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Quercetin induced HepG2 cells apoptosis through ATM/JNK/STAT3 signaling pathways

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Abstract: Liver cancer is the seventh most common malignant tumor in the world and is the second highest cause of death due to cancer. Quercetin, a flavonoid with low toxicity, widely exists in various fruits and vegetables. It has the potential to be a therapeutic agent against various cancers. This study aimed to demonstrate the anti-tumor effect of quercetin on HepG2 cells. Quercetin suppressed the HepG2 cell proliferation in a dose-dependent manner in cell viability assay. Induction of cell apoptosis was confirmed by apoptotic cells population (sub-G1 peak) detected by flow cytometer. A decrease in mitochondrial membrane potential and caspase-3 activation were also demonstrated in this study. Furthermore, quercetin induced HepG2 cell apoptosis through ROS-mediated phosphorylated ataxia-telangiectasia mutated, c-Jun N-terminal kinases, signal transducer, and activator of transcription 3 (STAT-3), and Bax signaling pathways. These results suggest that quercetin has the potential to become an effective drug against the tumor.

Introduction

Liver cancer is the seventh most common malignant tumor globally and ranks second in deaths due to cancer. Liver cancer cases are increasing most speedily, with an annual increase of 2%–3% from 2007 to 2016 in the USA. The 5-year survival rate has not increased remarkably in the past 20 years In China (Siegel *et al.*, 2020). Various etiologies and inducing factors are explicit (Forner *et al.*, 2018). Hepatitis B infection, hepatitis C infection, alcohol, diabetes and nonalcoholic fatty liver disease are the leading risk factors (Kakehashi *et al.*, 2020). In the clinic, ultrasound and alpha-fetoprotein are recommended for the preliminary screening of liver cancer. However, their underuse limits the detection rate (Yang and Heimbach, 2020). Regarding the therapy, hepatocellular carcinoma (HCC) is insensitive to radiotherapy and chemotherapy (Sheng *et al.*, 2018). Less than 30% of HCC patients are applicable for surgical treatment. Though radiofrequency ablation (RFA) and microwave ablation (MWA) are applied to the treatment of HCC, RFA is still confined to its heat dissipation effect (Han *et al.*, 2020), and the MWA's long diameter damages the normal liver tissue (Lin *et al.*, 2016). Neither chemical and surgical treatment nor interventional therapy could provide a good prognosis. Therefore, there is an urgency to find an effective way to treat HCC.

Quercetin, 3,3′,4′,5,7-pentahydroxyflavone (C15H10O7), is a kind of antioxidant (Shabbir *et al.*, 2021). Owing to being natural ingredients of the polyphenols that exist in various fruits and vegetables, it is widely accessible (Reyes-Farias and Carrasco-Pozo, 2019). Previous researchers have found that quercetin has lots of functions, such as anti-inflammatory, anti-oxidation, anti-diabetic, and anti-allergic properties (Badolato *et al.*, 2017). Quercetin also potentially has anti-tumor properties (Tang *et al.*, 2020). It inhibits the proliferation of colon cancer (Lin *et al.*, 2020) and can downregulate the expression of MMP-2/-9 protein to restrict the hyperplasia of breast cancer cells named MCF-7 (Ozkan and Bakar-Ates, 2020). Song *et al.* (2020a) found that the PI3K/Akt/mTOR signaling pathway could be blocked by quercetin for curing human prostate cell carcinoma PC-3 cells. In addition, compared to other present chemical drugs, quercetin was a fascinating anticancer compound owing to its low toxicity (Rauf *et al.*, 2018). *In vivo* toxicological experiments confirmed that low oral doses of quercetin did not cause significant changes in morphological, behavioral, hematological, serum biochemical,

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and histopathological parameters in Swiss albino mice, while high oral doses showed mild toxicity (Pal and Tripathi, 2020).

Reactive Oxygen Species (ROS) is a collective term for a series of derivatives of molecular oxygen, including superoxide anion radicals, singlet oxygen, hydroxyl radicals, and so on (Zhu *et al.*, 2016). Although ROS occurs as a common attribute of aerobic life (Sies and Jones, 2020), the redox imbalance is associated with many diseases, such as prostate cancer, Alzheimer's disease, cardiac and pulmonary diseases (Climent *et al.*, 2020; Tonnies and Trushina, 2017; Ward *et al.*, 2018). It is also related to many intracellular processes, such as cell apoptosis, DNA damage and mitochondria damage (Kang *et al.*, 2019; Sreedhar *et al.*, 2020; Srinivas *et al.*, 2019). The increasing ROS levels can activate anti-tumourigenic signaling, causing death to the oxidative stress induced-cancer cells (Moloney and Cotter, 2018). Quercetin induces apoptosis by producing intracellular ROS (Kim *et al.*, 2013; Kim *et al.*, 2017). The relationship between quercetin and oxidative stress has been reported (Bishayee *et al.*, 2015), but its specific mechanisms and pathways of anti-cancer effect are still not clear. This study was conducted to understand how quercetin-related changes induce cell apoptosis through ROS-associated signaling pathways.

Materials and Methods

Materials

HepG2 cells were bought from American Type Culture Collection (Manassas, Virginia). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco. Cell counting kit-8 (CCK-8), mitochondrial membrane potential assay kit with JC-1, caspase-3, cell cycle and apoptosis analysis kit, and ROS assay kit were purchased from Beyotime Biotechnology. Phosphorylated ataxia-telangiectasia mutated (P-ATM), signal transducer and activator of transcription 3 (STAT-3), c-Jun N-terminal kinases (JNK), Bax, and β-actin were obtained from Cell Signaling Technology. Quercetin was provided by Professor Dong Hao from Zhongkai University of Agriculture and Engineering.

Cell culture and viability assay

The cytotoxicity of quercetin was measured by CCK-8 assay (Gong *et al.*, 2018). First, HepG2 cells were incubated in DMEM supplemented with 10% FBS in 96-well plates at a density of 4×10^4 cells/well for 24 h in a 5% CO_2 atmosphere at 37°C. Then, quercetin was added at different concentrations to HepG2 cells incubated in DMEM with 1% FBS for 24 h. Then, 10 μL of CCK-8 solution was added per well and incubated for different durations to detect the absorbance. The absorbance, which indicated the cell viability, was measured at 450 nm via a microplate spectrophotometer. The assay was repeated thrice.

Determination of cell cycle and apoptosis

HepG2 cells with a density of 4×10^4 were planted in 6-well plates. After 24 h, the original medium was discarded, and quercetin with different concentrations (10/20/40 μmol/L) was added for 24 h. The pretreated HepG2 cells were trypsinized and resuspended with PBS, then immobilized with 70% precooled ethanol. After incubating with propidium iodide (PI) at 37°C, HepG2 cells were then analyzed by flow cytometry (Xiong *et al.*, 2019).

Mitochondrial membrane potential staining assay

HepG2 cells treated with different concentrations (10/20/40 μmol/L) of quercetin were washed with PBS. The prepared JC-1 staining solution was added to the above cells and incubated at 37°C, then washed with JC-1 buffer solution thrice. Finally, stained HepG2 cells were observed under a fluorescence microscope (Zeng *et al.*, 2018).

Caspase-3 activity

HepG2 was grown overnight to a density of 8×10^4 in a 10 cm petri dish. After 24 h of treatment with quercetin at the above different concentrations, cells were lysed by radioimmunoprecipitation assay (RIPA) buffer and the supernatant was obtained by centrifugation at 4°C. The cellular lysate liquid and testing buffer solution were kept on standby before the testing reagent was added to various samples. The absorbance was measured at 405 nm wavelength using a microplate spectrophotometer (Thermo Fisher Scientific, America) to determine the protein concentration of samples when analyzing the caspase-3 activity (Gu *et al.*, 2018).

Detection of reactive oxygen species generation

HepG2 cells were treated with quercetin at different concentrations for 24 h, then stained with (5(6)-Carboxy-2′,7′-dichlorofluorescein), amine-reactive green fluorophore (DCFA) probe and observed under an inverted fluorescence microscope (Leica, Germany). The level of reactive oxygen species (ROS) was determined by the fluorescence intensity. Excitation wavelength (488 nm) and emission wavelength (525 nm) were used to detect the fluorescence intensity (Deng *et al.*, 2018).

Western blotting

HepG2 was grown at a density of 8×10^4 in a 10 cm petri dish overnight. After 24 h of treatment with quercetin at the above different concentrations, cells were lysed by RIPA, and the supernatant was obtained by centrifugation at 4°C. Each protein was quantified by a BCA assay. X-ray film was used to monitor the target protein (Mishra *et al.*, 2017; Xu *et al.*, 2022).

Statistical analysis

All the data are shown as mean ± SD. Differences between two groups were analyzed by one-way ANOVA. A probability of $p < 0.05$ (*) or $p < 0.01$ (**) indicated statistical significance.

Results

In vitro anticancer activity of quercetin

The viability of quercetin-treated cells was measured by CCK-8 assay. As shown in Fig. 1A, HepG2 cells treated with 10 μM quercetin for 24 h exhibited diminished cell viability to 84%; at 20 and 40 μM concentration the cell viability was dramatically reduced to 56% and 47%, respectively. As shown in Fig. 1B, the effect of quercetin on HepG2 was manifested as a reduction in the number of viable cells, cytoplasm pyknosis, and loss of cell connections.

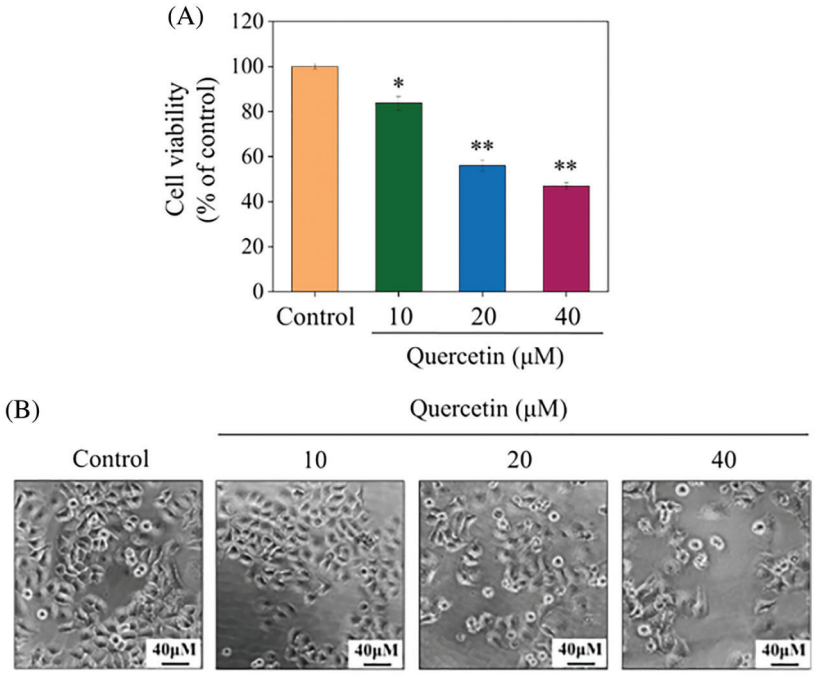

FIGURE 1. Effects of quercetin on the proliferation of HepG2 cells by CCK-8 assay. (A) The cell viability was monitored by quercetin at the different concentrations for 24 h. (B) Morphological changes in HepG2 cells were observed by phase-contrast microscopy. Bars with different characters are statistically different at $^{*}p < 0.05$ or $^{**}p < 0.01$ (n = 3).

Induction of cell apoptosis by quercetin

Apoptosis makes a momentous difference in many aspects, such as organ development, anti-tumor, and treatment of cardiovascular diseases (Codispoti *et al.*, 2019; Dong *et al.*, 2019; Pistritto *et al.*, 2016). The effect of quercetin on cell apoptosis was detected by flow cytometry. The flow cytometer can detect DNA fragmentation that takes the shape of a sub-G1 peak. The blue low peak ahead of the G0/G1 peak indicates the apoptosis proportion. According to Fig. 2, the control group showed 1.03% apoptosis rate. Compared with HepG2 cells treated with 10 μM (4.49%) and 20 μM (5.84%), the rate of apoptosis was higher (16.41%) when exposed to 40 μM quercetin.

Depletion of mitochondrial membrane potential (ΔΨm)

As shown in Fig. 3, a bright field revealed that quercetin decreased the number of alive cells and caused more death as its concentration increased. When the mitochondrial membrane potential was high, JC-1 monomers assembled to form J-aggregates emitting red fluorescence. In contrast, green fluorescence was generated when the potential was low, which is a symbolic event in the early stage of apoptosis. As shown in Fig. 3A, with the increase in concentration, the mitochondrial membrane potential in the cell gradually decreases. Fig. 3B shows the result of the quantization of green fluorescence intensity using IMAGE J. The green fluorescence increased gradually with concentration, suggesting that Quercetin could down-regulate mitochondrial membrane potential.

Induction of caspase cleavage by quercetin

Caspase-3 plays a pivotal role in the process of cell apoptosis (Jiang *et al.*, 2020). As shown in Fig. 4, the caspase-3 activity of the control group was 100%, while HepG2 treated with 10, 20, and 40 μM quercetin, exhibited 115%, 143%, and 231% caspase-3 activity. These data indicated that quercetin markedly activated caspase-3 activity and promoted the rate of apoptosis.

Induction of reactive oxygen species generation by quercetin

ROS plays an important role in cell apoptosis. The ROS generation was detected by DCFA fluorescence assay. As shown in Fig. 5A, a remarkably increased accumulation of the intracellular ROS was observed as the concentration of quercetin increased (control 100%, 10 μM 124%, 20 μM 137%, 40 μM 192%), consistent with a previous study (Kim *et al.*, 2014). The fluorescence intensity of the quercetin-treated HepG2 cells is shown in Fig. 5B.

Activation of reactive-oxygen species-mediated apoptotic signaling pathways by quercetin

Excess generation of ROS can cause DNA damage, induce the expression of pro-apoptotic proteins, and restrict the anti-apoptotic protein. The effects of quercetin on the expression

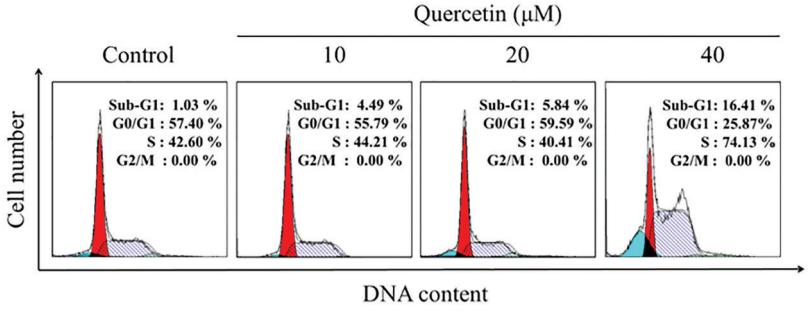

FIGURE 2. Flow cytometric analysis of HepG2 cells after treatment with quercetin for 24 h incubated with PI after immobilization with 70% precooled ethanol. The cell cycle distribution and apoptotic cell population were determined by a flow cytometer.

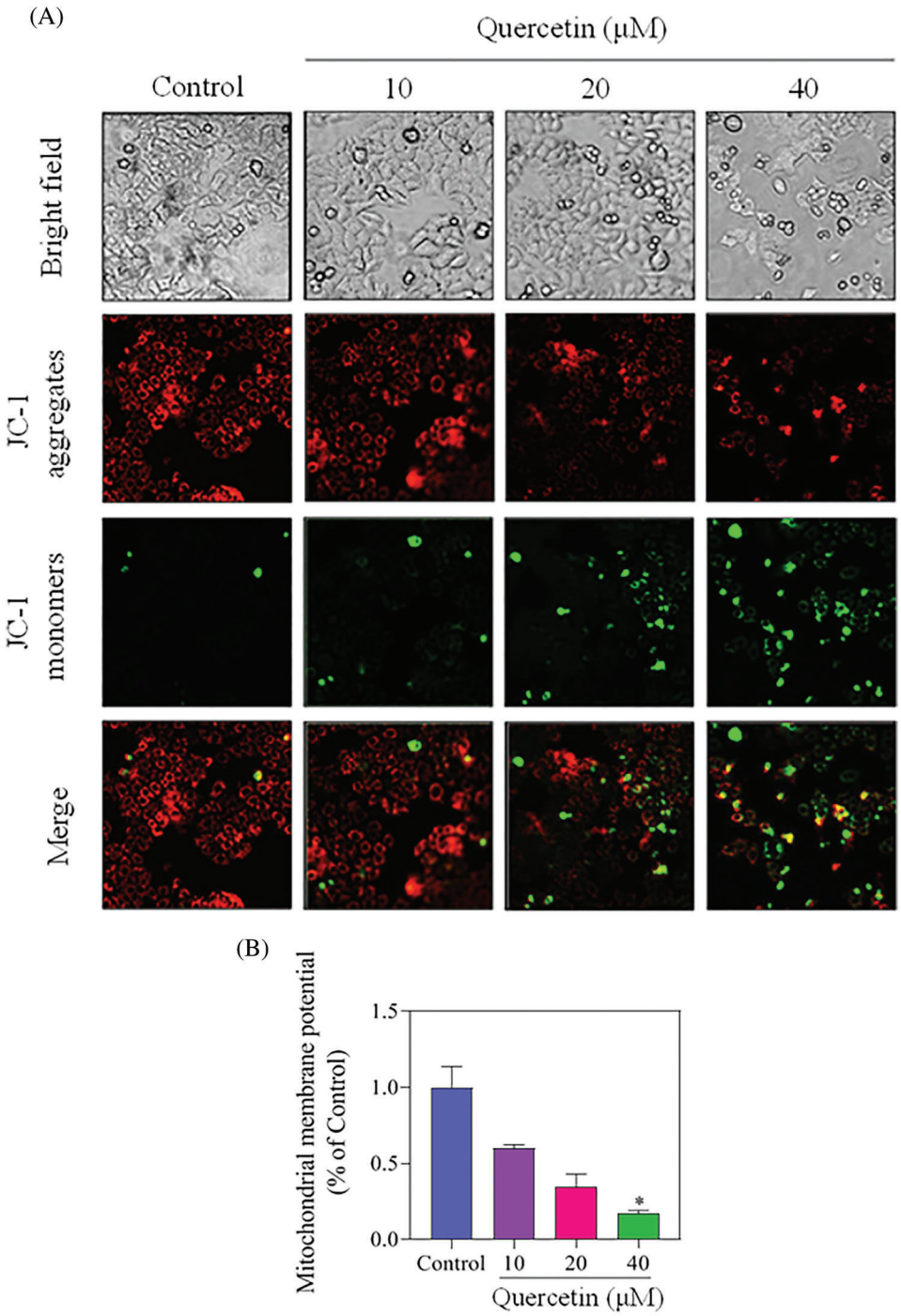

FIGURE 3. The mitochondrial membrane potential of quercetin-treated cells for 24 h was detected with JC-1 fluorescent probe under a fluorescent microscope. (A) Fluorescence microscope photograph of JC-1 staining. (B) IMAGE J software was used to quantify the green fluorescence of the JC-1 monomer (n = 3).

of these proteins were detected by western blotting. As shown in Fig. 6A, the expression of P-ATM, JNK, and Bax were distinctly activated, and STAT-3 was significantly suppressed with a gradually increased concentration of quercetin. Collectively, these data demonstrated that quercetin induced HepG2 cell apoptosis through ROS-mediated P-ATM, JNK, Bax, and STAT-3 signaling pathways, as shown in Fig. 6B.

Discussion

Quercetin is the major representative of the important bioactive flavonoids and possesses anti-inflammatory (Hou *et al.*, 2019), anti-oxidation (Song *et al.*, 2020b), anti-diabetic (Bule *et al.*, 2019), and anti-allergic properties. Quercetin exerts anti-inflammatory effects by reducing the expression of inflammatory factors such as interferon-γ and tumor necrosis factor-α on the skin of mice with specific dermatitis. Quercetin acts as an antioxidant by interacting with DNA. In terms of the anti-diabetic role, quercetin reduces serum glucose mainly through antioxidant action and regulation of liver gene expression. In terms of anti-allergy, quercetin alleviates the inflammatory response of allergic conjunctivitis by inhibiting the membrane-associated

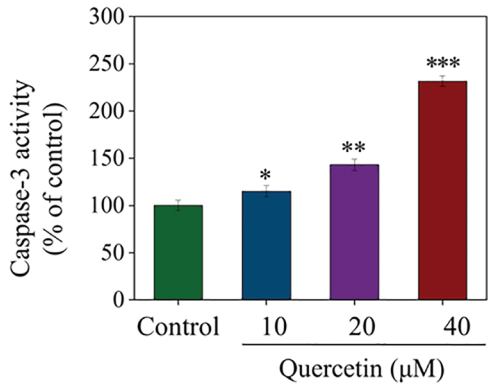

FIGURE 4. The activation of caspase-3 was triggered by different concentrations of quercetin, as examined via the synthetic fluorogenic substrate. Bars with different characters are statistically different at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ (n = 3).

protein tyrosine kinase (Lyn) (Ding *et al.*, 2020). In previous studies, the anti-tumor activity of quercetin was reported (Azeem *et al.*, 2022), which inhibits tumor cell proliferation and downregulates tumor protein expression (Ozkan and Bakar-Ates, 2020; Song *et al.*, 2020a). *In vitro* experiments showed that quercetin alone can induce apoptosis of 13 hepatocellular carcinoma cell lines. On the other hand, quercetin conjugator quercetin-3-O-glucoside (Q3G) shows a strong ability to inhibit DNA topoisomerase II and induces apoptosis of HepG2 cells by activating caspase-3. Q3G is also a potential anticancer agent for liver cancer, and its mechanism of action may be cell cycle arrest and apoptosis induction (Sudan and Rupasinghe, 2014).

Here, we have examined the effect of quercetin in inducing apoptosis of tumor cells. Assessment of the *in vitro* anticancer activity of quercetin revealed that as the concentration of quercetin increased, the cell viability decreased, and the number of cells with normal morphology decreased. This suggests that quercetin distinctly suppressed HepG2 cell proliferation. Cell cycle and apoptosis can be observed directly by flow cytometry. The increase in apoptotic peaks indicated that quercetin inhibited cell proliferation through apoptosis in a concentration-dependent manner. The early apoptosis can be reflected by the depletion of mitochondrial membrane potential and is revealed by green fluorescence in the JC-1 experiment. The results further demonstrate that quercetin contributed to HepG2 apoptosis and had an impact on mitochondria.

ROS is related to tumors, nervous diseases, cardiovascular diseases, etc. (Climent *et al.*, 2020; Kang *et al.*, 2019; Tonnies and Trushina, 2017; Liu *et al.*, 2021). It is involved in the process of apoptosis, DNA damage, and mitochondrial damage (Moloney and Cotter, 2018; Sreedhar *et al.*, 2020; Srinivas *et al.*, 2019). When HepG2 cells were treated with increasing concentration of quercetin, the production and fluorescence intensity of ROS increased, which collectively indicated that quercetin induced ROS generation. Excessive production of ROS can activate the anti-tumor signaling pathway, leading to oxidative stress-induced apoptosis. Western blotting showed that P-ATM, JNK, Bax, and STAT-3 were involved. The expression of P-ATM, JNK, and Bax was up-regulated, which suggested that they promote apoptosis through ROS generation downstream (Niu *et al.*, 2021; Dandoti, 2021). The expression of STAT-3 was decreased, indicating the inhibition of its cell growth and survival promotion function.

Research into new biomaterial technologies such as drug-supported nanomaterials and nano-antioxidants is increasing. Quercetin-loaded nano-lipid structure showed protective and antioxidant activity against paraquat-induced cytotoxicity, and quercetin nanoparticles showed a protective effect against aflatoxin B1-induced hepatotoxicity (Ahmadian *et al.*, 2020; Eftekhari *et al.*, 2018). Therefore, quercetin can be combined with nanomaterials for the synergistic treatment of liver diseases.

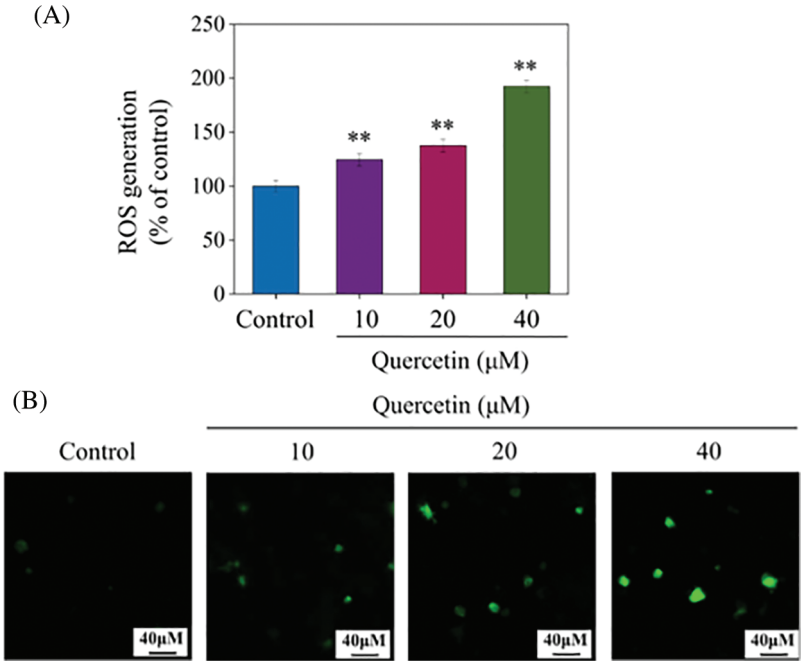

FIGURE 5. The overproduction of ROS induced by quercetin. (A) ROS levels were detected by 10 μM DCF fluorescence using a microplate spectrophotometer. (B) The intracellular fluorescence intensity was observed by a fluorescent microscope. Bars with different characters are statistically different at $^{*}p < 0.05$ or $^{**}p < 0.01$ (n = 3).

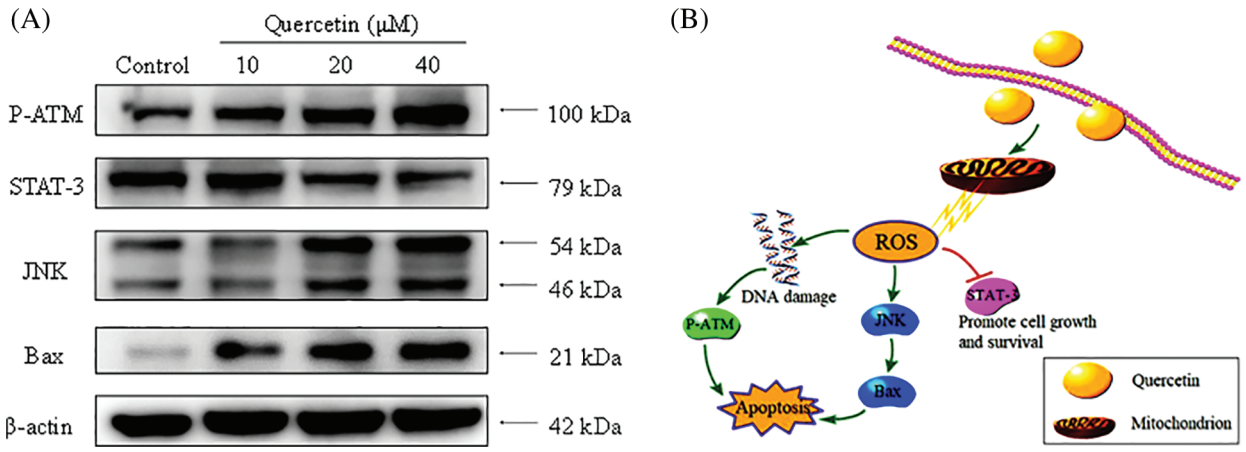

FIGURE 6. The apoptotic signaling pathways were activated by quercetin. (A) The HepG2 cells exposed to different concentrations of quercetin expressed the pro-apoptotic and anti-apoptotic protein tracked by western blotting. β-actin was used as the loading control. (B) The apoptotic signaling pathways induced by quercetin include ROS-mediated P-ATM, JNK, Bax and STAT-3 signaling pathways.

Conclusions

In summary, quercetin dramatically inhibited the dose-dependent proliferation of HepG2 cells. Additionally, the treatment enhanced the proportion of apoptotic cells, shown in the sub-G1 peak. The intracellular quercetin attacked mitochondria and caused repression of mitochondrial membrane potential, which triggered excess ROS production. The expression of the pro-apoptotic protein was activated through ROS downstream P-ATM, JNK, and Bax signaling pathways. Therefore, our study suggests that quercetin with anti-tumor properties may be a promising flavonol compound in the treatment of HCC.

Availability of Data and Material: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author Contribution: Draft manuscript preparation: WTL; Experiments carry out: DYC and JYS; Analysis of results: RLZ; Data collection RK; Study design: BZ, HD, and YHL. All authors reviewed the final version of the manuscript.

Ethics Approval: This project was approved by the Ethics Committee of Guangzhou Women and Children Medical Center (Approval No. 2017021803).

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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