

Investigating the inhibitory effect of β -sitosterol on liver fibrosis by modulating the p53/Caspase8/Caspase9/Caspase3 signaling pathway

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Abstract

Liver fibrosis (LF) is a chronic pathological process caused by hepatic pathological factors. Polygonatum sibiricum (PS) is a commonly used traditional Chinese medicine. Studies have shown that PS can protect the liver and lower blood glucose and blood lipids. β -sitosterol (BS) is one of the monomer components of PS and has anti-cancer, hypoglycemic and low toxicity properties. However, there are no clinically recognized drugs for the treatment of liver fibrosis. To explore the mechanism by which BS inhibits LF. In this study, we utilized pharmacological networks, molecular docking, molecular dynamics and basic experiments to investigate the mechanism of action of BS to improve LF. The mechanisms and pathways of action of BS to improve LF were explored by CCK-8, single-cell cloning, Western-blot, immunofluorescence, TUNEL, FITC-PI, HE, Masson, Sirius red staining and immunohistochemistry. Our study confirmed the possible mechanisms of BS in treating liver fibrosis. After treatment with BS, LX-2 cells underwent apoptosis, CCL₄-induced liver fibrosis in mice was suppressed, and liver fibrosis indexes were improved. Meanwhile, we found the possible mechanisms of BS treatment of liver fibrosis using network pharmacology and molecular docking and found that the ameliorative effect of BS on liver fibrosis was related to the p53/Caspase8/Caspase9/Caspase3 signaling pathway. Our results reveal the mechanism of BS in the treatment of liver fibrosis, suggesting that BS may be a possible drug for treating liver fibrosis and deserves further investigation.

1. Introduction

Chronic liver disease (CLD) has a critical threat to humankind, such as nonalcoholic fatty liver disease (NAFLD), which is characterized by inflammation and fibrosis [1]. A variety of pathological factors cause the occurrence of LF. Without timely intervention of related pathogenic factors, cirrhosis and even liver cancer will occur. And killed about 2 million people worldwide each year [2]. The pathology of LF performance of collagen fiber and the extracellular matrix (ECM) accumulate, lead to the fibrotic changes [3]. Inflammation promotes hepatic stellate cells (HSCs) activation, cytokines and chemokines and HSCs to differentiate into muscle fibroblasts collagen fibers, the activation of HSCs are important events in LF [4]. Upon activation of HSCs intracellular expression of α -smooth muscle actin (α -SMA), collagen type I alpha 1 chain (COL1A1)[5], collagen type III alpha 1 chain (COL3A1), matrix metalloproteinase 2 (MMP2)[6] is high thus promoting the development of LF. Inflammatory cells could heal wounds during injury and produce matrix metalloproteinase 9 (MMP9) to degrade fibrosis[7].

LF is reversible [8], Studies have shown that LF can be inhibited by TCM [9, 10]. So far, however, no clinical effects of the approved drug therapy of LF [11]. Therefore, looking for safe and effective anti-fibrosis drug is particularly important.

In recent years, TCM and related monomers have received extensive attention [12]. With the deepening of understanding of TCM, the research shows that TCM contains complex ingredients, can be used in the treatment of many diseases [13], a growing number of studies prove that TCM for CLD has certain curative effect [14–16]. PS is a traditional Chinese medicine with many effects, such as improving

immunity, preventing aging, protecting liver, and anti-cancer [17–19]. TCM also has the advantage of less side effects [20], PS to improve LF may have certain advantages, but there is no research prove that PS can inhibit LF, the principle of which is not clear. Although traditional Chinese medicine has the advantage of having many single components and targets, it is difficult to know the single components that play a positive role in the treatment of a certain disease due to the large number of single components. As a result, the concentration and amount used in the treatment of diseases in traditional Chinese medicine may be very large and toxic[21] compared with Western medicine, and two or more monomers may have antagonistic effects leading to a reduction in efficacy[22]. Therefore, the search for therapeutic monomers in traditional Chinese medicines is of great importance.

β -sitosterol(BS) is one of the main components of PS and studies have shown that BS has anticancer properties against cancers such as breast[23] and prostate[24]. BS is cardioprotective and antidiabetic[25] without any significant toxicity[26] and is easily extracted from plants and herbs, which suggests that BS has a great potential to be developed as a new drug.

Utilizing network pharmacology for query and prediction makes TCM treatment of diseases more precise and effective[27, 28]. The holistic view of treating diseases in TCM is consistent with the research ideas of network pharmacology [29], Network pharmacology for the development of TCM and research provides new idea and method. In this study, we used the network composition and targets for pharmacology for PS and predicted the possible PS mechanisms by which inhibit LF (FIG. 1). After a series of screening and simulation docking, we found that BS, a monomer component of PS, has a strong potential for inhibiting liver fibrosis.

2. Materials and Methods

2.1. Network pharmacology

2.1.2. Finding targets of PS and LF

Using TCMSP (<https://old.tcmsp-e.com/tcmsp.php>) and ETCM (<http://www.tcmip.cn/ETCM/index.php/Home/Index/index.html>), PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), HERB (<http://herb.ac.cn/>)[30–32] to find potential targets of PS. Using GeneCards database (<https://www.genecards.org/>), CTD (<https://ctdbase.org/>), DisGeNET (<https://www.disgenet.org/>) for LF related targets[27, 33].

2.1.3. Building the protein - protein interaction network diagram (PPI)

Venny 2.1.0 was used to find common targets for PS and LF. The targets were then entered into STRING 12.0 (<https://cn.string-db.org/>) to construct the protein-protein interaction networks (PPI). Visualization of the PPI using Cytoscape 3.9.1[34].

2.1.4. Enrichment analysis

Then targets input to DAVID (<https://david.ncifcrf.gov/tools.jsp>) to get the results of enrichment. Through R 4.1.3, GO and KEGG were visualized and analyzed to further speculate the potential mechanism of drugs on LF[35, 36].

2.2. Molecular docking

The TCMSP database was used to find and screen the PS monomer components, followed by downloading the molecular structural formulae of the monomer components via PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), and subsequently downloading the structural formulae of the relevant targets in the PDB (<https://www.rcsb.org/>). The docking simulations of the monomer components and targets were performed by AutoDockTools 1.5.7, and the molecular docking results were visualized and transformed by Pymol 2.4.0.

2.3. Drug preparation and reagents

2.3.1. In vitro experimental part

Beta-Sitosterol (purity > 80% CAS No.: 83-46-5) was purchased from MCE(MedChemExpress), dissolve in DMSO and dilute 1:1000 with DMEM medium before use. DMEM (Viva Cell C3113-0500), fetal bovine serum (LONSERA 5711-001S), trypsin (Biofroxx 9002-07-7), CCK-8(C0038), crystal violet staining solution (Beyotime C0121-100),TUNEL(Beyotime C1086), FITC-PI (Transgenbiotech R20901), fluorescent secondary antibody (Beyotime A0516), GAPDH (60004-1-Ig), COL3A1, COL1A1, MMP9, MMP2, α -SMA, Bcl-2, Bax, Caspase3, Caspase8, Caspase9, p53, Goat Anti-Mouse IgG-HRP, Goat Anti-Rabbit IgG-HRP. The antibodies were bought from Proteintech.

2.3.2. In vivo experimental part

CCl₄(MACKLIN 56-23-5), olive oil (MACKLIN 801-25-0), HE Stain kit (Beyotime C0105S), Masson Stain kit (Solarbio G1340), Sirius Red Stain kit (Solarbio G1472) AST detection kit and ALT detection kit were from Nanjing Jiancheng Bioengineering Institute, antigen repair solution (Proteintech PR30001), DAB Horseradish Peroxidase Color Development Kit (Beyotime P0202).

2.3.3. LX-2 cell culture protocol

From Wuhan Punosai Life Technology Co., LTD. Buy the life human source LX-2 cells. LX-2 cells contain 10% and 1% of the double resistance to serum DME in training. Cells are placed at 37 °C and 5% CO₂ in the constant temperature incubator (Thermo 3111) training. All experiments in the 4 generation of cell.

2.3.4. Cell viability assay

Add 8000 cells/hole LX-2 cells in 96-well plate. The next day, add BS (0, 5, 10, 20, 40, 80,160,320 μ g/mL) into the cells. After 24h of drug treatment, 100 μ L CCK-8 reagent (DMEM: CCK-8 = 10:1) was added to the cells, and the steps were referred to the instructions of CCK-8.

2.3.5. Cell cloning

Add 2000 LX-2 cells in 24-well plate. After 72 hours, add BS (0, 40, 80, 128 μ g/mL). After 72 hours, add 200 μ L 4% paraformaldehyde into 24-well plates for 20min. In the dark place add crystal violet dye solution for 10min, rinsed with PBS, then observed and photographed.

2.3.6. Cellular immunofluorescence

Add 2×10^5 LX-2 cells into 24-well plate. After 24h add 40ng/ml PDGF-BB[38], 40 μ g/mL and 128 μ g/mL BS into the plate. The next day add 4% paraformaldehyde. 0.3% Triton X-100 was added and left for 15min at room temperature. 5% BSA was added for 45min. Antibody (1:200) was added overnight at 4°C. The next day add rabbit IgG-HRP (1:500) for 1h at room temperature. Then use microscope (Thermo evo5000) to observe and take photos.

2.3.7. FITC-PI double staining to detect cell apoptosis

2×10^5 LX-2 cells were added into a 24-well plate and then placed in a constant temperature incubator. After 24h, the cells were treated with PDGF-BB, low BS (40 μ g/mL), and high BS (128 μ g/mL), respectively. (Refer to the instructions of the kit for specific steps).

2.3.8. Cell apoptosis was detected by TUNEL assay

2×10^5 LX-2 cells were added to each of the 24 well plates and then placed in a constant temperature incubator. PDGF-BB, low BS and high BS were added respectively, and then cultured in the incubator. After 24h, add 4% paraformaldehyde for 30min. (refer to the kit instructions for specific steps)

2.3.9. Western blot

LX-2 cells protein extraction (refer to the instructions for specific steps). Loading Buffer was added to the sample, 12% SDS-polyacrylamide gel was applied, and electrophoresis was started at 80V-120V voltage. PVDF membrane transfer was performed at the end of electrophoresis. PVDF membranes were put into 5% skim milk for 90min. PVDF membrane was placed in primary antibody and kept away from light overnight at 4°C. The PVDF membrane was removed and added rabbit IgG-HRP for 90min. Exposure was performed with chemiluminescent reagents and analyzed with Image Lab software. Western-blot method was consistent in vivo.

2.4. Animals

Female mice(C57BL/6) were purchased from (Chengdu Dashuo), weigh 20g $\pm$ 2g, were bred at scientific research center of Chengdu Medical College. Standard 12h light and dark environment circulation, constant temperature 22°C $\pm$ 2°C, provide food and water. All used in the experiment of mice with healthy body, good immunity. All animal experimental procedures were approved by the Animal Welfare and Ethics Committee of Chengdu Medical College. All animal experiments were performed in accordance with the relevant guidelines and regulations, including the ARRIVE guidelines for animal research.

2.4.1. Animals experiment group

Put 40 mice into 4 groups, normal group, LF group, low BS group, high BS group. Except for normal groups, the rest groups were injected CCl_4 (0.6mL/kg) [39] twice a week. After 5 weeks, the Low-dose BS group got BS (400 $\mu\text{g/kg}$) by gavage, and High-dose BS group was treated with BS (1280 $\mu\text{g/kg}$) by gavage, 3 times a week for 4 weeks. After 4 weeks, the mice were anesthetized for 5 min by intraperitoneal injection of 3% pentobarbital sodium (administration dose 0.1 mL/100 g), and then the eyeballs of the mice were removed for ocular blood sampling, followed by rapid dislocation of the head and neck and removal of the livers for further experiments.

2.4.2. Detection of AST/ALT in mouse serum

Collect eyeball blood of mice, serum was centrifuged at 3000 R /min for 3min and stored at -20°C . ALT/AST content was detected by AST/ALT detection kit.

2.4.3. Liver pathology was detected in mice

After killing the mice, the livers were removed. The liver tissues were put into 4% formaldehyde and paraffin sectioned after 48 hours. The liver tissues were processed by HE, Masson, Sirius red and immunohistochemical reagents for staining and immuno-methods, respectively, and finally observed and photographed under the microscope.

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0.0. All data were presented as mean $\pm$ standard deviation (SD). Analysis of variance One-way ANOVA was used to compare the two groups ($n \geq 3$). P value was less than 0.05, the difference was statistically significant.

3. Results

3.1. Network pharmacology to explore the possible ways in which PS inhibits LF

The full name of PS was entered into TCM database to search its targets, and 170 potential targets were found. The number of LF targets in the disease database is 1179. The drug targets and LF targets were intersected by venny 2.1.0, and 39 common targets were found. Then input the 39 common targets into the STRING 12.0 to construct protein interaction network (PPI), (Fig. 1A). Using Cytoscape 3.9.1 to analyze PPI data, we concluded that tp53(p53) is the core target. And the deeper color means the more important target (Fig. 1B). We input the top 26 key targets of Fig. 1B into DAVID database for enrichment analysis to determine the possible mechanism of PS on LF. The Biological process (BP) term found key targets mainly related to promoting apoptotic processes, response to drugs (Fig. 1C). The cellular component (CC) term found that the key targets were mainly related to the cytoplasm, nucleus, and mitochondria (Fig. 1D). The analysis results of the molecular function (MF) term showed that the key

targets were mainly related to protein binding, protein modification, and enzyme binding (Fig. 1E). It is obvious that the key targets have a strong relationship with the p53 pathway and cancer pathway (Fig. 1F). Figure 1G shows the PS-LF-target-pathway network diagram, where the red triangle is LF, the orange V-shape is PS, the green ellipse is the key targets, and the blue square is the KEGG pathways.

3.2. Database and molecular docking screening of monomer components of PS

The monomer components of PS were screened in terms of “oral bioavailability (OB)”, “drug similarity (DL)” and “blood-brain barrier penetration (BBB)” by TCMSP database, and three monomer components were found to meet the requirements, namely, β -sitosterol, sitosterol and diosgenin (Fig. 2A). Subsequently, the molecular docking method was used to simulate the docking of the monomer components with the pathway proteins: p53, Caspase8, Caspase9, and Caspase3 for more in-depth screening of the monomer components. It was found that β -sitosterol binds and forms stable hydrogen bonds with amino acid residues in four pathway proteins, amino acid residues ASP-100, GLU-297, ASN-259, and HIS-277, with binding energies of -6.15, -3.57, -3.6, and - 5.12 kcal/mol, respectively (Fig. 2B 1–4). Sitosterol can only bind to Caspase8 and Caspase3, and the relevant amino acid residues are ALA-274 and ASP-135, with binding energies of -2.87 and - 4.19 kcal/mol, respectively (Fig. [fig2](#)>2</link>C 1–2). Diosgenin binds Caspase8, Caspase9 and Caspase3 pathway proteins, the amino acid residues were LYS-151, SER-261 and ASP-190, where the binding The binding energies were - 4.71, -6.16 and - 6.47 kcal/mol, respectively (Fig. 2D 1–3). This suggests that β -sitosterol can bind to the four pathway proteins and form stable hydrogen bonds compared with the other two monomer components, so β -sitosterol may be one of the effective monomer components in flavonoids that can play a role in the inhibition of hepatic fibrosis, and therefore the subsequent studies will be centered on β -sitosterol.

3.3. LX-2 cells were inhibited by BS

The activation of HSCs is the beginning of the LF. To explore whether BS can inhibit HSCs. We add (0-320 μ g/mL) of BS into LX-2 cells to explore BS could inhibit LX-2 cells proliferation. The results of CCK-8 experiment showed the survival rate of LX-2 cells decreased with the increase of drug concentration (IC₅₀≈128 μ g/mL was used as the concentration of experimental group in subsequent experiments) (Fig. 4A). According to Cell cloning experiments, with the drug concentration increased, the proliferation of LX-2 cells decreased (Fig. 4B). Compared with the PDGF-BB(40ng/mL) group, the expression of COL3A1, COL1A1, MMP9, MMP2 and α -SMA in LOW BS(40 μ g/mL) and HIGH BS(128 μ g/mL) group was decreased (Fig. 4C). The results of immunofluorescence showed that the red fluorescence intensity gradually decreased, that means α -SMA of LX-2 was decreased (Fig. 4D). These results indicate that LX-2 cells could be inhibited by BS.

3.4. BS promotes LX-2 cells to undergo apoptosis

The results of network pharmacological analysis (Fig. 1C) were validated to explore whether BS could let LX-2 cells apoptosis. Fitc-pi staining and TUNEL fluorescence staining showed that the apoptosis rate of LX-2 cells in the drug-treated group (LOW and HIGH BS) was higher than that in the negative control group (PDGF-BB) (Fig. 5-1A-B). Western blot results showed that BS could increase the expression of Bax, decrease the Bcl-2 (Fig. 5 – 2 A). Immunofluorescence results and Western blot results are the same; Bax red fluorescence intensity is increased significantly, the Bcl-2 red fluorescence intensity is significantly reduced (Fig. 5 – 2 B). These results indicated that BS could promote LX-2 cells apoptosis.

3.5. BS through the regulation of p53/Caspase8/Caspase9/Caspase3 pathways promote LX-2 cells apoptosis

The results in Fig. 1F and Fig. 2B showed that the possible reason for apoptosis in LX-2 cells is that BS regulates the p53 pathway. According to Western blot, compared with negative control groups, protein expression levels of p53, cleaved-Caspase8, cleaved-Caspase9 and cleaved-Caspase3 in treatment groups were up-regulated. However, the protein expressions of Caspase8, Caspase9, and Caspase3 were down-regulated (Fig. 6A). Immunofluorescence results and Western blot results are the same; cleaved-Caspase3 red fluorescence intensity is increased significantly (Fig. 6B). These results indicated that BS could promote LX-2 cells apoptosis. As for pathway the BS through the regulation of p53/Caspase8/Caspase9/Caspase3 apoptosis signaling pathways promote LX-2.

3.6. Inhibitory effect of BS on LF in vivo

To explore the effect of BS on LF in vivo, we constructed LF model by injecting CCl₄ to mice. HE stains can learn that compared to normal group, the liver cells in model group (injected with CCl₄ only) are disorder, fatty change and liver cells have been vacuolated. In contrast, the liver cells of the treatment group (low BS and high BS) showed a relatively orderly arrangement and reduced the incidence of fatty degeneration (Fig. 7A). From Masson staining, we can see that the blood vessel wall and surrounding tissues produced obvious dark blue fibers in the model group (Fig. 7B). And in the treatment group the number of blue fibers has decreased, the LF conditions have been inhibited. From Sirius Red staining we can learn that the collagen fiber (in red color) in model group is more than in normal group, however in treatment groups the number of collagen fiber is less than model group (Fig. 7C). Compared with the normal group, the AST/ALT ratio in the serum of mice in the model group was significantly elevated; and with the increase of BS concentration, the AST/ALT ratio was significantly decreased (Fig. 7D).

3.7. Liver fibrosis protein expression in liver tissue

Immunohistochemical results showed that compared with the model group mice, the expression of COL1A1, COL3A1, and α -SMA antibodies in the liver tissues of the drug-treated group mice gradually decreased with the increase of drug concentration (Fig. 8A-C). Compared with the CCl₄ group, the

expression of COL3A1, COL1A1, MMP9, MMP2 and α -SMA in the liver tissues of the drug-treated group mice gradually decreased with the increase of drug concentration (Fig. 8D). The above results indicated that the LF situation in mice gradually improved with the increase of BS drug concentration.

4. Discussion

TCM treatment is considered the most common treatment in China. PS as a kind of medicine edible Chinese herbal medicine, has been active since was found in clinical treatment, it has a history of more than 2000 years [40]. Nowadays, many diseases are treated by PS in clinical practice [41]. A study shows that PS also has an anti-injury effect on the liver [42]. TCM also has the advantage of having many ingredients and targets compared to western drugs[43]. However, no studies have shown that PS can anti-LF.

We combined network pharmacology and molecular docking with basic experiments to investigate the underlying mechanisms of PS in inhibiting LF. Network pharmacology results showed that PS and LF had 39 common targets, indicating that LF may be regulated by PS and these targets were inhibited. It can be found in enrichment analysis that it is related to apoptosis pathway, p53 signaling pathway and other pathways. From the results of molecular docking and molecular dynamics (Fig. 2–3), it is known that BS can form hydrogen bonds with protein molecules in the p53 signaling pathway and bind them stably, suggesting that BS may be one of the effective monomer components for ameliorating liver fibrosis. And studies have pointed out that kill HSCs, can inhibit the development of LF [44, 45]. Bax and Bcl-2 protein as the regulation of cell apoptosis factor [46]. When Bax expression quantity increases, the Bcl-2 expression quantity cut will promote cell apoptosis [47]. α -SMA, COL1A1, COL3A1, MMP9 and MMP2 as phenotypic protein of LF and HSCs activation markers, if by drugs to lower their expression levels can slow the progression of LF [48–53]. Studies have shown that drugs induce HSCs to die by enhancing the expression of protein p53 [54], The increased expression of a series of apoptotic factors Caspase3, Caspase8, and Caspase9 can significantly promote the apoptosis of HSCs. We established a mouse model of LF by injection of CCl₄ to explore whether BS could inhibit LF. Serological indexes AST/ALT activity [55], Inhibition of LF was demonstrated by HE, Masson, Sirius Red staining and immunohistochemistry [56]. The results show that the BS can inhibit the LF.

We hypothesized from the network pharmacology, molecular docking and molecular dynamics results that BS may be one of the components of PS that can effectively ameliorate liver fibrosis. RMSD shows that BS is able to reach equilibrium quickly after binding to protein molecules and exhibits high binding stability, Rg and SASA show that BS binds to protein molecules and fluctuates stably without significant contraction and expansion during movement; in addition, BS also forms a certain number of hydrogen bonds with protein molecules, and the value of RMSF is also relatively low, and the results of these simulations suggest that BS is able to bind to protein. These simulation results indicate that BS can bind to protein molecules stably and work well. Basic experiments have shown that, BS can promote the expression of Bax, cleaved-Caspase3, cleaved-Caspase8 and cleaved-Caspase9, and inhibit the expression of Bcl-2, α -SMA, COL1A1, COL3A1, MMP9 and MMP2. Interestingly our results found that

inhibiting MMP9 expression instead inhibited the development of LF. This is contrary to previous findings[57, 58], suggesting that MMP9 is a double-edged sword, where increased expression improves liver fibrosis and decreased expression also improves LF, which may require a threshold to regulate MMP9 expression to better improve liver fibrosis. More importantly, we guess through the analysis of the basic experiment verify the network pharmacology and molecular docking: BS can through the regulation of p53/Caspase8/Caspase9/Caspase3 signaling pathway to promote hepatic stellate cell senescence and apoptosis and inhibiting LF (Fig. 9).

The disadvantages of today by CCC in clinical treatment of LF is that it is toxic to organs [59, 60]. Compared with the toxicity of CCC, TCM is safer. Beta-sitosterol is a non-toxic substance and its potential anticancer therapeutic properties are very strong[61]. beta-sitosterol has great value in the future treatment of diseases and it deserves to be researched and developed! There are other monomer components in PS that have not been experimentally verified, which is the deficiency of our study. In the future study, we will add other monomer components for further in-depth exploration to find more effective monomer components, for clinical treatment and prevention of LF provide safer and more effective ideas and solutions.

5. Conclusion

Our research by combining network pharmacology, molecular docking and basic experiment, our study analyzed the effect and possible mechanism of BS in inhibiting LF. The results indicate that BS may inhibit LF by regulating p53/Caspase8/Caspase9/Caspase3 signaling pathway to cause apoptosis of LX-2 cells. The results provide a more novel and reliable plan to treat LF in clinical.

Declarations

Author Contribution

Yuxin Zhang. and Fengmei Deng. conceived and designed the study. Yilun Liu. Haotian Shen. and Bin Tang. performed the network pharmacology, molecular docking, and molecular dynamics simulations. Weitong Li. and Yuting Fan. conducted the in vitro LX-2 cell experiments (including CCK-8, clone formation, immunofluorescence, TUNEL, and Western blot). Yuxin Zhang. carried out the in vivo animal experiments (CCl4-induced mice model, HE, Masson, and Sirius red staining). Yuxin Zhang. and Li Cheng. analyzed the data and wrote the main manuscript text. Yuxin Zhang. prepared all figures and tables. All authors reviewed, revised, and approved the final manuscript.

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Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of interests

No conflicts of interest.

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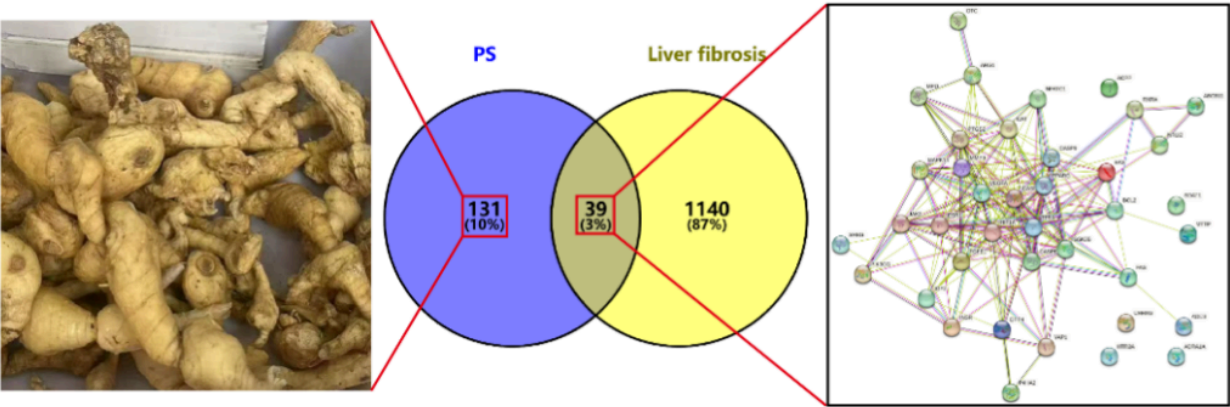
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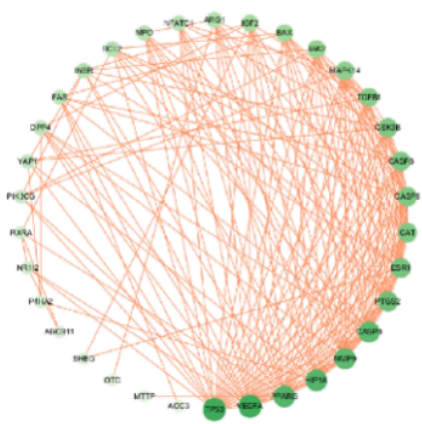
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Figures

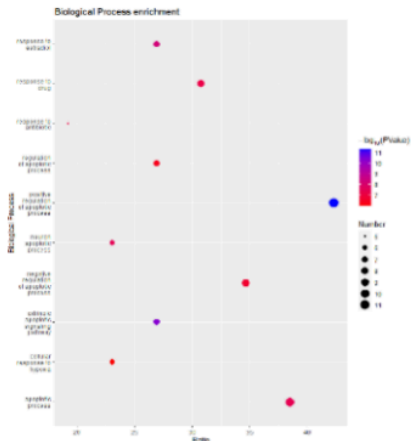
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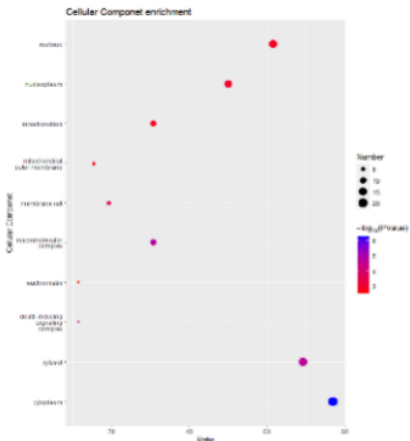
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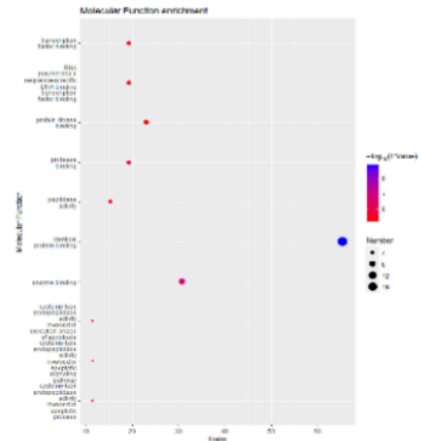
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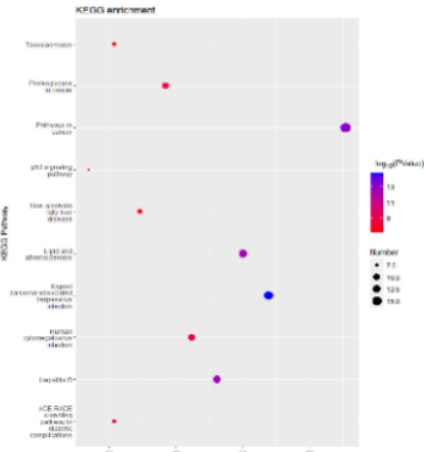
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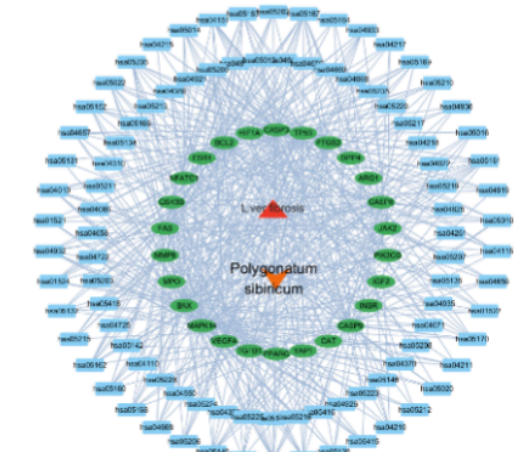


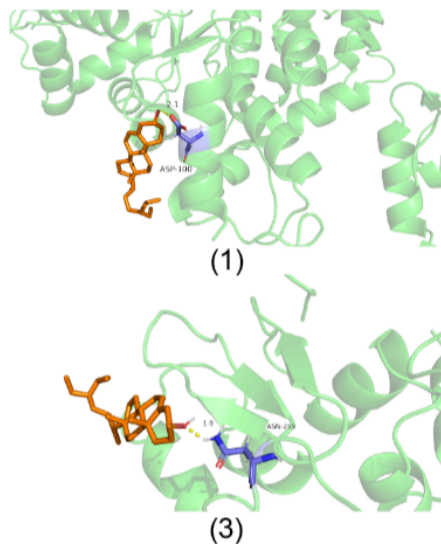
Figure 1

Network pharmacology analysis results. (A) Photograph of PS, Venny diagram and PPI of PS and LF targets. (B) Shows the relationship of common targets. (C) BP, (D) CC, (E) MF, (F) pathway: enrichment results of the important targets. (G) PS-LF-target-pathway visualization network. KEGG enrichment analysis was performed using the KEGG database (<https://www.kegg.jp/>).

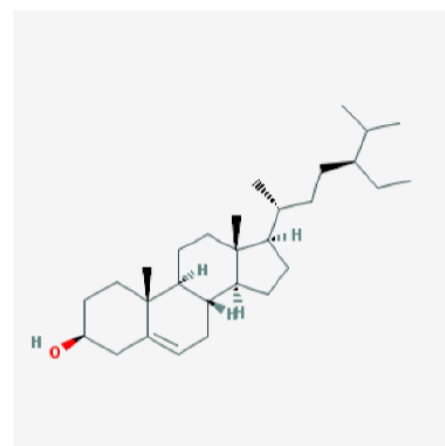
A

Mol ID	Molecule Name	MW	AlogP	Hdon	Hacc	OB (%)	Caco-2	BBB	DL	FASA-	HL	Save
MOL000358	beta-sitosterol	414.79	8.08	1	1	36.91	1.32	0.99	0.75	0.23	5.36	
MOL000359	sitosterol	414.79	8.08	1	1	36.91	1.32	0.87	0.75	0.22	5.37	
MOL000546	diosgenin	414.69	4.63	1	3	80.88	0.82	0.27	0.81	0.19	4.14	

B



E



C



D

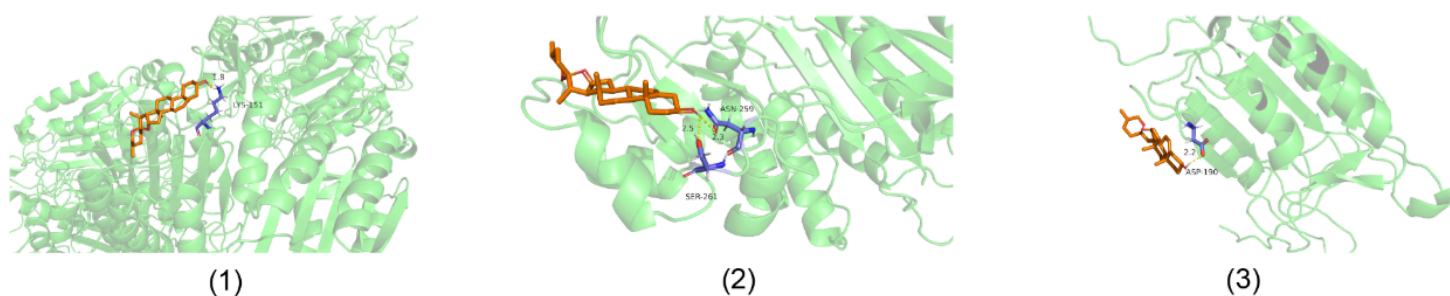


Figure 2

Monomer component screening and molecular docking results. (A) TCMSP database based on “OB”, “DL”, and “BBB” screening of three monomer components. (B) β -sitosterol and pathway proteins p53, Caspase8, Caspase9, and Caspase3 molecular docking results. (C) Results of molecular docking of sitosterol with pathway proteins Caspase8 and Caspase3. (D) Results of molecular docking of diosgenin with Caspase8, Caspase9, and Caspase3 molecules. (E) Chemical structure of BS.

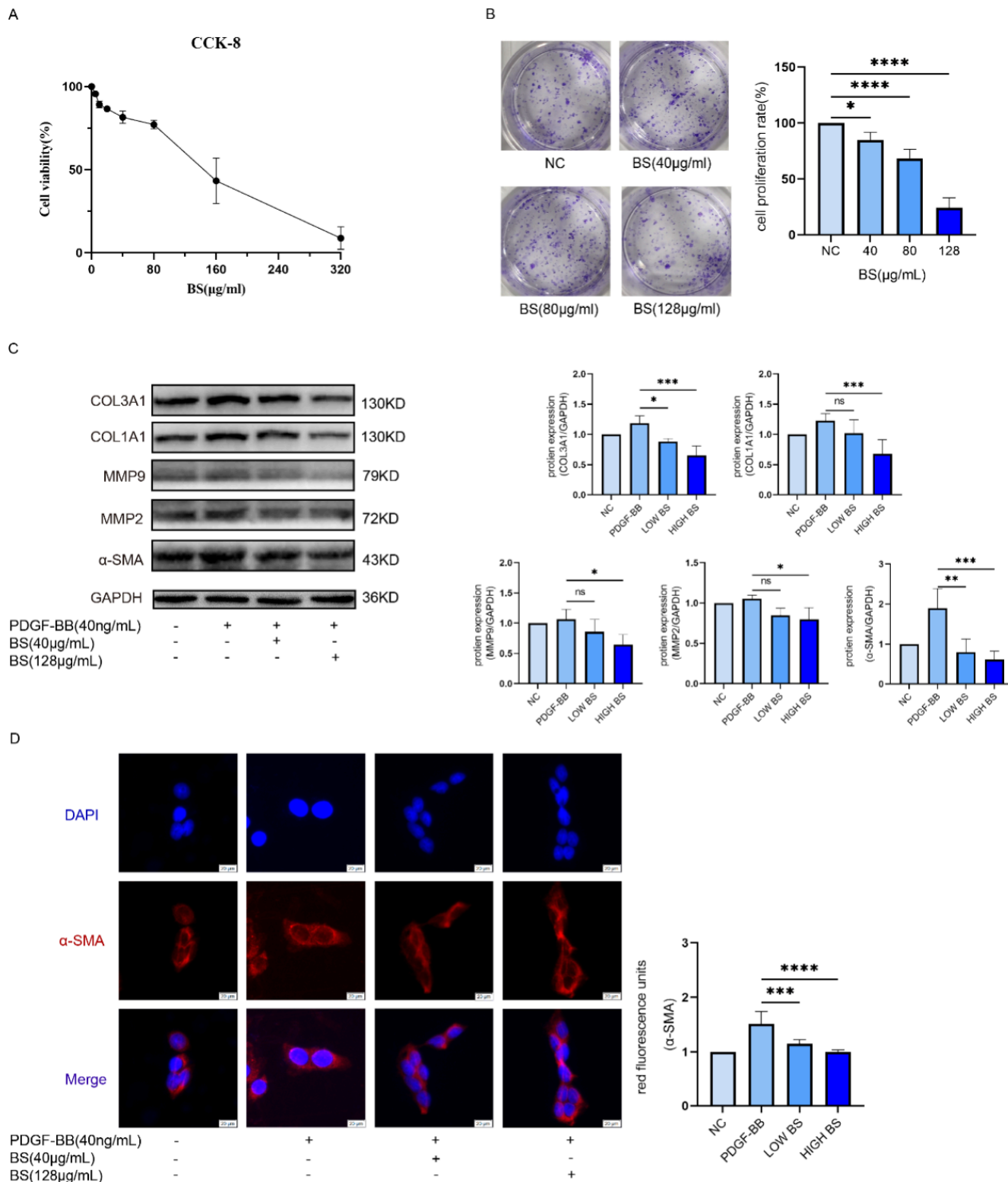
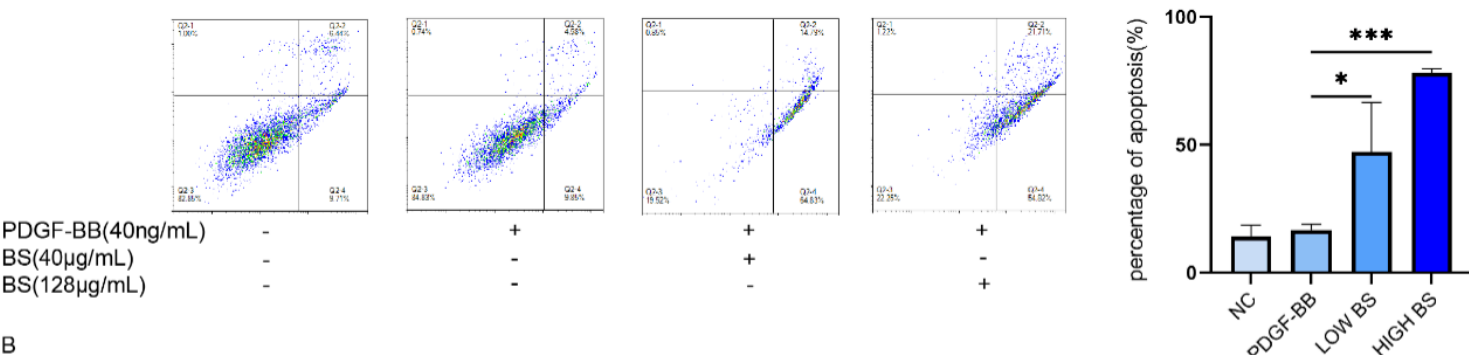


Figure 3

LX-2 cells were inhibited by BS. (A) The result of CCK-8 assay. (B) The result of cell monoclonal assay. (C) Western blot analysis of BS-regulated expression of COL1A1, COL3A1, MMP9, MMP2, and α-SMA in LX-2 cells. The samples derive from the same experiment or parallel experiments and gels/blots were processed in parallel. The blots shown were cropped from different gels and spliced together; the splicing boundaries are indicated by dividing lines. (D) The results of immunofluorescence method, red:

α -SMA, blue: nucleus; Scale bar, 20 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant).

A



B

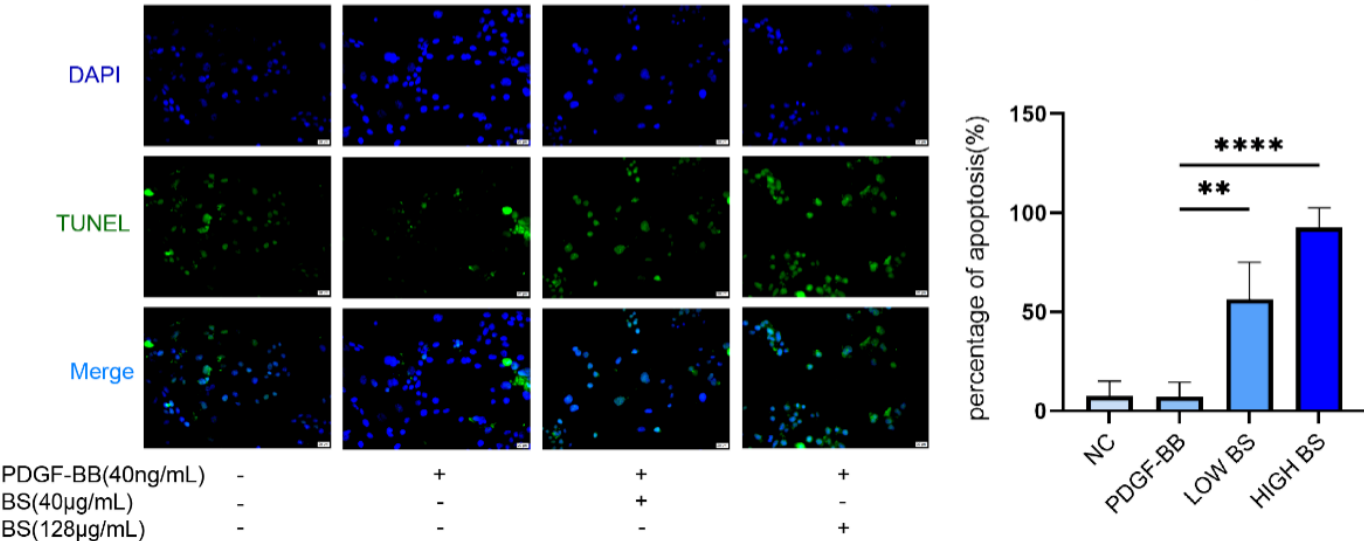


Figure 4

Fig. 5-1. BS promoted LX-2 cells apoptosis. (A-B) Using FITC-PI and TUNEL fluorescence staining detection LX-2 degree of apoptosis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant).

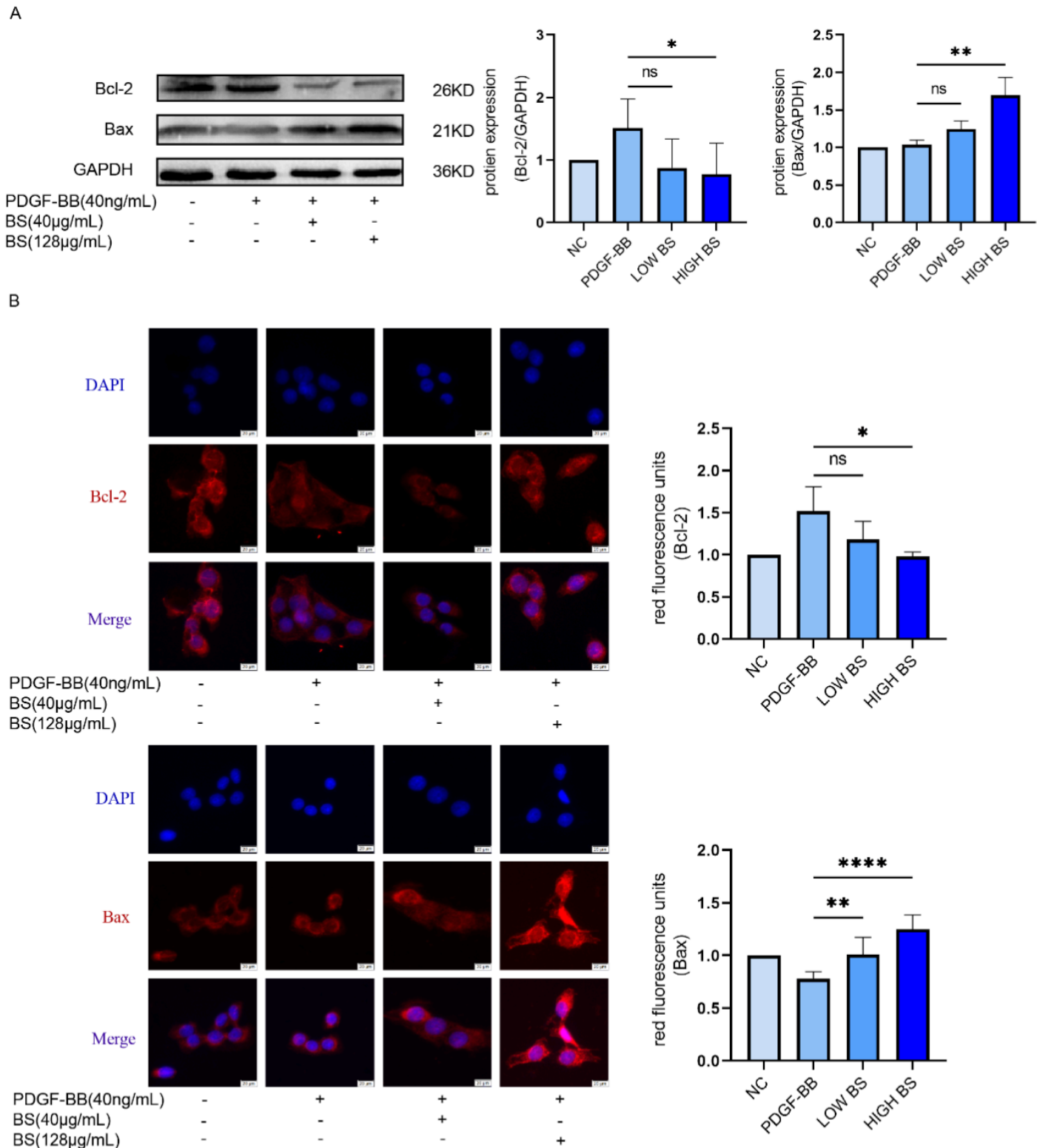
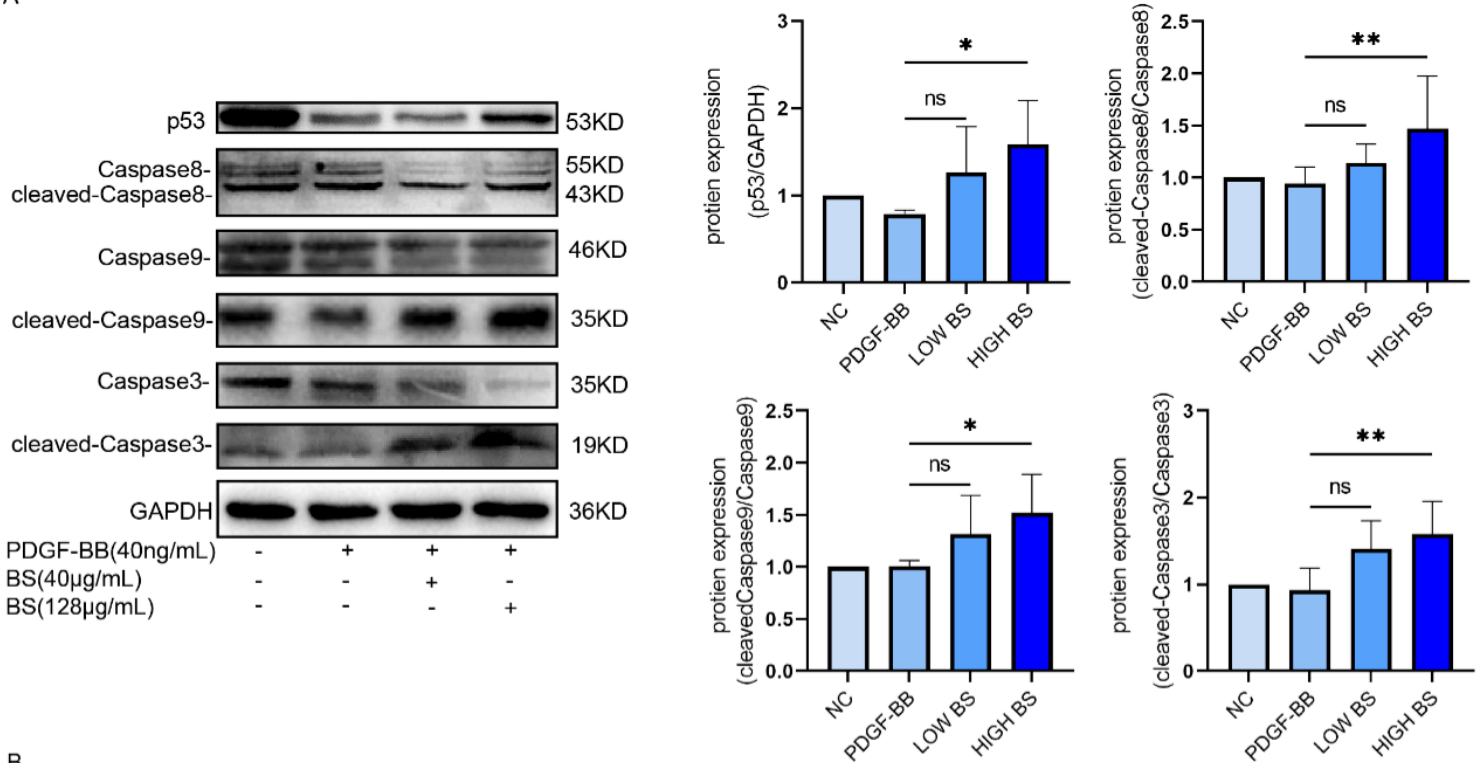


Figure 5

Fig. 5-2. (A) Western blot analysis of BS-regulated expression of Bax and Bcl-2 in LX-2 cells. The samples derive from the same experiment or parallel experiments and gels/blots were processed in parallel. The blots shown were cropped from different gels and spliced together; the splicing boundaries are indicated by dividing lines. (B) The immunofluorescence detected Bax and Bcl-2. Red: Bax, Bcl-2; Scale bar, 20μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant).

A



B

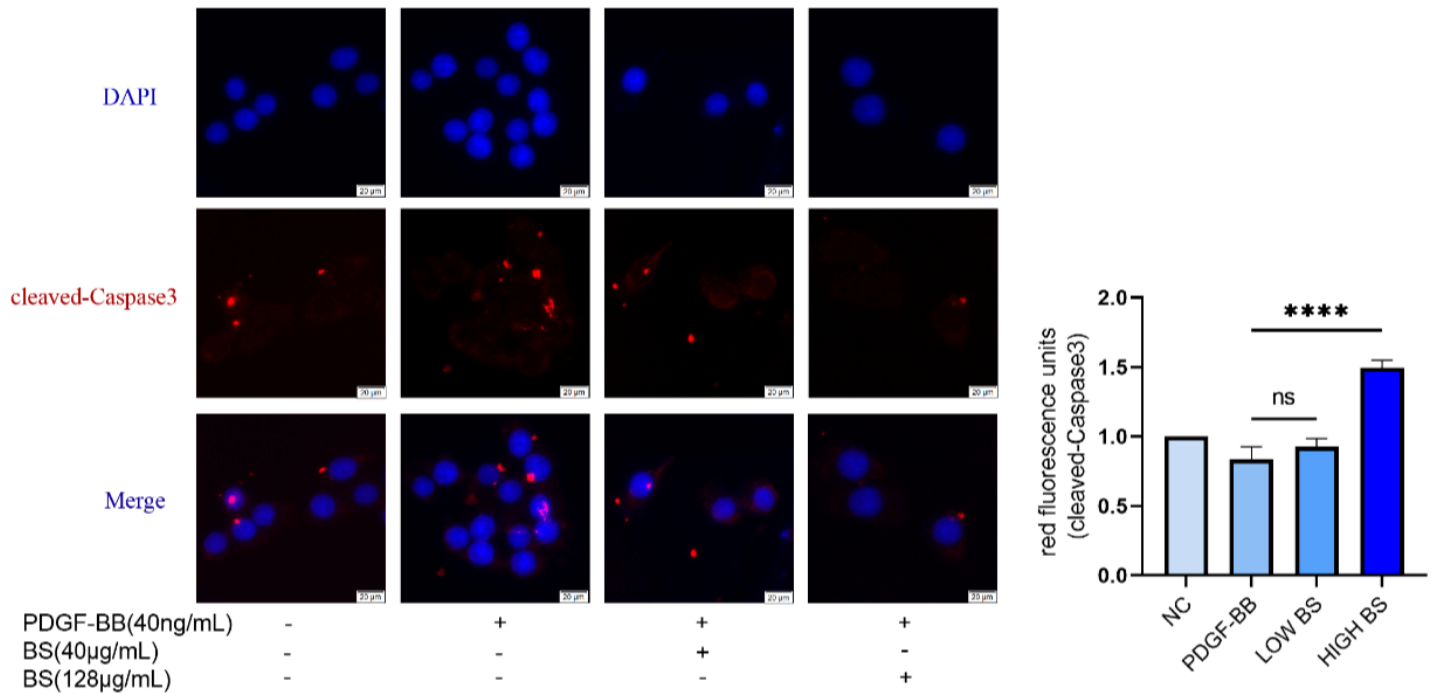


Figure 6

Fig. 6. BS through the regulation of p53/Caspase8/Caspase9/Caspase3 pathways promote LX-2 cells apoptosis. (A) Western blot detected the expression of pathway proteins p53, Caspase8, Caspase9, and Caspase3. The samples derive from the same experiment or parallel experiments and gels/blots were processed in parallel. The blots shown were cropped from different gels and spliced together; the splicing boundaries are indicated by dividing lines. (B) The immunofluorescence detected cleaved-Caspase3, blue: nucleus; Scale bar, 20 μm. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, NS (not significant).

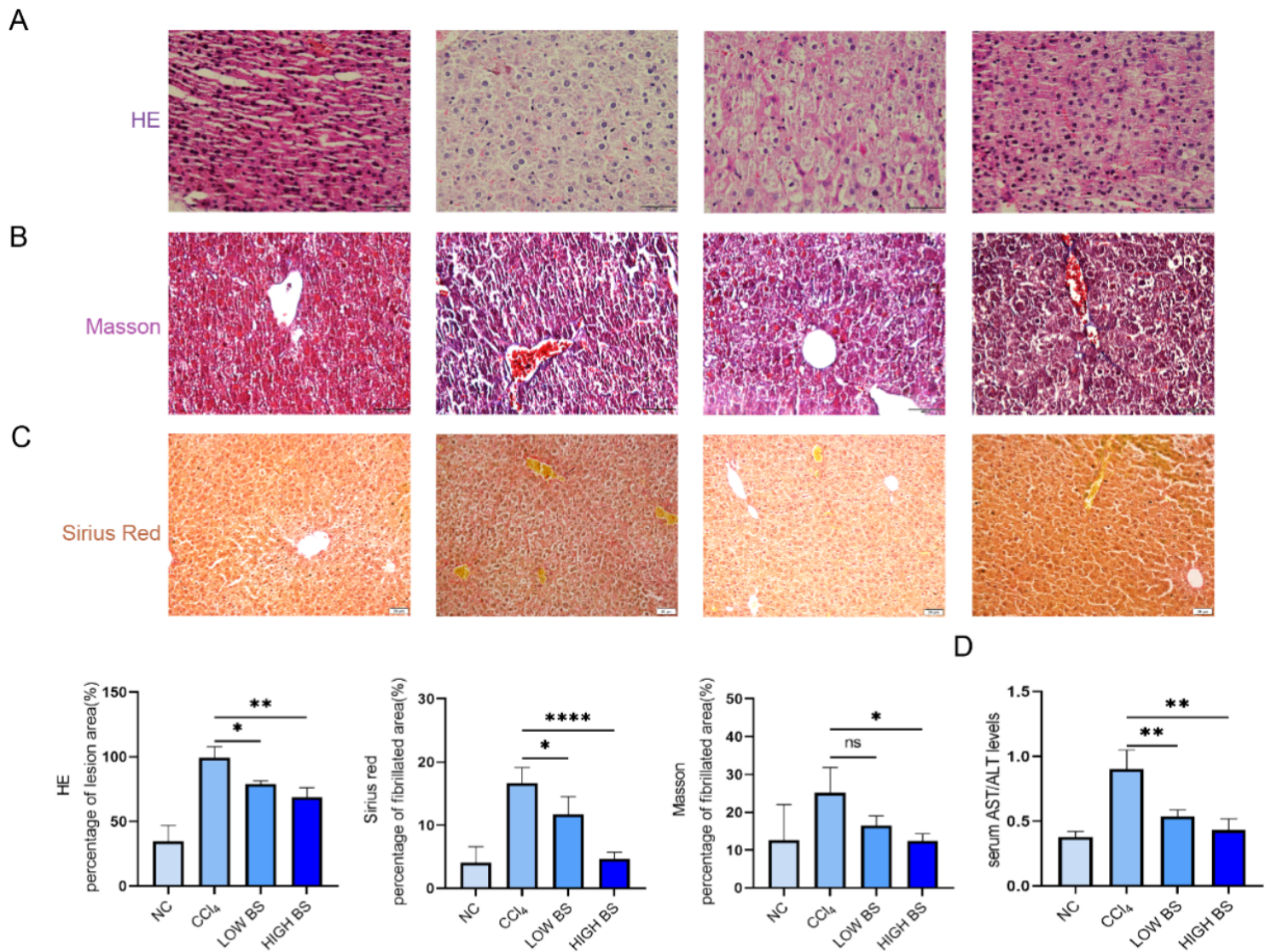


Figure 7

Fig. 7. Inhibitory effect of BS on liver fibrosis in vivo. (A-C) Staining of liver tissue with HE, Masson and Sirius Red to detect liver tissue lesions; (D) The AST/ALT ratio in the blood of mice was measured using the AST/ALT kit. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant).

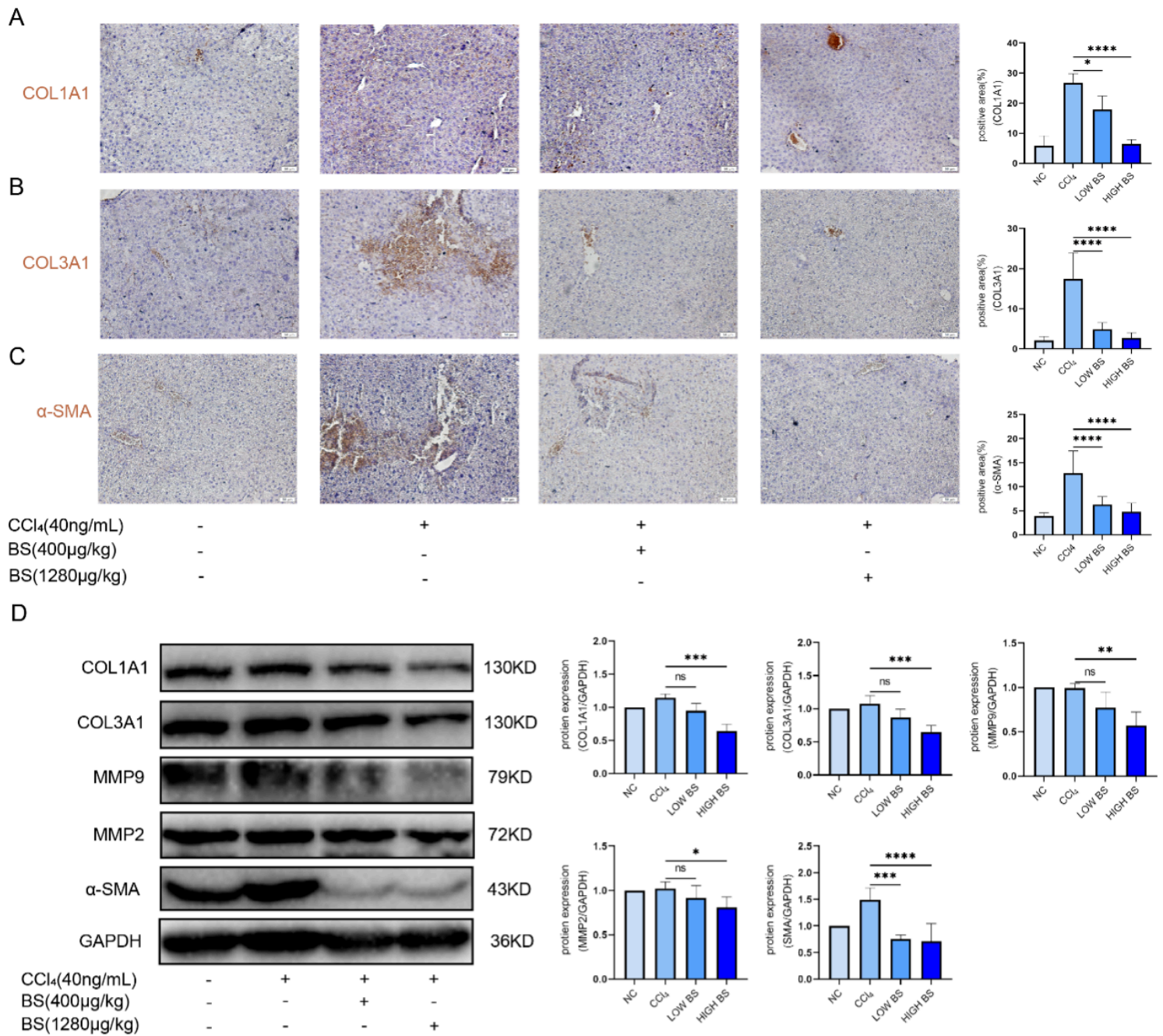


Figure 8

Fig. 8. (A-C) Immunohistochemical detection of COL1A1, COL3A1 and α -SMA expression in liver tissues; Scale bar, 50 μ m. (D) Protein expression of COL1A1, COL3A1, MMP9, MMP2, and α -SMA in mouse liver tissues was determined by Western blot. The samples derive from the same experiment or parallel experiments and gels/blots were processed in parallel. The blots shown were cropped from different gels and spliced together; the splicing boundaries are indicated by dividing lines. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS (not significant).

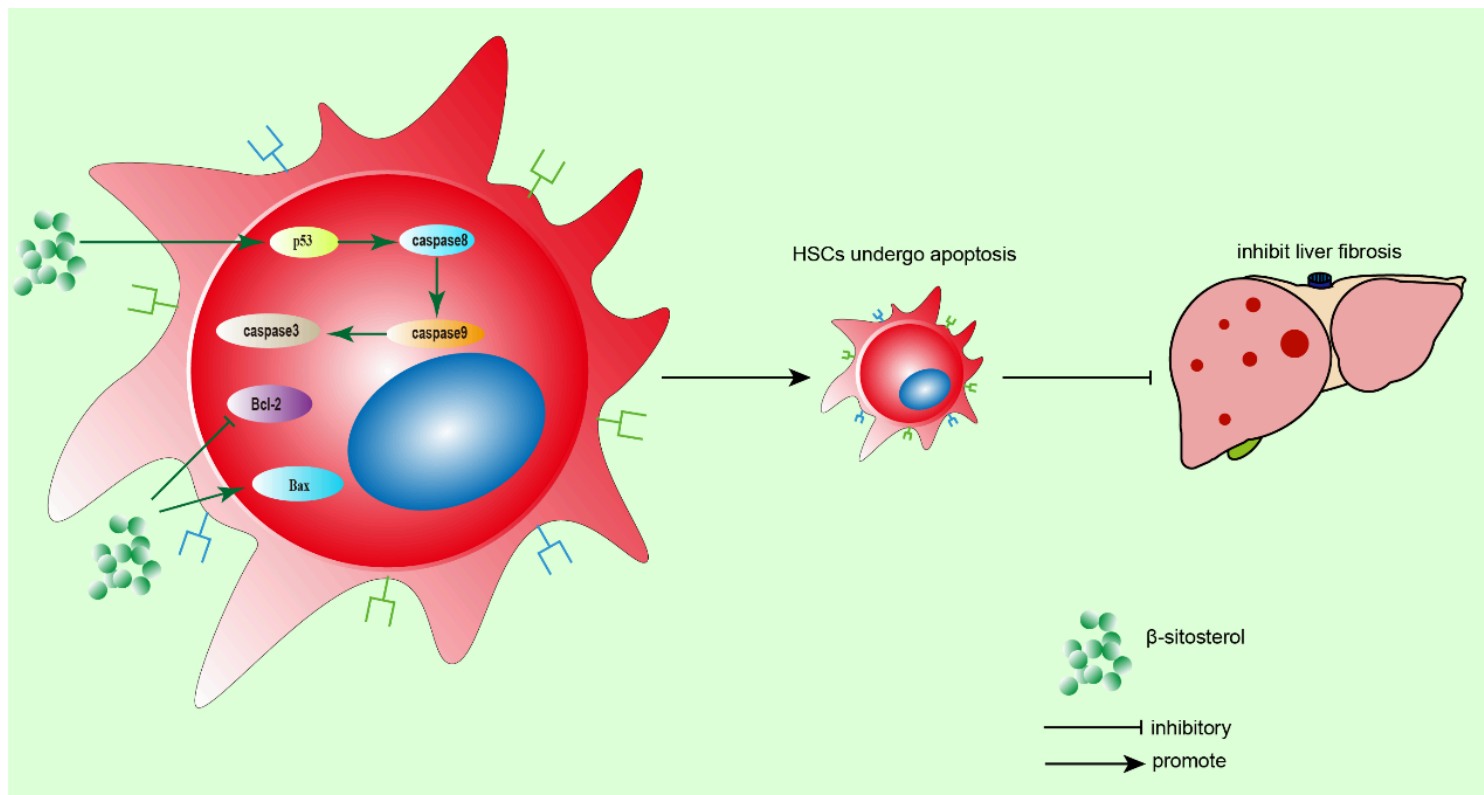


Figure 9

Fig. 9. Mechanisms of BS inhibition of liver fibrosis.

Supplementary Files

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