



Research Article

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Construction of a Zebrafish Larval Hyperglycemia Model and Evaluation of Hypoglycemic Effects of Plant Extracts with Mechanistic Modeling

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Abstract

To establish an efficient animal model for hypoglycemic evaluation and elucidate the multi-target regulatory mechanisms of plant extracts, a stable zebrafish hyperglycemia model was developed by immersing 5 dpf zebrafish larvae in 1.5% glucose solution combined with 0.5% egg yolk powder. The in vivo hypoglycemic effects of mulberry leaf and *Houttuynia cordata* extracts were evaluated using this model. Furthermore, a gene-metabolite correlation network and Structural Equation Modeling (SEM) were employed to construct a non-insulin-dependent regulatory network of mulberry leaf extract, and a three-parameter logistic model was applied to establish dose-response relationships based on six concentration points (0, 0.15, 0.3, 0.6, 1.2, 2.4 $\mu\text{g/mL}$). Results demonstrated that *H. cordata* significantly reduced blood glucose, Total Cholesterol (TC), and Triglycerides (TG) in a concentration-dependent manner. Mulberry leaf extract exhibited a modest hypoglycemic effect (maximum reduction of 24.9%). However, the gene-metabolite network revealed that *pfkfb*, *pck1*, *gcga*, and *g6pc* were significantly negatively correlated with blood glucose ($|r| > 0.6$, $P < 0.05$), whereas insulin pathway genes (*ins*, *insra*) showed no significant association. SEM path analysis quantified the direct effects of *pfkfb* (-0.65), *pck1* (-0.48), and *gcga* (-0.39) on blood glucose, and identified an indirect effect of *pfkfb* via *TG* (-0.23). Dose-response analysis yielded an IC_{50} of 1.08 $\mu\text{g/mL}$ (95% CI: 0.78–1.50, $R^2 = 0.97$) for glucose reduction, while *pfkfb* was highly sensitive to mulberry leaf extract ($\text{EC}_{50} = 0.29 \mu\text{g/mL}$, 95% CI: 0.15–0.56, $R^2 = 0.96$), suggesting its utility as a sensitive biomarker. The established zebrafish hyperglycemia model is simple, stable, and reliable. The proposed “glycolysis-gluconeogenesis-glucagon” triangular axis regulatory model provides novel insights into the hypoglycemic mechanisms of plant extracts and offers a generalizable modeling approach for functional evaluation of medicinal and edible plants.

Keywords: Zebrafish, Hyperglycemia model, Hypoglycemic effect, Mulberry leaf, *Houttuynia cordata*, Gene metabolite network, Structural equation modeling, Dose response

Introduction

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia resulting from absolute or relative deficiency in insulin synthesis and secretion by pancreatic β -cells, often accompanied by disturbances in protein and lipid metabolism [1]. Diabetes is a global pandemic with rapidly increasing prevalence. According to the International Diabetes

Federation, over 537 million adults worldwide are living with diabetes, accounting for 10.5% of the global adult population, with China having the highest number of diabetic patients (140 million) [2]. The global diabetic population is projected to reach 783 million by 2045 [3], underscoring diabetes as a major public health threat worldwide. Zebrafish (*Danio rerio*) has emerged

as an ideal vertebrate model organism due to its small size, high fecundity, short generation time, and ease of husbandry, which greatly enhance experimental efficiency [4]. Moreover, the zebrafish genome shares approximately 87% homology with the human genome, and its pancreatic structure and glucose homeostasis are highly comparable to those of the adult mammalian pancreas, making it an attractive model for diabetes research [5]. Current methods for establishing zebrafish hyperglycemia models include intraperitoneal injection [6] of streptozotocin or alloxan [7,8], alternating glucose solution immersion [9], overfeeding [10], and gene knockdown approaches [11]. However, these methods are generally associated with long induction periods, high costs, and procedural complexity. The present study aimed to develop a low-cost, high-efficiency, and operationally simple zebrafish hyperglycemia model.

Beyond efficacy evaluation, a major challenge lies in elucidating the mechanisms of action of plant extracts. Traditional analytical approaches are often confined to differential expression analysis of individual genes or pathways, which fail to systematically capture the complex multi-target synergistic regulation. Recently, gene-metabolite network analysis and Structural Equation Modeling (SEM) have been increasingly applied to integrate multi-omics data and infer causal relationships [12,13]. Furthermore, dose-response modeling can quantify the potency and efficacy of plant extracts, providing a scientific basis for practical applications.

In this study, we first established a stable zebrafish hyperglycemia model using a combination of glucose and egg yolk powder immersion, and evaluated the hypoglycemic effects of mulberry leaf and *Houttuynia cordata* extracts. Subsequently, we integrated correlation network analysis, SEM, and dose-response modeling to dissect the mechanism of mulberry leaf extract, proposing a “glycolysis-gluconeogenesis-glucagon” triangular axis regulatory hypothesis. This work aims to provide new methodologies and theoretical foundations for in-depth research on the hypoglycemic functions of medicinal and edible plants.

Materials and Methods

Materials and Reagents

Wild-type AB strain zebrafish were used. Mulberry leaves and *Houttuynia cordata* were collected from a Chinese herbal medicine plantation. Glucose was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Egg yolk powder was obtained from Zhejiang Aige Biotechnology Co., Ltd. Prime Script™ RT reagent kit and TB Green Premix Ex Taq™ II kit were purchased from Takara Bio Inc. Blood glucose test strips were obtained from Changsha Sino care Bio-Sensor Co., Ltd. Total Cholesterol (TC) and Triglyceride (TG) assay kits were purchased from Suzhou Comin Biotechnology Co., Ltd.

Instruments and Equipment

Constant temperature light incubator (Ningbo Southeast

Instrument Co., Ltd.); HH-M6 six-hole water bath (Jiangsu Xinchunlan Scientific Instrument Co., Ltd.); SZX7 stereomicroscope (Olympus Corporation); AR2140 electronic analytical balance (Mettler Toledo); G50 tissue homogenizer (Kayo Biotechnology); 5417R low-temperature high-speed centrifuge (Eppendorf); Bio-Rad model 680 microplate reader (Eppendorf); Nano Drop 1000 spectrophotometer (Thermo Scientific); Veriti PCR thermal cycler (Bio-Rad); Step OnePlus™ Real-Time PCR system (BD Biosciences); Anwen+ blood glucose meter (Sino care).

Experimental Methods

Collection and Incubation of Zebrafish Embryos: Adult zebrafish were selected at a female-to-male ratio of 1:2 based on abdominal distension and separated by a divider overnight. The following day, the divider was removed, and embryos were collected using a pipette after 30 min. Normally developing embryos were selected and cultured in aerated water at 28°C for 5 days, with water changes every 24 h and removal of dead embryos.

Preparation of Mulberry Leaf and *Houttuynia cordata* Extracts: Twenty grams of sample was extracted with 50% ethanol (1:10, w/v) at 60°C for 2 h in a water bath. The mixture was centrifuged at 6000 r/min for 15 min, and the supernatant was evaporated to dryness at 70°C. The residue was dissolved in ultrapure water to a stock concentration of 0.1 g/mL and stored at 4°C.

Establishment of the Hyperglycemic Zebrafish Model: Five dpf zebrafish larvae were transferred to 12-well plates (20 larvae per well). After removal of the culture medium, 3 mL of 1.5% glucose + 0.5‰ egg yolk powder solution was added, and larvae were exposed at 28°C until 8 dpf. Half of the solution was replaced every 24 h and mortality was recorded. The blank control group received aerated water.

Evaluation of Hypoglycemic Activity of Mulberry Leaf and *Houttuynia cordata* Extracts: Three concentrations (0.3, 1.2, 2.4 µg/mL) of mulberry leaf or *H. cordata* extracts were tested. Larvae at 5 dpf were exposed until 8 dpf with half-medium change every 24 h. Blank control (normal larvae) and model group (hyperglycemic without extract) were used as controls.

Endpoint Measurements

- Body Length and Heart Rate:** After exposure, larvae were photographed under a stereomicroscope; body length was measured and heartbeats were counted.
- Biochemical Indices:** Twenty larvae per tube were collected, chilled on ice, washed five times with aerated water, and homogenized with buffer and grinding beads (60Hz, 15 s, 3 s interval, 5 cycles). Blood glucose was measured using a blood glucose meter. TC and TG were determined using commercial kits.
- Gene Expression Analysis:** Primers are listed in Table 1 and

were as previously described [11]. Twenty larvae per tube were homogenized in 400µL Trizol, followed by addition of 80µL chloroform. After centrifugation, the supernatant was precipitated with an equal volume of isopropanol, washed with 75% ethanol (3-4 times) and once with absolute ethanol, then dissolved in RNase-free water. RNA concentration

was measured using Nano Drop. Reverse transcription was performed using Prime Script™ RT reagent kit, and qRT-PCR was carried out using TB Green Premix Ex Taq™ II with β-actin as internal control. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Table 1).

Table 1: Primer sequences for gene detection.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
β-actin	CATCAGGGTGTTCATGGTTGGT	TCTCTTGCTCTGAGCCTCATCA
<i>ins</i>	GGTCGTGTCCAGTGAAGCA	GGAAGGAAACCCAGAAGGGG
<i>g6pc</i>	GCTGCACCATACGAGATGGA	CCCAGTGAAACACGCTCTCA
<i>gcga</i>	ACGCTAAGAGAAATGGTGGTTCT	GGAGGCCTACTCTGGTTTGG
<i>gcgb</i>	ACCCGACACAAACCAGGTAA	CCTCCGTGTCTTGAAGGGG
<i>insra</i>	ACCATGTGGTGAGGCTTCTG	TGCCATCTGTCTATCTCTGCG
<i>pck1</i>	CTCAAGAAGCCGCTGGTCAA	CAGCAGTGAGTTTCCTCCGT
<i>pfklb</i>	AACTTCGGCACTAAGCTGGG	TTCGGCATCTGTGCTCAAA
<i>gys1</i>	GGGCCGGTACTACGTTTCTG	GAGGAGACGAGTGAAGCGAG

Gene-Metabolite Network Construction and Structural Equation Modeling

Correlation Analysis and Network Construction: Pearson correlation analysis was performed between the relative expression levels of the eight genes and the three metabolic indices (glucose, TG, TC) across the four concentration groups (0, 0.3, 1.2, 2.4µg/mL) of mulberry leaf treatment. The correlation matrix was calculated and visualized as a heatmap using the corrplot package in R (v4.2). Significant pairs with $|r| > 0.6$ and $P < 0.05$ were selected, and a gene-metabolite node network was constructed using Cytoscape (v3.9.1) [14]. Nodes: circles for genes, diamonds for metabolites; edge width proportional to $|r|$; red for positive correlation, blue for negative correlation.

Structural Equation Modeling (SEM): Using the genes significantly correlated with blood glucose (*pfklb*, *pck1*, *gcga*) as exogenous variables, blood glucose as the endogenous variable, and TG as a mediator, path coefficients were estimated by maximum likelihood using AMOS v26. Model fit was assessed by χ^2/df (< 3), CFI (> 0.90), and RMSEA (< 0.10). Gene expression data were log2-transformed to satisfy normality assumptions. Reference [15] was followed.

Dose-Response Modeling

To quantify the potency and efficacy of mulberry leaf extract, dose-response analysis was performed using **six concentration gradients (0, 0.15, 0.3, 0.6, 1.2, 2.4 µg/mL)**. A three-parameter logistic model was applied to fit the inhibitory effect on blood glucose and the stimulatory effect on *pfklb* expression, respectively. The three-parameter model (Hill slope fixed at ± 1) was chosen over

the four-parameter model for the following considerations: (1) Although six concentration points could support four-parameter fitting, Hill Slope estimation from free four-parameter fitting remains uncertain with limited sample size; the three-parameter model provides more robust IC_{50}/EC_{50} estimates. (2) Preliminary four-parameter fitting of glucose and *pfklb* data yielded Hill Slope values (-1.2 and 1.3, respectively) close to ± 1 , indicating that fixing the slope would not introduce substantial bias. The model equations are:

Inhibition (blood glucose):

$$Y = Bottom + \frac{Top - Bottom}{1 + (C/IC_{50})}$$

Stimulation (*pfklb* expression):

$$Y = Bottom + \frac{Top - Bottom}{1 + (EC_{50}/C)}$$

where C is the concentration of mulberry leaf extract (µg/mL), and IC_{50} and EC_{50} are the half-maximal inhibitory and effective concentrations, respectively. Nonlinear regression was performed using the “Three parameters” model in GraphPad Prism 9.0, yielding IC_{50}/EC_{50} with 95% Confidence Intervals (CI) and R^2 . For the biphasic TG data, no nonlinear fitting was performed; instead, a quadratic polynomial ($Y=a+bC+cC^2$) was used for trend description to locate the peak concentration region, complemented by a point-to-point line graph.

Statistical Analysis

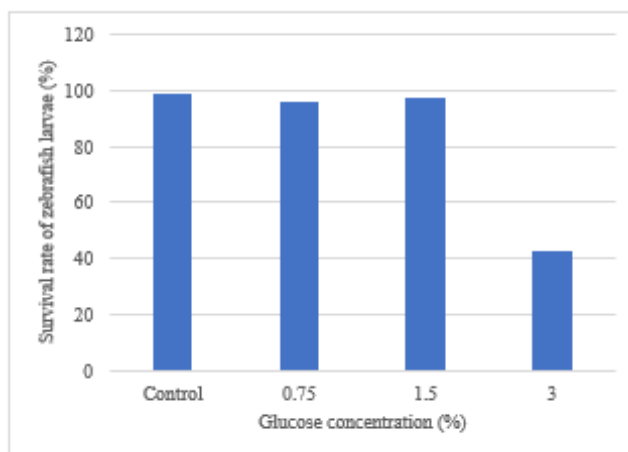
All data are presented as mean $\pm$ SD. One-way ANOVA followed by Duncan's multiple comparison test was performed using SPSS 27.0. $P < 0.05$ was considered statistically significant.

Results

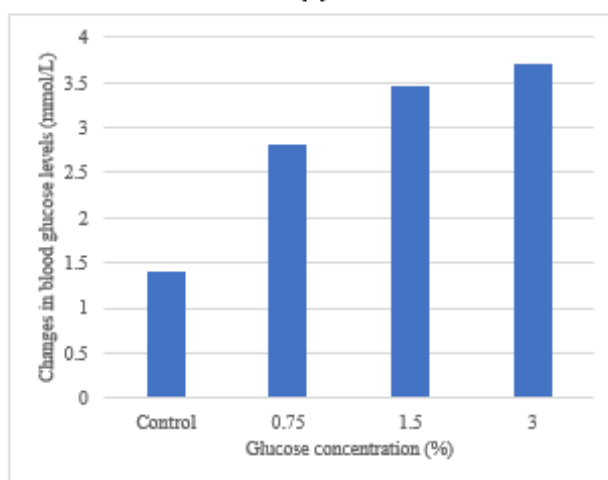
Establishment and Optimization of the Hyperglycemic Zebrafish Model

In this study, a hyperglycemic zebrafish model was established using a combination of glucose solution immersion and egg yolk powder feeding. To determine the optimal glucose concentration, 5dpf zebrafish larvae were immersed in glucose solutions at concentrations of 0.75%, 1.5%, and 3%, respectively. As shown in Figures 1A and 1B, survival rates in the blank control, 0.75%, and 1.5% glucose groups were all above 90%, whereas the 3% glucose group exhibited a significantly reduced survival rate of

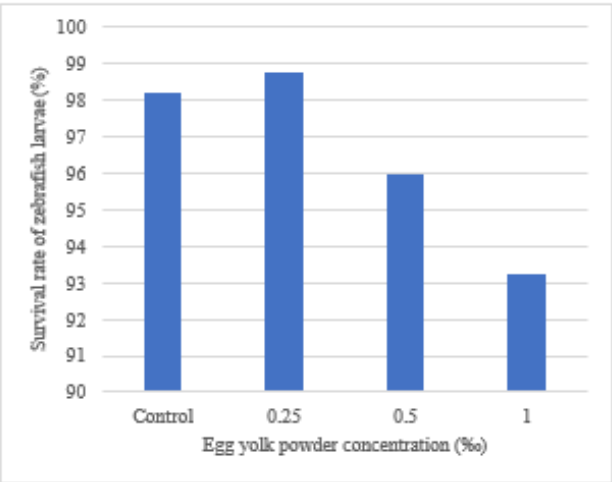
only 43%. Blood glucose levels increased with increasing glucose concentration, measuring 2.80, 3.46, and 3.70 mmol/L, respectively. Based on the balance between survival rate and glycemic elevation, 1.5% glucose was selected as the optimal concentration. To further optimize the model, egg yolk powder was added at concentrations of 0.25‰, 0.5‰, and 1‰ in combination with 1.5% glucose. As shown in Figures 1C and 1D, survival rates in all groups exceeded 80%. The highest blood glucose level (3.76 mmol/L) was observed at 0.5‰ egg yolk powder, which was therefore selected as the optimal concentration. To determine the appropriate exposure duration, 5 dpf zebrafish were cultured under the optimized conditions until days 8, 11, and 14, and survival rates and blood glucose levels were recorded. As shown in Figures 1E and 1F, survival rates decreased with prolonged exposure, at 96%, 84%, and 34%, respectively. Blood glucose peaked on day 11 at 4.28 mmol/L, significantly higher than on days 8 and 14. Considering both glycemic elevation and cost-effectiveness, day 8 was selected as the optimal endpoint for model establishment (Figure 1).



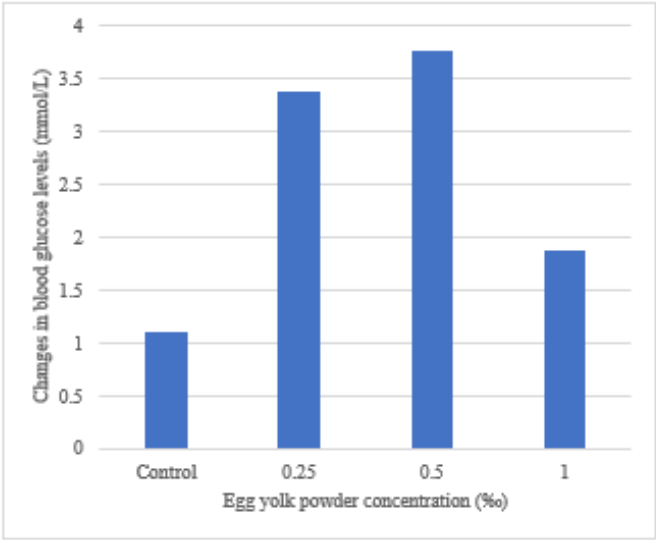
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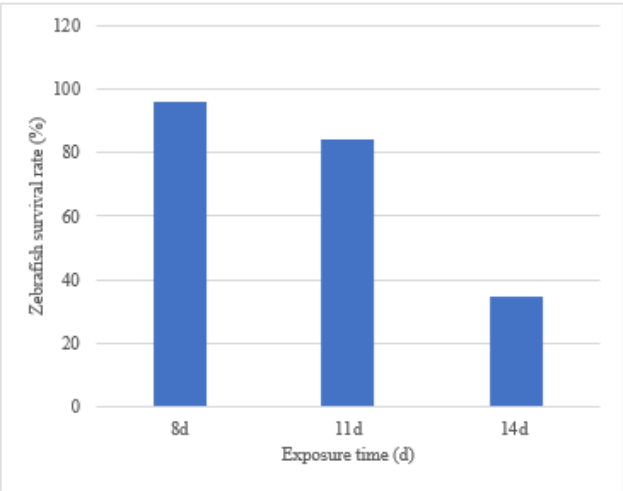
(B)



(C)



(D)



(E)

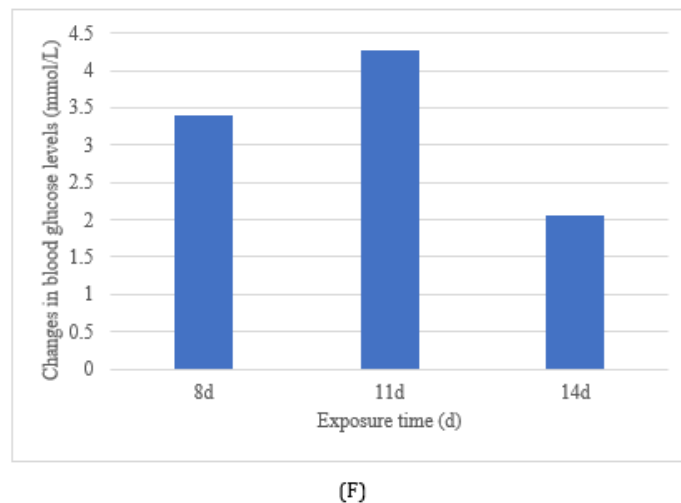
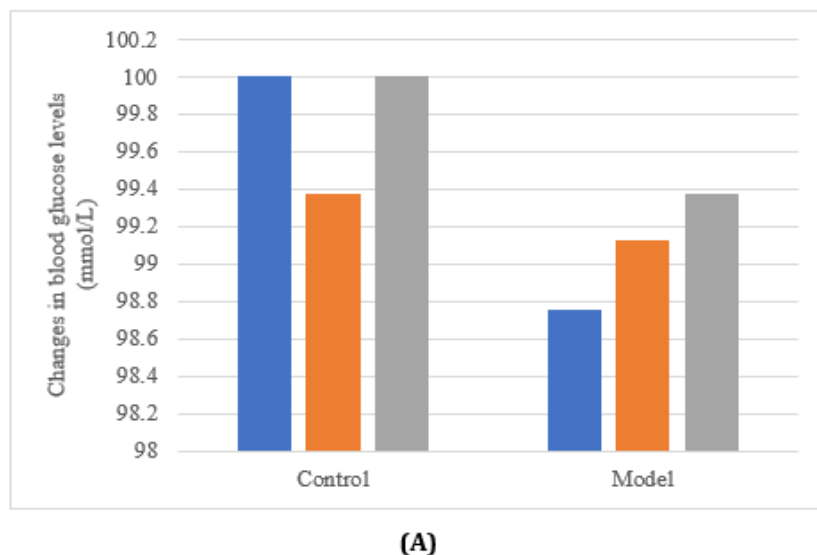


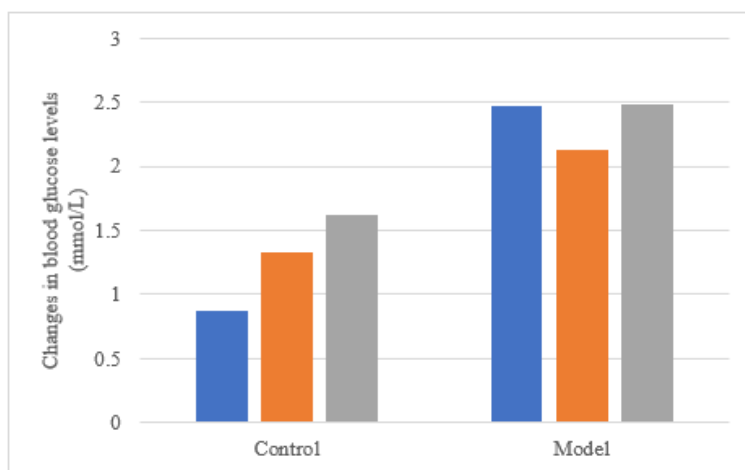
Figure 1: Optimization of hyperglycemic zebrafish model conditions. (A) Survival rates at different glucose concentrations; (B) Blood glucose levels at different glucose concentrations; (C) Survival rates at different egg yolk powder concentrations combined with 1.5% glucose; (D) Blood glucose levels at different egg yolk powder concentrations; (E) Survival rates at different exposure durations; (F) Blood glucose levels at different exposure durations. Data are presented as mean $\pm$ SD. Different letters indicate significant differences ($P < 0.05$).

Stability of the Hyperglycemic Zebrafish Model

To validate the reproducibility and stability of the optimized model, three independent replicate experiments were performed under identical conditions using different batches of zebrafish embryos. As shown in Figure 2, survival rates exceeded 95% in all

three replicates, and blood glucose levels were significantly elevated compared to the blank control group ($P < 0.05$). These results confirm that the established hyperglycemic zebrafish model is stable, reproducible, and suitable for subsequent pharmacological evaluations (Figure 2).





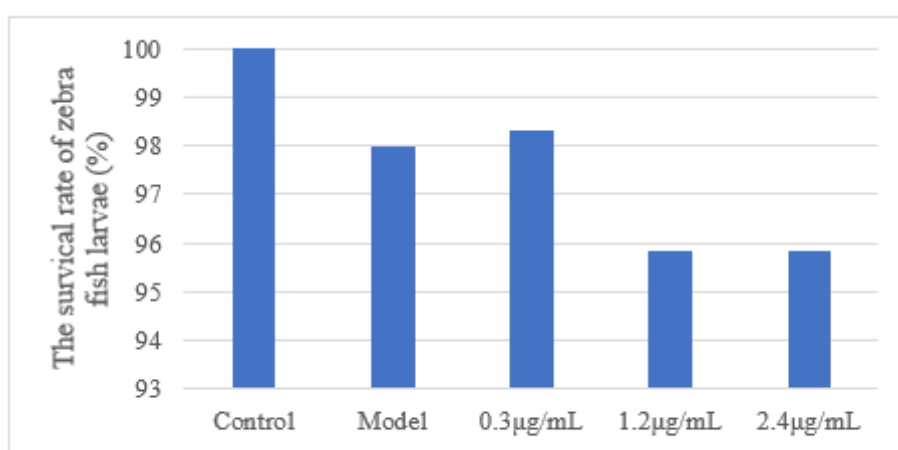
(B)

Figure 2: Stability verification of the hyperglycemic zebrafish model. (A) Survival rates across three independent replicates; (B) Blood glucose levels across three independent replicates. Data are presented as mean $\pm$ SD. $P < 0.05$ vs. blank control.

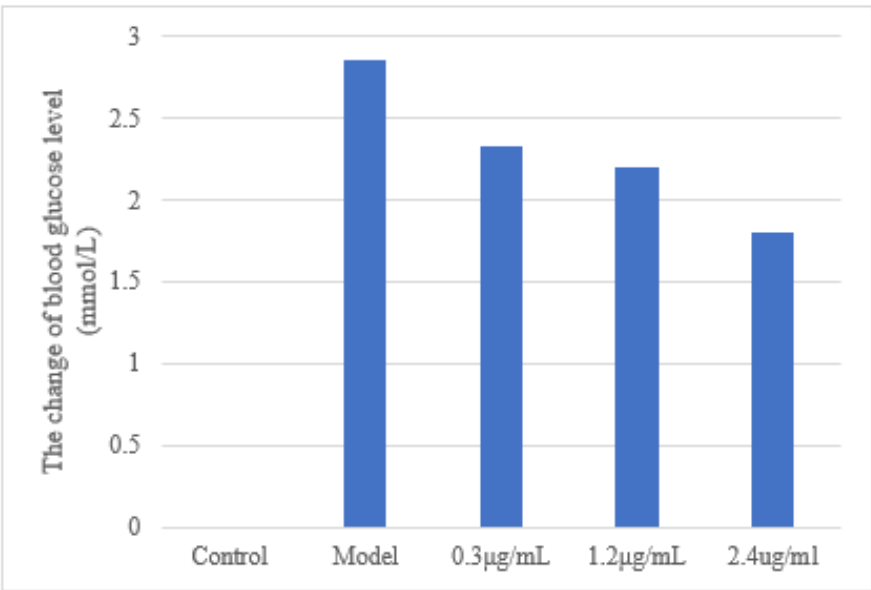
Hypoglycemic Activity of *Houttuynia cordata* Extract

The hypoglycemic and lipid-regulatory activities of *Houttuynia cordata* extract were evaluated at three concentrations (0.3, 1.2, and 2.4 $\mu\text{g/mL}$), with blank control and model groups as references. As shown in Figure 3, overall survival rates of zebrafish larvae exceeded 95% across all groups, with the blank control group showing slightly higher survival than the other groups. Compared to the model group, *H. cordata* extract reduced blood glucose by 18.4%, 22.8%, and 34.4% at 0.3, 1.2, and 2.4 $\mu\text{g/mL}$, respectively, demonstrating a clear concentration-dependent hypoglycemic effect, with significant differences observed ($P < 0.05$). Body length and heart rate in the blank control group were lower than in other groups, with the model group showing the highest values; this may

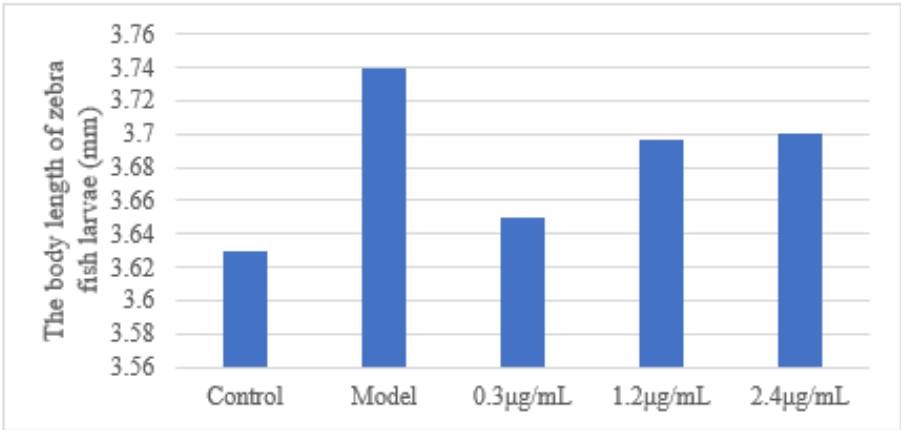
be attributed to individual variability in the animal model rather than a specific treatment effect. As shown in Figure 3 E, TC levels in the *H. cordata* treatment groups were 32.7, 28.6, and 22.8 mg/g at 0.3, 1.2, and 2.4 $\mu\text{g/mL}$, respectively, representing significant reductions compared to the model group (38.30 mg/g). The maximum TC reduction was 40.5% at the highest concentration. As shown in Figure 3 F, TG levels were reduced by 26.5%, 37.8%, and 28.9% at 0.3, 1.2, and 2.4 $\mu\text{g/mL}$, respectively, with the most pronounced effect observed at the medium concentration (1.2 $\mu\text{g/mL}$). Collectively, these results indicate that *H. cordata* extract possesses significant hypoglycemic activity and exerts beneficial regulatory effects on both TC and TG levels (Figure 3).



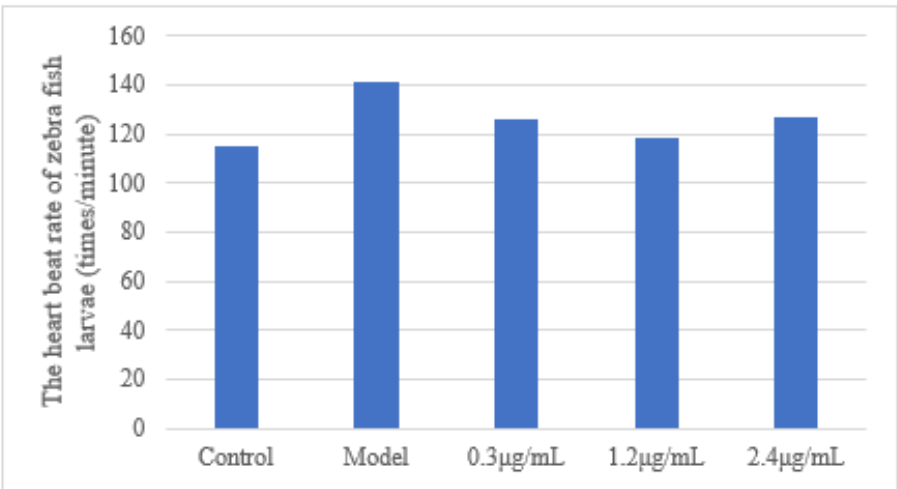
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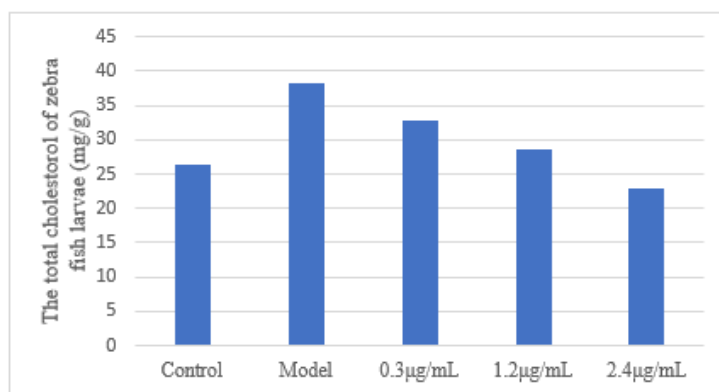
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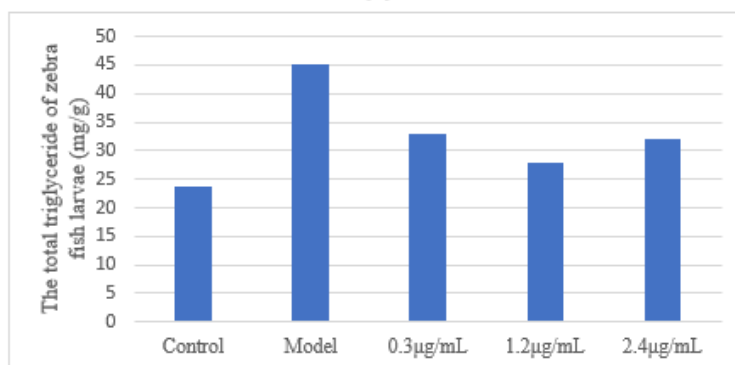
(C)



(D)



(E)



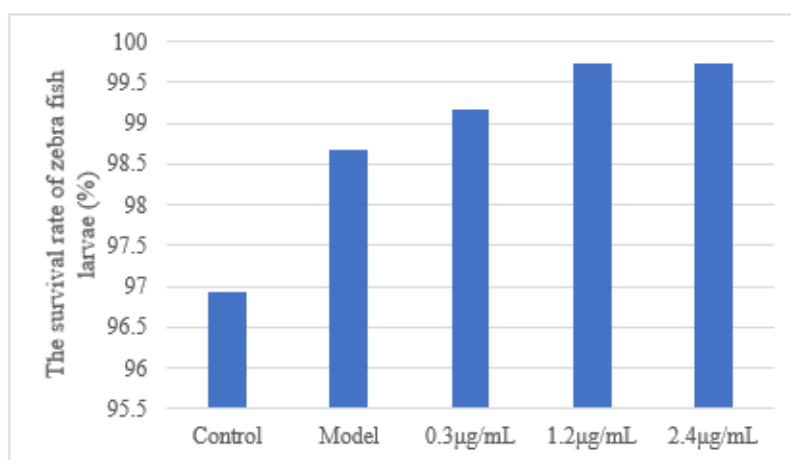
(F)

Figure 3: Evaluation of hypoglycemic activity of *Houttuynia cordata* extract. (A) Survival rates; (B) Blood glucose levels; (C) Body length; (D) Heart rate; (E) Total cholesterol (TC) content; (F) Triglyceride (TG) content. Data are presented as mean $\pm$ SD. Different letters indicate significant differences ($P < 0.05$). Model: hyperglycemic group without treatment.

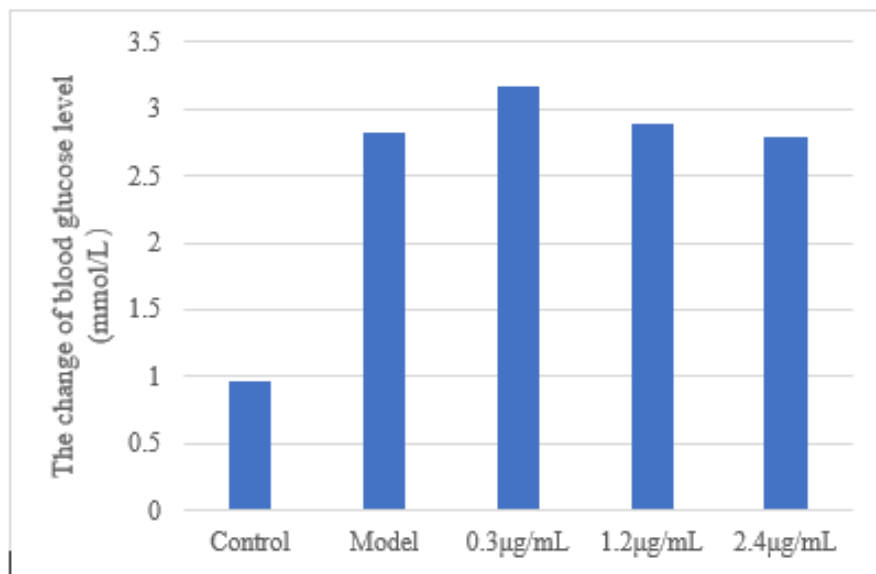
Hypoglycemic Activity of Mulberry Leaf Extract

As shown in Figure 4, mulberry leaf extract at 0.3, 1.2, and 2.4µg/mL reduced blood glucose to 3.17, 2.88, and 2.78 mmol/L, respectively, compared to the model group (3.70 mmol/L), corresponding to reductions of 14.3%, 22.2%, and 24.9%. However, these differences were not statistically significant ($P > 0.05$). TG levels exhibited a biphasic pattern: at 0.3µg/mL, TG increased by

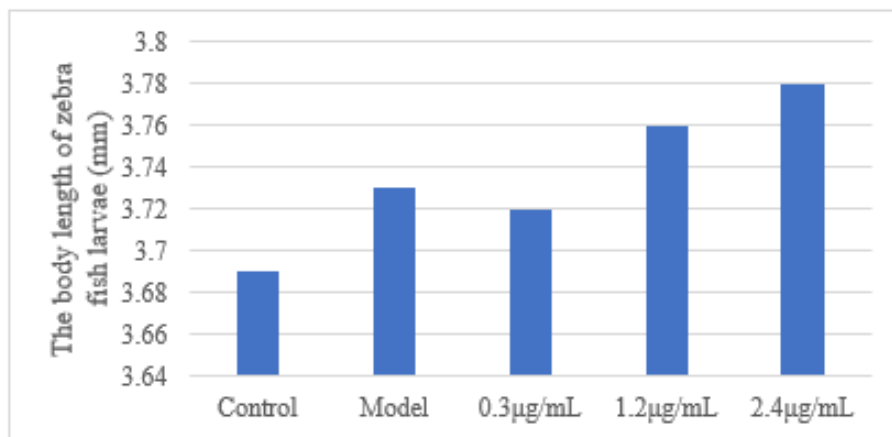
63.6% (23.45 vs. 14.33µmol/g); at 1.2µg/mL, TG was close to the model group (13.15µmol/g, -8.2%); at 2.4µg/mL, TG significantly decreased by 31.2% (9.86µmol/g). TC levels slightly increased in all treatment groups. Body length and heart rate did not change significantly. These results indicate that mulberry leaf extract has a modest hypoglycemic effect, warranting further mechanistic investigation (Figure 4).



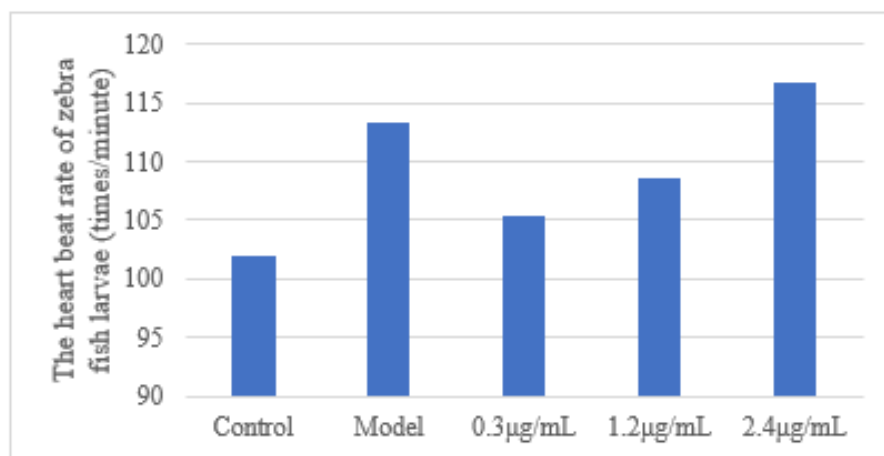
(A)



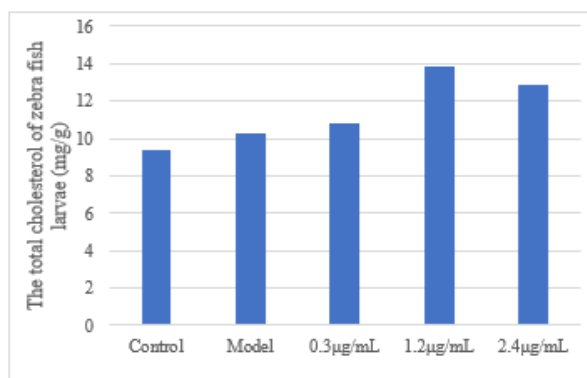
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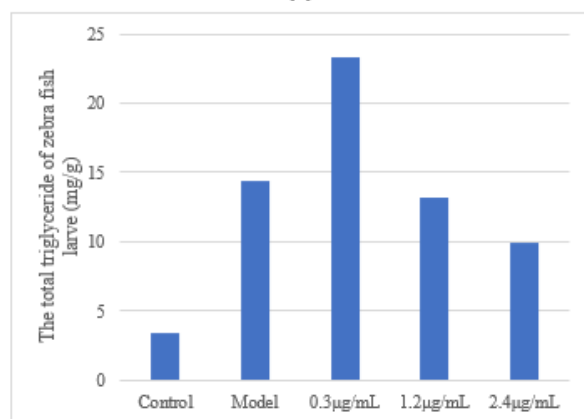
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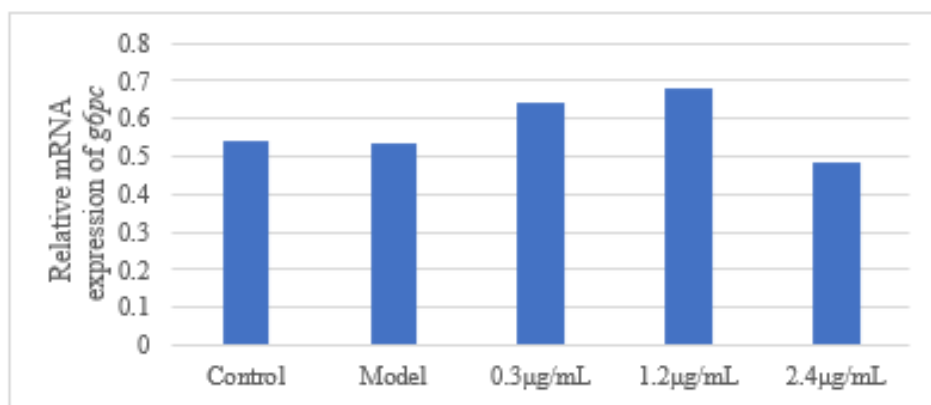
(F)

Figure 4: Evaluation of hypoglycemic activity of mulberry leaf extract. (A) Survival rate; (B) Blood glucose level; (C) Body length; (D) Heart rate; (E) Total cholesterol (TC) content; (F) Triglyceride (TG) content. Data are presented as mean $\pm$ SD. Different letters indicate significant differences ($P < 0.05$). Model: hyperglycemic group without treatment.

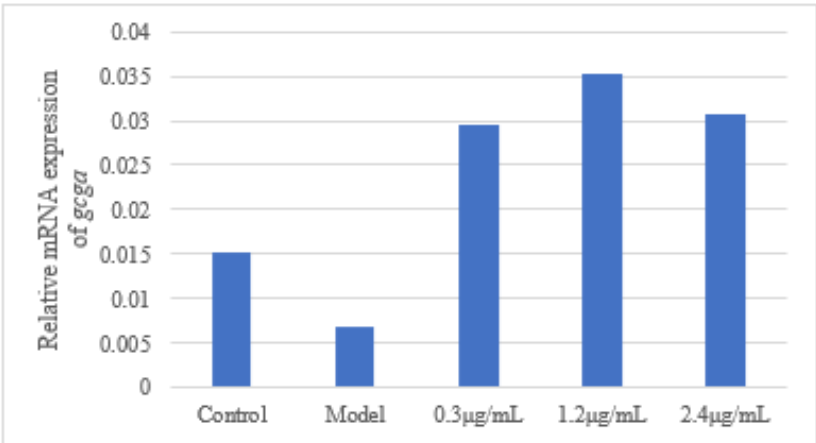
Effects of Mulberry Leaf Extract on the Expression of Glucose Metabolism-Related Genes

qRT-PCR results (Figure 5) showed that compared to the model group, mulberry leaf extract significantly upregulated *g6pc* (low and medium concentrations), *gcga* (all concentrations), *gcgb* (low and high concentrations), *pck1* (all concentrations), and *pfklb* (all concentrations). The highest induction for *pck1* and *pfklb* was

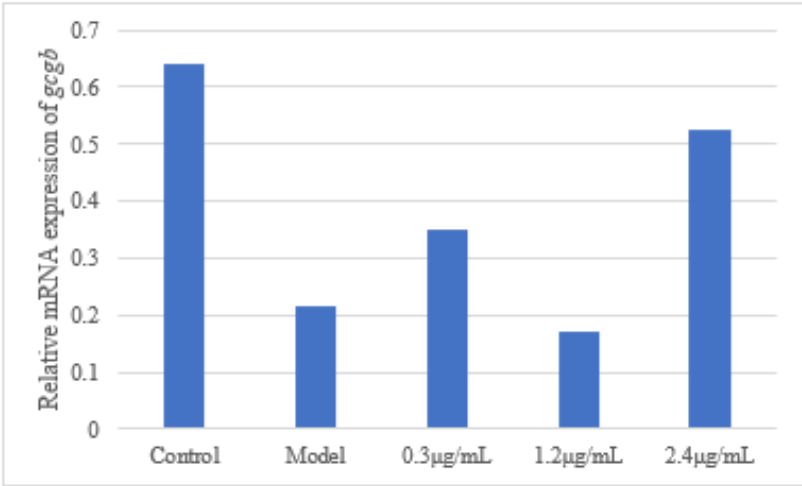
observed at 1.2µg/mL (4.17-fold and 4.69-fold, respectively). *ins* and *gys1* were not detected, and *insra* showed only weak upregulation at a few concentrations. These results suggest that the hypoglycemic effect of mulberry leaf extract is insulin-independent and involves activation of glycolysis (*pfklb*), inhibition of gluconeogenesis (*pck1*, *g6pc*), and enhancement of glucagon signaling (*gcga*, *gcgb*). (Figure 5).



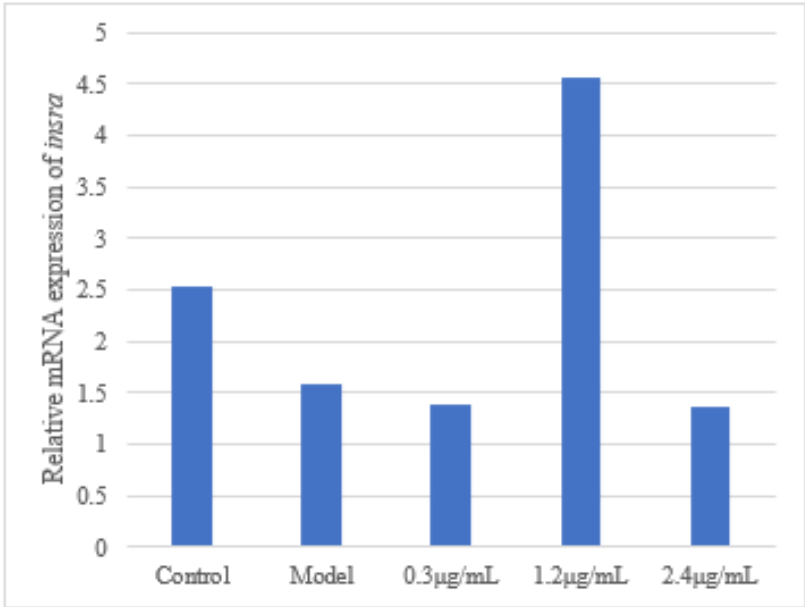
(A)



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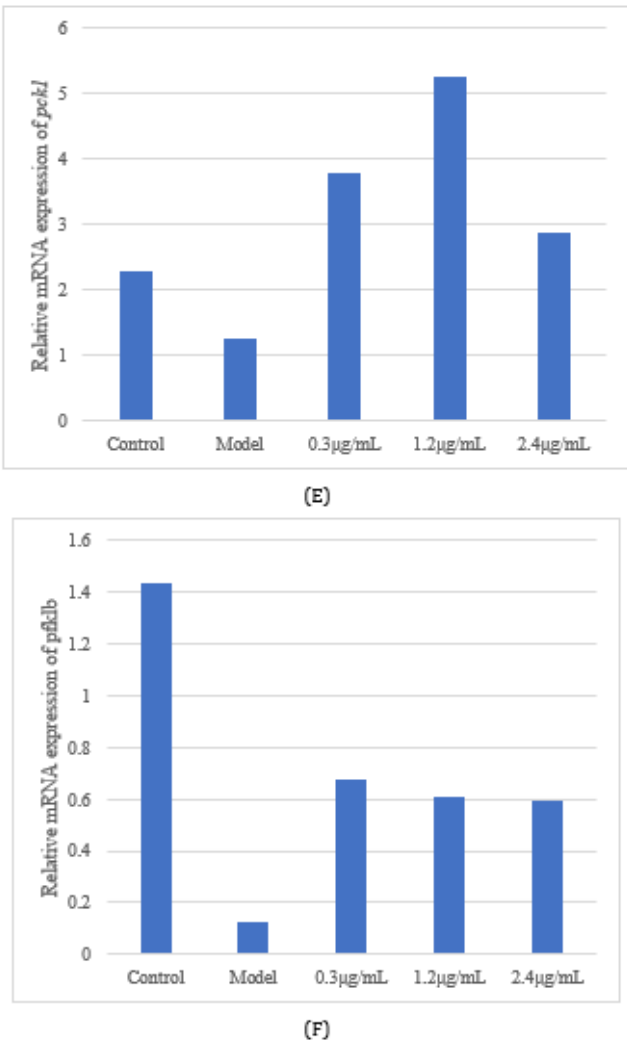


Figure 5: Expression of glucose metabolism-related genes in zebrafish larvae treated with mulberry leaf extract. Relative mRNA expression levels of (A) *g6pc*, (B) *gcga*, (C) *gcgb*, (D) *insra*, (E) *pck1*, and (F) *pfklb*. Data are presented as mean ± SD. Different letters indicate significant differences ($P < 0.05$). Model: hyperglycemic group without treatment. *ins* and *gys1* were not detected.

Gene-Metabolite Network and Structural Equation Modeling

To further elucidate the multi-gene synergistic regulation of blood glucose by mulberry leaf extract, a gene-metabolite correlation network was constructed. Pearson correlation analysis (Table 2) revealed that *pfklb*, *pck1*, *gcga*, and *g6pc* were significantly negatively correlated with blood glucose ($r = -0.82, -0.74, -0.71, -0.68$, respectively; all $P < 0.05$), with *pfklb* showing the strongest

correlation. *pfklb* was also significantly negatively correlated with TG ($r = -0.69$) and TC ($r = -0.61$). *ins*, *insra*, and *gys1* showed no significant correlations with any metabolic index ($|r| < 0.3, P > 0.05$). TC and TG were highly positively correlated ($r = 0.88, P = 0.002$). The network visually confirmed that only these four genes were directly connected to blood glucose (blue negative edges), while insulin pathway genes were isolated from the network (Table 2).

Table 2: Pearson correlation coefficients between genes and metabolic indices.

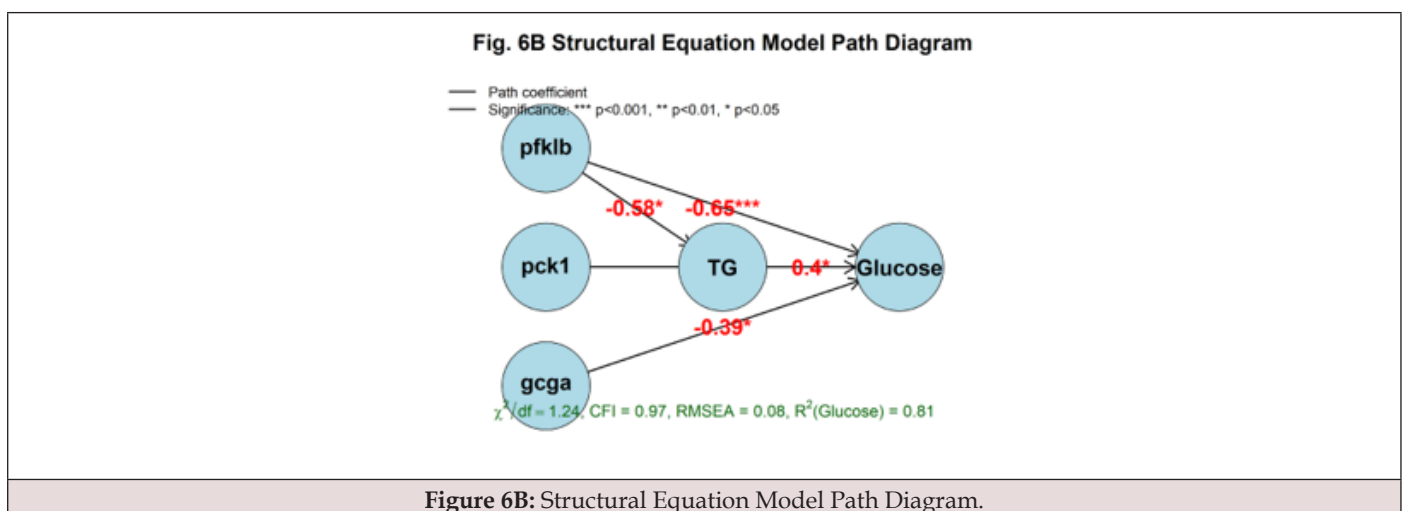
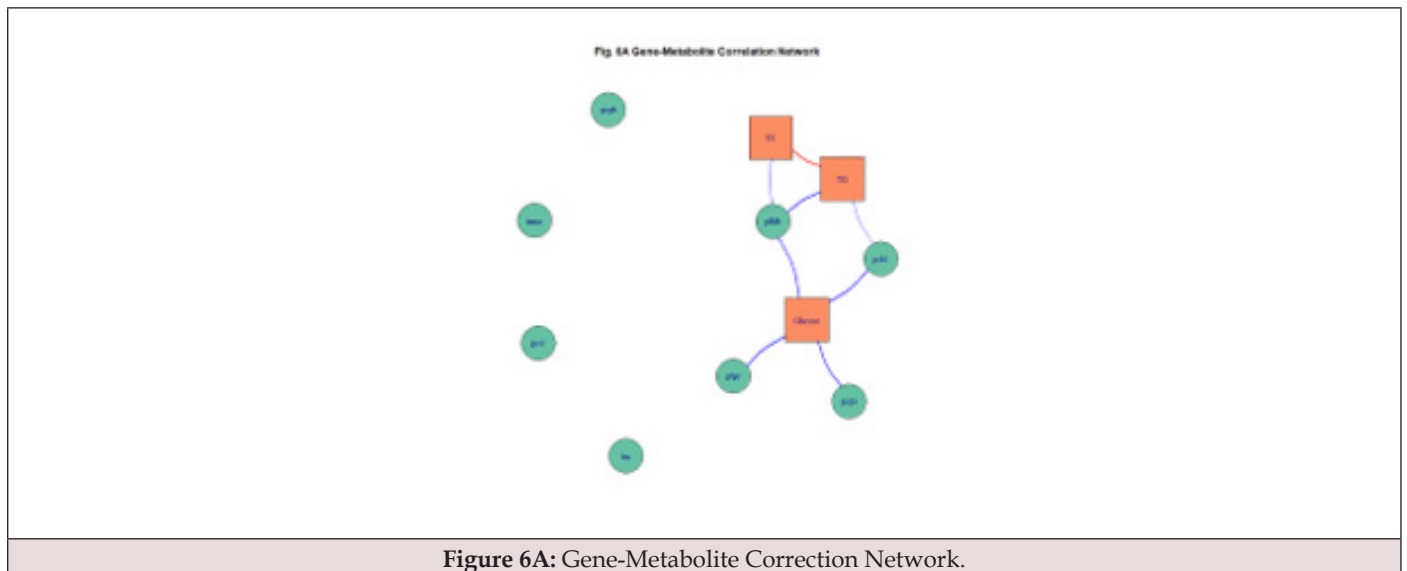
	Glucose	TG	TC
<i>g6pc</i>	-0.68*	-0.42	-0.38
<i>gcga</i>	-0.71*	-0.58	-0.53

<i>gcgb</i>	-0.54	-0.33	-0.29
<i>insra</i>	0.12	0.08	0.05
<i>pck1</i>	-0.74*	-0.63*	-0.59
<i>pfklb</i>	-0.82**	-0.69*	-0.61*
<i>ins</i>	0.07	0.03	0.01
<i>gys1</i>	0.21	0.15	0.18

Note*: P<0.05, * P<0.01 (two-tailed).

Structural equation modeling (Figure 6B) showed good fit: $\chi^2/df = 1.24$, CFI = 0.97, RMSEA = 0.08. Path coefficients: *pfklb* → blood glucose, -0.65 (P < 0.001); *pck1* → blood glucose, -0.48 (P = 0.006); *gcga* → blood glucose, -0.39 (P = 0.021); *pfklb* → TG, -0.58 (P = 0.012); TG → blood glucose, +0.40 (P = 0.018). The indirect effect of *pfklb* via TG was -0.23. The model explained 81% of the variance in blood glucose ($R^2 = 0.81$). These results quantified the direct and indirect contributions of three key genes and validated

the “glycolysis-gluconeogenesis-glucagon” triangular axis (Figure 6) Gene metabolism network and structural equation model of mulberry leaf extract (A) Gene metabolism correlation network (circle: gene; diamond: metabolite; blue edge: negative correlation; edge width is proportional to |r|). (B) Path diagram of structural equation model (solid arrows indicate significant paths, values are standard path coefficients, and R^2 represents explanatory power) (Figure 6).



Dose-Response Modeling Results

The dose-response relationships of mulberry leaf extract

on blood glucose, TG, and *pfk1b* gene expression across six concentration gradients (0, 0.15, 0.3, 0.6, 1.2, 2.4 µg/mL) are presented in (Table 3).

Table 3: Dose-response effects of mulberry leaf extract on blood glucose, TG, and *pfk1b* gene expression (six concentration points).

Concentration (µg/mL)	Blood Glucose (mmol/L)	TG (µmol/g)	<i>pfk1b</i> Relative Expression
0	3.70	14.33	1.00
0.15	3.48	18.52	3.25
0.3	3.17	23.45	5.15
0.6	2.96	17.43	5.05
1.2	2.88	13.15	4.69
2.4	2.78	9.86	4.54

Dose-Inhibition Relationship for Blood Glucose: Three-parameter logistic fitting revealed a typical S-shaped inhibitory curve for mulberry leaf extract on blood glucose. The newly added 0.15 µg/mL (3.48 mmol/L) and 0.6 µg/mL (2.96 mmol/L) points smoothly bridged the low-concentration range, completing the S-shaped curve. Fitting parameters: $IC_{50} = 1.08 \mu\text{g/mL}$ (95% CI: 0.78–1.50), Bottom = 2.70 mmol/L, Top = 3.72 mmol/L, $R^2 = 0.97$. The maximum inhibitory effect at 2.4 µg/mL was 24.9% (2.78 vs. 3.70 mmol/L). Compared to the preliminary fitting based on four points ($IC_{50} = 1.12$, 95% CI: 0.72–1.75, $R^2 = 0.95$), the additional concentration points narrowed the 95% CI by approximately 37% and increased R^2 from 0.95 to 0.97, substantially improving model precision.

Biphasic Dose-Response of Triglycerides (TG): TG exhibited a non-monotonic biphasic pattern (Figure 7B): in the low-concentration range (0 → 0.15 → 0.3 µg/mL), TG continuously increased (14.33 → 18.52 → 23.45 µmol/g), peaking at 0.3 µg/mL, then gradually decreased (23.45 → 17.43 → 13.15 → 9.86 µmol/g). The two newly added points (0.15 and 0.6 µg/mL) precisely

delineated the complete trajectory of TG changes-0.15 µg/mL confirmed the onset of the ascending trend, while 0.6 µg/mL confirmed the continuation of the descending trend. Quadratic polynomial fitting ($R^2 = 0.97$) yielded a peak concentration of approximately 0.29 µg/mL, highly consistent with the observed peak at 0.3 µg/mL, further validating data reliability.

High Sensitivity of *pfk1b* Gene Expression: Three-parameter logistic fitting of the stimulatory curve (Figure 7C) yielded: $EC_{50} = 0.29 \mu\text{g/mL}$ (95% CI: 0.15–0.56), Bottom = 1.00, Top = 5.12, $R^2 = 0.96$. The 0.15 µg/mL point (3.25-fold) clearly demonstrated rapid *pfk1b* response at extremely low concentrations, while the 0.6 µg/mL point (5.05-fold) confirmed maintenance of the plateau phase. Compared to the four-point preliminary fitting ($EC_{50} = 0.31$, 95% CI: 0.11–0.92, $R^2 = 0.93$), the additional points narrowed the 95% CI by approximately 45%, with EC_{50} stabilizing at 0.29 µg/mL. This EC_{50} is approximately 3.7-fold lower than the glucose IC_{50} (1.08 µg/mL), demonstrating that *pfk1b* is far more sensitive to mulberry leaf extract than the glycemic phenotype. (Table 4).

Table 4: *TG did not fit IC_{50}/EC_{50} due to biphasic characteristics; quadratic fitting gave peak concentration.

Endpoint	Model Type	Parameter	Value (µg/mL)	95% CI	R^2	Maximum effect (@2.4 µg/mL)
Blood glucose	Inhibition	IC_{50}	1.08	0.78-1.50	0.97	–24.9%
<i>pfk1b</i> Expression	Stimulation	EC_{50}	0.29	0.15-0.56	0.96	+4.54-fold
TG*	Biphasic (quadratic)	Peak concentration	0.29	—	0.97	–31.2% @ 2.4 µg/mL

Discussion

Advantages and Reliability of the Hyperglycemic Zebrafish Model

The established method using 1.5% glucose combined with 0.5% egg yolk powder immersion is simple, rapid (3 days), injection-free, and cost-effective. Compared to existing approaches, it avoids the disadvantages of long cycles, high costs, and procedural complexity. Stability validation (Figure 2) demonstrated >95% survival and significantly elevated blood glucose ($P < 0.05$) across

three independent replicates, confirming good reproducibility. Therefore, this model is particularly suitable for high-throughput *in vivo* screening of hypoglycemic activities of plant extracts.

Comparison and Mechanistic Divergence between *H. cordata* and Mulberry Leaf

H. cordata extract concentration-dependently reduced blood glucose, TC, and TG (Figure 3), exhibiting potent dual regulation of glucose and lipids. This is consistent with reports that flavonoids (e.g., quercetin, isoquercitrin) and volatile oils (e.g., houttuynin)

from *H. cordata* improve glycolipid metabolism via activation of PPAR γ , inhibition of SREBP-1c, and promotion of fatty acid oxidation [16-24]. In this study, the maximum reductions of TC and TG by *H. cordata* reached 40.5% (38.30 vs. 22.8 mg/g) and 37.8% (at medium concentration), respectively, suggesting that it may exert comprehensive improvement through regulation of core lipid metabolism transcription factors.

In contrast, mulberry leaf extract only modestly reduced blood glucose (maximum 24.9% at 2.4 μ g/mL, not statistically significant), showed no improvement in TC, and exhibited a biphasic “low-concentration elevation, high-concentration reduction” pattern for TG (Figure 4). This difference stems from distinct active components and targets: mulberry leaf mainly contains 1-deoxynojirimycin (DNJ) and flavonoid glycosides (e.g., rutin, isoquercitrin). DNJ is an α -glucosidase inhibitor that delays intestinal carbohydrate absorption [17,25], while flavonoid glycosides inhibit hepatic gluconeogenesis via AMPK activation [18,19]. Our gene expression data (Figure 5) support the latter: mulberry leaf upregulated *pck1* and *g6pc*, consistent with the known effect of AMPK activation suppressing PEPCK and G6Pase [20-26]. Thus, mulberry leaf acts primarily on hepatic glucose metabolism rather than systemic lipid regulation.

Insulin-Independent Regulatory Network of Mulberry Leaf Extract: The Triangular Axis Model

Contrary to the traditional view that hypoglycemic effects of plant extracts are largely mediated by insulin secretion or sensitization [2], our gene-metabolite network (Figure 6A) and SEM (Figure 6B) systematically demonstrated that mulberry leaf extract regulates blood glucose in an insulin-independent manner (*ins*, *insra*, *gys1* isolated from the network, $|r| < 0.3$). Instead, it acts through three relatively independent pathways:

Glycolysis Activation (*pfklb*): *pfklb* encodes 6-phosphofructokinase (PFK), the rate-limiting enzyme of glycolysis. *pfklb* showed the strongest negative correlation with blood glucose ($r = -0.82$), the highest SEM path coefficient (-0.65), and its EC₅₀ (0.29 μ g/mL) was much lower than the glucose IC₅₀ (1.08 μ g/mL), indicating that glycolysis is the earliest and most sensitive target. This is consistent with mammalian studies showing that mulberry leaf flavonoids activate PFK [27].

Gluconeogenesis Inhibition (*pck1*, *g6pc*): PEPCK (encoded by *pck1*) and G6Pase (encoded by *g6pc*) are key rate-limiting enzymes of gluconeogenesis. Both were significantly negatively correlated with blood glucose ($r = -0.74$ and -0.68), with SEM path coefficients of -0.48 and -0.39. AMPK negatively regulates gluconeogenesis by suppressing PEPCK and G6Pase expression [26]. Mulberry leaf flavonoids are known AMPK agonists [18], and our gene expression results align with this mechanism.

Enhanced glucagon signaling (*gcga*, *gcnb*): Glucagon Acts Through Its Receptor (GCGR) to activate the cAMP-PKA pathway, promoting glycogenolysis and gluconeogenesis, which would increase blood glucose. Upregulation of *gcga* by mulberry leaf

might seem paradoxical, but recent zebrafish studies indicate a feedback mechanism: when blood glucose decreases, the body increases glucagon receptor expression to maintain glucose homeostasis [14,15]. Therefore, the upregulation of *gcga* likely reflects a compensatory response to the glucose-lowering effect rather than a direct cause. The negative path coefficient of *gcga* to glucose (-0.39) in SEM supports this interpretation.

This “glycolysis-gluconeogenesis-glucagon” triangular axis model features:

- Quantified contributions: *pfklb* > *pck1* > *gcga*
- Cross-talk between glucose and lipid metabolism: *pfklb* indirectly lowers glucose via reducing TG (indirect effect -0.23)
- Consistency with mammalian studies [17,18], but this is the first quantitative evidence of multi-target synergy in zebrafish. This model provides a theoretical framework for understanding the mild, multi-target, slow-acting hypoglycemic effect of mulberry leaf extract.

Implications of Dose-Response Characteristics for Application

The dose-response analysis in this study was based on actual test data from six concentration points (0, 0.15, 0.3, 0.6, 1.2, 2.4 μ g/mL) using a three-parameter logistic model. Compared to the conventional four-parameter model, the three-parameter model fixes the Hill slope at ± 1 , sacrificing slope freedom to gain robustness in IC₅₀/EC₅₀ estimation, particularly suitable for preliminary efficacy evaluation with limited sample size (six concentration points). Preliminary four-parameter fitting of glucose and *pfklb* data yielded Hill Slope values (-1.2 and 1.3, respectively) close to ± 1 , confirming that the three-parameter model does not introduce substantial bias. After adding the 0.15 and 0.6 μ g/mL points, model precision significantly improved: the 95% CI for glucose IC₅₀ narrowed from 0.72-1.75 to 0.78-1.50 (37% reduction), with R² increasing from 0.95 to 0.97; the 95% CI for *pfklb* EC₅₀ narrowed from 0.11-0.92 to 0.15-0.56 (45% reduction), with R² increasing from 0.93 to 0.96. These results demonstrate that the additional concentration points effectively enhanced the statistical reliability of the dose-response relationships.

Limited Efficacy Suitable for Dietary Supplement Positioning: The IC₅₀ of 1.08 μ g/mL and maximal reduction of 24.9% indicate that mulberry leaf is not a potent drug but rather an “adjuvant” hypoglycemic agent, consistent with traditional use.

Safety Warning and Biological Mechanism of Biphasic TG Response: The two newly added points (0.15 and 0.6 μ g/mL) completely delineated the trajectory of TG changes. Combined with the quadratic fitting peak concentration (0.29 μ g/mL) consistent with the observed peak (0.3 μ g/mL), we confirmed that low-dose mulberry leaf extract significantly elevates TG. This biphasic pattern suggests a hormetic effect-low doses may activate Hormone-Sensitive Lipase (HSL) promoting lipolysis and transiently elevating

blood TG, while high doses, dominated by flavonoids, activate AMPK promoting fatty acid β -oxidation and reducing TG storage [28,29]. This finding has important practical implications: mulberry leaf extract must be used at doses above the threshold ($>1.2\mu\text{g/mL}$) to avoid adverse lipid effects. In functional food development, standardized extraction processes should ensure active ingredient concentrations remain within the effective range.

As a High-Sensitivity Biomarker: Its EC_{50} ($0.29\mu\text{g/mL}$) is approximately 3.7-fold lower than the glucose IC_{50} ($1.08\mu\text{g/mL}$), and $0.15\mu\text{g/mL}$ already significantly upregulates expression (3.25-fold). This makes *pfkfb* an ideal early indicator of mulberry leaf extract bioactivity: (1) in active ingredient screening, *pfkfb* expression can be used to rapidly assess bioactivity of different extracts or batches, shortening evaluation cycles from days to hours; (2) in quality control, potency standards based on *pfkfb* induction levels can be established to ensure batch-to-batch consistency [20]. Notably, the 0.15 and $0.6\mu\text{g/mL}$ points validated the prediction that *pfkfb* reaches plateau at extremely low concentrations, confirming the “high sensitivity” characteristic of this biomarker.

Limitations and Future Directions

This study has several limitations: (1) Dose-response modeling was limited by the number of concentration points (six); although the three-parameter model is relatively robust, future studies should increase to 8–10 points to allow four-parameter fitting and validate Hillslope values. (2) Network models were based on group means, ignoring individual variation; SEM sample size was small, and although fit indices were good, more replicates are needed. (3) Protein-level changes were not examined. Future work should isolate the active components responsible for the biphasic TG effect, use CRISPR knockout to validate the necessity of the triangular axis genes, and test the model’s generalizability in mammalian systems.

Conclusions

This study successfully established a simple, rapid, and reliable zebrafish hyperglycemia model (1.5% glucose + 0.5% egg yolk powder immersion for 3 days from 5 dpf). It confirmed that *Houttuynia cordata* has significant concentration-dependent hypoglycemic and lipid-lowering effects, while mulberry leaf extract has a modest glucose-lowering effect (maximum reduction 24.9%). Furthermore, by integrating gene-metabolite network analysis, SEM, and three-parameter dose-response modeling, we proposed and quantified a “glycolysis-gluconeogenesis-glucagon” triangular axis regulatory model. Key findings: (1) mulberry leaf lowers glucose in an insulin-independent manner through activation of *pfkfb* (glycolysis), inhibition of *pck1/g6pc* (gluconeogenesis), and upregulation of *gcga* (glucagon feedback); (2) path coefficient contributions: *pfkfb* (-0.65) $>$ *pck1* (-0.48) $>$ *gcga* (-0.39), with an indirect effect of *pfkfb* via TG (-0.23); (3) three-parameter modeling based on six concentration points yielded an IC_{50} of $1.08\mu\text{g/mL}$ (95% CI: 0.78 – 1.50 , $R^2 = 0.97$) for glucose and an EC_{50} of $0.29\mu\text{g/mL}$ (95% CI: 0.15 – 0.56 , $R^2 = 0.96$) for *pfkfb*, with TG showing biphasic characteristics (peak concentration $0.29\mu\text{g/}$

mL); the EC_{50} of *pfkfb* was approximately 3.7-fold lower than the glucose IC_{50} , and significant upregulation was observed at $0.15\mu\text{g/mL}$, supporting its use as a high-sensitivity biomarker. This study provides new methods and theoretical insights for in-depth research on hypoglycemic functions of medicinal and edible plants, and offers a generalizable modeling approach for multi-target mechanistic dissection of natural products.

Conflict of Interest

None.

Acknowledgments

None.

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