Gossypin induces apoptosis and autophagy via the MAPK/JNK pathway in HT-29 human colorectal cancer cells

JUN-MO MOON[1], SANG-WOO LEE[1], YUN-SEO JANG[1], SU-A LEE[1], SOO-HYUN JUNG[1], SANG-KI KIM[1,2], BYUNG-KWON PARK[1,2], YOUNG-SEOK PARK[1,2], BYEONG-SOO KIM[1,2], MYEON-SIK YANG[1] and JI-YOUN JUNG[1-3]

[1]Department of Companion and Laboratory Animal Science, Kongju National University, Yesan, Chungcheongnam 32439, Republic of Korea; [2]Research Institute for Natural Products, Kongju National University, Yesan, Chungcheongnam 32439, Republic of Korea; [3]Research Center of Crop Breeding for Omics and Artificial Intelligence, Kongju National University, Yesan, Chungcheongnam 32439, Republic of Korea

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Abstract. Gossypin, a flavone found in *Hibiscus vitifolius*, exhibits antioxidant, antidiabetic, anti-inflammatory and anticancer effects. The present study investigated the potential of gossypin to induce apoptosis and autophagy in HT-29 human colorectal cancer (CRC) cells, and assessed its association with the MAPK/JNK pathway. Cell viability assays, DAPI staining, flow cytometry, acridine orange staining, western blotting, hematoxylin and eosin staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and immunohistochemistry were performed. The results revealed an increased number of apoptotic bodies, higher apoptosis rates and enhanced autophagy in gossypin-treated HT-29 cells. To investigate autophagy during cell death, the effects of the early autophagy inhibitor 3-methyladenine (3-MA) and the late autophagy inhibitor hydroxychloroquine on cell viability and the expression of apoptosis-related proteins were assessed. Significant increases in cell viability were observed following 3-methyladenine pretreatment, as well as a decrease in the expression levels of Bcl-2 and an increase in Bax. The analysis of MAPK pathway proteins following treatment with gossypin revealed that the levels of phosphorylated (p-)JNK and p-p38 were significantly increased in a concentration-dependent manner. The JNK inhibitor SP600125 was used to confirm the role of the JNK pathway in gossypin-induced apoptosis and autophagy. Moreover, gossypin reduced the volume of HT-29 tumors in mice, and western blotting indicated the induction of apoptosis and autophagy in these tumors *in vivo*. Finally, TUNEL and immunohistochemistry experiments confirmed the induction of apoptosis and p-JNK upregulation in these tumors *in vivo*. In conclusion, the present study suggested that gossypin may induce MAPK/JNK-mediated apoptosis and autophagy in HT-29 CRC cells, highlighting the potential of gossypin as an anticancer agent.

Correspondence to: Professor Ji-Youn Jung, Department of Companion and Laboratory Animal Science, Kongju National University, 54 Daehak-ro, Yesan-eup, Yesan, Chungcheongnam 32439, Republic of Korea
E-mail: wangza@kongju.ac.kr

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Introduction

Cancer is one of the leading causes of death worldwide, accounting for a considerable number of mortalities among various human diseases (1). Colorectal cancer (CRC) accounts for ~10% of all cancer cases and ranks as the second leading cause of cancer-related deaths worldwide (2). While CRC predominantly affects individuals aged ≥50 years, the incidence and mortality rates among younger adults <50 years of age have steadily increased over the past 25 years (3). According to the World Health Organization (WHO), >1.9 million new cases of CRC were diagnosed globally in 2020, with >930,000 deaths attributed to CRC in the same year. The WHO estimates that by 2040, the incidence of CRC will increase by 63% to 3.2 million cases annually, with deaths rising by 73% to 1.6 million per year (2). In the United States, cancer is the second leading cause of death, with CRC ranking as the third most prevalent type of cancer (4). Surgical resection remains the primary treatment option for CRC (5); however, when CRC is not detected at an early stage or optimal surgical procedures are not feasible, patient prognosis often worsens. Moreover, resection of primary or metastatic tumors can sometimes promote tumor recurrence (6). In addition, chemical drugs, such as 5-fluorouracil (5-FU) and capecitabine, which are frequently employed as anticancer treatments for CRC, often lead to side effects including diarrhea, nausea and vomiting, and can lead to cardiac toxicity. Furthermore, patients with genetic disorders, such as dihydropyrimidine dehydrogenase deficiency, are at an increased risk of neurotoxicity following the administration of 5-FU and capecitabine (7,8). Consequently, research into natural compounds derived from plants or fruits, which

exhibit low toxicity *in vivo*, has gained momentum as a promising approach for treating chronic diseases such as cancer (9).

Gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside), a flavone found in *Hibiscus vitifolius*, is a secondary metabolite that contributes to plant pigmentation. Traditionally, gossypin has been recognized for its anxiolytic, antidiabetic, antioxidant, anti-inflammatory and anticancer properties (10-15). In addition, recent studies have also explored its potential cardioprotective effects with regard to ischemic heart disease (16,17).

Apoptosis is characterized by morphological changes, such as chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage. During apoptosis, cells break down into small, membrane-bound apoptotic bodies, which are removed through phagocytosis without triggering inflammatory responses, which is a major advantage of this process (18). Apoptosis is a self-regulatory mechanism that eliminates abnormal cells, such as those with DNA damage (19). In cancer, inducing apoptosis can selectively target and remove cancer cells without affecting surrounding healthy cells.

Autophagy is a physiological mechanism wherein cellular organelles are sequestered within autophagosomes, which then fuse with lysosomes for degradation. This process occurs in all cells and is regulated in response to stress or nutrient deprivation (20). Autophagy is mediated by various autophagy-related (ATG) proteins recruited to the cell membrane, and LC3 and Beclin 1 serve key roles in autophagosome formation (21,22). As well as in cancer, autophagy is an important process in aging, autoimmune diseases, Crohn's disease and rheumatoid arthritis. While generally considered a survival mechanism under conditions of cellular stress, previous studies have suggested that the regulation of autophagy in tumors, combined with anticancer drugs, can enhance tumor cell death (23,24).

The MAPK pathway is involved in cell survival, proliferation and metastasis (25). The three main MAPK signaling pathways include the ERK, JNK and p38 kinase pathways (26). ERK is involved in cell proliferation, apoptosis and cytoskeletal remodeling, JNK regulates cell proliferation and death through various targets (25,26), and p38 serves a central role in cell cycle progression, metastasis and differentiation (27).

The present study used *in vitro* and *in vivo* experiments to investigate whether gossypin induces apoptosis and autophagy in HT-29 CRC cells, and to determine whether these processes are mediated via the MAPK/JNK pathway.

Materials and methods

Reagents and antibodies. RPMI-1640 medium used for cell culture was purchased from Welgene, Inc. Fetal bovine serum (FBS) and penicillin were obtained from Gibco; Thermo Fisher Scientific, Inc. Gossypin (Fig. 1A; purity confirmed by HPLC: 98.5%) was purchased from Apollo Scientific. General reagents, dimethyl sulfoxide (DMSO), MTT, DAPI and the JNK inhibitor SP600125 were procured from MilliporeSigma. The FITC-Annexin-V detection kit (cat. no. 556547) was procured from BD Pharmingen; BD Biosciences. Primary antibodies against Bax (rabbit; 1:1,000; cat. no. 2772), Bcl-2 (rabbit; 1:1,000; cat. no. 4223), PARP (rabbit; 1:1,000; cat. no. 9542), JNK (rabbit; 1:1,000; cat. no. 9252), phosphorylated (p-)JNK (rabbit, 1:1,000, cat. no. 4668), ERK (rabbit; 1:1,000; cat. no. 9102), p-ERK (rabbit; 1:1,000; cat. no. 9101), p38 (rabbit; 1:1,000; cat. no. 9212), p-p38 (rabbit; 1:1,000; cat. no. 9211), mTOR (rabbit; 1:1,1000; cat. no. 2983), p-mTOR (rabbit; 1:1,000; cat. no. 2971), Beclin 1 (rabbit; 1:1,000; cat. no. 3738), and LC3 (rabbit; 1:1,000; cat. no. 2775), and the HRP-conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. 7074), were obtained from Cell Signaling Technology, Inc. Primary anti-β-actin (mouse; 1:1,000; cat. no. sc-47778) and HRP-conjugated secondary mouse IgG (1:2,000; cat. no. sc-516102) antibodies were purchased from Santa Cruz Biotechnology, Inc. Hydroxychloroquine (HCQ) and 3-methyladenine (3-MA) were acquired from Selleck Chemicals.

Cell culture and treatment. The human CRC cell line HT-29 (cat. no. 30038) was obtained from the Korean Cell Line Bank; Korean Cell Line Research Foundation. Cells were cultured in RPMI-1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin/neomycin at 37˚C in an atmosphere containing 5% CO_2. When the bottom surface of the culture flask reached 80-90% confluence, the cells were washed with PBS and passaged using a cell scraper. The medium was replaced every 2-3 days. Gossypin was dissolved in DMSO at concentrations of 30, 60, 90, 120 and 150 μM, and stored at -20˚C. HT-29 cells were treated with gossypin at 37˚C in 5% CO_2 for 24 h. For pretreatment of HT-29 cells, the autophagy inhibitors 3-MA (2 mM) and HCQ (20 μM) were dissolved in the medium and the cells were pretreated at 37˚C and 5% CO_2 for 2 h. In addition, the JNK inhibitor SP600125 (10 μM) was dissolved in DMSO, added to the medium and used to pretreat HT-29 cells at 37˚C and 5% CO_2 for 2 h.

MTT assay. The MTT assay was conducted to test the inhibitory effects of gossypin on HT-29 cell viability. HT-29 cells were seeded into a 96-well plate at a density of $2x10^4$ cells/ml and incubated for 24 h. Thereafter, the cells were treated with 0, 30, 60, 90, 120 and 150 μM gossypin and incubated at 37˚C in the presence of 5% CO_2 for 24 h. After treatment, 40 μl MTT reagent was added to each well, and the plate was incubated at 37˚C in 5% CO_2 for 2 h. Subsequently, the MTT solution was removed, and 100 μl DMSO was added to each well to dissolve formazan crystals. Absorbance was measured at 595 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

DAPI staining. DAPI staining was performed to observe apoptosis-associated morphological changes in the nuclei of gossypin-treated HT-29 cells. HT-29 cells were seeded in a 60-mm dish at a density of $1x10^5$ cells/ml and incubated at 37˚C in 5% CO_2 for 24 h. Cells were then treated with 0, 60 and 120 μM gossypin and incubated for another 24 h. Thereafter, the dishes were washed with PBS, fixed in 4% formaldehyde at room temperature for 15 min and washed again using PBS. DAPI solution was then added (2 ml) and the cells were observed using a fluorescence microscope (Zeiss AG).

Flow cytometry. Annexin V/propidium iodide (PI) staining was conducted to quantitatively analyze gossypin-induced apoptosis in HT-29 cells. HT-29 cells were treated with 0, 60 and 120 μM gossypin at 37˚C in 5% CO_2 for 24 h. Cells were then washed with PBS and harvested using a cell scraper, followed by centrifugation at 260 x g for 5 min at 4˚C.

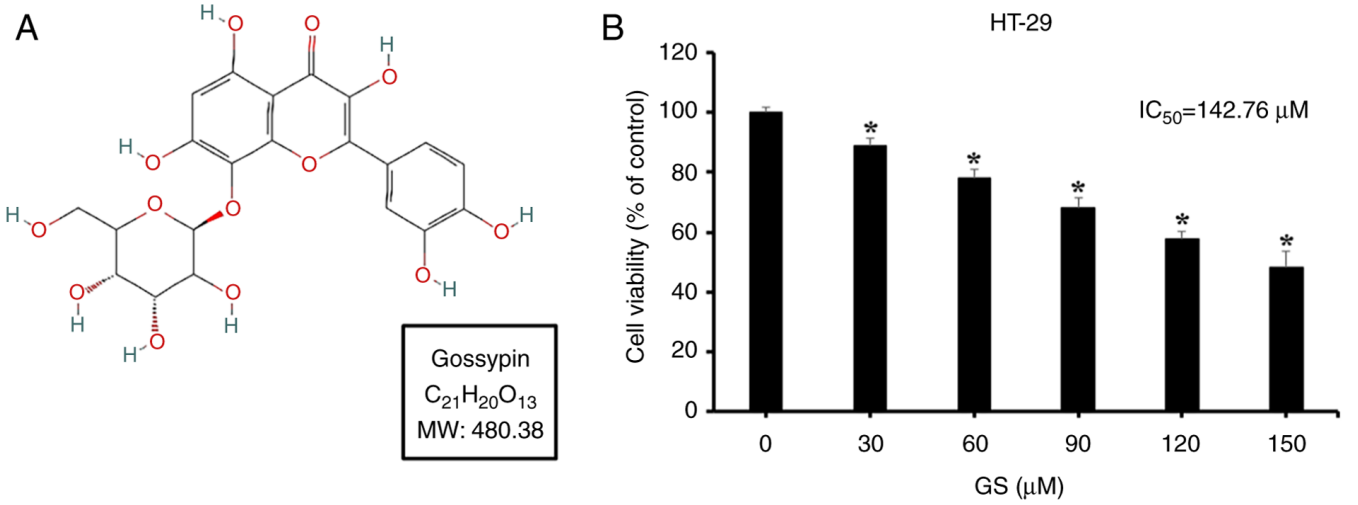

Figure 1. Effects of GS on the viability of HT-29 human colorectal cancer cells. (A) Chemical structure of GS (PubChem identifier: 45933927; https://pubchem.ncbi.nlm.nih.gov/compound/45933927). (B) HT-29 were treated with various concentrations of GS for 24 h. The inhibition of cell viability was measured using the MTT assay. Data are presented as the mean and standard deviation of three experimental repeats. *P<0.05 vs. control group. GS, gossypin; IC_{50}, half maximal inhibitory concentration.

The cell pellet was resuspended in 1X Annexin V binding buffer at a density of $1x10^6$ cells/ml, and the cells were then treated with FITC-conjugated Annexin V and PI (binding buffer:FITC-conjugated Annexin V/PI, 20:1, v/v) in the dark at room temperature for 15 min. Subsequently, the samples were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences) with BD FACSuite™ software version 1.0.6 (BD Biosciences).

Western blotting. Western blotting was performed to analyze the protein expression levels in gossypin-treated HT-29 cells. HT-29 cells were cultured in a 75T flask at 37˚C in 5% CO_2 for 24 h and then treated with gossypin at concentrations of 0, 60 and 120 μM at 37˚C in 5% CO_2 for 24 h. Cells were then harvested using a cell scraper and centrifuged at 260 x g for 5 min at 4˚C. The cell pellet was lysed using cell lysis buffer (PRO-PREP™ Protein Extraction Solution; Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C for 20 min. The lysate was centrifuged at 15,920 x g for 5 min, and the supernatant was collected. The protein concentration was measured using a Bradford protein assay. Protein samples (60 μg/lane) were loaded and separated by SDS-polyacrylamide gel electrophoresis on 12% gels and were transferred to nitrocellulose membranes. Membranes were blocked using 5% skim milk-TBS-Tween 20 (20 mM Tris·HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20) at room temperature for 2 h, followed by overnight incubation with primary antibodies in 5% skim milk-TBST at 4˚C. Membranes were then incubated with secondary antibodies in 5% skim milk-TBST at room temperature for 2 h, the protein bands were visualized using ECL detection reagents (Pierce; Thermo Fisher Scientific, Inc.) and band densities were analyzed using ImageJ Launcher software version 1.52 (National Institutes of Health).

Acridine orange staining. To assess whether gossypin induces autophagy in HT-29 cells, acridine orange staining was performed. After culturing HT-29 cells in a 75T flask until they reached 80-90% confluence, cells were seeded at $1x10^5$ cells/ml in 60-mm dishes and incubated for 24 h at 37˚C in 5% CO_2. The medium was then replaced, and the cells were incubated with gossypin at 0, 60 and 120 μM for 24 h at 37˚C in 5% CO_2. After incubation, the cells were washed with PBS and fixed in 4% paraformaldehyde solution at room temperature for 15 min. Subsequently, the cells were stained using 2 ml acridine orange stain solution (Thermo Fisher Scientific, Inc.) at room temperature for 12 min and were observed using a fluorescence microscope (Zeiss AG).

Xenograft model. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Kongju National University (approval no. IACUC-KNU_2024-07; Yesan, South Korea). A total of 10 BALB/c nude female mice (weight, 18-22 g; age, 4 weeks) were purchased from Nara Biotech Co., Ltd. The mice were housed under a 12-h light/dark cycle at 23±3˚C and 50±10% humidity. Food and water supplies were restricted for 2 h before and after administration; however, during the remaining time, food and water were available *ad libitum*. The humane endpoints were as follows: Tumor volume reached 10% of body weight, or if the mouse exhibited signs of significant pain and distress. No mice met these humane endpoints in the *in vivo* experiment and were humanely euthanized at the conclusion of the planned study. For the xenograft model, HT-29 cells ($1x10^7$ cells/ml) suspended in RPMI-1641 with 50% FBS were subcutaneously injected into both shoulders of the mice. After the tumors stabilized, gossypin (100 mg/kg, n=5) or vehicle control (0 mg/kg gossypin, PBS 10 ml/kg, n=5) was orally administered five times per week for 4 weeks. Tumor volumes were measured every 3 days using Vernier calipers (Mitutoyo Corporation) and were calculated using the following formula: Tumor volume (mm^3)=0.5 x [width (mm)]^2 x [length (mm)]. At the end of the experiment, mice were euthanized using CO_2 gas (30% vol/min for 3 min). Mice were exposed to CO_2, and their movement and breathing were observed to confirm that both had stopped. Afterward, the heartbeat was confirmed to have stopped, and CO_2 exposure was halted. Euthanasia

was verified by confirming that mice did not recover within 10 min. Euthanized mice were autopsied and the tumors were weighed.

Hematoxylin and eosin staining. The murine liver and kidneys tissue were fixed in 10% formaldehyde for 24 h at room temperature, embedded in paraffin and sectioned into 5-μm slices. Sections were deparaffinized using xylene, hydrated with ethanol, and were then stained with hematoxylin for 5 min and with eosin for 30 sec at room temperature. The tissues were observed using an optical microscope (Olympus Corporation).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The TUNEL assay was conducted using a TUNEL Apoptosis Detection Kit (cat. no. HY-K1091; MedChemExpress). Tumor tissues were fixed in 10% formaldehyde for 24 h at room temperature, embedded in paraffin and sectioned into 4-μm slices. Tumor tissue sections were deparaffinized using xylene and rehydrated using ethanol. After washing with PBS, the sections were treated with proteinase K (20 μg/ml, 100 μl per slide) for 30 min at room temperature. Endogenous peroxidase activity was inactivated using 0.3% hydrogen peroxide at room temperature for 30 min, followed by incubation with equilibration buffer and a mixture of biotinylated Nucl. Mix and rTdT at 37˚C for 1 h. Streptavidin HRP was then applied to each slide, followed by incubation at 37˚C for 30 min. After washing with PBS, the slides were stained with DAB solution at room temperature for 30 min, counterstained with methyl green at room temperature for 5 min, mounted and observed using an optical microscope (Olympus Corporation).

Immunohistochemistry. Immunohistochemistry was performed to analyze the expression of apoptosis-related proteins in tumor tissues. Tumor tissues were fixed in 10% formaldehyde for 24 h at room temperature, embedded in paraffin and sectioned into 4-μm slices. Tumor tissue sections were deparaffinized using xylene and rehydrated using ethanol. Antigen retrieval was conducted by immersing the slides in sodium citrate buffer (pH 6.0) and placing them in a water bath at 97˚C for 20 min, and then the slides were transferred to distilled water and allowed to cool at room temperature for 20 min. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. After washing with PBS, blocking was performed using 5% BSA (MP Biomedicals)-TBST at 37˚C for 1 h. Subsequently, the sections were incubated with primary antibodies against p-JNK (1:100 in 5% skim milk-TBST; cat. no. 4668; Cell Signaling Technology, Inc.) overnight at 4˚C. After washing with PBS, a HRP-conjugated secondary antibody (cat. no. 8114; Cell Signaling Technology, Inc.) was applied at room temperature for 2 h, and DAB (cat. no. 8059; Cell Signaling Technology, Inc.) staining was performed. Slides were counterstained with methyl green at room temperature for 5 min, mounted and observed using an optical microscope (Olympus Corporation).

Statistical analysis. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Comparisons among groups were performed using one-way ANOVA followed by Dunnett's or Tukey's test. Differences between two groups were analyzed using unpaired Student's t-test. Statistical analysis was performed using SPSS Statistics Version 27 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of gossypin on the viability of HT-29 CRC cells. To evaluate the effects of gossypin on cell viability, an MTT assay was conducted using HT-29 cells. The cells were divided into six groups and treated with gossypin concentrations of 0, 30, 60, 90, 120 and 150 μM for 24 h, Compared with the control group (0 μM), cell viability decreased in a concentration-dependent manner to 88.8, 78.2, 68.1, 57.5 and 48.0%, respectively, with a statistically significant difference observed starting at 30 μM (Fig. 1B). Based on the MTT assay results, concentrations of 60 and 120 μM were selected for subsequent experiments as moderate and high doses, respectively.

Induction of apoptosis by gossypin in HT-29 CRC cells. To determine whether the reduction in cell viability by gossypin was caused by apoptosis, DAPI staining was performed to observe morphological changes in cells. Apoptotic cells showed nuclear condensation and formation of apoptotic bodies, which were quantified. The proportion of apoptotic cells increased in a concentration-dependent manner to 0.8, 3.1 and 7.7%, respectively (Fig. 2A). To determine changes in the rate of apoptosis, Annexin V and PI were used for double staining, followed by flow cytometric analysis. The combined ratio of Annexin V-positive regions (upper-right and lower-right quadrants) showed a concentration-dependent increase, with values of 27.0, 36.0 and 51.3% across the respective concentration groups (Fig. 2B). Additionally, western blotting was performed to examine the expression levels of apoptosis-related proteins. The results indicated that, in HT-29 cells, increasing concentrations of gossypin led to enhanced cleavage of PARP, which contributes to DNA repair, an increase in the pro-apoptotic protein Bax, and a decrease in the anti-apoptotic protein Bcl-2 (Fig. 2C).

Induction of autophagy by gossypin in HT-29 CRC cells. To investigate whether gossypin induced autophagy, acridine orange staining was performed to detect acidic vesicular organelles, a characteristic of autophagy. The results showed an increase in autophagic vacuole-positive cells with increasing gossypin concentrations (Fig. 3A). Western blotting was also performed to examine the expression levels of autophagy-associated proteins. The results showed decreased expression of p-mTOR, an inhibitor of autophagosome formation, and increased levels of Beclin 1 and LC3-II, which are proteins critical for autophagosome formation (Fig. 3B).

Effects of autophagy inhibition on gossypin-induced apoptosis. To investigate the induction and inhibition of apoptosis when gossypin-induced autophagy was suppressed in HT-29 cells, the cells were pretreated with an early-stage autophagy inhibitor 3-MA and a late-stage autophagy inhibitor HCQ, and cell viability was assessed using the MTT assay for gossypin. Subsequently, cell viability, and the expression levels of

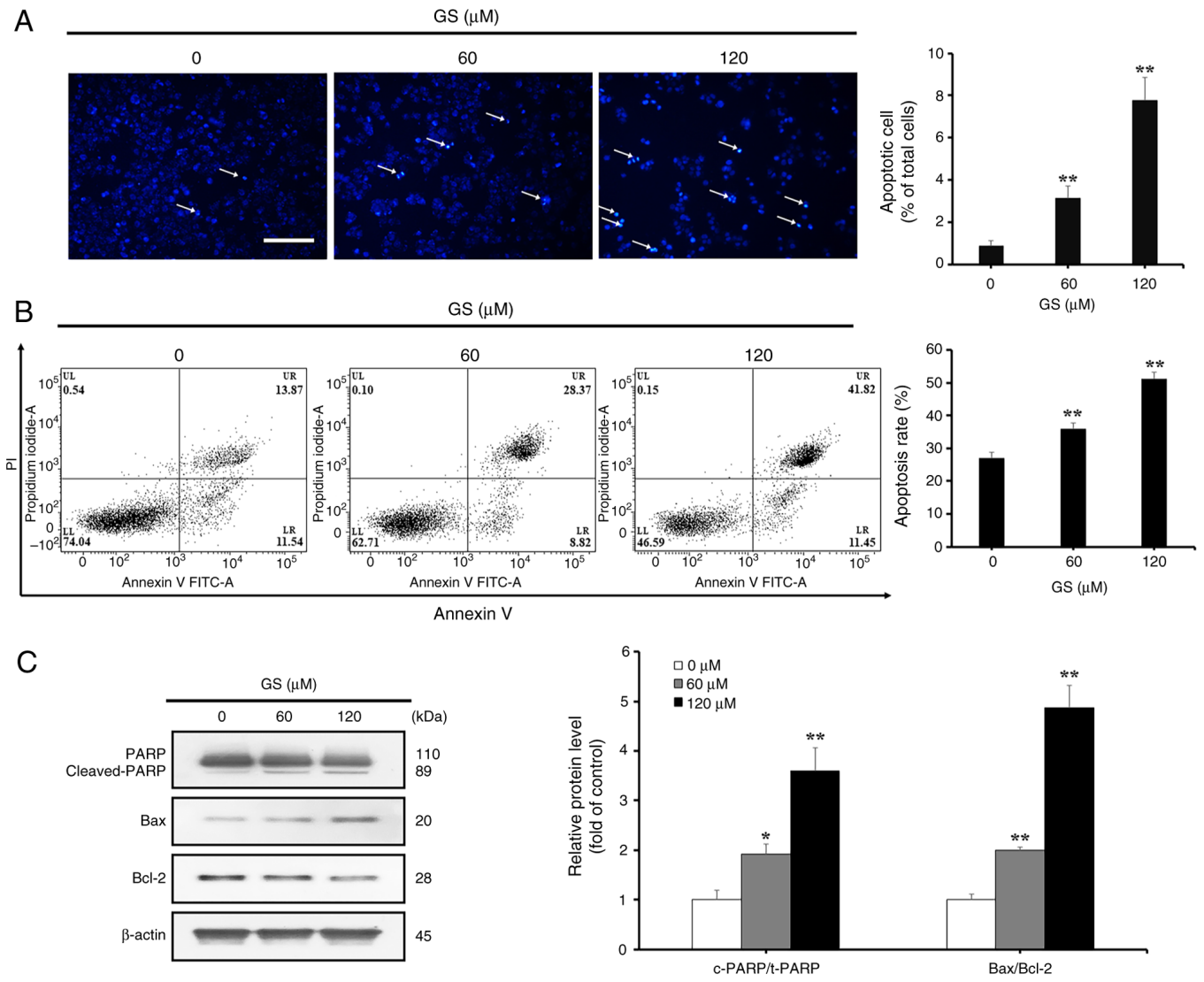

Figure 2. Effects of GS on the apoptosis of HT-29 colorectal cancer cells. (A) HT-29 cells were treated with GS (0, 60 and 120 μM) for 24 h, and the cells were then stained with DAPI. Positive cells were analyzed using a fluorescence microscope and the arrows indicate chromatin condensation (scale bar, 100 μm). The average number of DAPI-positive cells is presented as a percentage of total cells. (B) HT-29 cells were treated with GS (0, 60 and 120 μM) for 24 h, stained with Annexin V/PI and analyzed by flow cytometry. The percentage of apoptotic cells among total cells is shown. (C) HT-29 cells were treated with GS (0, 60 and 120 μM) for 24 h, and the expression levels of apoptosis-related proteins, PARP, Bax and Bcl-2, were measured by western blotting. β-actin was used as a loading control, and semi-quantification was performed using ImageJ. Control (0 μM) cells were subjected to treatment with an equal amount of dimethyl sulfoxide. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. control group. GS, gossypin; PI, propidium iodide.

autophagy- and apoptosis-related proteins were assessed. Cell viability was significantly increased in the group treated with 3-MA and gossypin compared with that in cells treated with gossypin alone, but no significant difference was observed with HCQ treatment (Fig. 4A). To determine whether the 3-MA-induced changes in cell viability were associated with apoptosis, flow cytometric analysis was conducted following pretreatment with this inhibitor. The results indicated that Annexin V positivity was significantly lower in the group pretreated with 3-MA compared with that in cells treated with gossypin alone (Fig. 4B). Furthermore, western blotting was performed to confirm that these changes were associated with apoptosis. Compared with in cells treated with gossypin alone, the application of 3-MA followed by gossypin resulted in decreased Bax and cleaved PARP expression, and increased Bcl-2 expression, indicating a tendency for apoptosis to be suppressed (Fig. 4C). These findings confirmed that gossypin-induced autophagy may contribute to HT-29 cell death.

The MAPK/JNK pathway in gossypin-induced apoptosis and autophagy. To determine whether gossypin-induced apoptosis in HT-29 cells involved the MAPK pathway, MAPK pathway proteins were analyzed using western blotting. The results showed decreased p-ERK expression, and increased p-JNK and p-p38 levels in response to gossypin (Fig. 5). To further investigate changes in apoptosis and autophagy After JNK inhibition using SP600125 in HT-29 cells, the resulting changes in cell viability and the expression levels of apoptosis- and autophagy-related proteins in response to gossypin were examined through western blotting. The resulting changes in cell viability, and the expression levels of apoptosis- and

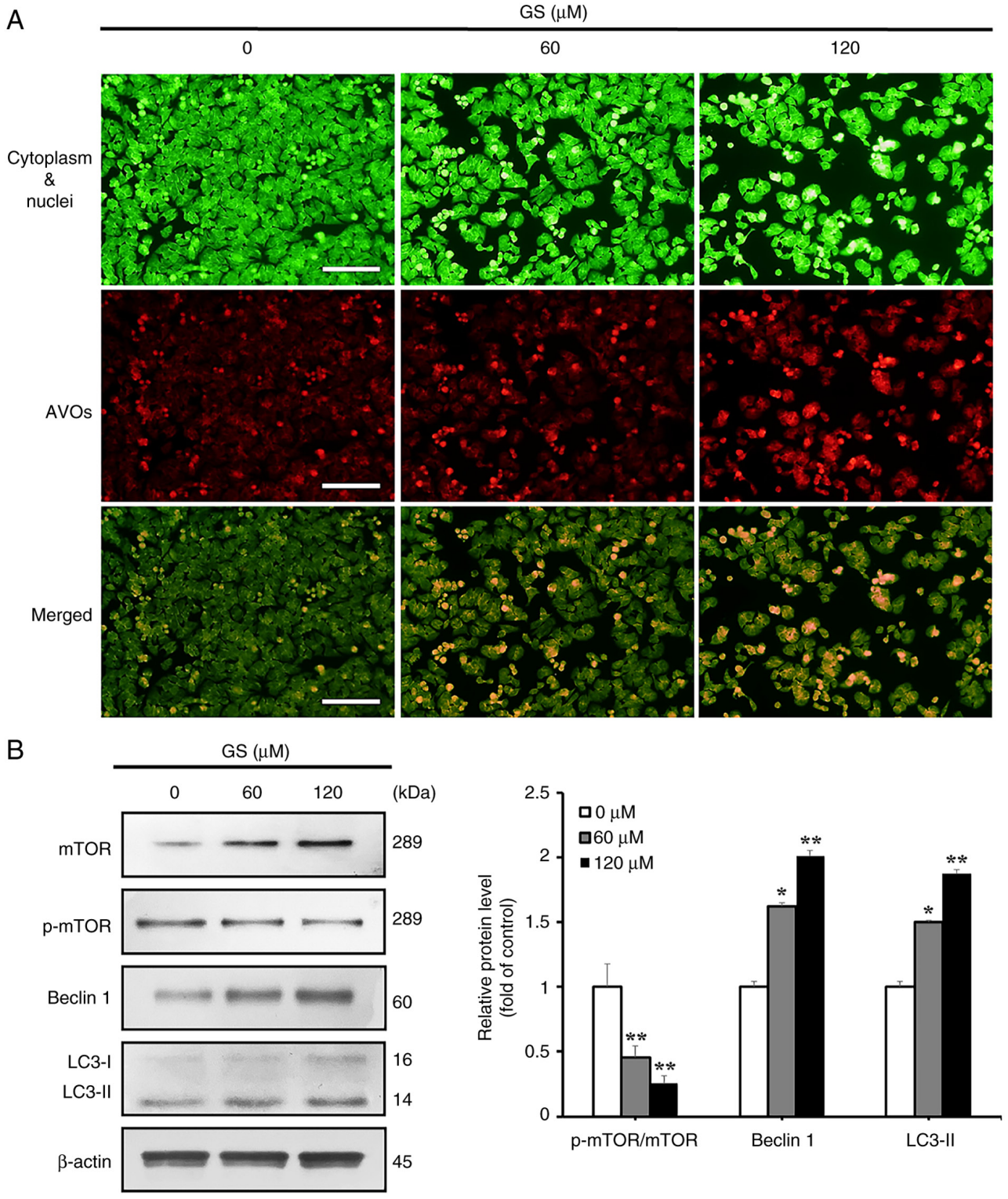

Figure 3. Effect of GS on the induction of autophagy in HT-29 colorectal cancer cells. HT-29 cells were treated with GS (0, 60 and 120 μM) for 24 h. (A) AVOs were visualized via fluorescence microscopy using acridine orange staining. The cytoplasm and the nuclei-were stained fluorescent green, and the AVOs were stained fluorescent red (scale bar, 100 μm). (B) Expression levels of autophagy-related proteins, mTOR, p-mTOR, Beclin 1 and LC3, were measured by western blotting. β-actin was used as a loading control and semi-quantification was performed using ImageJ. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. control group. AVOs, acidic vesicular organelles; GS, gossypin; p-, phosphorylated.

autophagy-related proteins were examined through western blotting. Cell viability was significantly increased in the SP600125-treated gossypin group compared with that in the gossypin-only group (Fig. 6A). The results further showed that, compared with in the group treated with gossypin alone, the group treated with SP600125 and gossypin exhibited decreased Bax expression and increased Bcl-2 expression, indicating suppression of apoptosis (Fig. 6B). Additionally,

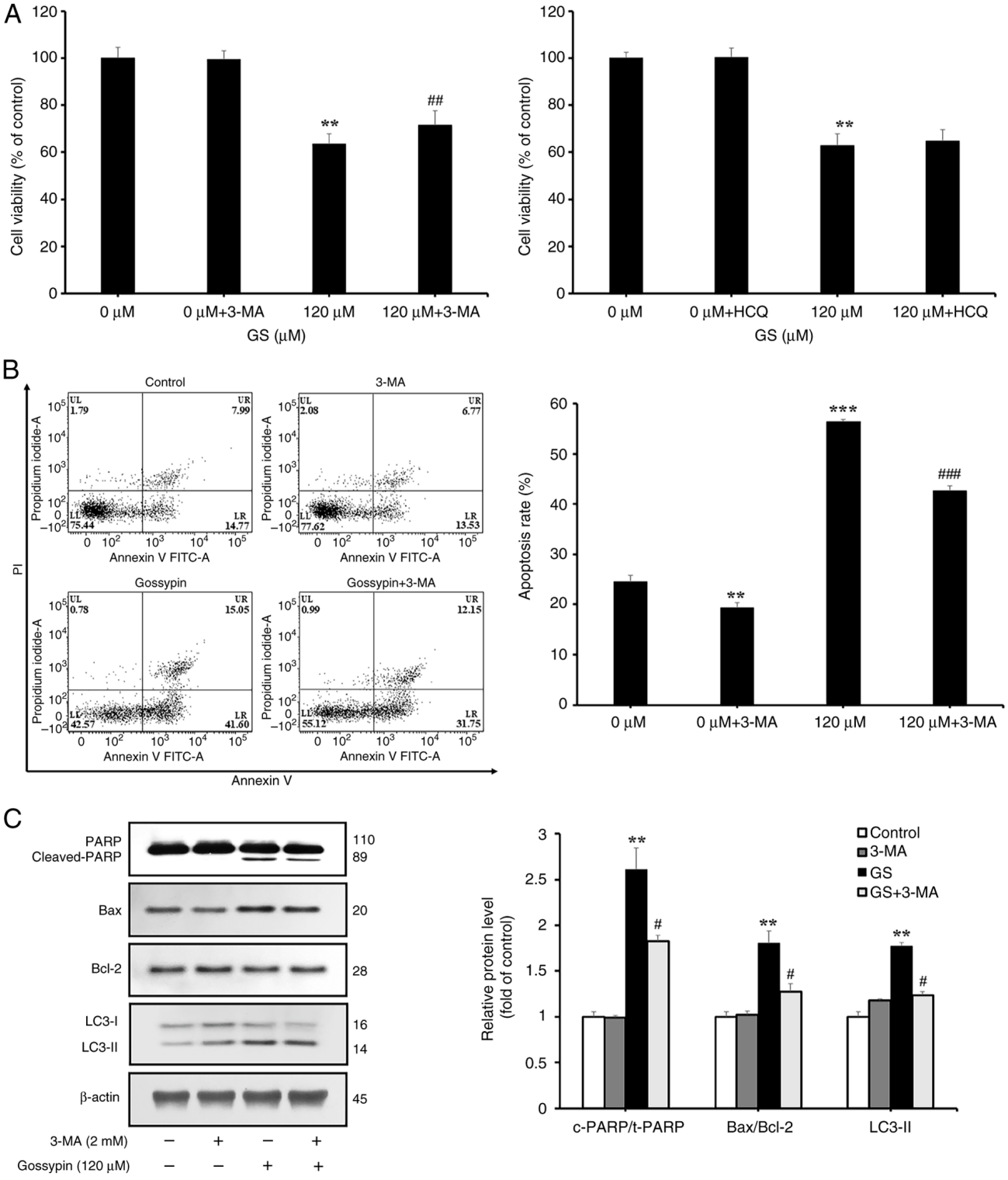

Figure 4. Effect of GS on autophagy in HT-29 colorectal cancer cells. (A) HT-29 cells were pretreated with 3-MA (2 mM) or HCQ (20 μM) for 2 h and then treated with GS (120 μM) for 24 h. Cell viability was measured using the MTT assay. (B) HT-29 cells were pretreated with 3-MA (2 mM) for 2 h and then treated with GS (120 μM) for 24 h. The cells were then stained with Annexin V/PI and were analyzed by flow cytometry. The bar graph represents the percentage of apoptotic cells among total cells. (C) Protein expression levels of Bax, Bcl-2 and LC3 were measured by western blotting. β-actin was used as a loading control and semi-quantification was performed using ImageJ. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. GS group. 3-MA, 3-methyladenine; GS, gossypin; HCQ, hydroxychloroquine; PI, propidium iodide.

a reduction in LC3-II expression confirmed the suppression of autophagy in this group. These results suggested that gossypin-induced apoptosis and autophagy in HT-29 cells may be mediated through the MAPK/JNK pathway.

Effects of gossypin on HT-29 xenograft tumors. The anticancer effects of gossypin observed *in vitro* were evaluated *in vivo* using an HT-29 xenograft model. HT-29 cells were subcutaneously injected into both shoulders of BALB/c nude mice. Once

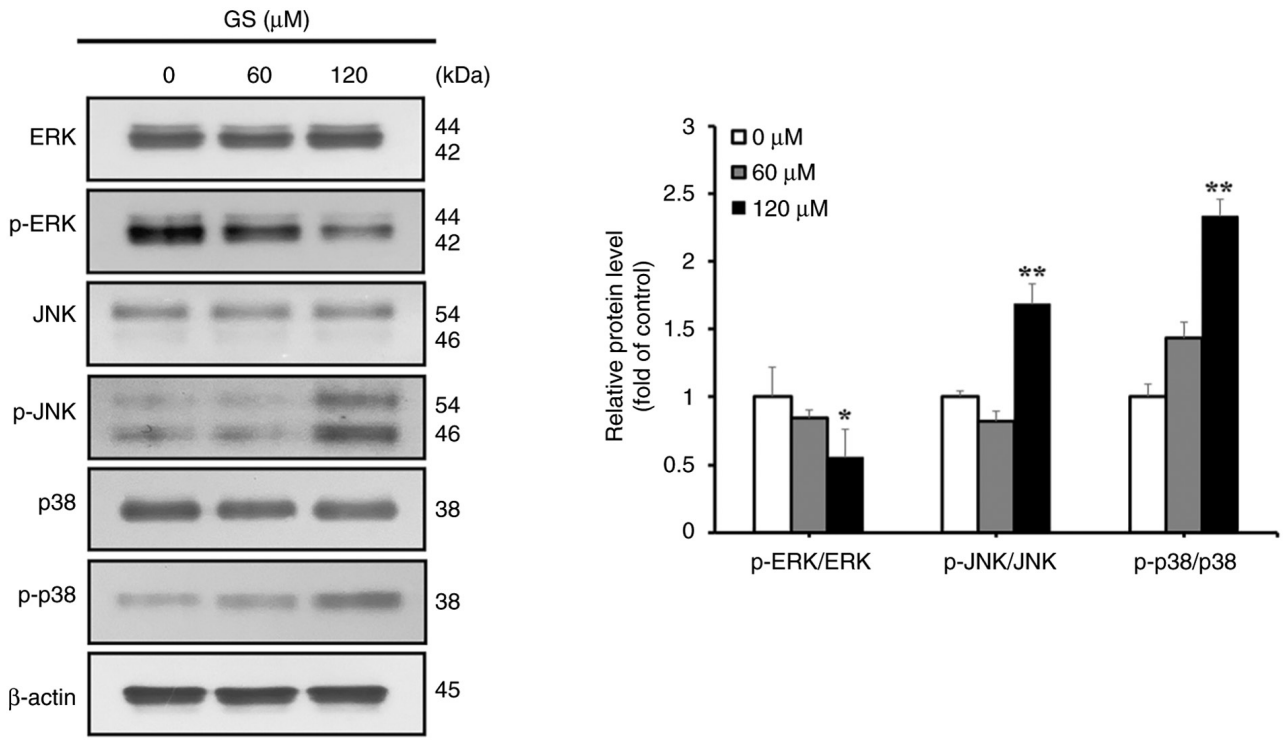

Figure 5. Effects of GS on the expression levels of MAPK pathway proteins. Expression levels of MAPK pathway-related proteins, ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were measured by western blotting. β-actin was used as a loading control and semi-quantification was performed using ImageJ. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. control group. GS, gossypin; p-, phosphorylated.

tumors reached ~90 mm^3, the mice were randomly assigned to two groups, and gossypin (0 and 100 mg/kg) was orally administered for 28 days (five times/week). Notably, tumor volume was significantly reduced in the gossypin-treated group (Figs. 7A and S1). Tumor weight exhibited a decreasing trend; however, this change was not statistically significant. Additionally, there was no notable difference in body weight between the control group and the gossypin group (Fig. 7B and C). Hematoxylin and eosin staining was performed to determine whether gossypin administration in mice induced toxicity in the liver and kidneys, which are toxicity-sensitive organs. No differences were observed in either organ between the gossypin-treated and control groups (Fig. 7D).

Gossypin-induced apoptosis and autophagy in tumors. Western blotting was performed to investigate whether gossypin induced apoptosis and autophagy in CRC tumors *in vivo*. The results showed an increased expression of key apoptosis-related proteins, including cleaved PARP and Bax, and a decreased expression of Bcl-2 in the gossypin-treated group; additionally, the levels of key ATG proteins, LC3-II and Beclin 1, were increased (Fig. 8A). A TUNEL assay was also conducted on CRC tumors. The gossypin-treated group exhibited a significantly higher number of TUNEL-positive cells than the control group; quantification of the images revealed that the treated group had more than three times the number of TUNEL-positive cells than the control group (Fig. 8B).

MAPK/JNK pathway activation in gossypin-treated tumors. Immunohistochemistry was performed to assess p-JNK expression in tumors. The results showed a clear increase in the number of p-JNK-positive cells in the gossypin-treated group compared with that in the control group; quantification of these p-JNK-positive cells revealed that the treated group had more than four times the number observed in the control group (Fig. 8C).

Discussion

Gossypin, a flavone found in *H. vitifolius*, has previously been studied for its diverse physiological effects and anticancer properties (10,11,13). However, its role in apoptosis, autophagy and MAPK pathways in human CRC cell lines remains underexplored. The present study investigated the anticancer effects of gossypin on the human CRC cell line HT-29, and assessed whether these effects were mediated through MAPK/JNK-induced apoptosis and autophagy.

To determine whether gossypin suppresses the viability of human CRC cells, HT-29 cells were treated with gossypin, and cell viability was assessed using an MTT assay. The results showed a significant concentration-dependent reduction in cell viability, with a half-maximal inhibitory concentration value of 142.76 μM. Regarding the treatment duration of gossypin in human HT-29 CRC cells, a duration of 24 h was selected, based on prior anticancer studies involving natural products (28-30). Previous studies have demonstrated that gossypin similarly reduces cell viability in various cancer cell lines, including lung (A549), breast (MCF-7), gastric (HGC27, AGS), melanoma (A375, WM1552C, WM793B, SKMEL-31, SKMEL-28), glioblastoma (U251) and prostate (PC-3) cells,

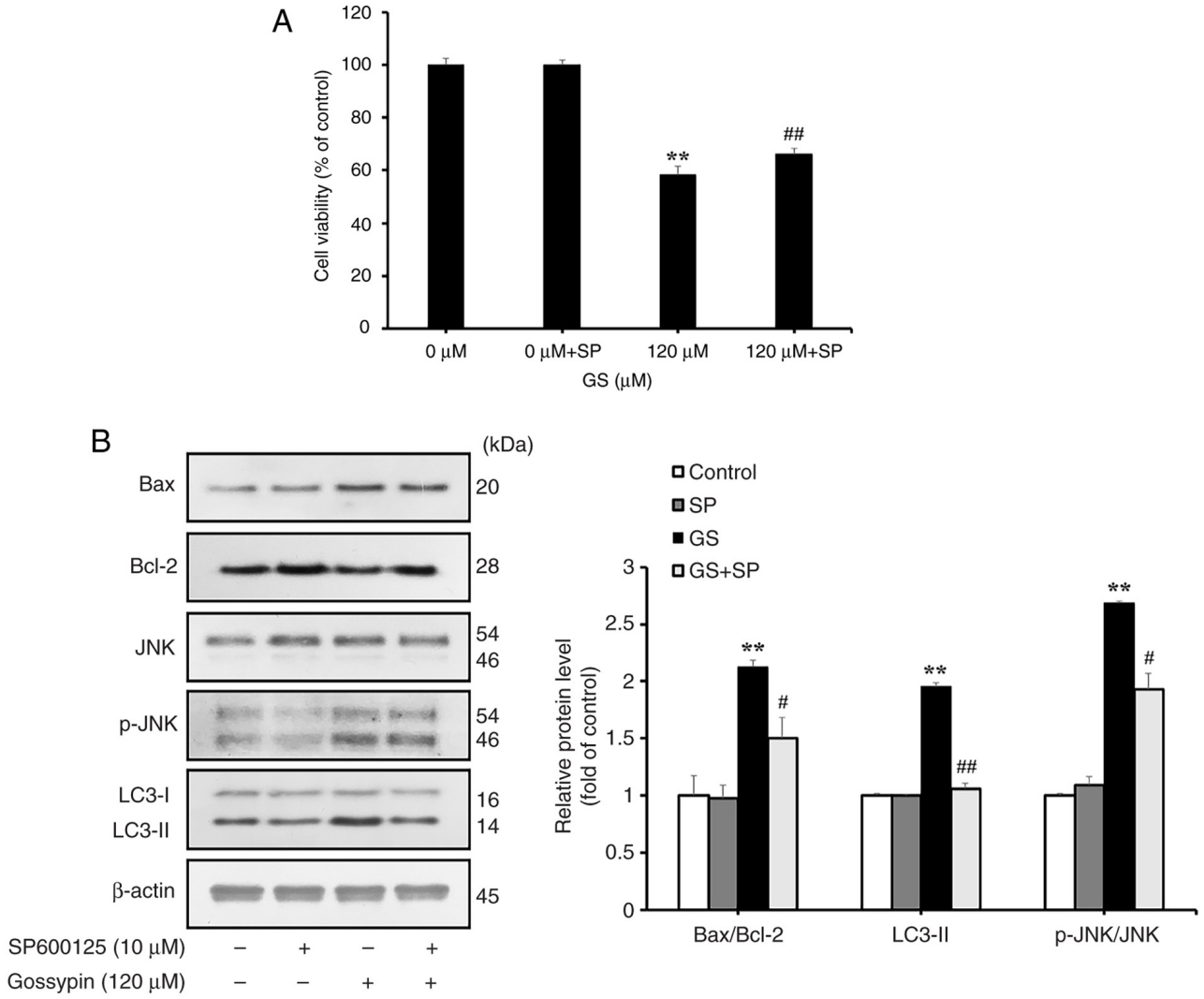

Figure 6. GS induces regulation of apoptosis and autophagy via JNK in HT-29 colorectal cancer cells. (A) HT-29 cells were pretreated with SP (10 μM) for 2 h and then treated with GS (120 μM) for 24 h. Cell viability was measured using the MTT assay. (B) Protein expression levels of Bax, Bcl-2, JNK, p-JNK and LC3 were measured by western blotting. β-actin was used as a loading control and semi-quantification was performed using ImageJ. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. GS treatment group. GS, gossypin; p-, phosphorylated; SP, SP600125.

suggesting its anticancer potential across a range of cell types (13-15,31-33). The toxicity of gossypin in nontumor cells has also been investigated in a previous study; in human astrocytes, gossypin showed no toxicity at concentrations ranging from 0 to 90 μM over 24, 48 and 72 h (32). However, toxicity assessments involving cell types other than tumors remain inadequate. The absence of these results presents a major limitation in this research, and further experiments involving gossypin and nontumor cells are crucial to evaluate its potential as an anticancer agent.

To assess whether the reduction in cell viability was mediated by apoptosis, DAPI and Annexin V/PI staining assays were performed. Apoptosis leads to chromatin and nuclear condensation, forming apoptotic bodies, which exhibit blue fluorescence when stained with DAPI (34). In the current study, HT-29 cells treated with gossypin exhibited a concentration-dependent increase in the number of apoptotic bodies. Apoptosis was also investigated using Annexin V, which binds to phosphatidylserine exposed on the cell surface during apoptosis, and PI, which stains the nucleus but cannot penetrate intact cell membranes, marking necrotic or late apoptotic cells (35,36). After treating HT-29 cells with gossypin and staining them with Annexin V/PI, flow cytometry revealed that the total apoptosis rate, combining early and late apoptosis, was significantly increased in a concentration-dependent manner. Previous studies using Annexin V/PI staining following gossypin treatment reported similar trends; apoptosis rates were shown to be increased with higher gossypin concentrations in human lung and gastric cancer cell lines (13,15). These findings suggested that gossypin dose-dependently induces apoptosis in HT-29 cells.

Additionally, apoptosis-related proteins were examined through western blotting. In mitochondrial pathway-mediated apoptosis, Bcl-2 family proteins serve a crucial role; this family includes pro-apoptotic proteins, such as Bax and Bak, and anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, which regulate the mitochondrial membrane. During apoptosis, decreases in Bcl-2 and Bcl-xL expression, and increases in

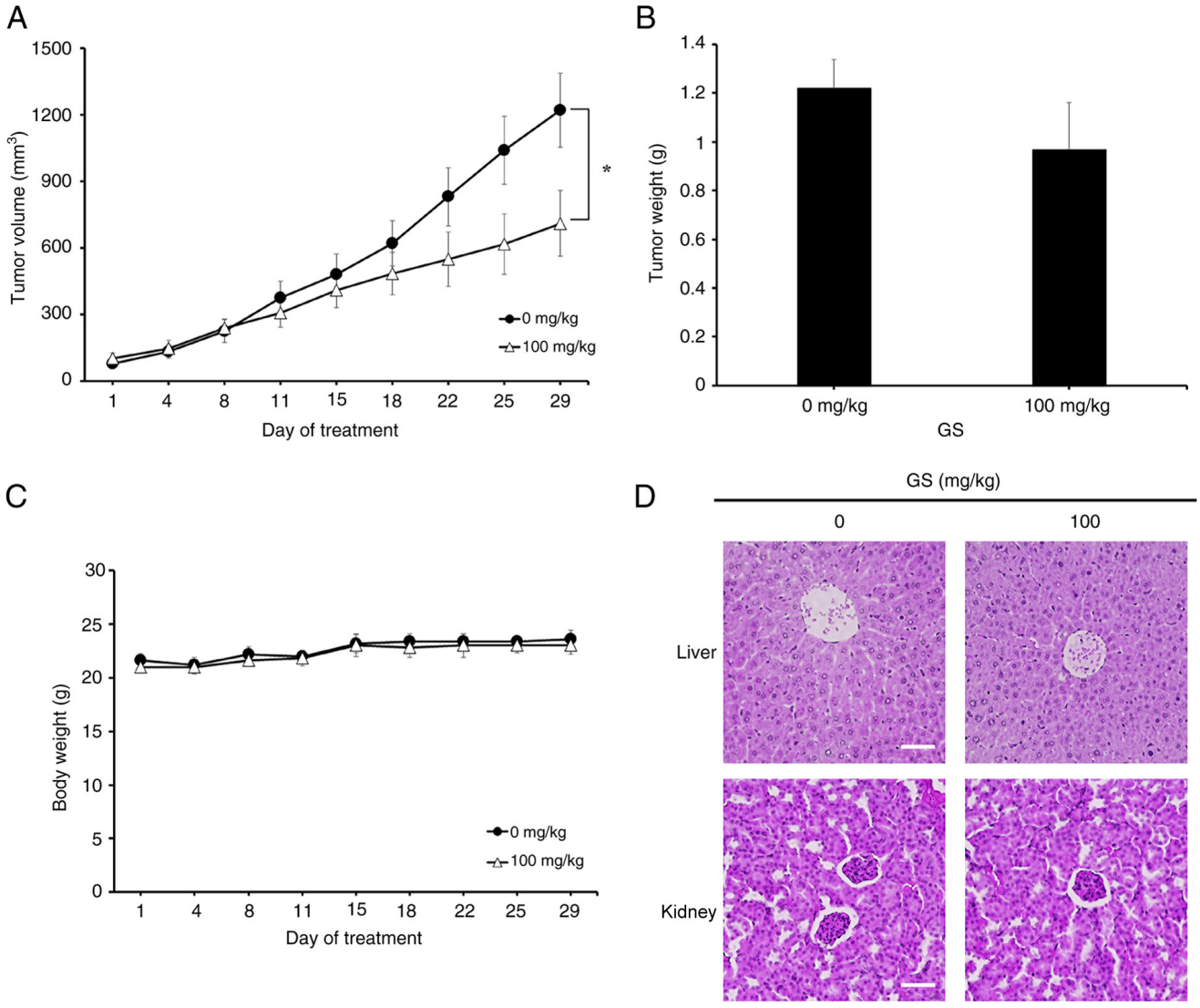

Figure 7. Effects of GS on HT-29 tumors *in vivo*. Nude mice bearing HT-29 cells as a xenograft model were treated with GS (0 and 100 mg/kg) for 28 days. (A) Tumor volume, (B) tumor weight and (C) body weight were measured. (D) Histological toxicity analysis of the liver and kidney in nude mice was performed using hematoxylin and eosin staining (scale bar, 50 μm). *P<0.05 vs. control group. GS, gossypin.

Bax and Bak expression, disrupt the mitochondrial membrane, enhancing its permeability. Due to this increased permeability, cytochrome *c* is released from the mitochondria, which, in conjunction with Aparf-1, triggers a caspase cascade that ultimately induces apoptosis (18). PARP, a protein essential for DNA repair, is cleaved by activated caspases during apoptosis (37,38). Western blotting after treating HT-29 cells with gossypin demonstrated a concentration-dependent increase in the expression levels of cleaved PARP and the pro-apoptotic protein Bax, alongside a decrease in the expression of the anti-apoptotic protein Bcl-2. These findings suggested that gossypin may induce mitochondrial pathway-mediated apoptosis in HT-29 cells by promoting PARP cleavage, decreasing Bcl-2 expression and increasing Bax levels.

Autophagy is a physiological process that degrades cellular organelles and molecules via lysosomes to maintain homeostasis. Autophagy is mediated by ATG proteins, which form autophagosomes that eventually fuse with lysosomes to create autolysosomes (39,40). Western blotting was performed in the present study to investigate whether gossypin induced apoptosis and autophagy in CRC tumors. Notably, acridine orange staining showed a dose-dependent increase in acidic vesicular organelles in HT-29 cells treated with gossypin. Additionally, western blotting demonstrated a decreased expression of p-mTOR, a factor that inhibits the initial formation of autophagosomes. Concurrently, there was an increased expression of Beclin 1, a protein that promotes autophagy and overall autophagosome formation, as well as an elevated conversion of LC3-I to LC3-II, which is critical for the early formation of autophagosomes (22,40). These findings indicated that gossypin may induce autophagy in the human CRC cell line HT-29.

The dual role of autophagy in cancer includes acting as both a tumor-suppressing mechanism through organelle removal and a tumor-promoting mechanism by recycling metabolic by-products to sustain tumor growth (20). To understand the mechanism of gossypin-induced autophagy in HT-29 cells, autophagy inhibitors were used to treat cells, followed by a cell viability assay and western blotting. Previous studies on HT-29 and HCT-116 CRC cells have demonstrated that inhibiting

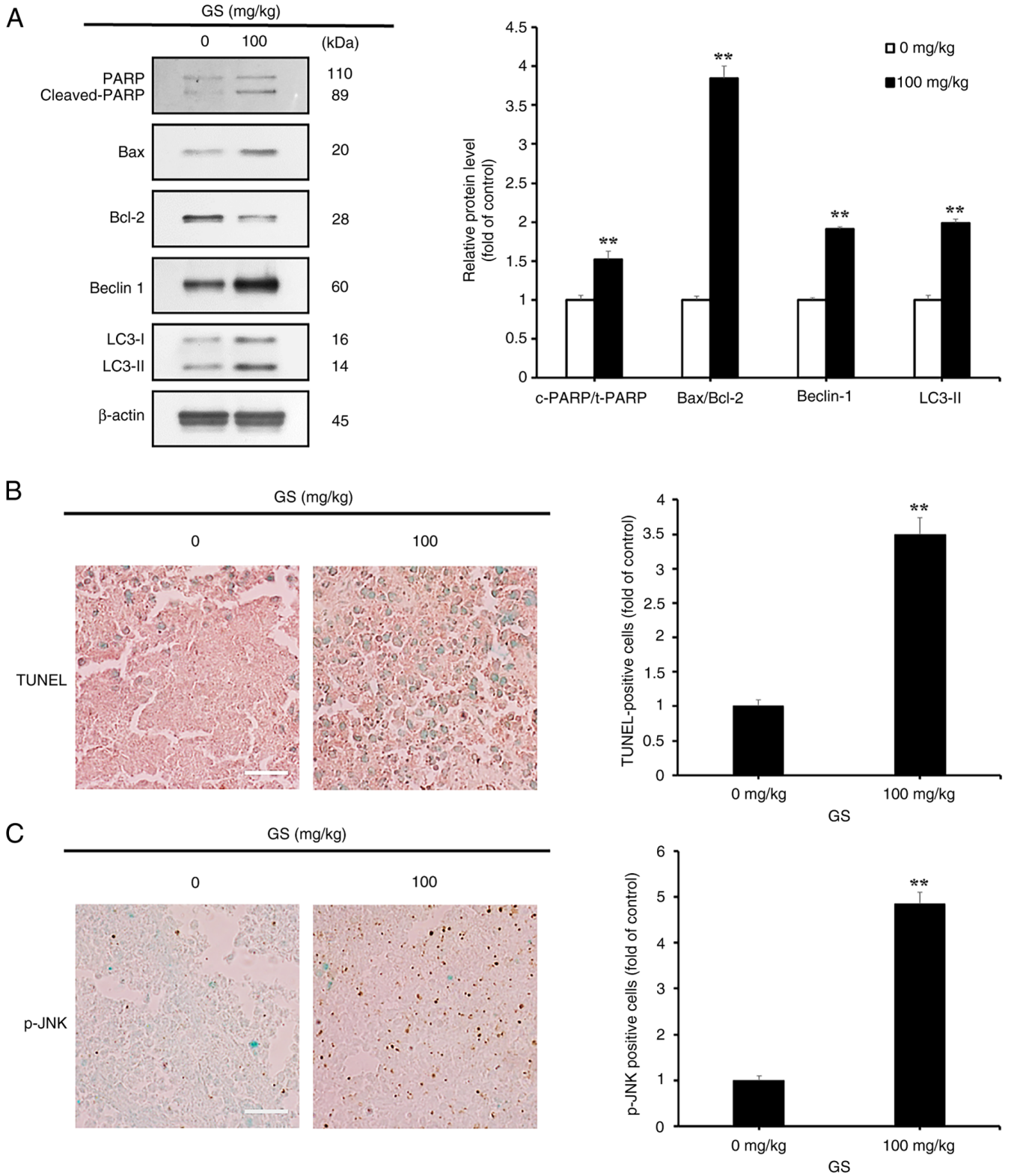

Figure 8. Effects of GS on JNK-mediated apoptosis and autophagy in tumors. (A) Tumor protein expression levels of PARP, Bax, Bcl-2, Beclin 1 and LC3 were measured by western blotting. β-actin was used as a loading control and semi-quantification was performed using ImageJ. The results are representative of three independent experiments and data are presented as the mean ± standard deviation. (B) Apoptosis was measured in tumor tissues using a TUNEL assay. (C) p-JNK expression was measured in tumor tissues by immunohistochemistry. TUNEL-positive and p-JNK-positive cells were observed under a light microscope (scale bar, 25 μm) and are shown as the average of three fields. **P<0.01 vs. control group. GS, gossypin; p-, phosphorylated; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

autophagy with 3-MA and chloroquine alters cell viability in response to treatment with *Ganoderma lucidum* polysaccharide; 3-MA treatment increases cell viability, whereas chloroquine treatment decreases it. Moreover, apoptosis induction was shown to be reduced in the 3-MA-pretreated group (41). In the present study, HCQ treatment did not

significantly affect cell viability, whereas treatment with 3-MA resulted in a marked increase in cell viability. To explore whether autophagy inhibition affected apoptosis, flow cytometry and western blotting were performed. The results indicated a trend toward reduced Annexin V positivity and decreased expression of apoptosis-related proteins in response to 3-MA. These findings suggested that gossypin-induced autophagy may serve a role in promoting cell death alongside apoptosis. Furthermore, the mechanisms of autophagy in cancer cells vary depending on multiple factors, including the administered drug, the cell type and the stage of autophagy, highlighting its complex and context-dependent nature.

The MAPK pathway has a critical role in cell proliferation, differentiation, apoptosis and angiogenesis (26). A previous study demonstrated that treatment of HepG2 human liver cancer cells with gossypin increases ERK phosphorylation over time, whereas JNK and p38 phosphorylation levels remain unchanged (42). In the current study, increasing gossypin concentrations induced phosphorylation of JNK and p38 in HT-29 cells but decreased ERK phosphorylation. Previous anticancer studies using natural compounds have demonstrated varying phosphorylation patterns of MAPK pathway proteins, suggesting that the phosphorylation of ERK, JNK and p38 may differ depending on specific conditions in cancer therapy (29,41-43). To investigate the role of gossypin-induced p-JNK expression in HT-29 cells, the JNK inhibitor SP600125 was used to treat cells, followed by analyses of cell viability and the expression of apoptosis- and autophagy-related proteins using western blotting. Inhibition of the elevated JNK expression in gossypin-treated HT-29 cells reduced the expression levels of proteins associated with apoptosis and autophagy. Previous studies on natural compounds have reported that the inhibition of elevated p-JNK expression in cancer cells leads to the suppression of cell death pathways, such as apoptosis, autophagy and paraptosis (28,29,44), suggesting that p-JNK is involved in these pathways. Therefore, it was hypothesized that the p-JNK expression upregulated by gossypin likely acted as a mediator of apoptosis and autophagy in HT-29 cells. To the best of our knowledge, the present study is the first to confirm the influence of the MAPK/JNK pathway in gossypin-treated cancer cells, and the findings provide foundational evidence that gossypin promotes cell death in cancer cells via p-JNK. However, it remains challenging to delineate the relationship between apoptosis and autophagy in relation to JNK expression induced by treatment with a fixed concentration of gossypin over only 24 h. Consequently, further investigations are necessary to explore how variations in JNK expression, influenced by different concentrations and treatment durations of gossypin, affect the interplay between apoptosis and autophagy, and whether these processes occur through distinct pathways. In addition, the current study exclusively cross-validated the role of JNK within the MAPK pathway. To demonstrate that gossypin exerts anticancer effects through the MAPK pathway in CRC cells, it is essential to investigate the relationship between decreased p-ERK levels and increased p-p38 levels in relation to apoptosis and autophagy.

To determine whether the anticancer effects of gossypin observed *in vitro* also occurred *in vivo*, xenograft experiments were conducted. A previous melanoma xenograft study demonstrated a significant reduction in tumor volume and an increase in TUNEL-positive cells in gossypin-treated groups (31). In the present study, gossypin treatment significantly suppressed tumor volume compared with that in the control group, although tumor weight only showed a decreasing trend. No differences were observed in body weight and the histopathological features of livers and kidneys between the murine control and treatment groups, suggesting that gossypin suppressed tumor growth *in vivo* without toxicity. Western blotting of tumor samples revealed that, consistent with the *in vitro* findings, apoptosis and autophagy were increased in the gossypin-treated group. Additionally, TUNEL assay results showed significant increases in the number of TUNEL-positive cells in tumors of the gossypin-treated group compared with that in the control group, consistent with previous findings. These results suggested that the suppression of CRC in the gossypin-treated group may be mediated by apoptosis and autophagy. Furthermore, the significant increase in the number of p-JNK-positive cells in the gossypin-treated group indicated that gossypin-induced apoptosis and autophagy in CRC tumors were mediated through the JNK pathway. However, the present *in vivo* toxicity evaluation of gossypin was conducted using immune-deficient BALB/c nude mice, rather than healthy BALB/c wild-type mice, and this evaluation was limited to the liver and kidneys, which are organs particularly sensitive to toxicity. Thus, further detailed and systematic experiments are essential to establish the overall *in vivo* safety of gossypin as an anticancer agent. In addition, the dose used in the present *in vivo* experiments was based on concentrations referenced in previous studies (31). Research on the pharmacokinetics and drug resistance associated with gossypin is lacking; therefore, comprehensive studies that address clinical limitations are essential to support the selection of gossypin as a potential anticancer agent.

In conclusion, the present study investigated gossypin-induced apoptosis and autophagy in human HT-29 CRC cells. Gossypin reduced the viability of HT-29 cells through apoptosis, and induced autophagy by decreasing p-mTOR levels and increasing Beclin 1 and LC3-II expression. Gossypin-induced autophagy in HT-29 cells activated pro-apoptotic proteins, promoting apoptosis as part of a cell death mechanism. Furthermore, the present results confirmed that the induction of apoptosis and autophagy was regulated via the MAPK/JNK pathway. Additional *in vivo* experiments demonstrated that gossypin also induced apoptosis and autophagy in HT-29 tumors by increasing p-JNK expression. Taken together, gossypin may induce apoptosis and autophagy in human HT-29 CRC cells via the MAPK/JNK pathway, suggesting the potential of gossypin as a natural anticancer agent for CRC therapy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JMM and JYJ designed and conducted experiments, and collected data. JMM wrote the manuscript. SWL, YSJ, SKK, BKP and YSP analyzed and interpreted data. SAL, SHJ, MSY, BSK and JYJ analyzed the results and reviewed the manuscript. JYJ edited the manuscript and acquired funding. All authors confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) (approval no. IACUC-KNU_2024-07) of Kongju National University (Yesan, South Korea).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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