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Combined Effects of Eugenol and Ascorbic Acid on Glycemic Regulation and Transcriptional Modulation of Metabolic and Inflammatory Genes in Alloxan-Induced Diabetic Rats

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ABSTRACT

Diabetes mellitus is characterized by hyperglycemia, oxidative stress, inflammation, and progressive β-cell dysfunction. Current therapies primarily focus on glycemic control and may not fully address these underlying mechanisms. Antioxidant compounds such as eugenol and L-ascorbic acid (vitamin C) have attracted attention as potential complementary approaches. However, their individual and combined effects on metabolic regulation and diabetes-related gene expression remain unclear. Therefore, this study aimed to investigate the effects of eugenol and vitamin C, alone and in combination, on glucose homeostasis and the expression of insulin-related, glucose transport-related, and inflammatory genes in pancreatic and hepatic tissues of diabetic rats. Rats were divided into five groups: healthy control, diabetic control, eugenol-treated (10 mg/kg/day; 0.25 mL of 10 mg/mL), vitamin C-treated (100 mg/kg/day; 0.25 mL of 30 mg/mL), and combination-treated groups. Treatments were administered for six weeks. Fasting blood glucose (FBG), body weight, and the expression levels of insulin-related (*Ins1/2*, *Insr*, *Pdx1*), glucose transport-related (*Glut1*, *Glut2*), and inflammation-related (Tnfα) genes in pancreatic and hepatic tissues were measured. The diabetic group showed persistently elevated blood glucose levels and reduced weight gain compared with the healthy controls. Vitamin C and eugenol treatments reduced glucose levels, with vitamin C showing a greater reduction (410 to 220 mg/dL) than eugenol (343.5 to 258.5 mg/dL). The vitamin C + eugenol group exhibited the largest absolute decline in FBG from baseline (471 to 275 mg/dL), although its final glucose concentration remained higher than that of the vitamin C-treated group (220 mg/dL). Body weight trends indicated that vitamin C supported mild weight gain, whereas eugenol was associated with weight loss, which was attenuated by combination therapy. Gene expression analysis revealed reduced *Ins1/2*, *Insr*, *Glut2*, and *Pdx1* expression in diabetic rats, while combination therapy partially restored the expression of some investigated genes. Elevated *Tnfα* expression in diabetes was markedly reduced by eugenol and vitamin C, indicating potential anti-inflammatory effects. However, combination therapy increased hepatic *Tnfα* expression, highlighting tissue-specific differences in gene expression responses. Overall, eugenol and vitamin C, particularly when administered together, were associated with improved glycemic profiles and modulation of key metabolic, β-cell-associated, and inflammatory gene transcripts. However, these transcriptional alterations require further validation at the protein and functional levels.

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia, resulting from impaired insulin secretion, insulin action, or both (Antar *et al.*, 2023). Globally, more than 530 million people are

affected, and this number is projected to rise substantially in the coming decades, placing a heavy burden on public health systems and economies (Magliano & Boyko, 2021). Beyond hyperglycemia, DM is associated with oxidative stress, chronic inflammation, and metabolic dysregulation, which collectively contribute to severe complications such as nephropathy, neuropathy, retinopathy, and cardiovascular disease (Antar *et al.*, 2023; Islam *et al.*, 2025; Petrie *et al.*, 2018). These multifactorial complications emphasize the need for therapeutic approaches that not only regulate blood glucose but also target the underlying molecular drivers of disease progression. Although several pharmacological agents, including insulin analogs, sulfonylureas, metformin, thiazolidinediones, and newer classes such as DPP-4 and SGLT2 inhibitors, are available for the management of DM, their effectiveness is often limited by adverse effects, high costs, and restricted efficacy against oxidative stress and inflammation (Gieroba *et al.*, 2025). Consequently, there is a growing interest in natural bioactive compounds with multi-targeted, low toxic, antioxidant, and anti-inflammatory potential as adjunct or alternative therapies in diabetes management (Dahlen *et al.*, 2021; Yagasaki and Muller, 2022). Among these compounds, Eugenol, a phenolic constituent of clove oil (*Syzygium aromaticum*) (Abdou *et al.*, 2021), has demonstrated multiple biological activities, including antioxidant, anti-inflammatory, and anti-diabetic effects (Anjum *et al.*, 2023; Damasceno *et al.*, 2024; Jiang *et al.*, 2025).

Evidence suggests that eugenol can improve insulin sensitivity, modulate insulin signaling pathways, suppress pro-inflammatory cytokines, and enhance glucose uptake and glycogen synthesis in liver and muscle tissues. Its mechanisms involve reactive oxygen species (ROS) scavenging, inhibition of NF-κB activation, and possible regulation of genes critical for insulin biosynthesis and glucose transport (Anjum *et al.*, 2023; Gojani *et al.*, 2023; Jiang *et al.*, 2025). Despite its potential, most studies have examined eugenol in isolation, and little is known about its impact on the co-ordinated expression of insulin- and inflammation-related genes in pancreatic and hepatic tissues.

L-ascorbic acid (vitamin C), a water-soluble antioxidant, also plays an important role in glucose homeostasis and redox balance (Gradinaru & Popa, 2025). In diabetic states, its levels are frequently depleted, contributing to increased oxidative stress and impaired insulin action. Supplementation with vitamin C has been shown to improve glucose utilization, enhance insulin sensitivity, and protect pancreatic β-cells from oxidative injury (Aragon-Vela *et al.*, 2026; Nosratabadi *et al.*, 2023). Furthermore, it can downregulate inflammatory mediators such as TNFα and interleukins, supporting its potential as an adjunct therapy (Wilson *et al.*, 2024; Wong *et al.*, 2020). Its synergistic interaction with other natural antioxidants has also been suggested, though limited data exist on its combined effect with phenolic compounds such as Eugenol.

Given their distinct but complementary mechanisms, eugenol modulating gene expression, insulin signaling, and ascorbic acid enhancing redox balance and β-cell protection, their combination may yield enhanced benefits that exceed the effects of either compound alone. However, the molecular basis of these potential complementary effects, particularly regarding gene expression patterns in key metabolic organs, remains poorly understood. To address this gap, the present study aimed to investigate the simultaneous effects of eugenol and ascorbic acid on blood glucose regulation, body weight, and the expression of genes involved in insulin biosynthesis (*Ins1/2*), insulin receptor signaling (*Insr*), pancreatic β-cell function (*Pdx1*), glucose transport (*Glut1*, *Glut2*), and inflammation (*Tnfα*) in the pancreas and liver of alloxan-induced diabetic rats. We also evaluated the correlation of blood glucose levels with body weight and gene expression to correlate molecular changes with physiological outcomes. By exploring both systemic outcomes and tissue-specific transcriptional responses, this work provides insights into the potential effects of natural antioxidants on metabolic regulation in diabetes and identifies molecular changes that warrant further functional investigation.

Materials and Methods

Chemicals and reagents

Eugenol (purity≥ 98%) and L-ascorbic acid (purity≥ 99%) were purchased from Sigma-Aldrich (Germany). Alloxan monohydrate was obtained from Sigma-Aldrich (Germany) and used for the induction of diabetes. RNA isolation kit (DenaZist Asia, Iran), DNase I kit (Fermentas, Germany), and cDNA synthesis kit (AddBio, South Korea) were used for RNA extraction, DNase treatment, and cDNA synthesis. Quantitative real-time PCR (qRT-PCR) reagents and primers specific to the genes of interest were also sourced from BioBasic (Canada) and Metabion (Germany), respectively.

Experimental animals

A total 50 male Wistar rats (180-220 g) were obtained from the animal facility at Kermanshah University of Medical Sciences (KUMS, Kermanshah, Iran). The animals were housed under standard laboratory conditions with a 12-hour light/dark cycle, controlled temperature (22-25 °C), humidity (50-60%), and were given ad libitum access to water and standard chow. All experimental procedures were approved by the Institutional Animal Care Committee of Biological Sciences of Razi University, Iran (IR.RAZI.REC.1399.045) and conducted in accordance with ARRIVE guidelines. The sample size (n= 8 per group) was determined based on previous similar studies and a power analysis calculation to ensure s= 0.05 α= 0.05, while adhering to the 3R principles of animal ethics (Festing, 2006). In addition, randomization and blinding procedures were implemented to minimize potential bias. Animals were randomly allocated to five experimental groups (n= 8 per group) and assigned coded identifiers. Investigators responsible for sample processing, quantitative real-time PCR (qRT-PCR) analysis, and data collection remained blinded to group allocation throughout the study.

Induction of diabetes

Diabetes was induced in the rats using alloxan monohydrate (150 mg/kg; a single intraperitoneal/i.p. injection). Since the rats did not have identical body weights, the alloxan solution was prepared at a fixed concentration (75 mg/ml), and the injection volume was individually adjusted according to each animal's body weight to ensure administration of the target dose (150 mg/kg/day). Alloxan was freshly dissolved in saline and administered once to overnight-fasted rats. To prevent alloxan-induced hypoglycemic shock, animals received 10% (w/v) sucrose solution as drinking water ad libitum for 24 hours (h) after alloxan injection (Kim, 2024). However, 8 rats died following alloxan administration. After 48 h, baseline fasting blood glucose (FBG) levels (Week 0) were measured using a glucose meter (Accu-Chek Active, Roche, Germany). Diabetes was confirmed by measuring blood glucose levels. Rats with blood glucose levels above 250 mg/dL were considered diabetic and included in the study. Control rats received normal saline instead of alloxan and were similarly handled.

Vitamin C and eugenol solutions

Vitamin C (≥ 99% purity) in powder form and eugenol (≥ 98% purity) were obtained from Sigma-Aldrich (Germany). A stock solution of vitamin C was prepared by dissolving 30 mg of the powder in one mL of sterile normal saline to obtain a final concentration of 30 mg/ml. For Eugenol, a working solution (10 mg/ml) was prepared by diluting 10 µl of eugenol solution in 990 µl of olive oil. Both solutions were prepared immediately before use under light-protected sterile conditions, sterilized by filtration, and administered fresh.

Experimental design

Baseline glucose levels (Week 0) were recorded prior to treatment. Diabetic rats were randomly assigned to experimental groups. Animals were divided into five groups (n= 8 rats per group): 1) Control group: Received normal saline (0.25 ml) and olive oil (0.25 ml) daily for 6 weeks and served as the normal, non-diabetic group; 2) Diabetic group: Diabetic rats received normal saline and olive oil, and served as the untreated diabetic group; 3) Eugenol-treated group: Diabetic rats were treated with eugenol (0.25 ml; 10 mg/kg/day) daily via oral gavage; 4) Ascorbic acid-treated group: Diabetic rats received ascorbic acid (0.25 ml; 100 mg/kg/day) daily via i.p. injection; 5) Combination-treated group: Diabetic rats were administered a combination of

eugenol (0.25 ml; 10 mg/kg/day) and ascorbic acid (0.25 ml; 100 mg/kg/day) daily via oral gavage and i.p. injection, respectively. The treatments were continued for a six-week period. The doses of eugenol and ascorbic acid were selected based on previously reported studies (Alharthy *et al.*, 2023; Iwata *et al.*, 2014; Shivavedi *et al.*, 2019) that evaluated their effects on glucose metabolism and gene expression. The animals were monitored daily for general health, and body weight and food intake were recorded weekly.

Blood glucose measurement

Fasting blood glucose levels were measured at specific intervals (at the end of 1, 3, 5, and 6 weeks) during the experiment. Rats were fasted overnight before blood collection, with free access to water. Blood samples were obtained via tail vein puncture using a sterile lancet. Glucose levels were immediately determined using a portable glucometer (Accu-Chek Active, Roche, Germany), following the manufacturer's instructions. Measurements were taken in the morning to minimize circadian variation. Changes were analyzed relative to baseline.

Body weight monitoring

The body weight of all rats was recorded weekly throughout the experimental period using a digital weighing balance (Sigma, Germany). Weights were measured in the morning before measuring blood glucose levels to ensure consistency. Changes in body weight were used to assess general health status and the impact of treatments.

Tissue collection and gene expression

After the treatment period (6 weeks), rats were anesthetized with a ketamine (100 mg/kg)-xylazine (10 mg/kg) (Bremer Pharma, Iran) mixture (1:10), and their liver and pancreatic tissues were rapidly excised and transferred into cryotubes, immediately snap-frozen in liquid nitrogen, and then stored at -80 °C for a long-term preservation and RNA extraction. Total RNA was isolated from both liver and pancreas tissues using a column total RNA extraction kit (DenaZist Asia, Iran), following the manufacturer's protocol. The integrity and quality of the RNA were determined using 1% agarose gel electrophoresis and ethidium bromide (Sigma-Aldrich, Germany) staining (Ricicova & Palkova, 2003). The possible genomic contaminations in the RNA samples were removed by DNase I kit (Fermentase, Germany) following the manufacturer's protocol for 30 min at 37°C (Aref *et al.*, 2024). The DNase I-treated RNAs were used for subsequent analysis.

For gene expression analysis, cDNA was synthesized from 1 µg of total RNA using the cDNA synthesis kit (DenaZist Asia, Iran). The cDNA synthesis was performed following the manufacturer's instructions with an incubation step of 10 minutes at 25°C, followed by 60 minutes at 50°C, and finally a 5-minute incubation at 80°C to terminate the reaction. Gene expression analysis was performed using quantitative real-time PCR (qRT-PCR). Specific primers were designed using Allele ID software (Version 6) for the target genes, including *Ins1/2*, *Insr*, *Glut1*, *Glut2*, *Pdx1*, and *Tnfα* (Table 1). All qRT-PCR reactions were performed using six biological replicates per experimental group, with each biological sample analyzed in at least two technical replicates. The qRT-PCR reactions were performed in triplicate using a SYBR Green PCR Master Mix (BioBasic, Canada). The cycling conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of amplification [95°C for 10 seconds, 51-59°C (depending on the specific primers of the target and reference genes) for 30 seconds, 72 °C for 30 seconds]. Amplification specificity was confirmed by melt curve analysis, and only single-peak amplification profiles were accepted for further analysis. In the present study, the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene was selected as the reference gene based on previous studies in diabetic animal models and its frequent application for qRT-PCR normalization (Hu *et al.*, 2026; Lotfy *et al.*, 2024; Naseem *et al.*, 2020). However, the expression stability of *Gapdh* was not independently validated under the specific experimental conditions of diabetes induction and antioxidant treatment, representing a limitation of the current study. Finally, expression levels of target genes were calculated using the 2^-ΔΔCt method (Schmittgen and Livak, 2008). The relative expression levels were calculated using the comparative Ct method, where ΔCt was calculated as Ct (target gene) -Ct (reference gene), and ΔΔCt was calculated as ΔCt (treated or diabetic group) -ΔCt (control group). Relative fold changes were determined as 2^-ΔΔCt.

Table 1. List of target genes, GenBank accession numbers, and primers used for qRT-PCR analysis.

Genes	GenBank ID	Oligomer (5'→3')
Gapdh	NM_017008	F: GCTGGTGCTGAGTATGTCGTGGAG R: GCGGAAGGGGCGGAGATGATG
Ins1/2	NM_019129.3	F: CCATCAGCAAGCAGGTCATTGTTC R: CGACGGGACTTGGGTGTGTAG
Insr	NM_017071.2	F: GATGCCACCAATCCTTCCGTTCCC R: GCTGTCCTCCGCCTGCCTCTC
Pdx1	NM_022852.3	F: GTGCCAGAGTTCAGTGCTAATCC R: ACTTCCCTGTTCCAGCGTTCC
Glut1	NM_011400.3	F: TGCTGCTCAGTGTCATCTTCATCC R: ATCTGCCGACCCTCTTCTTTCATC
Glut2	L28126.1	F: GGATCTGCTGACCTGTGAAAGT R: TCCACTACCCTGTACCTGCC
Tnfα	NM_012675	F: AGAACTCCAGGCGGTGTC R: GAGAAGATGATCTGAGTGTGAGG

F: Forward primer; R: Revers primer

It is worth noting that gene expression analysis was performed using biological samples obtained from individual animals, with each sample analyzed in technical replicates. Technical replicates were averaged before statistical analysis.

Statistical analysis

Statistical analyses were performed using SPSS software (version 25.0; IBM Corp., USA). Normality of data distribution was evaluated using the Shapiro-Wilk test prior to applying parametric methods. Intergroup comparisons were carried out using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Repeated-measures ANOVA was used to analyze longitudinal FBG data. All results are expressed as mean ± standard deviation (SD), and statistical significance was set at $p< 0.05$. Graphs were generated using GraphPad Prism (version 9.0; GraphPad Software, USA).

Results

Blood glucose levels

Blood glucose levels were monitored at weeks 0, 1, 3, 5, and 6 in all experimental groups (Fig. 1). Baseline glucose values (week 0) did not differ significantly among groups ($p> 0.05$). Accordingly, treatment efficacy was interpreted based on both the magnitude of glucose reduction over time relative to initial values and the final glucose concentrations measured at the end of the experiment. The control group maintained stable normoglycemic values (~92 mg/dL) throughout the study, whereas diabetic animals exhibited sustained hyperglycemia (~295 mg/dL), confirming successful diabetes induction. Following treatment initiation, all treated diabetic groups showed a gradual reduction in FBG levels compared with their initial values.

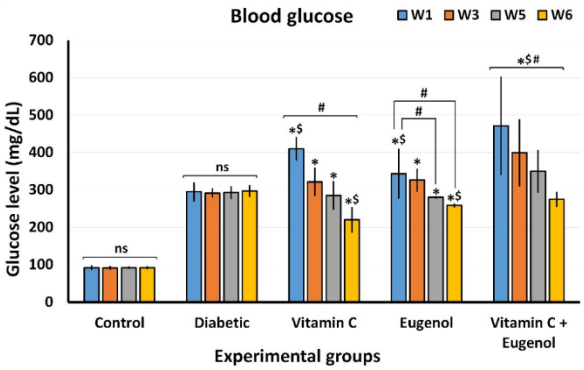

Fig. 1. Fasting blood glucose levels in the experimental groups during the 6-week study period: Baseline blood glucose was measured 48 h after alloxan administration and prior to treatment initiation (week 0, W0), and subsequent measurements were performed at week 1 (W1), week 3 (W3), week 5 (W5), and week 6 (W6). Data are presented as mean ± SD (n= 8 biological replicates per group). Statistical analyses were performed using two-way repeated-measures ANOVA followed by the appropriate post hoc multiple-comparison test. *$p< 0.05$ vs. Control; $$p< 0.05$ vs. Diabetic; #$p< 0.05$ vs. other treatment groups; ns, not significant.

The vitamin C-treated group exhibited a progressive decline in glucose levels from 410 mg/dL at week 1 to approximately 220 mg/dL at week 6. Similarly, eugenol administration

reduced glucose levels from approximately 343 mg/dL to 258 mg/dL during the same period. The combined vitamin C + eugenol treatment produced the largest numerical reduction from baseline values (471 mg/dL at week 1 to 275 mg/dL at week 6); however, the final glucose concentration remained higher than those reported for the vitamin C-treated and non-diabetic control groups.

Observations and body weight changes

The changes in body weight of rats were evaluated over time (weeks 1, 3, 5, and 6) across different experimental groups. Results demonstrated that in the control group, body weight steadily increased over the six weeks, showing normal growth progression (Fig. 2).

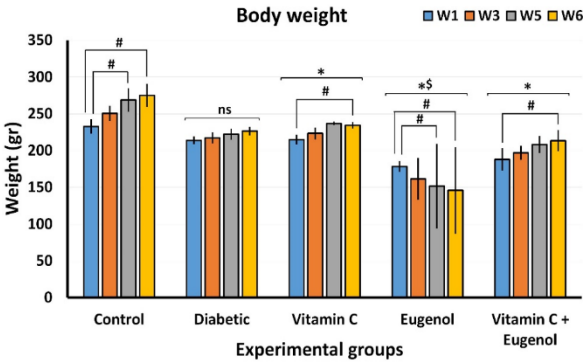

Fig. 2. Body weight changes in the experimental groups during the 6-week study period: Body weight was recorded at week 1 (W1), week 3 (W3), week 5 (W5), and week 6 (W6). Data are presented as mean ± SD (n= 8 biological replicates per group). Statistical analyses were performed using two-way repeated-measures ANOVA followed by the appropriate post hoc multiple-comparison test. *p< 0.05 vs. Control; $p< 0.05 vs. Diabetic; #p< 0.05 vs. other treatment groups; ns, not significant.

Similarly, the diabetic group exhibited a slight increase in body weight over time, but the gain was noticeably less than the similar reported value in the control group. The vitamin C-treated diabetic group exhibited a gradual and consistent increase in body weight, comparable to what observed in the untreated diabetic group, but without a significant improvement in weight gain. In contrast, the eugenol-treated group showed a clear downward trend in body weight from week 1 to week 6, with substantial weight loss. Interestingly, the vitamin C + eugenol combination group demonstrated a recovery in body weight over time. Although the initial weights were lower than the other groups, by week 6, the average weight approached the value recorded in the diabetic and vitamin C-only groups. These results demonstrated a possible protective or counterbalancing effect of ascorbic acid when combined with eugenol, mitigating the weight-reducing effects of eugenol alone.

Correlation of the blood glucose levels and body weight

The correlation between FBG levels and body weight was examined across four time points (weeks 1, 3, 5, and 6) in different rat groups: control, diabetic, eugenol-treated, vitamin C-treated, and vitamin C + eugenol combination-treated. In week 1, the diabetic group showed elevated glucose (295 mg/dL) and slightly reduced weight compared to the control group (Fig. 3A), while treatment groups, particularly eugenol and vitamin C + eugenol, exhibited significantly higher glucose levels (343.5 mg/dL) and lower body weights (188 g). By week 3, the control group maintained low glucose and high weight, while the diabetic group remained hyperglycemic but with a slight weight gain, reflecting typical diabetic symptoms (Fig. 3B). Rats treated with vitamin C showed partial improvement, with glucose around 326 mg/dL and body weight near 223.5 g, compared to week 1. The eugenol-treated group exhibited high glucose (326.5 mg/dL) and lower weight (161.5 g). In contrast, the combination treatment group (vitamin C + eugenol) showed a better metabolic response than eugenol alone, with reduced glucose (399.5 mg/dL) and improved weight (197 g), compared to week 1. In week 5, all groups demonstrated better weight profiles, with the diabetic and treatment groups gaining more weight (Fig. 3C). The eugenol group still exhibited high glucose (280 mg/dL) and low weight (152 g), but vitamin C and combination treatments showed further improvement compared to weeks 1 and 3. Finally, in week 6, a clearer trend emerges; the combination therapy group showed significantly lowered glucose (275 mg/dL) and higher weight (213.5 g), approaching the diabetic group. In contrast, the Eugenol-alone group remained the least favorable in both glucose and weight metrics (Fig. 3D). These results showed that combined eugenol and

vitamin C treatment provided a gradual yet consistent improvement in both glycemic control and body weight restoration over time in diabetic rats.

Gene expression analysis in the pancreas tissue

The results of our qRT-PCR indicated that the expression of *Ins1/2*, critical genes involved in insulin biosynthesis, was significantly downregulated (3.03-fold decrease) in the pancreas of diabetic rats compared to the normal (non-diabetic) group (Fig. 4A). Treatment with eugenol or vitamin C individually resulted in partial restoration of *Ins1/2* expression. However, the combination treatment with eugenol and vitamin C produced the most pronounced upregulation, with expression levels significantly higher than in the diabetic group, though not to the level of the control group.

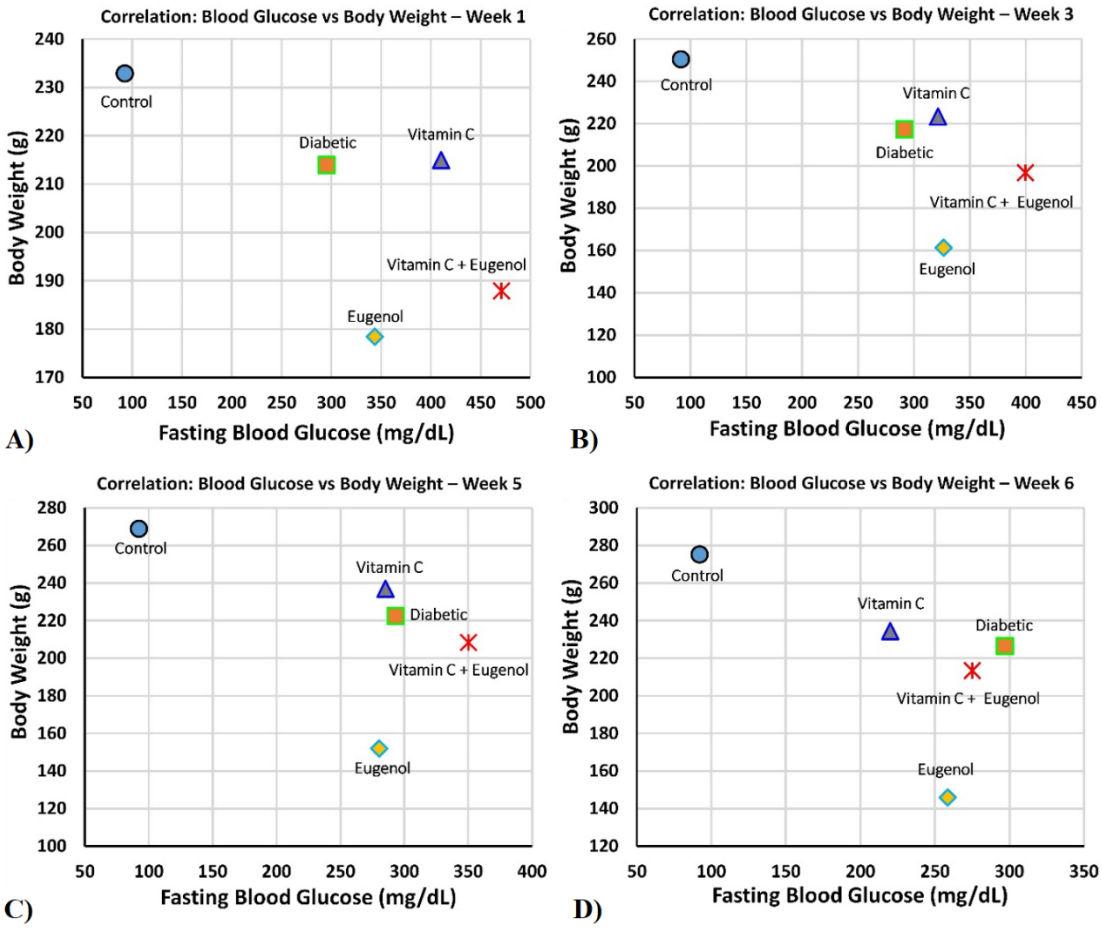

Fig. 3. Correlation between fasting blood glucose (FBG) levels and body weight in the experimental groups: A) correlation at week 1 (W1); B) correlation at week 3 (W3); C) correlation at week 5 (W5); D) correlation at week 6 (W6). Each panel shows the relationship between FBG concentration (mg/dL) and body weight (g) at the corresponding time point.

The expression of the *Insr* gene, a key player in insulin signaling, was also significantly downregulated in the diabetic (8.52-fold decrease) and eugenol (11.82-fold reduction) groups, compared to the control group (Fig. 4B). In the vitamin C-treated group, *Insr* expression was slightly restored compared to diabetic and eugenol groups, though not to the level of the control group. However, combination treatment using both eugenol and vitamin C resulted in a significant increase in *Insr* expression (2.29-fold). This pattern was associated with altered expression of insulin signaling-related genes.

The relative expression levels of the *Glut2* gene, a key glucose transporter primarily expressed in the liver and pancreas, were assessed across

different experimental groups in non-diabetic and alloxan-induced diabetic rats. Results indicated that the *Glut2* gene was expressed in the normal group (Fig. 4C). In contrast, in the untreated diabetic group, *Glut2* expression was markedly reduced (5.6-fold decrease). Supplementation with vitamin C alone led to a partial recovery in expression, though still significantly downregulated (3.57-fold reduction) compared to normal group. However, treatment with eugenol alone showed even further suppression (5.88-fold decrease). Notably, the combination treatment with eugenol and vitamin C resulted in a notable improvement, bringing *Glut2* expression closer to normal levels. Statistical analysis confirmed that the combination therapy (eugenol + vitamin C) differed significantly from the untreated and single-treatment groups, providing evidence that the combined treatment may exert a complementary effect in attenuating diabetes-induced suppression of *Glut2* expression.

The expression of *Pdx1* was investigated in pancreatic tissue under different treatment conditions in diabetic and non-diabetic (normal) rats. In the normal group, *Pdx1* expression was considered the baseline (1-fold), whereas in the untreated diabetic group, expression was drastically downregulated to 0.12-fold (Fig. 4D). Treatment with vitamin C led to a modest improvement in expression, which was not statistically different from the normal group. Treatment with eugenol and eugenol + vitamin C both resulted in significant but partial restoration of *Pdx1* expression, suggesting a better recovery compared to untreated diabetic rats, though not fully restored to normal levels. These results showed that while eugenol and vitamin C individually or in combination help restore *Pdx1* expression, the effect remained incomplete.

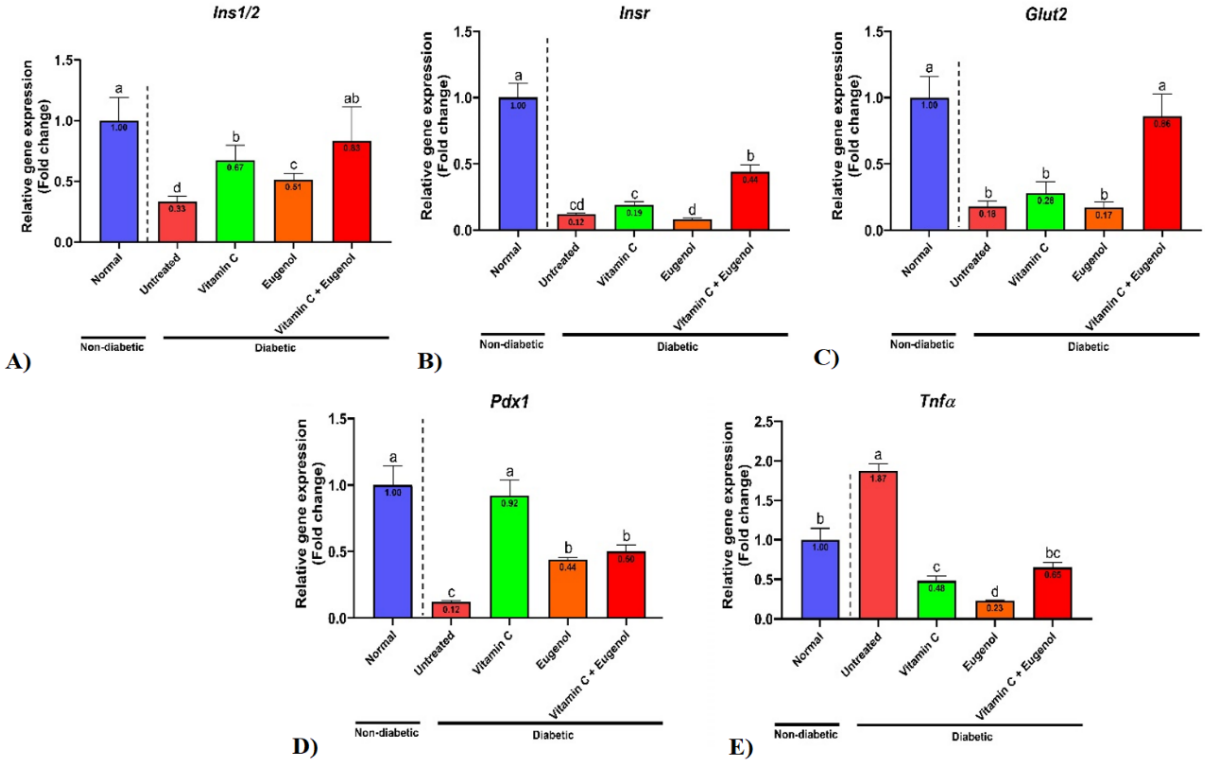

Fig. 4. Relative mRNA expression levels of pancreatic genes in the experimental groups: A) relative mRNA expression of Ins1/2; B) relative mRNA expression of Insr; C) relative mRNA expression of Glut2; D) relative mRNA expression of Pdx1; E) relative mRNA expression of Tnfα. Expression was evaluated in pancreatic tissue of normal, diabetic, vitamin C-treated, eugenol-treated, and vitamin C + eugenol-treated groups. Relative gene expression was determined by qRT-PCR using the 2^−ΔΔCt method and normalized to Gapdh expression. Data are presented as mean ± SD (n= 6 biological replicates per group). Different lowercase letters above the bars indicate statistically significant differences among groups ($p< 0.05$); groups sharing at least one common letter are not significantly different.

In the present study, the relative expression of the *Tnfα* gene was examined in the pancreas tissue of various experimental groups of rats. Our results showed that *Tnfα* gene expression was significantly upregulated (1.87-fold) in the untreated diabetic group compared with the normal group, which is consistent with the marked inflammatory response induced by alloxan-mediated diabetes (Fig. 4E). In contrast, treatment with vitamin C significantly reduced *Tnfα* expression (2.08-fold decrease). Interestingly, eugenol treatment resulted in the lowest *Tnfα* expression (4.36-fold reduction) among all diabetic groups. The combined treatment of eugenol and vitamin C also significantly reduced *Tnfα* expression levels (1.54-fold decrease) compared to untreated diabetic group, though not as much as eugenol alone.

Gene expression analysis in the liver tissue

The relative gene expression levels of the *Insr* gene were studied in the liver of rats across different experimental groups. The normal group showed the highest *Insr* expression (Fig. 5A).

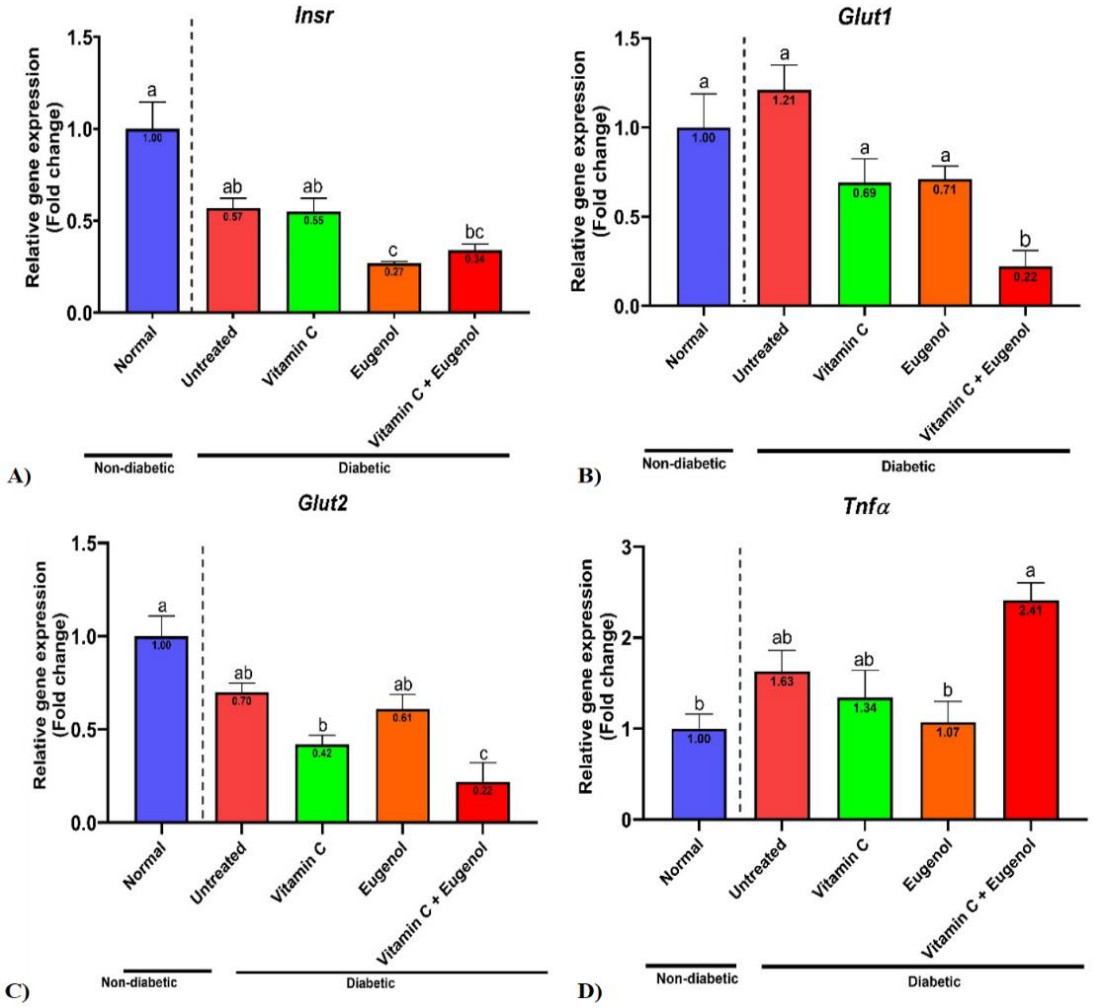

Fig. 5. Hepatic mRNA expression levels of glucose metabolism- and inflammation-related genes in the experimental groups: A) relative mRNA expression of Insr; B) relative mRNA expression of Glut1; C) relative mRNA expression of Glut2; D) relative mRNA expression of Tnfα. Expression was evaluated in liver samples from normal, diabetic, vitamin C-treated, eugenol-treated, and vitamin C + eugenol-treated groups. Relative transcript levels were determined by qRT-PCR using the 2^−ΔΔCt method and normalized to Gapdh expression. Data are presented as mean ± SD (n= 6 biological replicates per group). Different lowercase letters above the bars indicate statistically significant differences among groups ($p< 0.05$); groups sharing at least one common letter are not significantly different.

In contrast, the untreated diabetic group exhibited a significant downregulation of *Insr* expression (1.74-fold reduction). Treatment with vitamin C did not result in a notable improvement compared to the untreated diabetic group. Interestingly, eugenol-treated diabetic rats showed the most substantial reduction in *Insr* expression (3.7- fold decrease). However, co-treatment with eugenol and vitamin C slightly attenuated this suppression, although expression levels remained markedly lower than similar reports in the normal group. This increase was not statistically significant. These results showed that while diabetes suppressed *Insr* expression, eugenol exacerbated this effect, and co-administration with ascorbic acid offered only limited mitigation.
In the liver, the expression of *Glut1* is essential for glucose uptake and regulation. Our results showed that *Glut1* expression varied across the different experimental groups (Fig. 5B). In the non-diabetic control group (normal), *Glut1* expression was detected. Interestingly, the untreated diabetic group exhibited an increased expression of *Glut1* (1.21-fold). Treatment with either vitamin C or eugenol slightly reduced *Glut1* expression, compared to the untreated diabetic group. However, these reductions were not statistically significant. Remarkably, the combination treatment of eugenol and vitamin C led to a significant decrease (0.22-fold) in *Glut1* expression. This reflected a highly significant reduction compared to both the normal and untreated diabetic groups.
In this study, the relative expression levels of the *Glut2* gene were evaluated in the livers of rats across different experimental groups (Fig. 5C). In the normal group, *Glut2* expression was highest. The untreated diabetic group showed a noticeable decrease (0.7-fold) in *Glut2* expression. Treatment with vitamin C led to a further significant reduction (2.38-fold decrease) in *Glut2* expression. In contrast, eugenol treatment appeared to maintain *Glut2* expression at a slightly higher level than vitamin C, though still lower than the normal (1.64-fold reduction) and untreated diabetic groups. Notably, the combined treatment with eugenol and vitamin C induced the most pronounced downregulation of *Glut2* expression (4.55-fold reduction), indicating a highly significant difference compared with both the normal and untreated diabetic groups.
The relative gene expression of the *Tnfα* gene was also examined in the liver of rats across various experimental groups (Fig. 5D). In the normal group, *Tnfα* expression remained at a low baseline level. The untreated diabetic group showed an increase in *Tnfα* expression (1.63-fold), although this increase is not statistically significant compared to the normal group. Diabetic rats treated with vitamin C or eugenol individually showed a slight reduction in *Tnfα* expression, compared to the untreated diabetic group. However, the reductions did not appear to be statistically significant. Interestingly, the combination of eugenol and vitamin C resulted in a significant increase (2.41-fold) in *Tnfα* expression compared with the normal (non-diabetic) group; however, this increase was not significantly different from the untreated diabetic group.

Correlation of blood glucose and gene expression

The correlation between FBG levels and the expression patterns of *Ins1/2*, *Insr*, *Glut2*, *Pdx1*, and *Tnfα* genes in pancreatic tissue was evaluated among the different experimental groups (Fig. 6). The normal control group exhibited a blood glucose level of 92 mg/dL, with baseline expression levels of the investigated genes. In contrast, the untreated diabetic group showed a significant increase in FBG levels (297 mg/dL) accompanied by reduced expression of *Ins1/2* (0.33-fold), *Insr* (0.12-fold), *Glut2* (0.18-fold), and *Pdx1* (0.12-fold), while *Tnfα* expression increased to 1.87-fold compared with the control group. The vitamin C-treated group showed an FBG level of 220 mg/dL and exhibited reduced expression of *Ins1/2* (0.67-fold), *Insr* (0.19-fold), *Glut2* (0.28-fold), and *Pdx1* (0.92-fold). In this group, *Tnfα* expression decreased significantly to 0.48-fold compared with the untreated diabetic group. The eugenol-treated group showed an FBG level of 258.5 mg/dL, with decreased expression of *Ins1/2* (0.51-fold), *Insr* (0.085-fold), *Glut2* (0.28-fold), and *Pdx1* (0.44-fold). In addition, *Tnfα* expression was significantly reduced to 0.23-fold. The combined eugenol and vitamin C treatment group exhibited altered expression patterns of *Ins1/2*, *Insr*, *Glut2*, and *Pdx1* compared with the untreated diabetic group. Additionally, *Tnfα* expression was reduced to

0.65-fold in the combination-treated group. Correlation analysis revealed an inverse relationship between FBG levels and the expression of *Ins1/2*, *Insr*, *Glut2*, and *Pdx1*, whereas *Tnfα* expression showed a positive association with FBG levels in the untreated diabetic group. The relationship between FBG levels and the fold changes in the expression of *Insr*, *Glut1*, *Glut2*, and *Tnfα* genes was evaluated across the five experimental groups (Fig. 7). In the normal control group, FBG was 92 mg/dL, and the relative expression levels of the investigated genes were considered as the calibrator values (1.0). The untreated diabetic group exhibited an increased FBG level of approximately 297 mg/dL, accompanied by reduced expression of *Insr* (0.57-fold) and *Glut2* (0.70-fold), while *Glut1* and *Tnfα* expression levels increased to 1.21-fold and 1.63-fold, respectively, compared with the control group.

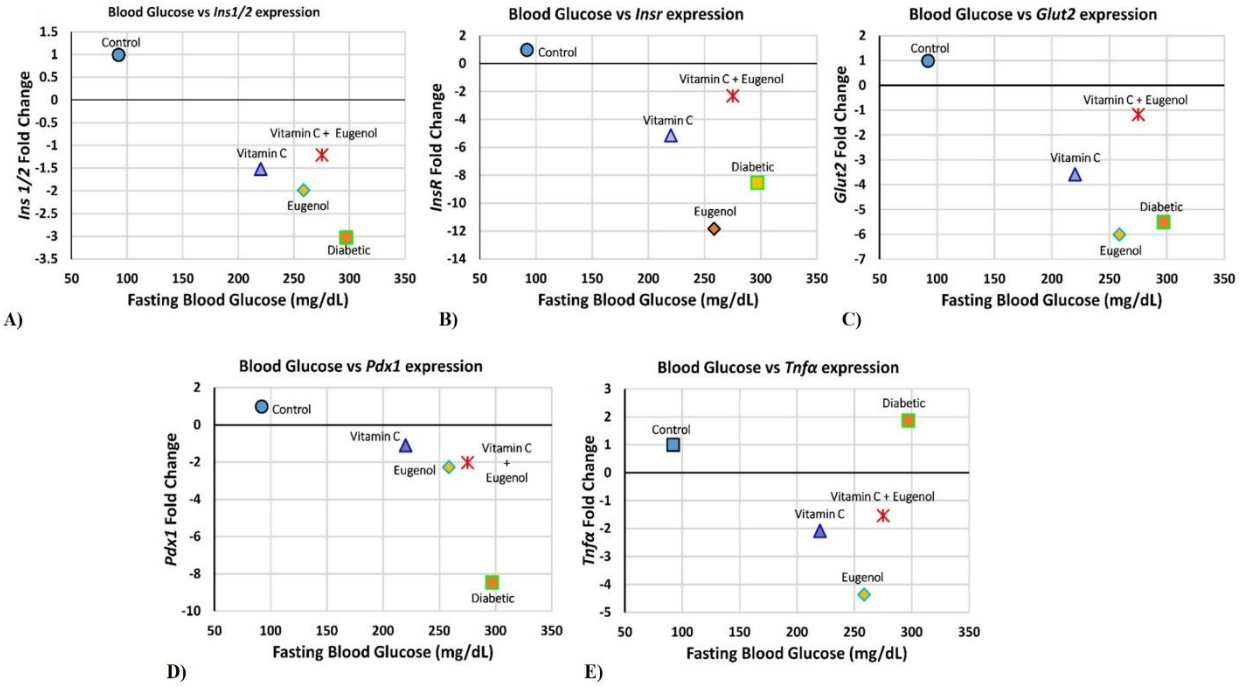

Fig. 6. Correlation between fasting blood glucose (FBG) levels and relative mRNA expression of pancreatic genes in the experimental groups: A) correlation with Ins1/2 expression; B) correlation with Insr expression; C) correlation with Glut2 expression; D) correlation with Pdx1 expression; E) correlation with Tnfα expression. Scatter plots illustrate the relationship between FBG concentration (mg/dL) and relative gene expression determined by qRT-PCR in pancreatic tissue of normal, diabetic, vitamin C-treated, eugenol-treated, and vitamin C + eugenol-treated groups.

In the vitamin C-treated group, FBG levels reached approximately 220 mg/dL. Expression analysis showed decreased levels of *Insr* (0.55-fold) and *Glut2* (0.42-fold), whereas *Tnfα* expression was 1.34-fold compared with the control group. The eugenol-treated group exhibited an FBG level of 258.5 mg/dL, with reduced expression of *Insr* (0.27-fold), *Glut1* (0.71-fold), and *Glut2* (0.61-fold), while *Tnfα* expression remained at 1.07-fold.

The combination treatment group (eugenol + vitamin C) showed an FBG level of 275 mg/dL and exhibited reduced expression of *Insr* (0.34-fold), *Glut1* (0.22-fold), and *Glut2* (0.22-fold). In this group, *Tnfα* expression increased to 2.41-fold compared with the control group. Overall, correlation analysis demonstrated a negative association between FBG levels and the expression of *Insr*, *Glut1*, and *Glut2*, whereas *Tnfα* expression showed a positive association with FBG levels across the experimental groups.

Discussion

Diabetes mellitus involves complex disturbances in glucose metabolism, insulin signaling, β-cell function, and inflammatory regulation, which are

controlled by multiple molecular pathways (Ahn, 2025; Antar *et al.*, 2023; Chandrasekaran & Weiskirchen, 2024). In the present study, we investigated the individual and combined effects of eugenol and ascorbic acid on glucose homeostasis, metabolic gene expression, and inflammatory marker in pancreatic and hepatic tissues of alloxan-induced diabetic rats. Our findings demonstrated that both compounds exerted beneficial but distinct effects, while combined treatment produced variable which impacts depending on the tissue and the target examined gene.

Alloxan-induced diabetes resulted in persistent hyperglycemia and impaired weight gain, which is consistent with previous reports describing ROS-mediated β-cell destruction and metabolic dysfunction and following alloxan exposure (Ighodaro *et al.*, 2017; Lenzen, 2008; Singh *et al.*, 2024). Ascorbic acid supplementation improved glycemic control and partially preserved body weight, supporting previous evidence regarding its antioxidant, anti-inflammatory, and insulin-sensitizing properties (Azarova *et al.*, 2023; El-Aal *et al.*, 2018; Ludvigsson, 2021; Price *et al.*, 2001). However, the effects of antioxidant supplementation in diabetes remain complex, as prolonged antioxidant administration may also influence metabolic pathways in a context-dependent manner. Eugenol, a phenolic compound with reported antioxidant, anti-inflammatory, and hypoglycemic activities (Anjum *et al.*, 2023; Damasceno *et al.*, 2024; Jiang *et al.*, 2025), reduced blood glucose levels in diabetic rats but showed a weaker effect on overall metabolic improvement and was associated with weight reduction, which is consistent with previous findings regarding its effects on glucose-metabolizing enzymes and hepatic glycogen metabolism (Srinivasan *et al.*, 2014). Notably, combined administration resulted in the greatest reduction in blood glucose levels, suggesting an improved glucose-lowering response compared with individual treatments.

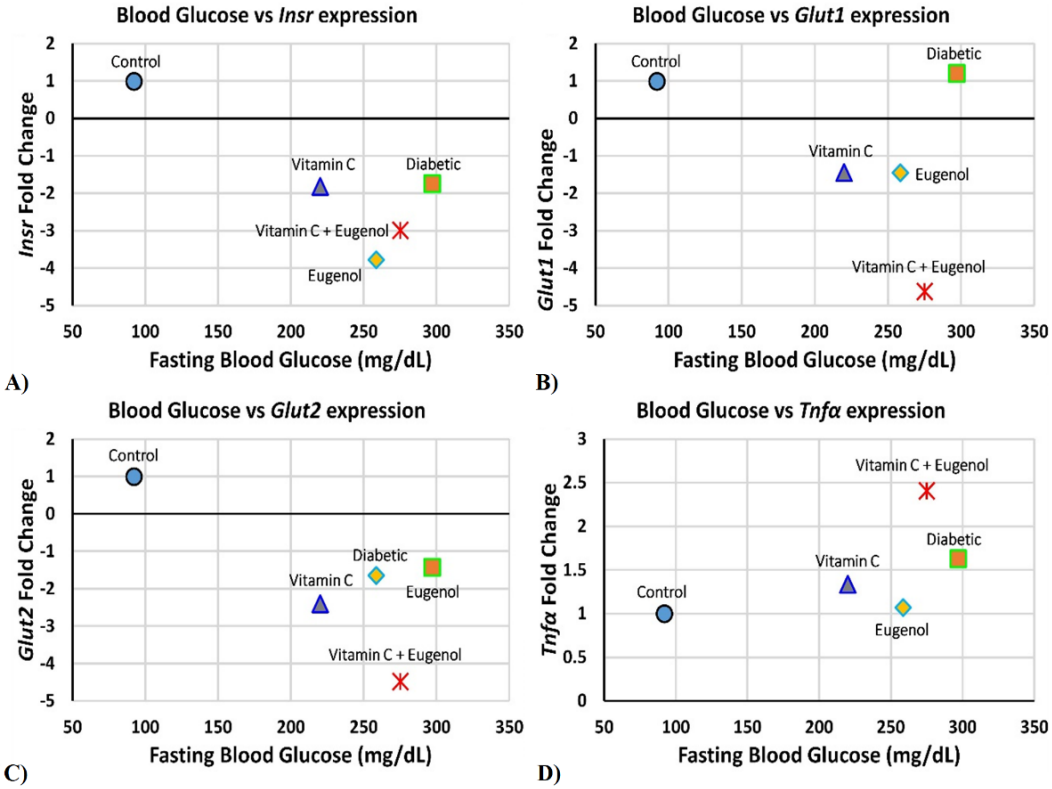

Fig. 7. Correlation between fasting blood glucose (FBG) levels and relative mRNA expression of hepatic genes in the experimental groups: A) correlation with Insr expression; B) correlation with Glut1 expression; C) correlation with Glut2 expression; D) correlation with Tnfα expression. Scatter plots illustrate the relationship between FBG concentration (mg/dL) and relative gene expression determined by qRT-PCR in liver tissue of normal, diabetic, vitamin C-treated, eugenol-treated, and vitamin C + eugenol-treated groups.

At the pancreatic level, diabetes markedly reduced the expression of *Ins1/2*, *Insr*, *Glut2*, and *Pdx1* genes, reflecting impaired insulin synthesis, β-cell function, and glucose sensing (Al-Adsani *et al.*, 2022; Karbaschi & Zardooz, 2023; Wang *et al.*, 2018). *Pdx1*, as a key regulator of β-cell differentiation and insulin transcription, is considered an important therapeutic target in diabetes (Ebrahim *et al.*, 2022; Zhang *et al.*, 2022). Both compounds partially restored the expression of several pancreatic genes, and the combined treatment produced higher *Ins1/2* and *Glut2* expression compared with individual treatments, indicating enhanced modulation of metabolic gene transcription. However, these transcriptional changes cannot be considered direct evidence of functional β-cell recovery, and further studies involving insulin secretion, protein expression, and histological analyses are required. Similarly, increased *Insr* expression following combination treatment suggests altered insulin-related transcription but does not confirm activation of downstream insulin signaling pathways.

Inflammatory gene analysis showed distinct elevation of *Tnfα* expression in diabetic pancreatic tissue, which is consistent with the established role of inflammation in diabetes progression (Cecerska-Heryc *et al.*, 2025; Darakjian *et al.*, 2021; Tsalamandris *et al.*, 2019). Eugenol showed the strongest suppression of *Tnfα* expression, supporting its anti-inflammatory activity. However, the combined treatment did not further enhance this effect, suggesting that interactions between bioactive compounds may vary depending on the molecular target.

The hepatic response differed considerably from pancreatic findings, emphasizing tissue-specific regulation. Diabetes reduced hepatic *Insr* and *Glut2* expression, while eugenol alone further decreased *Insr* expression, indicating that systemic metabolic benefits do not necessarily correspond to identical transcriptional responses in different organs. Combination treatment partially modified these effects but did not fully restore normal expression patterns. Changes in *Glut1* and *Glut2* expression after combined treatment may represent adaptive alterations in hepatic glucose handling; however, their functional implications require confirmation through protein and metabolic assays. Similarly, the increase in hepatic *Tnfα* expression in follow of combination treatment contrasts with pancreatic responses and suggests a distinct tissue-specific inflammatory regulation that requires further investigation.

Correlation analysis supported the relationship between metabolic status and gene expression alterations. The inverse association between FBG and pancreatic *Ins1/2*, *Insr*, *Glut2*, and *Pdx1* expression agrees with their roles in maintaining glucose homeostasis. In addition, the positive correlation between *Tnfα* expression and hyperglycemia supports the contribution of inflammation to diabetic progression (Du *et al.*, 2023; Klimontov *et al.*, 2023; Olson *et al.*, 2012). Differences between pancreatic and hepatic responses may arise from the distinct biological functions, antioxidant capacities, and metabolic environments of these tissues. Pancreatic β-cells are particularly sensitive to oxidative stress due to limited antioxidant defense mechanisms, which may explain their responsiveness to antioxidant compounds (Newsholme *et al.*, 2019). Conversely, the liver plays a central role in glucose metabolism and xenobiotic processing, potentially resulting in different responses to combined antioxidant exposure (Muoio & Newgard, 2008). Therefore, the effects of eugenol and ascorbic acid combination appear to be tissue- and gene-dependent rather than uniformly beneficial across all metabolic pathways.

It should also be acknowledged that qRT-PCR interpretation depends strongly on reference gene stability. Although *Gapdh* is widely used for normalization in diabetes-related studies, its expression may vary under specific metabolic conditions. Future studies should validate multiple housekeeping genes to ensure more accurate normalization of transcriptional data. Finally, because the present study was limited mainly to gene expression analysis, further investigations incorporating protein-level assessments, functional assays, insulin measurements, and pathway analyses are necessary to determine whether the observed transcriptional changes translate into improved metabolic function.

Conclusion

In conclusion, the present study demonstrates that eugenol and ascorbic acid modulate glycemic

control and the expression of genes involved in glucose metabolism and inflammation in alloxan-induced diabetic rats. The combined treatment showed a greater improvement in blood glucose regulation and produced distinct transcriptional responses in pancreatic and hepatic tissues, highlighting the tissue-dependent effects of these bioactive compounds. However, the observed changes in gene expression should be interpreted cautiously, as transcriptional modulation does not necessarily indicate functional restoration of metabolic pathways. Further studies incorporating biochemical, histological, and protein-level analyses are required to determine whether these molecular changes translate into improved β-cell function and metabolic outcomes. Additional investigations addressing dose optimization, treatment duration, and downstream signaling mechanisms will be essential to clarify the therapeutic potential and biological limitations of eugenol and ascorbic acid combination therapy in diabetes.

Author contribution statement

E.M. Resources, Investigation, Visualization, Data curation, and analysis; **S.S.** Supervision, Project administration, Methodology, and Manuscript writing; **M.G.** Consultant for animals working.

Conflict of interests

The authors declare no conflict of interest.

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