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Research article

# Stilbenoids from fenugreek seeds alleviate insulin resistance by regulating the PI3K/AKT/mTOR signaling pathway in a type 2 diabetes zebrafish model

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ABSTRACT

Insulin resistance (IR) is the main cause of type 2 diabetes mellitus (T2DM). The specific targets and underlying mechanisms responsible for the ameliorative effects of the stilbenoid compounds found in fenugreek seeds for ameliorating IR require further study. Here, we were predicted by using the network pharmacology prediction, molecular docking and molecular dynamics simulation approach the targets in common and the potential mechanismsof three stilbenoid compounds (rhaponticin, desoxyrhaponticin, and rhapontigenin) in relation to T2DM and IR. The results showed that the compounds may improve IR through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. Molecular docking studies revealed that they exhibit high binding affinity with the structural domains of peroxisome proliferator-activated receptor gamma (PPARG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PI3K, and AKT. These results suggest that PPARG and GAPDH may be the potential targets for these three compounds in the treatment of T2DM.Subsequently, experiments using the zebrafish T2DM model showed that the stilbenoid compounds had varying degrees of efficacy in improving IR through the PI3K/AKT/mTOR signaling pathway, and rhaponticin had the most promising effects. The findings implicate a potential mechanism of action for the three stilbenoid compounds in enhancing insulin resistance (IR) through modulation of the PI3K/AKT/mTOR pathway.

Abbreviations

| | |
|---|---|
| T2DM | Type 2 diabetes mellitus |
| IR | Insulin resistance |
| GO | Gene Ontology |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| RHAc | Rhaponticin |
| dRHAc | Resoxyrhaponticin |
| RHAg | Rhapontigenin |
| TG | Triglyceride |
| TC | Total cholesterol |
| HDL-C | High-density lipoprotein cholesterol |

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(*continued*)

| | |
|---|---|
| LDL-C | Low-density lipoprotein cholesterol |
| BCA | Bicinchoninic acid |
| PPI | Protein–protein interaction |
| PBS | Phosphate buffered saline |
| 2-NDBG | 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose |
| qRT–PCR | Real-time fluorescence quantitative analysis |
| AKT | Protein kinase B |
| PPARG | Peroxisome proliferator-activated receptor gamma |
| ERBB2 | Erythroblastic oncogene B2 |
| MMP9 | Matrix metalloproteinase 9 |
| PTGS2 | Prostaglandin-endoperoxide synthase 2 |
| SRC | Nonreceptor tyrosine kinase |
| HSP90AA1 | Heat shock protein 90 alpha family class A member 1 |
| ESR1 | Estrogen receptor 1 |
| BCL2 | B-cell lymphoma 2 |
| EGRF | Epidermal growth factor receptor |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| PI3K | Phosphatidylinositol 3-kinase |
| mTOR | Mammalian target of rapamycin |
| INS | Insulin |
| GCG | Glucagon |

## 1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from a complex interplay of various contributing factors [1]. The global prevalence of T2DM is substantial, accounting for approximately 90 % of all reported cases of diabetes [2]. Data provided by the International Diabetes Federation indicate that China has the highest prevalence of diabetes cases globally. The annual mortality rate in China due to diabetes and its associated complications is estimated to be approximately 10,000 individuals. Furthermore, there has been a significant rise in the prevalence of T2DM among the younger population [3].

The primary etiological factors contributing to T2DM are insulin resistance (IR) and impaired insulin secretion [4]. Currently, the pharmacological interventions used to manage T2DM include metformin, pioglitazone, sulfonylureas, and thiazolidinediones. However, these medications often have a variety of adverse reactions, such as hypoglycemia, lactic acidosis, peripheral edema, severe hepatotoxicity, and gastrointestinal complications due to prolonged administration. Therefore, novel hypoglycemic agents with potent glucose-lowering activity and minimal adverse effects are needed [5,6].

Fenugreek (*Trigonella foenum-graecum* L.) is a leguminous plant that is recognized as a valuable resource with dual applications in both food and medicine [7]. The Chinese Pharmacopoeia documents that fenugreek seeds possess the therapeutic properties of 'kidney warming, yang support, cold dispelling, and pain relief [8].' Fenugreek is rich in diverse bioactive constituents, including stilbenoids, flavonoids and coumarins [9]. The stilbenoid compound is classified as a polyhydroxyphenolic compound, wherein the basic parent nucleus consists of stilbenoid and hydroxyl groups replace different positions on the benzene ring [10]. Modern pharmacological studies have revealed that stilbenoid compounds exhibit a diverse range of biological and pharmacological activities, encompassing antioxidant [11], hypoglycemic [12], anti-inflammatory [13], and anticancer effects [14].

Previously, we successfully isolated three stilbenoids (Rhaponticin (RHAc), Desoxyrhaponticin (dRHAc), and Rhapontigenin (RHAg)) from fenugreek seeds using high-speed countercurrent chromatography [15]. These components exhibit the potential to enhance insulin sensitivity and mitigate mitochondrial dysfunction in 3T3-L1 adipocytes, while also demonstrating varying degrees of inhibition on the expression of proteins associated with adipocyte differentiation and lipid accumulation, ultimately leading to improved glucose uptake [7]. However, the precise mechanism underlying this improvement remains unclear.

The application of computer simulation technology has played a pivotal role in drug design in recent years. Molecular docking and molecular dynamics simulation technology provide important tools for revealing the potential mechanism of drug and receptor targets, exploring the spatial structure of drug targets and identifying disease-related non-synonymous single nucleotide polymorphisms (nsSNPs), and provide useful reference and theoretical support for further experiments [16–18]. In this study, network pharmacology prediction, molecular docking and molecular dynamics simulation were used to obtain the potential mechanisms of three stilbenoid compounds. Then, we established a zebrafish T2DM model by administering a high - fat diet combined with glucose induction. The in vivo evaluation of three stilbenoid compounds was conducted to assess their effects on glucose and lipid metabolism as well as insulin resistance, while investigating the potential mechanisms underlying their impact on T2DM.Our results provide valuable insights for future disease treatment and drug development.

## 2. Materials and methods

### 2.1. Network pharmacology analysis

#### 2.1.1. Acquisition of stilbenoids for disease targeting

The SMILES(Simplified Molecular Input Line Entry System) identifiers for RHAc, dRHAc, and RHAg were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). These identifiers were subsequently entered into the SwissTargetPrediction database (http://www.swis-stargetprediction.ch) to predict their potential targets. Target genes related to T2DM and IR were identified by searching the GeneCards database (https://www.ge-necards.org/) and OMIM database (https://www.omim.org/) using keywords "type 2 diabetes" and "insulin resistance." The targets were integrated in Excel, and the duplicated genes were eliminated. The resulting combined targets represent the disease targets of this study. We employed Venny software (https://bioinfogp.cnb.csic.es/tools/venny/) to identify the intersection between medicine active compound targets and disease targets, thereby identifying potential key targets for treating the disease among medicine compound.

#### 2.1.2. Protein–protein interaction (PPI) network construction and network topology analysis

The common targets of compounds and diseases were imported into the STRING database (https://string-db.org/), the object was set to (homo sapiens), the highest confidence was 0.900, and the free gene node was hidden to obtain the protein interaction relationship. Subsequently, these results were imported into Cytoscape 3.9.0 (National Institute of General Medical Sciences, Bethesda, MD, USA) to obtain network topology parameters. Finally, the top 10 core target genes were selected based on their degree values. The Degree value represents the protein interconnections, and the selected core gene occupies the most central position in the PPI graph.

#### 2.1.3. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses

The GO enrichment and KEGG pathway enrichment analyses were conducted using the open-source bioinformatics software Bioconductor (http://www.bioc-onductor.org/).ClusterProfiler,Stringin and Pathview packages were installed and run in the R software, and the GO and KEGG function enrichment analysis of biological processes was performed, and the visual display was performed through the wechat platform. The GO analysis was divided into three parts, The roles of target proteins in gene Function were annotated from three aspects: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). KEGG analysis was mainly pathway analysis, and the purpose was to clarify the main signaling pathways of drug treatment of diseases. Visualization was performed using bioinformatics platf-orms to elucidate the potential mechanisms underlying the effects of the three stilbenoids on T2DM.

#### 2.1.4. Molecular docking simulation

The macroscopic protein target receptors were obtained from the RCSB PDB database (http://www.rcsb.org/), and the small molecule compounds were acquired from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking simulations were performed using Discovery Studio (version 19.1, Accelrys Inc., San Diego, CA, 2019). The docking protein and small molecule files were respectively inputted into pymol for visualization of the results, while 2D rendering of the docking outcomes was obtained using Discovery Studio.

### 2.2. Zebrafish maintenance

Adult AB strain zebrafish were procured from Hubei Chuangxin Biotechnology Co., Ltd. (Wuhan, China). The zebrafish used in this study were maintained in a specialized water recirculation system specifically designed for zebrafish housing (Beijing Aisheng Technology Development Co., Ltd., Beijing, China). The water temperature was maintained within the range of 26–28 °C, the pH levels were carefully controlled at 7.0, and the light/dark cycle was 14/10 h.

Zebrafish embryos obtained through natural pairwise mating were cultured in E3 medium (Shanghai Feixi Biotechnology Co., Ltd., Shanghai, China) at a constant temperature of 28 °C under a continuous light source. All zebrafish procedures were ethically approved by the Animal Ethics Committee of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

### 2.3. Establishment of the T2DM model in zebrafish

Hyperglycemia in zebrafish was induced by administering a high-sugar and high-fat diet. glucose were purchased from Shanghai Yuan Ye Biotechnology Co., Ltd. (Shanghai, China). Wild-type adult zebrafish aged 5–6 months were randomly selected, encompassing both males and females. Fish in the experimental group were subjected to alternate soaking in glucose solutions of 2 % and 3 % and fed a high-fat diet four times daily, and water was refreshed every 12 h. Fish in the control group were immersed in standard system water and provided with a regular diet. The experiment was conducted over a duration of 14 days.

Zebrafish larvae, specifically those that were 20 days post-fertilization, were carefully selected for this study. Larvae in the experimental group underwent alternating immersion in 2 % and 3 % glucose solutions and were fed a high-fat diet administered four times daily, and water renewal occurred every 12 h. Larvae in the control group were cultured in standard system water and fed a regular diet. The experiment was conducted over a duration of 10 days.

The common fish feed and 10 % (w/w) cholesterol were dissolved in diethyl ether through stirring for the preparation of a high-fat diet. Subsequently, complete evaporation of the diethyl ether was performed to obtain a concentrated high-cholesterol diet.

### 2.4. Therapeutic intervention using stilbenoids

After successfully establishing the zebrafish T2DM model, adult zebrafish were anesthetized with tricaine. The treatment group included the positive control metformin and three stilbenoids compounds (RHAc, dRHAc and RHAg). Eight fish were included in each group. After anesthesia, one group of fish received an intraperitoneal injection of metformin (Biotechnology Co., Ltd. ,Shanghai, China)(positive control) at a dose of 5 mg/kg. Fish in the three treatment groups received intraperitoneal injections of RHAc, dRHAc, or RHA at doses ranging from 2.5 to 100 mg/kg (10 μL per fish). Fish in the control group received an injection of 0.9 % NaCl solution. In the zebrafish larvae T2DM model, larvae were exposed to a solution containing 50 μM metformin (positive control) [19] or 2.5–100 μM of the three stilbenoids. Each experimental group consisted of thirty zebrafish larvae. The treatment duration for both adult zebrafish and larvae was 6 days.

### 2.5. Measurement of biochemical parameters

At the conclusion of the experiment, zebrafish were subjected to a 12 h overnight fasting period prior to sampling. Subsequently, they were anesthetized and euthanized. Following death, adult fish were promptly extracted to drain excess water from their bodies. Muscular tissue was extracted in a controlled low-temperature environment, and tissues loaded in 2 mL EP tubes were immediately stored in liquid nitrogen. After being euthanized, zebrafish larvae were washed three times with phosphate buffered saline (PBS) and gently blotted dry before being assembled into batches of 10 larvae and immediately stored in liquid nitrogen.

The muscle tissue and larvae were homogenized at a low temperature, weighed, and added to ice-cold PBS (pH = 7.4) at a solid/liquid ratio of 1:9. Following homogenization, the fish samples were centrifuged at 4 °C for 15 min at 12,000 rpm. The supernatant was used for the quantification of biochemical parameters, including glucose, TG, TC, HDL-C and LDL-C(Nanjing Jiancheng Bioengineering Institute,Nanjing, China) contents, using corresponding assay kits. Protein content was determined using the bicinchoninic acid method.

### 2.6. Oil Red O and 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NDBG) analysis

At the experimental endpoint, zebrafish larvae were subjected to a 24 h fasting period, followed by anesthesia and fixation in 4 % paraformaldehyde at 4 °C for 12 h. Subsequently, the samples were rinsed with PBS and treated with a 60 % isopropanol solution for 30 min to enhance permeability. The samples were stained with Oil Red O solution for 12 h in the dark [20]. Following staining, the stained larvae were subjected to a gentle wash with 60 % isopropanol for a duration of 2–3 min in order to eliminate any residual coloration. Subsequently, the larvae were observed under a dissecting microscope. (Shunyu Optical Technology Co., LTD., Ningbo, China).

For 2-NBDG staining, zebrafish larvae were incubated with a solution containing 40 μM 2-NBDG for a duration of 60 min, followed by thorough washing using a saline solution (0.9 %) for a period of 20 min [21]. Subsequently, the stained larvae were visualized, and images were captured using a fluorescence microscope (Shunyu Optical Technology Co., Ltd., Zhejiang, China).

### 2.7. Histopathological analysis

Fresh liver tissue from adults was fixed with 4 % paraformaldehyde tissue fixative solution for more than 24 h. After removing the tissue from the fixative, we trimmed the target tissue with a scalpel in the ventilation cupboard and placed each labeled sample in a dehydration box. Specimens were embedded in paraffin by graded dehydration in ethanol. Subsequently, 3 μm sections were

**Table 1**
Primers used for qRT−PCR.

| Gene name | Upstream and downstream primer sequences |
|---|---|
| ***ACT F*** | ***5′-CATCAGGGTGTCATGGTTGGT-3′*** |
| ***ACT R*** | ***5′-TCTCTTGCTCTGAGCCTCATCA-3′*** |
| ***INS F*** | ***5′-GAGCCCCTTCTGGGTTTCC-3′*** |
| ***INS R*** | ***5′-AAGTCAGCCACCTCAGTTTCCT-3′*** |
| ***INSRA F*** | ***5′-GGAGCCCCACTCGTCTAACAAA-3′*** |
| ***INSRA R*** | ***5′-CGCCGTTGTGAATGACGTATTC-3′*** |
| ***GCG F*** | ***5′-AAGCGAGGAGACGATCCAAA-3′*** |
| ***GCG R*** | ***5′-TCCAACACACACCAGCAAATG-3′*** |
| ***PI3K F*** | ***5′-GAGATTTTCTCGGCCCTGGCT-3′*** |
| ***PI3K R*** | ***5′-ACTCTTCCCATCTGTGTGAGGC-3′*** |
| ***AKT2 F*** | ***5′-CGGAGGTCCTGAAGATGC-3′*** |
| ***AKT2 R*** | ***5′-CTTGAAGGGTGGAACGAG-3′*** |
| ***mTORC1 F*** | ***5′-GAAGGTGGAAGTGTTTGAGC-3′*** |
| ***mTORC1 R*** | ***5′-TAGCGAGCGTGTGTAGTTG-3′*** |
| ***PPARG F*** | ***5′-GCCATCAGCGAAGTCAC-3′*** |
| ***PPARG R*** | ***5′-CAGGGTCCCGTCTTTATT-3′*** |
| ***GAPDH F*** | ***5′-GATGGTCATGCAATCACAGTCTA-3′*** |
| ***GAPDH R*** | ***5′-ATCATACTTGGCAGGTTTCTCAA-3′*** |

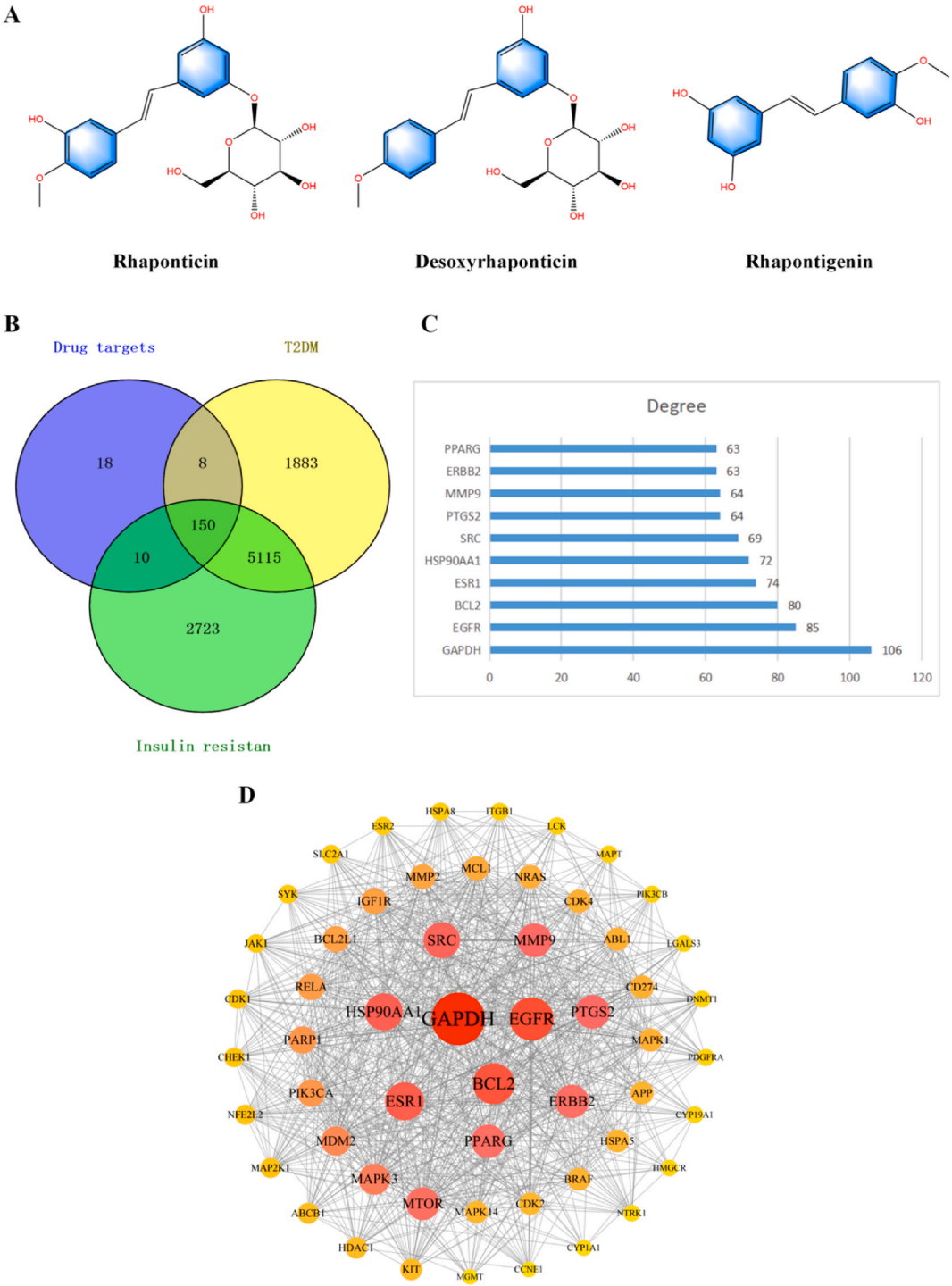



**Fig. 1.** Network pharmacology prediction.(A) The structural formula of RHAc, dRHAc and RHAg.(B)Direct targets of RHAc, dRHAc and RHAg.(C) PPI and network topology analysis.(D)Degree value of the top ten targets. (E)GO enrichment analysis.(F)KEGG pathway analysis.(G)Network of 'T2DM-Stilbenoids-target-pathway'.

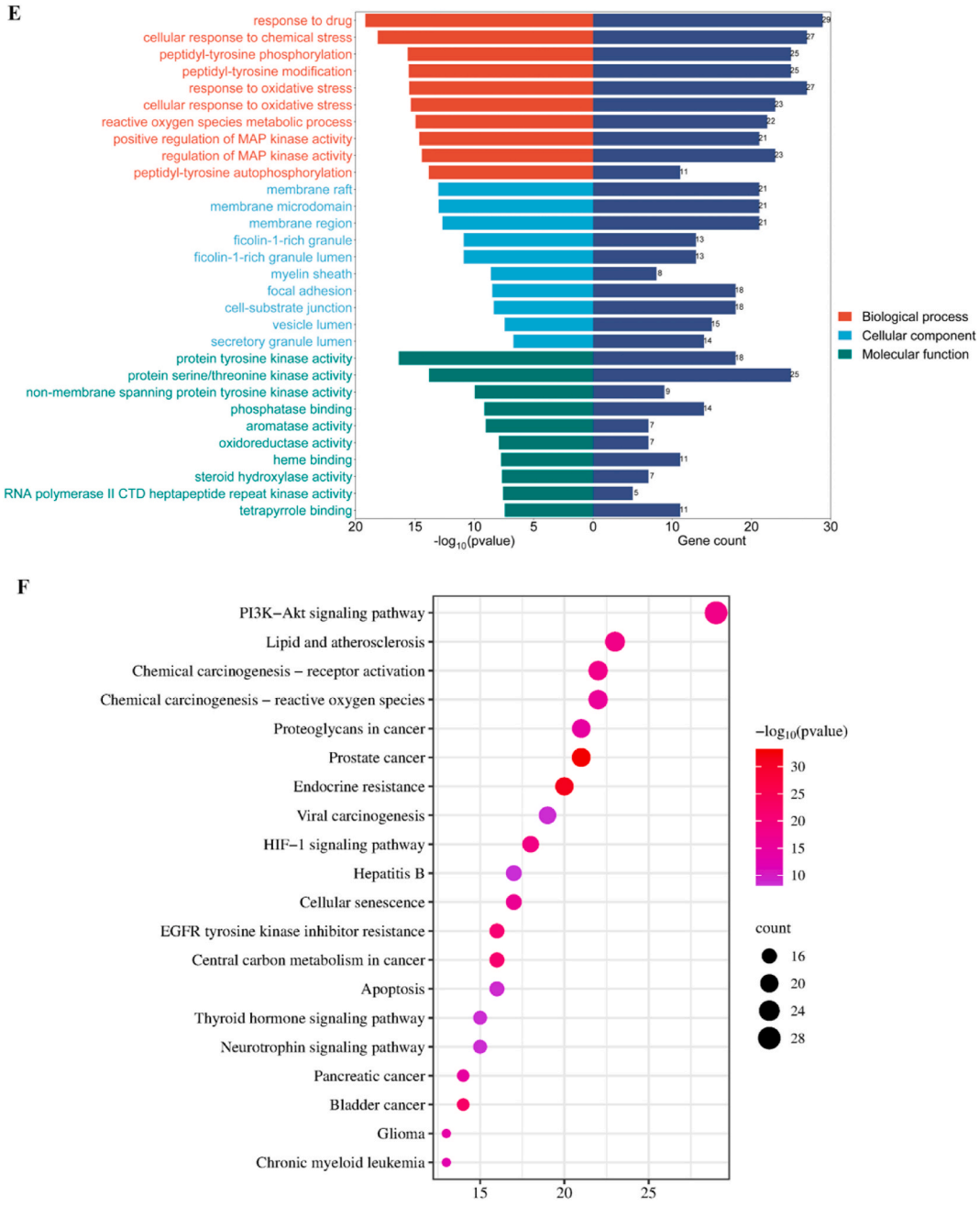



**Fig. 1.** (*continued*).

rehydrated and stained with hematoxylin and eosin. Photographic recordings were made using a Nikon Eclipse 80i microscope (Nikon, Japan).

### 2.8. Real-time fluorescence quantitative analysis (qRT−PCR)

Zebrafish larvae were euthanized. To further assess the impact of the three compounds on insulin resistance, we subsequently compared the mRNA expression levels of *actin(ACT, as a reference gene), insulin(INS), insra(INSRA) and glucagon(GCG)*. Furthermore, we examined and compared the mRNA expression levels of phosphatidylinositol 3-kinase (*PI3K),* protein kinase B (*AKT2), mammalian target of rapamycin (mTORC1), peroxisome proliferator-activated receptor gamma (PPARG), and* glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* in each group to gain deeper insights into the underlying mechanisms of these candidate drugs. After sampling,a small amount of corresponding zebrafish larvae from each group was collected and transferred into a 2 mL centrifuge tube.

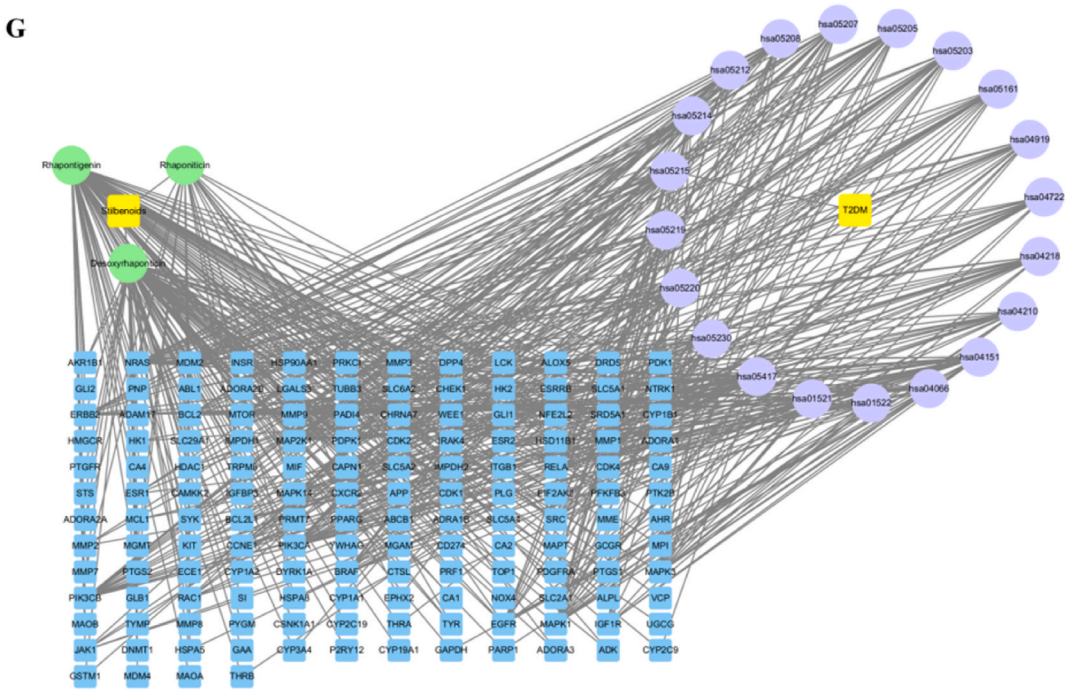



**Fig. 1.** (*continued*).

Subsequently, TRIzol (Takara, Beijing, China) reagent was added for grinding and lysis, followed by mRNA extraction in accordance with the manufacturer's instructions. The concentration of the RNA extraction liquid was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription for cDNA synthesis was performe using a reverse transcription kit (Pars Toos, Mashhad, Iran). Adding the ChamQ Universal SYBR qPCR Master Mix (Vazyme,Nanjing, China),qRT−PCR wasconducted in a 96-well plate. Primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China) (Table 1).

### *2.9. Western blot analysis*

We compared the expression levels of target proteins between control zebrafish larvae and stilbenoids-treated zebrafish larvae to assess the effects of treatment with the drug candidates. Approximately 10 mg of each sample was extracted and homogenized in 100 μL of protein lysis buffer. After 30 min, the mixture was centrifuged at 12,000 rpm for 10 min. The resulting supernatant was collected, and its protein concentration was measured using the BCA(Biyuntian Biotechnology Co., Ltd, Shanghai, China) assay. Proteins were adjusted to the same concentration and later loaded onto 8 % SDS−PAGE gels and then transferred to polyvinylidene difluoride membranes. After transmembrane transfer, the cells were blocked in 5 % skim milk for 1 h before incubation with primary antibodies against PPARG,AKT, *p*-AKT,PI3K,p-PI3K,mTOR,p-mTOR and GAPDH at 4 °C, followed by incubation with secondary antibodies for 1 h at room temperature.

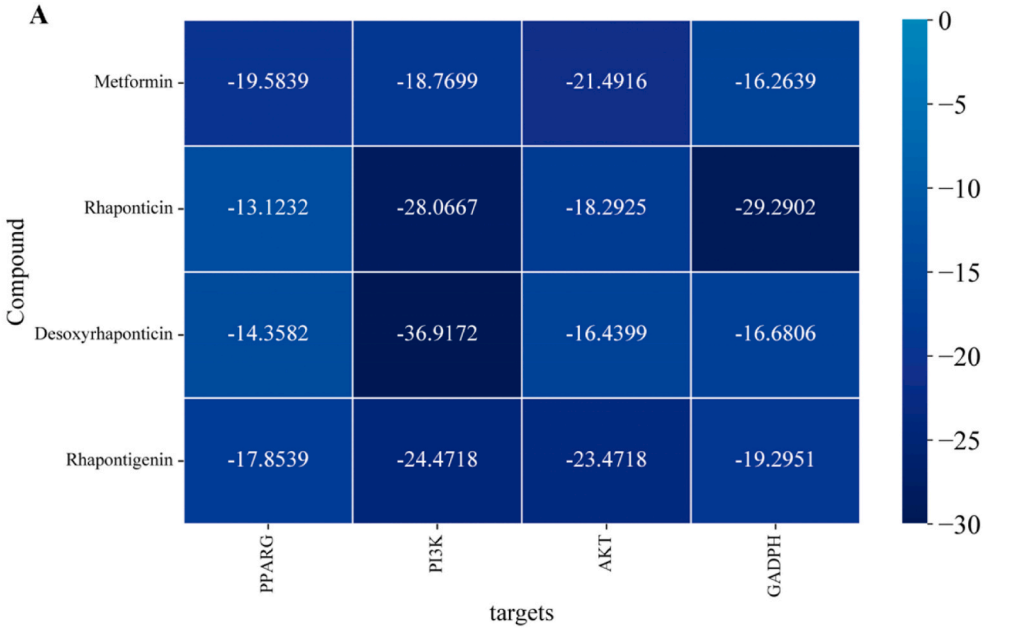
### *2.10. Statistical analysis*

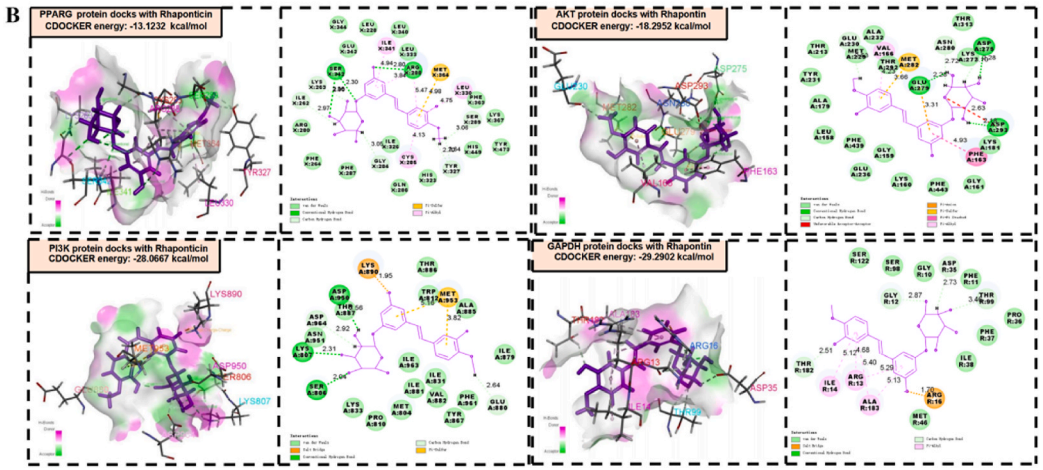
Experimental data were analyzed using GraphPad Prism 8.0 software (San Diego, CA, USA). All the data are presented as the mean standard deviation (ax ± s). T test or one-way analysis of variance was used for comparisons among multiple groups. A significance level of $P < 0.05$ was considered statistically significant.

## 3. Results

### *3.1. Network pharmacology analysis*

The structural formula of RHAc, dRHAc and RHAg were shown in Fig. 1A. Based on the screening results obtained from the database, we found 7156 target genes associated with T2DM and 7998 target genes related to IR. By integrating the target profiles of RHAc, dRHAc, and RHAg with the T2DM and IR targets, we identified 150 direct targets (Fig. 1B). The top 10 core targets were selected based on their degree values, and PPARG, erythroblastic oncogene B2 (ERBB2), matrix metalloproteinase 9 (MMP9), prostaglandin-endoperoxide synthase 2 (PTGS2), nonreceptor tyrosine kinase (SRC), heat shock protein 90 alpha family class A member 1 (HSP90AA1), estrogen receptor 1 (ESR1), B-cell lymphoma 2 (BCL2), epidermal growth factor receptor (EGRF), and GAPDH were


A
Metformin
Rhaponticin
Desoxyrhaponticin
Rhapontigenin
Compound
-19.5839
-18.7699
-21.4916
-16.2639
-13.1232
-28.0667
-18.2925
-29.2902
-14.3582
-36.9172
-16.4399
-16.6806
-17.8539
-24.4718
-23.4718
-19.2951
PPARG
PI3K
AKT
GADPH
targets
0
−5
−10
−15
−20
−25
−30


B
PPARG protein docks with Rhaponticin
CDOCKER energy: -13.1232 kcal/mol
AKT protein docks with Rhapontin
CDOCKER energy: -18.2952 kcal/mol
PI3K protein docks with Rhaponticin
CDOCKER energy: -28.0667 kcal/mol
GAPDH protein docks with Rhapontin
CDOCKER energy: -29.2902 kcal/mol

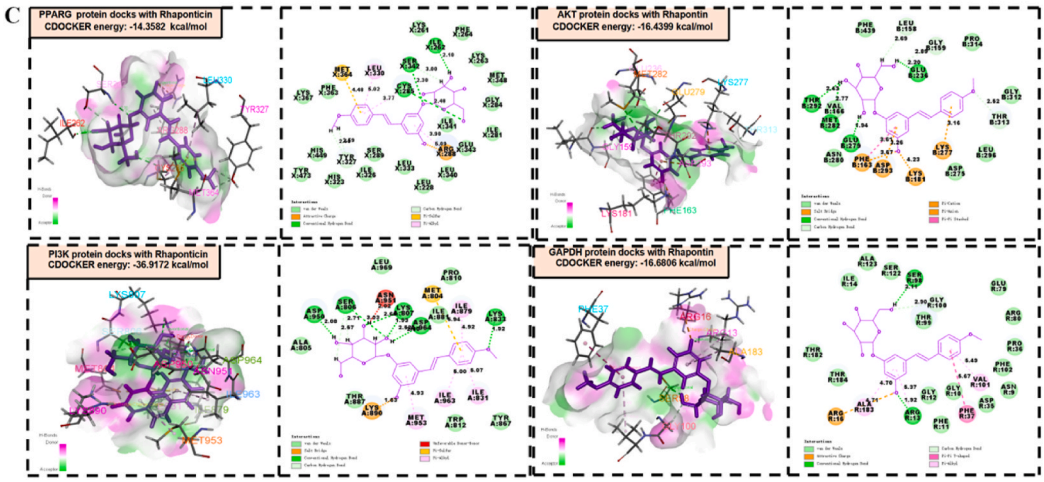

C
PPARG protein docks with Rhaponticin
CDOCKER energy: -14.3582 kcal/mol
AKT protein docks with Rhapontin
CDOCKER energy: -16.4399 kcal/mol
PI3K protein docks with Rhaponticin
CDOCKER energy: -36.9172 kcal/mol
GAPDH protein docks with Rhapontin
CDOCKER energy: -16.6806 kcal/mol

**Fig. 2.** Heat map of the optimal docking score between the individual compounds and target proteins based on the binding energy(A)Visualization of the molecular docking results of RHAc (B), dRHAc (C) and RHAg (D).

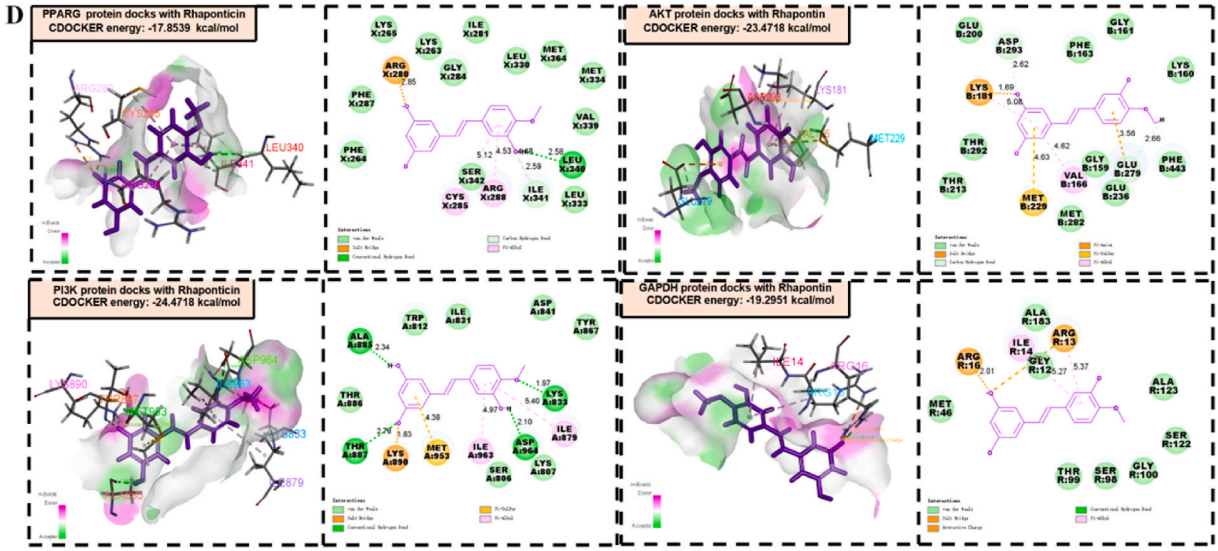



**Fig. 2.** (*continued*).

identified as common direct targets of the three stilbenoid compounds (Fig. 1C). Fig. 1D illustrates the PPI networks for RHAc, dRHAc, and RHAg.

In the GO enrichment analysis, 3787 biological processes, 326 cellular components, and 494 molecular functions key targets were identified. The top 20 entries were identified based on their statistical significance as determined by P values and gene counts. As depicted in Fig. 1E, BP primarily encompassed phenomena such as cellular response to chemical stress, response to oxidative stress, activation of protein kinase activity, regulation of reactive oxygen species metabolic process, and positive regulation of protein serine/threonine kinase activity. The main cellular components were membrane rafts, membrane microdomains, membrane regions, ficolin-1-rich granules, cytoplasmic vesicle lumens, apical parts of cells, cell projection membranes, and various protein complexes, including the cyclin-dependent protein kinase holoenzyme complex and the protein kinase complex. 'The molecular functions were predominantly protein tyrosine kinase activity, protein serine/threonine, nonmembrane spanning protein tyrosine kinase activity, tetrapyrrole binding, protein phosphatase binding, nuclear receptor activity, ligand-activated transcription factor activity, metalloendopeptidase activity, and oxygen binding.

In the KEGG enrichment analysis, 258 pathways were identified as being enriched. The pathways primarily associated with T2DM and its complications included the PI3K/AKT signaling pathway, lipid and atherosclerosis pathway, hypoxia-inducible factor 1 signaling pathway, endocrine resistance pathway, insulin signaling pathway, and T2DM pathway. Additionally, we observed pathways associated with apoptosis, cancer, and viral infections, such as bladder cancer, central carbon metabolism in cancer, hepatocellular carcinoma, acute myeloid leukemia, p53 signaling pathway, Kaposi sarcoma-associated herpesvirus infection and hepatitis C. Fig. 1F lists the top 20 representative pathways based on the enrichment gene count and P value. Fig. 1G illustrates the networks of 'T2DM-Stilbenoids-target-pathway'.

### 3.2. Molecular docking

Molecular docking is a widespread approach to observe receptor-ligand interactions. In general, a lower binding energy of the ligand to the receptor indicates a more stable bound conformation [22]. Based on the above PPI and KEGG prediction results, we selected PPARG (PDB ID:1FM6),PI3K(PDB ID:1E8Z),AKT (PDB ID:3D0E) and GADPH(PDB ID:1U8F) for molecular docking with RHAc, dRHAc, and RHAg. The selected compounds were subjected to analysis using the CDOCKER module in Discovery Studio 2019 software. The corresponding results are presented in Fig. 2(A-D). The three compounds exhibited favorable binding energies with all four proteins. The residue interactions of RHAc, dRHAc, and RHAg with PPARG, PI3K, AKT, and GAPDH were illustrated in Fig. 2(B-E). It can be seen from the figure that the three compounds mainly interact with the residues of the four proteins through van der Waals force, hydrogen bond and hydrocarbon bond. The interacting residues are shown in Table S1 in the Appendix. Differences in compounds and protein binding sites lead to alterations in the binding energy phenomenon. Among the three compounds, RHAc and dRHAc interacted with SER-324 in PPARG through hydrogen bonds, while RHAg interacted with SER-324 through van der Waals forces. We further conducted molecular dynamics (MD) simulations of the three compounds and PPARG protein (Fig. S1). Although the molecular docking results indicated a higher binding energy between RHAg and PPARG, the MD simulation results demonstrated that RHAc formed a more stable binding with PPARG, potentially accounting for its superior glucose-lowering effect.

### 3.3. Biochemical indicators in the adult zebrafish T2DM model

After establishing the zebrafish model of adult-onset T2DM, the concentration gradients of the three compounds were sequentially administered at doses of 2.5 mg/kg, 5 mg/kg, 10 mg/kg, 50 mg/kg, and 100 mg/kg in an ascending order (Fig. S2). Following comparison, the compounds concentration of 2.5 mg/kg was selecte. Consequently, after a six-day administration period, the optimal dosage was determined to be 2.5 mg/kg, we measured the levels of glucose, TC, TG, HDL-C, and LDL-C in adult zebrafish (Fig. 3A–E).

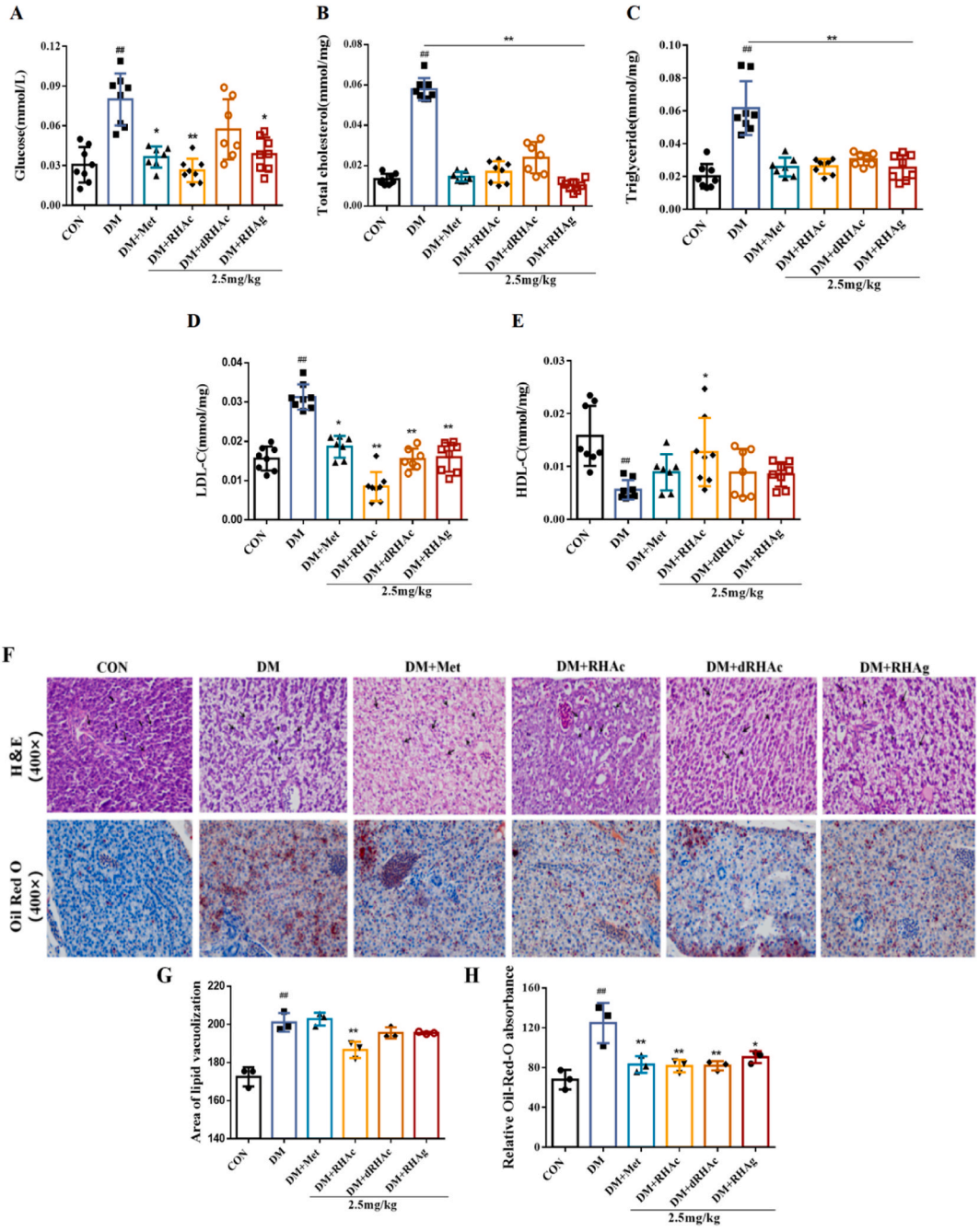



**Fig. 3.** (A) Glucose levels of adult zebrafish in each group. (B) TC levels of adult zebrafish in each group. (C) TG levels of adult zebrafish in each group. (D) LDL-C levels of adult zebrafish in each group. (E) HDL-C levels of adult zebrafish in each group. (F) Effect of stilbenoids on the liver tissue morphology and Oil red O staining of adult zebrafish.(G)Area of lipid vacuolization.(H)Relative absorbance of Oil-Red-O stain Values are expressed as the mean ± SD in each group. #$p < 0.05$ and ##$p < 0.001$ model group versus Con group. *$p < 0.05$ and **$p < 0.001$ treatment group versus model group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Adult zebrafish in the model group exhibited elevated levels of glucose, TC, TG, and LDL-C and lower levels of HDL-C compared to the control. These findings suggested the presence of glucose and lipid metabolism disorders in these zebrafish. In the RHAc, dRHAc, and RHAg groups all exhibited significantly reduced glucose, TC, TG, and LDL-C levels and increased HDL-C levels compared to the control group. These findings suggested that all three stilbenoids effectively ameliorated glucose and lipid metabolism disorders induced by high-sugar and high-fat conditions in zebrafish.

The liver tissue sections from adult zebrafish were subjected to HE and Oil red O staining to assess the histological and accumulation of lipids changes that occurred during the experiment (Fig. 3F–H). In the control group, the hepatic tissue exhibited normal morphology, which was characterized by closely arranged hepatocytes with centrally located spherical nuclei. In contrast, the T2DM group exhibited a significant presence of hepatocyte degeneration and vacuolization in the pathological sections. The hepatocytes exhibited an irregular arrangement, primarily attributed to the deposition of lipid droplets within the zebrafish liver tissue. However, after treatment with RHAc, dRHAc, or RHAg, the lipid droplets and vacuoles within the intercellular spaces of the liver cells exhibited varying degrees of reduction, leading to a more organized arrangement. Among the treatment groups, the livers of fish treated with RAHc exhibited reduced hepatotoxicity and minimal side effects. These results suggested that the three stilbenoids positively influenced liver tissue morphology by mitigating hepatocyte degeneration and enhancing overall structural integrity, with RHAc demonstrating superior efficacy compared to the other two compounds tested. The Oil red O staining results further demonstrated an increase in lipid accumulation in the liver tissue of the model group, which was significantly attenuated following administration of RHAc, dRHAc, and RHAg.

### 3.4. Biochemical indicators in the zebrafish larvae T2DM model

The T2DM model of zebrafish larvae was successfully induced by exposure to high glucose and high fat for a duration of 10 days, with the concentration gradient of the three compounds ranging from 2.5 μM to 80 μM (Fig. S3). Following comparison,a compound concentration of 5 μM was selected, after 6 days of administration of RHAc, dRHAc, or RHAg, larvae had decreased glucose, TC, TG, and LDL-C levels and increased HDL-C levels. Thus, all three stilbenoids ameliorated the glucose and lipid metabolism disorders induced by high-sugar and high-fat conditions in zebrafish larvae (Fig. 4A–E).

Following 2-NDBG staining, the fluorescence intensity in zebrafish larvae was primarily concentrated in the abdominal region. In comparison to the control group, the model group exhibited significantly lower fluorescence intensity. However, after treatment with metformin, RHAc, dRHAc, or RHAg, the green fluorescence intensity increased to varying degrees (Fig. 4F and G). This suggests that the stilbenoids enhanced the uptake and consumption of glucose in zebrafish larvae. RHAc resulted in a significant improvement in the uptake and consumption of glucose compared to the other compounds.

Whole-body Oil Red O showed that compared to the control group, zebrafish larvae in the model group exhibited deeper staining and increased lipid deposition in the liver and yolk sac. After administration of RHAc, dRHAc or RHAg, the staining of the yolk sac and

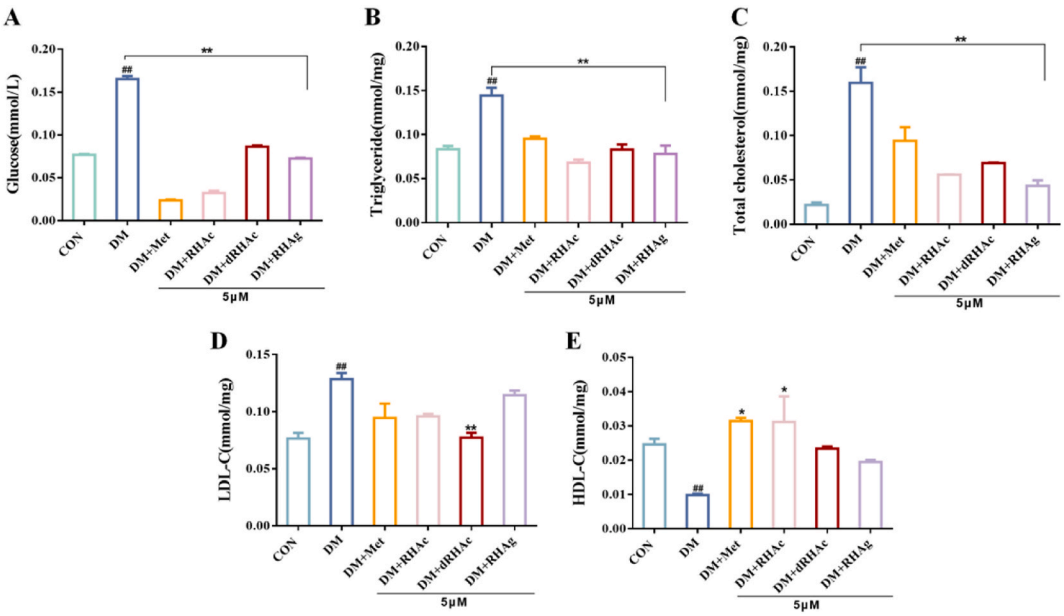



**Fig. 4.** (A) Glucose levels of zebrafish larvae in each group. (B) TG levels of zebrafish larvae in each group. (C) TC levels of zebrafish larvae in each group. (D) LDL-C levels of zebrafish larvae in each group. (E) HDL-C levels of zebrafish larvae in each group.(F)2-NDBG staining of zebrafish larvae. (G)Mean fluorescence intensity of 2-NDBG.(J)Whole-mount Oil red O staining of zebrafish larvae (the yellow area indicates the liver). Values are expressed as the mean ± SD in each group. #$p < 0.05$ and ##$p < 0.001$ model group versus Con group. *$p < 0.05$ and **$p < 0.001$ treatment group versus model group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

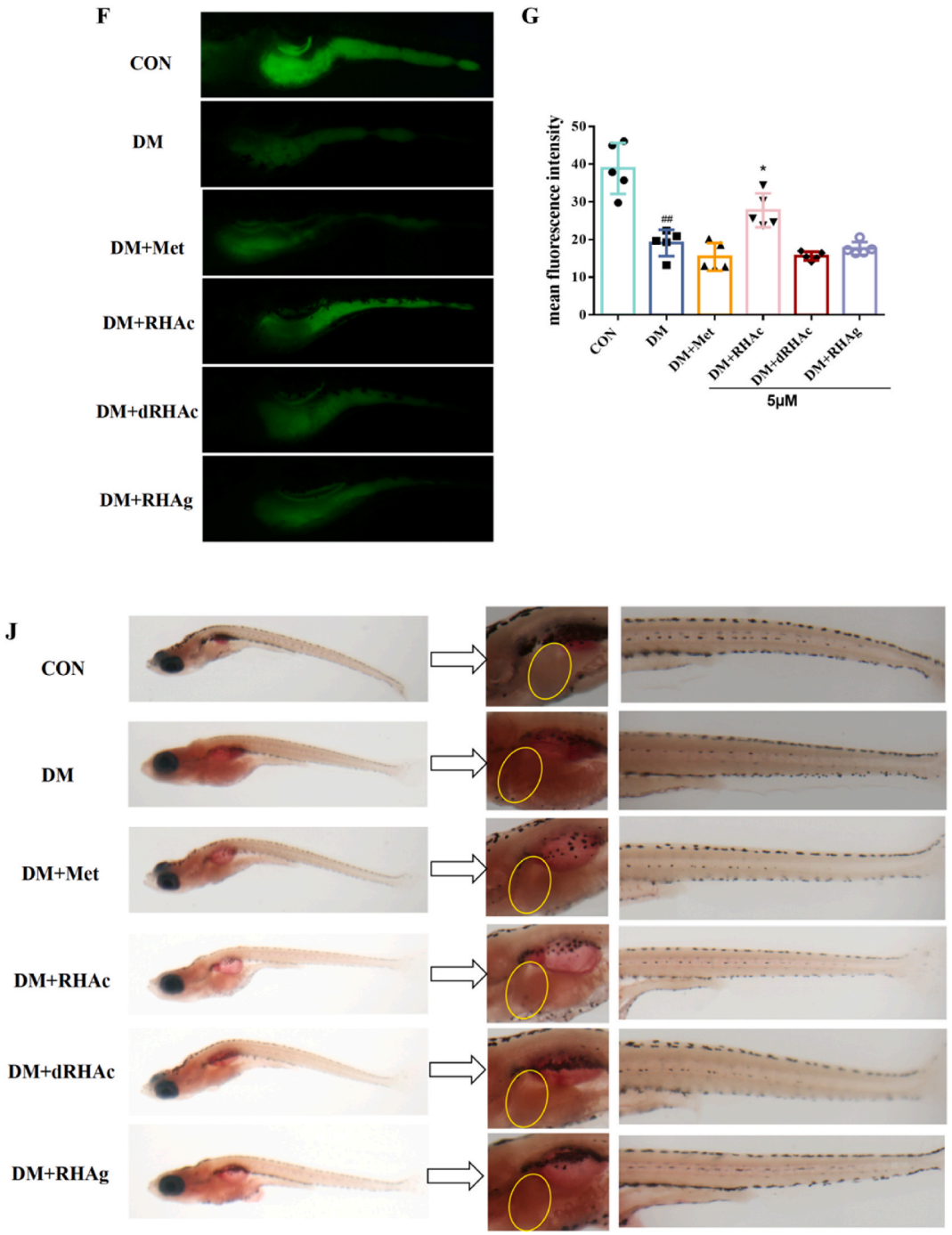



**Fig. 4.** (*continued*).

liver in the larvae decreased to different extents, indicating that the three stilbenoids reduced lipid accumulation (Fig. 4J).

### 3.5. RT-qPCR and western blotq analysis

The expression levels of *INS*, *INSRA* and *GCG* in zebrafish larvae exhibited an upward trend following induction with a high-sugar and high-fat diet (Fig. 5A–C). Surprisingly, under conditions of significantly elevated glucose levels in the body, *Glucagon* expression was upregulated despite its expected regulatory role during low glucose states [23]. This finding further substantiates the presence of aberrant glucose and lipid metabolism in zebrafish following exposure to a high-sugar and high-fat regimen. Following the

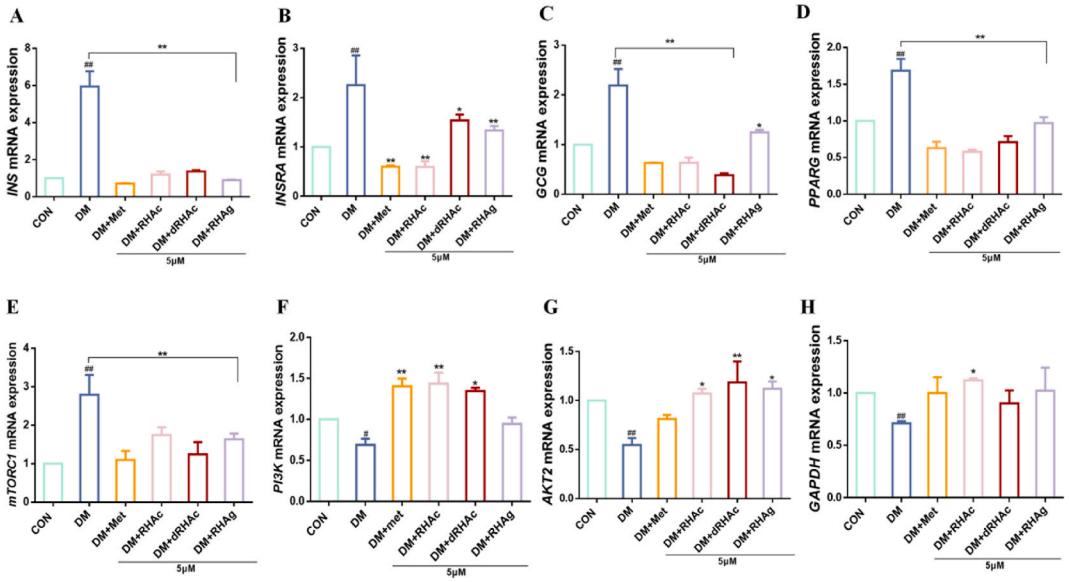



**Fig. 5.** Effect of stilbenoids on relevant gene expression.(A) mRNA expression of *INS* in zebrafish larvae.(B) mRNA expression of *INSRA* in zebrafish larvae.(C) mRNA expression of *GCG* in zebrafish larvae.(D) The mRNA expression level of *PPARG* in zabrafish larvae.(E) The mRNA expression level of *mTORC1* in zebrafish larvae.(F) The mRNA expression level of PI3K in zebrafish larvae.(G)The mRNA expression level of *AKT2* in zebrafish larvae. (H) The mRNA expression level of *GAPDH* in zebrafish larvae.Values are expressed as the mean ± SD in each group. #p < 0.05 and ##p < 0.001 model group versus Con group. *p < 0.05 and **p < 0.001 treatment group versus model group.

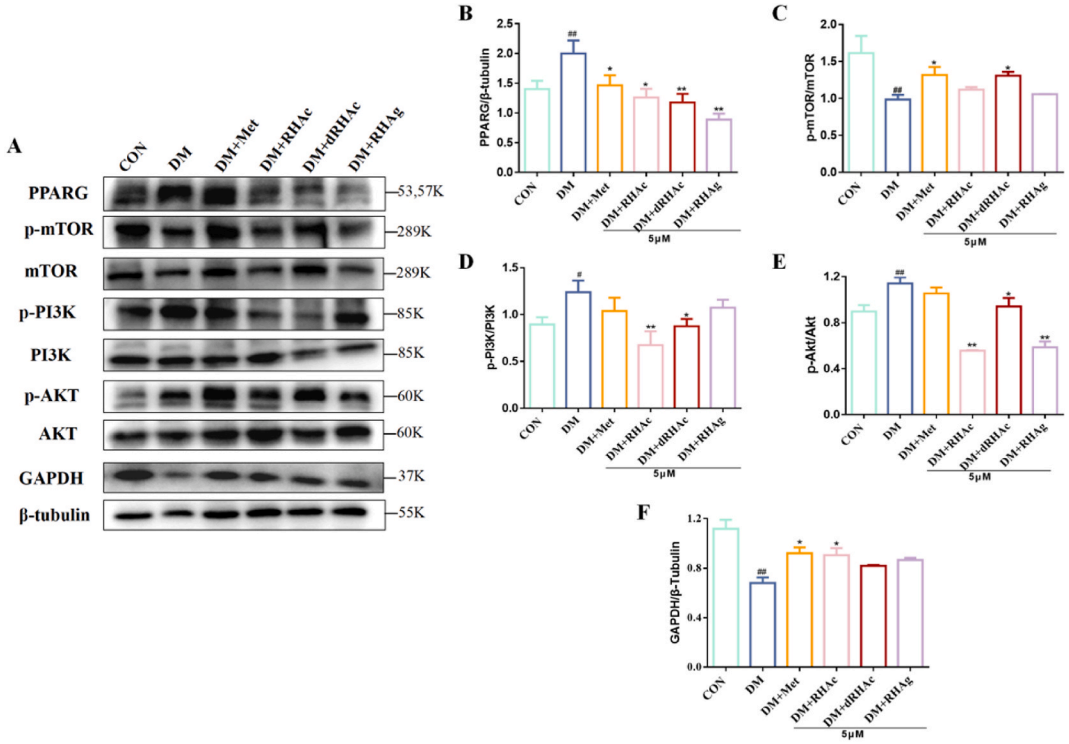



**Fig. 6.** Stilbenoids changes the expression of PPARG, mTOR,PI3K, AKT and GAPDH in zebrafish larvae. (A)Protein banding. (B)Gray analysis diagram of PPARG (C)Gray analysis diagram of mTOR.(D)Gray analysis diagram of PI3K(E)Gray analysis diagram of AKT.(F)Gray analysis diagram of GAPDH.

administration of metformin, RHAc, dRHAc or RHAg, the mRNA expression levels of *INS*, *INSRA* and *GCG* decreased to varying degrees, suggesting that these compounds have the potential to ameliorate aberrant glucose and lipid metabolism in zebrafish.

In order to further investigate the hypoglycemic potential mechanism of stilbenoids, we assessed the impact of styrene compounds on PPARG, mTOR, PI3K, AKT and GAPDH at both gene and protein levels. The expression levels of genes related to the PI3K/AKT signaling pathway, PPARG, mTORC1, and GAPDH were assessed in zebrafish larvae (Fig. 5D–H). Consistent with expectations, the model group exhibited increased expression of *PPARG* and *mTORC1*, as well as reduced expression of *PI3K, AKT*, and *GAPDH*. However, intervention with stilbenoids ameliorated these alterations. The validation of this result was further extended to the protein level. As depicted in Fig. 6A–F, the administration of stilbene in zebrafish larvae significantly decreased the expression of PPARG, as well as the phosphorylation-related expression of PI3K and AKT. At the same time, the phosphorylation of mTOR and the expression of GAPDH were increased. This result indicated that the three Stilbenoids compounds were found to enhance insulin sensitivity by modulating the PI3K/AKT/mTOR signaling pathway at least in part.

## 4. Discussion

Type 2 diabetes is a chronic metabolic disease characterized by persistent hyperglycemia [24]. IR is recognized as the primary etiological factor in T2DM, which is defined clinically as the inability of a known quanity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population [25]. Consequently, improving IR holds the potential to effectively prevent and manage T2DM. Currently, long-term use of drugs for the treatment of T2DM often leads to certain adverse reactions [26,27]. Therefore, increasing attention has been devoted to exploring the anti-insulin resistance effects of bioactive compounds derived from medicinal and food plants of the same family, aiming for their potential therapeutic applications [28]. It has been discovered that plant-derived stilbenoids exhibit significant hypoglycemic activity. Pterostilbene improves glycemic control in insulin-resistant obese rats by increasing hepatic glucokinase activity and increasing skeletal muscle glucose uptake [29]. In vitro studies have also shown that pterostilbene protects islet β cells from oxidative stress and apoptosis [30]. ChoiSB et al. [12] demonstrated that RHAc significantly augmented the uptake of sugar by 3T3-L1 adipocytes. Therefore, the potential of stilbenoids in the development and application for treating T2DM and its complications is promising. We previously isolated three polyphenolic stilbenoids (RHAc, dRHAc, and RHAg) from fenugreek seeds and demonstrated their beneficial regulatory effects on glucose and lipid metabolism in 3T3-L1 adipocytes cells.

In this study we used computer simulation technology for predictive analysis to identify potential targets of RHAc, dRHAc, and RHAg in relation to targets related to T2DM and IR. The top ten targets identified based on degree values comprised PPARG, ERBB2, MMP9, PTGS2, SRC, HSP90AA1, ESR1, BCL2, EGFR and GAPDH. The KEGG enrichment analysis results suggested that RHAc, dRHAc, and RHAg may improve IR through the PI3K/AKT signaling pathway.

Among the top ten targets, ERBB2, SRC, HSP90AA1, EGFR, and BCL2 play pivotal roles in orchestrating cellular survival/death, proliferation, differentiation, and repair in response to metabolic cues [31–35]. Previous studies have demonstrated the crucial involvement of Src family kinases in the pathogenesis of diabetic nephropathy [36]. The EGFR protein exerts regulatory control over glucose uptake, glycolysis, amino acid metabolism, lipid synthesis, and mitochondrial function through the activation of multiple signaling pathways including PI3K-Akt and Ras-MAPK [37]. Certain members of the BCL-2 family, such as Bcl-xL/Bcl-2asociated death promoter (BAD), activate glucokinase (GK) through phosphorylation. Meanwhile, BNIP3 (BCL2 interacting protein 3) regulates hepatic adaptation to starvation and satiation states by modulating fatty acid oxidation (FAO) and gluconeogenesis [35]. In addition to the aforementioned proteins, ESR1 and PTGS2 may exert significant influence on diabetes through hormonal regulation [38,39]. In diabetic patients, hyperglycemia induces alterations in MMP9 expression, resulting in lower levels compared to non-diabetic individuals [40]. PPARG adipokines and GAPDH, a key enzyme in glycolysis, play important roles in the pathogenesis of obesity, diabetes and other diseases [41,42].

To further elucidate the potential mechanisms underlying the hypoglycemic effects of these stilbenoids, we generated a zebrafish model of T2DM in adults and larvae by a high-sugar and high-fat diet. The zebrafish serves as an exemplary model organism in the field of scientific research. The similarity between the zebrafish and the human genome reaches 87 %. The zebrafish pancreas and insulin-sensitive target tissues, such as the liver and muscle, exhibit evolutionary conservation, with key mechanisms related to glucose metabolism displaying remarkable similarity to those observed in certain mammals [43,44]. Furthermore, crucial genes associated with glucose metabolism, including hexokinase, are actively expressed in zebrafish. Although the characterization of certain liver metabolic enzymes (e.g., CYP450) in zebrafish remains incomplete [45], hindering a comprehensive understanding of the correlation between zebrafish and human drug metabolism, zebrafish still offers significant advantages for investigating diabetes pathogenesis and facilitating drug development and screening. In this study, The T2DM zebrafish model was successfully established by employing 2 % and 3 % glucose and high-fat diet, enabling the evaluation of both the hypoglycemic effects of the compounds and their underlying mechanisms.

The glucose-lowering effects of three stilbenoids were assessed in an adult zebrafish model of T2DM at doses ranging from 2.5 to 100 mg/kg (Fig. S1). Despite the mortality observed in one zebrafish each from the metformin-treated group and the dRHA-treated group, the remaining zebrafish exhibited satisfactory growth even at high doses of 50 mg/kg and 100 mg/kg. Therefore, we posit that the mortality of zebrafish is not attributed to the drug; rather, it can be attributed to the persistence of a high concentration of glucose solution in zebrafish post-drug administration. Due to the presence of interindividual variability, variations in zebrafish tolerance towards high glucose concentrations or drug absorption capacity exist, ultimately resulting in zebrafish mortality. Moreover, several studies have demonstrated that sex disparities contribute to distinct glycemic fluctuations, suggesting that females may exhibit reduced responsiveness to hyperglycemia owing to estrogen-related alterations [46]. However, this finding had no impact on the

outcomes of our study. In this study, we found that the stilbenoids led to varying degrees of improvement in glucose, TG, TC, HDL-C, and LDL-C levels. HE staining of adult fish tissue sections revealed that the three compounds had a significant hepatoprotective effect compared to both the model group and the positive drug group, suggesting their potential for reducing hepatotoxicity with minimal side effects. The results of Oil red staining further demonstrated the efficacy of the three compounds in reducing lipid accumulation. The results obtained in this study are consistent with the findings reported by Chen J [47] et al. In diabetic mouse model, intragastric administration of RHAc significantly reduced blood glucose concentration, as well as levels of TG, LDL, and cholesterol. Furthermore, RHAc treatment also attenuated fibrosis and steatosis in hepatocytes of mice.

The advantageous characteristics of transparency and ease of microscopic observation in Zebrafish larvae [48] make them a suitable model for evaluating the global glucose-lowering effects of RHAc, dRHAc, and RHAg. The T2DM larvae were cultured with a glucose solution containing concentrations ranging from 2.5 μM to 80 μM of RHAc, dRHAc, and RHAg for a duration of 6 days (Fig. S2). At the conclusion of the experiment, all zebrafish larvae exhibited robust growth and displayed normal morphology. The data from this study indicate that zebrafish exhibit good tolerance to low doses of the three stilbenoids, despite the absence of safety information regarding their long-term administration. The levels of glucose, TG, TC, LDL-C and HDL-C in zebrafish larvae were found to be consistent with those observed in adult fish. To further assess the impact of the three stilbenoids on glucose and lipid metabolism, eight zebrafish larvae were selected from each group were subjected to the whole body Oil Red O and 2-NDBG staining. The larvae showed varying degrees of improvement in lipid deposition and glucose uptake after Stilbenoids treatment. Among the three compounds tested, RHAc had the most pronounced effect due to its reduced hydrolytic ability. The impact of RHAc was found to be the most significant among the three compounds. We speculate that this may be attributed to the substitution of one benzene ring connected with the double bond ethylene by an oxyglucoside bond, resulting in a reduction in its hydrolysis ability. Additionally, the replacement of the other benzene ring with hydroxyl and methoxy groups, along with the attachment of hydroxyl and methyl groups to the benzene ring as electron withdrawing systems forming conjugated systems, further enhances drug activity expression. Consequently, RHAc exhibits a stronger therapeutic effect than dRHAc and RHAg [49,50].

When we measured the expression of genes related to T2DM and IR in zebrafish, we found that the model group exhibited increased expression of insulin and its receptor insra, concomitant with elevated glucagon levels. Subsequent treatment with individual stilbenoids restored insulin and glucagon levels to normal. However, the regulation of glucagon is expected to occur in response to hypoglycemia. This aberrant expression pattern implies the potential involvement of an insulin and glucagon imbalance in the pathogenesis of T2DM [23,51].

The PI3K/AKT signaling pathway is necessary for normal metabolism due to its own characteristics, and its imbalance can lead to the development of obesity and T2DM [52]. Insulin initially binds to the alpha subunit of the insulin receptor located on the cellular surface, inducing phosphorylation of tyrosine residues on IRS. Subsequently, activated IRS translocates to the cell membrane and triggers PI3K activity, resulting in the generation of PIP3 as a second messenger on the plasma membrane. PIP3 then interacts with intracellular signaling proteins Akt and PDK1, leading to Ser308 phosphorylation of Akt by PDK1 and subsequent activation. Activated Akt modulates its downstream target proteins FoxO, GSK-3, mTOR, and other substrate receptors through phosphorylation events that regulate glucose uptake, glycogen synthesis, gluconeogenesis, and protein synthesis [53,54]. In our previous study, we discovered that three stilbenoids compounds can enhance AKT and AMPK phosphorylation to reduce glucose in 3T3-L1 cells [7]. In this study, molecular docking results demonstrated favorable binding energies between the three stilbenoids and PI3K and AKT. Experimental validation showed that the three stilbenoid compounds improved IR at least in part through PI3K/AKT signaling pathway.

mTOR is a key kinase downstream of PI3K/AKT, which regulates tumor cell proliferation, growth, survival and angiogenesis. mTORC1 is required for β-cell survival and proliferation under physiological conditions, and there is evidence that its excessive activation in patients with diabetes [55]. The expression of mTORc1 was further assessed in the present study, revealing a significant increase in the model group and subsequent decrease following drug administration. These findings suggest that the three stilbenoids compounds may improve IR via modulation of the PI3K/AKT/mTOR signaling pathway.

PPARG exerts significant effects on insulin regulation and regulation of adipocyte maturation and differentiation, as well as various other factors involved in this process. PPARG also plays a crucial role in maintaining insulin sensitivity across multiple tissues [56]. The loss of PPARG results in adipose tissue fat atrophy and IR, and muscle PPARG knockout mice exhibit IR [57]. Additionally, PPARG promotes the differentiation of adipocyte progenitor cells into mature adipocytes [58]. Our molecular docking analysis indicated that the three stilbenoids had favorable binding energies with PPARG, suggesting the potential for using PPARG as a therapeutic target for diabetes treatment. The qRT-PCR and Western blot results revealed a significant increase in PPARG expression within the model group, which was subsequently decreased following administration of the three stilbenoid compounds. This suggests that these compounds exert partial regulation over lipid metabolism by inhibiting PPARG expression.

GAPDH has long been acknowledged as a pivotal enzyme in energy metabolism, playing a crucial role in the generation of ATP and pyruvate through glycolysis. Studies have demonstrated that downregulation of GAPDH expression in zebrafish liver cells and zebrafish results in noticeable increases in lipid deposition and decreases in glycogen content, which can subsequently lead to disruptions in glucose and lipid metabolism [59]. After GAPDH inhibition in mice, upper glycolytic metabolites accumulate within hours, and lower glycolytic intermediates are depleted within minutes [60]. Based on our findings, we postulate that GAPDH may serve as a pivotal regulatory factor in the disruption of glucose and lipid metabolism. Our molecular docking results demonstrated the robust binding affinities of all three stilbenoids with GAPDH, suggesting that GAPDH could be a potential therapeutic target for diabetes treatment utilizing these stilbenoids. In this study, there is a significant decrease in the expression of GAPDH in the model group, which was subsequently restored upon administration of the three stilbenoids compounds. This suggests that these compounds effectively regulate gluconeogenesis by activating GAPDH expression.

In this study, we found that the three polyphenolic stilbenoids isolated from fenugreek seeds significantly ameliorated IR in vivo

and that RHAc had the most promising effects. Network pharmacology predictions suggested that PPARG and GAPDH may serve as potential targets for treating T2DM using these three stilbenoids. Experimental validation further supported the premise that the stilbenoids tested improve IR by modulating the PI3K/AKT/mTOR pathway. After the administration of three stilbenoid compounds, the PI3K/AKT signaling pathway was activated, leading to the overexpression of mTOR is phosphorylated. Consequently, there was a decrease in the expression of PPARG, a downstream lipid metabolism regulatory gene. This reduction may partially account for the observed decrease in lipid accumulation in zebrafish. Simultaneously, GAPDH expression, a key enzyme involved in gluconeogenesis/glycolysis pathway, was upregulated to enhance glucose uptake and utilization, ultimately improving IR.

## 5. Conclusion

During the drug development process, the identification of novel drug targets and the discovery and optimization of lead compounds play a pivotal role. Currently, in the initial phase of drug development, numerous lead compounds with clinical potential are derived from natural products. Metformin, which was isolated from Galega officinalis in the early stage, was found to have hypoglycemic effect. After a series of comprehensive studies and rigorous clinical trials, it has ultimately evolved into the preferred first-line oral hypoglycemic medication for effectively managing T2DM [61]. In summary, the present study demonstrates that three stilbenoids compounds (RHAc, dRHAc, RHAg) derived from fenugreek seeds improve IR via the PI3K/AKT/mTOR signaling pathway. However, further investigation is warranted to elucidate the precise mechanisms by which stilbenoid compounds regulate targets associated with glucose and lipid metabolism in order to improve IR. We will focus future research on the impact of these compounds on IR through their potential targets and on the interrelationships among targets and pathways. Therefore, this study provides valuable data and serves as a theoretical foundation for clinical drug research and development. Simultaneously, it presents a promising avenue for utilizing natural products in the treatment of diabetes.

## Ethics declarations

This study was reviewed and approved by the Ethical Review Committee of Northwest Institute of Plateau Biology, Chinese Academy of Sciences, with the approval number: 2023–07. Informed consent was obtained from all subjects involved in the study.

## Data availability statement

Data will be made available on request.

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## CRediT authorship contribution statement

**Yidan Gao:** Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Yun Wu:** Supervision, Methodology. **Fangfang Tie:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Honglun Wang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32007.

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