

# Jiedu Xiaozheng Yin decoction inhibits hepatoma cell proliferation by inducing apoptosis via the mitochondrial-mediated pathway

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**Abstract.** Jiedu Xiaozheng Yin decoction (JXY) is a type of Chinese traditional medicine, which has been used to treat various types of cancer. The present study explored the mechanisms underlying the anticancer activity of JXY. The effects of ethyl acetate extraction of JXY (EE-JXY) were evaluated on the HepG2 human hepatoma cell line *in vitro* and *in vivo*. Following treatment of the HepG2 cells with EE-JXY for 24 h, cell viability, apoptosis, mitochondrial membrane potential, caspase enzyme activity and the expression levels of apoptotic-associated proteins (Bcl-2 and Bax) were detected by MTT, flow cytometry, ELISA and western blotting respectively. In addition, HepG2 cells were subcutaneously transplanted into BALB/c nude mice, and the tumor bearing mice were treated with either EE-JXY (0.06 g/kg) or normal saline for 21 days. Tumor volume and weight were measured and recorded. The apoptotic index, and the expression levels of Bax and cytochrome *c* were determined with immunohistochemical staining. Treatment with EE-JXY inhibited the proliferation of HepG2 cells, and reduced cell viability in a dose- and time-dependent manner. Furthermore, EE-JXY induced HepG2 cell apoptosis, as demonstrated by a loss of plasma membrane asymmetry and externalization of phosphatidylserine, collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, and an increased ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2. Furthermore, EE-JXY inhibited tumor growth and increased the apoptotic index of tumors in tumor-bearing mice. In conclusion, the results of the present study suggest that JXY inhibits HepG2 cell proliferation through mitochondrion-mediated apoptosis, which may partially explain its anticancer activity.

## Introduction

The use of herbs for the prevention and treatment of disease has been practiced for thousands of years in various countries, including China, Japan and Korea (1,2). Particularly in cancer treatment, polyherbal Chinese traditional medicine (TCM) formulas are widely used as an adjuvant therapy to surgery, chemotherapy and radiotherapy (3,4). Certain TCM herbs and formulas have previously been reported to target cancer by regulating immune function, inhibiting angiogenesis, inducing apoptosis and reversing the multi-drug resistance of tumor cells (5-7). Since the identification of increasingly clarified molecular mechanisms, and the low toxicity of medicinal herbs, TCM has recently gained increased global attention.

The regulation of apoptosis in malignant cells is an area of extensive investigation in cancer research. Apoptosis may be induced by either intrinsic stimuli, such as cytokine deprivation and DNA damage; or extrinsic stimuli, such as death ligand-receptor engagement. Intrinsic and extrinsic apoptotic signaling eventually leads to the activation of cysteine-dependent aspartate-directed proteases, which are known as caspases, and nucleases, resulting in cellular destruction (8,9). The mitochondrial pathway is the main intrinsic apoptotic pathway, and is responsible for mitochondrion-dependent apoptosis. Pro-apoptotic and anti-apoptotic proteins regulate the permeability of the mitochondrial outer membrane (MOM) (10,11). Numerous plant-derived compounds, including matrine, quercetin and glaucocalyxin, have been shown to induce apoptosis via the mitochondria-mediated pathway in various cancer cell lines (12-14). In addition, unidentified components in the aqueous extracts of *Bryonia dioica*, *Hedyotis diffusa* Willd and *Prunella* have also been reported to induce apoptosis of tumor cells via the mitochondrion-dependent pathway (15-17). Furthermore, certain TCM antitumor formulas, such as Yi Guan Jian, Chan-Yu-Bao-Yuan-Tang and Ge-Jee-Bok-Ryung-Hwan, have been shown to induce apoptosis (18-20). Therefore, promoting cell apoptosis by regulating the mitochondrial pathway is an important antitumor mechanism of TCM.

Jiedu Xiaozheng Yin (JXY) is a formula that contains four anti-inflammatory and detoxification herbs: *Hedyotis diffusa* Willd (HDW) 30 g, *Sophora flavescens* (SF) 15 g, *Psuedobulbus cremastrae* (PC) 15 g and *Spica prunellae*

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15 g. Jiedu means clearing heat and detoxification; Xiaozheng means removing the mass (tumor); and Yin means water solution or decoction. According to the TCM theory, the accumulation of carcinogens and heat (fever symptoms) are key causative factor of tumorigenesis; therefore, it is concordant that anti-inflammation and detoxification are main principles in anticancer treatment (21). A previous study by our group demonstrated that the ethyl acetate fraction of JXY (EE-JXY) can inhibit the growth of HepG2 hepatoma cancer by arresting cells at G<sub>1</sub> phase of the cell cycle, and inhibiting angiogenesis by downregulating the expression levels of vascular endothelial growth factor (VEGF)-A and VEGFR-2 *in vivo* and *in vitro* (22,23). However the precise mechanisms regarding its antitumor activity remain largely unknown. Therefore, the present study aimed to investigate the molecular mechanisms of JXY-induced apoptosis in the HepG2 hepatoma cell line.

## Materials and methods

**Materials and reagents.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1), iBlot® Western Detection Stack/iBlot® Dry Blotting system, and caspase-3 and -9 colorimetric protease assay kits were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Rabbit anti-human monoclonal antibodies targeting Bcl-2 (cat. no. 2876) and Bax (cat. no. 2774) were obtained from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-mouse Bax monoclonal antibody (cat. no. MS-714) and rabbit anti-mouse cytochrome *c* monoclonal antibody (cat. no. MS-1273) were purchased from Fuzhou Maixin Biotech Co., Ltd. (Fuzhou, China). TumorTACS *in situ* Apoptosis Detection kit was obtained from Roche Palo Alto LLC (Palo Alto, CA, USA). Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Preparation of EE-JXY.** JXY is a TCM formula. Each dose of JXY contains: 30 g HDW, 15 g *Spica prunellae*, 15 g SF and 15 g PC. The four herbs were purchased from Guo Yi Tang Hospital of Fujian University of Traditional Chinese Medicine (Fuzhou, China). EE-JXY was prepared according to the methods outlined in our previous study (22). EE-JXY was dissolved in normal saline (NS, 6 mg/ml) for oral administration in mice.

**Cell culture.** The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The cells were subcultured at 80-90% confluence.

**Detection of apoptosis by flow cytometry.** Following incubation of the cells with various concentrations of EE-JXY (0, 0.05, 0.1, 0.2 and 0.4 mg/ml) for 24 h, the rate of apoptosis

was determined by flow cytometry, using fluorescence-activated cell sorting with a FACSCalibur™ (BD Biosciences). The apoptotic cells were identified using the Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit. The ratios of Annexin V<sup>+</sup>/PI<sup>-</sup> and Annexin V<sup>+</sup>/PI<sup>+</sup> cells were calculated using CellQuest™ software (version 3.3; BD Biosciences), which indicated the rates of early and late stage apoptosis, respectively.

**Measurement of mitochondrial membrane potential ( $\Delta\psi_m$ ) by flow cytometry.** JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, which is indicated by a fluorescence emission shift from green to red, and can be used as an indicator of mitochondrial potential. Briefly, 1x10<sup>6</sup> treated HepG2 cells were resuspended following trypsinization in 1 ml DMEM. The cells were then incubated with 10 µg/ml JC-1 at 37°C, in an atmosphere containing 5% CO<sub>2</sub>, for 30 min. Both red and green fluorescence emissions were analyzed by flow cytometry after JC-1 staining.

**Analysis of caspase activation.** The activity of caspase-3 and -9 was determined by colorimetric assay using caspase-3 and -9 activation kits respectively, according to the manufacturer's instructions. Briefly, following treatment with various concentrations of EE-JXY for 24 h, the HepG2 cells were lysed with lysis buffer (Beyotime Inc., Shanghai, China) on ice. The lysed cells were then centrifuged at 16,000 x g for 10 min. The protein concentration of the clarified supernatant was determined using the Bradford assay and 100 µg of each protein sample was incubated with 50 µl of the colorimetric tetrapeptides: Asp-Glu-Val-Asp (DEAD)-p-nitroaniline (pNA), which is the specific substrate of caspase-3; or Leu-Glu-His-Asp (LEHD)-pNA, which is the specific substrate of caspase-9, at 37°C in the dark for 2 h. The absorbance of the samples was measured at 405 nm in an ELISA plate reader (EXL800; BioTek Instruments, Inc., Winooski, VT, USA). The data were normalized to the activity of the caspases in control cells, and are presented as 'fold of control'.

**Western blot analysis.** A total of 2x10<sup>5</sup> HepG2 cells were seeded into six-well plates in 2 ml DMEM and treated with various concentrations of EE-JXY for 24 h. The treated cells were then lysed with mammalian cell lysis buffer (M-PER; Thermo Fisher Scientific, Waltham, MA, USA) containing protease (EMD Millipore, Billerica, MA, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich). The cell lysates were separated by 12% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes using the iBlot® Western Detection Stack/iBlot® Dry Blotting system. The PVDF membranes were then blocked with SuperBlock T20 Tris-Buffered Saline (TBS) Blocking Buffer (Thermo Fisher Scientific) for 30 min and washed in TBS containing 0.25% Tween-20 (TBST). The membranes were subsequently incubated overnight at 4°C with the primary antibodies. After further washing with TBST, the membranes were incubated with a rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h (1:50). The membranes were visualized using SuperSignal Pico Substrate (Thermo Fisher Scientific), and images were captured using a Kodak Image Station 400R (Kodak, Rochester, NY, USA).

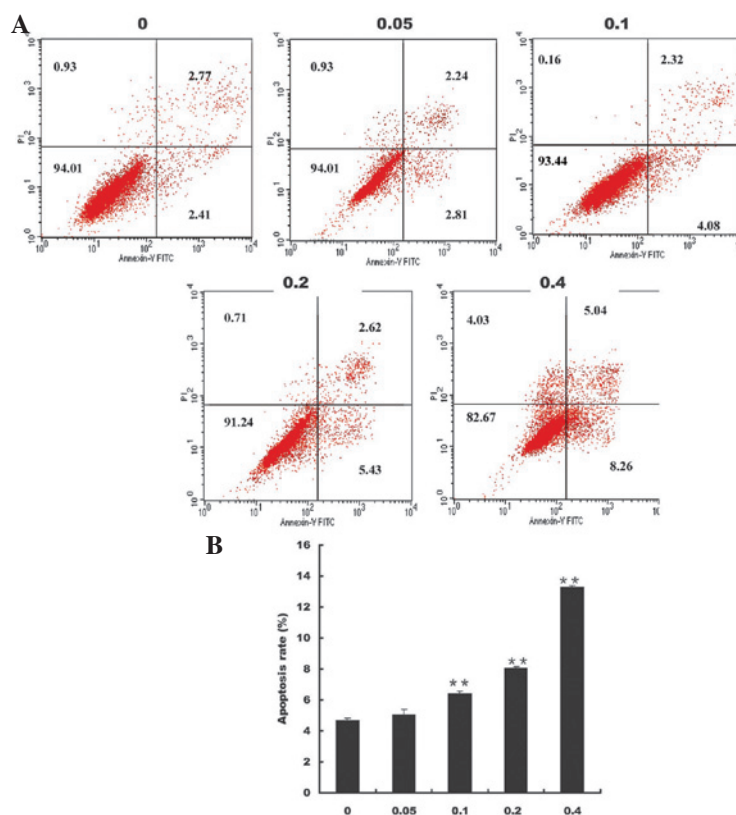


Figure 1. Effects of EE-JXY on apoptosis of HepG2 human liver carcinoma cells. (A) HepG2 cells were treated with the indicated concentrations of EE-JXY for 24 h, stained with Annexin V/PI, and analyzed by FACS. Representative FACS scatter-grams of Annexin V/PI staining display four different cell populations: Double-negative staining (LL, lower left) indicating live cells; Annexin V-positive/PI-negative staining (LR, lower right) indicating cells in early apoptosis; Annexin V/PI double-positive staining (UR, upper right) indicating cells in late apoptosis; Annexin V-negative and PI-positive staining (UL, upper left) indicating dead cells. Data shown are representative of three independent experiments. (B) Quantification of FACS analysis. Data shown are presented as the mean  $\pm$  standard deviation (UR+LR) from three independent experiments. \*\* $P < 0.01$ , compared with the control group. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; PI, propidium iodide; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.

**Tumor apoptosis assay.** A total of 16 male BALB/c nude mice, weighing between 18 and 22 g were purchased from the School of Basic Medical Science, Peking University (Beijing, China). The animals were maintained in a pathogen-free facility ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ,  $55\% \pm 5\%$  humidity, 12-h light/12-h dark cycle). Mice were sacrificed by  $\text{CO}_2$  inhalation. The animals were injected with a HepG2 cell suspension ( $1 \times 10^6$  cells per mouse) in the right flank. After seven days the mice were randomly divided into two groups: Mice in the EE-JXY group were orally administered EE-JXY (0.06 g/kg), and mice in the control group were administered the same volume of saline containing 0.1% dimethyl sulfoxide. Tumor sections from the mice were fixed with 4% paraformaldehyde for 48 h. The 5- $\mu\text{m}$  tumor sections were analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining, using a TumorTACS *In Situ* Apoptosis Detection kit (Roche Palo Alto LLC), in order to detect fragmented DNA, according to the manufacturer's instructions. Microscopic immunohistochemical images were captured using an Olympus microscope (Olympus Corporation, Tokyo, Japan) and Motacam 5000 C camera from Motic Instruments, Inc. (Richmond, BC, Canada) and analyzed by Motic Med 6.0 software. The number of positive cells and the total number of cells were counted in five arbitrarily selected microscopic fields, at  $\times 100$  magnification (each  $7,050 \mu\text{m}^2$  in size). A dark brown nucleus represented

the positive staining of apoptotic tumor cells with TUNEL. The apoptotic index (AI) was calculated according to the following formula:  $\text{AI} = \text{number of positive cells} / \text{number of total cells}$ . All procedures on mice were performed according to the Animal Care Guidelines issued by the Ministry of Science and Technology of the People's Republic of China. The present study was approved by the Animal Care Committee of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

#### Immunohistochemical staining for Bax and cytochrome c.

Tumor samples were fixed in 10% buffered formalin for 24 h and were conventionally processed into paraffin-embedded tumor sections. The sections were then subjected to antigen retrieval and blocking of endogenous peroxidase activity using commercial kits (Fuzhou Maixin Biotech Co., Ltd.; cat. nos. MVS-0101 and BLK-0001, respectively). For immunostaining, the sections were incubated with primary mouse monoclonal Bax antibody (1:100) or cytochrome c antibody (1:150). The sections were then incubated with biotinylated appropriate secondary antibody followed by conjugated HRP-streptavidin (Fuzhou Maixin Biotech Co., Ltd.). Subsequently 3,3'-diaminoben-zidine (Sigma-Aldrich) was added to the sections, which were incubated at room temperature and counterstained with diluted Harris hematoxylin (Sigma-Aldrich). The cells were quantified by counting the

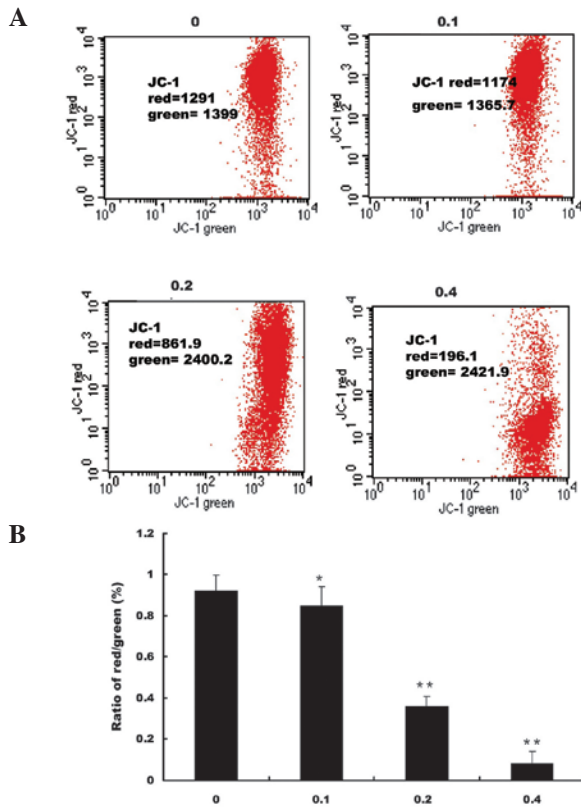


Figure 2. Effects of EE-JXY on mitochondrial membrane potential in HepG2 human liver carcinoma cells. (A) HepG2 cells were treated with the indicated concentrations of EE-JXY for 24 h and stained with JC-1. The mean JC-1 fluorescence intensity was detected using FACS. Data shown are representative of three independent experiments. (B) Quantification of FACS analysis. Data is presented as the mean  $\pm$  standard deviation from three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with the control cells. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide; EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; PI, propidium iodide; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.

number of positive cells and the total number of cells in five arbitrarily selected fields from each tumor at 100x magnification. Data are presented as the percentage of positive cells.

**Statistical analysis.** All data represent the mean of three determinations. The data were analyzed using SPSS package for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). Statistical analyses of the data were performed with a Student's t-test and one-way analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**EE-JXY induces apoptosis of HepG2 cells.** In our previous study, the results of an MTT assay demonstrated that treatment with EE-JXY markedly inhibited cell growth (22). In the present study, apoptosis of HepG2 cells was analyzed by FACS analysis using Annexin V/PI staining. Annexin V-positive/PI-negative and Annexin V-positive/PI-positive populations (labeled as LR or UR in the fig. 1) identify cells undergoing early and late apoptosis, respectively. Following treatment of the cells with 0.1, 0.2 and 0.4 mg/ml EE-JXY for 24 h, the percentage of apoptotic cells was markedly increased ( $P < 0.01$ , Fig. 1).

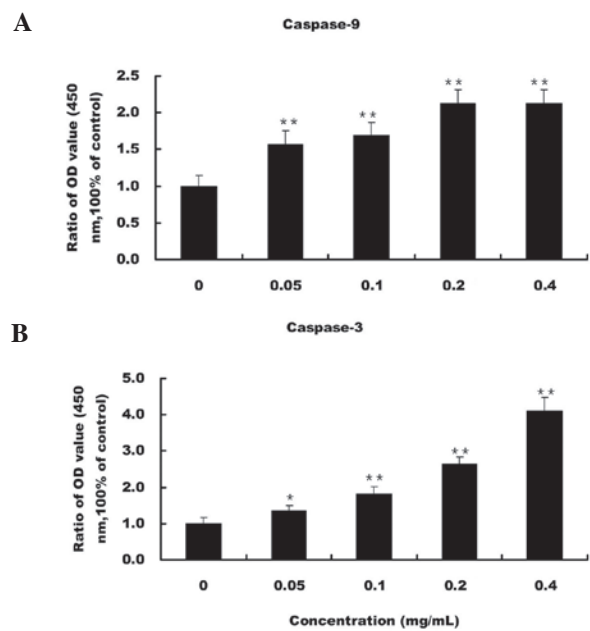


Figure 3. Effects of EE-JXY on the activity of caspases in HepG2 human hepatic carcinoma cells. HepG2 cells were treated with the indicated concentrations of EE-JXY for 24 h. (A) Caspase-9 and (B) caspase-3 activity was determined using a colorimetric assay. The data were normalized to the caspase activity within the control cells (treated with 0.5% DMSO vehicle) and are presented as the 'fold of control'. Data are presented as the mean  $\pm$  standard deviation from three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with the control cells. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; OD, optical density.

**EE-JXY induces mitochondrial potential ( $\Delta\psi_m$ ) loss.** FACS analysis with JC-1 staining was used to determine the alterations in mitochondrial membrane potential following treatment with EE-JXY. JC-1 is a lipophilic, cationic dye that selectively enters mitochondria, and differences in fluorescence can be detected by FACS using green and red channels. JC-1 fluorescence shifted from a JC-1-green-bright/JC-1-red-bright signal in untreated HepG2 cells to a JC-1-green-bright/red-dim signal in cells treated with EE-JXY. The percentage of cells with a reduced ratio of JC-1-green-bright/red-dim following treatment with 0.05, 0.1 and 0.2 mg/ml EE-JXY was 0.85 ( $P < 0.05$ ), 0.36 ( $P < 0.01$ ) and 0.08% ( $P < 0.01$ ), respectively, as compared with the saline-treated control cells (Fig. 2).

**EE-JXY activates caspase-9 and caspase-3.** The present study detected the activation of caspase-9 and caspase-3 by colorimetric assay using the following specific chromophores: DEVD-pNA, which is a specific substrate of caspase-3, and LEHD-pNA, which is a specific substrate of caspase-9. Treatment with EE-JXY dose-dependently induced the activation of caspase-9 and caspase-3 in the HepG2 cells ( $P < 0.05$  and  $P < 0.01$  respectively, as compared with the control cells; Fig. 3).

**EE-JXY decreases the expression levels of Bcl-2 and increases the expression levels of Bax.** The anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax are able to mediate cell death or survival, through regulation of mitochondria (24). In the present study a western blot analysis was conducted to examine the protein expression levels of Bcl-2 and Bax in



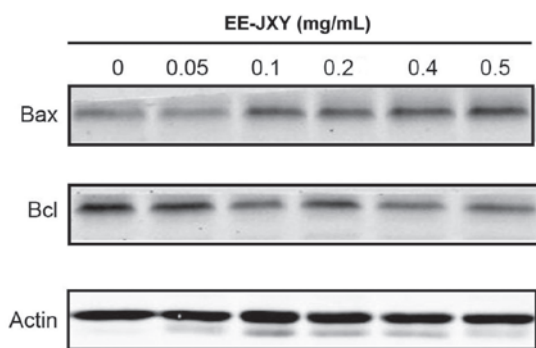


Figure 4. Effects of EE-JXY on the protein expression levels of Bax and Bcl-2 in HepG2 human hepatic carcinoma cells. HepG2 cells were treated with the indicated concentrations of EE-JXY for 24 h. Protein expression levels of Bax and Bcl-2 were determined by western blotting. Actin was used as an internal control. Results are representative of three independent experiments. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin.

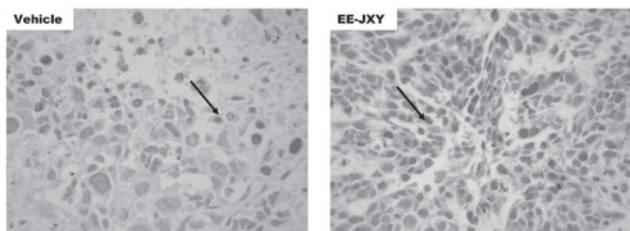
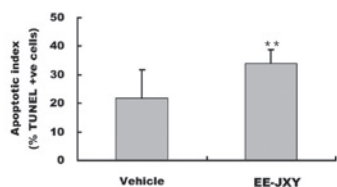


Figure 5. Effects of EE-JXY on the apoptotic index of tumor samples. Tumor tissues were processed for IHC staining for TUNEL. The photographs are representative images captured at magnification, x200. Quantification of the IHC assay is presented as a percentage of positively-stained cells. Data are presented as the mean  $\pm$  standard deviation from six mice in each group. \*\* $P < 0.01$ , versus the vehicle control group. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; IHC, immunohistochemistry.

the EE-JXY-treated HepG2 cells. Treatment with EE-JXY markedly increased the protein expression levels of Bax and reduced the protein expression levels of Bcl-2 in the HepG2 cells, in a dose-dependent manner (Fig. 4).

**EE-JXY induces tumor apoptosis in vivo.** In our previous study, *in vivo* results demonstrated that treatment with EE-JXY reduced tumor volume and weight by ~39%, as compared with the control group (22). To further confirm the *in vitro* pro-apoptotic effects of EE-JXY, the present study compared the AI of tumors between the two groups. The AI of the EE-JXY group was 35%, which was significantly higher as compared with the control group ( $P < 0.01$ , Fig. 5). Furthermore, immunohistochemical staining showed that the percentage of Bax-positive tumor cells in the EE-JXY group was 32% higher, as compared with in the vehicle group ( $P < 0.01$ , Fig. 6). The expression levels of cytochrome *c* were also increased in the cytoplasm of EE-JXY group ( $P < 0.01$ , Fig. 7).

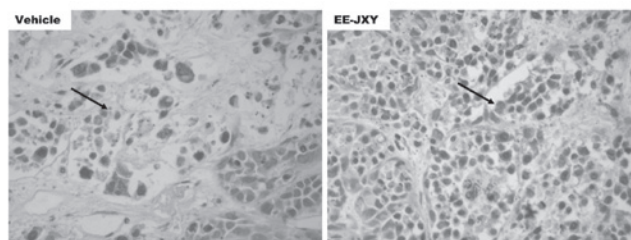
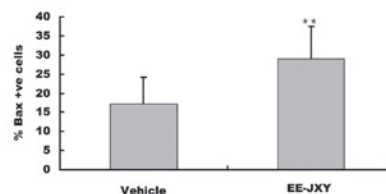


Figure 6. Effects of EE-JXY on the expression levels of Bax in tumor samples. The protein expression levels of Bax were determined by IHC staining. Quantification of the IHC assay is presented as the percentage of positively-stained cells. Data are presented as the mean  $\pm$  standard deviation from six mice in each group. \*\* $P < 0.01$ , versus the vehicle control group. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; IHC, immunohistochemistry.

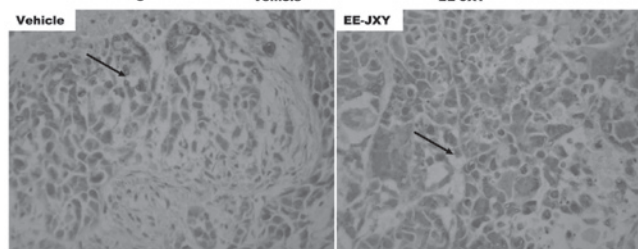
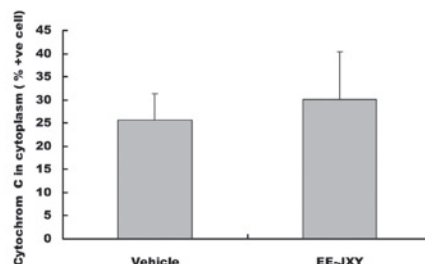


Figure 7. Effects of EE-JXY on the expression levels of cytochrome *c* in the cytoplasm. The protein expression levels of cytochrome *c* were determined by IHC staining. Quantification of the IHC assay is presented as the percentage of positively-stained cells. Data are presented as the mean  $\pm$  standard deviation from six mice in each group. \*\* $P < 0.01$ , versus the vehicle control group. EE-JXY, ethyl acetate fraction of Jiedu Xiaozheng Yin; IHC, immunohistochemistry.

## Discussion

JXY is a polyherbal formula that was initially established by Professor Jian Du, according to the principles of TCM theory. JXY is composed of HDW, *Prunella*, SF and PC. It has been used to treat malignancies of the liver, lung and stomach (25). The present study demonstrated that treatment with JXY inhibited tumor cell growth in a dose- and time-dependent manner, and JXY-induced apoptosis was accompanied by a collapse of the mitochondrial membrane potential ( $\Delta\psi_m$ ) and activation of caspase proteins. Furthermore, JXY inhibited the growth of xenografted HepG2 tumors in nude mice, and this was

accompanied by no significant side effects. Concordant with the *in vitro* findings, treatment with JXY promoted apoptosis and increased the expression levels of Bax and cytochrome *c* in tumor tissue *in vivo*.

Mitochondria are capable of inducing apoptosis through the release of numerous caspase activators. Among these activators, cytochrome *c* activates caspases by forming a complex with apoptotic protease activating factor-1 and procaspase-9, which triggers the activation of caspase-9 and subsequently cleaves the effector caspase-3 (26). These results suggest a direct link between the mitochondria and JXY-induced apoptosis.

The mitochondrial pathway is the main intrinsic apoptotic pathway. It is regulated by pro-apoptotic proteins, such as Bax and Bak, and anti-apoptotic proteins, such as Bcl-2 and Bcl-x<sub>l</sub>. These proteins control the permeability of the MOM through homo- and hetero-association. Activation of either of these pro-apoptotic proteins is sufficient to induce MOM permeabilization (MOMP) (27,28). MOMP leads to the release of pro-apoptotic proteins, including cytochrome *c* and Diablo/Smac, which subsequently trigger the activation of the caspase cascade (29). A rapid collapse of mitochondrial transmembrane electrical potential ( $\Delta\psi_m$ ) has previously been reported in TCM-induced apoptosis of cancer cells (30).

JXY is composed of four medicinal herbs. According to the TCM theory, these four herbs may have synergistic effects in inhibiting tumor growth. Previous pharmacological studies have demonstrated that each herb exhibits distinct antitumor effects. Extraction of HDW and *Prunella* has previously been demonstrated to induce cell apoptosis, via the mitochondrion-dependent pathway (17,31-33) and via regulation of c-Jun N-terminal kinase expression (34). Woo *et al* (35) reported that 2 $\alpha$ ,3 $\alpha$ -dihydroxyurs-12-ene-28-oic acid from *Prunella* induced apoptogenic activity in Jurkat T leukemia cells, which was mediated by a loss of  $\Delta\psi_m$ , mitochondrial cytochrome *c* release, and subsequent activation of caspase-9 and caspase-3, leading to the activation of caspase-7 and caspase-8. Apoptosis was also shown to be regulated by Bcl-2 and Bax. A previous study demonstrated that disruption of the mitochondrial membrane and the release of cytochrome *c* was regulated by the ratio of active anti- and pro-apoptotic Bcl-2 family members (36). Pro-apoptotic factors, including Bcl-2, prevent mitochondrial membrane disruption, whereas Bax promotes these events (37). Feng *et al* (38) reported that oleanolic acid from *Prunella vulgaris* increased the rate of apoptosis of SPC-A-1 cells, by increasing the ratio of Bax/Bcl-2 (39). Furthermore, SF has been shown to induce the apoptosis of SGC7901 cells by loss of mitochondrial membrane potential, reduction in the Bcl-2/Bax ratio, and significant activation and cleavage of caspase-3 (39). To determine whether the levels of Bcl-2 family proteins were altered in JXY-treated HepG2 cells, the present study examined the protein expression levels of Bcl-2 and Bax. Increased protein expression levels of Bax and decreased protein expression levels of Bcl-2 were observed following treatment of the HepG2 cells with various doses of JXY, and these alterations occurred in a dose-dependent manner. These results confirm that JXY may induce apoptosis via the mitochondrial pathway.

Cytochrome *c* is a pro-apoptotic family protein, which is usually decreased in tumors (40,41). Zhang *et al* (42) previously

reported that matrine and oxymatrine, two major components in SF, induced apoptosis by causing a collapse in mitochondrial membrane potential, inducing cytochrome *c* release from the mitochondria, reducing the ratio of Bcl-2/Bax, and increasing activation of caspase-3 (42,43).

In conclusion, the present study provides novel evidence suggesting that JXY is an effective and safe therapy for the treatment of cancer. However, the potential for development of JXY as an adjuvant agent in hepatoma cancer chemotherapy requires further study.

## Acknowledgements

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