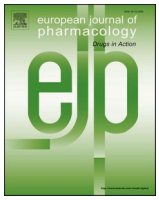
European Journal of Pharmacology 980 (2024) 176865

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar



Vitexin promotes the anti-senescence effect via inhibiting JAK2/STAT3 in D-Galactose-induced progeria mice and stress-induced premature senescence

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ARTICLE INFO

Keywords:
Vitexin
Progeria mice
Aging
Senescence-associated secretory phenotype
JAK2/STAT3

ABSTRACT

Vitexin is a natural flavonoid glycoside compound extracted from the leaves and seeds of Vitex negundo. It is widely distributed in the leaves and stems of numerous plants and exhibites remarkable anti-tumor, anti-inflammatory, and anti-hypertensive properties. However, whether vitexin presents the anti-aging and senescence prevention effect has not been fully elucidated. The purpose of this study is to investigate the effect of vitexin on progeria mice and cellular senescence, as well as its underlying molecular mechanisms. To generate a premature aging/senescence model *in vivo* and *in vitro*, we used D-galactose (D-gal), hydrogen peroxide (H_2O_2), and adriamycin (ADR), respectively. Our findings demonstrated that vitexin potentially delays D-gal-induced progeria mice; similar effects were observed in stress-induced premature senescent fibroblasts in culture. Interestingly, this effect of vitexin is closely correlated with the reduction of the senescence-associated secretory phenotype (SASP) and the inhibition of the SASP-related JAK2/STAT3 pathway. Furthermore, we determined that vitexin meets the pharmacological parameters using the freely available ADMET web tool. Collectively, our findings demonstrate that vitexin possesses anti-senescence and anti-aging properties due to the inhibition of SASP and suppression of JAK2/STAT3 signaling pathway.

1. Introduction

Aging is an inevitable natural process accompanied by the onset of cellular senescence. Pathological conditions in senescent cells contribute to increased aging and age-related diseases (Childs et al., 2017). Researchers are seeking new therapeutic medications and targets to eradicate or mitigate the negative consequences of these senescent cells during aging (Zhang et al., 2023). The stress-induced aging/senescence model has been adopted to investigate the effects of various bioactive ingredients on senescence and its underlying mechanisms both *in vivo* and *in vitro* (Xia et al., 2023; Yuan et al., 2022). A progeria-like mouse model was established using chronic injection with D-galactose (D-gal) *in vivo* (Wei et al., 2005). Hydrogen peroxide (H_2O_2) (Han et al., 2016) and adriamycin (ADR) (Huang et al., 2022) have been commonly used as chemotherapeutic agents to induce cellular senescence *in vitro*.

Vitexin (Apigenin-8-C-β-D-glucopyranoside) is a flavonoid compound extracted from the leaves and seeds of the Chinese herb, *Vitex negundo*, as well as from hawthorn, buckwheat, bamboo, mung bean, and passiflora (Babaei et al., 2020). Vitexin has been used in clinical treatment of cardiovascular diseases, primarily improving blood circulation and removing blood stasis (Fang et al., 2023). Accumulating evidence indicates that vitexin exhibits preclinical antitumor activity and

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https://doi.org/10.1016/j.ejphar.2024.176865
Received 12 March 2024; Received in revised form 26 July 2024; Accepted 29 July 2024
Available online 30 July 2024

alleviates ethanol-induced liver injury (Liu et al., 2018; Wang et al., 2019; Yuan et al., 2020). Additionally, vitexin has various bioactivities including antioxidant (Babaei et al., 2020), anti-inflammatory(Zhao et al., 2021), anti-myocardial ischemia (Yang et al., 2021), anti-epilepsy (Luo et al., 2018), anti-nociceptive (Zhu et al., 2016), and neuro-protective effects (Malar et al., 2018; Mustapha and Mat Taib, 2023). Vitexin has been proved to be an excellent antioxidant due to its ability to donate electrons and scavenge free radicals. In recent years, there has been increasing interest in natural anti-aging products, and vitexin has garnered significant attention for its strong antioxidant properties. Some studies have reported that vitexin treatment improved the body movement of aged nematodes, suggesting that vitexin positively affects the healthspan of nematodes (Lee et al., 2015). However, the mechanisms responsible for its effects on cellular senescence and aging have not been fully elucidated.

Vitexin has been shown to increase cell viability and suppress inflammatory cytokines (TNF-α, IL-6) and ROS levels (Chen et al., 2016). Proinflammatory cytokines are the main components of senescence-associated secretory phenotype (SASP) (Han et al., 2022), and the upregulation of SASP and ROS are hallmark of aging/senescence (Lopez-Otin et al., 2013). Therefore, in this study, we investigated the role and mechanism of vitexin in inhibiting SASP in an stress-induced aging/senescence model. Our previous studies confirmed the role of JAK2/STAT3 pathway in SASP production and senescence development. STAT3 mediates a paracrine positive-feedback loop to reinforce and amplify local SASP production and downstream senescence (Han et al., 2020b). To alleviate stress-induced senescence and aging, a novel medication or compound that can inhibit JAK2/STAT3 pathway is required.

In this study, we investigated the effects and mechanisms of vitexin in D-galactose-induced progeria mice and stress-induced premature senescence by detection of senescence markers, SASP factors, and the activation in JAK2/STAT3 pathway. Additionally, we used the ADMET web tool to analyze the pharmacokinetic properties of vitexin.

2. Materials and methods

2.1. Chemicals and reagents

Vitexin (Cat# HY-17471A) and doxorubicin (adriamycin, ADR) (Cat# HY-15142) were purchased from MedChemExpress (Shanghai, China). D-galactose (Cat# A600215) were purchased from Sangon Biotech (Shanghai, China). Antibodies used included β-actin (Cat# NC011) was from Zhuangzhibio (Xi'an, China). p21 (Cat# CY5543) was from Abways Technology (Shanghai, China). p16 (Cat#ab51243) was purchased from Abcam (MA, USA). JAK2 (Cat#WL02188) and pJAK2 Y1007/1008 (Cat#WL02997) were from Wanleibio (Xi'an, China). STAT3 (Cat#4904) and pSTAT3 Y705 (Cat#9145) were from Cell Signaling Technology (Danvers, MA).

2.2. Animal experiments

6 week-old male mice (C57BL/6, 22 ± 2 g) were obtained from Cyagen and housed in a Xi'an medical university animal center maintained at 23–25 °C with a 12-h light/dark cycle and free access to drinking water and food. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Xi'an medical university (Xi'an, China, approval No. XYLS2020102). D-gal-induced aging mouse model was performed as described previously (Azman and Zakaria, 2019). The mice were randomly assigned into three groups (n = 7) after a week of acclimation: control group (saline i.p, solvent p.o.), D-gal model group (treatment D-gal at 150 mg/kg/day via intraperitoneal injection, solvent p.o.), and D-gal plus vitexin group (administered D-gal plus vitexin at 60 mg/kg/day). The mice were administered either a PBS or D-gal intraperitoneal injection for 14 weeks or were oral administration of vitexin for 13 weeks starting from the 2nd week of D-gal injection. Mice were sacrificed at the end of the experiments, and lung, liver and brain tissues were collected, snap-frozen in liquid nitrogen, and maintained at −80 °C until further use. The serum and liver tissue assessment were collected for assessment of MDA levels. The liver and kidney tissues were collected for RT-qPCR and immunoblotting.

2.3. Y maze

Y maze assay was performed as previously described (Huang et al., 2022). The three arms of the Y maze were randomly set as the start arm, the new arm, and the other arm. Before test, the novel arm was blocked with a partition, the mouse was put in the starting arm and allowed to move freely between starting arm and other arms to adapt for 10 min. After 1 h, opened the new arm, put the mice in platform from the starting arm, let it move freely in the three arms for 5 min, the route of mice autonomous movement was recorded. After each test, the Y-maze platform was cleaned with 75% alcohol and water. The starting arm, novel arm and other arms of different mice were randomly arranged during each test. The calculation method is as follows: suppose that the new arm is A, the starting arm is B, the other arm is C, and the movement route of the mice during the test is ABCACACAB. During the test, the mice path passes through 9 arms, and the number of shuttles is 9; consecutive ABC, BCA, and CAB are counted as 1 point. The spontaneous alternation score (%) = [(Number of alternations)/(Total arm entries-2)] × 100.

2.4. MDA assay

To test the lipid peroxidation, we use Lipid Peroxidation (MDA) Assay Kit (Cat# A003-1, Nanjing Jiancheng Bioengineering Institute). The procedure was followed by the manufacturer's instruction.

2.5. Cell culture

NIH3T3 (murine fibroblast lines) cells and MRC-5 (human normal embryonic lung fibroblast) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37 °C.

2.6. Cellular senescence models and vitexin treatment

For H_2O_2-induced cellular senescence, a H_2O_2 treatment protocol was performed as previously described (Han et al., 2016, 2020b). In brief, NIH3T3 cells were trypsinized and suspended in phosphate buffer solution (PBS) at a 1×10^6 cells/mL density and exposed to 400 μM (NIH3T3) or 250 μM (MRC-5) H_2O_2 in an Eppendorf tube at 37 °C for 45 min. During H_2O_2 treatment, the tube was turned upside down gently every 5–10 min. After H_2O_2 treatment, the cell suspension were rotated at 800 rpm for 5 min and supernatants were removed. Then the cells were cultured with complete medium and accepted different concentration vitexin treatment for 72 h. Vehicle control cells were incubated with 0.001% DMSO.

For ADR-induced senescence, cells were treated with 0.5 μM (NIH3T3) or 0.1 μM (MRC-5) ADR for 48 h, respectively. Media was changed after 48 h and cells were treated with different concentration vitexin for 72 h. Vehicle control cells were incubated with 0.001% DMSO.

2.7. Cell viability assay

H_2O_2-treated and control NIH3T3 cells were incubated in complete medium with vitexin (0–400 μM) for 3 days. Cell viability was determined by CCK8 assay. The results were expressed as mean ± SD of triplicates.

2.8. Senescence-associated β-galactosidase (SA-β-gal) staining

Intracellular senescence-associated-β-galactosidase (SA-β-gal) activity was assayed using a SA-β-gal staining kit (Cat#C0602, Beyotime, Beijing, China) according to the manufacturer's instruction, and senescent cells were identified as bluish green stained cells under a phase-contrast microscope. The percentage of SA-β-gal-positive cells in total cells was determined by counting 1000 cells in eight random fields, for each group. The results were expressed as mean ± SD of triplicates.

For SA-β-gal staining of tissues (liver, lung, brain and fat) *in vitro*. Tissues were fixed with 10% paraformaldehyde for 24 h. Then, using gradient concentrations of sucrose for dehydration, and tissues was embedded in OCT, sectioned at 10 μm thickness. The SA-β-gal staining was performed using SA-β-gal staining kit as above description. In addition, the fat tissue was cut into little pieces, and the SA-β-gal staining were performed with 24 well culture plate in the staining solution at 37 °C for 12 h.

2.9. EdU incorporation

EdU incorporation was detected by using the Cell-Light™ EdU Apollo® 567 *in vitro* kit (RiboBio, Cat#C10310-1) according to the manufacturer's instructions. Cells were labelled with 10 μM EdU for 2 h. After 4% paraformaldehyde fixation, cells were rinsed once with PBS and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Then, cells were rinsed once with PBS and stained with Apollo 567 staining solution for 30 min. The staining mix was prepared fresh each time and was used for staining cells immediately. After staining, the cells were washed several times with PBS with 0.5% Triton X-100. Cells were counterstained with Hoechst 33342 and imaged by fluorescence microscopy. Fluorescent images were analyzed using Image J. The percentage of EdU-positive cells in total cells was determined by counting more than 600 cells in eight random fields for each group. The results were expressed as mean ± SD of triplicates.

2.10. ELISA assay

Medium was changed 24 h before collecting culture supernatant for ELISA analysis. The cell culture supernatant was centrifuged at 1000 g, 15 min at 4 °C. Quantification of IL-1β in cell culture supernatant was assessed by Mouse Quantikine ELISA Kit (IL-1β: Cat#CSB-E08054m, CUSABIO, Wuhan, China) according to manufacturer instructions. Cells were counted to determine the cell number and the measured IL-1β levels were normalized to the cell number.

2.11. Immunoblotting

Whole cell lysates were extracted in RIPA lysis buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate, 1 mM PMSF) supplemented with phosphatase inhibitor cocktail. The lysates were rotated at 12000 rpm 4 °C for 30 min, and supernatants were loaded. The protein concentration was determined by BCA method (Cat#CW20011S, CWBIO, Beijing, China). The lysates containing 30 μg of proteins were loaded on the SDS-polyacrylamide gel and separated by electrophoresis, followed by blotting on a polyvinylidene difluoride membrane (Cat#IPVH00010, Millipore, Germany). The target proteins were probed by corresponding primary antibodies and then incubated with the secondary antibody. Immunological signals were surveyed via an electrochemical luminescence method, using the Immobilon Western Chemilum HRP Substrate kit (Cat#WBKLS0500, Millipore) and ChemiDoc™ Touch Imaging system. The band intensities were quantified by Image J software. Every experiment was repeated at least three times, and representative data are shown.

2.12. RNA extraction and RT-qPCR

Total RNA was isolated from cultured cells using TRIzol (Takara, Shiga, Japan) and 1 μg of total RNA was used for reverse transcription by PrimeScript™ RT reagent Kit with gDNA Eraser (Cat# RR047A, Takara, Shiga, Japan). Then quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using TB Green® Premix Ex Taq™ II (Cat# RR820A, Takara, Shiga, Japan). The sequences of primers for real-time PCR analysis are as follows: 18S rRNA forward: TTGACGGAAGGGCACCACCAG, reverse: GCACCACCACCACGGAATCG. *p21* forward: GTGGCCTTGTCGCTGTCTT, reverse: GCGCTTGGAGTGATAGAAATCTG. *p16* forward: CTGCAAGAGACTTCCATCCAG, reverse: AGTGGTATAGACAGGTCTGTTGG. *Il-1α* forward: CGAAGACTACAGTTCTGCCATT, reverse: GACGTTTCAGAGGTTCTCAGAG. *Il-1β* forward: GCAACTGTTCCTGAACTCAACT, reverse: ATCTTTTGGGGTCCGTCAACT. *Il-6* forward: ACTCACCTCTTCAGAACGAATTG, reverse: CCATCTTTGGAAGGTTCAGGTTG. *Il-8* forward: CTGGTCCATGCTCCTGCTG, reverse: GGACGGACGAAGATGCCTAG. The 18S rRNA served as the internal normalization control. Experiments were repeated three times.

2.13. ADMET Prediction

Vitexin's physicochemical properties, water solubility, absorption, metabolism, excretion and medicinal chemistry were predicted using the freely available Web tool SwissADME (http://www.swissadme.ch/index.php), ADEMTlab 3.0 (https://admetlab3.scbdd.com) and ADMET (https://db.yaozh.com/pharmwings).

2.14. Statistical analysis

Data are expressed as means ± SD from at least 3 biological replicates. Statistical analysis was performed using Prism 7 (Graphpad Software Inc.). Student's *t*-test was used to analyze differences between two groups, and differences between multiple groups were assessed by one-way analysis of variance followed by the Bonferroni post hoc test. $p < 0.05$ is considered to be significant and $p < 0.01$ was considered highly significant.

3. Results

3.1. Vitexin alleviates aging in progeria mice model

The chemical structure of vitexin is shown in Fig. 1A. To determine the effect of vitexin on aging *in vivo*, a progeria-like model was established using chronic D-gal injection, vitexin was administered orally one week later, following the procedure shown in Fig. 1B. As expected, D-gal treatment markedly induced progeria-like phenotypes, including increased fur graying and cognitive decline (Y maze), whereas vitexin exhibited a protective role (Fig. 1C and D). In addition, the level of malondialdehyde (MDA) increased in the serum and liver tissue of D-gal-induced aging mice, but decreased after long-term vitexin treatment (Fig. 1E). More importantly, no pathological damage was observed in the liver, kidney, and lung tissues after vitexin treatment, as analyzed via hematoxylin-eosin (HE) staining, indicating that vitexin was not toxic to the mice (Fig. S1). Next, we evaluated the effect of vitexin on senescence markers, including senescence-associated β-galactosidase (SA-β-gal) activity, and the expression levels of senescence-associated proteins p21 and p16 in various tissues. Compared to D-gal-induced aging mice, vitexin treatment significantly decreased the SA-β-gal activity in epididymal fat (Fig. 2A), liver, lung, and brain tissues (Fig. 2B). Additionally, the expression of senescence-associated proteins p21 and p16 was increased in D-gal-induced aging mouse tissues, but it was considerably decreased in vitexin-treated mice (Fig. 2B–D). Taken together, these results indicate that vitexin can improve age-related behavioral phenotypes and alleviate tissue aging in a progeria mice model.

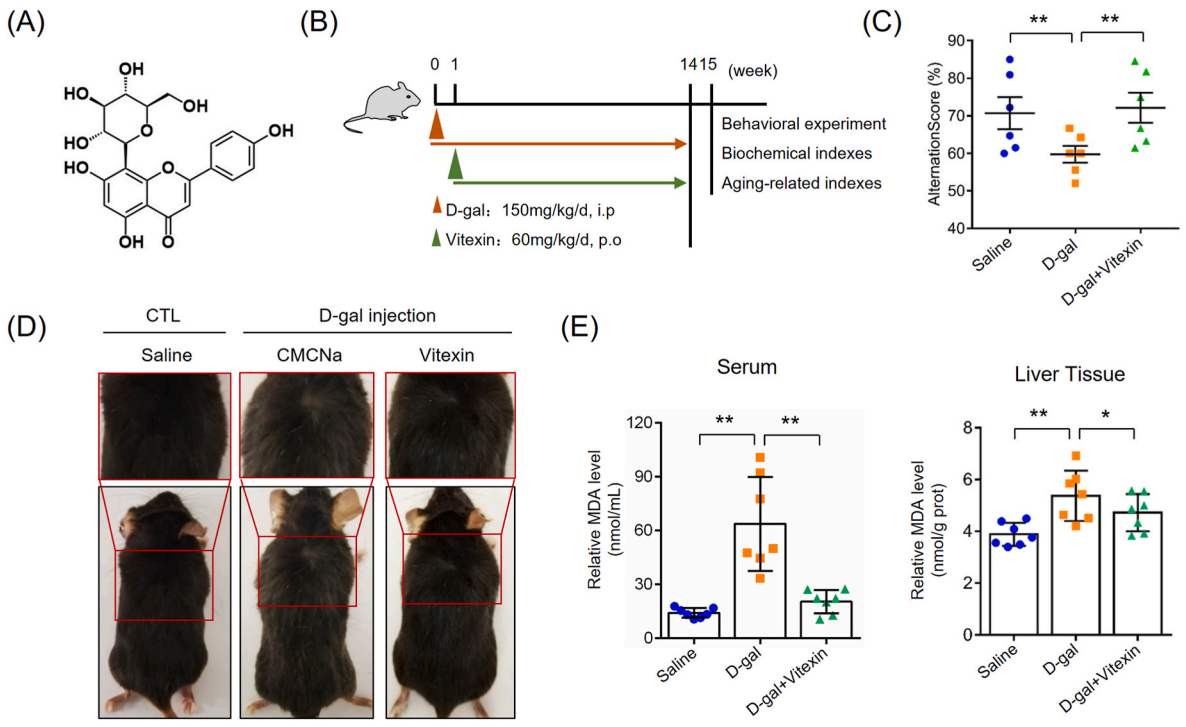

Fig. 1. Vitexin Improves Age-related Behavioral Phenotypes in D-gal-Induced Aging mice. (A) Chemical structure of vitexin. (B) Schematic diagram of the animal experiment plan. Mice received intraperitoneal injections of D-gal (150 mg/kg/day, i.p.) daily for 14 weeks to induce progeria, and vitexin (60 mg/kg/day, p.o.) was administered daily for 13 weeks for treatment. (C) The alternation score of saline, D-gal and vitexin group mice (n = 6/group) were analyzed by Y-maze test. (D) Effects of vitexin on changes in mouse hair phenotype. (E) Malondialdehyde (MDA) levels in serum and liver tissue. Data are plotted as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the indicated sample.

3.2. H_2O_2 and ADR treatment induces cellular senescence

Next, to investigate the effect of vitexin on cellular senescence, we established ADR-induced and H_2O_2-induced SIPS (stress-induced pre-senescence) cell models in NIH3T3 and MRC-5 cells, respectively. As shown, ADR-treated NIH3T3 cells gradually became enlarged, flattened and largely positive for SA-β-gal staining (Fig. 3A and B). The arrest of cell growth was apparent (Fig. 3A and B), and the protein levels of p21 and p16 were elevated (Fig. 3C). Similarly, H_2O_2 treatment also increased the number of SA-β-gal positive cells, decreased EdU incorporation, and the upregulated the levels of p21 and p16 proteins (Fig. 3A–C). Additionally, this SIPS cell model was successfully established using MRC-5 human lung fibroblast cells (Fig. 3D–F). These results demonstrate that the H_2O_2 or ADR treatment protocol we used can reliably induce cellular senescence in fibroblasts. Therefore, the following experiments were performed within 3 days after H_2O_2 or ADR treatment.

3.3. Vitexin prevents stress-induced pre-senescence

Using the established cell model for senescence, we first examined the effect of vitexin on the cell viability of NIH3T3 and MRC-5 cells. Proliferating and senescent cells were treated with 0–400 μM vitexin for 3 days, and cell viability was evaluated using the CCK8 assay. As shown in Fig. S2, the cell viability of senescent NIH3T3 did not significantly decrease up to 200 μM. Vitexin reduced the viability and caused cell death in proliferating MRC-5 to a greater extent than in senescent cells at 200 μM. However, the cell viability of both proliferating and senescent NIH3T3 and MRC-5 cells decreased at 400 μM (Fig. S2). Therefore, we selected concentrations of 50 μM and 100 μM for the following experiments. Then, we evaluated the effect of vitexin on ADR-induced cellular senescence by SA-β-gal staining, EdU incorporation, and the levels of senescence-associated proteins p21 and p16. As shown in Fig. 4A and B, the ratio of SA-β-gal-positive cells was remarkably reduced upon treatment with vitexin, with a greater reduction at the higher concentration of 100 μM. Consistently, the protein levels of p21 and p16 decreased in ADR-induced senescent cells with vitexin treatment (Fig. 4C). We also tested the effect of vitexin on H_2O_2-induced cellular senescence. As expected, the anti-senescence effect of vitexin (50 and 100 μM) was confirmed by decreased positive of SA-β-gal staining (Fig. 4D and E), an increased EdU incorporation (Fig. 4D, E), and a reduction in the levels of p21 and p16 protein (Fig. 4F). Notably, vitexin also showed a senescence-inhibitory effect in MRC-5 cells, as evidenced by a remarkable reduction in the ratio of SA-β-gal-positive cells and decreased expressions of p21 and p16 proteins (Figs. S3A–C). These results indicate that vitexin prevents stress-induced pre-senescence.

3.4. Vitexin inhibits senescence via downregulation of JAK2/STAT3-mediated SASP production

Based on network pharmacology and our previous study, it has been reported that JAK2/STAT3 pathway mediates SASP production, which plays an important role in senescence development (Han et al., 2020b). We investigated whether the effects of vitexin on cellular senescence and mouse aging were associated with JAK2/STAT3 pathway. The activation of JAK2 and STAT3 is indicated by enhanced phosphorylation at JAK2 (Y1007/Y1008) and STAT3 (Y705). Our results showed that the protein levels of phosphorylated JAK2 and STAT3 were decreased in vitexin-treated senescent NIH3T3 cells (Fig. 5A and B), indicating that vitexin downregulated JAK2/STAT3 activity in senescent cells. Besides, we assessed whether vitexin can affect senescence-associated secretory phenotype (SASP) production. Compared with ADR and H_2O_2 groups, treatment of senescent NIH3T3 cells with vitexin (100 μM) for 3 days significantly decreased the mRNA levels of SASP factors, including interleukin-1α (Il-1α), interleukin-1β (Il-1β), interleukin-6 (Il-6), and interleukin-6 (Il-8), whereas vitexin showed subtle effects on SASP

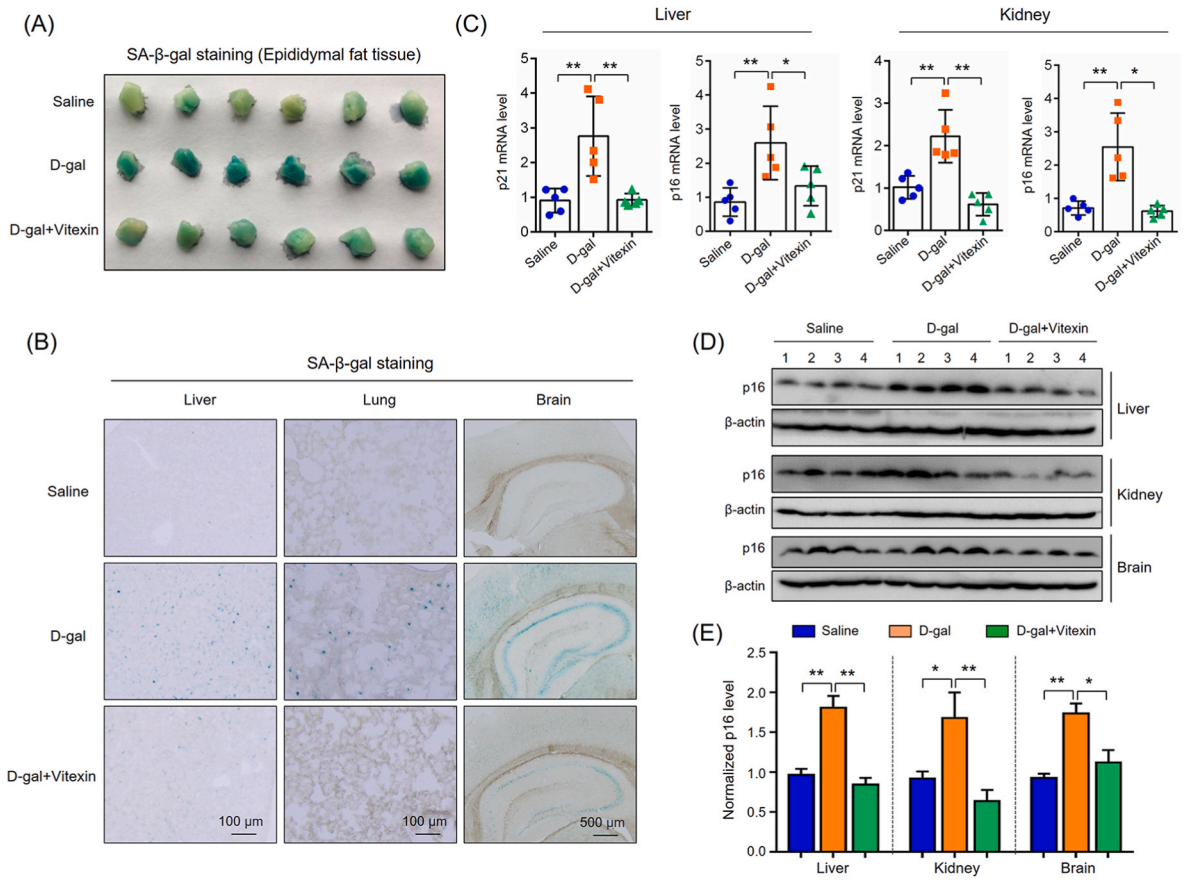

Fig. 2. Vitexin Alleviates Tissue Aging in Progeria Mice Model. (A) Representative images of SA-β-gal staining in epididymal fat tissue. (B) Representative images of SA-β-gal staining in liver, lung, and brain tissues. Scale bars, 100 μm or 500 μm. (C) RT-qPCR analysis of p21 and p16 mRNA levels in liver and kidney tissues. (D) Representative images of immunoblot assays for p16 in liver, kidney, and brain tissues. (E) Quantification of p16 expression levels using Image J software. β-actin served as an internal control. Data are plotted as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the indicated sample.

expression at a lower concentration (50 μM) (Fig. 5C). The ELISA results showed that the levels of IL-1β in the cell culture supernatants from vitexin-treated cells (ADR + vitexin and H_2O_2+vitexin) were significantly decreased compared to the ADR and H_2O_2 groups (Fig. 5D).

Next, the effect of vitexin on JAK2/STAT3-mediated SASP production was evaluated *in vivo* using the D-gal-induced progeria mouse model. Phosphorylated STAT3 expression was remarkably elevated in the D-gal-induced aging model mice, but a significant decrease was observed in the vitexin-treated mice (Fig. 5E, F). Additionally, vitexin reduced the expression of SASP factors in the liver and kidney tissues of mice (Fig. 5G). Collectively, these data indicate that the mechanism underlying the vitexin-mediated anti-aging effect is partially via the JAK2/STAT3 pathway.

3.5. *ADME Prediction for vitexin*

To estimate the pharmacokinetic properties of vitexin, we performed in silico analysis using the freely available web tool SwissADME (http://www.swissadme.ch/index.php), ADEMTlab 3.0 (https://admetlab3.scbdd.com) and ADMET (https://db.yaozh.com/pharmwings). We obtained the following interesting results (Table S1): vitexin has good water solubility, can be absorbable by the gastrointestinal tract, and can slightly penetrate the blood-brain barrier (BBB). Additionally, the plasma protein binding (PPB) of vitexin is low, indicating a high therapeutic index. It is either a substrate or an inhibitor of glycoprotein or cytochromes P450 (CYP) isoforms. The predicted plasma clearance of vitexin is low, and it is an intermediate short half-life drug. Furthermore, vitexin satisfies the Lipinski Rule (MW ≤ 500; logP ≤5; Hacc ≤10; Hdon ≤5, 1 violation), the Pfizer Rule (logP<3; TPSA>75), and the Golden Triangle (200 ≤ MW ≤ 500; logP ≤4), suggesting a favorable ADMET profile. However, its absolute bioavailability is low.

4. Discussion

In the present study, we demonstrated that vitexin can alleviate D-gal-induced premature aging and inhibit stress-induced senescence, and this effect is largely mediated by the inhibition of the JAK2/STAT3 pathway and subsequent SASP inhibition. To the best of our knowledge, this is the first report demonstrating the anti-aging effect of vitexin in mammals and elucidating the molecular basis for this effect. It is reasonable to consider that vitexin could be a potential drug for preventing aging and age-related diseases.

Previous studies have suggested the beneficial pharmacologic actions of vitexin, indicating that it can inhibit the formation and beta-amyloid peptide-induced toxicity (Guimarães et al., 2015), reduce neural cell death through downregulation of proinflammatory and apoptotic signaling pathways (Lima et al., 2018; Malar et al., 2018). The pharmacokinetic characteristics of vitexin, such as physicochemical properties, drug-likeness, and medicinal chemistry, encouraged us to further explore its biological significance. In this study, we clarified the aging protective role of vitexin, establishing a causal relationship verification through both *in vitro* and *in vivo* experiments. Although data from naturally aged mice would be more ideal, our results from D-gal-injected mice are still valuable since D-gal-induced aging mouse model is preferred due to its convenience, minimal side effects, and higher survival rate throughout the experimental period. This model effectively simulates age-related declines in physical power and cognition ability, elevated oxidative stress and DNA damage, increased

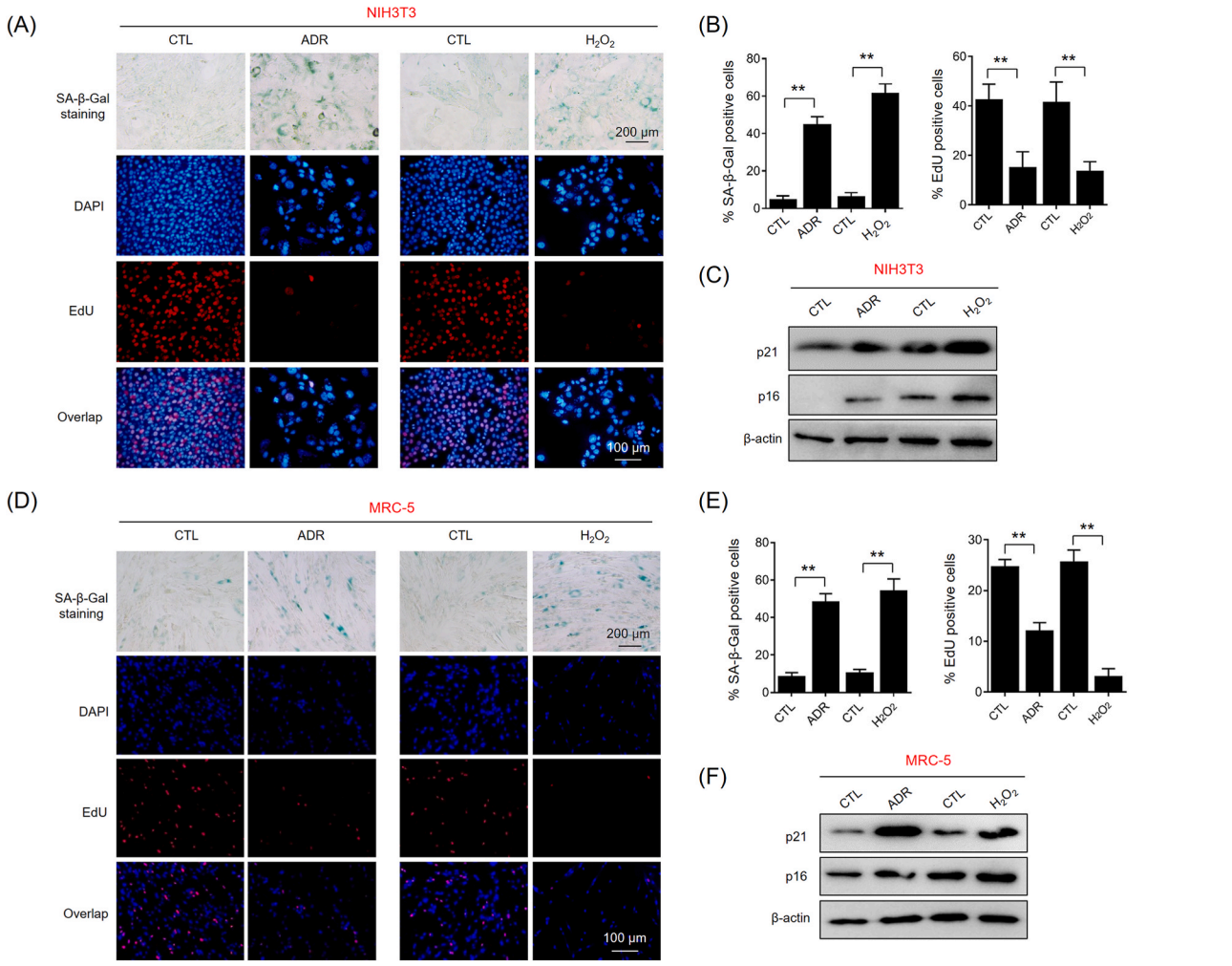

Fig. 3. Oxidative Stress Induces Senescence. (A) Representative images of SA-β-Gal staining and EdU incorporation in ADR-treated and H_2O_2-treated NIH3T3 cells. Scale bars, 200 μm or 100 μm. (B, E) Percentages of SA-β-Gal- and EdU-positive cells in a total of more than 600 cells. (C, F) Representative images of immunoblot assays for p21 and p16. (D) Representative images of EdU incorporation and SA-β-Gal staining in ADR-treated and H_2O_2-treated MRC-5 cells. Scale bars, 200 μm or 100 μm. Data are plotted as mean ± *SD*. $^{**}p < 0.01$ compared to the indicated sample.

expression of p16 and proinflammation factors in tissues, and obvious degenerative alterations in organs. To assess the effect of vitexin on aging *in vitro*, we employed a sub-lethal dose of hydrogen peroxide (H_2O_2) and adriamycin (ADR) to induce cellular senescence. Previous studies have validated the rationale of this model for stress-induced premature senescence (SIPS), which closely mimics the characteristics observed in replicative senescent cells (Han et al., 2020a; Huang et al., 2022).

In our study, we observed a significant reduction in serum and liver tissue levels of the MDA following vitexin treatment, consistent with its known anti-oxidative property. Our findings indicate that vitexin can ameliorate the overall aging phenotype in premature aging mice by reducing the number of SA-β-gal-positive cells and the expression of senescence markers such as p16 and p21. Although the pharmacological analysis showed that vitexin has low blood-brain barrier penetration (Table S1), our research demonstrated its ability to inhibit brain aging and improve cognitive impairment associated with aging. Given that the senescent tissues and cells express SASP, which includes various pro-inflammatory factors (cytokines, chemokines, proteases, growth factors, and bioactive lipids), thereby inducing inflammation in adjacent healthy cells and throughout tissues and body (Acosta et al., 2013; Zhou et al., 2024). Long-term exposure to SASP reinforces the cell cycle arrest in senescent cells and promotes their accumulation, further exacerbating systemic inflammation (Rodier et al., 2009). We suspected that vitexin may mitigate brain aging by reducing age-related systemic inflammation. Our data based on RT-qPCR assay confirmed this hypothesis, showing that vitexin significantly reduced the mRNA expression of several pro-inflammatory SASP factors (Il-1α, Il-1β, and Il-8) by 40–60% in the kidney and liver tissue of mice (Fig. 5E).

There are two potential strategies for delaying the occurrence of aging and various age-related diseases. One approach involves the clearance of already formed senescent, known as “senolytics” (Chaib et al., 2022). Eliminating senescent cells periodically in ageing mice has been shown to extend lifespan, improve tissue health, and mitigate age-related diseases by reducing SASP and associated inflammation. The second strategy is to prevent cellular senescence before tissue aging, termed "senotherapy" (Birch and Gil, 2020). In our study, cell viability assays indicated that the IC50 of vitexin in senescent cells was similar to that in proliferating cells for both NIH3T3 and MRC-5 cells. Vitexin showed no obvious cytotoxic effects within the range of 0–200 μM both in senescent and proliferating cells, suggesting that vitexin does not act as a senolytics. Our findings suggest that vitexin (50 and 100 μM) dose-dependently inhibits stress-induced pre-senescence and down-regulates SASP production, presenting anti-aging properties by inhibiting cellular senescence rather than promoting senescent cells elimination.

Regarding the mechanism by which vitexin inhibits senescence and alleviates aging. It is reported that JAK2/STAT3 pathway is activated

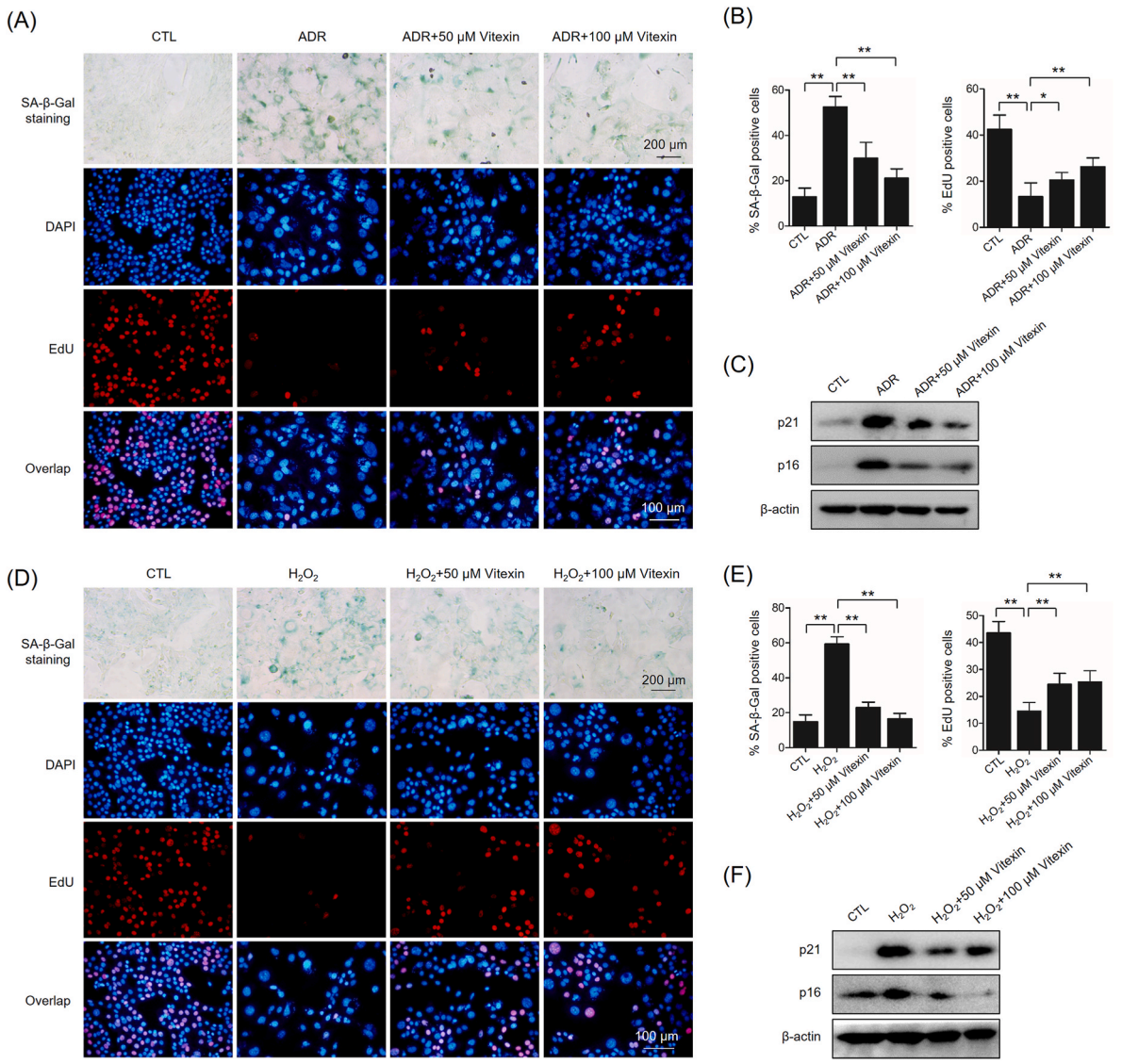

Fig. 4. Vitexin Inhibits Stress-Induced Pre-senescence. (A, D) Representative images of SA-β-Gal staining and EdU incorporation in NIH3T3 cells. Scale bars, 200 μm or 100 μm. (B, E) Percentages of SA-β-Gal- and EdU-positive cells in a total of more than 600 cells. (C, F) Representative images of immunoblot assays for p21 and p16. Data are plotted as mean ± *SD*. $*p < 0.05$, $**p < 0.01$ compared to the indicated sample.

during senescence and aging. SASP can be suppressed by inhibiting the JAK pathway in senescent cells, and JAK1/2 inhibitors have been shown to reduce inflammation and alleviate frailty in aged mice (Xu et al., 2015). IL-6, a major component of SASP, signals through the JAK2/-STAT3 pathway and stabilizes cellular senescence (Ortiz-Montero et al., 2017; Wu et al., 2020). Our previous studies also demonstrated that STAT3 inhibitors suppress SASP-induced inflammation and senescence *in vitro* (Han et al., 2020b). JAK2/STAT3 inhibition could prove to be a way to alleviate frailty or cellular senescence-related chronic diseases in late life (Xu et al., 2015). In the present study, we found that JAK2/-STAT3 signaling is involved in SASP reduction following vitexin treatment, as evidenced by significant decreases in phosphorylated JAK2 and STAT3 levels in senescent cells and aged mice (Fig. 5). Herein, we propose that the molecular basis of the vitexin's anti-aging effect involves JAK2/STAT3-mediated SASP reduction and mitigation of systemic inflammation.

In summary, using the D-gal-induced aging mice and stress-induced premature senescence model, we explored vitexin's role in aging/senescence repression and demonstrated a mechanism involving JAK2/STAT3 inhibition and SASP reduction. Further investigation is necessary to elucidate the detailed molecular mechanisms, but our findings suggest that vitexin may represent a promising new anti-aging drug. Clinical studies are warranted to explore the protective effects of vitexin against aging and age-related diseases. Given its low gastrointestinal tract absorption and oral bioavailability, future research should focus on enhancing its dissolution rate and absorption.

Funding

This work was supported by the National Natural Science Foundation of China (grant number 32300986), Natural Science Basic Research Plan in Shaanxi Province of China (grant number 2023-JC-YB-731), Scientific Research Program Funded by Education Department of Shaanxi Provincial Government (grant number 23JS051), College Students' Innovation and Entrepreneurship Training Program in Shaanxi Province (grant number S202211840052), Innovation Support Plan in Shaanxi Province of China (grant number 2021TD-48).

CRediT authorship contribution statement

Xiaojuan Han: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding

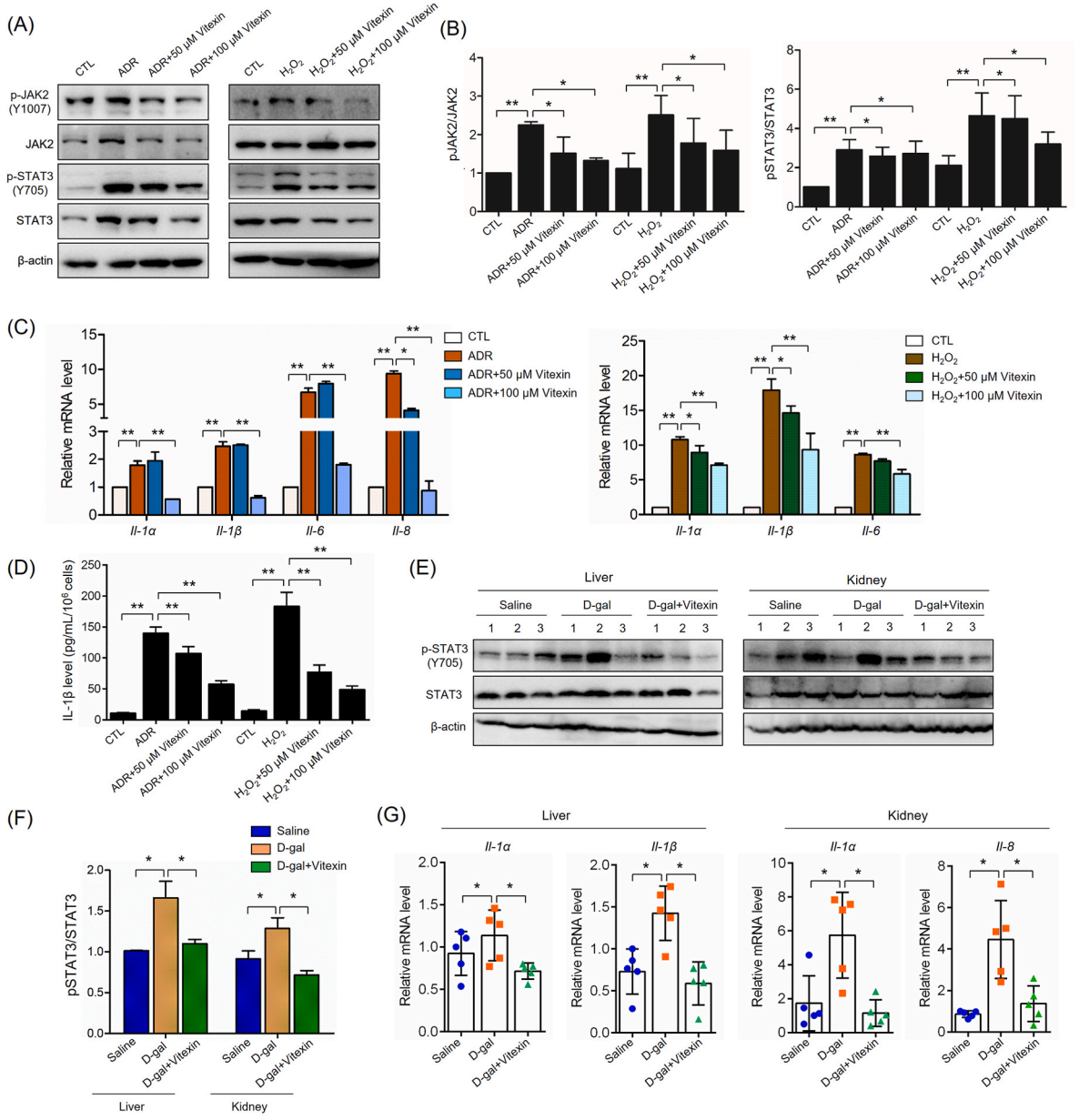

Fig. 5. Vitexin Decreases SASP via Inhibition of JAK/STAT3 Pathway in Stress-Induced Pre-senescence and D-gal-Induced Aging Mice. (A) Representative images of immunoblot assays for pJAK2, JAK2, pSTAT3, and STAT3. (B) Quantification of protein expression levels using Image J software. (C) mRNA levels of senescence-associated secretory phenotype (SASP) factors analyzed by RT-qPCR.(D) The level of IL-1β from cell culture supernatants was examined by ELISA. (E) Representative images of immunoblot assays for pSTAT3 and STAT3 in liver and kidney tissue. (F) Quantification of protein expression using Image J software. (G) mRNA levels of SASP factors analyzed by RT-qPCR in liver and kidney tissue. Data are plotted as mean ± *SD*. $*p < 0.05$, $**p < 0.01$ compared to the indicated sample.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank the support of Academician workstation of Chen Zhi-nan in Xi'an City, Xi'an International Science and Technology Cooperation Base International Joint Research Center on Target Biomedicines and Youth Innovation Team of Shaanxi Universities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2024.176865.

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