impact of bioflavonoids on the viability of RIN5F cells

The study examined the effects of different concentrations of individual phenolic compounds hesperetin, genistein and 6-gingerol (100, 200, 400 µg/mL) on the cell viability after a 24-hour incubation period, and the obtained results are provided in **Fig. 1**. The concentrations of 100 and 200 µg/mL were determined to be non-toxic to the cells, as there was minimal reduction in cell viability at these doses. Furthermore, the impact of combinations of extracts at various ratios (1:1:1, 1:2:1, 1:3:1, 1:1:2, 1:1:3, 2:1:1, 3:1:1, 1:2:2, 1:2:3, 1:3:2, 1:3:3, 2:2:1, 2:2:3, 3:2:3, 3:2:2 µg/mL) was examined. It was observed that the extracts were not cytotoxic to RIN-5F cells at concentrations of 100 and 200 µg/mL. Interestingly, only two ratios of extracts (1:1:1 and 1:2:1 µg/mL) were found to be non-toxic when used in combination (**Fig 2**). Additionally, cell confluency was reduced under high glucose concentration (25mM), as depicted in **Fig 3**.

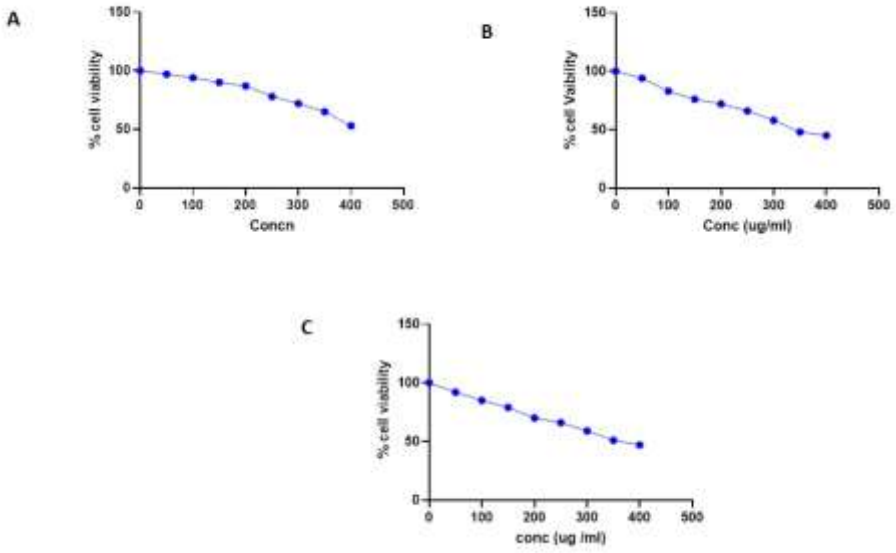

Fig 1: MTT assay of individual extract A. Hesperetin B. Genistein C. 6-Gingerol

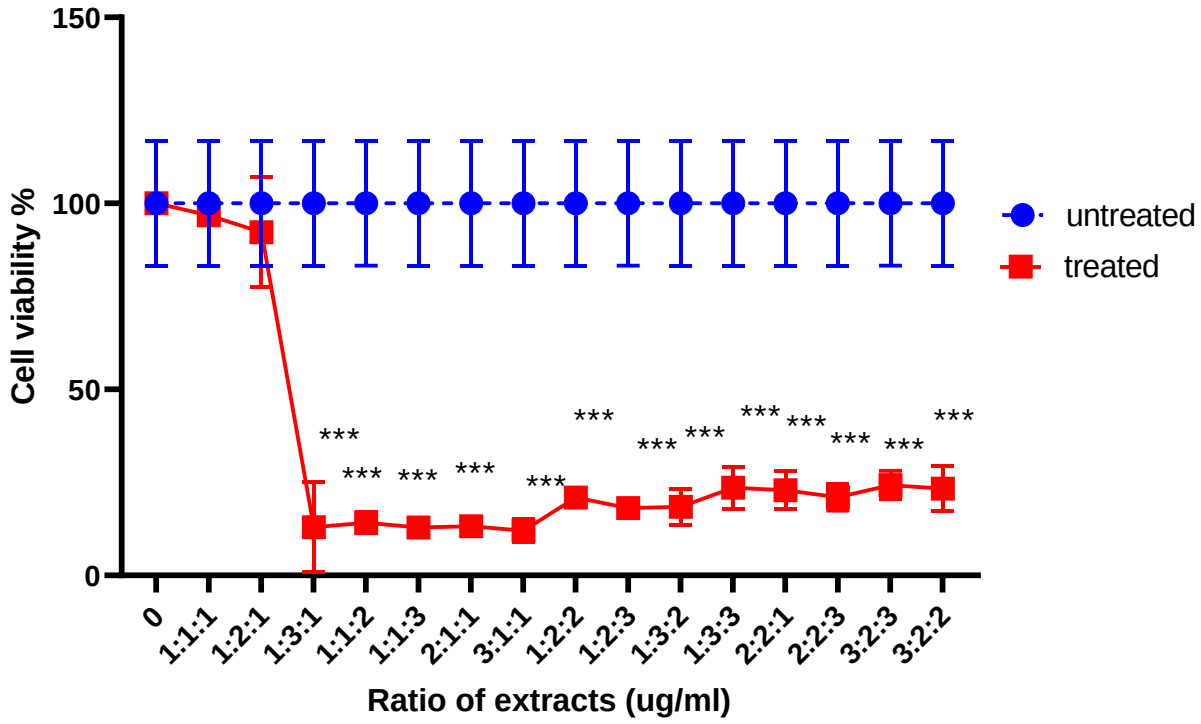

Fig 2: Cell viability of RIN-5F Cells after 24 hours treatment with combined phenolic compounds using the MTT assay. Control or untreated wells were given medium alone. Data is represented as mean ± standard deviation . ***P < 0.01 vs control group.

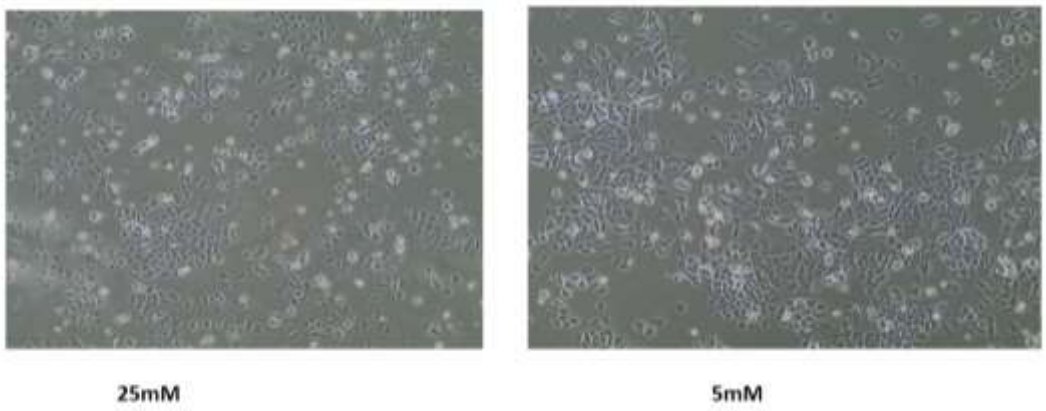

Fig 3: Confluency of cells at high glucose (25Mm) and low glucose (5Mm)

2. Effect of streptozotocin on RIN-5F cells viability

The effects of phenolic compound combinations (1:1:1 and 1:2:1 µg/mL) were assessed using pre-treatment, simultaneous treatment, and post-treatment schedules against STZ (1 to 40 µM) induced cell toxicity in RIN-5F cells.

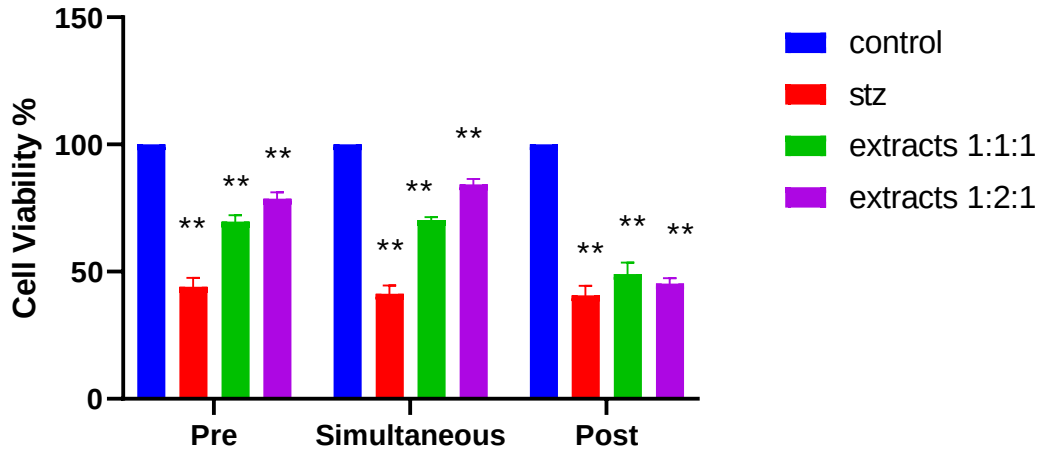

Fig 4:. Effect of phenolic compounds on viability of cells post, pre and simultaneous treatment of STZ. Data was expressed as the mean ± standard deviation, **P < 0.01 vs untreated control.

3. **Effect of phenolic compounds on glucose stimulated insulin secretion**

Exposure to the combination of phenolic compounds led to a notable rise in the insulin concentration in the cell culture medium at all administered doses under both glucose concentrations of 5 mM and 25 mM. The most substantial stimulation of insulin secretion occurred in RIN-5F cells treated with the phenolic compound combination at a ratio of 1:1:1 µg/ml.

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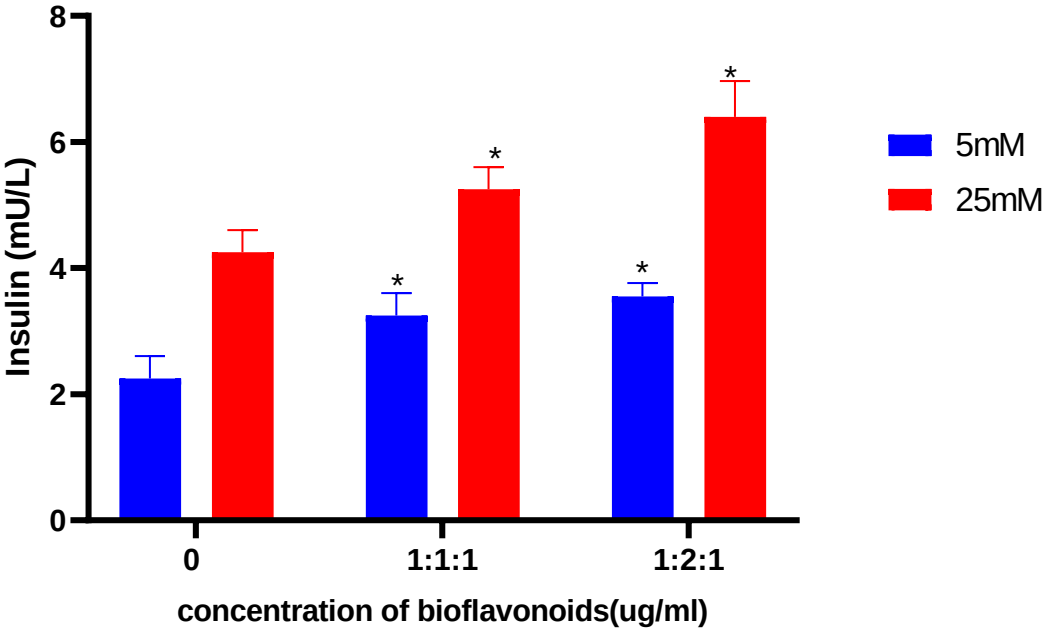

Fig 3: Effect of phenolic compound combination on glucose stimulated insulin release (GSIS) in RIN-5F cells. Data was expressed as mean ± SD. *P < 0.01 vs untreated control.

In vivo studies

Effect of phenolic compounds on glycemic status

After eight weeks, fasting blood glucose was significantly higher ($p<0.001$) in the diabetic control group compared to the normal group. Furthermore, in the diabetic control group, serum insulin levels were notably lower compared to those in the normal group. Treatment with the bioflavonoids

over eight-week period resulted in a significant decrease ($p<0.001$) in fasting blood glucose levels compared to the diabetic control group (**Fig 4).** Moreover, serum insulin levels were found to be increased and HOMA-IR in the treatment group compared to the diabetic control group (**Fig 5**).

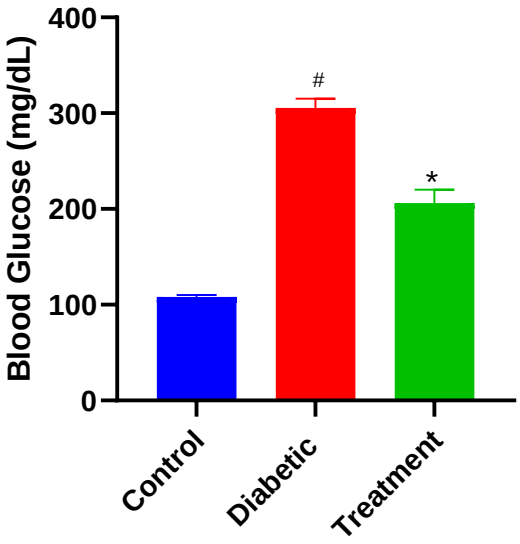

Fig 4: Effect of Bioflavonoid combination on blood glucose. Data were presented as mean ± SEM (n=8; each group). # $p<0.001$ vs normal *$p<0.001$ vs. Diabetic control

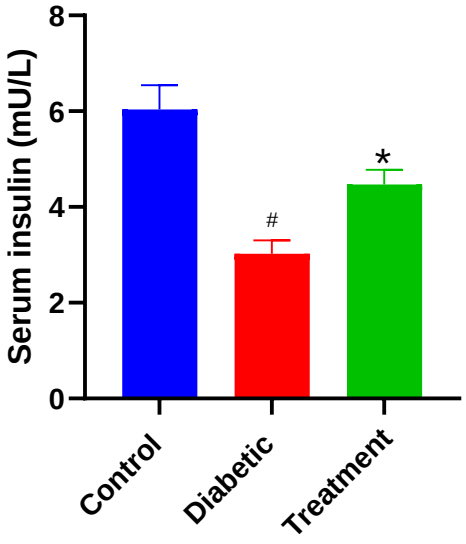

Fig 5: Effect of phenolic compounds on Serum insulin. Data were presented as mean ± SEM (n=8; each group). # $p<0.001$ vs normal control, *$p<0.001$ vs. Diabetic control, One Way Analysis of Variance (Tukey's Multiple Comparison).

Effect of phenolic compound interventions on OGTT

OGTT in the diabetic control group exhibited significantly higher glucose concentrations at all the tested time points compared to normal rats (fig. 6). Treatment with bioflavonoid combination significantly lowered the glucose concentration as compared to diabetic rats. The area under the curve values for the diabetic control group also showed significantly higher values ($p<0.001$)

compared to normal rats . Treatment with bioflavonoid combination significantly decreased the area under cure as compared to diabetic rats

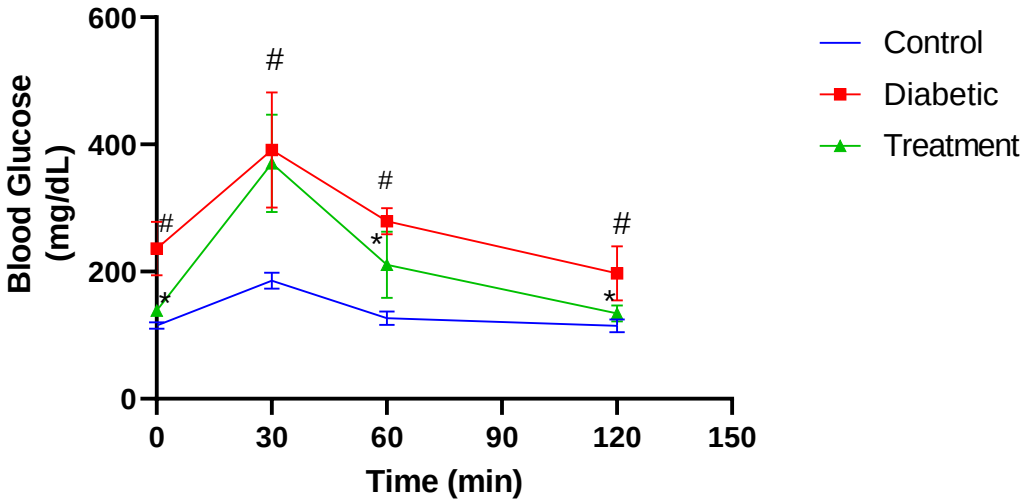

Figure 6: Effect of bioflavonoid combination on parameters of oral glucose tolerance test (OGTT). Concentration vs time plot. Each value represents mean ± SEM (n=8; each group). # p<0.001 vs normal control, *p<0.05 vs diabetic control, One Way Analysis of Variance (Dunnett's Multiple Comparison).

Effect of phenolic compounds on hepatic glycogen, Hb and HBA1c

In both normal and experimental type 2 diabetic rats, the effects of phenolic compounds on total hemoglobin, glycosylated hemoglobin (HbA1c), and liver glycogen levels were investigated. Significantly lower levels of hepatic glycogen, total hemoglobin, and an increase in glycosylated hemoglobin were observed in the type 2 diabetic rats compared to normal rats ($p < 0.05$). However, oral administration of phenolic compounds resulted in a significant increase in hepatic glycogen and total hemoglobin levels and a notable decrease (by 54.8%) in glycosylated hemoglobin levels compared to untreated diabetic rats ($p < 0.05$)**.(Fig 7)**

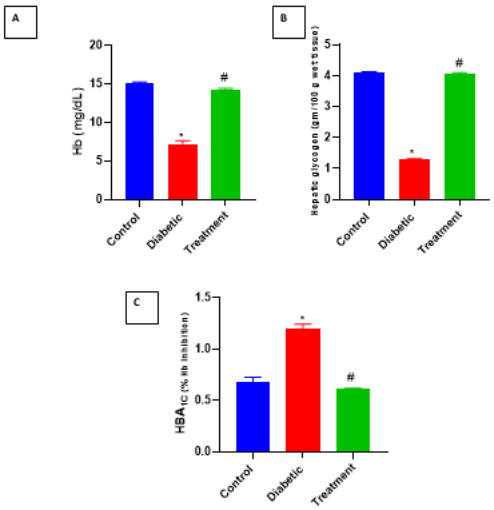

Fig 7: Effect of phenolic compounds on A. Hb B. hepatic glycogen C. HBA1c. Each value represents mean ± SEM (n=8; each group). * p<0.001 vs normal control, # p<0.05 vs diabetic control, One Way Analysis of Variance (Dunnett's Multiple Comparison).

DISCUSSION

Persistent elevation in blood glucose levels and the subsequent rise in reactive oxygen species (ROS) Contribute to the decline in β-cell function, exacerbating insulin resistance and ultimately leading to elevated blood glucose levels, as highlighted in previous studies(Kaneto et al., 2010) (Legaard et al., 2022). Insulin resistance, a key predictor of type 2 diabetes onset, is exacerbated as the disease progresses, with glucotoxicity playing a significant role in accelerating pancreatic β-cell dysfunction and diabetes onset(D'Angelo et al., 2022).

Elevated blood glucose levels trigger the production of free radicals via glucose autoxidation and protein glycation, contributing to oxidative stress and cardiovascular complications observed in STZ-induced diabetes (Bhatti et al., 2022). In our investigation, the bioflavonoid combination notably enhanced glucose-stimulated insulin secretion in RIN-5F cells. The magnitude of this effect depended on the severity of hyperglycemia, independent of cellular stress or mitochondrial dynamics alterations (Galizzi and Di Carlo, 2022). Natural products can stimulate islet cell insulin release and improve insulin sensitivity, thereby enhancing glucose uptake. Treatment with the bioflavonoid combination restored β-cell and functional mitochondrial content close to normal levels.

Glycogen levels function as a marker for insulin-like activity.In diabetics, glycogen content in skeletal muscle and liver is significantly lower than in non-diabetics due to insulin's role in stimulating glycogen synthase activity, which is inactive in the absence of insulin(Vats et al., 2003). Conversely, insulin inhibits glycogenolysis, leading to reduced liver glycogen content in insulin deficiency conditions. Treatment with phenolic compounds notably increased hepatic glycogen levels in diabetic rats, possibly due to glycogen synthase system reactivation from increased insulin secretion. Additionally, insulin has been reported to enhance glycogen synthase activity (Witters and Avruch, 1978).

Our study demonstrated diabetes development, as evidenced by elevated blood glucose, decreased insulin levels, increased HOMA-IR scores, and area under the curve in OGTT. While our bioflavonoid combination effectively mitigated hyperglycemia, the effect was modest, with approximately 25% and 15% reductions in blood glucose and HOMA-IR scores, respectively. These findings underscore the insulin-enhancing potential of the flavonoid combination, independent of glycemic control mechanisms.

Conclusion

Flavonoids represent a significant class of bioactive compounds found in secondary metabolites. Our study has emphasized the antidiabetic potential of specific flavonoids, both in vitro and in vivo. The current study results indicate that these flavonoids exert in vitro antidiabetic effects by enhancing insulin secretion in GSIS. Moreover, these phenolic compounds also enhance insulin levels in tye II diabetic rats and also enhanced the hepatic glycogen levels. These findings underscore the opportunity for further exploration to develop nutritional products and semi-synthetic analogs possessing potent antidiabetic properties with minimal adverse effect.

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