

## Original Article

# Xiaoai Jiedu recipe promotes ferroptosis against hepatocellular carcinoma progression via NF- $\kappa$ B signaling axis

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**Abstract:** Xiaoai Jiedu recipe (XJR), a classical compound prescription used in traditional Chinese medicine, is frequently administered as an adjuvant therapy for patients with cancer. However, its anti-hepatocellular carcinoma (HCC) activity and the molecular mechanisms underlying this effect remain incompletely defined. This study investigated whether XJR suppresses HCC progression and explored the potential involvement of NF- $\kappa$ B-associated ferroptosis regulation. An HCC xenograft mouse model and HepG2 cells were used to determine whether XJR restrains HCC progression by promoting ferroptosis in association with inhibition of NF- $\kappa$ B signaling. Immunohistochemistry (IHC), immunofluorescence (IF) staining, and western blotting were performed to detect NF- $\kappa$ B pathway activity and ferroptosis-related targets, including KEAP1, Nrf2, GPX4, and xCT. ELISA, transmission electron microscopy (TEM), and fluorescent probes, including DCFH-DA, JC-1, FerroOrange, and Liperfluor, were used to assess oxidative stress, mitochondrial membrane potential, labile ferrous iron, and lipid peroxidation in HCC models. Histopathological and cellular assays, including H&E staining, TUNEL fluorescence, Annexin V-FITC/PI staining, and Ki67 staining, showed that XJR reduced proliferative activity and increased tumor cell injury and death. XJR increased the lipid peroxidation marker MDA and reduced the antioxidant indicators GSH and SOD in serum and HepG2 cells. TEM and fluorescent probe assays further showed progressive mitochondrial injury, oxidative stress, labile iron accumulation, and lipid peroxidation, consistent with increased ferroptotic susceptibility. IHC, IF, and western blotting indicated that XJR reduced NF- $\kappa$ B p65 nuclear translocation and decreased the phosphorylation ratios of p-NF- $\kappa$ B/NF- $\kappa$ B and p-I $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$ . XJR also upregulated KEAP1 and downregulated Nrf2, GPX4, and xCT. Pharmacological inhibition of NF- $\kappa$ B with BAY11-7082 produced similar changes and further enhanced the effects of XJR in HepG2 cells, supporting the involvement of the NF- $\kappa$ B/Nrf2/xCT/GPX4 axis in XJR-associated ferroptosis. These findings suggest that XJR exerts anti-HCC effects, at least partly, by promoting ferroptosis through inhibition of the NF- $\kappa$ B signaling pathway and downstream antioxidant defenses. The results provide preclinical evidence supporting further investigation of XJR as an adjuvant candidate for HCC therapy.

**Keywords:** Hepatocellular carcinoma (HCC), NF- $\kappa$ B, ferroptosis, traditional Chinese medicine (TCM), Xiaoai Jiedu recipe (XJR)

## Introduction

Hepatocellular carcinoma (HCC) ranks among the globally prevalent fatal cancers, characterized by rapid disease progression, frequent postoperative recurrence, and unsatisfactory clinical outcomes [1]. Current treatment strategies, including surgical resection, chemotherapy, and targeted therapy, are limited by substantial side effects, drug resistance, and high

postoperative recurrence rate, highlighting the urgent need to identify effective anti-HCC drugs and actionable molecular targets [2, 3]. Unrestrained tumor proliferation and impaired cell death are major contributors to the high mortality associated with HCC.

Ferroptosis is a distinct form of iron-dependent programmed cell death driven by excessive lipid peroxidation and has become a viable

therapeutic strategy for HCC [4, 5]. The cystine-glutamate antiporter xCT together with GPX4 constitute the core defensive machinery against ferroptosis. These components are frequently upregulated in HCC, where they reduce lipid peroxide accumulation and enable tumor cells to evade ferroptotic death [6]. The Nrf2/KEAP1 axis is a major antioxidant pathway that regulates the expression of ferroptosis-related genes, including GPX4 and xCT. Persistent Nrf2 activation is strongly associated with ferroptosis resistance and malignant deterioration in HCC. Multiple signaling networks regulate ferroptosis, and the NF- $\kappa$ B cascade has been implicated as a central regulator of ferroptosis resistance in HCC [7, 8].

Accumulating evidence has established a close link between chronic inflammation and malignant transformation [9]. Persistent hepatic inflammation is a core pathogenic factor that fuels the initiation and progression of HCC [10]. NF- $\kappa$ B signaling is constitutively activated in the HCC microenvironment and has been associated with ferroptosis resistance [11, 12]. In resting cells, I $\kappa$ B proteins bind to NF- $\kappa$ B and retain it within the cytoplasm, thereby maintaining NF- $\kappa$ B in an inactive state. Upon external stimulation, I $\kappa$ B is phosphorylated and degraded, resulting in nuclear translocation of the NF- $\kappa$ B p65 subunit and activation of genes involved in inflammation, cell survival, and redox adaptation [13, 14]. Previous studies have indicated that persistent NF- $\kappa$ B activation may reinforce the Nrf2/GPX4/xCT antioxidant defense network, thereby reducing lipid reactive oxygen species (ROS) accumulation and limiting ferroptotic cell death. Conversely, pharmacological inhibition of NF- $\kappa$ B can sensitize HCC cells to ferroptosis by weakening antioxidant defenses and promoting lipid peroxidation [15, 16], making NF- $\kappa$ B a promising therapeutic target.

Traditional Chinese medicine (TCM) offers potential advantages for HCC management because of its multi-component and multi-target characteristics and relatively low systemic toxicity [17, 18]. Xiaohai Jiedu Recipe (XJR), a classic TCM prescription, has been clinically used to enhance immune function, alleviate chemotherapy-related adverse reactions, and improve the quality of life of patients with cancer [19]. Previous studies have reported the anti-HCC effects of XJR *in vitro* and *in vivo* [20, 21]. However, whether the anti-tumor effects of XJR involve the NF- $\kappa$ B/ferroptosis regulatory

axis remains unclear. We therefore hypothesized that XJR inhibits HCC proliferation and progression by enhancing ferroptotic susceptibility through suppression of the NF- $\kappa$ B signaling cascade. To test this hypothesis, we evaluated the anti-HCC effects of XJR and examined the associated molecular changes.

## Methods

### *Materials and reagents*

XJR was provided by Anhui Bozhou Traditional Chinese and Western Medicine Co., Ltd. and Suzhou Tianling Traditional Chinese Medicine Decoction Pieces Co., Ltd. Detailed analytical data supporting the chemical characterization of the herbal formulation have been reported in our previous publication [9]. The herbal composition of XJR includes Spreading Hedyotis Herb (Batch No.: 2021082201), Appendiculate Cremastra Pseudobulb (Batch No.: CG08001), Fruit of Fiveleaf Akebia (Batch No.: CR02801), Stiff Silkworm (Batch No.: 2022033103), Centipede (Batch No.: CD03101), Heterophylly Falsestarwort Root (Batch No.: 2022022301), and Dwarf Lilyturf Root Tuber (Batch No.: CG01601). The total ion flow chromatograms under positive and negative ion modes are presented in [Supplementary Figure 1](#). The composition of herbal ingredients in XJR is provided in [Supplementary Table 1](#). Antibodies against NF- $\kappa$ B p65 (8242), phospho-NF- $\kappa$ B p65 (3033), I $\kappa$ B- $\alpha$  (4812), phospho-I $\kappa$ B- $\alpha$  (2859), KEAP1 (8047), Nrf2 (12721), GPX4 (52455), GAPDH (5174), and  $\beta$ -actin (4970) were sourced from Cell Signaling Technology (Danvers, USA). An antibody against xCT (R382036) was obtained from HUABIO (Zenbio, China). A prestained protein ladder (WJ103) was obtained from Yamei Biotech (Shanghai, China).

### *Animals*

Animal experiments in this study were performed in accordance with the ethical guidelines and were approved by the Experimental Animal Ethical Committee of Nanjing University of Chinese Medicine (SYXK 2014-0001). The experimental procedures were approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine (Approval Number: 202403A061). Four- to six-week-old male BALB/c-nu mice (20  $\pm$  2 g) were purchased from Qinglongshan Animal Breeding Center (Nanjing, China). The animals were



raised under specific pathogen-free environment, with free access to standard chow and sterile drinking water.

To establish the HCC xenograft model,  $5 \times 10^6$  H22 cells were subcutaneously inoculated into the right flank of each mouse [10]. When the tumor diameter reached 5-6 mm, tumor-bearing mice were randomized into four groups: the Control group, which received an equivalent volume of normal saline, and the low- (1 g/kg/d XJR), middle- (3 g/kg/d XJR), and high- (9 g/kg/d XJR) dose XJR intervention groups ( $n=6$  for each). All mice received continuous intragastric administration once daily for 14 consecutive days. Twenty-four hours after the last treatment session, all experimental mice were euthanized, and tumor tissues and peripheral blood specimens were collected for subsequent biochemical, histopathological, and molecular analyses. Before blood collection from the orbital arteriovenous plexus, the animals were anesthetized with inhaled isoflurane to minimize pain (induction 2-3%, maintenance 1.5-2%). For euthanasia, the mice were deeply anesthetized with inhaled isoflurane, followed by cervical dislocation to ensure rapid and humane death.

## Serum preparation

Twenty-four healthy male Sprague-Dawley rats (6-8 weeks old, 220-250 g) were obtained from Qinglongshan Animal Breeding Center (Nanjing, China). Experimental rats were randomly assigned to two groups and subjected to daily intragastric gavage over an eight-day treatment period. Animals in the control group were administered an equivalent dose of sterile normal saline each day, while the XJR intervention group was treated with 9 g/kg XJR once a day, which was chosen as the high-dose concentration according to *in vivo* experimental results. Twenty-four hours after the final administration, blood samples were collected and centrifuged to isolate supernatant serum. The serum was heat-inactivated at 56°C for 30 min and sterilized by filtration through a 0.22-μm microporous membrane. Finally, the pretreated serum was diluted with serum-free medium to a final working concentration of 15% for subsequent cellular experiments [11].

## Cell lines and culture

The HCC lines (HepG2 and H22 cells) were purchased from the Cell Bank of the Shanghai

Branch of the Chinese Academy of Sciences. Cells were authenticated by STR profiling and tested negative for mycoplasma contamination by a PCR-based assay. HepG2 cells were pre-treated with NF-κB inhibitor (BAY11-7082) (10 μM; Selleck, USA), or the ferroptosis inhibitor ferrostatin-1 (Fer-1; 0.5 μM; Selleck, USA) which was used as a reference control [12]. The cells were cultured in DMEM basal medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

## Histological and immunohistochemical analysis

Excised tumor specimens were fixed in 4% paraformaldehyde, dehydrated through a graded ethanol series, embedded in paraffin, and serially sectioned for histopathological analysis. Hematoxylin and eosin (H&E) staining was performed to visualize tumor histomorphological alterations by light microscopy. In addition, immunohistochemistry (IHC; Sangon Biotech, Shanghai, China) was performed to detect the proliferation marker Ki67, NF-κB, and ferroptosis-related proteins in the Nrf2/xCT/GPX4 axis in xenograft tumor tissues.

## Enzyme-linked immunosorbent assay (ELISA)

Whole-blood samples were centrifuged at 3500 r/min for 20 min to obtain serum. For cell samples, the collected samples were mixed with an appropriate volume of PBS, followed by thorough homogenization and additional centrifugation. The levels of glutathione (GSH; YFXEM00126, mouse), superoxide dismutase (SOD, YFXEM00394, mouse), malondialdehyde (MDA, YFXEH00392, mouse), GSH (YFXEM-00212, human), SOD (YFXEM00218, human), and MDA (YFXEH00299, human) in mouse serum and HepG2 cell samples were measured using ELISA kits (YFXBIO, China).

## Transmission electron microscopy (TEM)

The ultrastructural morphological characteristics of HCC xenograft tissues were examined using transmission electron microscopy (TEM; JEOL JEM-1400Flash) at low magnification ( $\times 3,000$ ; scale bar, 5 μm) and high magnification ( $\times 10,000$ ; scale bar, 1 μm). Briefly, tumor tissues were rapidly dissected into cubic fragments (1 \* 1 \* 1 -mm<sup>3</sup>) immediately after animal

sacrifice. The tissue blocks were pre-fixed in TEM fixative overnight at 4°C. After post-fixation with 1% osmium tetroxide for 2 h at room temperature, the tissue specimens were embedded in epoxy resin. Subsequent ultrathin sections were prepared and double-stained with lead citrate and ethanol-diluted 2% uranyl acetate prior to TEM imaging.

## *Flow cytometry for apoptosis assay*

Cellular apoptosis induced by XJR was quantitatively assessed using an Annexin V-FITC/PI Apoptosis Detection Kit and flow cytometry (FACSC, BD Instruments, USA). Cells were seeded in six-well plates at a density of  $1.0 \times 10^4$  cells/well and treated with the designated XJR concentrations for 24 h. The cells were then collected and resuspended in 500 µL binding buffer, followed by the addition of 5 µL of Annexin V-FITC and equivalent volume of PI staining solution. Following a 20-min room-temperature incubation away from light, flow cytometry was promptly applied to quantify apoptotic cell percentages.

## *Western blotting*

Total cellular protein was extracted from HepG2 cells and subcutaneous tumor tissues using RIPA lysis buffer containing mixed protease and phosphatase inhibitor cocktails. Equal amounts of extracted protein (20 µg of total protein per lane) were separated via SDS-PAGE electrophoresis before being electroblotted onto PVDF membranes. The membranes were blocked using 5% skim milk and then incubated overnight at 4°C with primary antibodies against NF-κB p65, p-NF-κB p65, IκBα, p-IκBα, KEAP1, Nrf2, GPX4 and xCT (1:1,000 dilution). The membranes were then incubated with HRP-conjugated secondary antibodies (1:10,000 dilution). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system.

## *Immunofluorescence staining*

HepG2 cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA to reduce nonspecific antibody binding. The samples were incubated overnight at 4°C with primary antibodies against NF-κB p65, Nrf2, and GPX4, followed by incubation with the corresponding fluores-

cence-conjugated secondary antibodies. DAPI solution was used for nuclear counterstaining, and fluorescence images were acquired from the different groups and examined using a fluorescence microscope.

## *Measurement of mitochondrial reactive oxygen species (ROS)*

Intracellular ROS levels were detected using DCFH-DA (Beyotime). After the indicated treatments, HepG2 cells were co-incubated with 10 µM DCFH-DA and Hoechst 33342 at 37°C for 25 min. Finally, the intracellular ROS fluorescence signals of each group were captured using a ZEISS fluorescence microscope (Germany).

## *Measurement of mitochondrial membrane potential (MMP) ( $\Delta\psi_m$ )*

MMP was detected using a commercial JC-1 fluorescence staining kit (Beyotime) according to the manufacturer's instructions. After the indicated interventions, HepG2 cells were incubated with 1 mL of JC-1 working solution per well for 20 min. Subsequently, cell nuclei were counterstained with Hoechst. The fluorescence signals reflecting MMP changes were ultimately visualized and captured using a fluorescence microscope.

## *Detection of ferrous ion ( $Fe^{2+}$ )*

Intracellular ferrous iron ( $Fe^{2+}$ ) levels were assessed using the FerroOrange fluorescent probe (Dojindo, Japan) according to the manufacturer's instructions. After three washes with serum-free DMEM, the cells were incubated with  $1 \mu\text{mol} \cdot \text{L}^{-1}$  FerroOrange working solution at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$  for 30 min. Fluorescence images reflecting cellular  $Fe^{2+}$  levels were subsequently captured using a fluorescence microscope.

## *Assessment of LPO*

Intracellular lipid peroxidation (LPO) was determined using the Liperfluo fluorescent probe (Dojindo, Japan) following the manufacturer's protocols. After washing with serum-free DMEM, the cells were cultivated with  $5 \mu\text{mol/L}$  Liperfluo working solution diluted in serum-free medium at 37°C for 30 min. Fluorescence images were subsequently captured using a

## XJR induces ferroptosis via NF-κB axis in HCC

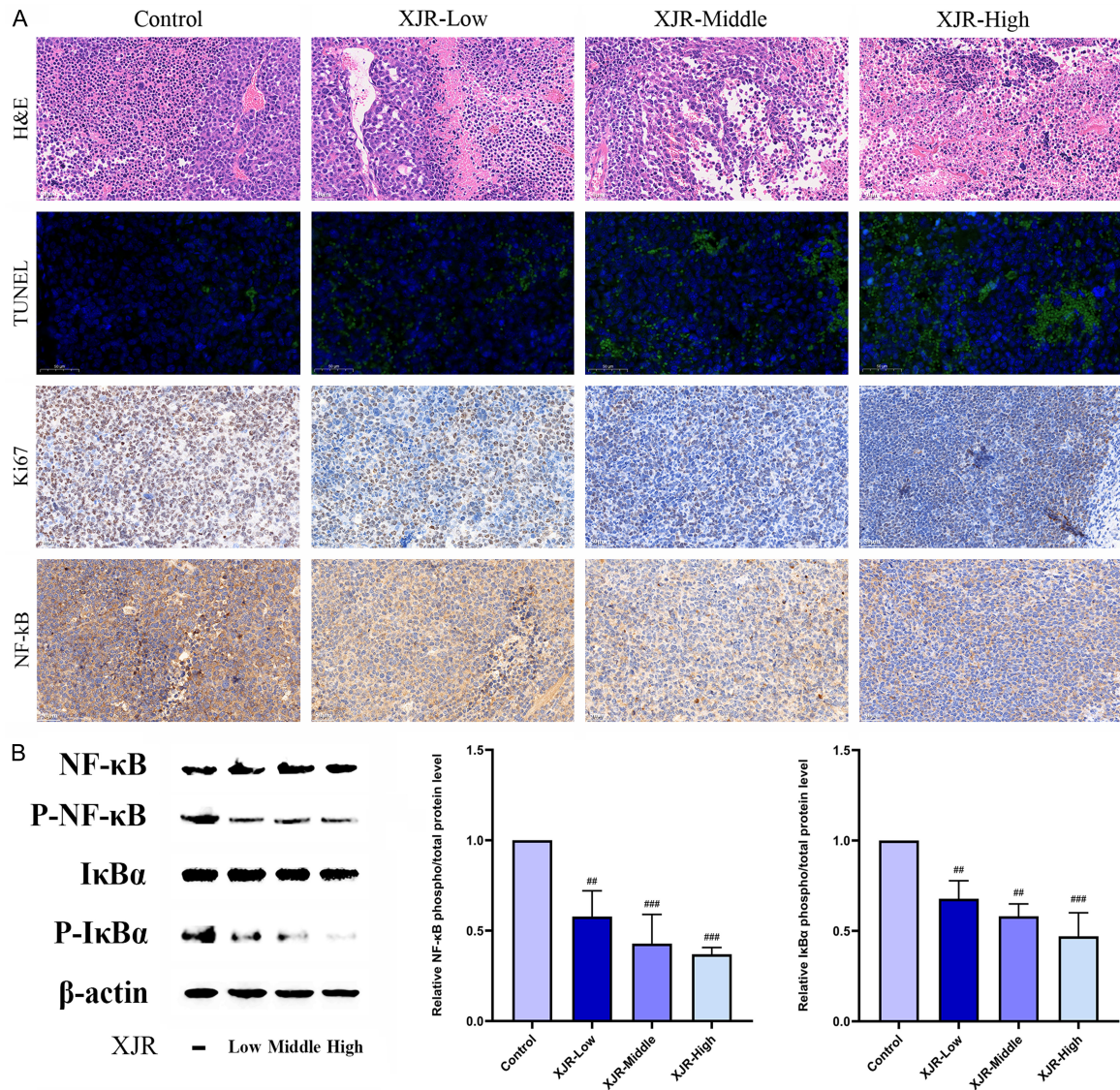


**Figure 1.** XJR inhibited HCC cell proliferation and the NF-κB signaling pathway. A. Representative H&E staining, immunohistochemistry staining of Ki67, and TUNEL assay in tumor pathological sections from mice in each group; magnification  $\times 400$ . B. Effects of XJR on the protein levels of p-NF-κB/NF-κB and p-IκBα/IκBα in tumor tissue of each group, with quantitative analysis. Notes: <sup>##</sup> $P < 0.01$  and <sup>###</sup> $P < 0.001$  vs. Control group.

Zeiss Axio Vert.A1 inverted fluorescence microscope.

### Statistical analysis

Experimental data and quantitative diagrams were generated using GraphPad Prism 9.0. Data were expressed as mean  $\pm$  standard deviation (SD) from at least three independent experiments. Data distribution was assessed using the Shapiro-Wilk test before applying parametric tests. Comparisons between two groups were performed using an unpaired Student's t-test, whereas multiple-group com-

parisons were performed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. A two-sided  $P < 0.05$  were regarded as statistically significant.

### Results

#### *XJR suppressed proliferation and induced tumor cell death in HCC*

H&E staining of xenograft tumor sections revealed histopathological difference among the groups (**Figure 1A**). In the Control group, tumor cells were densely arranged and exhibit-



ed marked malignant atypia, including enlarged hyperchromatic nuclei, elevated nucleocytoplasmic ratio, and frequent mitotic figures. Necrosis and vacuolar degeneration were rarely observed, consistent with vigorous proliferative capacity of the tumor cells. The XJR-Low group largely retained a dense cellular architecture comparable to that of the control group but showed focal cytoplasmic vacuolation and mild karyopyknosis, suggesting early cellular injury. In the XJR-Middle group, tumor cell density declined moderately alongside looser cellular arrangement and widened intercellular spaces. Focal vacuolar degeneration and sporadic necrotic lesions were observed, accompanied by widespread karyopyknosis and karyorrhexis, indicating aggravated cell death. The XJR-High group exhibited extensive coagulative necrosis, presenting as diffuse pale eosinophilic regions. Most tumor cells showed structural collapse, whereas only a small number of atypical cells and mitotic figures remained, indicating the most severe tumor injury across all cohorts. Collectively, these morphological alterations demonstrated that XJR suppressed tumor growth and promoted tumor cell injury and death in a dose-dependent manner.

Consistent with the morphological alterations in H&E staining, TUNEL staining demonstrated increased tumor cell death across the treatment cohorts, with a dose-dependent increase in the proportion of TUNEL-positive cells. Ki67 immunohistochemistry was performed to assess the in situ proliferative activity of xenograft tumor cells. Compared with the control group, the proportion of Ki67-positive cells progressively decreased in XJR-Low, XJR-Middle and XJR-High groups. Collectively, the TUNEL and Ki67 results indicate that XJR exerted prominent anti-proliferative and pro-cell-death effects against HCC in a concentration-dependent manner, with the high-dose treatment showing the greatest therapeutic effect (**Figure 1A**).

## *XJR inhibited NF-κB signaling in HCC xenograft tumors*

IHC for NF-κB p65 revealed extensive nuclear localization of NF-κB p65 in tumor cells of the control group. Increasing doses of XJR progressively reduced NF-κB p65 nuclear localization (**Figure 1A**). Consistent with the IHC results,

western blot analysis of xenograft tumor tissues showed that XJR suppressed the phosphorylation levels of IκBα and NF-κB p65, as reflected by progressive decreases in the p-p65/total p65 and p-IκBα/total IκBα ratios with increasing XJR dosage compared with the control group (**Figure 1B**). Taken together, these findings indicate that XJR suppresses canonical NF-κB pathway activation in a dose-dependent manner, which may contribute to reduced tumor proliferation and increased tumor cell injury in HCC xenografts.

## *XJR disrupted Nrf2-dependent antioxidant defense and promoted oxidative stress in xenograft tumors*

ELISA quantification of serum oxidative stress markers showed marked biochemical changes after XJR treatment. Compared with the control group, serum MDA, a marker of lipid peroxidation, was significantly increased, whereas the antioxidant indicators GSH and SOD progressively declined with increasing XJR doses, exhibiting a clear dose-dependent tendency (**Figure 2A**).

Ultrastructural alterations of HCC xenograft tissues were characterized using TEM. Low-magnification images were used to assess overall cellular morphology, and high-magnification images were used to evaluate subcellular organelle lesions in the boxed regions (**Figure 2B**). In the Control group, HCC cells exhibited regular nuclear contours, uniformly distributed chromatin, compact cytoplasm, and narrow intercellular spaces. The mitochondria were largely intact, with densely and orderly arranged cristae. Few cytoplasmic lipid vacuoles or electron-dense inclusions were observed, indicating intact mitochondrial homeostasis and negligible spontaneous ferroptosis in untreated tumor cells.

With increasing XJR doses, tumor cells displayed progressively greater nuclear chromatin condensation, cytoplasmic vacuolation, and electron-dense inclusions. Mild pathological changes were observed in the XJR-Low group, including slight nuclear shrinkage, focal heterochromatin aggregation, sporadic cytoplasmic vacuolation, and marginally widened intercellular spaces. At higher magnification, the mitochondria showed mild swelling with partial cris-



XJR induces ferroptosis via NF- $\kappa$ B axis in HCC

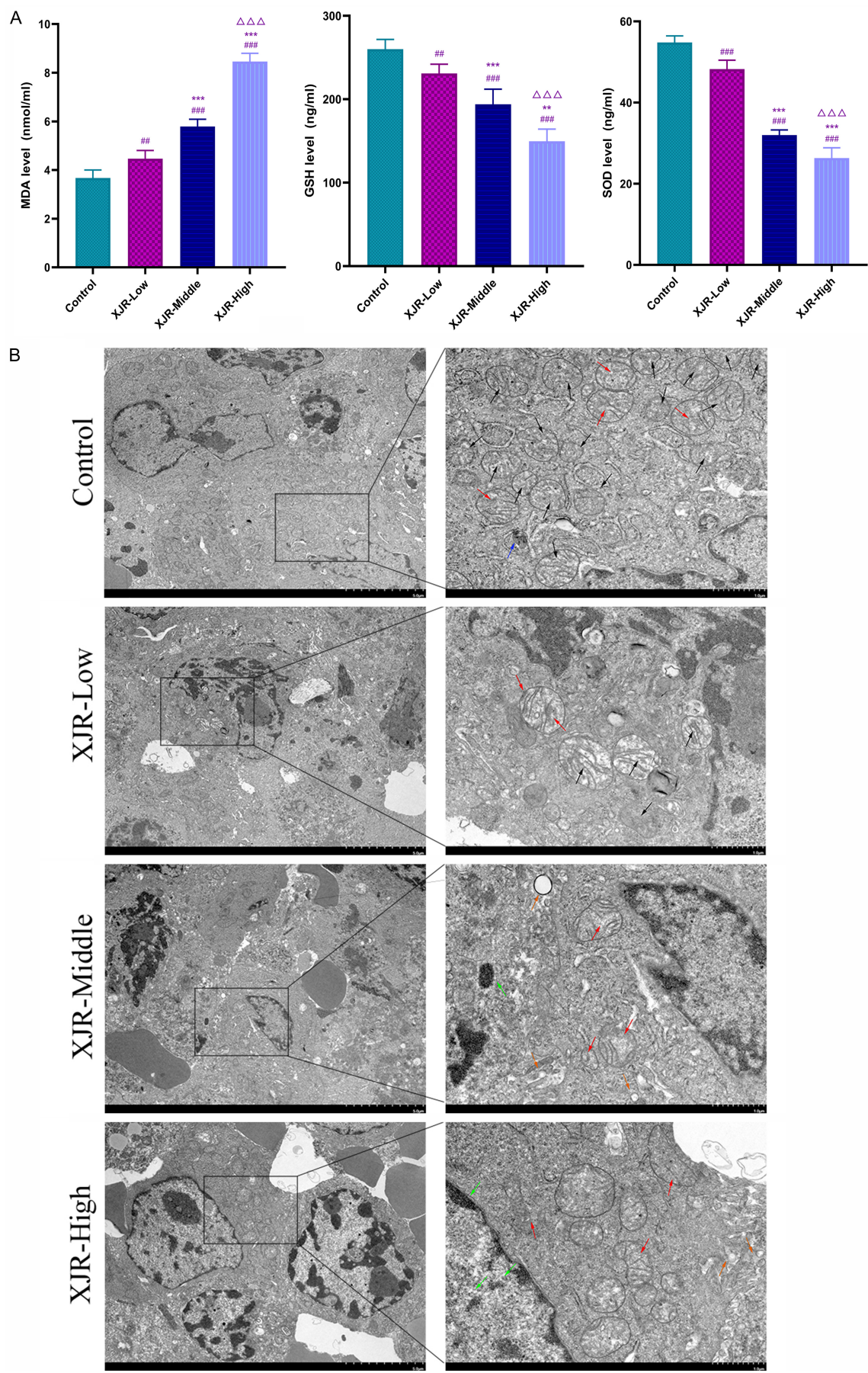


**Figure 2.** XJR promoted progressive oxidative stress to drive ferroptosis in HCC xenograft tumors. A. MDA, GSH and SOD levels in mouse serum detected by ELISA. B. Representative TEM images of HCC xenograft tissues in each group. Left panels: low magnification (×3,000; scale bar, 5 μm); right panels: high magnification (×10,000; scale bar, 1 μm). Notes: Black arrows: normal mitochondria; red arrows: damaged mitochondria; orange arrows: lipid vacuoles; green arrows: iron deposits. MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; TEM, transmission electron microscopy. <sup>##</sup>P < 0.01 and <sup>###</sup>P < 0.001 vs. Control group; <sup>\*\*</sup>P < 0.01 and <sup>\*\*\*</sup>P < 0.001 vs. XJR-Low group; <sup>ΔΔΔ</sup>P < 0.001 vs. XJR-Middle group.

tae rarefaction, alongside sparse autophagic vesicles and tiny lipid vacuoles, demonstrating faint initiation of lipid peroxidation.

In the XJR-Middle group, these lesions were more pronounced. Irregular nuclear indentation and massive peripheral chromatin condensation were evident, accompanied by increased cytoplasmic vacuolation and enlarged intercellular spaces. High-magnification TEM revealed moderately swollen mitochondria with a pale matrix and disrupted cristae. Lipid vacuoles and electron-dense inclusions were more frequently observed in the low-dose group, indicating aggravated intracellular iron overload as well as lipid peroxidation.

Notably, the most severe cellular damage was observed in the XJR-High group. Tumor cells exhibited marked nuclear deformation and diffuse chromatin pyknosis, with extensive cytoplasmic vacuolation and large-scale disruption of intercellular contacts. At ultrastructural level, massive mitochondria were severely swollen and vacuolated, with substantial loss or dissolution of cristae, accompanied by massive accumulation of lipid vacuoles and iron deposits.

Collectively, XJR triggered progressive nuclear injury, mitochondrial damage, mitophagy-related changes, and lipid/iron deposition-like ultrastructural alterations in a dose-dependent manner in vivo. These findings are consistent with enhanced oxidative and ferroptosis-associated cellular injury during XJR-mediated inhibition of HCC progression.

Consistently, IHC analysis of xenograft tumor tissues showed that the staining intensities of Nrf2, GPX4 and xCT decreased with increasing XJR doses (**Figure 3A**). Western blot analysis further demonstrated increased KEAP1 protein expression and decreased Nrf2, GPX4 and xCT expression in the XJR-treated groups compared with the control group (**Figure 3B**). Together, these in vivo findings suggest that XJR modulates the KEAP1/Nrf2 antioxidant pathway,

reduces the expression of the ferroptosis-defense proteins GPX4 and xCT, weakens anti-oxidant defense capacity, and may thereby promote lipid peroxidation in HCC tumor tissue.

## *NF-κB Inhibition enhanced XJR-associated oxidative stress, iron accumulation, and lipid peroxidation in HepG2 cells*

To evaluate the cytotoxic effects of XJR and the NF-κB inhibitor BAY11-7082 in HCC cells, TUNEL assay and Annexin V-FITC/PI flow cytometry were performed to assess apoptosis-associated changes (**Figure 4**). Only weak green fluorescence was detected in the control group, indicating low basal DNA fragmentation. Treatment with XJR or BAY11-7082 alone increased TUNEL positivity compared with the untreated control cells. The combination treatment produced the strongest TUNEL signal and the highest proportion of Annexin V-positive cells, suggesting that NF-κB inhibition enhanced XJR-associated apoptotic cell injury.

ELISA results showed that XJR treatment significantly increased MDA levels and decreased GSH and SOD levels in HepG2 cells compared with the control group. Single administration of BAY11-7082 triggered similar changes in these oxidative biomarkers. Moreover, combined treatment with XJR and BAY11-7082 exerted a synergistic effect, further increasing MDA levels and decreasing GSH and SOD levels (**Figure 5A**).

The fluorescence intensities of DCFH-DA, which reflects total intracellular ROS (**Figure 5B**), FerroOrange, which detects labile ferrous iron (**Figure 5D**), and Liperfluor, which indicates lipid peroxidation (**Figure 5E**), were all elevated after treatment with XJR or BAY11-7082 alone. The combination treatment produced stronger fluorescence signals than either monotherapy. JC-1 staining showed increased green fluorescence, indicating mitochondrial membrane depolarization in both single-treatment groups, whereas combined intervention exacerbated mitochondrial damage to a greater extent (**Figure 5C**).



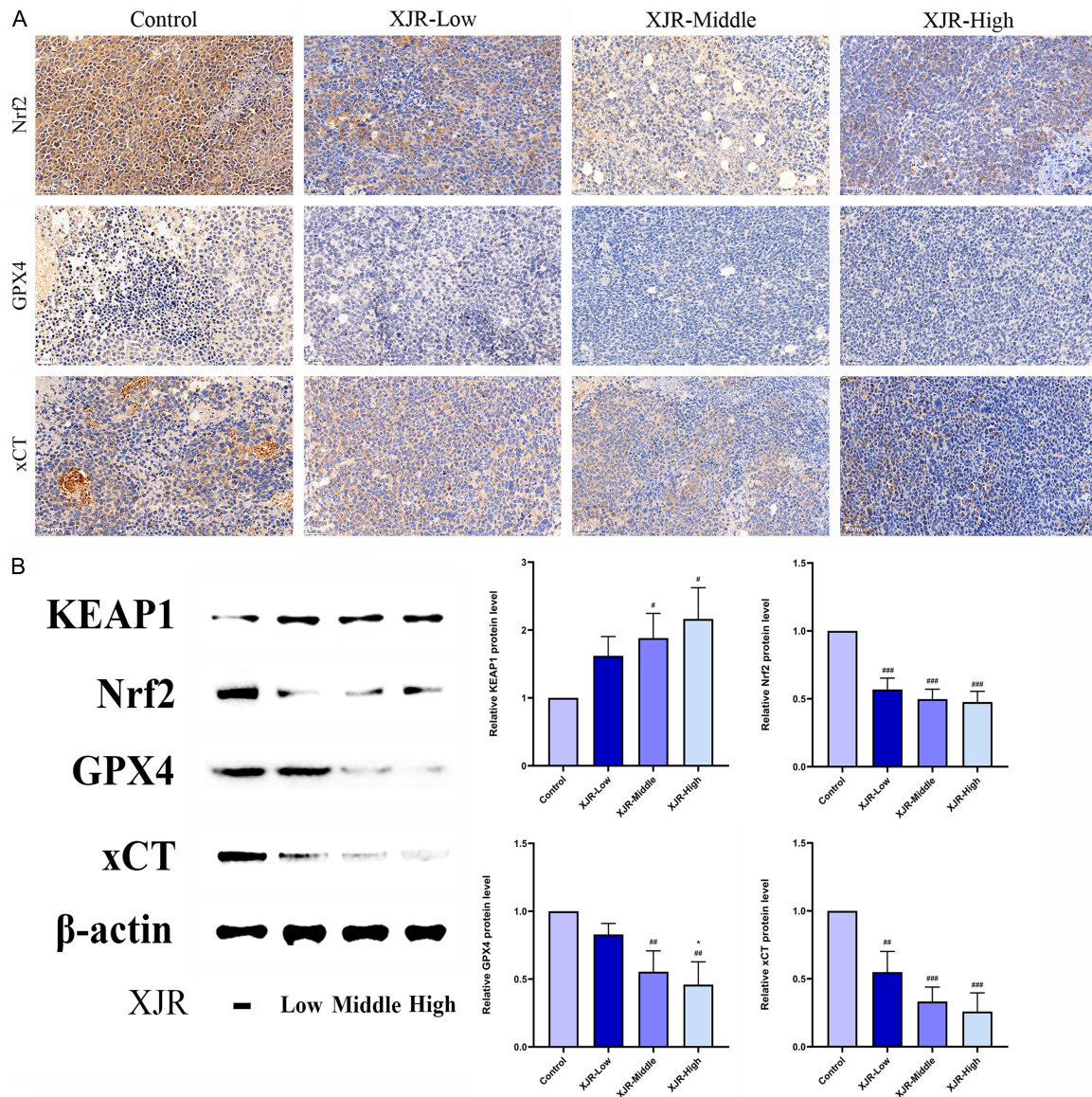


**Figure 3.** XJR disrupted Nrf2-dependent antioxidant defense and facilitated oxidative stress in xenograft tumor. A. Immunohistochemical staining of Nrf2, GPX4 and xCT in tumor tissue; magnification  $\times 200$ . B. Effects of XJR on the protein levels of KEAP1, Nrf2, GPX4 and xCT in tumor tissue of each group, with quantitative analysis. Notes: #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. Control group; \*P < 0.05 vs. XJR-Low group.

Collectively, these findings indicate that pharmacological suppression of NF-κB with BAY11-7082 enhances XJR-associated oxidative stress, labile iron accumulation, lipid peroxidation, and mitochondrial membrane depolarization in HepG2 cells.

*XJR promoted ferroptosis by inhibiting NF-κB-mediated ferroptosis resistance*

As shown in **Figure 6A**, western blot analysis of HepG2 cell lysates demonstrated that either

XJR or the NF-κB inhibitor BAY11-7082 alone reduced the p-NF-κB/NF-κB and p-IκBα/IκBα ratios, increased KEAP1 expression, and concurrently reduced the anti-ferroptosis proteins Nrf2, GPX4 and xCT compared with the control group. These molecular changes were further enhanced by combined treatment with XJR and BAY11-7082.

Consistent with the western blot findings, immunofluorescence analysis further showed that XJR reduced NF-κB p65 nuclear translocation.



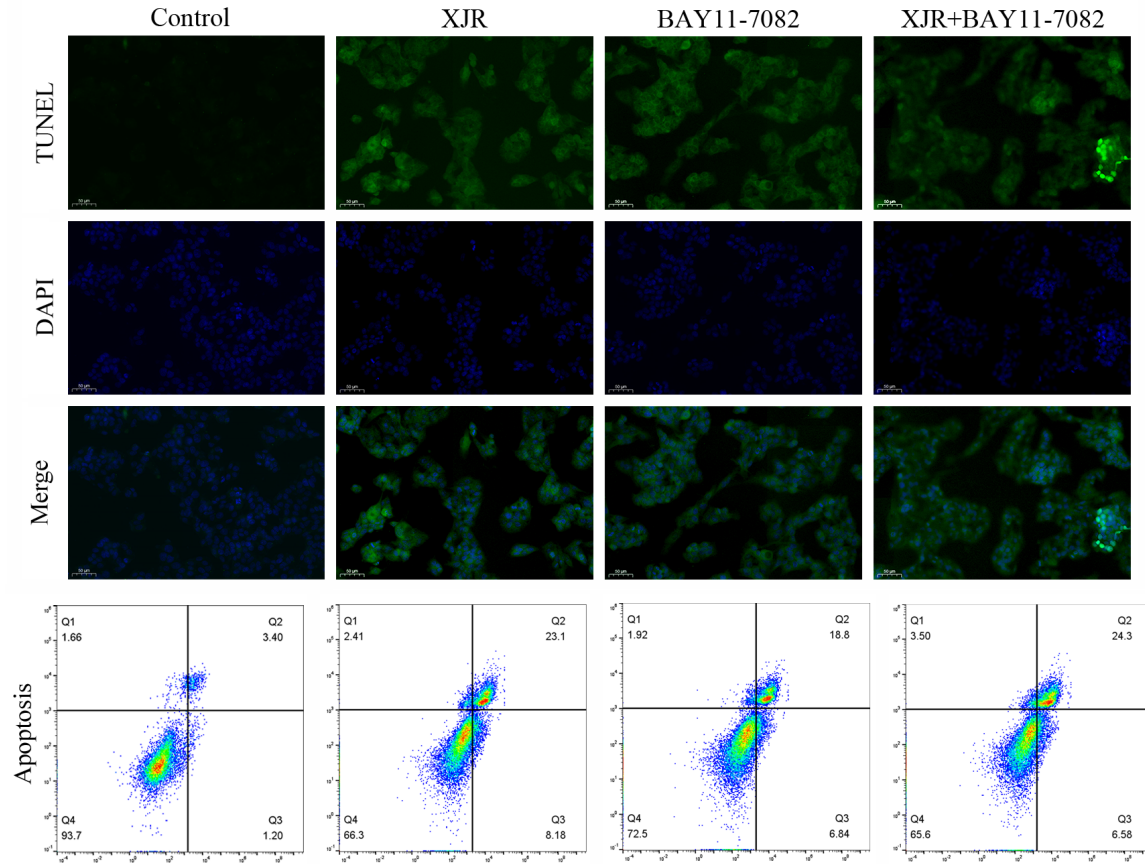


**Figure 4.** XJR combined with BAY11-7082 synergistically induced apoptosis in HepG2 cells. Representative images of TUNEL staining and flow cytometry analysis of apoptosis. Upper panel: Representative TUNEL immunofluorescence images (green: TUNEL-positive apoptotic cells; blue: DAPI-stained nuclei); magnification  $\times 40$ . Lower panel: flow cytometric quantification of apoptosis by Annexin V-FITC/PI/PI staining.

tion, decreased nuclear Nrf2 accumulation, and reduced cytoplasmic GPX4 abundance, and co-administration with BAY11-7082 strengthened these inhibitory effects (**Figure 6B-D**). Collectively, these in vitro findings suggest that XJR suppresses the cytoprotective Nrf2/xCT/GPX4 antioxidant cascade in association with inhibition of NF- $\kappa$ B signaling.

#### *Ferrostatin-1 partially attenuated XJR-induced ferroptosis*

To further verify the involvement of ferroptosis in the anti-HCC effects of XJR, ferroptosis rescue experiments were performed using the ferroptosis inhibitor Ferrostatin-1 (Fer-1).

Western blot results showed that combined treatment with Fer-1 and XJR decreased KEAP1 protein expression and upregulated the protein levels of Nrf2, GPX4 and xCT relative to XJR monotherapy, indicating that Fer-1 supplemen-

tation partially reversed the regulatory effect of XJR (**Figure 7A**). The fluorescence intensities of DCFH-DA (**Figure 7B**), FerroOrange (**Figure 7D**), and Liperfluo (**Figure 7E**) were markedly reduced after incubation with Fer-1, whereas subsequent XJR treatment partially restored these signals. JC-1 staining showed reduced green fluorescence after addition of Fer-1, indicating attenuation of mitochondrial membrane depolarization; however, XJR co-treatment partially reversed this effect (**Figure 7C**).

#### **Discussion**

HCC is a clinically heterogeneous malignancy and remain one of the leading causes of cancer-related death worldwide [13]. Only a small subset of patients with early-stage HCC are eligible for potentially curative treatment, such as surgical resection and liver transplantation. Furthermore, currently available systemic therapies provide limited efficacy for patients with

## XJR induces ferroptosis via NF- $\kappa$ B axis in HCC

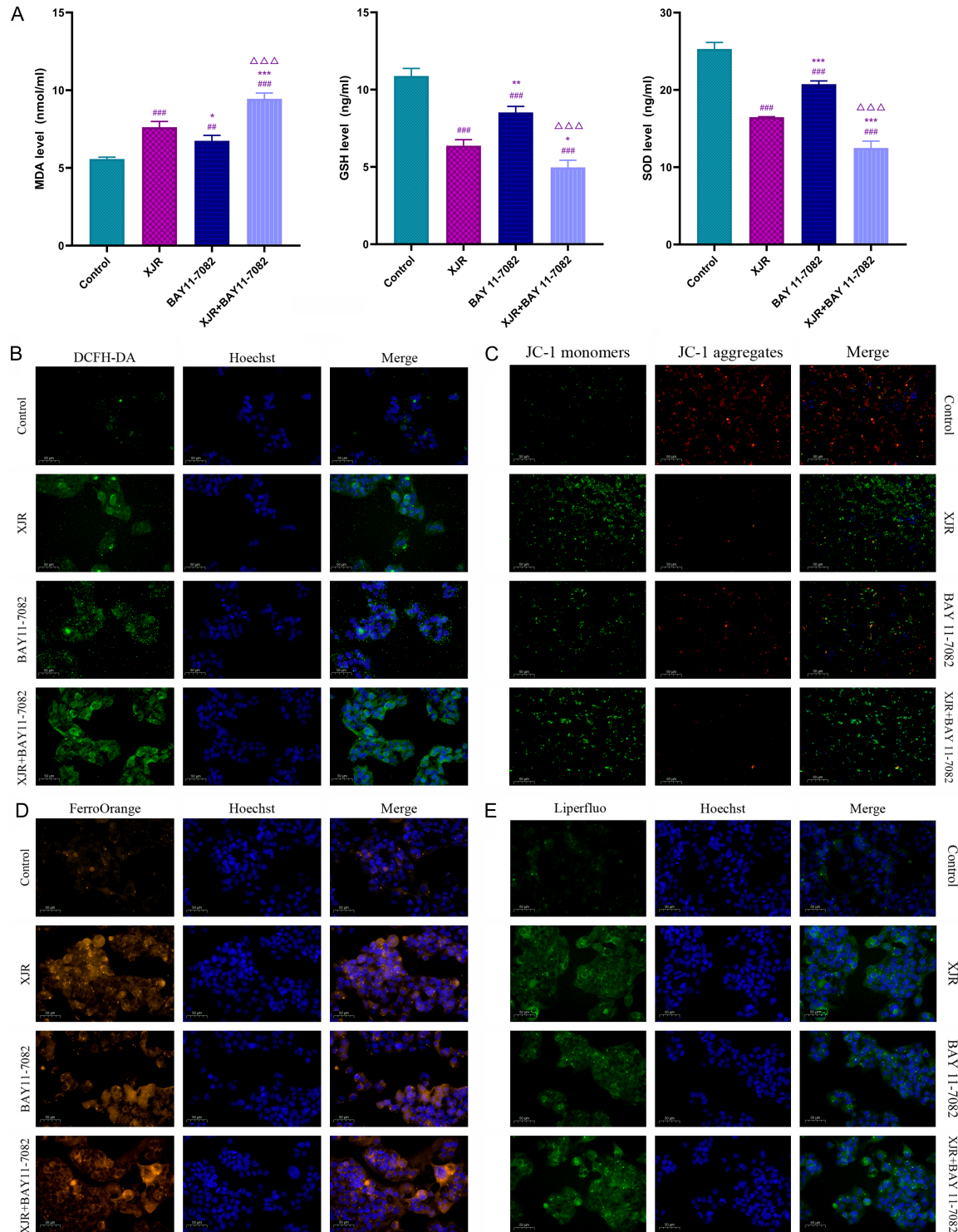


**Figure 5.** NF- $\kappa$ B inhibition enhanced XJR-associated oxidative stress, iron accumulation, and lipid peroxidation in HepG2 cells. A. MDA, GSH, and SOD levels in HepG2 cells detected by ELISA. B. ROS production detected using the fluorescent probes DCFH-DA; magnification  $\times 40$ . C. Changes in MMP in HepG2 cells measured using the JC-1 kit; magnification  $\times 40$ . D. Fe<sup>2+</sup> accumulation examined using the FerroOrange probe; magnification  $\times 40$ . E. LPO in XJR-treated cells detected by Liperfluo staining; magnification  $\times 40$ . Notes: MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; ROS, reactive oxygen species; ELISA, enzyme-linked immunosorbent assay; MMP, mitochondrial membrane potential; LPO: lipid peroxidation. <sup>##</sup>P < 0.01 and <sup>###</sup>P < 0.001 vs. Control group; <sup>\*</sup>P < 0.05, <sup>\*\*</sup>P < 0.01 and <sup>\*\*\*</sup>P < 0.001 vs. XJR group;  <sup>$\Delta\Delta$</sup> P < 0.001 vs. BAY11-7082 group.

# XJR induces ferroptosis via NF-κB axis in HCC

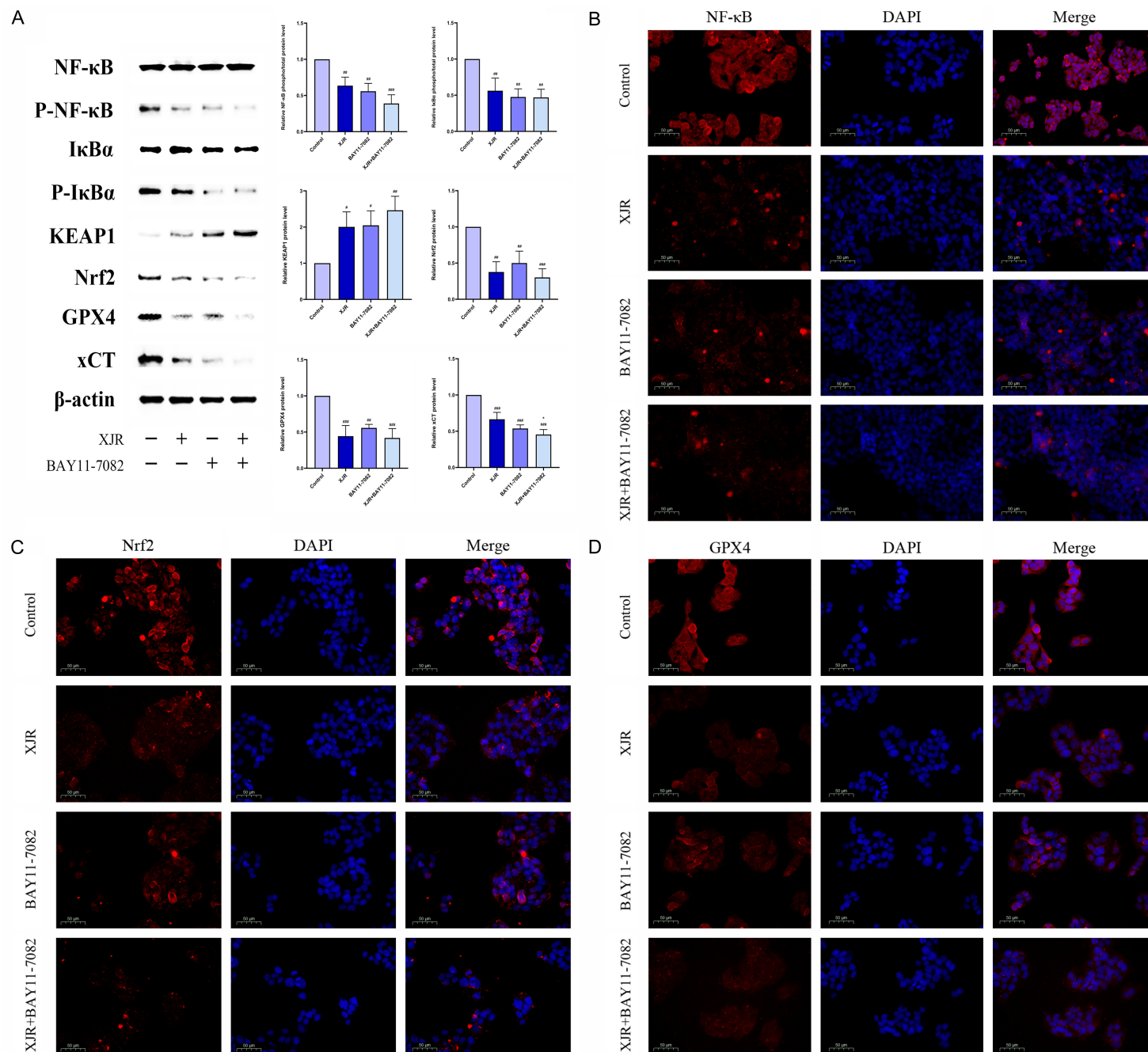




**Figure 6.** XJR and BAY11-7082 synergistically inhibited the NF-κB-Nrf2/xCT/GPX4 cascade in HepG2 cells. A. Effects of XJR on the protein levels of p-NF-κB/NF-κB, p-IκBα/IκBα, KEAP1, Nrf2, GPX4 and xCT in each group, with quantitative analysis. B-D. Immunofluorescence staining of NF-κB, Nrf2 and GPX4; magnification ×40. Notes: #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. Control group; \*P < 0.05 vs. XJR group.

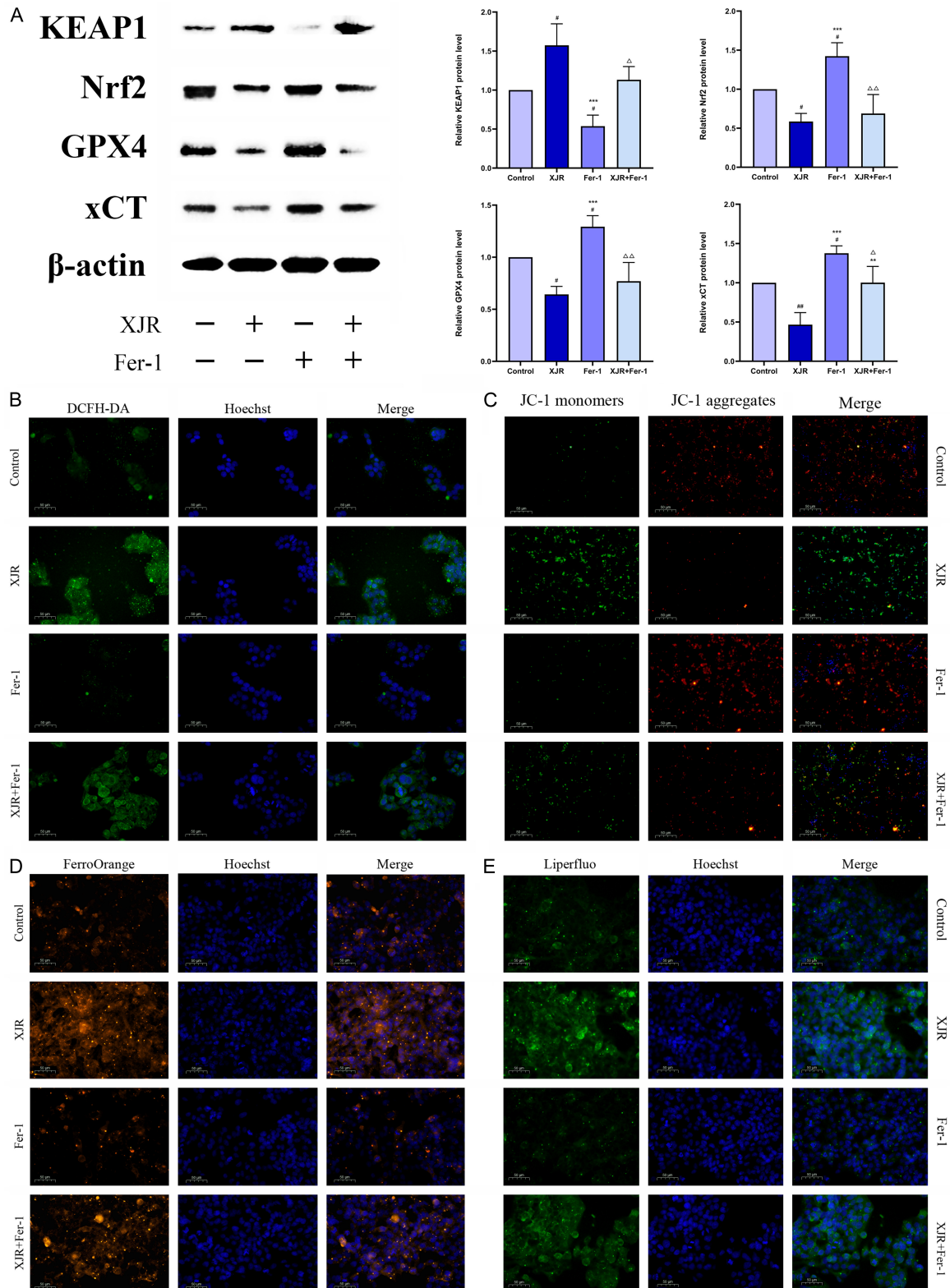


**Figure 7.** XJR counteracted Ferrostatin-1-mediated suppression of ferroptosis in HCC. A. Effects of XJR on the protein levels of KEAP1, Nrf2, GPX4 and xCT in each group, with quantitative analysis. B. ROS production detected using the fluorescent probe DCFH-DA; magnification ×40. C. Changes in MMP in HepG2 cells measured with the JC-1 kit; magnification ×40. D. Fe<sup>2+</sup> accumulation examined using the Ferrorange probe; magnification ×40. E. LPO in XJR-treated cells detected by Liperfluo staining; magnification ×40. Notes: ROS, reactive oxygen species; LPO, lipid peroxidation; MMP, mitochondrial membrane potential. #P < 0.05 and ##P < 0.01 vs. Control group; \*\*\*P < 0.001 vs. XJR group; ΔP < 0.05 and ΔΔP < 0.01 vs. Fer-1 group.

advanced HCC, underscoring the need to develop new therapeutic strategies and candidate agents. XJR has been used in clinical practice as a TCM formula for HCC and has been reported to alleviate clinical symptoms and improve treatment-related outcomes.

This study provides evidence that the anti-HCC activity of XJR is associated with regulation of the NF-κB/ferroptosis axis, supporting further investigation of XJR as a potential adjuvant strategy for HCC. From the perspective of TCM theory, HCC is often interpreted within a framework involving phlegm, blood stasis, toxin, and deficiency. XJR is traditionally used to detoxify, resolve phlegm and blood stasis, and reinforce healthy qi. The present molecular findings offer a possible biological interpretation of these traditional concepts by linking XJR treatment to impaired antioxidant defense and increased ferroptotic susceptibility. We investigated the anti-HCC effects of XJR and its associated molecular mechanism using HepG2 cells and an H22 subcutaneous xenograft model. Although these models differ in species origin and experimental context, their combined use enabled the effects of XJR to be examined in both cellular and in vivo tumor settings. Our results showed that XJR inhibited tumor cell proliferation and suppressed tumor growth in vivo. Mechanistically, the findings suggest that XJR promotes ferroptosis-related oxidative injury, at least partly, through inhibition of NF-κB pathway.

To clarify the involvement of NF-κB in XJR-associated ferroptosis, we used the NF-κB inhibitor BAY11-7082 as a pharmacological validation tool. BAY11-7082 is widely used to investigate NF-κB-dependent biological processes [14, 15]. BAY11-7082 alone reproduced several biological effects observed after XJR treatment, including increased MDA, decreased GSH/SOD, and increased ROS, labile iron, and lipid peroxidation, together with reduced NF-κB pathway activity and decreased Nrf2, xCT, and GPX4 expression. Combined treatment with

XJR and BAY11-7082 further enhanced these changes, suggesting that suppression of NF-κB signaling is closely associated with, and may potentiate, XJR-associated ferroptosis-associated injury. However, additional rescue experiments are needed to determine the extent to which NF-κB inhibition is required for the effects of XJR.

Cumulative evidence indicates that the NF-κB signaling cascade is an indispensable molecular link between chronic inflammation and HCC progression [16, 17]. Unlike transient physiological inflammatory induction, pathological NF-κB activation may be sustained by disrupted protein homeostasis, dysregulated kinase signaling, and impaired negative-feedback mechanisms. Persistent NF-κB activation coordinates a spectrum of oncogenic processes, including tumor-cell proliferation, metabolic remodeling, immune evasion, and multidrug resistance. Furthermore, NF-κB acts as a cytoprotective signaling node under therapeutic stress and may contribute to resistance to radiotherapy, conventional chemotherapy, and ferroptosis-based therapies [18].

Ferroptosis is a distinct form of programmed cell death characterized by iron-dependent lipid peroxidation and excessive ROS accumulation, ultimately leading to loss of membrane integrity and cell death [19]. Multiple signaling pathways and biological processes cooperatively regulate the initiation and execution of ferroptosis. Compared with normal cells, HCC cells may be more susceptible to ferroptotic damage owing to their elevated basal ROS levels and distinctive metabolic signatures, such as enhanced iron dependency [20]. Accumulating preclinical evidence supports an important role for ferroptosis in tumor progression, particularly in HCC [21]. Pharmacological induction of ferroptosis can inhibit HCC cell proliferation and tumor progression and may enhance tumor sensitivity to immunotherapy and antitumor immune responses. Accordingly, pharmacological induction of ferroptosis has

emerged as a promising therapeutic modality for HCC treatment [22]. TCM exerts anti-HCC effects by inducing ferroptosis in malignant hepatocytes through multi-component and multi-target mechanisms. Such pleiotropic effects may provide valuable clues for the development of novel ferroptosis-inducing agents [23].

A limitation of the present study lies in the difficulty in completely discriminating ferroptosis from apoptosis based solely on TUNEL and Annexin V/PI assays, because these two assays primarily detect DNA fragmentation and changes in plasma membrane integrity, respectively, and are not specific for apoptosis. Although multiple ferroptosis-related findings indicated that ferroptosis contributed substantially to the cell death induced by XJR, concurrent activation of apoptosis cannot be excluded. Crosstalk between ferroptosis and apoptosis may exert synergistic antiproliferative effects of XJR in HepG2 and H22 cells. Further pharmacological intervention using specific inhibitors of ferroptosis and apoptosis are needed to determine their respective contributions to XJR-mediated tumor suppression.

In addition, the specific active components of XJR responsible for NF-κB inhibition and ferroptosis promotion remain unidentified. Although BAY11-7082 provided pharmacological support for the involvement of NF-κB signaling, pathway-specific rescue experiments, such as NF-κB activation, p65 overexpression, or restoration of Nrf2/GPX4/xCT activity, were not performed. Moreover, the *in vivo* immunomodulatory effects of XJR were also not fully investigated. Future studies should identify the bioactive components of XJR, perform mechanistic rescue experiments, and examine the crosstalk among ferroptosis, inflammation, and anti-tumor immunity.

In summary, this study describes a potential anti-HCC mechanism of XJR. XJR inhibited canonical NF-κB signaling, increased KEAP1 expression, reduced Nrf2 activity and the expression of xCT and GPX4, weakened antioxidant defenses, and increased iron accumulation and lipid peroxidation. These changes were associated with ferroptosis-associated cellular injury and suppression of HCC progression. These findings provide preclinical evidence supporting further investigation of XJR as a

ferroptosis-modulating agent for HCC treatment.

## Conclusion

XJR suppressed HCC growth *in vivo* and inhibited HCC cell proliferation while inducing ferroptosis-associated cell death *in vitro*. These effects are associated with inhibition of the NF-κB signaling axis, suppression of Nrf2-dependent antioxidant defenses, downregulation of GPX4 and xCT, and increased ferroptosis-related oxidative injury. Targeting NF-κB-dependent antioxidant defense may represent an important mechanism underlying the anti-HCC activity of XJR.

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## Disclosure of conflict of interest

None.

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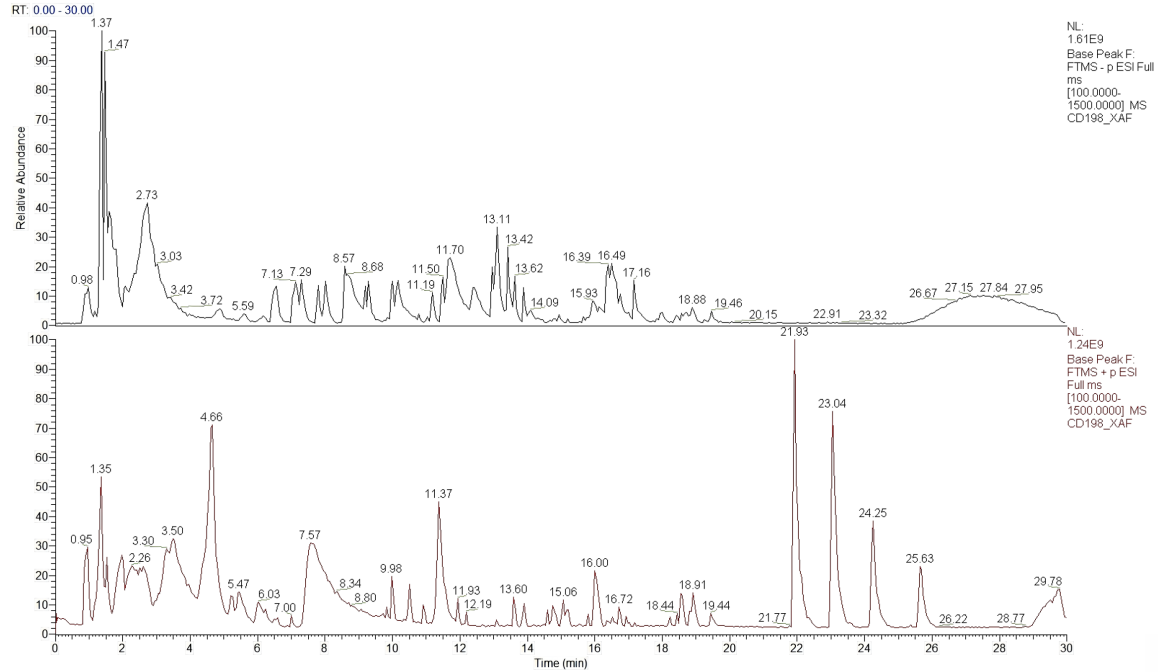
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# XJR induces ferroptosis via NF-κB axis in HCC



**Supplementary Figure 1.** Total ion flow chromatogram of positive and negative ions.

**Supplementary Table 1.** Formulation of Xiaoi Jiedu recipe (XJR)

| Pinyin name     | English name                          | Latin Pharmaceutical Name                       | Doses | Origin                                                                                                                                                             |
|-----------------|---------------------------------------|-------------------------------------------------|-------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Baihuasheshecao | Spreading Hedyotis Herb               | Herba Hedyotidis                                | 20 g  | The dried whole herb of <i>Scleromitron diffusum</i> (Willd.) R.J.Wang.                                                                                            |
| Shancigu        | Appendiculate<br>Crematrae Pseudobulb | Crematrae Pseudobulbus<br>Pleiones Pseudobulbus | 10 g  | The dried pseudobulb of <i>Crematra appendiculata</i> (D. Don) Makino.                                                                                             |
| Bayuezha        | Fruit of Fiveleaf Akebia              | Fructus Akebiae                                 | 12 g  | The Dried fruit of <i>Akebia quinata</i> (Thunb. ex Houtt.) Decne.                                                                                                 |
| Jiangcan        | Stiff Silkworm                        | Bombyx Batryticatus                             | 10 g  | The dried body of the larvae of <i>Bombyx mori</i> L that has been killed due to infection (or inoculation) of the fungus <i>Beauveria bassiana</i> (Bals.) Vuill. |
| Wugong          | Centipede                             | Scolopendra                                     | 3 g   | The dried body of <i>Scolopendra subspinipes mutilans</i> L. Koch                                                                                                  |
| Taizisheng      | Heterophylly Falsestarwort<br>Root    | Pseudostellariae Radix                          | 15 g  | The dried tuberous root of <i>Pseudostellaria heterophylla</i> (Miq.) Pax et Hoffm.                                                                                |
| Maidong         | Dwarf Lilyturf Root Tuber             | Ophiopogonis Radix                              | 12 g  | The dried tuberous root of <i>Ophiopogon japonicas</i> (Thunb.) Ker-Gawl.                                                                                          |