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Crocetin, the Key Hypoglycemic Agent in *Gardenia jasminoides* Identified by Spectrum-Effect Relationship, Targets GRK5 to Phosphorylate AKT2 and Improve Glucose Homeostasis

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ABSTRACT: Natural remedies are often used as standalone treatments or as complementary approaches to modern medicine for controlling type 2 diabetes. *Gardenia jasminoides*, a substance used in both food and medicine, has been clinically employed in traditional Chinese medicine to treat hyperglycemia. Here, we first identified crocetin as the compound responsible for the primary hypoglycemic activity of *G. jasminoides* and revealed that G protein-coupled receptor kinase 5 (GRK5) is the key target through which crocetin improves glucose homeostasis and insulin resistance. To identify the bioactive components in *G. jasminoides*, we conducted high-resolution mass spectrometry analysis and bioactivity-guided fractionation, confirming that crocetin is the primary bioactive constituent. We demonstrated the direct binding of crocetin to GRK5 via cellular thermal shift (CETSA) and surface plasmon resonance (SPR) assays. Further knockdown experiments confirmed the essential role of GRK5 in enhancing crocetin-mediated improvements in glucose metabolism. Additionally, we explored direct downstream targets of GRK5 and demonstrated for the first time that AKT2 is a novel GRK5-interacting protein. Collectively, these findings reveal that crocetin is a promising glucose-regulating phytochemical that targets GRK5 to phosphorylate AKT2, thereby improving glucose homeostasis.

Keywords: *Gardenia jasminoides*, Crocetin, glucose homeostasis, GRK5

1. Introduction

Diabetes is a widespread global epidemic affecting 476 million people worldwide, with 463 million people being diagnosed with type 2 diabetes mellitus (T2DM), and the number continues to rise annually [1]. As a complex metabolic disorder, T2DM is characterized primarily by hyperglycemia and insulin resistance in target tissues [2, 3]. Currently, various types of hypoglycemic agents are used to treat T2DM, including insulin

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sensitizers, insulin secretagogues, and glucosidase inhibitors [4]. However, most hypoglycemic drugs in use today have side effects to varying degrees, such as gastrointestinal adverse reactions and renal or hepatic impairment [5, 6]. Among the established therapeutic strategies for T2DM, α -glucosidase inhibitors have gained the approval of multiple treatment regimens [7]. These inhibitors work by suppressing α -glucosidase activity, which delays glucose absorption and slows the postprandial rise in blood glucose levels [8]. Therefore, α -glucosidase serves as a suitable standard for bioactivity-guided research.

Gardenia jasminoides, the mature dried fruit of the *Gardenia* genus in the *Rubiaceae* family, has long been recognized for its medicinal value in classical texts such as the Compendium of Materia Medica, particularly for its efficacy in “alleviating thirst”. Modern pharmacological research reveals a pathological link between this traditional efficacy and insulin resistance-mediated metabolic disorders, with potential mechanisms involving the regulation of glucose and lipid homeostasis [9]. Here, we predicted the hypoglycemic active components of *G. jasminoides* by establishing a high-performance liquid chromatography fingerprint and measuring the α -glucosidase inhibition rate. By combining nontargeted metabolomics with network pharmacology, we identified the key target GRK5. Guided by this target, we investigated the potential hypoglycemic active components of *G. jasminoides*. Bioactivity-guided fractionation identified crocetin as the primary active component. Mechanistically, crocetin targets GRK5 to improve glucose metabolism and identifies AKT2 as a novel direct downstream target of GRK5. Overall, this study exemplifies “drug-to-target” and “target-to-drug” approaches, which will facilitate the development of therapeutic agents for T2DM.

2. Materials and methods

2.1 Fingerprinting and α -glucosidase activity inhibition test

The *G. jasminoides* S1–S10 batches were identified as the dried mature fruits of *G. jasminoides* of the *Rubiaceae* family, and the 10 batches of different varieties of *G. jasminoides* were numbered S1–S10. 10 batches of *G. jasminoides* were weighed, crushed through a sieve, extracted via the cold immersion method with 15 times the amount of 70% ethanol, and extracted 3 times in total. Both the 1st and 2nd batches were extracted for 48 h, and the 3rd batches were extracted for 72 h. The filtrate was merged, the pressure was reduced to concentrate to the viscous form, the mixture was freeze-dried, and the lyophilized powder of *G. jasminoides* crude extract was obtained. Ten batches of lyophilized powder (100 mg) were placed in a 4.5 mL EP tube, water was added to dilute the sample mixture to a mass concentration of 50 mg/mL, and after ultrasonication, the mixture was passed through a 0.22 μ m filtration membrane to obtain the test solution. This assay was employed as a preliminary bioactivity-guided screening method to identify the fractions and corresponding chemical components from *G. jasminoides* with the most potent in vitro anti-hyperglycemic potential, thereby guiding the isolation of the lead compound for subsequent mechanistic studies.

2.2 High-performance liquid chromatography conditions

An Agilent Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) was used, the flow rate was 0.8 mL/min, the detection wavelength was 254 nm, the injection volume was 5 μL, the column temperature was 40 °C, mobile phase A was 0.2% formic acid aqueous solution, mobile phase B was acetonitrile, and gradient elution was used. 0.01–10 min, 5% A–6% A, 95–94% B; 10–40 min, 6%–7% A, 94–93% B; 40–55 min, 7%–13% A, 93–87% B; 55–65 min, 13%–14% A, 87%–86% B; 65–85 min, 14%–20% A, 86–80% B; 85–125 min, 20%–30% A, 80–70% B; 125–145 min, 30%–50% A, 70–50% B; 145–155 min, 50%–100% A, 50–0% B; 155–165 min, 100% A, 0% B.

2.3 Knockout of target components

Under the optimized HPLC conditions, the crude extract powder of *G. jasminoides* was prepared (50 mg/mL), and 50 μL was injected into each needle. The combined collection of activity-related peak eluent was the solution of the target knockout component, and the remaining eluent was its corresponding negative solution, which was concentrated under reduced pressure and passed through a 0.22 μm filtration membrane. The samples containing the target knockout component (denoted Px⁺) and the corresponding negative samples (denoted Px⁻).

2.4 LC–MS/MS conditions for knocking out the target components

Chromatographic conditions: column: Waters XBridge BEH C18 (2.1 mm×100 mm, 2.5 μm); mobile phase A: 5 mmol/L aqueous ammonium formate; mobile phase B: acetonitrile; flow rate: 0.25 mL/min; and injection volume: 2 μL.

The mass spectrometry conditions used were as follows: ion source parameters: electrospray ionization, positive/negative ionization mode (positive electrospray ionization/negative electrospray ionization, ESI⁺/ESI⁻); spray voltage, 5500 V; ion source temperature, 400 °C; air curtain gas, 0.24 MPa; atomization gas, 0.34 MPa; and auxiliary gas, 0.38 MPa.

2.5 Determination of the α-glucosidase inhibitory activity of *G. jasminoides* extract and knockout target components

The crude *G. jasminoides* extract powder/acarbose mixture was added to test solutions of different concentrations for subsequent experiments. The assay was carried out on 96-well plates, the reaction system was the same as that of Zhang et al. [10], and the data were processed according to the following formula: Inhibition rate = [1 - (OD sample - OD sample blank)/(OD negative - OD blank)] × 100%.

2.6 LC–MS sample preparation and LC–MS/MS analysis procedure

Sample Prep: 50 mg of *G. jasminoides* extract in a 1.5 mL tube + 800 μL of precooled MeOH:H₂O (7:3) + 20 μL of IS. The samples were ground (steel beads, 50 Hz, 5 min), ultrasonicated at 4 °C for 30 min, static at -20 °C for 1 h, and then centrifuged (14,000 g, 4 °C, 15 min). The (0.22 μm) supernatant was filtered for MS, and pooled QC (20 μL/sample) was used.

LC–MS: Waters 2D UPLC/QExactive HRMS. Column: Hypersil GOLD aQ (100×2.1 mm, 1.9 μm); 40 °C; 0.3 mL/min. Mobile phase: A = 0.1% FA H₂O, B = 0.1% FA ACN. Gradients: 5% B (0–2 min), 5–95%

B (2–22 min), 95% B (22–27 min), and 5% B (27.1–30 min). ESI ($\pm$): sheath gas 40, aux gas 10; capillary 320 °C, aux heater 350 °C; spray voltage (+) 3.80 kV, (–) 3.20 kV; m/z 150–1500.

2.7 Prediction of potential active ingredients

Nontargeted metabolomics data were initially filtered (Match >80, Level 1–2). Potential *G. jasminoides* active ingredients were screened via TCMSP (OB $\geq$ 30%, DL $\geq$ 0.10) and SwissADME. Targets for these compounds were predicted via TCMSP and SuperPred (probability >50%), followed by target name standardization via UniProt.

2.8 Prediction of core targets of *G. jasminoides* and diabetes mellitus

Diabetes targets were retrieved from disease databases (GeneCards, OMIM, TTD, PharmGkb, and DrugBank) using “Diabetes” as the keyword. Venny 2.1.0 identified targets intersecting *G. jasminoides* compounds (previously screened) and diabetes. These potential therapeutic targets were analyzed via the STRING database to generate a protein–protein interaction (PPI) network. The network file was imported into Cytoscape, and the CytoHubba plugin's MNC algorithm ranked nodes to identify key proteins. The top 15 proteins from the MNC analysis represent the core network for the anti-diabetic action of *G. jasminoides*.

2.9 Cell culture

HepG2 cells were cultured in high-sugar DMEM containing a mixture of FBS (10%; Gibco) and penicillin–streptomycin (1%; Solarbio) and were incubated at 37 °C and 5% CO₂. In the blank control group, the original medium was discarded (the blank control was set to 2 groups for subsequent validation experiments on the model of insulin resistance), new DMEM was added, and the medium in the model group was replaced with DMEM containing different concentrations of GlcN. After 24 h of induction in the incubator, the glucose content in the supernatant of the cells was detected via the GOD-POD method (Solarbio, BC2505).

2.10 Cellular thermal shift assay (CETSA)

HepG2 cells were inoculated in 6-well plates at a density of 2.5×10^5 /mL. A blank control group and a crocetin-treated group were set up. After compound treatment, the cells were incubated at 37 °C in a 5% CO₂ incubator for 48 h. The supernatant was discarded, and the cells were gently washed with precooled PBS to remove residual medium. The lysates were divided into 1.5 mL centrifuge tubes for each treatment group, heated with a gradient (45–63 °C) for 5 min, allowed to stand at room temperature for 5 min, and then centrifuged (4 °C, 12000 g) to separate the soluble protein supernatant from the denatured aggregated protein precipitates. Immunoblotting was performed after the addition of SDS protein buffer.

2.11 Surface plasmon resonance (SPR) analysis

The binding interactions between Crocetin and GRK5 were examined via SPR with a Biacore200 biosensor device. The binding experiments were conducted at a controlled temperature of 25 °C and a flow rate of 30 μ L/min. Various concentrations of the compounds (ranging from 0.3125 to 10 μ M) were prepared in running buffer composed of 40 mM Tris and 10 mM glycine hydrochloride (pH 2.0). These mixtures were

then passed over the immobilized targets at the indicated gradient concentrations. The kinetic dissociation constant (KD) for each interaction was determined via the Biacore Insight evaluation software version provided.

2.12 Transfection

HepG2 cells were inoculated in DMEM at a density of 60–80%. siRNAs were diluted in serum-free DMEM and combined with Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, 11668019) according to the manufacturer's protocols. The cells were collected 48 h later for Western blotting. *siGrk5* was generated by GenePharma with the following sequences: sense, CUCCGUGGCAGAAUAUGAATT; antisense, UUCAUAUUCUGCCACGGAGTT.

2.13 Co-IP assay

After the HepG2 cells were treated with the IP lysis buffer from the Co-IP Kit (Protein A/G Magnetic Beads; MCE, HY-K0202K), the supernatant was incubated overnight at 4 °C with either the GRK5 antibody (Santa Cruz Biotechnology, sc-518005), AKT2 (Cell Signaling Technology, #3063), or IgG control. The resulting antigen–antibody immunocomplexes were then added to premagnetized beads and incubated at 4 °C for 4 h. Following incubation, the complexes were washed with IP lysis buffer and subjected to magnetic separation. The immunoprecipitated proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected via the designated primary antibodies.

2.14 Western blot

Western blotting was performed following a standard protocol, as previously described [11]. The membranes were immersed in a chemiluminescent substrate (Mei5bio, MF074-01) and analyzed via a Fluor Chem M imaging system (ProteinSimple). Protein blots were quantified via ImageJ. The primary antibodies used in this study were against GRK5, AKT2, p-AKT2 (Ser474; Cell Signaling Technology, #8599), PI3K (Cell Signaling Technology, #4249), GLUT4 (Zenbio, #347063), and beta-actin (GenuIN, 3793-HRP).

2.15 Statistical analysis

The experiments were independently repeated at least 3 times and statistically analyzed via Prism software and SPSS software. The data are expressed as the means $\pm$ standard deviations (SDs). Statistical significance was calculated by one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant. Differences between individual group means were compared using Tukey's honestly significant difference (HSD) post-hoc test.

3. Results

3.1. The spectrum–effect relationship and component knockout method suggest that crocetin plays a role in improving glucose metabolism

To identify the active components in *G. jasminoides*, we first imported HPLC chromatograms from 10 batches of *G. jasminoides* sourced from different origins into the “Chinese Herbal Chromatographic

Fingerprint Similarity Evaluation System 2012 Edition” software. Using the chromatogram of sample S10 as the reference spectrum, we performed multipoint calibration, identifying 22 major characteristic peaks, sequentially numbered P1 to P22 (Fig. 1(a)). Guided by biological activity, we measured the α -glucosidase inhibitory activity in ethanol extracts from the fruits of the 10 *G. jasminoides* batches, with the IC₅₀ as the evaluation metric (Figs. 1(b) and 1(c)). By using the inverse of the IC₅₀ as the dependent variable and the total peak area as the independent variable, we established a spectral-effect model with DPS 7.05 software and PLS-DA analysis. This analysis revealed that the common peaks P3, P5, P7, and P22 were positively correlated ($R > 0.2$) with α -glucosidase inhibitory activity (Fig. 1(d)).

Next, we aimed to determine the chemical structures of the major characteristic peaks in *G. jasminoides* to identify specific components responsible for improving glucose metabolism. Each major peak was further analyzed via high-resolution mass spectrometry for formal identification. Structural characterization of the knockout components was achieved through secondary mass spectrometry fragment patterns, molecular and ionic signatures, the elution order of chromatographic peaks, and literature comparison. The following components were identified: geniposidic acid (P1), deacetyl asperulosidic acid methyl ester (P4), gardenoside (P5), scandioside methyl ester (P6), genipin 1-gentiobioside (P9), geniposide (P10), tea alcohol (P11), tea alcohol (P12), rutin (P13), cyclopenta[c]pyran-4-carboxylic acid (P15), crocin (P17), and crocetin (P22) (Fig. 1(e), Figs. S1(a)–(k) in Appendix A). On the basis of this information, we evaluated the inhibitory effects of individual peak components against α -glucosidase and found that P5 and P22 presented the strongest inhibitory activity (Figs. 1(f)–1(h)), suggesting that gardenoside and crocetin may be the primary functional factors responsible for the improvement in glucose metabolism in *G. jasminoides*.

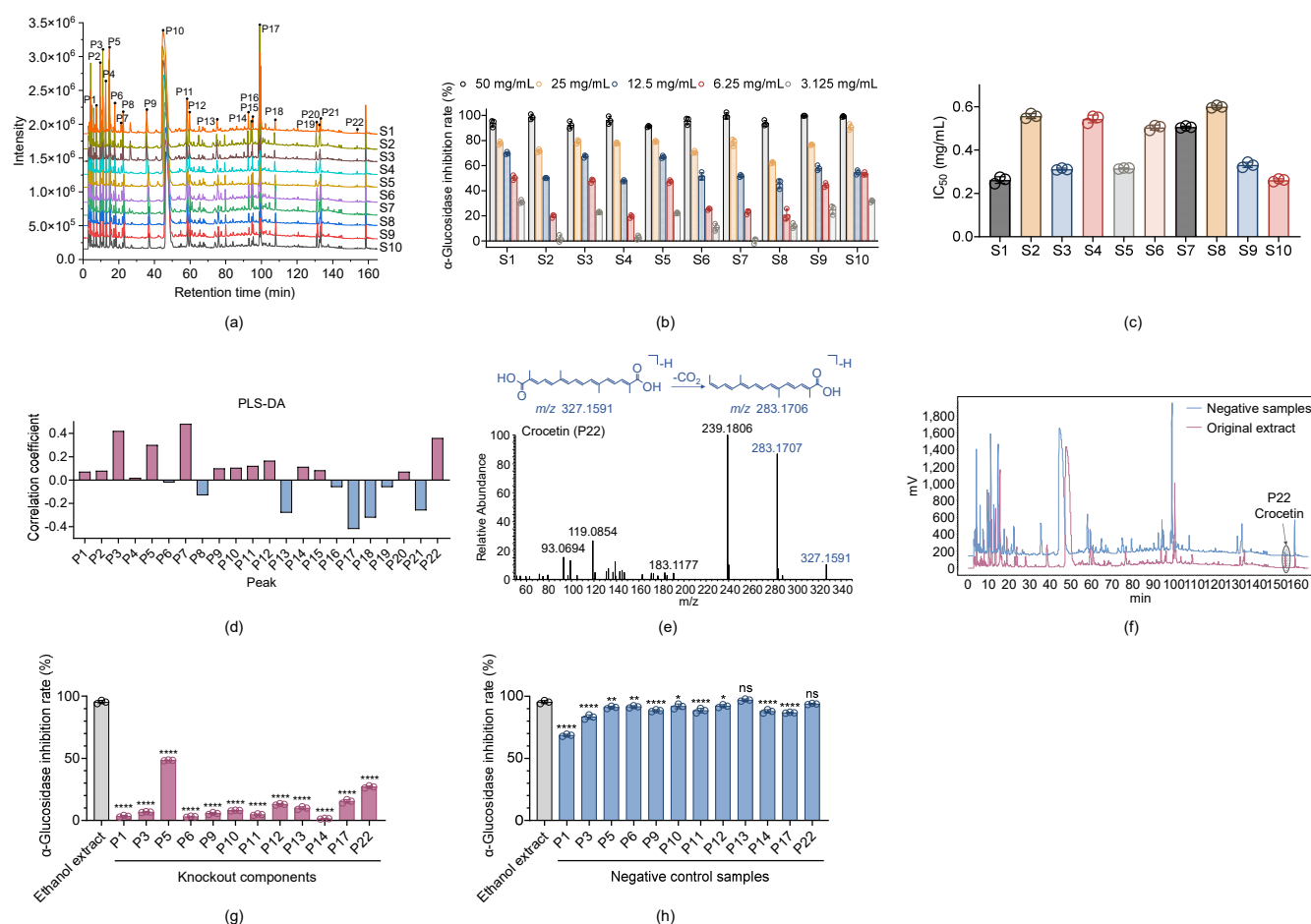


Fig. 1. The spectrum-effect relationship and component knockout method suggest that crocetin plays a role in improving glucose metabolism. (a) HPLC fingerprint of *G. jasminoides*. (b) Inhibition rates of α -glucosidase activity in 10 batches of *G. jasminoides* ($n = 3$). (c) The half maximal inhibitory concentration (IC_{50}) of α -glucosidase activity inhibition in 10 batches of *G. jasminoides* ($n = 3$). (d) Partial least squares regression model standardized regression coefficient plot. (e) High-resolution mass spectra and mass spectrometry cleavage pathway of the knockout component (P22). (f) HPLC chromatograms of *G. jasminoides* extract and crocetin knockout negative samples. (g and h) Inhibitory effects of the ethanol extract of *G. jasminoides*, corresponding knockout components, and negative control samples on α -glucosidase ($n = 3$). Compared with ethanol extract, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns (no significant difference).

3.2. GRK5 is the primary active target through which *G. jasminoides* improves glucose metabolism

The innovative drug discovery approach involves constructing a three-dimensional associative model that links “molecular networks, disease phenotypes, and drug effects” to precisely identify disease-causing genes and intelligently predict drug targets. Therefore, we further employed LC–MS/MS identification and systematically integrated five database resources—DrugBank, OMIM, TTD, PharmGKB, and GeneCards—to construct a multidimensional “component–target” network model. This approach explores the material basis and molecular mechanisms underlying the effects of *G. jasminoides* extract on diabetic pathological processes.

Following the LC–MS/MS identification of the *G. jasminoides* extract, we screened for compounds with match values > 80 and level values ≥ 2 in the database, resulting in 330 compounds identified in both positive and negative ion modes (Appendix B). After dual screening for oral bioavailability ($OB \geq 30\%$) and druggability ($DL \geq 0.18$), 27 active components and their corresponding 483 potential targets were identified

(Fig. 2(a)). A total of 6,634 diabetes-related targets were retrieved from the five databases (DrugBank, OMIM, TTD, PharmGKB, and GeneCards) (Fig. 2(b)). The intersection of these targets with the 483 targets associated with the active components of the *G. jasminoides* extract yielded 221 potential hypoglycemic targets (Fig. 2(c)). We then constructed a “component–target” network model based on the 27 active components from *G. jasminoides* and the 221 action targets. Through network topology analysis, GRK5 emerged as one of the highest-degree targets (Fig. 2(d)), suggesting that GRK5 is a key therapeutic target for *G. jasminoides*. Additionally, disease-target and biological-target intersection targets (confidence interval > 0.7) were imported into the STRING database for protein interaction analysis, where GRK5 again emerged as a key target (Fig. 2(e)). On this basis, compounds (geniposidic acid, genipin 1-gentiobioside, geniposide, rutin, crocin and crocetin) that regulate GRK5 were screened (Fig. 2(f)) as candidates for bioactivity-guided compound selection in subsequent steps.

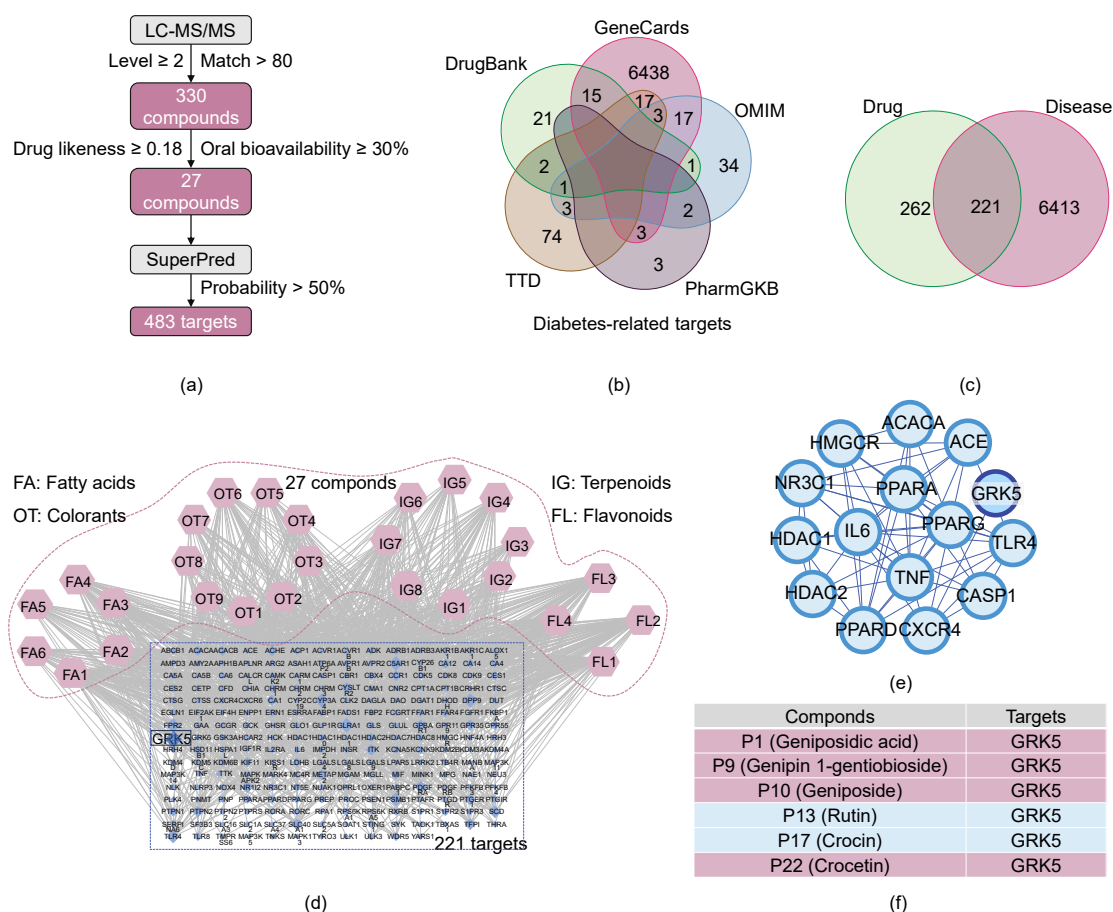


Fig. 2. GRK5 is the primary active target through which *G. jasminoides* improves glucose metabolism. (a) Selection and target prediction of effective components in *G. jasminoides*. (b) Intersection of diabetes targets. (c) The intersection of diabetes targets and *G. jasminoides* effective component targets. (d) “Active ingredient–target” network modeling. (e) PPI protein cytoHubba core network analysis of diabetes therapeutic targets. (f) The core targets of active ingredients in *G. jasminoides* and the intersection of active ingredient targets with the intersection compounds identified by LC–MS.

3.3. Crocetin is identified as the primary functional factor in *G. jasminoides* for improving glucose metabolism

As compounds that regulate GRK5, geniposidic acid, genipin 1-gentiobioside, geniposide, rutin, crocin, and crocetin were selected for further activity screening. First, the MTT colorimetric assay was employed to

examine the effects of these compounds on HepG2 cell viability. Crocetin had no significant effect on cell viability at concentrations ranging from 12.5 to 50 $\mu\text{mol/L}$. The other five compounds also had no significant effect on cell viability at concentrations ranging from 12.5 to 100 $\mu\text{mol/L}$. These compounds were then selected for activity screening at safe doses (Figs. 3(a)–(f)). Using glucose secretion levels as an evaluation metric, evidence has indicated that crocetin has the most potent effect. Compared with the model group, crocetin at 6.25 $\mu\text{mol/L}$ significantly reduced glucose secretion ($P < 0.0001$) by 30% under glucosamine (GlcN) stimulation, outperforming the positive control rosiglitazone (Rosi) at 10 $\mu\text{mol/L}$.

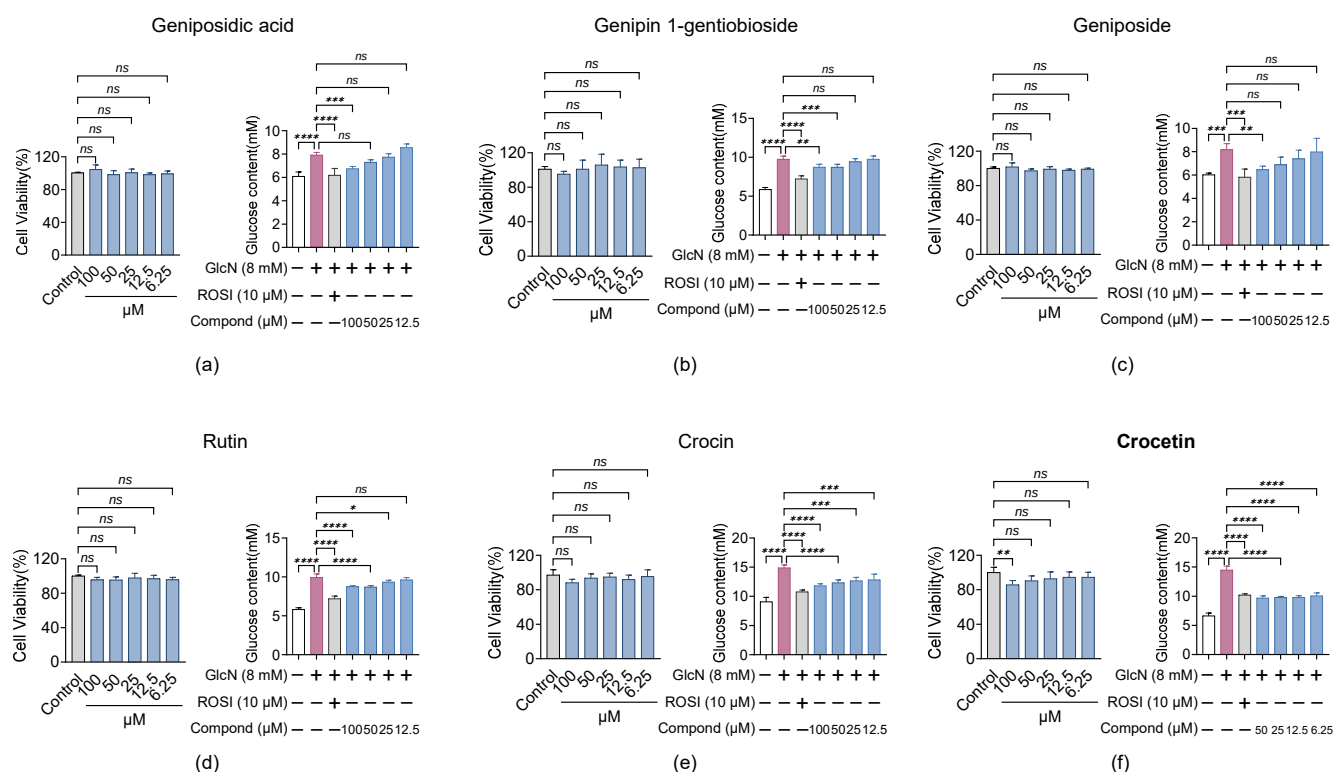


Fig. 3. Crocetin has been identified as the primary functional factor in *G. jasminoides* for improving glucose metabolism. Effects of geniposidic acid (a), genipin 1-gentiobioside (b), geniposide (c), rutin (d), crocin (e) and crocetin (f) on HepG2 cell viability and glucose consumption ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns (no significant difference).

3.4. Crocetin targets GRK5 activation and improves insulin resistance in vitro

To elucidate the mechanism underlying the hypoglycemic activity of crocetin, we first aimed to confirm that its target protein is GRK5. To this end, we performed cellular thermoshifted Western blot (CETSA-WB) assays to detect whether crocetin physically binds to GRK5. GRK5 expression levels decreased with increasing temperature, indicating a reduction in the thermal stability of the GRK5 protein at elevated temperatures. crocetin treatment significantly increased this thermal stability, with higher GRK5 expression levels observed in the crocetin-treated group than in the DMSO-treated group, confirming that crocetin binds to GRK5 (Figs. 4(a) and 1(b)). Next, we conducted surface plasmon resonance (SPR) experiments to reveal the binding and dissociation of GRK5 from immobilized AKT2, confirming that GRK5 directly interacts with AKT2. SPR measurements demonstrated a strong binding affinity between crocetin and GRK5, with a dissociation constant (KD) of 5.39×10^{-6} M, corroborating the CETSA-WB results (Fig. 4(c)).

To further elucidate the effects of crocetin on GRK5, we examined GRK5 protein expression levels in HepG2 cells before and after crocetin treatment. Compared with those in the control samples, the GRK5 protein levels in the GlcN-stimulated samples were significantly lower. After treatment with crocetin, GRK5 protein levels were significantly increased in a dose-dependent manner, indicating that crocetin activates GRK5, thereby improving glucose homeostasis (Figs. 4(d) and (e)).

Insulin resistance is a hallmark pathological feature of T2DM. GRK5 has been shown to be a positive regulator of insulin sensitivity, with impaired glucose tolerance and insulin sensitivity observed in GRK5^{-/-} mice, alongside disrupted AKT signaling pathways [12]. As a core component of insulin signaling, abnormal PI3K/AKT2 function is closely associated with the development of T2DM and insulin resistance [13]. Therefore, to further investigate the hypoglycemic mechanism of crocetin, we evaluated its effects on pathway-related proteins in IR-HepG2 cells. Compared with the control group, the model group presented significantly lower protein expression levels of PI3K, p-AKT2, and GLUT4. Following crocetin treatment, while it had no effect on AKT2 protein expression, crocetin promoted the expression of PI3K, p-AKT2, and GLUT4 (Figs. 4(f)-(i)). These findings suggest that crocetin may improve insulin resistance in HepG2 cells by activating GRK5 and the PI3K/AKT2 signaling pathway.

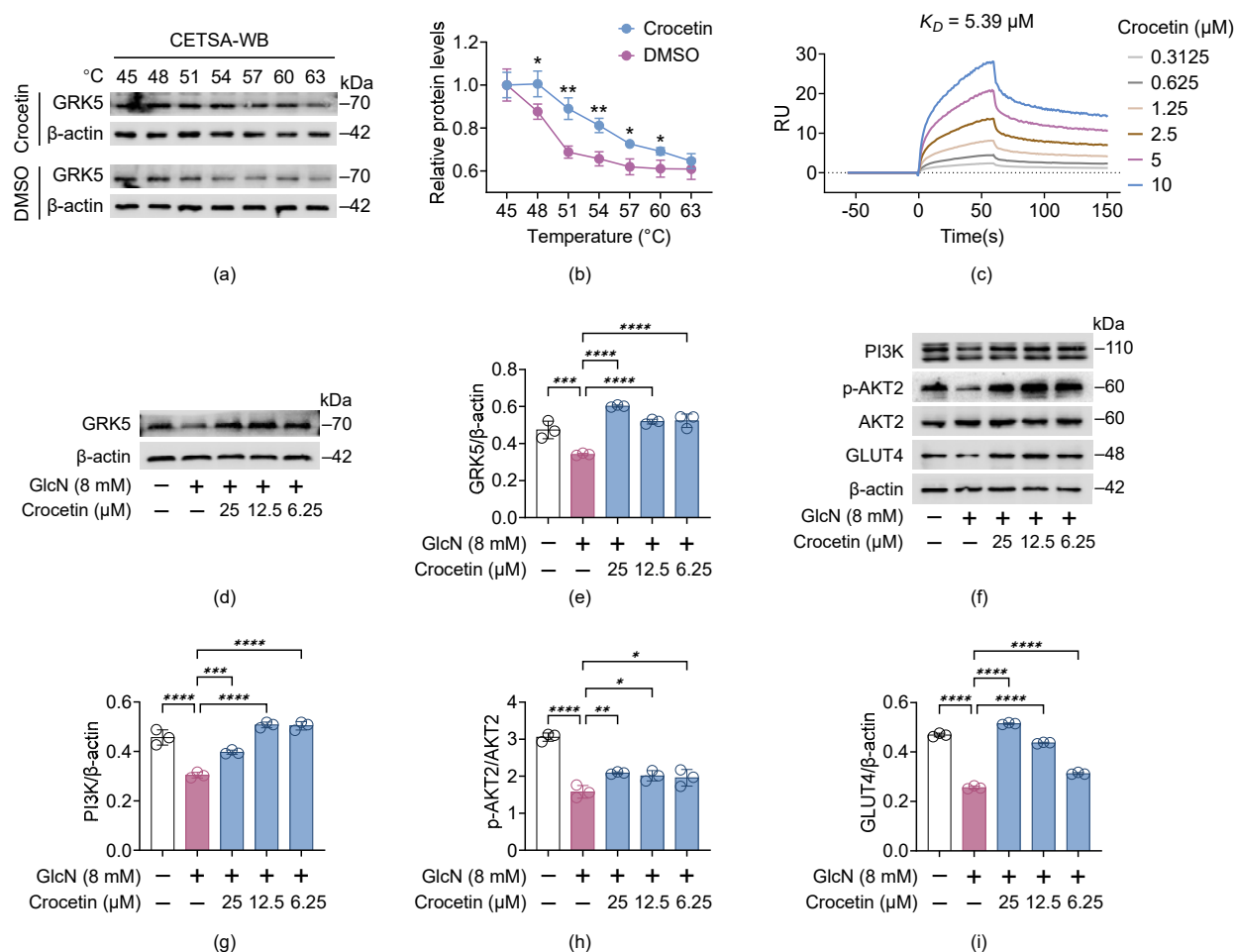


Fig. 4. Crocetin targets GRK5 activation and improves insulin resistance. (a and b) Crocetin improves the thermal stability of GRK5, as determined via a CETSA. (c) SPR analysis was performed to measure the affinity between crocetin and the protein GRK5. (d-i) Western blots and quantitative evaluation of the protein expression of GRK5, PI3K, AKT2, and GLUT4 in HepG2 cells exposed to GlcN and crocetin (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns (no significant difference).

3.5. GRK5 is essential for the improvement of glucose metabolism by crocetin

To further confirm the pivotal role of GRK5 in the enhancement of insulin resistance and glucose homeostasis by crocetin, we first knocked down GRK5 in HepG2 cells via siRNA, establishing a causal relationship between the effects of GRK5 and crocetin on glucose homeostasis. Western blot analysis revealed a significant reduction in GRK5 protein levels posttransfection (Figs. 5(a) and (b)). Compared with those in the GlcN group, GRK5 knockdown markedly increased glucose secretion and significantly decreased the protein expression levels of PI3K, p-AKT2, and GLUT4, further exacerbating glucose metabolism disorders and the progression of insulin resistance in the GlcN background (Figs. 5(c)-(g)). Importantly, adding crocetin to GRK5-knockdown cells no longer reduced glucose secretion or increased GLUT4 protein expression. Furthermore, the ability of GRK5 to increase PI3K and p-AKT2 protein expression was significantly attenuated (Figs. 5(c)-(g)), indicating that GRK5 is essential for the improvement of glucose metabolism by crocetin. Taken together, the combination of Crocetin-induced endogenous GRK5 upregulation and the dependence of Crocetin's effect on AKT2 on GRK5 expression provides compelling evidence for a functionally necessary link between Crocetin, GRK5, and AKT2 activation.

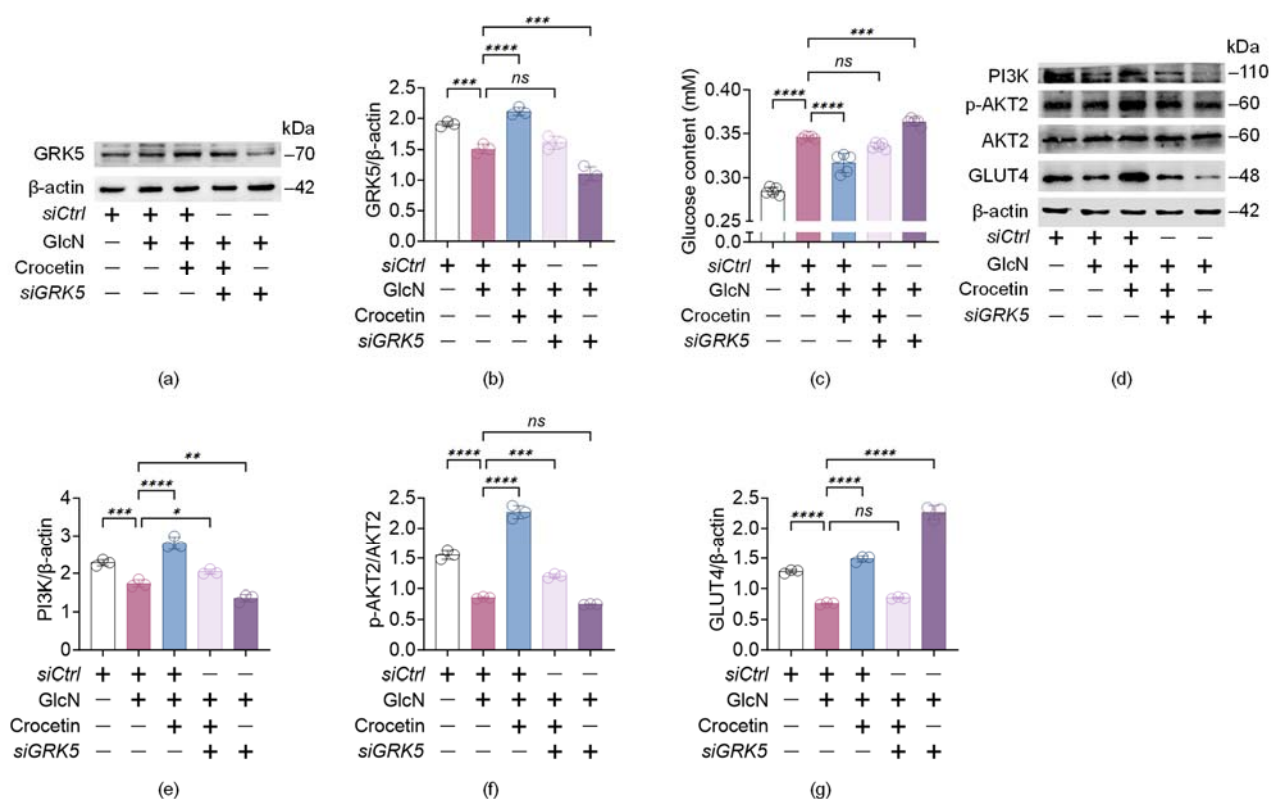


Fig. 5. GRK5 is essential for the improvement of glucose metabolism by crocetin. (a and b) Western blotting was used to detect the successful knockdown of GRK5 by siGRK5 ($n = 3$). (c) Effect of GRK5 knockdown on glucose depletion in HepG2 cells ($n = 3$). (d-g) Effect of GRK5 knockdown on the expression of proteins related to the PI3K/AKT signaling pathway ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns (no significant difference).

3.6. Identification of AKT2 as a novel GRK5-interacting protein

Having established GRK5 as a key mediator of the effects of crocetin on insulin resistance and glucose homeostasis, we investigated potential GRK5-binding partners to elucidate the molecular mechanism by

which GRK5 mediates the interaction between crocetin and AKT2. High-throughput affinity capture-MS screening of GRK5 interactors was performed via the BioGRID database. Potential GRK5 interactors were visualized via concentric circles, where proximity to the center (GRK5) indicates greater interaction strength. AKT2, identified as one of the proteins closest to the center, was determined to be a binding partner of GRK5 (Fig. 6(a)). Furthermore, immunoprecipitation (Co-IP) assays confirmed the physical interaction between GRK5 and AKT2 (Fig. 6(b)). Collectively, these results indicate that AKT2 is a novel binding partner of GRK5.

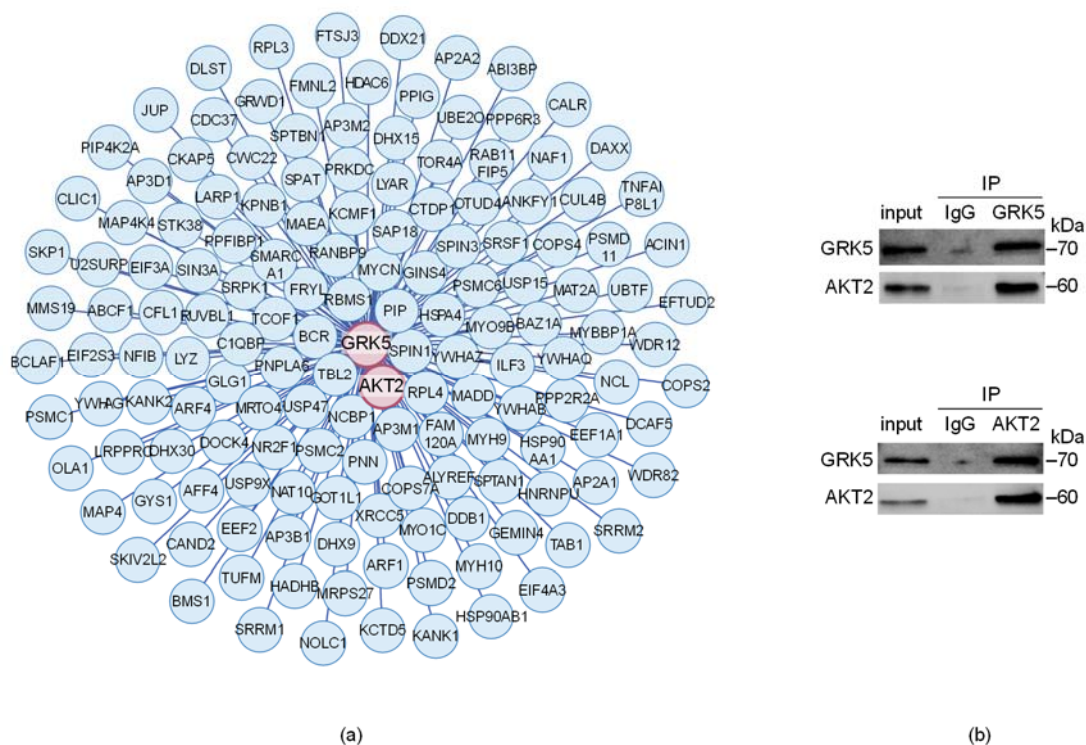


Fig. 6. AKT2 was identified as a novel GRK5-interacting protein. (a) High-throughput affinity capture-MS screening of GRK5 interactors was performed via the BioGRID database (<https://biogrid.org/>). (b) Co-IP analysis of the interaction between AKT2 and GRK5 in HepG2 cells. Anti-IgG served as a negative control.

4. Discussion

This study identified crocetin as a novel and promising glucose-regulating phytochemical that activates GRK5. GRK5 directly interacts with the key signaling kinase AKT2, thereby regulating GLUT4 and improving glucose homeostasis. These findings indicate that the crocetin-GRK5-AKT2 pathway may serve as a therapeutic target for improving T2DM.

Traditional Chinese medicine fingerprinting is an effective quality control method that comprehensively reflects the chemical information contained in herbal medicines, embodying their characteristics of “holism” and “complexity.” This method has been widely applied in quality control, authenticity verification, and studies of the spectral effects of traditional Chinese medicines [14]. However, research on the spectral effect relationship of *G. jasminoides* remains limited [15], and previous studies have relied solely on predictions of spectral-effect relationships without validation [16, 17]. Our study combines spectral-effect relationship prediction with validation through knockout peak component analysis. The

compounds identified via knockout technology in this study have all been reported in the literature on the chemical composition of gardenia, demonstrating the reliability of our mass spectrometry analysis results and research approach.

GRK5 belongs to the GRK family, which consists of highly homologous serine/threonine kinases that share a common protein structure. GRK5 responds to various stimuli by shuttling between the cytoplasm and nucleus to regulate receptor activity on the plasma membrane or gene expression within the nucleus [18]. Additionally, GRK5 phosphorylates GPCRs and modulates downstream signaling pathways, influencing the pathogenesis of multiple chronic degenerative diseases, including cardiovascular disease, cancer, T2DM, and rheumatoid arthritis [19-21]. Genome-wide association studies (GWASs) have identified GRK5 as a susceptibility gene for T2DM [12, 22]. The metabolic importance of GRK5 was first demonstrated in GRK5 knockout mice, which presented elevated blood glucose, insulin, and triglyceride levels and increased insulin resistance [19]. Furthermore, β -cell-specific GRK5 knockout mice presented impaired glucose tolerance, reduced β -cell mass, and glucose intolerance [23]. Currently, data linking GRK5 to human metabolic diseases are limited. A genome-wide association study revealed a significant association between the single-nucleotide polymorphism (SNP) rs10886471 in the third intron of the GRK5 gene and T2DM [24]. Collectively, these studies indicate a stronger association between the GRK5 gene and T2DM. Thus, our study revealed that knocking down GRK5 in IR-HepG2 cells exacerbates the progression of glucose metabolism disorders and insulin resistance, further confirming the central role of GRK5.

It is important to clarify the role of the α -glucosidase inhibition assay within our study's design. This assay was utilized as an initial, efficient screening platform for the bioactivity-guided fractionation of the *G. jasminoides* extract. α -glucosidase inhibitors are a clinically validated class of anti-diabetic drugs, making the inhibition of this enzyme a relevant proxy for potential hypoglycemic activity [7, 8]. This approach successfully led us to identify crocetin as the component with the highest correlation between concentration and inhibitory effect. However, our subsequent mechanistic investigations revealed that crocetin's primary mode of action in improving glucose homeostasis in insulin-sensitive cells is not through direct enzyme inhibition but via the modulation of a crucial intracellular signaling pathway involving GRK5 and AKT2. This highlights a common paradigm in natural product discovery: a primary screen can successfully identify a potent bioactive molecule, while deeper mechanistic studies may uncover a novel and perhaps more significant cellular target. Thus, the α -glucosidase assay served its purpose as an effective discovery tool, rather than an indicator of the final mechanism of action.

In this study, we focused on six major bioactive components of gardenia: geniposidic acid, genipin 1-gentiobioside, geniposide, rutin, crocin, and crocetin. We evaluated their regulatory effects on glucose metabolism in a HepG2 cell insulin resistance model. The experimental data revealed that crocetin exhibited the strongest glucose-consuming effect at equivalent concentrations, suggesting its pronounced hypoglycemic potential. The PI3K/AKT pathway is a classic insulin metabolic pathway [25, 26]. Dysfunction in this pathway impairs the ability of the liver to properly uptake and assimilate glucose,

leading to increased gluconeogenesis, reduced glycogen synthesis, enhanced glycogen release, insulin resistance (IR), and elevated blood glucose levels [27, 28]. Research has also indicated that crocetin exerts antidepressant effects in mice via the PI3K/AKT pathway [29]. Li et al. reported that crocetin has anti-inflammatory effects by inhibiting the expression of the PI3K/AKT signaling pathway, thereby protecting the retina [30]. All these studies demonstrate the potential of crocetin to act on the PI3K/AKT pathway. Therefore, in this study, we identified the PI3K/AKT signaling pathway as a key molecular mediator closely associated with crocetin and GRK5 in IR-HepG2 cells. Crocetin improved insulin resistance via the PI3K/AKT pathway, whereas GRK5 was identified as a critical mediator of the enhancement of insulin resistance and glucose homeostasis by crocetin.

A classical question in such studies is whether GRK5 overexpression alone is sufficient to activate AKT2. While this is a valid experimental approach, we reason that our current “endogenous upregulation + loss-of-function” paradigm offers strong and physiologically relevant evidence for the axis. Furthermore, it is important to contextualize this within ongoing debates about GRK5's mode of action. GRK5 may act as a direct kinase, a scaffold protein, or an indirect modulator of upstream receptors like IGF1R for AKT regulation [31]. A positive result from GRK5 overexpression would not distinguish among these models. Therefore, while our data firmly establish the functional necessity and coupling within the Crocetin-GRK5-AKT2 axis, the precise biochemical mechanism—whether through direct phosphorylation, scaffolding, or an indirect route—remains a fascinating and important question for future research. Such studies could employ *in vitro* kinase assays, co-immunoprecipitation with specific mutants, or detailed spatial imaging.

Another fundamental question arising from our work is how crocetin binding leads to the functional modulation of GRK5. Our data demonstrate a direct interaction with a K_D of 5.39 μM , but the downstream consequences of this binding event at the protein level are not yet fully understood. We hypothesize that crocetin functions as a positive allosteric modulator of GRK5. Binding of crocetin to a putative pocket on GRK5 could induce a conformational change that stabilizes an “active” state of the kinase. This 'active' state may not necessarily involve an increase in its catalytic activity towards canonical GPCR substrates, but rather could enhance its ability to function as a scaffold. For instance, the conformational shift could expose a previously occluded binding interface for AKT2, thereby increasing the affinity of the GRK5-AKT2 interaction, which we observed in our Co-IP experiments. To resolve this, future studies employing techniques such as hydrogen-deuterium exchange mass spectrometry (HDX-MS) could map the conformational changes in GRK5 upon crocetin binding. Furthermore, *in vitro* kinase assays using a known GRK5 substrate (e.g., a GPCR C-terminal tail peptide) in the presence and absence of crocetin would clarify if crocetin directly enhances GRK5's catalytic kinase activity. Furthermore, the specificity of crocetin for GRK5 over other members of the GRK family was not assessed in this study. The GRK family consists of seven members, with GRK4, GRK5, and GRK6 forming a subfamily with high sequence homology, particularly in the kinase domain [32]. It is therefore possible that crocetin may also interact with other GRK

isoforms, which could contribute to its overall biological effect or potential off-target activities. To establish GRK5 as a selective target, future investigations are required. These should include competitive binding assays or direct binding studies, such as SPR, using purified recombinant GRK2, GRK6, and other family members. Determining the binding affinity of crocetin across the GRK family will provide a comprehensive selectivity profile and further validate the therapeutic potential of targeting GRK5.

The effective concentrations of crocetin observed in our cellular models (6.25–25 μM) provide a valuable target for its biological activity. A key translational question is whether these concentrations are achievable in vivo following oral administration of *G. jasminoides* extracts. Notably, the low effective dose range (6.25–25 μM) may partially offset bioavailability challenges, as concentrations near the lower end (6.25 μM) are sufficient for significant glucose regulation—potentially reducing the risk of dose-dependent toxicity seen with higher-dose regimens of existing drugs (e.g., lactic acidosis with high-dose metformin). As a hydrophilic dicarboxylic acid, crocetin may face challenges related to intestinal permeability and first-pass metabolism, potentially resulting in low oral bioavailability. While the complex matrix of the whole extract could influence its absorption profile, dedicated pharmacokinetic studies are necessary to determine the actual plasma and tissue concentrations achievable with practical oral doses. Future research should focus on elucidating the absolute oral bioavailability of crocetin from standardized extracts and exploring formulation strategies, such as nano-encapsulation or phospholipid complexes, to potentially enhance its absorption and systemic exposure. Addressing these pharmacokinetic aspects will be crucial for evaluating the therapeutic potential and designing effective dosage regimens for crocetin or *G. jasminoides* extracts in the management of metabolic disorders.

A key limitation of the present study is its reliance on in vitro models. While our findings in HepG2 cell provide strong evidence for the molecular mechanism of crocetin, the physiological relevance and therapeutic potential must be confirmed in vivo. Future studies are planned using established diabetic animal models, such as streptozotocin (STZ)-induced diabetic mice or db/db mice. These studies will aim to verify the hypoglycemic effects of crocetin, assess its impact on insulin resistance and glucose tolerance, and analyze the expression and phosphorylation levels of GRK5, AKT2, and other key pathway proteins in relevant tissues like the liver and skeletal muscle. Such in vivo validation is essential to translate our mechanistic insights into a potential therapeutic strategy for type 2 diabetes [33, 34]. In addition, this study demonstrates the functional importance of the Crocetin-GRK5-AKT2 axis primarily through loss-of-function and endogenous activation models. Future studies employing complementary approaches, such as gain-of-function experiments in more defined systems or detailed interactome analyses, could further solidify and expand upon these findings. Specifically, elucidating the exact molecular interface between GRK5 and the AKT activation machinery represents a key next step.

In summary, we demonstrate that “spectrum effect” prediction combined with “component knockout” validation serves as a valuable tool for identifying previously undescribed compounds with pharmacological activity documented in traditional medicines. Furthermore, by integrating molecular network predictions

from network pharmacology with physical validation of molecular targets, we identified crocetin as an active compound and clarified its mechanism of action: it targets and activates GRK5, which directly interacts with the key signaling kinase AKT2. Moreover, crocetin (6.25 $\mu\text{mol/L}$) demonstrated superior glucose-regulating effects compared with those of rosiglitazone (10 $\mu\text{mol/L}$), highlighting its future therapeutic potential for treating T2DM through GRK5-dependent improvements in insulin resistance and glucose clearance. Critically, this GRK5-AKT2 axis represents a mechanistic departure from current drug classes: while rosiglitazone (a thiazolidinedione, TZD) acts via PPAR γ activation—inducing adipogenesis, fluid retention, and cardiovascular risks—crocetin’s targeted modulation of GRK5 avoids these liabilities. The lower effective concentration of crocetin (6.25 $\mu\text{mol/L}$ vs. 10 $\mu\text{mol/L}$ for rosiglitazone) further suggests a potentially wider therapeutic window, which may mitigate off-target adverse effects commonly associated with TZDs (e.g., weight gain, bone fractures) and other T2DM drugs (e.g., gastrointestinal distress with metformin, genitourinary infections with SGLT2 inhibitors) [35]. Given rosiglitazone’s black-box warnings for myocardial infarction, crocetin’s natural origin and pathway-specific action offer a promising alternative for patients with insulin resistance intolerant to conventional therapies.

5. Conclusion

Our study reveals that crocetin is a novel and promising phytochemical for glucose regulation in vitro. Mechanistically, crocetin targets and activates GRK5, which directly interacts with the key signaling kinase AKT2, thereby activating the PI3K/AKT signaling pathway. This activation improves insulin resistance, regulates GLUT4, and enhances glucose homeostasis (Fig. 7). These findings suggest that the crocetin-GRK5-AKT2 pathway may serve as a therapeutic target for improving type T2DM.

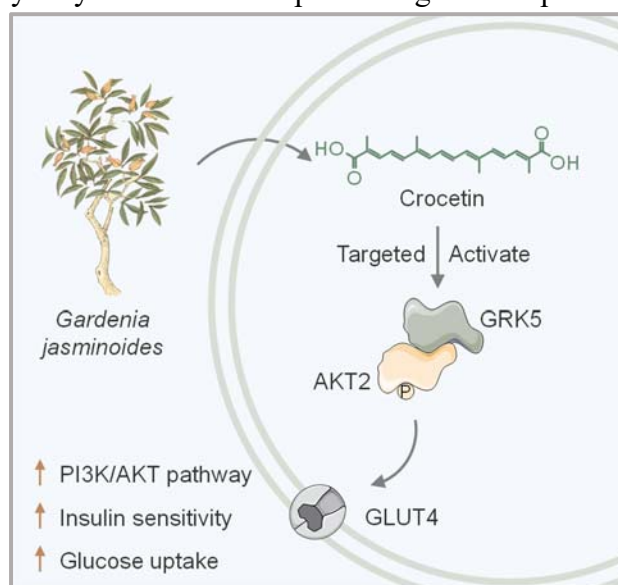


Fig. 7. Schematic illustration of the potential mechanism by which crocetin from *G. jasminoides* targets GRK5 to activate the AKT2 signaling pathway in HepG2 cells, improving insulin resistance and glucose homeostasis.

Declaration of competing interest

We declare that we have no financial or personal relationships with individuals or organizations that could inappropriately influence our work. There are no professional or personal interests in any product,

service, or company that could be perceived as influencing the position presented in the manuscript or the review thereof.

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Data availability

The data will be made available upon request.

Appendix A. Supplementary data

Supplemental information can be found online.

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