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Research Article
Induction of Apoptosis in the Human HepG2 Hepatoma Cell Line Through the Extrinsic Pathway by Delphinium Alkaloid A

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Abstract

Background and Objective: Hepatocellular carcinoma (HCC) is a primary malignant neoplasm derived from hepatocytes and HCC is currently the second most common cause of cancer-related death worldwide, HCC causes large numbers of deaths around the world each year. Delphinium alkaloid A is a novel C19-diterpenoid alkaloid that has been identified from *Delphinium giraldii* Diels (*D. giraldii*). In this study, a novel natural product from *D. giraldii* was used to explore its antitumor effect on death receptor pathway-mediated apoptosis in HepG2 cells as well as the underlying mechanism. **Materials and Methods:** The cytotoxicity of delphinium alkaloid A in HepG2 cells was analyzed by the MTT method. The morphology of HepG2 cells stained with Hoechst 33258 was assessed by fluorescence microscopy. Flow cytometry was used to analyze the cell cycle progression. The expression levels and activities of proteins related to the extrinsic pathway of apoptosis were subsequently analyzed. **Results:** The IC_{50} value of Delphinium alkaloid A in HepG2 cells was 48.67 μg mL^{-1}. After treatment with delphinium alkaloid A (25, 50 and 100 μg mL^{-1}), HepG2 cells exhibited an obvious apoptotic morphology, which included cell protuberance, concentrated cytoplasm and apoptotic bodies. Moreover, the cell cycle was altered and apoptosis peaked. The expression of FasL, Fas, TRAIL, TRAIL-R1, TRAIL-R2 and FADD in HepG2 cells was unregulated, while the expression of Pro-Caspase-8 and Pro-Caspase-3 was down regulated. Moreover, the activities of Caspase-8 and Caspase-3 were increased. **Conclusion:** Delphinium alkaloid A could induce HepG2 apoptosis via a death receptor-mediated pathway.

Key words: HepG2 cells, apoptosis, alkaloid, death-receptor, pathway

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary malignant neoplasm and HCC is currently the second most common cause of cancer-related death worldwide, HCC causes 662,000 deaths around the world each year[1]. Despite surgical management and the use of nonsurgical therapeutic modalities, the incidence of HCC is still increasing[2]. In recent years, natural plant-derived drugs or active components have drawn worldwide attention due to their therapeutic effects on HCC[3]. Two main pathways are primarily involved in cell apoptosis, namely, the extrinsic and intrinsic pathways, these pathways are mediated by mitochondria and death receptors, respectively and each pathway interacts and ultimately leads to apoptosis[4,5].

Delphinium giraldii Diels (Ranunculaceae) (*D. giraldii*) is a common species of Delphinium that is distributed in the middle zone of China[6]. The *D. giraldii* has been used clinically to treat rheumatism and to promote sedation[7]. Alkaloids, including norditerpenoids and diterpenoids are the characteristic bioactive ingredients of this plant[8,9]. Delphinium alkaloid A is a novel C19-diterpenoid alkaloid that has been identified in *D. giraldii*. Delphinium alkaloid A exhibited an anti-inflammatory effect in a previous report[10]. This study explored the antitumor effect of delphinium alkaloid A on death receptor-mediated apoptosis in HepG2 cells as well as the underlying mechanism. Moreover, this experiment will broaden the use and application of Delphinium alkaloid A as a potential antitumor drug.

MATERIALS AND METHODS

Study area: This study was carried out in the Fourth Affiliated Hospital of China Medical University, Shenyang, China from July, 2019 to October, 2020.

Reagents: Delphinium alkaloid A was obtained from Dr Liu, Changchun Sci-Tech University and the purity of the compound was greater than 98% as determined by HPLC. Hydroxycamptothecin, RPMI 1640, FBS, Hoechst 33258, PI, MTT, rabbit anti-human β-actin, Fas, FasL, Pro-caspase-8 and Pro-caspase-3, mouse anti-human DR4, DR5, TRAIL, FADD, FITC-goat anti-mouse antibodies and Caspase-8 and Caspase-3 activity kits were used.

Apparatus: The equipment used in this study included a CKX 41 fluorescence inverted microscope, RM2126 ultramicrotome, mini electrophoresis meter, microplate reader, EPICS XL flow cytometer, Sanyo CO_2 incubator.

Cell culture: HepG2 cells were provided by ATCC. The cells were grown in a tissue culture flask with RPMI 1640 medium and 10% heat-inactivated fetal bovine serum in a humidified atmosphere in 5% CO_2 at 37°C.

Effect of delphinium alkaloid A on HepG2 cell proliferation: A total of 100 µL cells (5×10^4 mL^{-1}) were seeded into a 96-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and co-cultured for 72 hrs, there were 12 parallel wells treated with each concentration. After the medium was removed from each well in which MTT solution had been added and incubated at 37°C for 4 hrs, dimethyl sulfoxide was added to each well. The spectrophotometric absorbance of each well was measured with a microplate reader at 570 nm.

Effect of delphinium alkaloid A on HepG2 cell morphology: A total of 1 mL cells (1×10^5 mL^{-1}) were seeded into a 6-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and cocultured for 48 hrs. The cells were fixed in paraformaldehyde (4%, v/v) at 4°C for 30 min, washed with PBS twice and stained with Hoechst 33258 for 30 min. Then, the staining solution was discarded by centrifugation and the cells were observed using a fluorescence microscope.

Effect of delphinium alkaloid A on the cell cycle of HepG2 cells: A total of 1 mL cells (1×10^5 mL^{-1}) was seeded into a 6-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and cocultured for 72 hrs. The cells were fixed in cold ethanol (70%, v/v) at 4°C for 12 hrs, washed with PBS twice and stained with PI for 30 min. Then, the staining solution was discarded by centrifugation and the cells were assessed by flow cytometry.

Effect of delphinium alkaloid A on apoptotic protein expression: To assess Fas, FasL, Pro-Caspase-8 and Pro-Caspase-3 expression in HepG2 cells by western blot, a total of 1 mL of cells (1×10^5 mL^{-1}) was seeded into a 6-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and cocultured for 24 hrs. Cytoplasmic extracts were prepared with cell lysis buffer and collected by centrifugation. A total of 40 µg of protein was separated in a 10% (w/v) polyacrylamide gel and blotted onto nitrocellulose membranes. The nitrocellulose membranes were blocked for 2 hrs and incubated with primary antibodies for 12 hrs. Subsequently, the membranes were washed and incubated with secondary

antibodies for 2 hrs. In all the experiments, Ponceau staining was carried out to ensure equal loading and the bands were visualized by a western blot system.

To assess TRAIL-R1, TRAIL-R2, TRAIL and FADD expression in HepG2 cells by flow cytometry, a total of 1 mL of cells (1×10^5 mL^{-1}) were seeded into a 6-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and cocultured for 24 hrs. The cells were collected and fixed with paraformaldehyde for 40 min and then, 1 mL Triton X-100 (0.1%, v/v) was added for 15 min to permeabilize the cells. Then, the cells were collected by centrifugation and blocked with 1 mL BSA (1%, v/v) for 1 h. Mouse-anti-human TRAIL-R1, TRAIL-R2, TRAIL and FADD antibodies were incubated for 1 h at 37°C. Subsequently, FITC anti-mouse antibodies were added and incubated for 30 min at room temperature and then the supernatant fluid was discarded. Then, 500 µL of PBS solution was added and the cells were assessed using flow cytometry.

Effect of delphinium alkaloid A on the activities of Caspase-8 and Caspase-3 in HepG2 cells: A total of 0.5 mL cells (1×10^5 mL^{-1}) were seeded into a 24-well plate and initially cultured for 24 hrs at 37°C. Then, different concentrations of delphinium alkaloid A were added and cocultured for 24 hrs. The cells were collected and analyzed with caspase kits at 405 nm.

Statistical analysis: Statistical analysis was carried out using SPSS 20.0. The calculated results of the data are expressed as Mean±Standard Deviation (SD). The statistical analyses were performed by using ANOVA to compare the different groups.

RESULTS

Delphinium alkaloid A effect on HepG2 cell proliferation: After delphinium alkaloid A treatment for 72 hrs, the MTT results showed a concentration-dependent inhibitory effect on the proliferation of HepG2 cells from 0.57-77.56% with the increase of dose. The Optical Densities (OD) of the treated groups were significantly different from those of the control group ($p<0.01$) and the IC_{50} was 48.67 mg L^{-1}. The results are shown in Table 1.

Delphinium alkaloid A effect on HepG2 cell morphology: After delphinium alkaloid A treatment for 48 hrs, the HepG2 cell morphology was altered. The cell morphology of the control group, 25, 50, 100 µg mL^{-1} group and HCPT group were shown in Fig. 1(a-e), respectively. Apoptosis-like

Table 1: Inhibitory rate of on HepG2 cells by MTT assay ($\bar{x}\pm s$, n = 12)

Groups	Concentration (µg mL^{-1})	Number	OD	Inhibition rate (%)	IC_{50} (µg mL^{-1})
Control	-	12	0.742±0.099	-	-
Delphinium alkaloid A	5	12	0.743±0.098*	0.57	48.67
	10	12	0.700±0.047**	27.55	
	20	12	0.510±0.082**	44.60	
	40	12	0.396±0.057**	50.28	
	80	12	0.358±0.036**	56.68	
	160	12	0.305±0.063**	77.56	

*vs: Control $p<0.05$, **vs: Control $p<0.01$

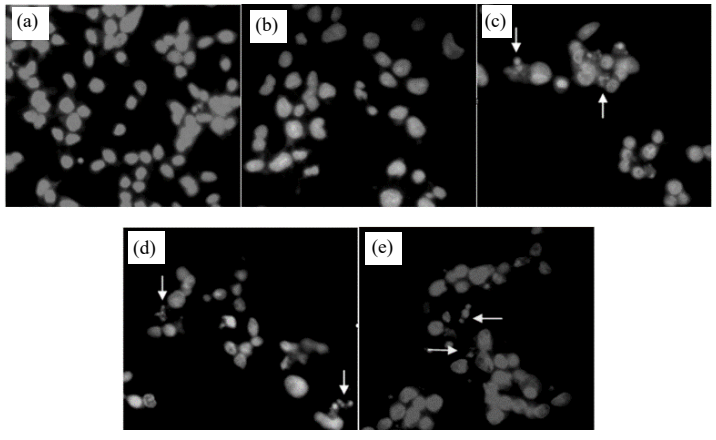

Fig. 1(a-e): Effect of delphinium alkaloid A on HepG2 cells morphology

(a) Cells were treated with RPMI1640 , (b) Cells were treated with 25 µg mL^{-1} delphinium alkaloid A, (c) Cells were treated with 50 µg mL^{-1} delphinium alkaloid A, (d) Cells were treated with 100 µg mL^{-1} delphinium alkaloid A and (e) Cells were treated with 10 µg mL^{-1} HCPT

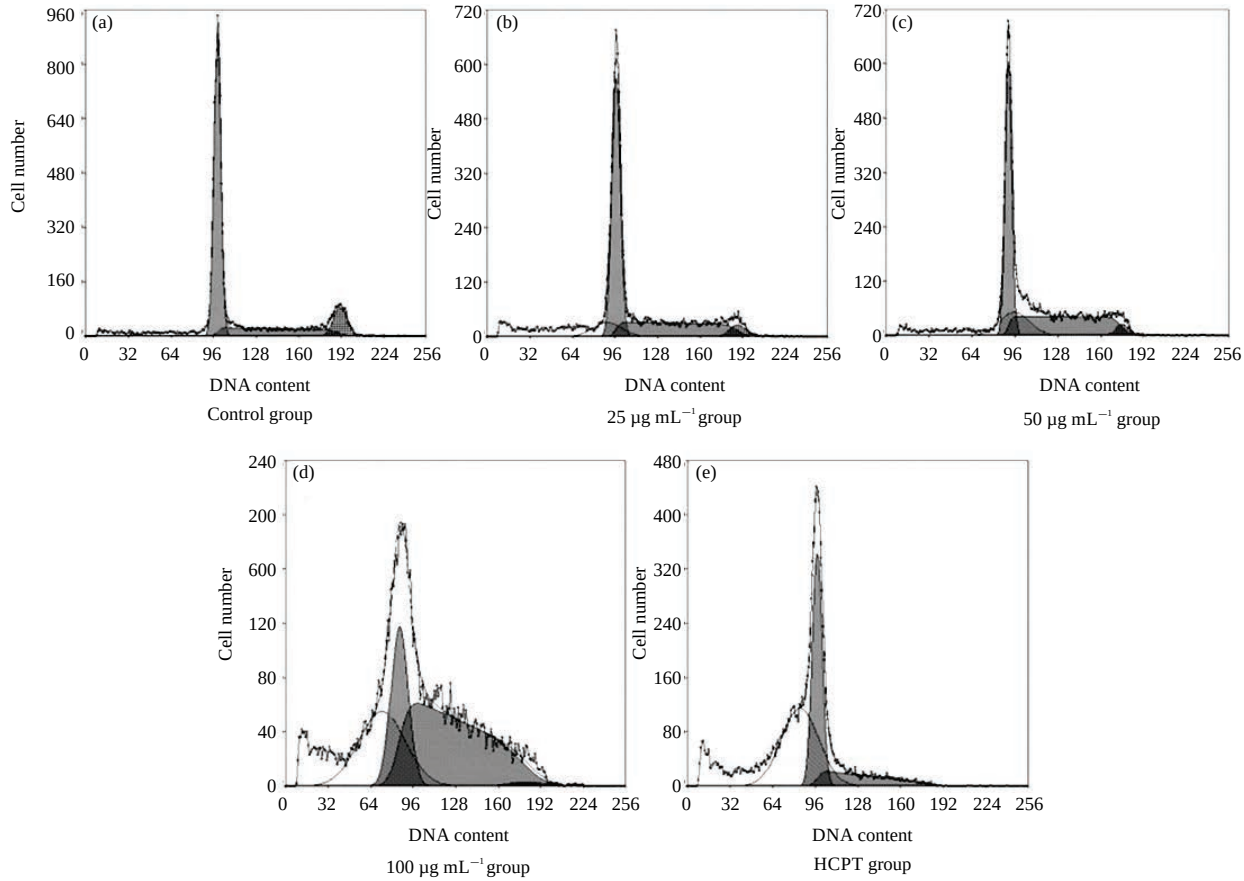

Fig. 2(a-e): Effect of delphinium alkaloid A on cell cycle of HepG2 cells

(a) Cells were treated with RPMI1640, (b) Cells were treated with 25 μg mL^{-1} delphinium alkaloid A, (c) Cells were treated with 50 μg mL^{-1} delphinium alkaloid A, (d) Cells were treated with 100 μg mL^{-1} delphinium alkaloid A and (e) Cells were treated with 10 μg mL^{-1} HCPT

Table 2: Effect of delphinium alkaloid A on cell cycle in HepG2 cells (n = 3)

		Cell cycle (%)			
Groups	Dose (μg mL^{-1})	G0/G1	S	G2/M	Apoptosis rate (%)
Control	-	65.16±1.24	22.71±0.51	12.14±0.37	-
Delphinium alkaloid A	25	59.31±0.86	35.37±0.34	5.32±0.72	9.93±0.52
	50	50.21±0.81	45.85±0.62	3.94±0.54	15.65±0.34
	100	29.62±0.46	68.75±0.91	1.63±0.10	26.94±0.81
HCPT	10	72.46±1.12	27.54±0.83	0	48.02±0.89

morphology, such as cytoplasmic condensation, cell swelling and microvillus disappearance, appeared and apoptotic bodies (indicated by the arrow) markedly appeared in the 50, 100 μg mL^{-1} group and HCPT group.

Delphinium alkaloid A effect on the cell cycle of HepG2 cells: After delphinium alkaloid, A treatment for 72 hrs, the cell cycle of HepG2 cells was altered. The cell cycle results of the control group, 25, 50, 100 μg mL^{-1} group and HCPT group were shown in (Fig. 2a-e). The percent of cells in the G0/G1 and G2/M phases decreased from 65.16±1.24-29.62±0.46% and 12.14±0.37-1.63±0.10%, while that in the S phase increased from 22.71±0.51-68.75±0.91%, which showed that cell cycle arrest in the S phase shown in Table 2.

Delphinium alkaloid A effect on apoptotic protein expression in HepG2 cells: After delphinium alkaloid A treatment for 24 hrs, the western blot results in Fig. 3a showed that the expression of Fas and FasL was increased and this expression was significantly different from that in the control group ($p<0.01$) as shown in Fig. 3b. The expression of TRAIL-R1of control group, 25, 50, 100 μg mL^{-1} group and HCPT

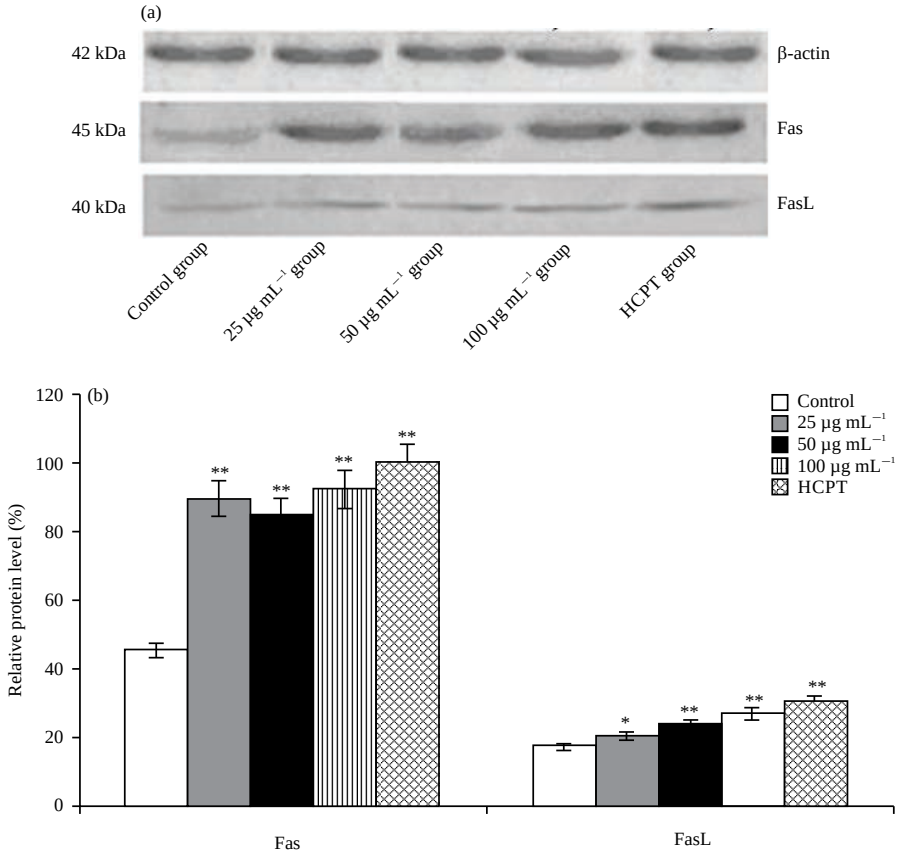

Fig. 3(a-b): (a) Western blot results of Fas and Fasl expression of HepG2 cells and (b) Effect of delphinium alkaloid A on Fas and Fasl expression of HepG2 cells
*p<0.05 vs control, **p<0.01 vs control

Table 3: Delphinium alkaloid A on the expression of TRAIL-R1, TRAIL-R2, TRAIL and FADD in HepG-2 cells (n= 3)

		Mean fluorescence intensity/($\bar{x}\pm$ s)			
Groups	Dose (μg mL^{-1})	TRAIL-R1	TRAIL-R2	TRAIL	FADD
Control	-	15.43±0.40	16.29±0.64	15.49±0.24	11.68±0.19
Delphinium alkaloid A	25	15.97±0.21	16.38±0.24	16.63±0.20*	16.49±0.12**
	50	16.57±0.42*	17.93±0.35*	16.85±0.37**	16.63±0.08**
	100	18.80±0.35**	18.65±0.42**	19.12±0.36**	18.76±0.33**
HCPT	10	16.34±0.66*	17.24±0.40*	17.67±0.24**	17.05±0.21**

*p<0.05 vs control, **p<0.01 vs control

group were shown in (Fig. 4a-e). The expression of TRAIL-R2 of the control group, 25, 50, 100 μg mL^{-1} groups and HCPT group were shown in (Fig. 5a-e). The expression of TRAIL of the control group, 25, 50, 100 μg mL^{-1} groups and HCPT group were shown in (Fig. 6a-e). The expression of TFADD of the control group, 25, 50, 100 μg mL^{-1} group and HCPT group were shown in Fig. (7a-e). The flow cytometry results showed that the expression of TRAIL-R1, TRAIL-R2, TRAIL and FADD was significantly increased in the treated group compared with that in the control group (p<0.01). As shown in Table 3, the mean fluorescence intensity of TRAIL-R1, TRAIL-R2, TRAIL and FADD were increased from 15.43±0.40-18.80±0.35, from 16.29±0.64-18.65±0.42, from 15.49±0.24-19.12±0.36 and 11.68±0.19-18.76±0.33, respectively. The western blot results in Fig. 8a further showed that the expression of Pro-Caspase-8 and Pro-Caspase-3 was significantly decreased in the treated group compared with that in the control group (p<0.01). The results were shown in Fig. 8b.

Delphinium alkaloid A effect on the activities of Caspase-8 and Caspase-3 in HepG2 cells: After delphinium alkaloid, A treatment for 24 hrs, the contents of pNA of Caspase-8in the

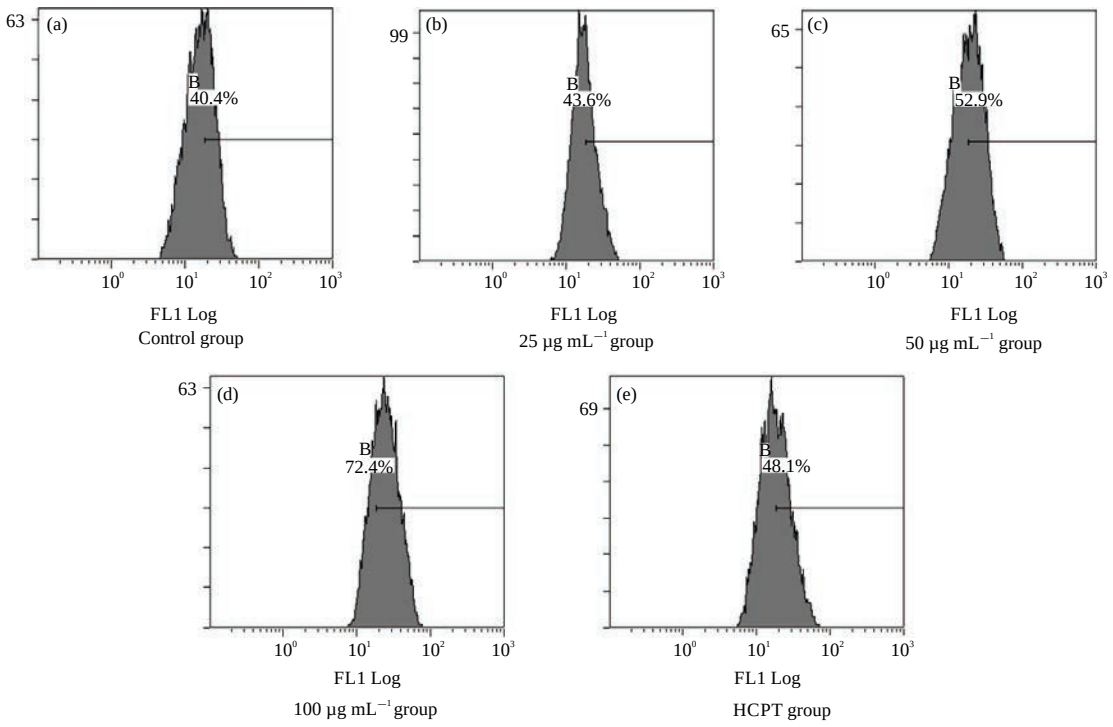

Fig. 4: Effect of delphinium alkaloid A on the expression of TRAIL-R1 of HepG-2 cells by flow cytometry

(a): Cells were treated with RPMI1640, (b): Cells were treated with 25 μg mL^{-1} delphinium alkaloid A, (c): Cells were treated with 50 μg mL^{-1} delphinium alkaloid A, (d): Cells were treated with 100 μg mL^{-1} delphinium alkaloid A and (e): Cells were treated with 10 μg mL^{-1} HCPT

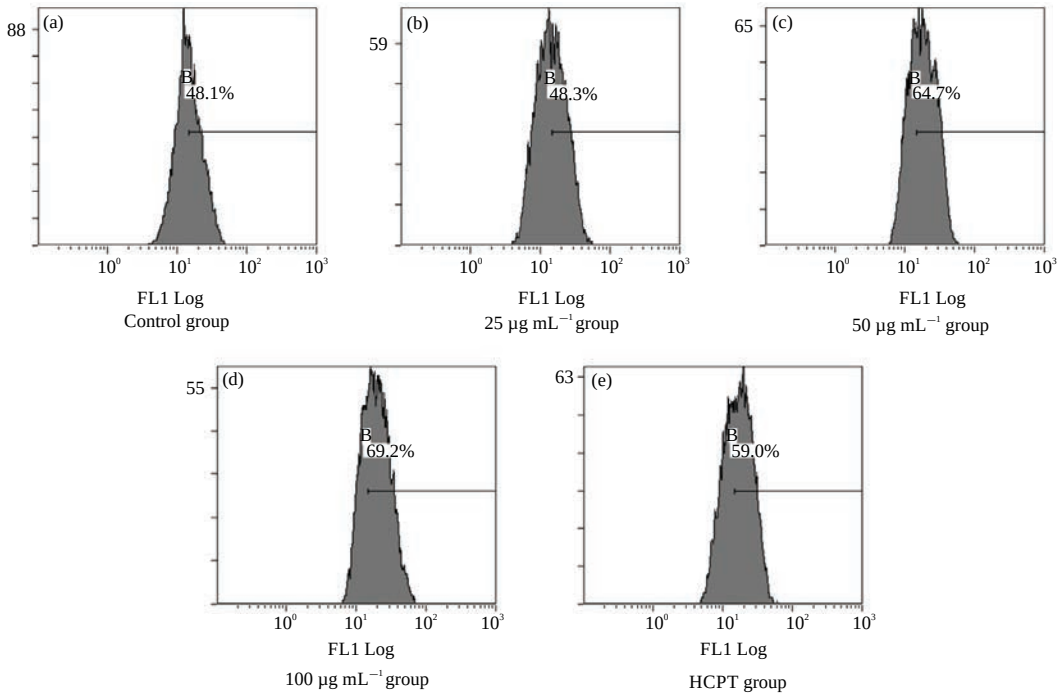

Fig. 5: Effect of delphinium alkaloid A on the expression of TRAIL-R2 of HepG-2 cells by flow cytometry

(a): Cells were treated with RPMI1640, (b): Cells were treated with 25 μg mL^{-1} delphinium alkaloid A, (c): Cells were treated with 50 μg mL^{-1} delphinium alkaloid A, (d): Cells were treated with 100 μg mL^{-1} delphinium alkaloid A and (e): Cells were treated with 10 μg mL^{-1} HCPT

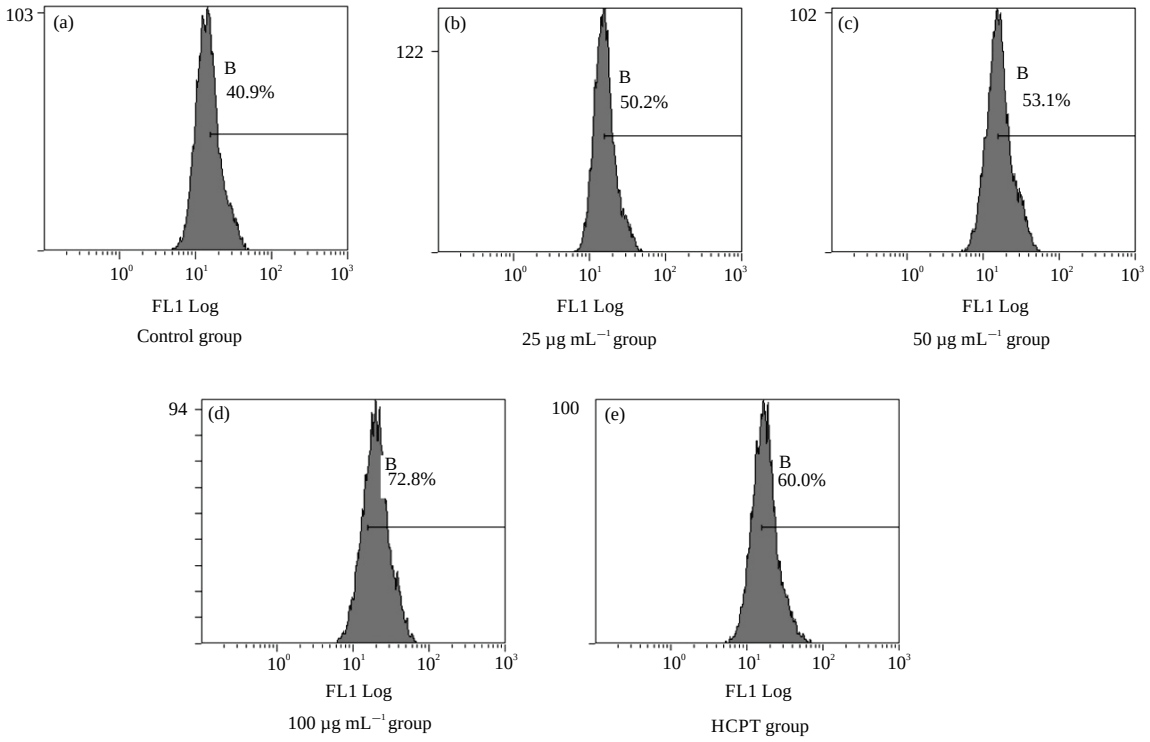

Fig. 6: Effect of delphinium alkaloid A on the expression of TRAIL of HepG-2 cells by flow cytometry

(a): Cells were treated with RPMI1640, (b): Cells were treated with 25 μg mL^{-1} delphinium alkaloid A, (c): Cells were treated with 50 μg mL^{-1} delphinium alkaloid A, (d): Cells were treated with 100 μg mL^{-1} delphinium alkaloid A and (e): Cells were treated with 10 μg mL^{-1} HCPT

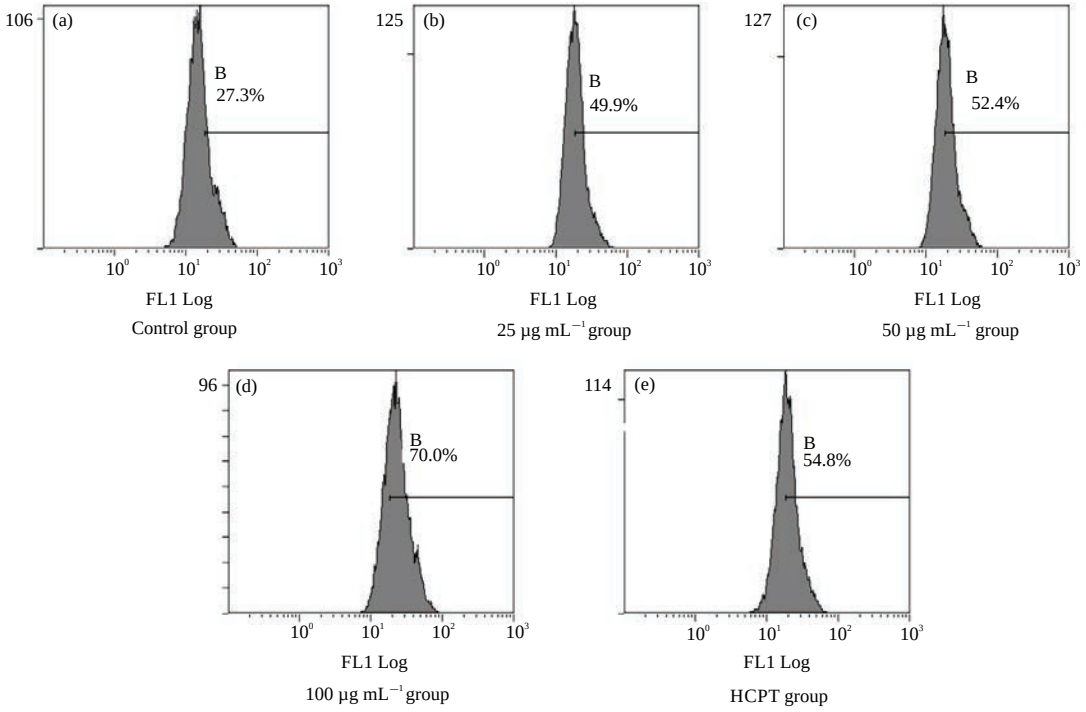

Fig. 7: Effect of delphinium alkaloid A on the expression of FADD of HepG-2 cells by flow cytometry

(a): Cells were treated with RPMI1640, (b): Cells were treated with 25 μg mL^{-1} delphinium alkaloid A, (c): Cells were treated with 50 μg mL^{-1} delphinium alkaloid A, (d): Cells were treated with 100 μg mL^{-1} delphinium alkaloid A and (e): Cells were treated with 10 μg mL^{-1} HCPT

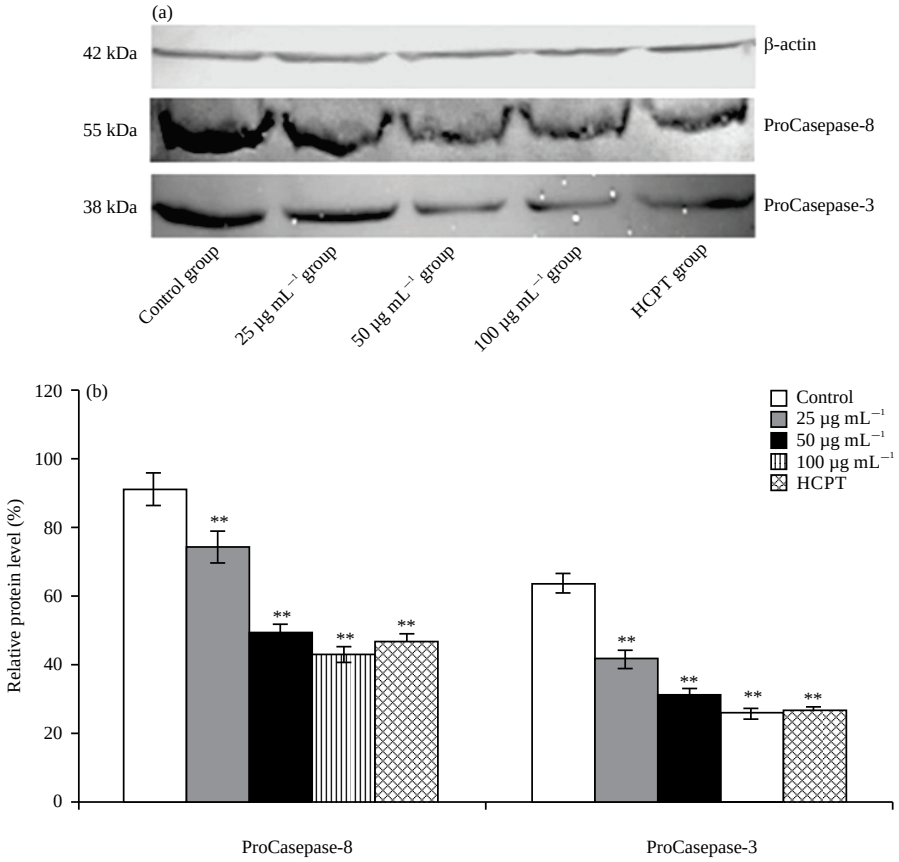

Fig. 8(a-b): Western blot results of (a) ProCaspase-8 and ProCaspase-3 expressions of HepG2 cells and (b) Effect of delphinium alkaloid A on ProCaspase-8 and ProCaspase-3 expressions of HepG2 cells
**p<0.01 vs control

Table 4: Delphinium alkaloid A on caspase-8 and caspase-3 activity of HepG-2 cells (n = 3)

		Caspase-8		Caspase-3	
Groups	Dose (μg mL^{-1})	pNA (μM)	Activity (%)	pNA (μM)	Activity (%)
Control	-	20.73±0.61	-	11.05±0.91	-
Delphinium alkaloid A	25	25.88±1.21*	24.77	6.19±0.11**	46.79
	50	38.59±0.91**	86.02	7.10±1.21**	55.03
	100	44.04±1.52**	112.29	9.22±1.52**	74.24
HCPT	10	55.84±0.61**	169.20	30.11±1.82**	173.02

*p<0.05 vs control, **p<0.01 vs control

HepG2 cells increased from 20.73±0.61-44.04±1.52 μM and this value was significantly different compared with that in the control group (p<0.01) are shown in Table 4. In addition, the activity of Caspase-8 and Caspase-3 was markedly increased to 112.29-74.24%, respectively.

DISCUSSION

Natural products have been considered an important source that could contribute to the development of potential chemotherapeutic agents. Delphinium alkaloid A was isolated from *Delphinium giraldii* Diels, the plant and its compounds were not reported to inhibit HepG2 cells[11]. The present investigation was carried out to evaluate the anticancer activity of delphinium alkaloid A on HepG2 cells for the first time and to elucidate its mechanism.

Apoptosis is a rigorous, active and orderly process of cell death that is regulated by many genes to maintain the stability of the intracellular environment[12]. The death receptor pathway is one of the 3 major apoptotic pathways and when death receptors bind to their respective ligands, cell apoptosis can occur[13]. Many studies have demonstrated that solid

tumours and haematological malignancies express Fas and FasL and in some cell lines, the Fas system is involved in drug-induced apoptosis[14,15]. The results showed that delphinium alkaloid A could increase the levels of Fas and FasL in HepG2 cells.

TRAIL is a member of the Superfamily of Tumor Necrosis Factors (TNFs). The mechanism by which TRAIL and its receptors induce apoptosis is as follows in this study[16]. When TRAIL-R1 and TRAIL-R2 bind to TRAIL, apoptosis signals can be transmitted into the cells. Then, the death domain on the C-terminus of FADD can interact with that on TRAIL-R1 and TRAIL-R2. Moreover, the death domain on the N-terminus of FADD can bind to the death-effect or domain on pro-Caspase-8 and finally, the Death-Inducing Signaling Complex (DISC) of TRAIL-TRAIL-Rs-FADD-pro-Caspase-8 can be formed. Pro-Caspase-8 cleaves itself to produce active Caspase-8, which can activate two pathways to transducer apoptosis signals. In the first pathway, Caspase-8 directly activates Caspase-3, Caspase-6 and Caspase-7, inducing apoptosis through the death-receptor pathway[17]. In the other pathway, Caspase-8 is connected to mitochondria by activating Bid, thus inducing apoptosis through mitochondria[18,19]. The FCM results showed that delphinium alkaloid A increased the expression of TRAIL-R1, TRAIL-R2, TRAIL and FADD in HepG2 cells in a dose-dependent manner. This result suggested that delphinium alkaloid A can promote the formation of DISC in HepG2 cells, inducing apoptosis in these cells through a death receptor-mediated pathway.

CONCLUSION

Delphinium alkaloid A, a novel alkaloid, can upregulate the expression of the death receptor proteins Fas, TRAIL-R1 and TRAIL-R2 and can also increase the levels of FasL and TRAIL, these effects can help to form the death-inducing signalling complex and then increase the activities of Caspase-8 and Caspase-3. Thus, delphinium alkaloid A can also induce HepG2 apoptosis through an extrinsic (death receptor-mediated) pathway, suggesting that delphinium alkaloid A is a valuable agent for cancer treatment.

SIGNIFICANCE STATEMENT

This study explored that delphinium alkaloid A could induce HepG2 apoptosis via a death receptor-mediated pathway, which will provide a new treatment idea for hepatoma. At the same time, the cell cycle and the expression of many proteins in cells were studied, which laid a solid foundation for further research.

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