

Kurarinol A from *Sophora flavescens* as potential anti-liver fibrosis agent that inhibits the activation of LX-2 cells by regulating TGF- β /Smads signaling pathway

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Abstract

Objective This work aims to discover bioactivity against liver fibrosis for the lavandulyl flavonoids from one botanical source of *S. flavescens*. Further use of transcriptomics technology to explore the molecular mechanism of anti-hepatic fibrosis.

Methods In this study, a model of LX-2 cells activation induced by transforming growth factor- β 1 (TGF- β 1) was established. A total of 35 free phenolics were isolated from *S. flavescens* to form a small compound library. These compounds on the proliferation of LX-2 cells were screened using MTS method. Furthermore, cell scratch, transcriptomics technology, real-time quantitative polymerase chain reactions (RT-qPCR) and Western blotting were used to evaluate the inhibitory effect of lavandulyl flavonoids on the proliferation and activation of LX-2 cells, and to explore the mechanism of lavandulyl flavonoids in improving liver fibrosis.

Results The results showed that a total of 11 compounds had a significant inhibitory effect on the proliferation of LX-2 cells and their IC_{50} was between 4-40 μ M by MTS assay. Among them, 8 compounds were reported for the first time. Particularly, kurarinol A (**1**, IC_{50} 12.65 μ M) showed noticeable inhibitory activities. Furthermore, The results of cell scratch test showed that KA inhibited the migration of LX-2 cells. The migration process was carried out in a dose-dependent manner at 24 and 48 hours. Then, KA remarkably inhibit the mRNA and protein levels of liver fibrosis markers (α -SMA, fibronectin and collagen I), and could effectively inhibit the development of liver fibrosis. Additionally, transcriptome analysis revealed that there were 106 differentially expressed genes (DEGs) with remarkably expression differences by the treatment of KA were identified in the LX-2 cells. The mechanism studies elucidated that KA exerted protective activities involved in modulating the TGF- β /Smads signaling pathway. Among them, KA inhibited the gene and protein of TGF- β 1, Smad2, Smad3, and Smad4 levels, respectively.

Conclusion KA can improve liver fibrosis. The mechanism of its anti-hepatic fibrosis was achieved by regulating the TGF- β /Smads signaling pathway. KA could be an effective anti-fibrosis agent.

Introduction

Hepatic fibrosis is recognized as a complex and advanced pathological state, distinguished by the aberrant proliferation of connective tissue and the excessive deposition of extracellular matrix (ECM) proteins within the hepatic microenvironment. It is widely acknowledged that this condition emanates from a spectrum of chronic hepatic injuries, including but not limited to alcohol-induced liver disease, nonalcoholic steatohepatitis, cholestatic disorders, and infections by hepatotropic viruses [1, 2]. The prevalence and mortality associated with liver fibrosis are escalating annually. Left untreated, there is a risk of progression to advanced liver diseases, including cirrhosis, the development of portal hypertension, and the potential for hepatocellular carcinoma [3, 4]. Nevertheless, a definitive pharmaceutical for hepatic fibrosis remains elusive, with numerous candidate treatments still in the investigative and developmental phases. [5]. Therefore, inhibiting liver fibrosis was a key step in the

treatment of acute and chronic liver diseases, protecting liver function, and preventing malignant liver lesions. The development of related drugs has become an urgent need in the medical field.

A key phenomenon in the pathogenesis of liver fibrosis is the activation of hepatic stellate cells (HSCs), which can be induced by a multitude of factors including hepatocellular injury, inflammatory processes, and the aberrant fibrotic tissue microenvironment [6]. HSCs possess the capacity for proliferation and migration, and are responsible for the synthesis of extracellular matrix proteins including fibronectin and collagen I, as well as the secretion of the contractile protein α -smooth muscle actin (α -SMA) [7]. Central to the pathogenesis of liver fibrosis is the activation and subsequent proliferation of HSCs, which are acknowledged as the principal contributors to the fibrotic process [8, 9]. Therefore, exploring the inhibition of HSCs activation, inducing apoptosis and preventing extracellular matrix deposition has become the main strategy for the treatment of liver fibrosis.

Transcriptome analysis has emerged as a pivotal instrument in disease research, particularly for deciphering the etiology of a spectrum of illnesses. Its strength lies in its ability to uncover biomarkers and shed light on the comprehensive mechanisms at play. Lately, this approach has gained significant traction, with an escalating number of investigations leveraging transcriptome profiling to dissect the intricate molecular pathways involved in hepatic conditions [10–12]. Therefore, this study used transcriptomics to explore the mechanisms involved.

Small molecules derived from natural products represent a vital wellspring in the realm of drug discovery. A variety of compounds with anti-fibrotic potential, such as Silibinin, Puerarin, Baicalin, and Tanshinone IIA, have emerged from the study and extraction of natural materials [13, 14]. In a prior study conducted by our research team, a novel lavandulyl flavonoid from *S. flavescens*, kurarinol A exhibited potential anti-liver injury effects in *vitro* and in *vivo* [15, 16]. In further studies, a model of LX-2 cells activation induced by transforming growth factor- β 1 (TGF- β 1) was established. It was found that ethyl acetate extract had a certain anti-hepatic fibrosis effect in LX-2 cells. Subsequently, A total of 35 free phenolics were isolated from *S. flavescens* to form a small compound library. These compounds on the proliferation of LX-2 cells were screened using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method. Furthermore, flow cytometry, cell scraping, real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting were used to evaluate the inhibitory effect of the screened compound KA on the proliferation and activation of LX-2 cells. After determining that KA can effectively inhibit the proliferation and activation of LX-2 cells, transcriptomics was used to elucidate its anti-hepatic fibrosis mechanism. The purpose of this study is to clarify the anti-fibrotic effect of KA and its potential mechanism, and to provide a theoretical basis for its future application in the treatment of liver fibrosis.

Materials and methods

Reagents and chemicals

KA and 34 other compounds were isolated and identified from the roots of *Sophora flavescens* Aiton. The traditional medicinal herb known as Ku Shen originates from the root system of *Sophora flavescens* Aiton, which belongs to the Fabaceae family. The nomenclature of this plant species was confirmed on May 14, 2024, by consulting the authoritative resource at <http://www.theplantlist.org>. The picking time, address, identification and specimen preservation location were explained in detail in previous articles [15, 16].

Dulbecco's modified eagle medium (DMEM medium), the dual antibiotic mixture (penicillin-streptomycin), 0.25% Trypsin-EDTA and dimethyl sulfoxide (DMSO) were purchased from Gibco Company. Fetal Bovine Serum (FBS) was obtained from Procell Life Science&Technology Co.,Ltd. (Wuhan, China). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was provided by Promega Co. (Wisconsin, USA). BCA protein assay kit, phosphatase inhibitor, protease inhibitor, UELandy super ECL plus and RIPA lysis buffer were acquired from Solarbio Technology Co., Ltd. (Beijing, China). Total RNA isolation kit and FastKing gDNA dispelling RT supermix were gained from Tiangen biotech Co., Ltd. (Beijing, China). TB Green premix Ex Taq II was purchased from TaKaRa Co. Ltd. (Japan). PCR Primers were obtained from Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). TGF-β1, Silibinin and SB431542 were provided by Absin Biotechnology Co., Ltd. (Shanghai, China). GAPDH, Fibronectin, α-SMA, Collagen-I antibody and Goat Anti-Rabbit IgG(H + L) HRP secondary antibody were gained from Proteintech Group, Inc. (Wuhan, China). TGF-β1, Smad 2/3, p-Smad 2/3 and Smad 4 were bought from ABclonal Technology Co.,Ltd. (Wuhan, China).

Cell culture

The LX-2 cell strain was procured from the Wuhan Pricella Biotechnology Co.Ltd (CL-0560). Cultivation of these cells was performed in Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% Fetal Bovine Serum (FBS) and 1% penicillin. The cells were incubated under controlled environmental conditions consisting of a 5% CO₂ in 95% air mixture, within a humidified chamber maintained at 37°C.

Cell proliferation and viability assay

The proliferation of cells was evaluated employing the CellTiter 96® AQueous One Solution assay (MTS). To assess the inhibitory effects of 35 compounds on the proliferation of LX-2 cells, cells were seeded at a density of 4×10^3 cells per 100 μL in 96-well plates. After allowing 24 h for the cells to reach 80% confluence, TGF-β1 at a concentration of 10 ng/mL, prepared in 2% Fetal Bovine Serum (FBS), was introduced to stimulate the cells for an additional 24 h. Subsequently, the compounds labeled 1–35 and silibinin, each at a final concentration of 40 μM and also prepared in 2% FBS, were added for a 48 h culture period. Then, 10 μL MTS solution was added to the medium directly, incubated for 3 h, and the optical density (OD) reading was taken at 490 nm with a microplate photometer (Bio-Tek, USA).

Cell scratch assay

LX-2 cells were seeded in six-well plates at $8 \times 10^4/2$ mL/well in DMEM medium containing 10% FBS. Upon the formation of a monolayer, sterile pipettes were employed to scratch the LX-2 cells, followed by a thorough wash with phosphate-buffered saline (PBS) to remove residual cellular material. Then LX-2 cells were stimulated with 10 ng/mL TGF- β 1 for 24 h, and then treated with KA (2.5, 5, 10 μ mol/L) and silibinin for 48 h. The injury site was captured at intervals of 0, 24, and 48 hours using an inverted microscope. The migration of cells was assessed through ImageJ software, which facilitated the measurement of the wound area. The extent of cell migration was determined by applying the formula: $[W(0\text{ h}) - W(48\text{ h}/24\text{ h})]/W(0\text{ h}) \%$.

Transcriptomic Analysis

The number of detected sample cells was 2×10^6 , which were control, model and KA (10 μ M) group, with 4 repeated samples in each group. RNA sequences analysis was performed by Guangzhou Kidio Biotechnology Co., Ltd as previously described. Concisely, total RNA extraction was performed utilizing the Trizol kit from Invitrogen, adhering to the manufacturer's instructions. RNA quality was ascertained through analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and validated by RNase-free agarose gel electrophoresis. Subsequently, the concentrated mRNA was divided into smaller pieces through the use of a fragmentation buffer. This was followed by a reverse transcription process, transforming the mRNA into complementary DNA (cDNA), with the aid of the NEBNext Ultra RNA Library Prep Kit for Illumina sequencing platforms. Purified cDNA fragments underwent end repair and were ligated with Illumina adapter sequences. The ligation mix was purified using AMPure XP Beads. The cDNA library was then PCR amplified and sequenced utilizing the Illumina Novaseq6000 system at Gene Denovo Biotechnology Co (Guangzhou, China). Raw sequencing reads were refined utilizing Cutadapt to eliminate substandard sequences. Thereafter, the selection of differentially expressed genes (DEGs) was based on stringent criteria, including a $|\log_2$ fold change| exceeding 1 and p-values less than 0.05, for in-depth analysis.

RT-qPCR analysis

After the cells were treated with the drug treatment method of LX-2 cells scratch test in the previous section, the total RNA was extracted for RT-qPCR detection. RNA was extracted from cellular samples using a Total RNA extraction kit. cDNA was then generated using a FastKing gDNA Removal RT SuperMix. Subsequently, RT-qPCR was conducted with the TB Green Premix Ex Taq II. The expression levels of the target mRNA were standardized relative to GAPDH. Primer sequences for the analysis are detailed in Table 1.

Table 1
Primer sequences used for RT-qPCR analysis.

Gene	Forward	Reverse
GAPDH	GGAGTCCACTGGCGTCTTCAC	GCTGATGATCTTGAGGCTGTTGTC
Collagen α	GCCTCTGCTCTCCGACCTCTC	GCTTTGTGCTTTGGGAAGTTGTCTC
α -SMA	CTTCGTTACTACTGCTGAGCGTGAG	CCCATCAGGCAACTCGTAACTCTTC
Fibronectin	ACGACTGTGGACCAAGTTGATGAC	CTGTGCTGCTACCTTCTACTGATGG
TGF- β 1	AGCAACAATTCCTGGCGATACCTC	TCAACCACTGCCGCACAACCTC
Smad2	CCTGCTCCTGACCACAACTCTG	TCATCCCTTCCCACCCACTCC
Smad3	AGGCTGCTTGTTAGGACTGTTTAC	CGCTGTGTTGAGGTTTGGTTCTG
Smad4	AGGATCAGTAGGTGGAATAG	TCTAAAGGTTGTGGGTCTGC

Western blotting

Following the drug treatment as described in the preceding section for the LX-2 cells scratch assay. LX-2 cells were lysed to extract proteins utilizing RIPA buffer. The protein concentration was determined through the BCA protein assay. Subsequent steps were executed in accordance with previously outlined methods [16]. GAPDH was used as a loading control.

Statistical analysis

Data analysis and graphical representation were conducted using GraphPad Prism, with results presented as mean $\pm$ standard deviation (SD). Comparisons of statistical data across various groups were conducted through a one-way analysis of variance (ANOVA), and $p < 0.05$ was deemed to indicate statistical significance.

Results

Inhibition of LX-2 cells proliferation and activation by ethyl acetate extract of *S. flavescens* and its 35 monomer compounds.

A large number of literatures have pointed out that the proliferation of hepatic stellate cells is the core element in the development of liver fibrosis. Therefore, it is of great significance to explore the mechanism of inhibiting the proliferation of hepatic stellate cells for the treatment of liver fibrosis. In the study, in order to screen the inhibitory effects of 35 compounds (40 μ M) and EtOAc extract (25 μ g/mL) from *S. flavescens* on the proliferation of LX-2 cells. TGF- β 1 (10 ng/mL) was used to stimulate the proliferation of LX-2 cells for 24 h (Fig.S1, Supplementary information), which was widely used to simulate the proliferation and activation of LX-2 cells [17, 18]. As shown in Fig. 1B (Table S2, Supporting

information), TGF- β 1 stimulation significantly increased LX-2 cell viability, while some 40 μ M compounds and EtOAc extract (25 μ g/mL) from *S. flavescens* significantly reduced TGF- β 1-stimulated LX-2 cell viability to less than 50%, and no significant cytotoxicity was observed (Fig. 1A, Table S1, Supporting information). The results showed that a total of 11 compounds had a significant inhibitory effect on the proliferation of LX-2 cells and their IC₅₀ was between 4–40 μ M by MTS assay (Fig.S2, Supporting information). Among them, 8 compounds (kurarinol A, kurarinol B, kushenol Q, sophoraflavanone B, kurarinol, kushenol A, sophoflavesceno, noranhyoicaritin) were reported for the first time.

Intriguingly, research has identified that the EtOAc extract of *S. flavescens* possesses an inhibitory effect on the proliferation of LX-2 cells, with a concentration of 25 μ g/mL significantly reducing the viability of TGF- β 1-stimulated LX-2 cells to below 50%. Furthermore, the majority of the isolated pure compounds also exhibited suppressive activity on LX-2 cells proliferation. Worthy of note, compound **1**, identified as kurarinol A (KA), exerts a pronounced inhibitory effect on the proliferation of LX-2 cells, ranking among the most potent compounds with an IC₅₀ value of 12.65 μ M under the modeled conditions (Fig.S2, Supporting information). Consequently, in order to elucidate the intimate relationship between the therapeutic efficacy of the EtOAc extract of *S. flavescens* and its constituent compounds, Western blot analysis was utilized to comparatively assess the inhibitory actions of both the extract and kurarinol A (KA) on the protein expression levels of the hepatic fibrosis biomarker, fibronectin (FN). The findings indicate that the EtOAc extract of *S. flavescens* and KA both effectively suppress the protein expression of FN. Specifically, KA at concentrations of 5 and 10 μ M exhibited enhanced therapeutic efficacy over the EtOAc extract at doses of 15 and 30 μ g/mL, respectively (Fig.S3, Supporting information). This suggests a strong association between the pharmacological potency of the EtOAc extract and that of its isolated monomeric compounds. Consequently, further investigation into the anti-fibrotic efficacy of compound **1** (kurarinol A) derived from the EtOAc extract of *S. flavescens* is deemed to be of considerable significance for the advancement of small molecule therapeutics in the treatment of liver fibrosis.

KA inhibited the proliferation and migration of activated LX-2 cells

Through the IC₅₀ (12.65 μ M) value of KA, RT-qPCR experiment was used to explore the appropriate drug concentration. Under the condition of TGF- β 1 modeling, the mRNA level of Fibronectin in LX-2 cells was detected by 0, 2.5, 5, 10, 20, 40 μ M KA, and the concentration of KA was determined to be 2.5, 5 and 10 μ M (Fig. 2A). Therefore, 2.5, 5 and 10 μ M of KA were selected for subsequent research. The current investigation utilized the MTS assay to evaluate the influence of multiple KA concentrations on the viability of LX-2 cells post-activation. The results showed that TGF- β 1 increased the viability of LX-2 cells, indicating that LX-2 cells was activated, while KA inhibited the viability of LX-2 cells in a dose-dependent manner (Fig. 2A).

In addition, to assess the effect of KA on LX-2 cells migration, an in vitro wound healing assay was conducted. This involved the generation of a scratch wound using a sterile pipette tip and subsequent

photography at 0, 24, and 48 hours to track the migration progress of the LX-2 cells. The wound area was utilized to assess the relative mobility. The findings indicated that TGF- β 1 enhanced the migration of LX-2 cells, indicating that LX-2 cells were activated, while KA inhibited the migration of LX-2 cells. The migration process was carried out in a dose-dependent manner at 24 and 48 h (Fig. 2B). Therefore, KA inhibits LX-2 activation by inhibiting proliferation and migration.

KA inhibited the expression of fibrosis biomarkers Fibronectin, α -SMA and collagen α 1(I) in LX-2 cells stimulated by TGF- β 1

In order to examine the influence of kurarinol A (KA) on cytokines implicated in fibrosis, an evaluation of the gene and protein expression profiles of fibronectin, α -smooth muscle actin (α -SMA), and collagen I was performed following a 48 h treatment with KA. The findings indicated that KA notably reduced the elevated levels of Fibronectin, α -SMA, and Collagen α 1(I) gene expression triggered by TGF- β 1, as depicted in Fig. 3A. In line with the RT-qPCR outcomes, KA also decreased the enhanced protein levels of Fibronectin, α -SMA, and Collagen α 1(I), which were induced by TGF- β 1, in a concentration-dependent manner. (Fig. 3B). Therefore, KA inhibited the expression of fibrosis cytokines in activated LX-2 cells.

Transcriptomics analysis of KA protective mechanisms

Transcriptomic profiling, recognized for its capacity to capture broad-scale gene expression dynamics, was employed in this investigation to identify and anticipate the underlying regulatory pathways associated with the disease pathology. Figure 4A illustrates the Principal Component Analysis (PCA), highlighting a substantial separation in the gene expression patterns between the control, disease model, and KA-treated groups, indicating distinct gene expression profiles among these groups. (Fig.S4, Supporting information). The histogram indicated that treatment with TGF- β 1 led to a notable up-regulation of 444 genes and suppression of 2947 genes in comparison to the control cohort. Following the introduction of KA, comparative analysis to the model group revealed an up-regulation of 216 genes and a down-regulation of 1119 genes. (Fig. 4B). Subsequently, an analysis employing a Venn diagram was conducted to delineate the 106 differentially expressed genes (DEGs) that were common to the trio of groups. (Fig. 4C). Additionally, the heatmap delineation disclosed a biphasic gene expression pattern within the normal, model, and KA groups: one cluster of genes underwent a sequential downregulation, upregulation, and downregulation; the opposing cluster manifested a converse pattern. (Fig. 4D). Following this, an investigation into the biological roles and signaling cascades linked to the DEGs was conducted utilizing the Gene Ontology (GO) repository. The GO annotations suggest that the DEGs are likely to be associated with a spectrum of biological processes (BP), cellular components (CC), and molecular functions (MF) (Fig. 4F). Additionally, the KEGG pathway analysis identified the involvement of the 106 DEGs in key biological pathways, including Hypertrophic Cardiomyopathy, interactions mediated by Cell Adhesion Molecules, the process of Leukocyte Transendothelial Migration, and the TGF-beta signaling pathway (Fig. 4E). Of particular interest, the TGF-beta signaling pathway is postulated to exert significant physiological influence within the context of hepatic fibrosis, potentially constituting the principal biological process engaged by the hepatoprotective effects of kurarinol A (KA). Collectively, the

findings imply that modulation of the TGF-beta signaling pathway may underlie the hepatic anti-fibrotic potential of KA.

KA inhibits TGF- β 1-induced LX-2 activation by regulating TGF- β /Smads signaling pathway

Central to the TGF- β /Smads signaling mechanism is the phosphorylation event of Smad2/3. Upon phosphorylation, Smad2/3 proteins complex with Smad4, translocate to the nucleus, and initiate transcription of genes harboring the requisite Smad binding elements (SBEs). This process culminates in the upregulation of extracellular matrix (ECM) protein expression [19]. In order to evaluate the capacity of KA to suppress the TGF- β /Smads signaling pathway, a series of experiments were executed to measure the expression profiles of key components within this pathway, employing both (RT-qPCR) and western blotting methodologies. As illustrated in Fig. 5B, the levels of TGF- β 1, Smad4, and the ratio of phosphorylated Smad2/3 to total Smad2/3 were considerably elevated in the model group, but were markedly reduced by KA treatment. In addition, RT-qPCR experiments showed that the mRNA results of TGF- β 1, Smad2, Smad3 and Smad4 were consistent with the trend of western blotting results (Fig. 5A). The findings suggest that KA inhibits the activation of LX-2 cells through its effect on dampening the TGF- β 1/Smads signaling pathway.

Discussion

Chronic liver damage of diverse etiologies can lead to hepatic fibrosis, a condition that, untreated, may escalate to cirrhosis and hepatocellular carcinoma. Presently, the therapeutic armamentarium lacks a specific medication for hepatic fibrosis, with a plethora of candidate drugs in various stages of research and development. As such, an unmet clinical demand persists for a potent pharmaceutical agent capable of effectively addressing liver fibrosis [20, 21]. Interestingly, KA, as a lavandulyl flavonoid compound isolated from the traditional Chinese medicine *Sophora flavescens*, have shown a hepatoprotective effect on CCl₄-induced acute liver injury in mice through its antioxidant and anti-inflammatory activities [16]. Hepatic disorders exhibit a spectrum of progressive stages, ranging from initial liver injury to fibrosis, cirrhosis, and, in severe cases, hepatocellular carcinoma. The gradation of severity and the underlying pathophysiological mechanisms vary significantly across the different phases of liver disease [22]. Therefore, it is not clear whether KA can reduce liver fibrosis. Interestingly, this study found that KA can inhibit the excessive accumulation of liver fibrosis markers (α -SMA, fibronectin and collagen I) in activated LX-2 cells and improve liver fibrosis by regulating the TGF- β /Smads signaling pathway.(Fig. 6).

Central to the pathogenesis of liver fibrosis is the activation and subsequent proliferation of hepatic stellate cells (HSCs), which are considered a key factor. The present study is designed to assess the efficacy of KA in ameliorating liver fibrosis potentially by inhibiting these cellular activities [23, 24]. Initially, a cell model of liver fibrosis was successfully established by TGF- β 1-induced LX-2 cells. Subsequently, the results showed that a total of 11 compounds had a significant inhibitory effect on the

proliferation of LX-2 cells and their IC₅₀ was between 4–40 μM by MTS assay. Among them, three compounds (Xanthohumol (**8**), Isoliquiritigenin (**9**) and Luteolin (**24**)) have been reported to have anti-hepatic fibrosis activity [25–27], and KA, as a new structural compound with anti-hepatic injury effect isolated from *Sophora flavescens*, has been focused on and studied in depth from two aspects of inhibiting the proliferation and activation of HSCs.

Upon activation, HSCs demonstrate the capacity for proliferation and migration, and are responsible for the biosynthesis of key ECM proteins such as fibronectin and collagen I, in conjunction with the secretion of the contractile protein α-SMA. The presence of α-SMA is widely regarded as a diagnostic marker for HSCs activation. The synthesis and secretion of collagen I and fibronectin by fibroblasts, when in excessive amounts, typify the pathological alterations associated with the development of liver fibrosis [28]. In the current study, cell scratch, RT-qPCR and Western blotting results showed that KA could inhibit the migration of activated LX-2 cells and the mRNA and protein levels of Fibronectin, Collagen I and α-SMA in a dose-dependent manner. These results indicate that KA can directly act on activated HSCs, thereby reducing liver fibrosis *in vivo*. In pursuit of a more profound understanding of the mechanisms by which KA suppresses the activation of LX-2 cells, the investigators have harnessed the power of transcriptomic technologies. This approach aims to uncover the molecular targets and biological pathways that are implicated in KA's therapeutic efficacy against hepatic fibrosis. Finally, it was found that the mechanism of KA against liver fibrosis was closely related to TGF-β/Smads signaling pathway. The TGF-β1/Smads signaling pathway is pivotal in the activation of HSCs and is central to the pathogenesis of liver fibrosis, characterized by the aberrant deposition of ECM components. TGF-β is acknowledged as a key cytokine that orchestrates the transition of HSCs into a more fibrogenic state. Activation of TGF-β1 triggers the phosphorylation of Smad2/3, resulting in the formation of phosphorylated Smad2/3 (p-Smad2/3). This active form then complexes with Smad4, leading to nuclear translocation and subsequent activation of gene transcription for ECM proteins [29, 30]. Therefore, exogenous molecules that disrupt TGF-β/Smads signaling to inhibit HSCs activation may be potential anti-hepatic fibrosis drugs. The study conducted by the research team has provided evidence that KA exerts an inhibitory effect on the protein levels of TGF-β1 and Smad4, and alters the ratio of phosphorylated to total Smad2/3 within LX-2 cells. This suggests a potential mechanism by which KA could suppress the activation of HSCs, specifically LX-2 cells, by impeding the TGF-β1/Smads signaling pathway. Consequently, this intervention may lead to an improvement in the condition of liver fibrosis.

In summary, this study explored the effect of KA on LX-2 cells activation and proliferation through a preliminary *in vitro* experiment. This pioneering work has, for the first time, established that KA can attenuate the activation of LX-2 cells and curb the unwarranted buildup of ECM by interfering with the TGF-β/Smads signaling pathways, thereby potentially ameliorating liver fibrosis. The implications of these results are that KA may be a promising candidate for the therapeutic intervention of liver fibrosis. Consequently, the study contributes novel and scientifically rigorous insights, providing a foundation and catalyst for future research into the anti-fibrotic properties of KA.

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Author's contribution **CXJ**, Investigation, Data Curation, Writing-Original Draft, WritingReview & Editing, Validation; **TB**, Validation, Formal analysis, Investigation; **HMH**, Validation, Investigation, Visualization; **ZX**, Methodology, Investigation; **LSG**, Project administration, Funding Acquisition; **LZ**, Methodology, Visualization; **ZZ**, Methodology, Investigation; **LQD**, Methodology, Project administration, Writing-Review & Editing; **LY**, Supervision, Conceptualization, Writing-Review & Editing, Funding acquisition. All authors reviewed the manuscript.

Abbreviations

TGF-β1	transforming growth factor-β1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt
RT-qPCR	real-time quantitative polymerase chain reactions
KA	Kurarinol A
ECM	extracellular matrix
HSCs	hepatic stellate cells
FN	fibronectin
α-SMA	α-smooth muscle actin
DMEM	Dulbecco's modified eagle
FBS	Fetal Bovine Serum
OD	optical density
PBS	phosphate buffer solution
DMSO	dimethyl sulfoxide
PCA	

Principal Component Analysis

SBEs

Smad binding elements

DEGs

differentially expressed genes

BP

biological process

CC

cellular component

MF

molecular function

GO

Gene Ontology

Declarations

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conflicts of interest All authors have disclosed that they do not have any conflicts of interest.

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Figures

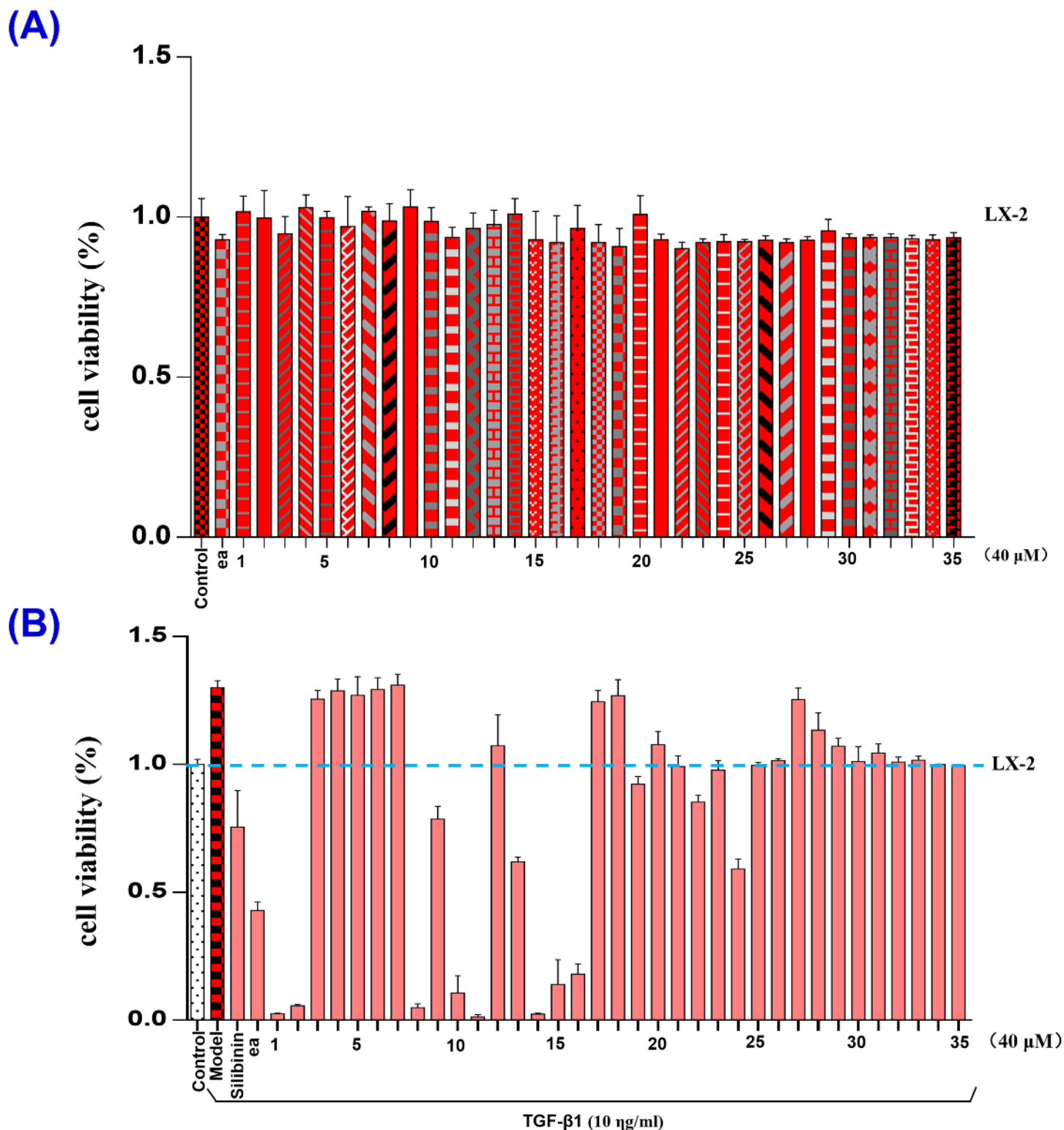
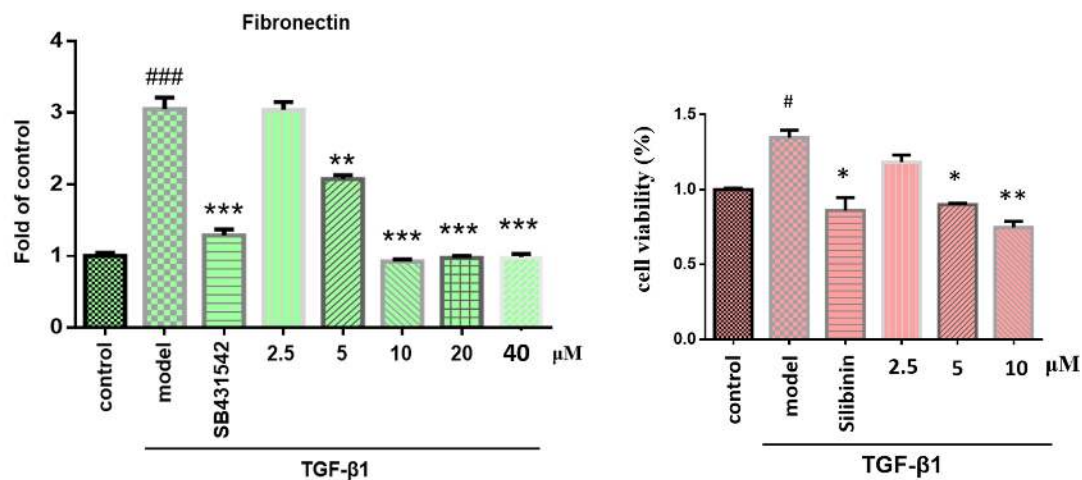


Figure 1

(A) The cytotoxicity of **1-35** compounds on LX-2 cells was tested by MTS assay. **(B)** Using the LX-2 cells proliferation model induced by TGF- β 1. MTS assay was used to screen the active compounds from **1-35** compounds that inhibit the proliferation of LX-2 cells. Silibinin (40 μ mol/L) was the positive drug. The results were expressed as percentage of the control group. Data were presented as mean $\pm$ SD (n=3).

(A)



(B)

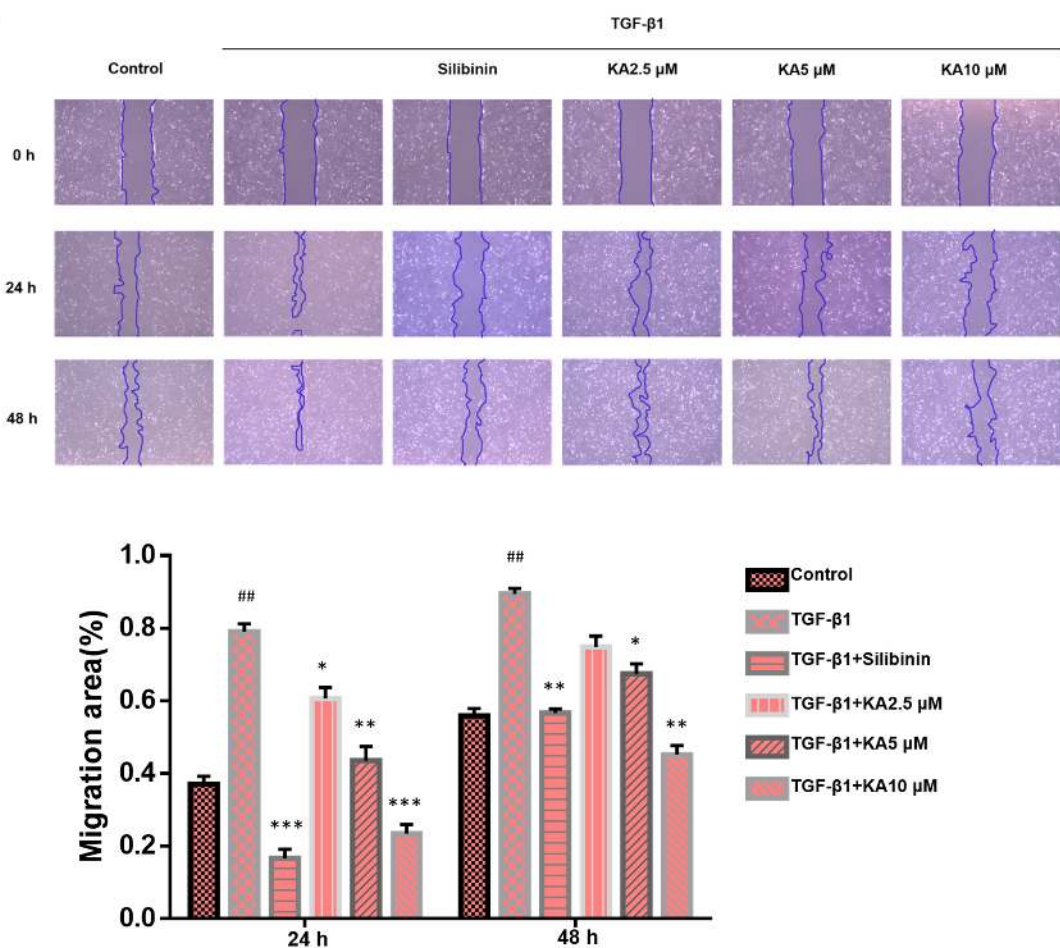


Figure 2

(A) The effects of different concentrations of KA on the proliferation and Fibronectin mRNA of LX-2 cells induced by TGF-β1. (B) Cell migration was monitored at intervals of 0, 24, and 48 hours post-KA administration (100×). The migration efficiency was quantified by calculating the relative migration rate. The results were pooled from three separate experiments. The data were presented as the mean value

with standard deviation (SD). (### $P < 0.001$ compared with control; ** $P < 0.01$, and *** $P < 0.001$ compared with the TGF- β 1 model group)

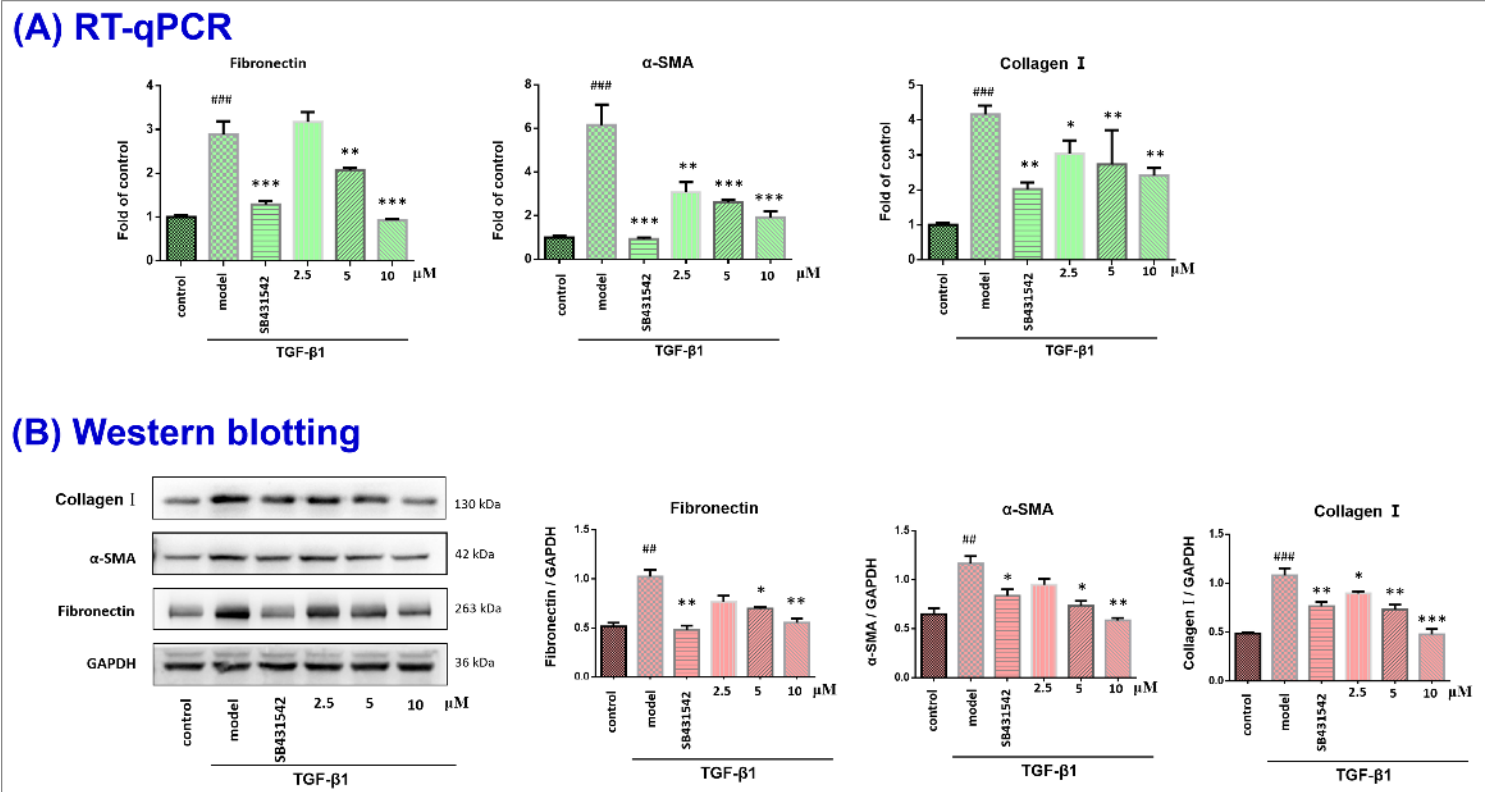


Figure 3

KA inhibited the expression of fibrotic cytokines Fibronectin, α -SMA and Collagen I at gene and protein levels. **(A)** Fibronectin, α -SMA and Collagen I mRNA expression. **(B)** Fibronectin, α -SMA and Collagen I protein expression. SB431542 (10 μ M) is used as the positive drug. Data from the three independent trials. Data were represented by mean $\pm$ SD. (### $P < 0.001$ compared with control; ** $P < 0.01$, and *** $P < 0.001$ compared with the TGF- β 1 model group)

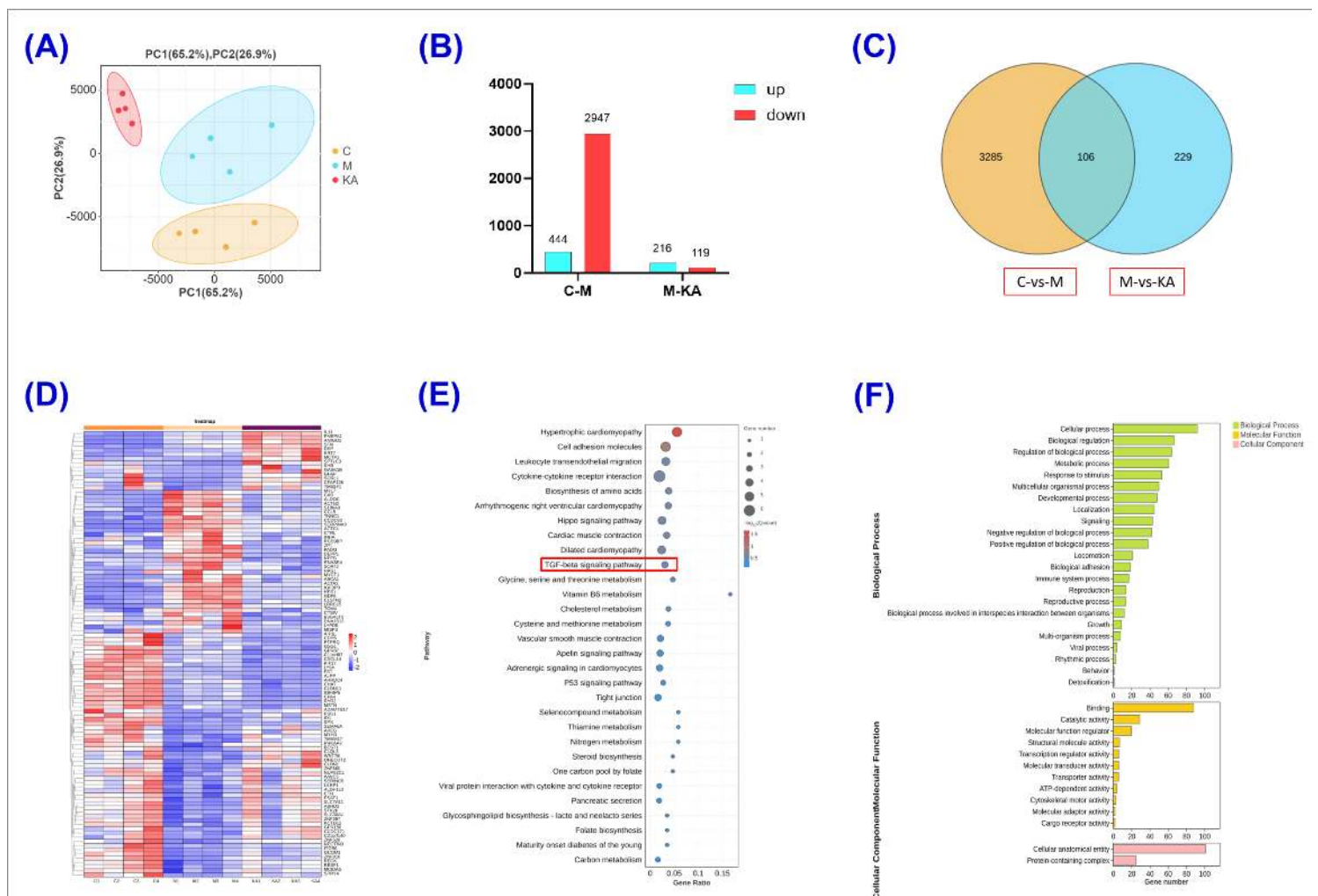
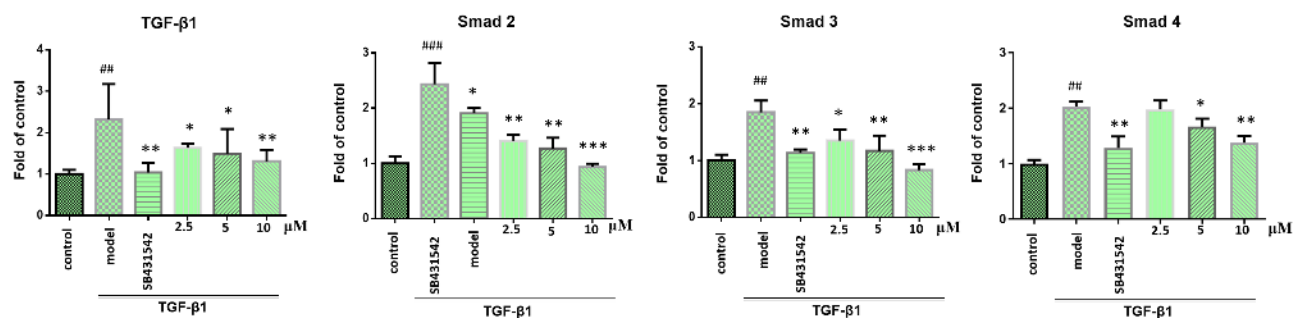


Figure 4

Transcriptome analysis. (A) PCA analysis. The PCA plot illustrates the relative positioning of observations, where close proximity denotes a high degree of similarity, and greater separation indicates divergence. The groups are delineated by the following labels: C1-C4 for the normal control group, M1-M4 for the model group, and KA1-KA4 for the group receiving the high dosage of KA. (B) An analysis of the differential gene expression was conducted, comparing the number of upregulated and downregulated unigenes in the Control versus Model groups and in the KA versus Model groups. (C) Venn diagram. A comprehensive analysis was performed to identify differentially expressed genes (DEGs) across the control, model, and KA treatment cohorts. (D) Heatmap. In the dataset, each gene is represented by a unique row, with each column corresponding to a specific sample. Genes with increased expression levels are indicated by the color red, and those with decreased levels by blue. The control group is designated C1 through C4, the model group is labeled M1 through M4, and the high-dose KA treatment group is identified as KA1 through KA4. (E) KEGG analysis. The color intensity of the dots is indicative of the statistical significance as denoted by the P-value, whereas the dots' size is proportional to the quantity of genes. (F) GO analysis. The y-axis corresponds to the GO annotation categories, and the x-axis represents the tally of genes. The color scheme applied is green to designate biological processes, pink for cellular components, and yellow to illustrate molecular functions.

(A) RT-qPCR



(B) Western blotting

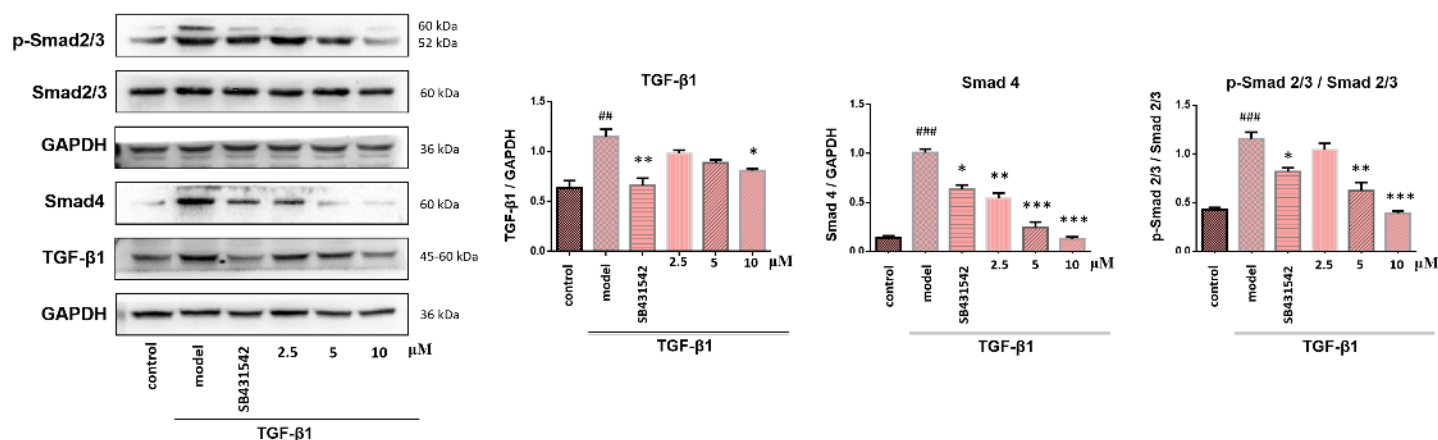


Figure 5

KA inhibited TGF-β1-induced TGF-β/Smads pathway activation. **(A)** Expression of mRNA for TGF-β1, Smad2, Smad3 and Smad4. **(B)** Expression of protein for TGF-β1, Smad2/3 and Smad4. SB431542 was used as positive drug. Data from the three independent trials. Data were represented by mean±SD. (^{###} $P < 0.001$ compared with control; ^{**} $P < 0.01$, and ^{***} $P < 0.001$ compared with the TGF-β1 model group)

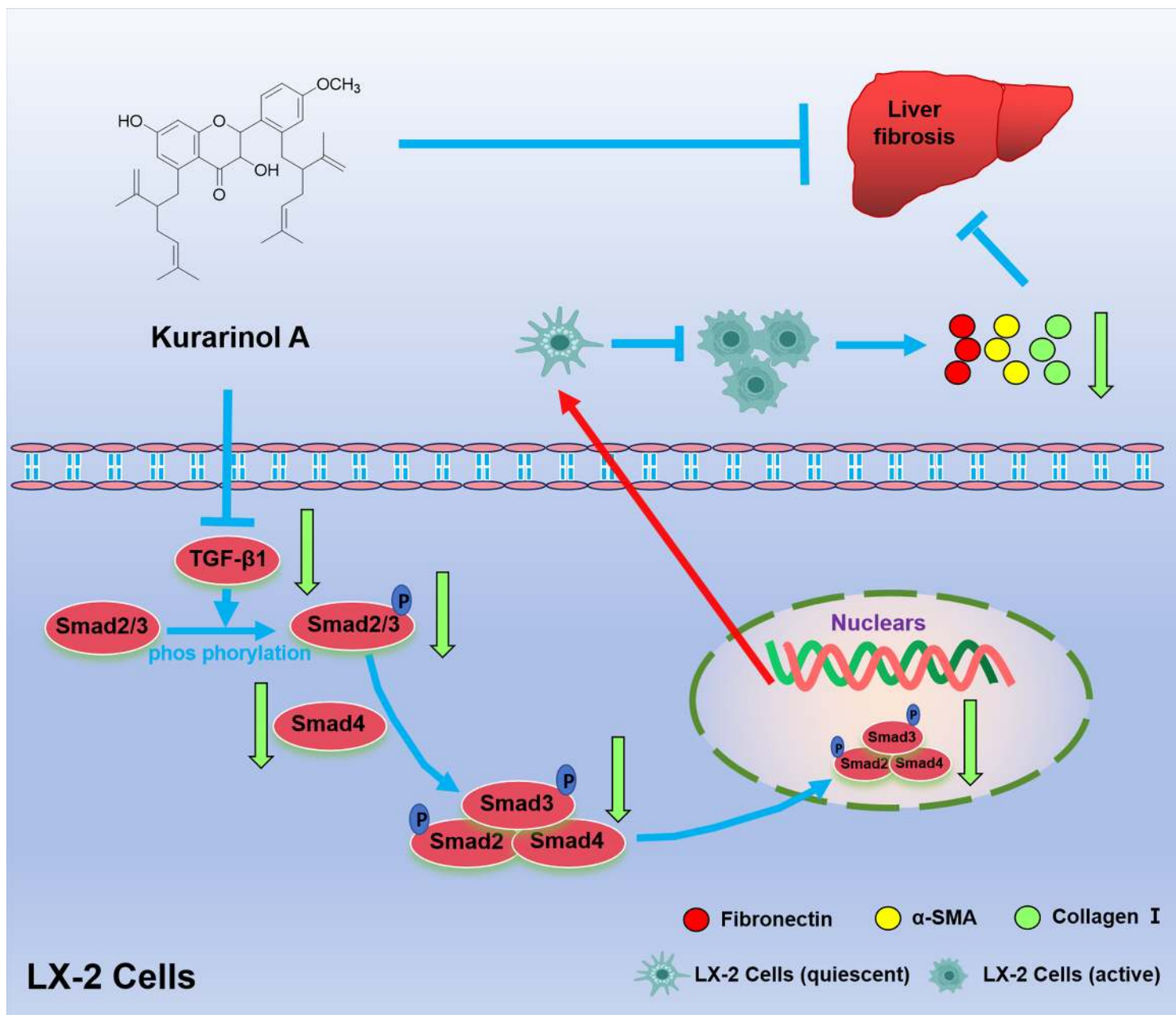


Figure 6

The mechanism by which KA attenuates HSCs activation and proliferation. The activation of LX-2 cells was regulated by TGF- β /Smads signaling pathway.

Supplementary Files

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- [Supportinginformation1.doc](#)