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Brucea javanica oil inhibits proliferation of hepatocellular carcinoma cells and induces apoptosis via the PI3K/AKT pathway

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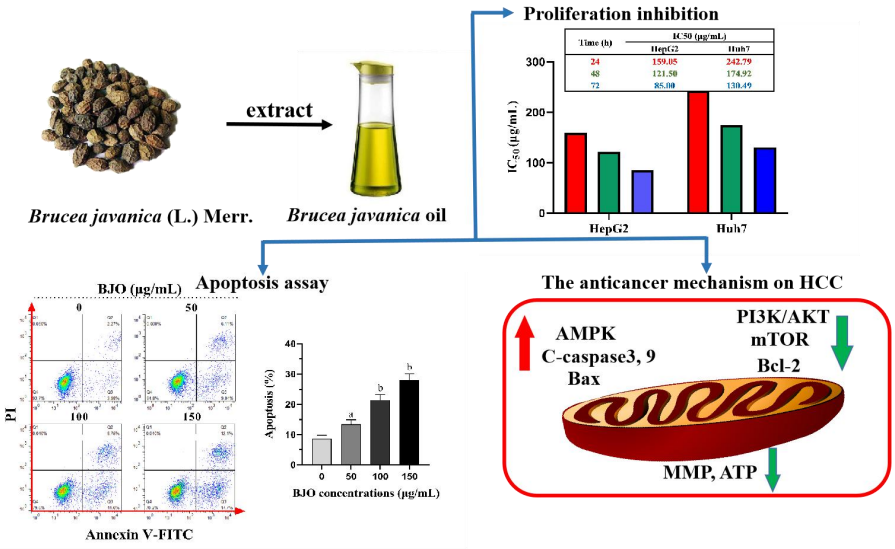
Highlights

Brucea javanica oil, extracted from traditional Chinese medicine Ya-dan-zi (*Brucea javanica* (L.) Merr.), inhibits proliferation of hepatocellular carcinoma cells and induces apoptosis through the PI3K/AKT pathway by upregulating C-caspase3, C-caspase9, Bax, and adenosine monophosphate-activated protein kinase, and downregulating Bcl-2 and mechanistic target of rapamycin.

Tradition

Ya-dan-zi (*Brucea javanica* (L.) Merr.), a dry fruit widely distributed in Southeast Asia, has been recorded in the ancient book of Chinese medicine, *Bencao Gangmu Shiyi* (*A Supplement to Compendium of Materia Medica*), written by the famous medical scientist named Zhao Xuemin in 1765 C.E. (Qing Dynasty of China). *Brucea javanica* oil, extracted from its mature seeds, has proven effective at treating hepatocellular carcinoma by promoting apoptosis, although the underlying mechanism remains to be determined.



Abstract

Background: *Brucea javanica* oil (BJO), distributed primarily in Southeast Asia, has long been utilized as a therapeutic agent for treating malignancies. However, its anticancer mechanisms are not clearly understood. The objective of this study was to examine the mechanisms underlying its treatment of hepatocellular carcinoma cells. **Methods:** CCK8 assay was used to evaluate cell viability. Hoechst33342 staining and flow cytometry analyses were used to examine apoptosis. Mito-Tracker Red CMXRos kit was used to measure the membrane potential of mitochondria. ATP assay kit was used to evaluate ATP levels. Western blots were used to assess the presence of AKT, adenosine monophosphate-activated protein kinase, Caspase3, Caspase9, Bax, and Bcl-2. **Results:** BJO inhibited the proliferation of hepatocellular carcinoma cells HepG2 in a time- and dose-dependent manner. It induced apoptosis, with the percentage of cells treated with 50–150 μg/mL BJO increasing from 8.01% to 28.02% in a concentration-dependent manner ($P < 0.05$, when 50 μg/mL of BJO group compared with the control group; $P < 0.001$, when 100 or 150 μg/mL of BJO group compared with the control group). After exposed to BJO, the expression of C-caspase3, C-caspase9 and Bax upregulated while that of Bcl-2 downregulated. BJO suppressed the PI3K/AKT pathway and promoted phosphorylation of adenosine monophosphate-activated protein kinase, while repressing the phosphorylation of mechanistic target of rapamycin. Compared with treatment by BJO alone, the PI3K/AKT agonist 740Y-P increased the survival rate of HepG2 cells ($P < 0.01$) and attenuated the inhibitory effect of BJO on cell apoptosis ($P < 0.05$). **Conclusion:** BJO is capable of inhibiting proliferation of HepG2 cells and inducing apoptosis via the PI3K/AKT pathway.

Keywords: *Brucea javanica* oil, Hepatocellular carcinoma, HepG2 cell, Cell proliferation, Cell apoptosis, PI3K/AKT

***Author contributions*:**

Yan-Peng Du contributed by conducting the laboratory research, analyzing the data and drafting the manuscript. Zhan Ye and Zhao-Jun Zheng contributed by analyzing the data and editing the manuscript. You-Dong Li and Jing Chen contributed by conducting the laboratory research and editing the manuscript. Farah Zaaboul contributed by editing the manuscript. Yong-Jiang Xu and Yuan-Fa Liu designed the study, supervised the laboratory research, and critically revised the manuscript for improving the content.

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***Abbreviations*:**

BJO, *Brucea javanica* oil; HCC, hepatocellular carcinoma; mTOR, mechanistic target of rapamycin; AMPK, adenosine monophosphate-activated protein kinase; TSC2, tuberous sclerosis complex 2; MMP, mitochondrial membrane potential.

***Competing interests*:**

The authors declare no conflicts of interest.

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Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. In 2018, the World Health Organization's Global Cancer Observatory reported 841,000 total cases and 782,000 deaths [1]. Unhealthy lifestyle habits, such as smoking, abusing alcohol, and eating foods contaminated with aflatoxin, as well as viral infections like hepatitis B and C, are recognized as the leading causes of HCC [1]. Nowadays, the most effective approach for treating HCC is hepatectomy; however, for patients with middle- or late-stage HCC are unfortunately not viable candidates [2]. Additionally, the rate of reoccurrence is quite high due to drug resistance, so finding novel therapies is a priority. In recent years, *Brucea javanica*, a plant commonly used in traditional Chinese medicine, has proven effective in HCC prevention [3, 4].

Brucea javanica (L.) Merr. is widely distributed in Southeast Asia and has been used in Chinese folk medicine for centuries to treat various diseases, such as malaria, dysentery, and many cancers [5, 6]. Its medicinal effects were first recorded in the ancient book of Chinese medicine, *Bencao Gangmu Shiyi* (*A Supplement to Compendium of Materia Medica*), written by the scientist, Zhao Xuemin, in 1765 C.E. *Brucea javanica* oil (BJO), extracted from the mature seeds of *B. javanica*, has been used since 1978 to treatment tumors.

Previous reports have identified fatty acids, quassinoids, triterpenoids, alkaloids, terides, and pregnanone in BJO, which are believed to be responsible for its diverse bioactivities [7, 8]. Numerous studies have reported that the use of BJO in a variety of cancer prevention or treatment regimens, including non-small or small cell lung cancer, bladder cancer, gastric cancer, and esophageal cancer is a safe and effective treatment option [6, 9, 10]. Importantly, BJO has also been reported to promote apoptosis in HCC cells [11, 12]; however, its underlying mechanisms have yet to be determined.

In cancer cells, death receptor-mediated apoptosis and mitochondrial-mediated apoptosis are two typical types of programmed cell death [13]. The mitochondrial-mediated pathway has been accepted as the most common type [14] and is regulated by the Bcl-2 family. Both pro-apoptotic and anti-apoptotic proteins regulate the permeability of the mitochondrial membrane to trigger cell apoptosis through cytochrome C release [14, 15]. The PI3K/AKT signaling pathway, a vital metabolic signaling pathway, is essential in regulating cell proliferation, apoptosis, migration, autophagy, and metabolism [16, 17]. It regulates apoptosis through the Bcl-2 family [18] and activates mechanistic target of rapamycin (mTOR) by inhibiting tuberous sclerosis complex 2 (TSC2) [19]. As an intracellular energy sensor, adenosine monophosphate-activated protein kinase (AMPK) can be suppressed by PI3K/AKT [20] and stimulate catabolism when intracellular energy supply is low, or in the event that conditions or mutations no longer make the cell viable [21]. Moreover, activation of AMPK can inactivate mTOR, thereby inhibiting anabolism [22] and furthering repression of cell proliferation and the initiating apoptosis [23, 24].

In this research, we investigated the biological activity of BJO and its underlying mechanisms in the treatment of HCC. Specifically, the inhibitory and apoptotic effects of BJO in HCC were examined, and apoptosis-related proteins (C-caspase3, C-caspase9, Bax, and Bcl-2) were quantified using Western blots. The expression levels of AKT, AMPK, and mTOR were determined after the administration of multiple doses of BJO. Protein levels, survival rates, and apoptotic activities were assessed following administration of the chemical activator, 740Y-P, to gain further insight into its molecular mechanisms.

Methods

Chemicals and reagents

Brucea javanica seeds were purchased from the Chinese herbal market of Bozhou, fetal bovine serum from Lonsa Science SRL (Uruguay) and 740Y-P from MedChemExpress (USA). The CCK8, Hoechst 33342 Kit, ATP assay kit, Mito-Tracker Red CMXRos kit, Annexin V-FITC apoptosis detection kit and ECL system detection kit were purchased from Beyotime Biology Co., Ltd. (China); AKT, p-AKT, p-AMPK, GAPDH antibodies were purchased from Abcam (UK), and Caspase3, Caspase9, Bax, Bcl-2, mTOR, p-mTOR antibodies from Wanlei Biotechnology Co., Ltd. (China).

Preparation of BJO

Following the methods outlined in previous studies [25], the extraction process was as follows: *Brucea javanica* seeds were pulverized and sieved, then placed in a conical flask with a rubber stopper (1 L). Anhydrous ethanol was added (24: 1 (mL/g)) and extracted at 70 kHz in an ultrasonic cleaning bath (KQ-300DE, Kunshan Ultrasonic Instrument Co., Ltd., China). The liquid was filtered twice, and then evaporated with the rotary (RE-3000 rotary evaporators, Shanghai Yarong Biochemical Instruments Co., Ltd., China) at 65 °C. Petroleum ether was added for extraction, the supernatant was removed, and then evaporated on the rotary at 45 °C. The original solution with BJO at 100 mg/mL was prepared by a phacoemulsification. Briefly, refined soy phospholipids (1.5%, w/v) were dissolved in water, and then mixed with BJO (10%, w/v). The mixture was subjected to an ultrasonic to obtain the original BJO solution. The working solution was prepared by diluting the original solution to the corresponding

concentration in the cell medium.

Cell lines and cultures

All cells (HepG2, Huh7, and L02) were purchased from Shanghai Institute of Biochemistry and Cell Biology (China). The cell medium contained 10% fetal bovine serum and 1% penicillin/streptomycin, and the cells were cultured at 37 °C in a 5% CO_2 environment.

Cell viability assay

Cell viability was evaluated by a CCK8 assay. Briefly, cells in logarithmic phase were inoculated in 96-well plates at 5×10^3 cells/well. After treatment with BJO at various concentrations (0–200 μg/mL) for 24, 48 and 72h, 10 μL of the CCK8 solution of was added and incubated at 37 °C, 5% CO_2 for 2 h. Absorbance was measured by a microplate reader (BioTek, USA).

Hoechst 33342 staining

Morphology of the apoptotic nuclei was examined using Hoechst 33342 staining. After the HepG2 cells were treated with BJO (0, 50, 100, or 150 μg/mL) for 24 h, the cells were stained with Hoechst 33342 staining solution at 37 °C, 5% CO_2 for 20 min, and then immediately observed with a fluorescence microscope (Axio Vert A1, Carl Zeiss, Germany).

Flow cytometry analysis of apoptosis

Apoptosis was detected by flow cytometry. Briefly, the cells were collected after treatment with BJO (0, 50, 100, or 150 μg/mL) for 24 h. Then, 1×10^5 cells were resuspended with Annexin V-FITC conjugate solution, followed by 5 μL of Annexin V-FITC and 10 μL of PI staining solution. The samples were evaluated using a flow cytometer (BD FACSCalibur, USA). Annexin V-FITC-positive/PI-negative was deemed as early apoptosis, and Annexin V-FITC-positive/PI-positive was deemed as late apoptosis; the total proportion of apoptotic cells was the sum of early and late apoptosis.

Mitochondrial membrane potential (MMP) assay

MMP was evaluated with the Mito-Tracker Red CMXRos kit. Following the description by manufacturer, the Mito-Tracker Red CMXRos working solution was added to the HepG2 cells treated with BJO (0, 50, 100, or 150 μg/mL) and incubated at 37 °C for 25 min, then immediately observed with a fluorescence microscope (Image Xpress Micro XLS, USA).

Detection of ATP Level

ATP levels were detected by ATP assay kit, whose principle is that ATP is required to provide energy when firefly luciferase (also known as luciferase) catalyzes luciferin to produce fluorescence. After receiving treatment by BJO (0, 50, 100, or 150 μg/mL), the cells were fully lysed at 4 °C, and the supernatant was collected after centrifuging at 1.2×10^4 g for 5 min. Then 100 μL of ATP detection working solution was added to the detection well, after keeping it at room temperature for 4 min, 20 μL of the sample was added, and detected with a luminometer (BioTek Synergy H4, BioTek USA) after 2 s.

Western blot analysis

After being treated by BJO (0, 50, 100, or 150 μg/mL), HepG2 cells were lysed, 10% SDS-PAGE was employed to separate the proteins, then they were transferred to a PVDF membrane. After blocking with 5% skim milk or BSA for 1 h, the membranes were incubated overnight at 4 °C with different primary antibodies, including anti-Caspase3, anti-Caspase9, anti-Bax, anti-Bcl-2, anti-mTOR, anti-AKT, anti-p-AMPK, anti-p-mTOR, anti-p-AKT, and anti-GAPDH. They were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the protein signal was detected by chemiluminescence detector (LI-COR C-Digit, LI-COR Biosciences, USA).

Statistical analysis

All the data are expressed in the form of mean ± SD, and all experiments were carried out independently three times. Duncan's multiple range test was conducted using SPSS; $P < 0.05$ was considered significant.

Results

BJO decreases the survival rate of HCC cells

HepG2 and Huh7 cells were treated with different concentrations (0–200 μg/mL) of BJO, respectively, and the anticancer effects of BJO on HCC cells were detected after 24, 48, and 72 h. As shown in Figure 1 and Table 1, the proliferation of HepG2 and Huh7 cells were inhibited in a dose- and time-dependent manner, and the IC_{50} values of BJO on HepG2 cells at 24, 48, and 72 h were 159.05, 121.50, and 85.00 μg/mL, and for the Huh7 cells, findings were 242.79, 174.92, and 130.49 μg/mL, respectively. Interestingly, BJO presented no significant effects on the survival rate of normal hepatocyte L02 cells at each corresponding concentration, indicating it did not have cytotoxic effects on normal hepatocytes.

BJO triggers apoptosis in HepG2 cells

After treatment with BJO (0, 50, 100, or 150 μg/mL) for 24 h, apoptosis was evaluated using Hoechst staining and annexin V-FITC/PI staining. To illustrate, Hoechst 33342 is a nuclear dye penetrating the cell membrane, and pyknotic nuclei in cells undergoing apoptosis will be stained bright blue by Hoechst. As shown in Figure 2A, the number of pyknotic nuclei in HepG2 cells increased as the dose of BJO increased. Pyknosis, the primary change to the nucleus during apoptosis, signifies death of cells [26]. The Annexin

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V-FITC/PI staining showed that the rising BJO concentrations induced dose-dependent increases in the number of early apoptotic and late apoptotic cells (Figure 2B). The number of apoptotic cells increased from 8.01% to 28.02% ($P < 0.05$, when 50 μg/mL of BJO group compared with the control group; $P < 0.001$, when 100 and 150 μg/mL of BJO groups compared with the control group) (Figure 2C). Moreover, C-caspase3, C-caspase9, and the pro-apoptotic protein, Bax were upregulated, whereas the anti-apoptotic protein, Bcl-2, was downregulated (Figure 2D). These results suggest that BJO induces apoptosis via the mitochondrial-meditated pathway in HCC cells.

Table 1 The IC_{50} of BJO on HepG2 and Huh7 cells.

Time (h)	IC_{50} (μg/mL)	
	HepG2	Huh7
24	159.05	242.79
48	121.50	174.92
72	85.00	130.49

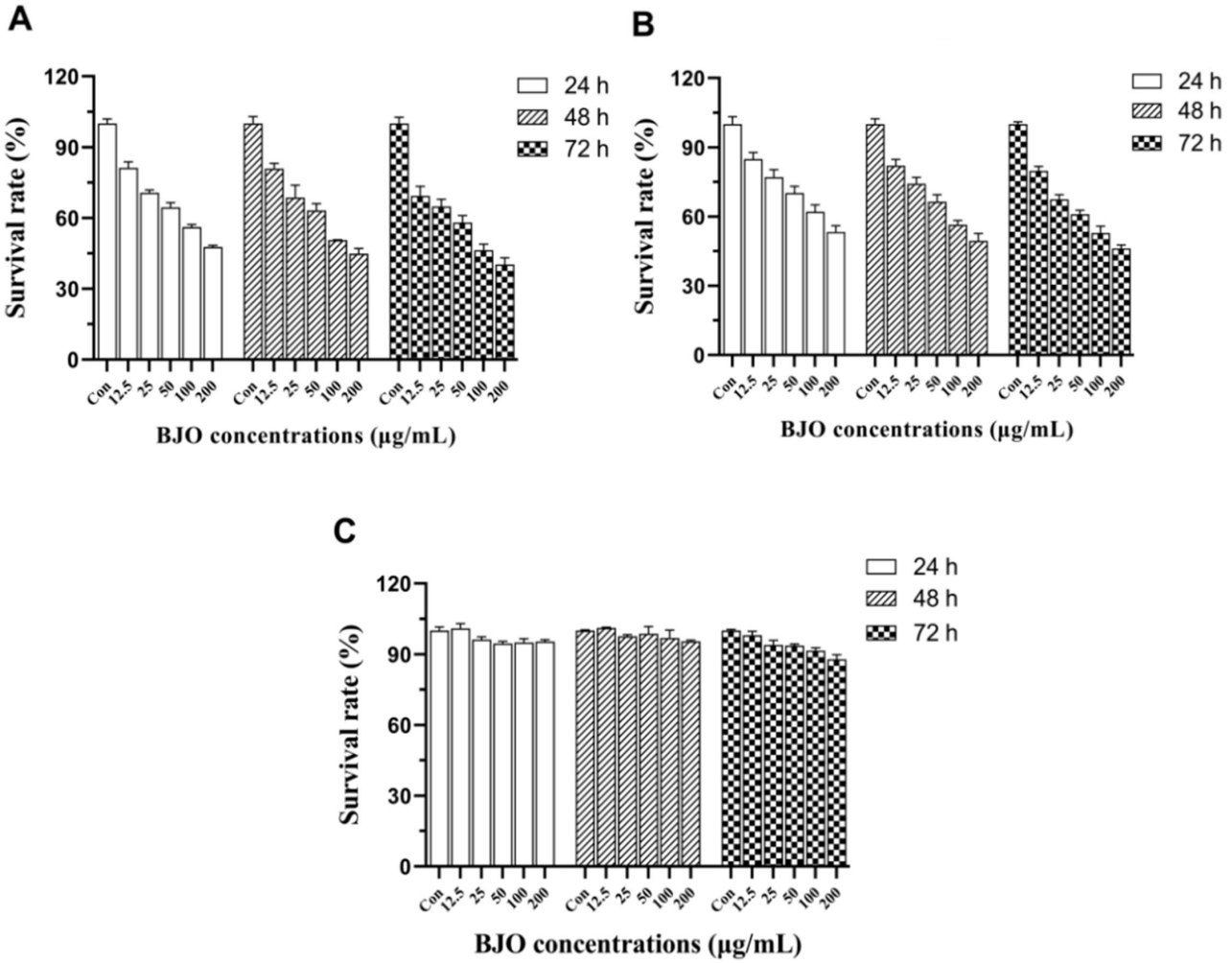

Figure 1 Effects of BJO on the survival rate of HepG2 (A), Huh7 (B), and L02 cells (C). The cells were treated with different concentrations of BJO (0, 12.5, 25, 50, 100, or 200 μg/mL, respectively) for 24 h, 48 h, and 72 h. Cell viability was evaluated by the CCK8 assay. Data are shown as the mean ± SD from three independent experiments.

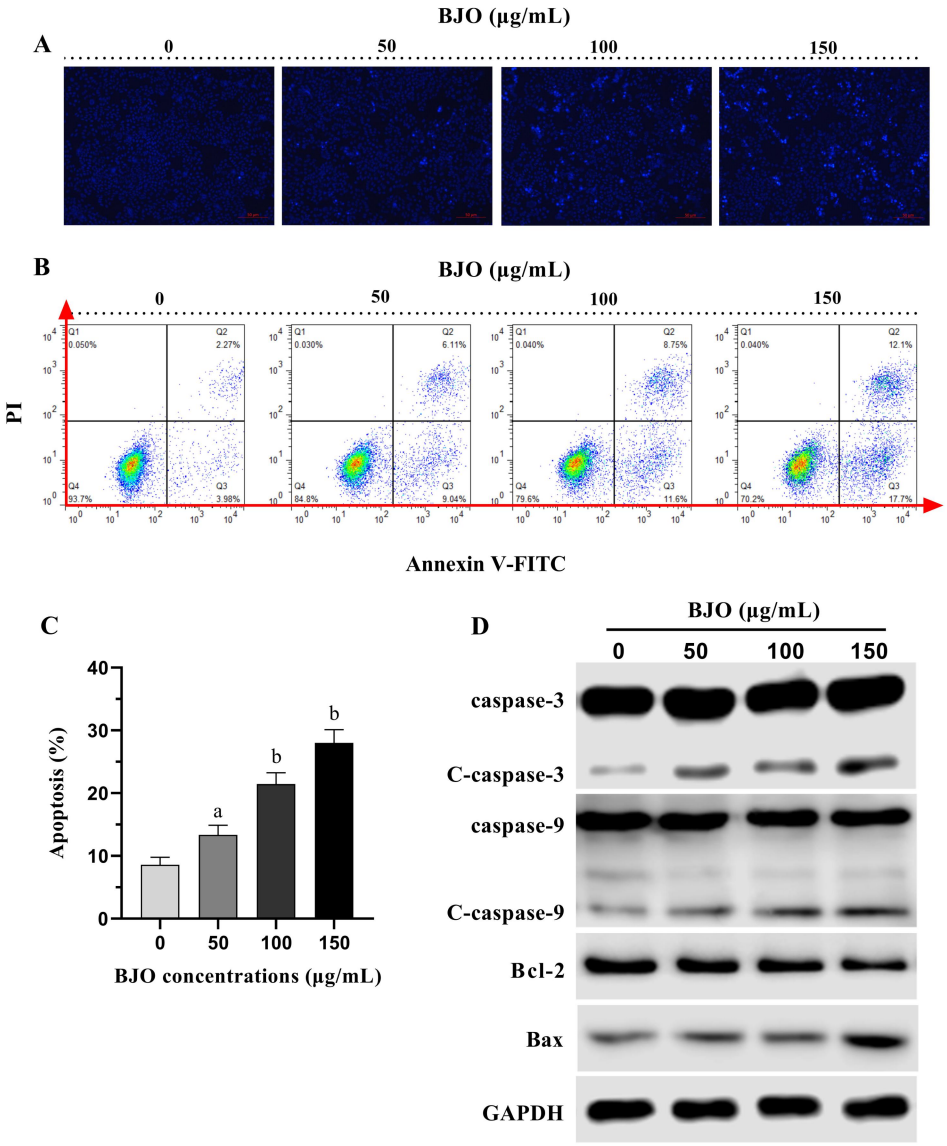

Figure 2 BJO induces HepG2 cell apoptosis. HepG2 cells were treated with different concentrations of BJO (0, 50, 100, or 150 μg/mL, respectively) for 24 h. (A) Hoechst 33342 staining images. (B) Detection of apoptosis by flow cytometry with Annexin V/PI staining. (C) Statistical analysis of the apoptotic cells. Data are shown as the mean ± SD from three independent experiments. a: $P < 0.05$ vs. control group, b: $P < 0.001$ vs. control group. (D) Apoptosis-related protein expression levels were detected by Western blot analysis.

BJO downregulates MMP and ATP levels in HepG2 cells

To further verify whether the mitochondrial-mediated pathway was involved in BJO-induced apoptosis, we observed the morphology of mitochondria in BJO-treated HepG2 cells using Mito-Tracker Red CMXRos staining, and the results showed the red fluorescence intensity gradually diminished after increasing the concentration of BJO (Figure 3A). Mito-Tracker Red CMXRos is an X-rosamine (Chloromethyl-X-rosamine, a CMXRos) derivative with cell permeability, which can specifically label mitochondria showing biological activities in cells. Such a dye emits bright red fluorescence inside mitochondria of normal cells. Therefore, the dwindling of red fluorescence will be indicative of apoptosis and continue as MMP decreases, since the stability of MMP is a key factor for securing physical biofunctions of mitochondria [27]. With regard to ATP levels, results showed that when compared with the control, the ATP level in BJO-treated HepG2 cells presented a downward trend ($P < 0.05$, when 50 μg/mL of BJO group compared with the control group; $P < 0.001$, when 100 and 150 μg/mL of BJO groups compared with the control group) (Figure 3B). Collectively, these suggest that BJO leads to mitochondrial dysfunction by damaging MMP, which in turn reduces ATP production. This also tells us that the mitochondrial-mediated pathway is involved in

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BJO-induced apoptosis.

Effects of BJO on the PI3K/AKT pathway

The PI3K/AKT pathway, as a vital cellular signaling pathway, regulates cell proliferation, cycle, apoptosis, migration, autophagy, and metabolism. However, abnormal activation of this pathway is known to be closely correlated with the development of tumors [16, 17]. To evaluate the effect of BJO on the PI3K/AKT pathway, we analyzed the activation level of AKT in BJO-treated HepG2 cells. The results showed that the (phosphorylated) p-AKT/AKT ratio decreased after increasing the concentration of BJO, and BJO inhibited the phosphorylation of AKT in a dose-dependent manner (Figure 4A). These results suggest that BJO suppresses the PI3K/AKT pathway.

Inhibition of PI3K/AKT can reduce the inactivation of AMPK [20], and a decreased ATP level usually also results in an increased AMP/ATP ratio, further enhancing the activation level of the AMPK pathway [28]. Moreover, PI3K/AKT and AMPK may regulate the activation level of mTOR, which plays a pivotal role in cell growth, proliferation, and apoptosis [29, 30]. To assess the effect of BJO on AMPK and mTOR activity, we analyzed the activation level of the two target proteins in BJO-treated HepG2 cells. As a result, BJO facilitated AMPK phosphorylation in a dose-dependent manner and repressed the p-mTOR/mTOR ratio after increasing the concentration of BJO (Figure 4B). Previous studies have uncovered that inhibiting the PI3K/AKT pathway can attenuate activation of mTOR [31]. Yet, AMPK activation can also suppress the phosphorylation of mTOR, thereby inhibiting its activation [23].

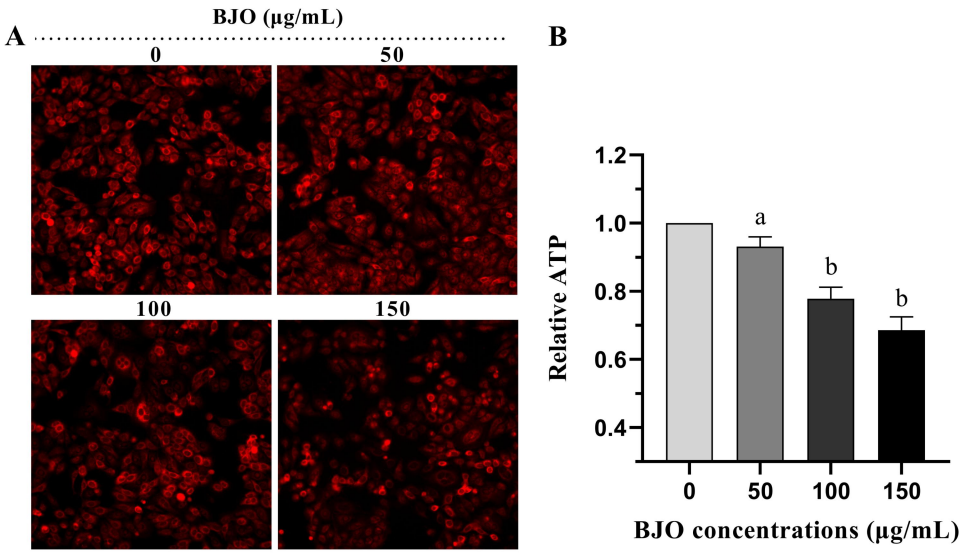

Figure 3 Effects of BJO on MMP and ATP levels in HepG2 cells. (A) HepG2 cells were treated with different concentrations of BJO (0, 50, 100, or 150 μg/mL, respectively) for 24 h and MMP was measured with the Mito-Tracker Red CMXRos kit. (B) ATP levels were detected by an ATP assay kit, Data are shown as the mean ± SD from three independent experiments. a: $P < 0.05$ vs. control group, b: $P < 0.001$ vs. control group.

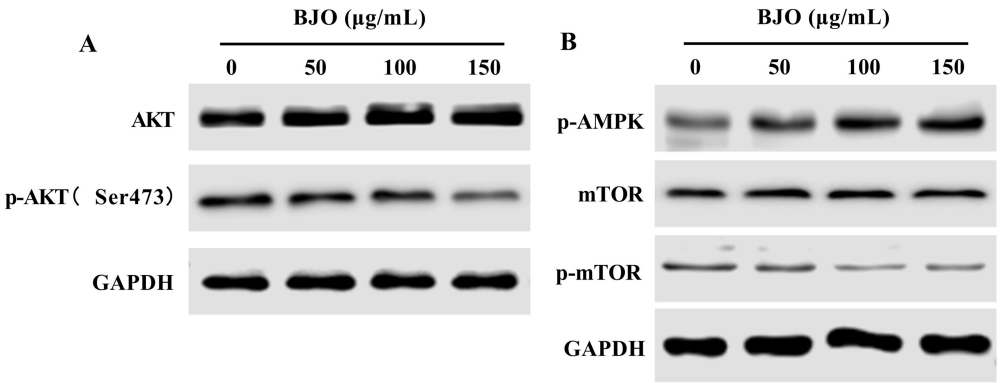

Figure 4 Effects of BJO on the PI3K/AKT pathway in HepG2 cells. (A) HepG2 cells were treated with different concentrations of BJO (0, 50, 100, or 150 μg/mL, respectively) for 24 h and the protein levels of AKT and p-AKT (Ser473) were measured by Western blot analysis. (B) Protein levels of p-AMPK and p-mTOR were measured by Western blot analysis.

BJO inhibited cell proliferation and triggered apoptosis in HepG2 cells via the PI3K/AKT pathway

As previously addressed, BJO may exert its anticancer activity through the PI3K/AKT pathway. Furthermore, to verify whether the induction of apoptosis and inhibition of cell proliferation were triggered by the PI3K/AKT signaling pathway, we employed a PI3K/AKT activator, 740Y-P. It was found that the survival rate increased ($P < 0.01$ compared with treatment by BJO alone) and the rare of apoptosis decreased ($P < 0.05$ compared with treatment by BJO alone) in the BJO + 740Y-P group compared with the BJO treatment group (Figure 5A–5C). In addition, 740Y-P reversed the lowered p-AKT/AKT ratio and Bcl-2 levels, as well as the elevated levels of p-AMPK, C-caspase3 and Bax that were induced by BJO (Figure 5D). These results suggest that BJO inhibits cell proliferation and induces apoptosis in HepG2 cells by repressing the PI3K/AKT signaling pathway (Figure 6).

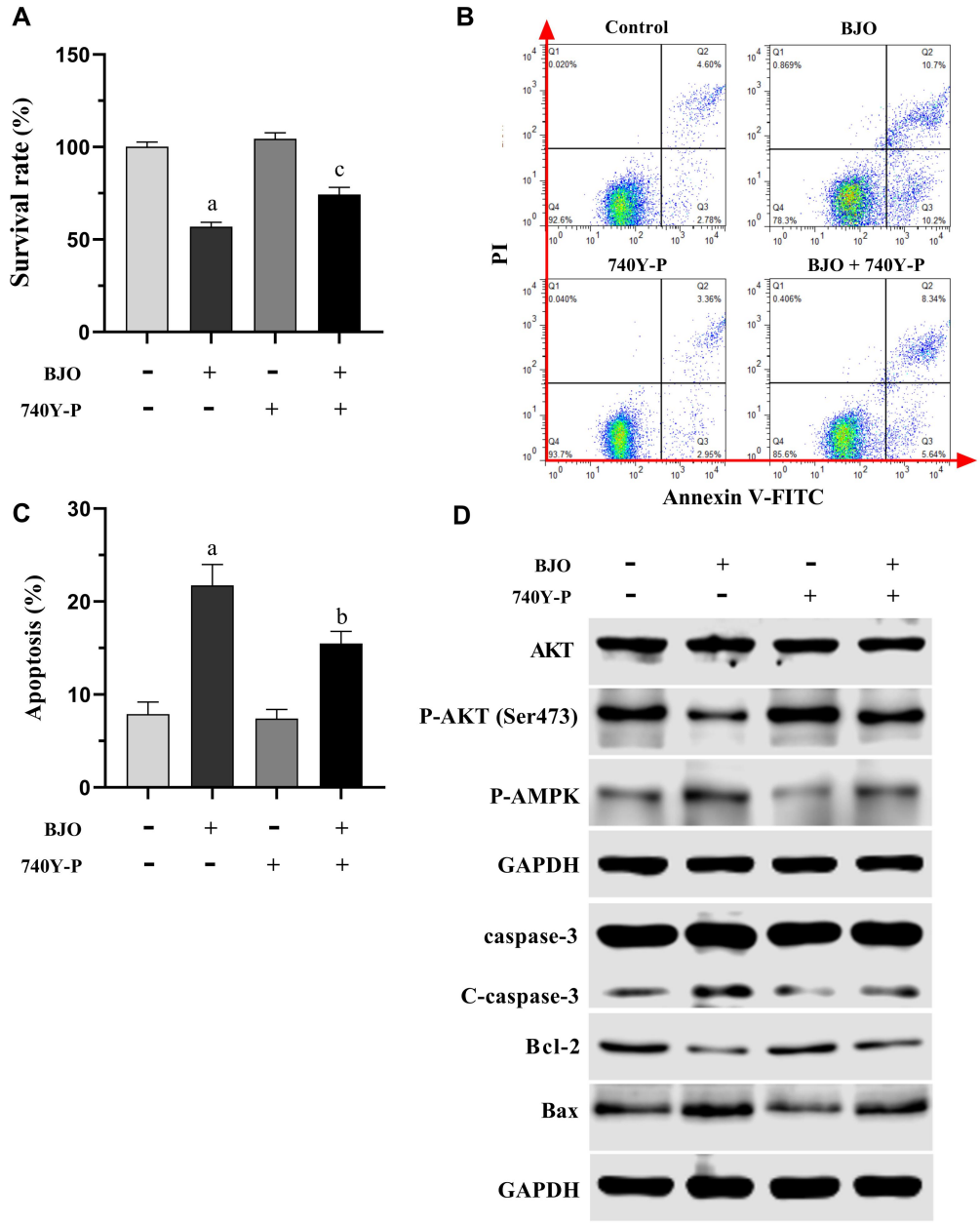

Figure 5 BJO inhibits proliferation and induces apoptosis via the PI3K/AKT pathway in HepG2 cells. (A) HepG2 cells were treated with 100 μg/mL of BJO alone or combined with 15 μM of 740Y-P for 24 h, then cell survival rate was evaluated by the CCK8 assay. (B and C) Apoptosis of HepG2 cells was detected by flow cytometry analysis using Annexin V-FITC/PI staining. (D) Protein levels of p-AKT (Ser473), p-AMPK and apoptosis-related proteins were detected by Western blot analysis. Data are shown as the mean ± SD from three independent experiments. a: $P < 0.001$ vs. control group, b: $P < 0.05$ vs. cells treated with BJO alone, c: $P < 0.01$ vs. cells treated with BJO alone.

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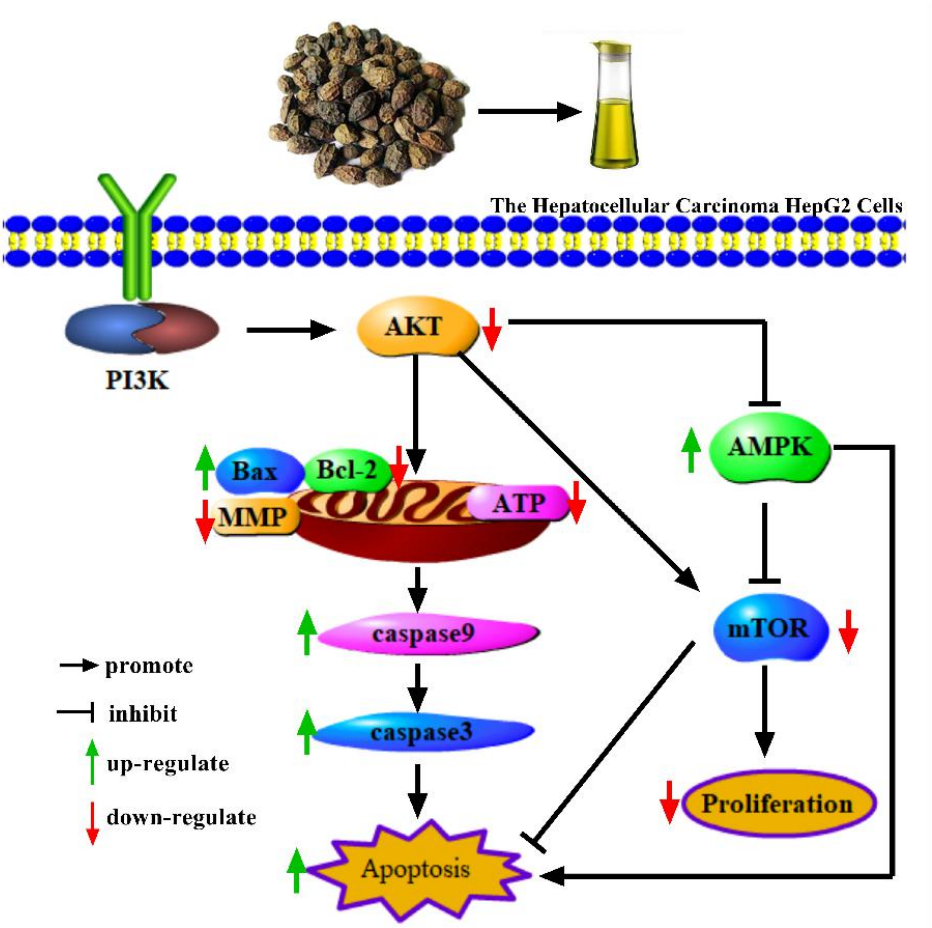

Figure 6 The proposed underlying mechanism of BJO in inhibiting proliferation of HepG2 cells and inducing apoptosis via the PI3K/AKT signaling pathway.

Discussion

BJO, derived from a medicinal Chinese plant, has been proven to be clinically effective at treating a variety of tumors. For example, Wang et al. have found that BJO inhibits the proliferation of the non-small cell lung cancer cell line, A549 and the small cell lung cancer cells line, H446, as well as initiates G0/G1 cell cycle arrest and fosters apoptosis [9]. Wu JR et al. have reported that BJO, when combined with chemotherapy, is effective at treating gastric cancer [32]. However, so far, there has been relatively little research on the impact of BJO on HCC cells and its underlying mechanisms. In this paper, we found that BJO could effectively curtail the proliferation of HepG2 cells and trigger apoptosis by depressing the PI3K/AKT pathway.

Suppressing proliferation and facilitating apoptosis is a two-pronged approach in anticancer therapies. Increasing bodies of evidence have revealed that both can be achieved through the PI3K/AKT pathway [18, 33, 34]. PI3Ks, composed of the catalytic subunit, P110 and the regulatory subunit, P85, are activated when P85 interacts with growth factors. This promotes the conversion of phosphatidylinositol-4, 5-bisphosphate to phosphatidylinositol-3, 4, 5-trisphosphate. Subsequently, AKT and PDPK1 are recruited by phosphatidylinositol-3, 4, 5-trisphosphate, which constitutes a docking site for PH-containing proteins. As a result, AKT is phosphorylated and activated by PDPK1, triggering a wide range of downstream signaling events [35].

The PI3K/AKT signaling pathway is hyperactivated in various tumors, inhibiting apoptosis of cancer cells by modulating levels of Bcl-2 and Bax [17, 18]. Apoptosis, a cellular defense mechanism and one type of programmed cell death, can eliminate excess and uncontrolled cells from tissues [36]. Cancer cells, mutant cells that should be recognized and eliminated by the body's defense system, can often proliferate uncontrollably, destroying healthy organ and increasing risk of mortality. Numerous studies have shown that suppressing activation of the PI3K/AKT pathway can trigger apoptosis in cancer cells. For example, Silencing Rab31 induces apoptosis in HCC

cells by inhibiting the PI3K/AKT pathway and decreasing the Bcl-2/Bax ratio [18]. Polyphenols isolated from *Allium cepa* L. also inhibit cell growth and foster apoptosis by decreasing the activity of the PI3K/AKT pathway in gastric cancer cells [37]. Similarly, in our study, we found that BJO inhibited the PI3K/AKT pathway and triggered mitochondrial-mediated apoptosis by releasing a high amount of C-caspase3 and C-caspase9, upregulating Bax and downregulating Bcl-2. The elevated Bax/Bcl-2 ratio can increase mitochondrial membrane permeability with the collapse of MMP, and then cytochrome C will be released from mitochondria, activating caspase9 (the initiator of apoptosis) followed by caspase3 (the executioner of apoptosis) to finally promote apoptosis [36, 38].

In addition, studies have revealed that the PI3K/AKT pathway, as a regulator of ATP levels and AMPK, can elevate ATP levels and reduce AMPK activity [19, 20], and that AMPK can be activated when ATP production is low [21]. Numerous findings have revealed that AMPK activation induces apoptosis. For instance, hexavalent chromium (Cr (VI)) initiates mitochondrial-dependent apoptosis through the interdependent activation of AKT, ERK1/2, and AMPK [39]. AMPK hypoactivity is also critical in repressing *Moutan cortex radicis*-induced cell apoptosis in human gastric cancer cells [40]. In our study, we found that BJO-induced apoptosis was synchronized with enhanced phosphorylation of AMPK. This demonstrates the existence of a specific association between AMPK and BJO-induced cell apoptosis, but the exact mechanism underlying their interaction warrants further investigation.

mTOR regulates protein and lipid synthesis, ribosome biogenesis, autophagy and apoptosis through its effects on cell growth and proliferation [29]. AKT activates mTOR by inhibiting TSC2, a negative regulator of mTOR [19]. For example, Shahad W. Kattan, et al. have reported that the PI3K/AKT/mTOR pathway involves the anti-proliferative and apoptotic activities of androstane derivatives in human HCC cells [30]. Contrastingly, AMPK inhibits the phosphorylation of mTOR by activating TSC2 [19, 23]. Our experimental results showed that BJO suppressed phosphorylation of mTOR in a dose-dependent manner. mTOR upregulates anabolic activities such as protein and lipid synthesis, so the diminished mTOR activation indicates that cell growth and proliferation are inhibited. Previous research has shown that AMPK activation, along with mTOR inhibition, can promote catabolism, further reducing energy consumption by generating more ATPs and thwarting anabolic processes [22], as well as suppressing proliferation and inducing apoptosis [21, 41]. As the interaction between PI3K/AKT and mTOR were clarified above, we speculate that BJO exerts its anticancer activity through the PI3K/AKT pathway in HepG2 cells. The detailed diagram of the mechanism is shown in Figure 6.

Conclusion

The present study investigated the biological effects of the BJO on HepG2 cells, and the results show that BJO suppresses cell growth, likely through facilitating apoptosis and inhibiting cellular proliferation. In specific, BJO up-regulated the expression levels of C-caspase3, C-caspase9 and Bax, while down-regulated that of Bcl-2 of HepG2 cells. Moreover, phosphorylation of AKT and mTOR were decreased, while that of AMPK was increased, indicating that BJO likely induced apoptosis by interacting with the PI3K/AKT signaling pathway. This molecular mechanism was further verified using 740Y-P. These findings imply BJO has great potential in being utilized for the prevention and the treatment of HCC.

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