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Original Article

Isorhamnetin from Astragalus membranaceus exerts anti-glioma effects via PI3K/Akt signaling pathway

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Abstract: This study examined the antitumor effects and molecular mechanisms of isorhamnetin in glioma treatment with a particular focus on its regulation of the PI3K/Akt signaling pathway, for developing targeted therapies. Using network pharmacology, we identified isorhamnetin could be used as a potential active ingredient in the regulation of glioblastoma (GBM) through the PI3K/Akt pathway. Bibliometric analysis was then used to review the current state and the developing trends in isorhamnetin research for anti-tumor applications. It was confirmed that isorhamnetin exerts its effects via the PI3K/Akt pathway. Our in vitro experiments showed further that isorhamnetin inhibits the proliferation and migration of glioma cells in a dose-dependent manner. Furthermore, isorhamnetin downregulated proteins related to the PI3K/Akt pathway, thereby suppressing its signaling activity. Isorhamnetin has antitumor effects against glioma by modulating the PI3K-Akt signaling pathway, providing experimental and mechanistic evidence for the potential use of isorhamnetin as a complementary GBM therapy.

Keywords: Network pharmacology, bibliometrics, glioma, isorhamnetin, PI3K/Akt signaling pathway

Introduction

Gliomas are malignant tumors usually present in the glial tissue of the central nervous system, and 81% of malignant brain tumors that arise are gliomas [1]. Although its clinical prognosis is modulated by several factors such as tumor grade, anatomic location, patient's age, and molecular markers, the 5-year relative survival rate is still found to be about 5% with a significant impact on human health [2-4]. The current standard treatment plan consists of maximal safe surgical resection with concomitant postoperative chemotherapy (the Stupp regimen) [5]. However, the survival benefit for patients remains limited. This poor outcome is attributed to multiple challenges including the blood-brain barrier, complex tumor microenvironment, large tumor heterogeneity and drug tolerance contributing to a small survival benefit of just 2.5 months [6, 7]. Despite progress in the field of molecular targeted therapy and immunotherapy for gliomas, limitations such as low response rates and drug resistance have remained [7, 8], leading to an overall poor prognosis. Exploring effective treatment approaches for GBM is of particular importance.

Natural products play a crucial role in anticancer drug development due to their unique structures and biological activities. In recent years, several natural products (such as curcumin, resveratrol, and quercetin), have been approved for clinical trials in oncology, demonstrating potential therapeutic effects in cancers [9, 10]. As a representative tonic herb in Traditional Chinese Medicine (TCM), *Astragalus membranaceus* (astragalus) is often used as an adjunct in comprehensive cancer therapy [11]. Clinical and pharmacological studies have confirmed that the active ingredients of *Astragalus*, including polysaccharides, saponins, and flavonoids, exhibit anti-inflammatory, immunomodulatory and cardiovascular protective effects [12]. Recent studies also reveal their potential to inhibit tumor cell proliferation and promote

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apoptosis in various cancers, including breast and lung cancer [13, 14]. Although the antitumor effects have been preliminarily demonstrated, current knowledge regarding the specific active components of astragalus and their mechanisms in inhibiting glioma remains limited.

Network pharmacology can effectively predict potential active ingredients and core targets within the complex system of traditional Chinese medicine, which has important potential to reveal tumor mechanisms and facilitate new drug discovery [15, 16]. However, most current studies on the inhibitory effects of astragalus on glioma remain confined to network pharmacology analyses. The specific active components that exert their effects through certain pathways and the underlying molecular mechanisms remain unclear [17, 18]. Bibliometric analysis, through the quantitative assessment of relevant literature, enables a systematic identification of research hotspots and evolutionary trajectories related to drugs and their components in disease studies. This approach effectively delineates cross-disciplinary areas, such as the relationships between drug components and biological pathways [19]. Although network pharmacology can systematically analyze the potential associations among active ingredients, targets and pathways, and bibliometrics can objectively reveal research hotspots and development trends, both have the limitation of lacking experimental verification. By integrating network pharmacology for computational prediction, bibliometrics for literature evidence, and in vitro experiments for experimental verification, an integrated research paradigm combining computation, literature analysis, and experimentation was established to more reliably clarify the material basis and molecular mechanism of drug action.

Based on this, the present study aims to identify key components in astragalus with anti-glioblastoma (GBM) activity and their potential mechanisms through a network pharmacology-based screening system. Bibliometric analysis will identify relevant literature, and subsequent in vitro experiments will validate the molecular mechanisms affecting proliferation, apoptosis, and migration in GBM cells. This research will provide novel strategies and theoretical foundations for the targeted treatment of GBM using active components from astragalus.

Materials and methods

Screening of chemical components of astragalus related to glioma

A network pharmacological analysis, based on the methodology of Xiao et al. [20], was performed to identify the chemical components of astragalus with potential anti-glioma activity. The active components of astragalus were identified from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database using screening criteria of oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18. Potential target genes of these components were predicted accordingly. We obtained glioma-related target gene information from the GeneCards, OMIM, DrugBank, TTD, and PharmGkb databases. The overlap between these datasets was examined to identify potential gene targets of astragalus components in glioma treatment. The overlapping targets were input into STRING 11.5 to develop a PPI network (interaction score > 0.9, Homo sapiens) emphasizing interactions with astragalus active components and disease targets. Cytoscape was used to visualize the relationship network. Target genes underwent GO functional and KEGG pathway enrichment analyses using R version 4.1.2. Biological processes and signaling pathways with statistical significance ($P \leq 0.05$) were identified to elucidate the potential mechanisms by which the active components of astragalus exert anti-glioma effects.

Bibliometrics research

The R package “bibliometrix” (v4.3.0, URL: https://www.bibliometrix.org) was utilized to perform a systematic analysis of academic literature on isorhamnetin within the field of tumor research. Data were extracted from PubMed, encompassing relevant publications from 2014 to 2024, and were standardized and stored in “PubMed export file” format. The publication numbers, citation rates, and collaboration indices were examined to understand the field development trends over time. Using keyword co-occurrence networks, we uncovered the knowledge structures. Finally, strategic coordinate maps were adopted to illustrate the evolution of research themes.

Molecular docking

Molecular docking was conducted using Autodock Vina. The molecular structure of isorhamnetin was retrieved from PubChem Compound (https://pubchem.ncbi. nlm.nih.gov/). The PDB structures of PI3K, Akt1, Bcl-2, and Bax were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Prior to docking, all water molecules were removed from the protein structures, and polar hydrogen atoms were added to ensure accurate binding interactions. A grid box of 30 × 30 × 30 points was generated to encompass the active sites of each protein, allowing for sufficient flexibility and movement of the ligand during the docking process.

Cell experiments

Cells and reagents: U251 and U87MG cells, sourced from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, USA) with 10% fetal bovine serum (FBS, BI, Israel). The Cell Counting Kit 8 (CCK8) assay was obtained from Beyotime (USA). Isorhamnetin was acquired from MedChemExpress (MCE, https://www.medchemexpress.com). The Annexin V/PI apoptosis detection kit was also obtained from Beyotime (Shanghai, China). Primary antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts) for anti-Bcl-2, anti-PI3K, anti-Akt, anti-p-PI3K, and anti-p-Akt, and from Hua Bioengineering Co., LTD. (Shanghai, China) for anti-β-actin.

Cell viability assay: Cell viability was determined via the CCK-8 assay. U251 and U87MG cells were plated in 96-well plates at a density of 5×10^3 cells per 100 μL per well. Following the designated treatment and incubation period, 10 μL of CCK-8 solution was added to each well and incubated for 30 minutes before measuring absorbance at 450 nm.

Detection of cell proliferation ability: The effects of isorhamnetin on GBM cell proliferation were evaluated using a clonogenic assay. Briefly, cells were seeded in 6-well plates (1,000 cells/well). After attachment, cultures were treated with varying concentrations of isorhamnetin (0, 25, 50, or 100 μmol/L) and incubated for 14 days. Colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for visualization and counting after PBS washing. All the experiments were done in triplicates.

Cell migration and invasion assays: Wound healing assay was used to examine the influence of isorhamnetin intervention on the migration ability of glioma cells. U87MG and U251 cells were seeded in 6-well plates and grown until about 80-90% confluence. A 200 μL pipette tip was used to create a standardized wound in the center of each well. After washing, the medium was replaced with experimental medium containing 2% FBS supplemented with different concentrations of isorhamnetin. Cells were observed and photographed at various time points (0 and 24 hours) using a microscope at 50× magnification. The invasive ability of the cells was assessed using a Transwell invasion assay. Cells (1.5×10^4/well) were resuspended in serum-free medium containing isorhamnetin (0-100 μM) and seeded in the upper chamber. The lower chamber was filled with medium containing 20% FBS as a chemoattractant. Following a 48-hour incubation, invading cells were fixed using 4% paraformaldehyde and stained with crystal violet. Invasion was quantified by counting the stained cells in four random fields (100× magnification), with triplicate measurements for each condition.

Detection of cell apoptosis level: Apoptosis was assessed via the Annexin V-FITC/PI apoptosis assay kit. After three washes with ice-cold PBS, U251 and U87MG cells were resuspended, stained, and analyzed for apoptosis via flow cytometry according to the manufacturer's instructions (Beckman Coulter, Atlanta, GA, USA).

Western blotting: Western blotting was applied to analyze protein expression. Cells were treated with isorhamnetin (0, 25, 50, 100 μmol/L) for 48 hours, lysed in RIPA buffer (Beyotime, China), and centrifuged at 12,000×g for 15 min at 4°C to extract total protein. Protein concentration was quantified with a BCA assay kit (Thermo Scientific, USA). Proteins were equally loaded, separated using 10% SDS-PAGE, and transferred to 0.45 μm PVDF membranes at 330 mA. Membranes were blocked using 5% non-fat milk for 1 hour at room temperature,

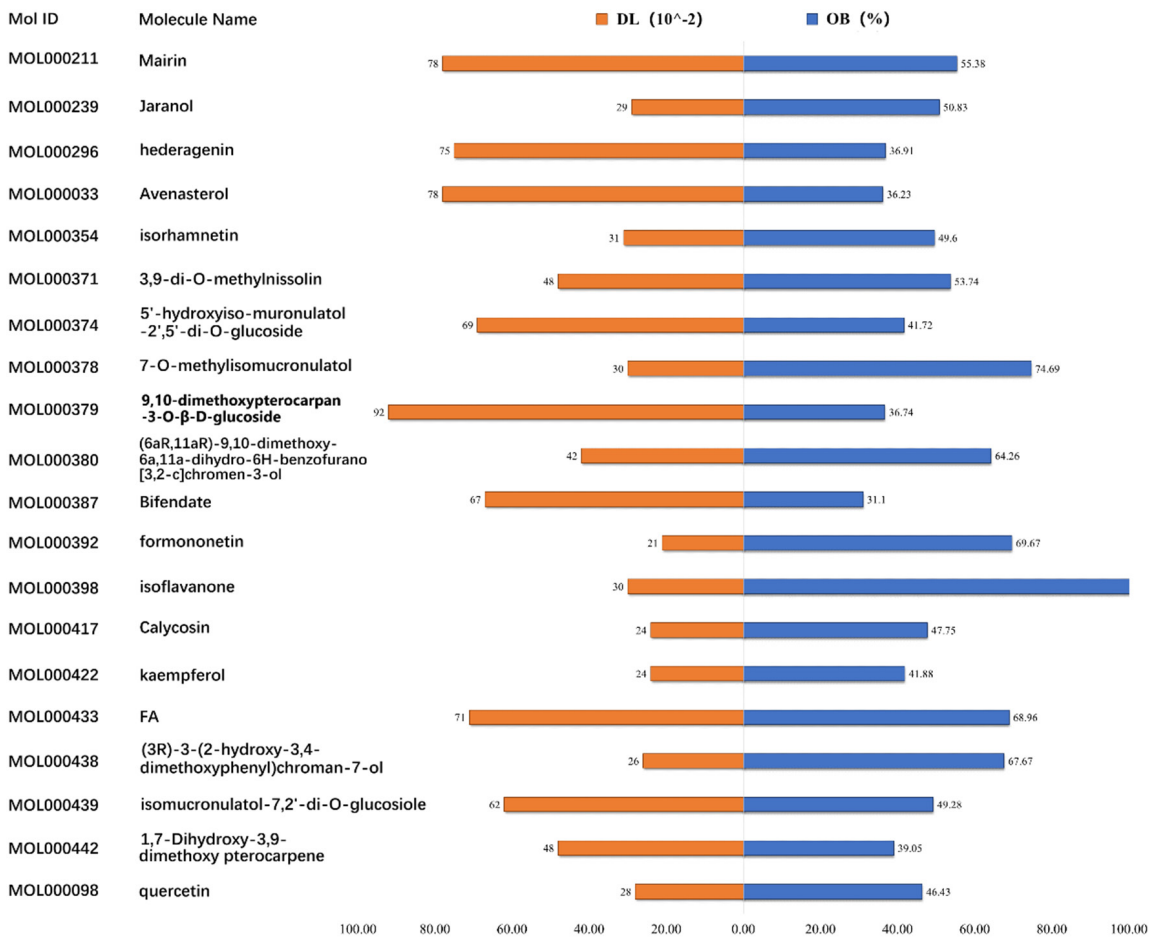


Figure 1. Active ingredients of astragalus membranaceus. The orange and blue bars represent the DL (10^-2) and OB (%) values of the ingredients, respectively.

followed by overnight incubation with primary antibodies (1:1000) at 4°C. Following TBST washing, the membranes were incubated with HRP conjugated secondary antibodies (1:5000) for 1 hour at room temperature. After the experiment, we used ultrasensitive ECL substrate (BL520A, Biosharp) to detect the signal and ImageJ software to quantify the band intensities.

Statistical analysis

Data analysis was performed using GraphPad Prism 8.0, where results were expressed as mean ± standard deviation (SD). Comparisons between two groups were conducted using independent Student's t-test. For comparisons between several groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. Statistical significance was defined as less than 0.05 *P*-value.

Results

Network pharmacology suggests active components of Astragalus and potential signaling pathways

Using the TCMSP database, twenty bioactive components of astragalus were described, which leading to the identification of 180 potential target genes (**Figure 1**). Concurrently, 5,101 glioma-associated genes were compiled from five authoritative databases (DrugBank, GeneCards, OMIM, PharmGkb, and TTD) (**Figure 2A**). Venn diagram analysis revealed 132 overlapping targets between astragalus components and glioma-related genes (**Figure 2B**), suggesting potential therapeutic interactions. A compound-target network was used to visualize these targets (**Figure 2C**). Analysis of protein-protein interactions using the STRING platform revealed 15 central targets (**Figure 2D**).

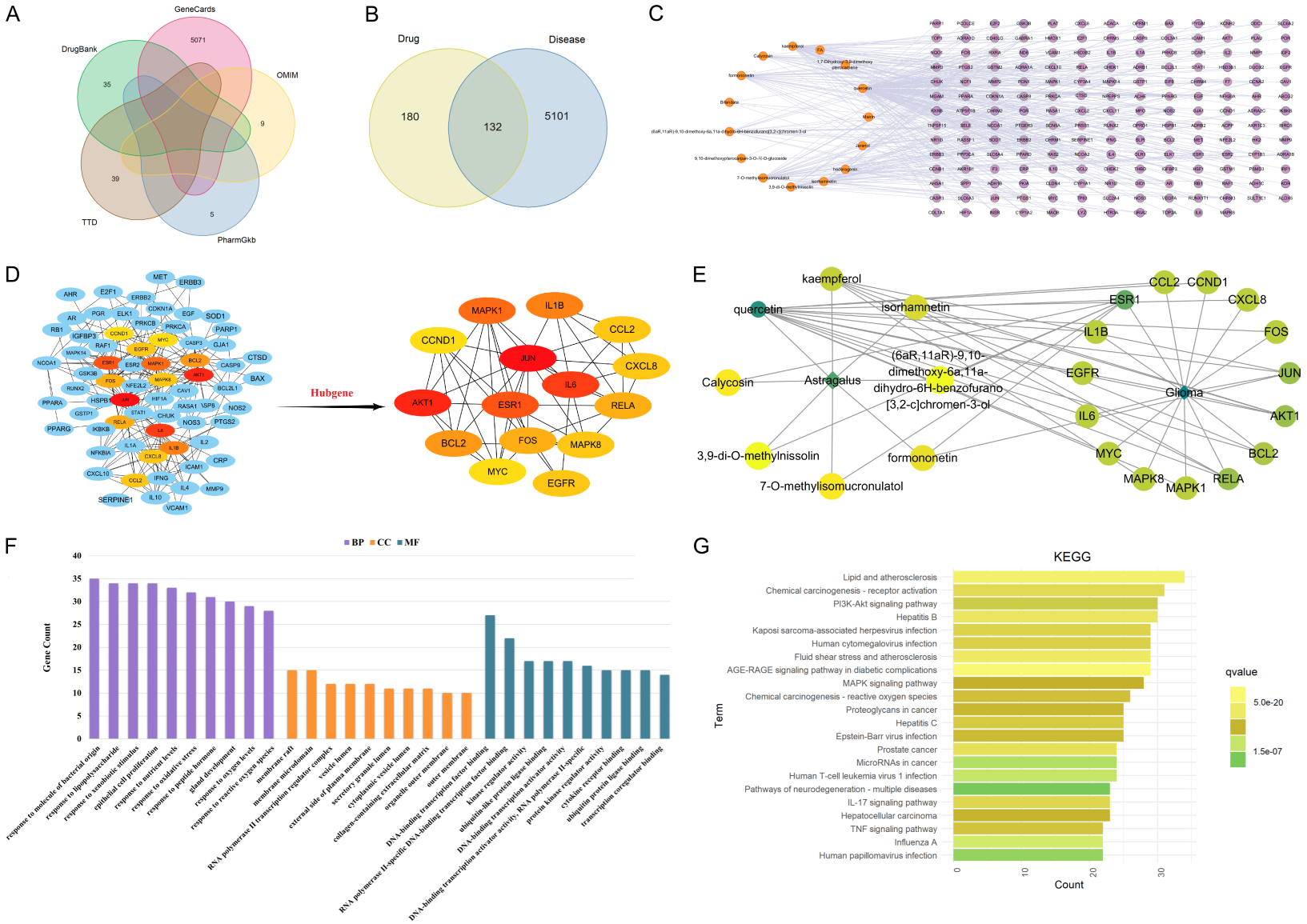


Figure 2. Network pharmacological results of the active components of Astragalus membranaceus. A. Venn plot of the intersection of multiple database targets; B. Venn plot of the intersection of drugs and disease targets; C. Network diagram of astragalus components and their target sites of action; D. Identification of 15 hub targets through PPI network analysis; E. Interaction network between astragalus bioactive components and the 15 hub genes; F, G. GO enrichment analysis diagram and KEGG functional analysis diagram of the main targets.

Subsequent network construction illustrated relationships between active components and these key targets (**Figure 2E**). GO enrichment and KEGG pathway analyses identified the top 10 significantly enriched GO terms (**Figure 2F**) and the top 20 enriched KEGG pathways (**Figure 2G**) for the target genes. Network pharmacology analysis suggested that isorhamnetin, a bioactive component of astragalus, may modulate glioma progression through PI3K-Akt pathway regulation, particularly via interactions with Akt1 and Bcl-2.

Bibliometric analysis indicates isorhamnetin's antitumor activity through the PI3K pathway

We conducted a bibliometric analysis to elucidate the anti-tumor effects of isorhamnetin. The results showed a steady growth in isorhamnetin-related research in recent years (**Figure 3A**). Co-occurrence network revealed that keywords such as tumor, apoptosis, and antioxidant clustered in cluster 1 (green region), supporting its potential anti-tumor properties (**Figure 3B**). Although studies on isorhamnetin and tumors remain limited, their number has shown an annual upward trend (**Figure 3C**), with antitumor effects emerging as a growing research focus (**Figure 3D**). Keywords analysis of antitumor studies reveals that current research primarily focuses on network pharmacology and molecular docking. The presence of the PI3K-Akt signaling pathway among the keywords suggests that isorhamnetin might exert its anti-tumor effects through this pathway (**Figure 3E**). However, no studies have yet explored isorhamnetin's role in glioma. Integrating network pharmacology and bibliometric analysis, we propose that isorhamnetin may inhibit glioma progression through the PI3K signaling pathway.

To assess the affinity of isorhamnetin for target genes of the PI3K pathway, we conducted the molecular docking assay. The results showed that isorhamnetin bound to the target proteins of the PI3K pathway through hydrogen bonding and strong electrostatic interaction, and isorhamnetin had a low binding energy of -7.709~-70.264 kcal/mol with PI3K, Akt1, Bcl-2 and Bax (**Figure 4**), indicating that the binding was highly stable.

Effects of isorhamnetin on the biological functions of glioma cells

Based on clues from network pharmacology and bibliometric studies, we investigated the potential anti-glioma effects of isorhamnetin and its possible mechanism through the PI3K signaling pathway.

CCK8 assays demonstrated that isorhamnetin treatment significantly decreased the viability of U87MG and U251 cells in a manner dependent on both time and dose (**Figure 5A**, **5B**). Colony formation assays further demonstrated that isorhamnetin markedly inhibited the proliferative capacity of both cell lines (**Figure 5C**, **5D**). Flow cytometry analysis showed that both early and late apoptotic cell populations significantly increased, leading to higher total apoptotic rates after isorhamnetin treatment (**Figure 5E**, **5F**). We used Transwell and wound healing assays to determine the effect of isorhamnetin on glioma cell invasion and migration. Wound healing experiments showed that isorhamnetin (≥ 50 μmol/L) significantly decreased the cell migration rate (**Figure 6A-D**). Similarly, in the Transwell tests, there were fewer cells passing through the Matrigel-coated membrane when treated with 50 μmol/L isorhamnetin (**Figure 6E-H**). The results suggest that isorhamnetin is an effective agent to inhibit glioma cell proliferation, induce apoptosis, and inhibit glioma cell migration and invasion.

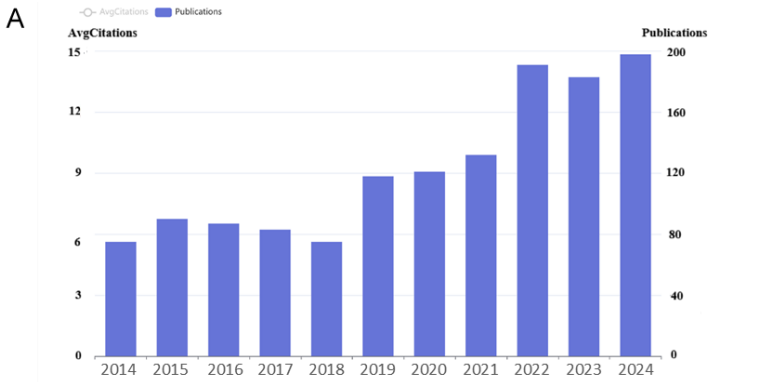
Isorhamnetin inhibits PI3K/Akt signaling pathway activation

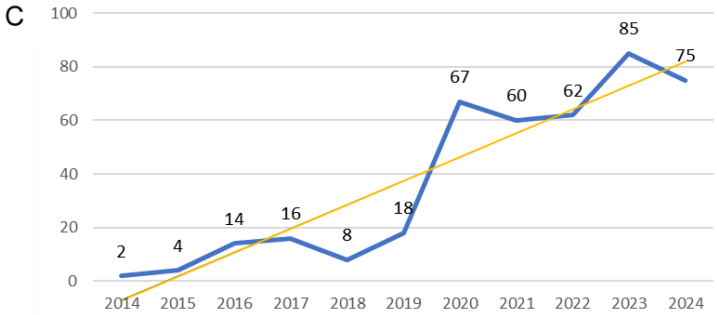
To investigate whether inhibitory effects of isorhamnetin on glioma cell migration and proliferation are related to the PI3K/Akt signaling pathway, we performed WB analysis.

Our study showed that the levels of p-PI3K and p-Akt proteins decreased in a dose-dependent manner (**Figure 7A-D**). Isorhamnetin inhibited PI3K phosphorylation in a dose-dependent manner (**Figure 7**), with r = -0.908, -0.885, P < 0.001, and the p-PI3K/PI3K ratio in U87MG, U251 decreased by 63.81±6.65%, 69.33± 10.90%, respectively. At the same time, we noticed a significant reduction in the Bcl-2 and an increase in the Bax expression (**Figure 7E-H**). The results show that isorhamnetin partially inhibits glioma cell tumorigenesis by suppressing the activation of the PI3K/Akt pathway.

Discussion

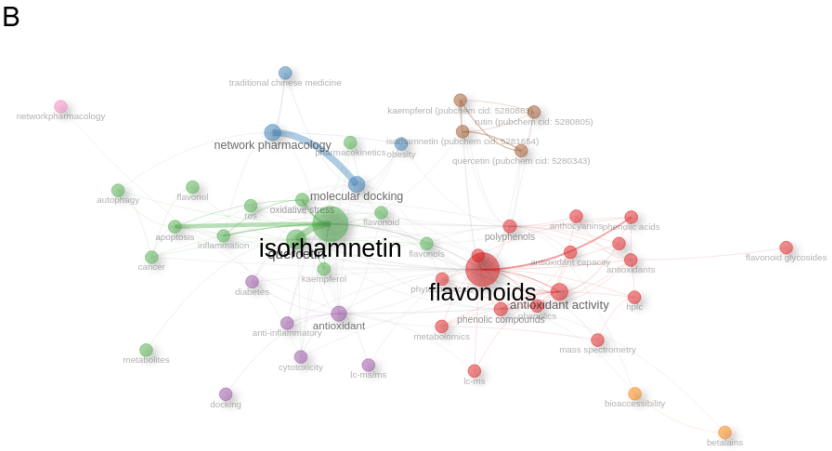
This study combined network pharmacology, bibliometric analysis and in vitro experiments to systematically explain the anti-glioma mech-





D **Top 10 Keywords with the Strongest Citation Bursts**

Keywords	Year	Strength	Begin	End	2014 - 2024
phenolic compounds	2016	0.97	**2016**	2017	
epithelial-mesenchymal transition (emt)	2019	1.44	**2019**	2020	
lung cancer	2019	1.44	**2019**	2020	
achyrocline satureioides	2020	0.82	**2020**	2021	
gastric cancer	2015	1.45	**2021**	2022	
acute toxicity	2021	0.85	**2021**	2022	
enzyme inhibition	2021	0.85	**2021**	2022	
endometrial cancer	2021	0.85	**2021**	2022	
cell cycle	2018	0.31	**2021**	2022	
antitumor activity	2017	0.99	**2022**	2024	



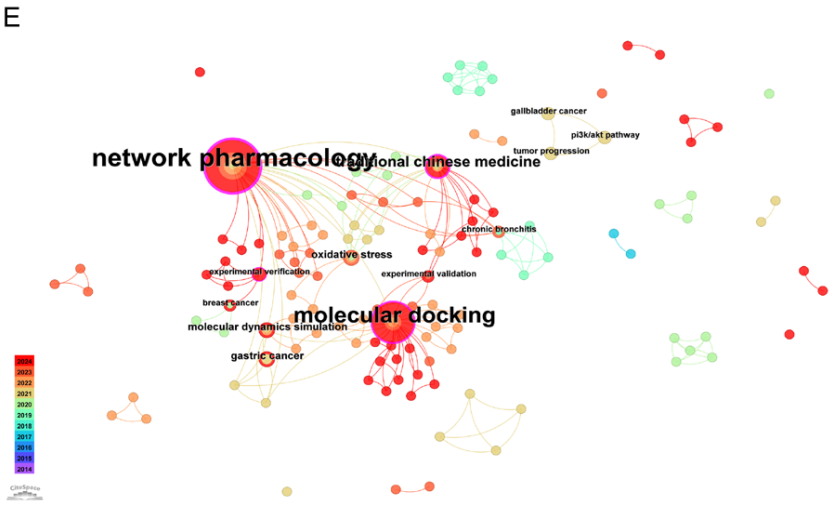


Figure 3. Results of Bibliometric Analysis on Isorhamnetin in Tumor Research. A. Publication trends of isorhamnetin-related studies over the past decade. B. Co-occurrence network of research themes in isorhamnetin studies. C. Annual publication count of isorhamnetin-related cancer research (2014-2024). D. Top 10 keywords with strongest citation bursts: blue bars indicate keyword appearance periods, red bars denote burst periods. E. Keyword co-occurrence analysis.

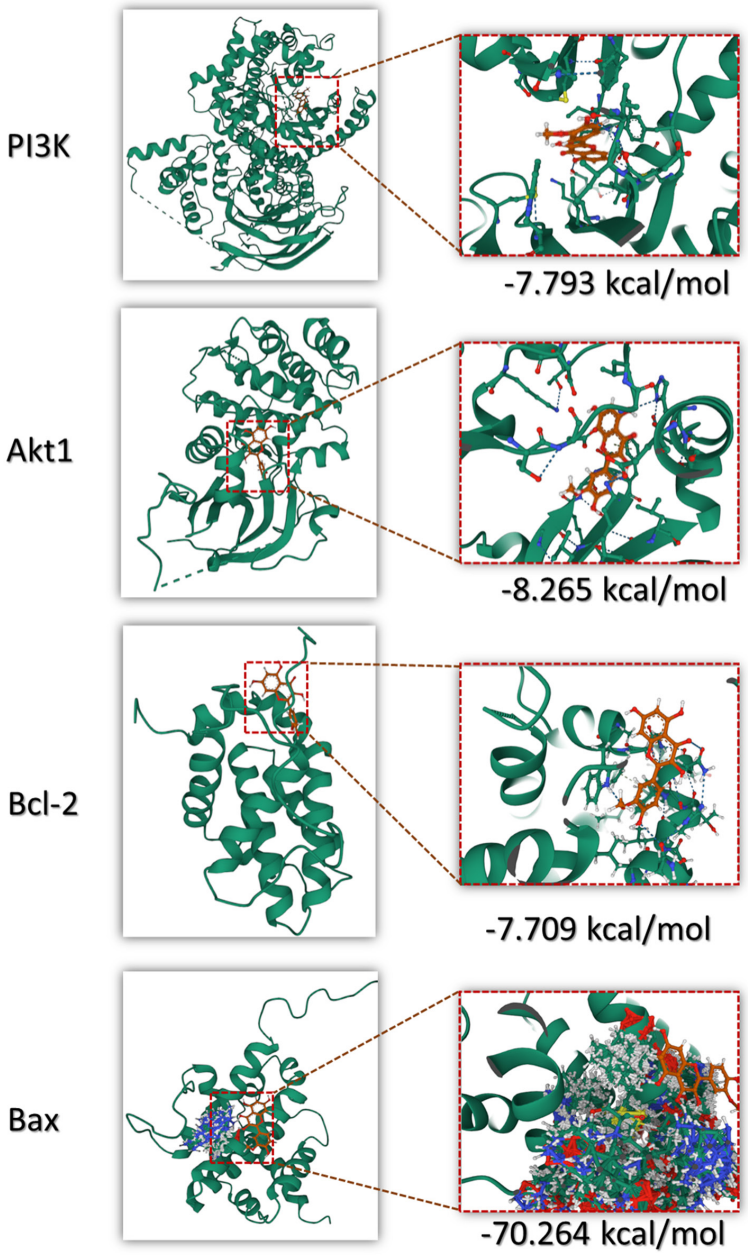


Figure 4. Molecular docking diagram of isorhamnetin and target molecules in the PI3K-Akt pathway.

anism of isorhamnetin to provide complementary strategies for the treatment of glioma. Combining network pharmacology and Bibliometric analysis, we identified isorhamnetin could be used as a potential active ingredient in the regulation of glioblastoma (GBM) through the PI3K/Akt pathway, which was validated by in vitro experiments.

Network pharmacology has emerged as a promising approach in modern TCM and herbal formula research. It establishes connections between multi-pharmacological networks and gene networks of human diseases. Through the association of comprehensive databases, network pharmacology can be used to analyze the complex relationship among biological systems, drugs and diseases, firstly to reveal the drug mechanisms and elucidate the multifaceted characteristics of TCM. This gives a theoretical and technical basis for drug discovery [21]. In recent years, the approach of network pharmacology is increasingly being used to understand the mechanisms of action of herbal compounds. It can give the systems-level perspective to the molecular mechanisms of TCM [22, 23]. Therefore, this method provides good support for the studying of the association between active compounds contained in astragalus and glioma. Our results from network pharmacology suggests that isorhamnetin, an active compound in astragalus, can exert its effects through the PI3K/Akt pathway. As a quantitative method to analyze scientific literature, bibliometric analysis can objectively and comprehensively depict the evolutionary trajectory and knowledge structure of research fields, reveal the change of research hotspots, collaborative networks, knowledge structures, and guide future research [24, 25]. Our bibliometric analysis of isorhamnetin's association with oncology gives us some hints of isorhamnetin involvement in the PI3K/Akt signaling pathway. Based on the network pharmacological and bibliometric findings, we performed in vitro experiments to confirm our results. The results confirmed that isorhamnetin induced apoptosis and inhibits proliferation of GBM cells via the PI3K/Akt pathway. Compared with previous single-dimensional studies, the triple

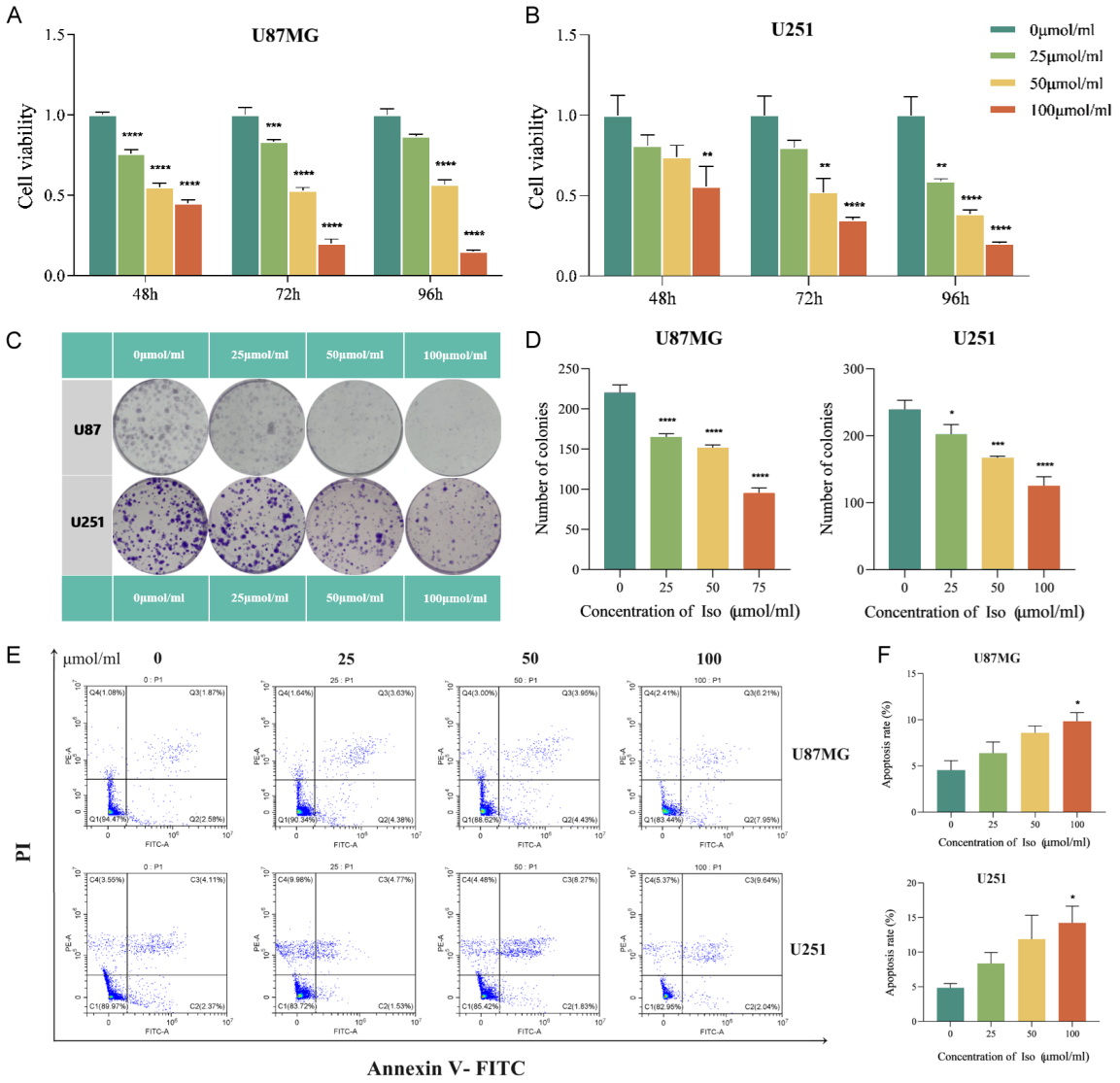


Figure 5. Impact of isorhamnetin on glioma cell viability and proliferation. (A, B) CCK8 assay results for U87MG (A) and U251 (B) cells exposed to varying isorhamnetin concentrations (0, 25, 50, 100 μM) over 48, 72, and 96 hours. (C, D) U87 and U251 cells underwent clonogenic assays under a 4× magnification microscope (C) and quantitative analysis (n = 3) (D) after treatment with varying concentrations of isorhamnetin (0, 25, 50, 100 μM). (E, F) Flow cytometry was used to assess apoptosis in U87 and U251 cells exposed to varying isorhamnetin concentrations (0, 25, 50, 100 μM) for 48 hours (E), followed by quantitative analysis (n = 3) (F).

validation model of network pharmacology - bibliometrics and in vitro experiments - improves the reliability of predicting the pharmacological effects of natural products.

Isorhamnetin is a natural compound, commonly found in astragalus, sea buckthorn, and ginkgo, with a broad spectrum of pharmacological effects, including cardiovascular protection, anti-inflammatory, antioxidant, antibacterial, and antiviral effects [26, 27]. Beyond these, it also has anti-tumor effects as it inhibits cell proliferation and promotes apoptosis [28-30]. The underlying mechanisms include the regulation of a number of key signaling pathways such as PI3K/Akt, Ras/MAPK, and Wnt/β-catenin [27, 31]. Recent studies have found that dysregulation of signaling pathways, such as PI3K/Akt, in glioma is closely linked to malignant progression [32]. Abnormal PI3K/Akt pathway activation is strongly correlated with glioma growth, invasion and resistance to chemotherapy, highlighting its potential as a therapeutic target [33-35]. As an important kinase downstream of

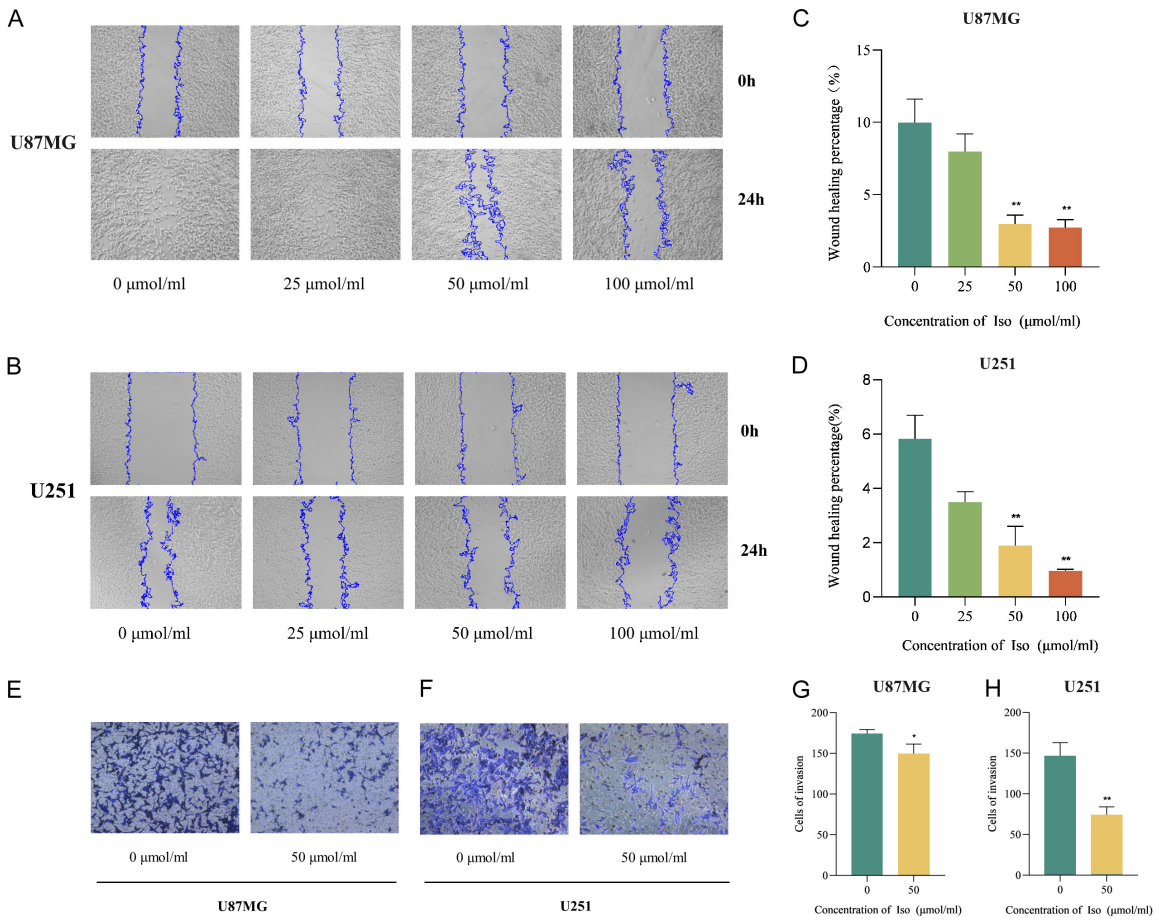


Figure 6. Effects of isorhamnetin on glioblastoma cell invasion and migration. (A-D) Images from wound healing assays after 48-hour treatment with varying isorhamnetin concentrations (0, 25, 50, 100 μM) isorhamnetin of U87 (A) and U251 (B) cells under a 4× magnification microscope, along with quantitative analysis of wound healing areas for U87 (C) and U251 (D) cells. (E-H) Images from Transwell assays showing U87 (E) and U251 (F) cells treated with 50 μM isorhamnetin for 48 h under a 10× magnification microscope, and quantitative analysis of migrating U87 (G) cells and U251 (H) cells (n = 3/group, t-test).

PI3K, Akt kinase via its phosphorylation plays a central role in the transmission of pro-survival signals and is therefore an important therapeutic target and a downstream effector of PI3K signaling [36, 37]. In our study, we found that isorhamnetin had significant effects of suppression on the phosphorylation levels of PI3K and Akt, while the total protein expression was unaffected in both cases. Consistent with reports in melanoma and breast cancer [28, 29], the treatment triggered apoptosis, thus inhibiting the proliferation and migration of glioma cells. This anti-tumor effect was associated with alterations of apoptosis-related proteins (increased Bax and decreased Bcl-2). Specifically, isorhamnetin can cause G2/M phase cell cycle arrest. At the molecular level, it first lowered the phosphorylation level of Akt along with downregulating Bcl-2 and upregulating Bax expression. In combination, these changes are responsible for the occurrence of apoptosis [38-40]. Consistent with existing literature, this study also validates the broad spectrum antitumor potential of isorhamnetin by inhibiting the PI3K/Akt signaling pathway. Its mechanism of action shows its synergistic effect with the reported PI3K/Akt inhibitors (e.g., NVP-BEZ235 and MK-2206), which further supports the development prospects of isorhamnetin as a candidate drug for glioma therapy.

This research is limited in a number of ways. Only cellular experiments are covered in the present study, and the effectiveness of isorhamnetin and its blood-brain barrier penetrability have not been confirmed in the animal

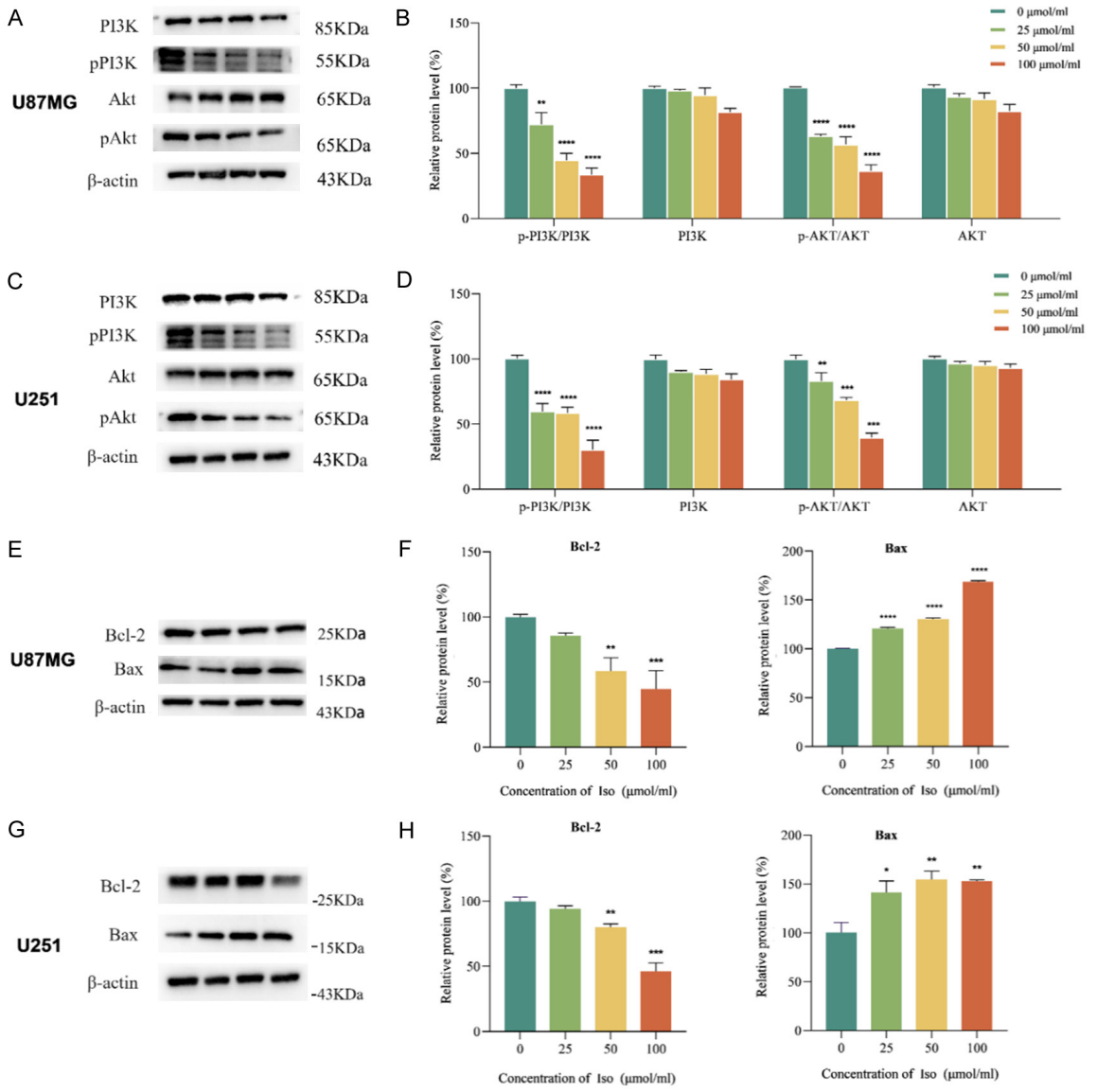


Figure 7. WB analysis of isorhamnetin's inhibition of the PI3K/Akt signaling pathway in glioma cells. (A-D) WB analysis was conducted to assess the expression levels of PI3K, p-PI3K, Akt, and p-Akt in U87 and U251 cells following 48-hour treatment with isorhamnetin at concentrations of 0, 25, 50, and 100 μM (A, C), along with corresponding quantitative evaluations (B, D). (E, F) The protein levels of Bcl-2 and Bax were examined in the same cell lines and WB conditions (E, G), accompanied by quantitative analysis (F, H).

models so far. We have found an association between isorhamnetin and PI3K-Akt pathway. Nevertheless, the causal relation and interaction with other pathways need to be explored in more detail. Future research is required to elucidate the mechanism in specific pathway inhibitors or activators in in vivo and advanced in vitro systems. However, through the incorporation of network pharmacology, bibliometrics, and in vitro effects, this study explains systematically how isorhamnetin controls apoptosis and proliferation in glioma cells, which will become the conceptual basis of future pharmaceutical development.

Conclusion

This study integrates network pharmacology, bibliometrics, and in-vitro experiments to illustrate that isorhamnetin, which is the active ingredient in astragalus, suppresses the growth, migration, and invasion of GBM cells through the PI3K/Akt signaling pathway, ultimately inducing apoptosis and displaying anti-tumor

effects. The findings suggest that isorhamnetin is a viable candidate for GBM therapy.

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Disclosure of conflict of interest

None.

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