

# Antihepatocarcinoma Effect of Solanine and Its Mechanisms

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**Abstract:** **Objective** To explore the antitumor effect of solanine and its mechanisms. **Methods** The *in vivo* antitumor effect of solanine was observed using models developed through *in vivo* transplantation of tumor cells; *In vitro* lines of sensitive antitumor cells were selected from the digestive system using MTT assay; The effect of solanine on cell morphology was observed using transmission electronic microscopy; The morphology of apoptotic cells was observed using Annexin V/PI double staining and laser confocal scanning microscopy (LCSM); The rate of cell apoptosis was measured using Annexin V/PI double staining and flow cytometry; The concentration of intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) was determined using Fluo-3/AM staining and LCSM; The membrane potential of cellular mitochondria was determined using TMRE staining and LCSM; The protein expression of Bcl-2 and Bax was measured using immunological marking and LCSM; And the activity of caspase-3 was measured using the colorimetric method. **Results** Solanine could inhibit the growth of tumor weight in  $S_{180}$  tumor-bearing mice and prolong the survival time of  $H_{22}$  tumor-bearing mice. MTT assay revealed that HepG2 cells were quite sensitive to solanine because solanine could induce morphological changes in HepG2 cells, with the rate of early apoptosis being 4%, 8.5%, and 20.1%, for HepG2 cells treated for 24 h with solanine at concentration of 0.4, 2, and 10  $\mu\text{g/mL}$ , respectively. Solanine could raise the  $[Ca^{2+}]_i$  and lower the membrane potential. It could reduce the protein expression of Bcl-2 while increase that of Bax, thus increasing the activity of caspase-3. **Conclusion** The obvious antitumor activity of solanine in human hepatocarcinoma is demonstrated. This inhibitory effect is achieved through solanine decreasing the Bcl-2/Bax ratio, thus increasing  $[Ca^{2+}]_i$ , which could enhance the enzymatic activity of the caspase family, thus inducing the apoptosis of HepG2 cells.

**Key words:** apoptosis; caspase; hepatocarcinoma;  $S_{180}$ ; solanine

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## Introduction

Solanine is found mainly in the tuber of potato, *Solanum tuberosum* L., and in the whole plant of nightshade, *S. nigrum* Linn., in Solanaceae. The content of this substance is rather high in green peel and sprouts of potato as well as in eggplant, especially in immature eggplants but also in mature eggplants at lower concentration. Nightshade is called by a large variety of names in folk medicine in China, and its whole plant contains many steroid alkaloids, including solamargine, solasonine, and solanine, as well as sponin and other substances, and has an antitumor effect. It has a high inhibitory effect on tumors in animals, with a marked cytotoxic effect (Ji, 1995). Its ethanol extract is capable

of inhibiting the growth of breast cancer and inducing apoptosis in tumor cells (Son *et al.*, 2003). The nightshade extract also has a strong anti-inflammatory effect on animals because it could facilitate the formation of antibodies (Liu and Peng, 1994). Antitumor effect of solamargine has been reported (Liu *et al.*, 2004), while solanine has an antikaryokinetic effect, and is capable of inducing apoptosis in HeLa cells from human cervical cancer (Fang *et al.*, 2010). Earlier studies by our team have revealed that the total alkaloids of nightshade could significantly prolong the survival time of  $H_{22}$  tumor-bearing mice (Ji, Wang, and Gao, 2005a), significantly lower the membrane fluidity and level of membrane proteins of  $H_{22}$  tumor-bearing mice (Ji, Wang,

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and Gao, 2005b), lower the RNA/DNA ratio in tumor cells in S<sub>180</sub> and H<sub>22</sub> tumor-bearing mice, block protein synthesis in tumor cells, and thus inhibit the growth of tumor cells (Ji, Wang, and Gao, 2005c). This paper examines the *in vivo* antitumor effect of solanine, and sensitive cell lines are selected *in vitro* from the digestive system. At the same time, the mechanisms for the antitumor effect of solanine are discussed from the perspective of the induction of apoptosis in sensitive cells.

## Materials and methods

### Drugs

Cytoxan was purchased from Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China); Solanine was provided by Heilongjiang Institute for Food and Drug Control; Camptothecin (CAM) was from Zhejiang Hisun Pharmaceutical Co., Ltd.; RPMI 1640 medium and pancreatin were from Gibco; Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.; Caspase-3 activity test kit was from Beyotime Institute of Biotechnology; Murine antihuman Bcl-2 antibody I and murine antihuman Bax antibody I were from Santa Cruz; Goat antimouse IgG-FITC antibody II was from Beijing Zhongshan Biotechnology Co., Ltd.; TMRE fluorescence probe was from Biotium, US.

### Apparatuses

CO<sub>2</sub> Incubator (NBS, US), Super-clean Worktable (Purification Equipment Plant of Sujing Group, Suzhou, China), Inverted Microscope (Olympus, Japan), Microplate Reader (Bio-Rad, US), Laser Confocal Scanning Microscope (LCSM, Leica, Germany), Transmission Electronic Microscope (TEM, Hitachi, Japan), Flow Cytometer (FC, Beckman-Coulter, US), and Horizontal High-speed Centrifuge (Beijing Medical Centrifuge Factory, China).

### Test animals and tumor cell lines

White mice of the Kunming strain, weighing (20.0 ± 2.0) g, half male and half female were provided by Section of Laboratory Animals, Harbin Institute of Veterinary Studies (00101003). S<sub>180</sub> and H<sub>22</sub> tumor cell lines were purchased from Institute of Cancer Studies, Cancer Hospital of Harbin Medical University; human hepatocarcinoma cell line HepG2, human gastrocarcinoma cell line SGC-7901, and human large intestine cancer cell line LS-174 were provided by

Institute of Cancer Studies of Heilongjiang Cancer Hospital.

### Effect of solanine on survival time of S<sub>180</sub> and H<sub>22</sub> tumor-bearing mice

**Development of tumor models** Mice were put to death by disjuncting their cervical vertebrae 7 d after being inoculated with tumor cells, and then fixed on wax plates. After sterilization, the skin of mice was cut open and peeled off. Some abdominal dropsy was drawn with a syringe and put in a sterile chamber surrounded by ice cubes for storage. In addition, a small amount of abdominal dropsy was drawn and put in a test tube, to be used for observing and counting the cells. One drop of the remaining abdominal dropsy in the syringe was dripped onto a carrying sheet glass, smeared and dyed using Wright's staining, after which the cells were classified and counted. The sample was used only when 97% or more of the cells were cancerous. The concentration of the abdominal dropsy was adjusted to 1 × 10<sup>7</sup> cells/mL with normal saline by 1:4. For the group with S<sub>180</sub> sarcoma, each animal was inoculated with 0.2 mL of the dropsy through injection at the armpit; And for the group with H<sub>22</sub> abdominal dropsy tumor, each animal was inoculated with 0.2 mL of the dropsy through ip injection.

**Pharmacodynamic experiment** The mice were randomly divided into five groups with 10 animals each, including a positive control, a negative control, and three groups treated with different doses of solanine (9.375, 18.75, and 37.5 mg/kg). Twenty-four hours after inoculation, drugs were administered once a day for consecutive 7 d, through ip injection for the S<sub>180</sub> group and sc injection for the H<sub>22</sub> group. Animals in the negative and positive control groups were administered with saline and Cytosine, respectively, while those in the three treated groups were administered with different doses of solanine. For the groups with solid tumor, the animals were put to death on the day after drug administration was terminated, and their hypodermic tumors were cut down and weighed. The therapeutic effect for solid tumor is expressed as the percentage of tumor weight that had been inhibited. The formula for calculating the inhibitory rate (*I*) is:

$$I = \frac{W_{\text{control}} - W_{\text{treated}}}{W_{\text{treated}}} \times 100\%$$

Where  $W_{\text{control}}$  means average weight for control, while  $W_{\text{treated}}$  indicates average weight for treated group

For the groups with abdominal dropsy, the average survival time (in number of days) was recorded for the treated and control groups, and the treated groups were observed for 60 d. For individuals who survived longer than 60 d, the survival time was also fixed as 60 d. The following formula is used to calculate the rate of life prolongation ( $R$ ):

$$R = \frac{T_{\text{treated}} - T_{\text{control}}}{T_{\text{control}}}$$

Where  $T_{\text{control}}$  means average survival time for control, while  $T_{\text{treated}}$  indicates average survival time for treated group

#### Cell incubation

HepG2 human hepatocarcinomatous cells, SGC-7901 human gastrocarcinomatous cells, and LS-174 human large intestine cancer cells were inoculated separately with suitable concentration in culture flasks, to which RPMI1640 containing 10% FBS was added. The flasks were incubated in a CO<sub>2</sub> incubator at a constant temperature of 37 °C, with 5% CO<sub>2</sub> and saturated humidity, with culture transfer performed once every 2–3 d. For the culture transfer, first the medium was discarded, the culture was rinsed for three times with PBS and then digested with pancreatin, and after that fresh medium was added which was blown and beaten to allow for thorough mixing. The cell concentration was adjusted to a suitable level, and then the culture was transferred to new culture flasks, to which a suitable amount of medium was added.

#### MTT selection for cytotoxic effect on tumors in the digestive system

Cells were taken during the logarithmic growth phase, and the concentration was adjusted to  $1 \times 10^4$  cells/mL. The cells were transferred to a 96-well culture plate at 100 μL/well, with 10 parallel wells for each kind of cells. The culture plates were then incubated in a CO<sub>2</sub> incubator with 5% CO<sub>2</sub> and at 37 °C. After the incubation, the cells were taken out and the medium was discarded, then 100 μL of 1 mg/mL MTT was added to each well. Hereafter cells were incubated for over 4 h, after which the liquid was discarded and 150 μL of DMSO was added to each well. The cultures were then vibrated on a microvibrator for 5 min so that the blue granules in them were thoroughly dissolved. The culture plate was then set in the microplate reader for absorbance ( $A$ ) to be measured at 570 nm. The inhibitory rate and IC<sub>50</sub> were then calculated.

#### Experiment on induction of apoptosis in HepG2 cells

**Drug administration** HepG2 cells were taken during the logarithmic growth phase, and after the concentration was adjusted to  $1 \times 10^6$  cells/mL, the cells were inoculated in a 6-well culture plate at 1 mL/well. The culture was then incubated for 24 h in a CO<sub>2</sub> incubator, after which solanine at different concentration was added to reach final concentration of 10, 2, and 0.4 μg/mL, respectively. CAM was added to the positive control to reach a final concentration of 1.15 μg, while culture medium of equal volume was added to the negative control, after which the cultures were again incubated.

**Morphology of apoptotic HepG2 cells observed using TEM** The cells were collected after they had been incubated for 24 h, rinsed once with PBS, and fixed with pre-cooled 2% glutaral at 4 °C for 2 h. After being fixed with 1% osmic acid for 1.5 h, the cells were dehydrated with common acetone, embedded in epoxy resin Epon812, cut into ultrathin sections, double stained with uranyl acetate and lead citrate, and observed with TEM.

**Morphology of apoptotic HepG2 cells examined with Annexin V-FITC/PI double staining and LCSM** Cells were collected after 24 h of incubation, rinsed once with PBS, after which the cells were again collected and  $1 \times 10^5$  resuspended cells were taken for the experiment. Annexin V-FITC binding buffer (195 μL) was added to re-suspend the cells, and 5 μL of Annexin V-FITC was added and the culture was gently mixed. The culture was then incubated for 10 min away from light at room temperature, then it was centrifuged for 5 min at  $1000 \times g$ . Then the supernatant was discarded, and 190 μL of Annexin V-FITC binding buffer was added to re-suspend the cells. Propidium iodide stain (10 μL) was added and gently mixed with the culture, after which the culture was set in an ice bath and kept away from light. The culture was then centrifuged for 5 min at  $1000 \times g$ , soon after the cells were collected, and 100 μL of Annexin V-FITC binding buffer was added to re-suspend the cells. A slide was smeared with this preparation and then observed under LCSM.

**Rate of early apoptosis analyzed using Annexin V-FITC/PI double staining and FC** Cells were

collected after 24 h of incubation, rinsed once with PBS, then  $1 \times 10^5$  re-suspended cells were collected and centrifuged for 5 min. The supernatant was discarded, and 195  $\mu\text{L}$  of Annexin V-FITC binding buffer was added gently to re-suspend the cells. Then 5  $\mu\text{L}$  of Annexin V-FITC was added and the culture was gently mixed, after which the culture was incubated for 10 min away from light at room temperature. After the culture was centrifuged, 190  $\mu\text{L}$  of Annexin V-FITC binding buffer was added gently to re-suspend the cells. Propidium iodide stain (10  $\mu\text{L}$ ) was added and gently mixed with the culture, then the culture was set in an ice bath and kept away from light. Soon after the culture was centrifuged and the cells were collected, then 100  $\mu\text{L}$  of Annexin V-FITC binding buffer was added gently to re-suspend the cells. The culture was then filtered through a 300-mesh sieve before it was mounted onto the FC for the rate of early apoptosis to be measured, with  $1 \times 10^5$  cells being examined.

#### Mechanisms for apoptosis of HepG2 cells

##### Effects of solanine on $[\text{Ca}^{2+}]_i$ of HepG2 cells (1)

**Drug administration** The test animals were divided into five groups as mentioned above. The final concentration of solanine for the treated groups was 2, 0.4, and 0.08  $\mu\text{g}/\text{mL}$ , respectively. A culture medium of equal volume was added to the negative control, while CAM with final concentration of 0.08  $\mu\text{g}/\text{mL}$  was added to the positive control. The cultures were then incubated again for 48 h with 5%  $\text{CO}_2$  and at 37  $^\circ\text{C}$  in a  $\text{CO}_2$  incubator. **(2) Effects on  $[\text{Ca}^{2+}]_i$**  After 48 h of incubation, the medium was drawn with a syringe, after which the cultures were rinsed thrice with PBS. Treatment fluid for Fluo-3/AM (molecular probes) fluorescence probe was added to achieve final concentration of 2  $\mu\text{g}/\text{mL}$ , and the cultures were incubated in a warm water bath at 37  $^\circ\text{C}$  away from light and then rinsed thrice, after which 200  $\mu\text{L}$  of PBS was added to cover all the cells, and LCSM was used to examine the fluorescence intensity (FI) of intracellular  $\text{Ca}^{2+}$  at an excitation wavelength of 488 nm and a radiation wavelength of 540–570 nm.

**Effect on membrane potential of HepG2 mitochondria observed using LCSM** After 48 h of incubation, the cells were taken from the incubator, the medium was drawn with a syringe, and the cultures were rinsed thrice with PBS. TMRE treatment fluid

(200  $\mu\text{L}$ ) was added to achieve final concentration of 2  $\mu\text{mol}/\text{L}$ , and the cultures were incubated at 37  $^\circ\text{C}$  away from light. Then the stain was sucked and the cells were gently rinsed for three times with PBS, then 200  $\mu\text{L}$  of PBS was added to cover all the cells in the wells, and LCSM was used to examine the FI at an excitation wavelength of 543 nm and a radiation wavelength of 550–590 nm.

**Effect on expression of Bcl-2 and Bax in HepG2 cells** Cell incubation and drug administration were performed as described above. After 24 h of incubation, the cells were collected and fixed with 4% formaldehyde polymerisatum for 30 min, then TPBS was added to re-suspend the cells. Murine antihuman antibody I treatment fluid was added to the cultures which were then incubated at 4  $^\circ\text{C}$  for the night. The cultures were rinsed with PBS, and goat antimouse IgG-FITC was added, then the cultures were incubated for 2 h at room temperature. The cultures were rinsed twice with PBS, and the cells were re-suspended, and the FI was measured using LCSM.

**Effect on caspase-3 activity in HepG2 cells** Cell incubation and drug administration were performed as described above. Caspase-3 activity was determined following directions on the test kit. After the cultures were incubated for 120 min away from light,  $A_{405}$  was measured on the microplate reader. The *p*-nitroaniline (10 mmol/L) provided by the test kit was diluted to 0, 10, 20, 50, 100, and 200  $\mu\text{mol}/\text{L}$ , respectively, and used as standard solutions. For each concentration, 100  $\mu\text{L}$  of the sample was taken for its  $A_{405}$  to be measured on the microplate reader, and a standardized curve was plotted.  $A_{405}$  for a sample minus  $A_{405}$  for the blank is the absorbance produced by the pNA when catalyzed by caspase-3 in the sample. The activity of caspase-3 is indirectly reflected by pNA in the sample as calculated by comparing with the standard curve.

## Results

### *In vivo* antitumor effect of solanine

The results are shown in Tables 1 and 2. It could be seen that solanine could significantly lower the tumor weight of  $S_{180}$  tumor-bearing mice and prolong their survival time.

### IC<sub>50</sub> for tumor inhibition in three cell lines

Experimental results (Table 3) show that solanine

**Table 1** Effect of solanine on tumor weight of  $S_{180}$  tumor-bearing mice by ip administration ( $\bar{x} \pm s$ ,  $n = 10$ )

Groups	Dosage / ( $\text{mg}\cdot\text{kg}^{-1}$ )	Tumor weight / g	Inhibitory rate / %
negative control	equal volume	$1.486 \pm 0.173$	—
Cytosan	20	$0.720 \pm 0.139^{**}$	51.55
solanine	9.375	$1.025 \pm 0.230^*$	31.03
	18.75	$0.912 \pm 0.216^*$	38.63
	37.5	$0.736 \pm 0.141^{**}$	50.47

\* $P < 0.05$  \*\* $P < 0.01$  vs negative control, same as below

**Table 2** Effect of solanine on rate of life prolongation in  $H_{22}$  tumor-bearing mice by ip administration ( $\bar{x} \pm s$ ,  $n = 10$ )

Groups	Dosage / ( $\text{mg}\cdot\text{kg}^{-1}$ )	Survival time / d	Rate of life prolongation / %
negative control	equal volume	$12.7 \pm 2.3$	—
Cytosan	20	$20.4 \pm 1.2^*$	60.63
solanine	9.375	$18.4 \pm 2.2^*$	44.88
	18.75	$21.1 \pm 1.9^*$	66.14
	37.5	$23.3 \pm 2.7^*$	83.46

**Table 3** Cytotoxic effect of solanine on HepG2, SGC-7901, and LS-174 cells ( $n = 10$ )

Compounds	$IC_{50} / (\mu\text{g}\cdot\text{mL}^{-1})$		
	HepG2	SGC-7901	LS-174
solanine	14.47	> 50	> 50
CAM	1.03	19.19	13.96

is rather sensitive to HepG2 hepatocarcinomatous cells.

### Experiments on HepG2 apoptosis

**Apoptosis observed using TEM** The results are shown in Fig. 1. It could be seen that the tumor cells in the negative control group (plate A) have nuclei of regular shapes with the nucleoli clearly shown as well as rough endoplasmic reticulum and are rich in mitochondria with clear ridges and membranes; Cells in the positive control group (plate B) show morphological changes typical of apoptotic cells, with decreased cellular processes, shrunk cytoplasm, heavily stained nuclei, massed and margined chromatin, vacuolated mitochondria, and apoptotic bodies as well; After being treated with solanine for 24 h, HepG2 cells in the high-, medium-, and low-dosage groups all show morphological changes typical of apoptotic cells, with highly condensed and margined chromatin, shrunk nuclei, chromosomes broken into fragments, bubbles

forming in the cytoplasm, apoptotic bodies forming, clearly swollen mitochondria in which ridges had disappeared, part of the mitochondria vacuolated, and the number of apoptotic bodies increasing with the increase in solanine concentration, as shown in plates C, D, and E in Fig. 1.

### Morphology of apoptotic cells examined with Annexin V-FITC/PI double staining and LCSM

As shown in Fig. 2, after 24 h of treatment with solanine at different concentration, some of the cells became heavily stained, with their membranes emitting a green fluorescence. The results suggested that phosphatidylserine (PS) became extroverted 24 h after solanine was administered (as shown in plates C, D, and E), which is typical morphology of apoptotic cells. The positive control (plate B) also showed clear apoptotic morphology. HepG2 cells in the negative control group (plate A) were lightly stained with Annexin V-FITC, showing no apoptotic morphology.

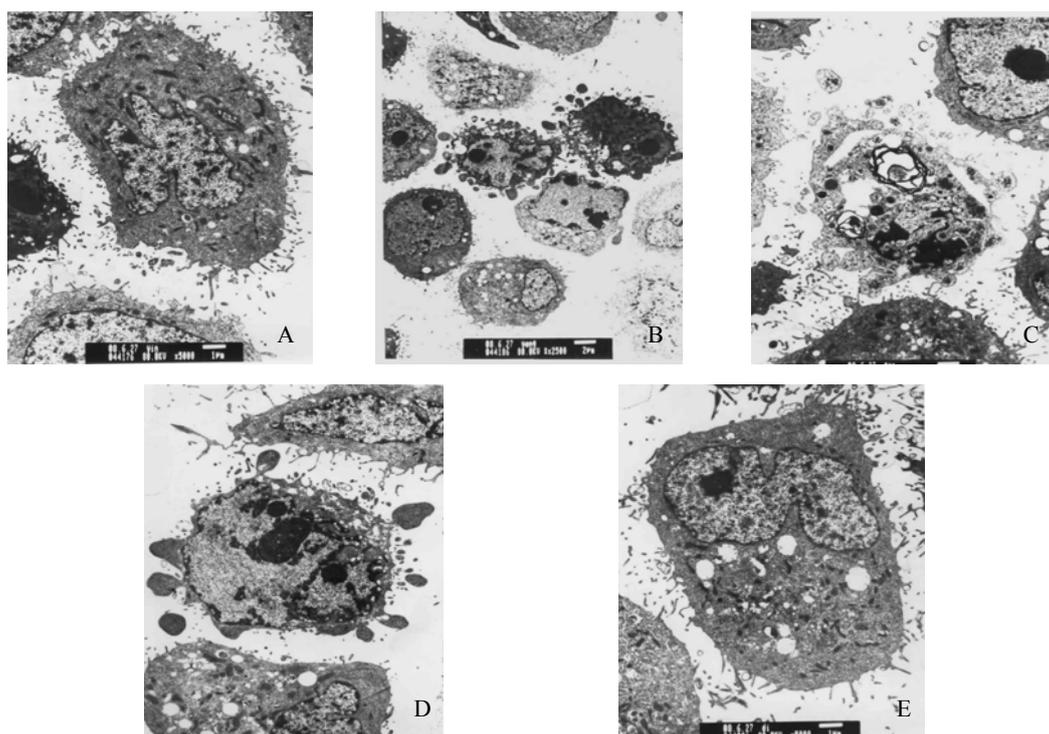
### Rate of early apoptosis analyzed using Annexin V-FITC/PI double staining and FC

As Fig. 3 showed, after the cultures were stained with Annexin V-FITC and PI, normal living cells were not found to be stained (as in the lower left corner of the plate); Cells in the early stage of apoptosis were stained with Annexin V-FITC only, being negative for PI staining (as in the lower right corner of the plate). After 24 h of treatment of HepG2 cells with solanine of 0.4, 2, and 10  $\mu\text{g}$ , the rate of early apoptosis was 4.0%, 8.5%, and 20.1%, respectively, showing that solanine could induce apoptosis in tumor cells, and the rate of apoptosis increases with increasing concentration of solanine, or, in other words, was dose-dependent to some extent.

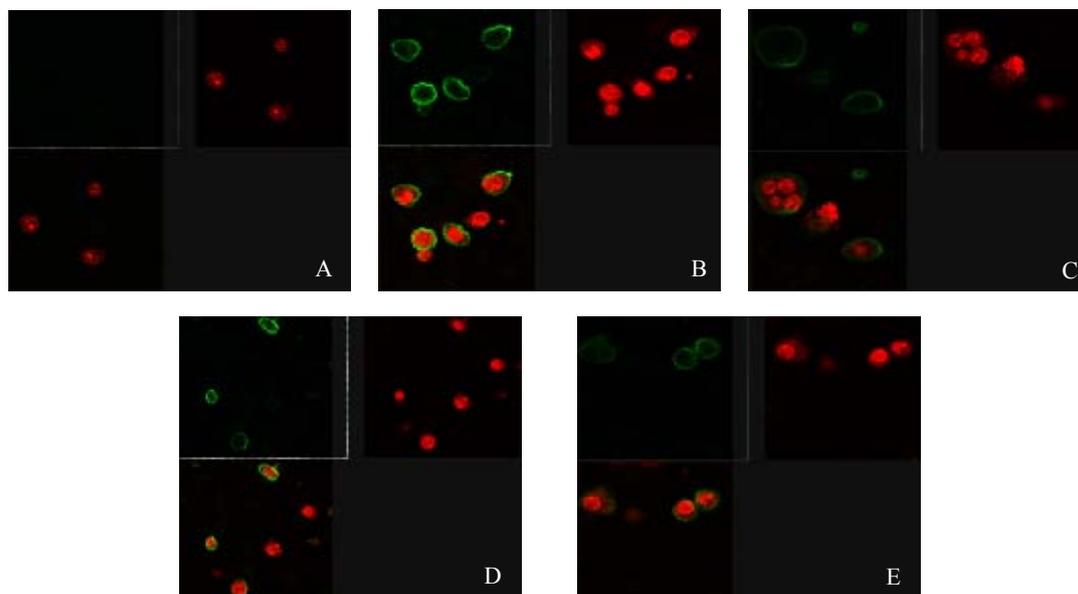
### Effect of solanine on mitochondrial channels of HepG2 Cells

**Effect of solanine on  $[\text{Ca}^{2+}]_i$  of HepG2 cells** HepG2 cells were double stained with Fluo-3/AM, and then observed under the LCSM to see the effect of solanine on  $[\text{Ca}^{2+}]_i$ , or FI, which indirectly reflects  $[\text{Ca}^{2+}]_i$  in different groups. The results are presented in Table 4, which shows that  $[\text{Ca}^{2+}]_i$  was relatively low in the negative control, while treatment with solanine raised  $[\text{Ca}^{2+}]_i$  in all the treated groups, with a dose-dependent manner.

### Changes in membrane potential of HepG2



**Fig. 1** TEM photographs of negative control (A), CAM (B), 10 (C), 2 (D), and 0.4  $\mu\text{g}\cdot\text{mL}^{-1}$  (E) solanine groups after 24 h of treatment



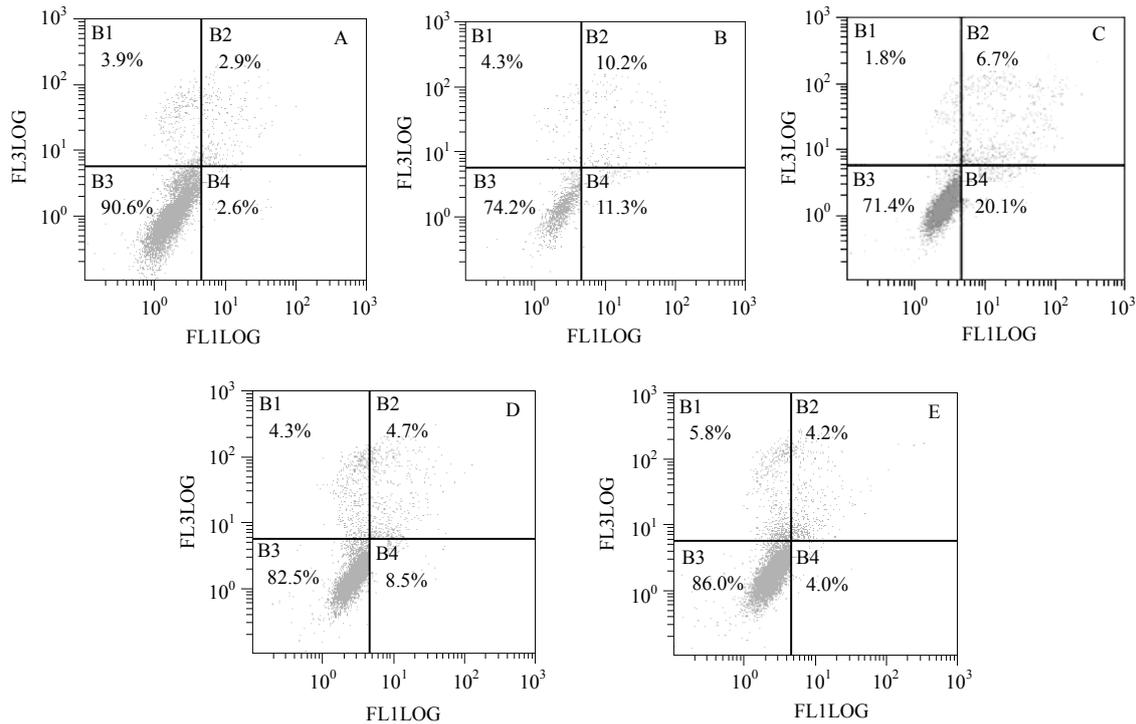
**Fig. 2** LCSM photographs of negative control (A), CAM (B), 10 (C), 2 (D), and 0.4  $\mu\text{g}\cdot\text{mL}^{-1}$  (E) solanine groups after 24 h of treatment (Annexin V-FITC/PI double staining)

**mitochondria during apoptosis induced by solanine**

The results are shown in Table 5. Compared with the negative control, solanine in concentration of 0.08, 0.4, and 2  $\mu\text{g}/\text{mL}$  significantly lowered the membrane potential in HepG2 cells ( $P < 0.01$ ). In the positive control, CAM also lowered the membrane potential in HepG2 cells significantly.

**Effect of solanine on expression of Bcl-2 and Bax proteins in HepG2 cells**

The experiment results show that 24 h after HepG2 cells were treated with solanine, the protein expression of Bcl-2 is significantly lower than that in the negative control, while the expression of Bax is significantly higher (Table 6). This means that the Bcl-2/Bax ratio



**Fig. 3** FC results of negative control (A), CAM (B), 10 (C), 2 (D), and 0.4  $\mu\text{g}$  (E) solanine groups after 24 h of treatment

**Table 4** Effect on  $[\text{Ca}^{2+}]_i$  in HepG2 cells ( $\bar{x} \pm s$ )

Groups	Final concentration / ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	<i>n</i>	FI
negative control	—	18	$23.98 \pm 10.06$
CAM solanine	0.08	16	$26.94 \pm 8.20$
	0.4	19	$158.75 \pm 14.52^{**}$
	2	20	$209.85 \pm 10.86^{**}$

**Table 5** Effect of solanine on membrane potential in HepG2 cells ( $\bar{x} \pm s$ )

Groups	Final concentration / ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	<i>n</i>	FI
negative control	—	53	$75.70 \pm 31.24$
CAM solanine	0.08	16	$47.17 \pm 15.98^{**}$
	0.4	37	$32.50 \pm 19.99^{**}$
	2	33	$28.49 \pm 9.71^{**}$

gradually decreased with the increase in dosage.

#### Effect of solanine on caspase-3 activity in HepG2 cells

The experiment results show that 24 h after HepG2 cells had been treated with solanine, caspase-3 activity in the cells was clearly higher than that in the

negative control, with the levels of activity in the 10, 2, and 0.4  $\mu\text{g}$  solanine groups differing significantly from the negative control (Table 7).

**Table 6** Effect of solanine on expression of Bcl-2 and Bax proteins in HepG2 cells (*n* = 20)

Groups	Drug concentration / ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	FI	
		Bcl-2	Bax
negative control	—	$36.84 \pm 10.17$	$7.28 \pm 9.56$
CAM solanine	1.15	$6.94 \pm 13.47^{**}$	$36.31 \pm 6.57^{**}$
	10	$5.78 \pm 12.28^{**}$	$45.47 \pm 9.34^{**}$
	2	$12.25 \pm 15.16^{**}$	$25.27 \pm 10.96^{**}$
	0.4	$27.40 \pm 11.45^{**}$	$15.55 \pm 14.28^*$

**Table 7** Effect of solanine on caspase-3 activity in HepG2 cells

Groups	Drug concentration / ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	Caspase-3 activity / mg prot
negative control	—	$1.37 \pm 9.96$
CAM solanine	1.15	$5.23 \pm 12.37^{**}$
	10	$5.45 \pm 14.25^{**}$
	2	$4.72 \pm 13.56^{**}$
	0.4	$3.47 \pm 17.28^{**}$

## Discussion

In this study, *in vivo* antitumor effect of solanine

was first observed by developing a mouse tumor model through transferring of S<sub>180</sub> and H<sub>22</sub> tumor cells. It was discovered that solanine could significantly lower the tumor weight in S<sub>180</sub> tumor-bearing mice and prolong the survival time of H<sub>22</sub> tumor-bearing mice, which means solanine has some inhibition on tumor in mice. In its clinical applications in Chinese materia medica, the nightshade is often used in the therapeutic treatment of tumors in the digestive system, therefore on the basis of *in vivo* study on its antitumor effect, we used MTT assay to observe the cytotoxic effect of solanine on three major types of tumor in the digestive system, namely, human hepatocarcinoma HepG2 cells, human gastrocarcinoma SGC-7901 cells, and human large intestine cancer LS-174 cells. The experiment results show that solanine has rather good cytotoxic effect on HepG2 cells.

From existing literature we know that most alkaloids have their cytotoxic effect by inducing apoptosis in tumor cells. So in ensuing experiments we focused on HepG2 cells, examining in depth the antitumor mechanisms of solanine from the perspective of apoptosis. First we morphologically determined the nature of the apoptosis involved by using TEM and Annexin V-FITC/PI double staining with LCSM, and then studied quantitatively the early apoptosis induced by solanine by using Annexin V-FITC/PI double staining with FC. The results show that solanine could induce morphological changes in HepG2 cells that are typical of apoptosis, with the rate of early apoptosis being 4%, 8.5%, and 20.1% 24 h after HepG2 cells were treated with solanine at concentration of 0.4, 2, and 10  $\mu\text{g/mL}$ , respectively.

Although it has been only less than four decades since the concept of apoptosis was formally proposed by Kerr, Wyllie, and Currie (1972), it has been an area of intense research efforts. Especially since the 1990s, research on apoptosis has been propelled to the forefront of life sciences, resulting in a high tide of research involving almost all areas in biomedical sciences, so that a tremendous amount of literature has accumulated in a few years on the morphological features of apoptotic cells, intracellular biochemical changes, factors that regulate genes for apoptosis, cause apoptosis, as well as the relationships between these factors and certain diseases. Membrane potential of

mitochondria in cells (Hwang *et al*, 2006; Lugli *et al*, 2005) and  $[\text{Ca}^{2+}]_i$  (Lepine, Sulpice, and Giraud, 2005; Pretorius and Bornman, 2005) are factors closely related to apoptosis as well as complementary with each other themselves.

Mitochondria are the intracellular  $\text{Ca}^{2+}$  stores of the cell, and changes in their membrane potential could cause  $\text{Ca}^{2+}$  and other ions to be released from or flow into mitochondria. In the same way, the release or inflow of  $\text{Ca}^{2+}$  and other ions are direct causes for the changes in membrane potential. The essence of membrane potential change is the opening of the permeability transition (PT) channels in the membrane. The opening of these channels precedes the release of  $\text{Ca}^{2+}$ , which is followed by the changes in membrane potential.

The dissipation of transmembrane potential of mitochondria in the process of apoptosis is mainly due to changes in the permeability of the inner membrane of mitochondria, because dynamic pores for PT (each composed of several proteins) are formed at points of contact between the outer and inner membranes of the mitochondrion (Zhang *et al*, 2004). PT channels are made up of proteins from various parts of the mitochondrion and those from cytoplasm (Wen and Han, 2002; Zhang *et al*, 1997). These include a protein in the cytochylema (hexokinase), proteins on the outer membrane of the mitochondrion (peripheral benzodiazepine receptors and voltage-dependent anion channels), a protein in the interstice between the inner and outer membranes of the mitochondrion (creatine kinase) (Lu, 2000), a protein on the inner membrane of the mitochondrion (ADP-ATP carrier) (Ying, 2000), and a protein in the mitochondrial matrix (cyclophilin D) (Zhong and Wu, 1999). Any substance, e.g. protoporphyria IX, the ligand for benzodiazepine receptors, that could specifically induce the formation of PT channels in mitochondria, could cause apoptosis (Xu, 2000; Chen, 1999; Wang, 2000; Yang *et al*, 1999). PT channels are a kind of channels with high electric conductivity. The lowering or even disappearance of membrane potential would suggest that PT channels are open.

The opening of PT pores could lead to the occurrence of two events (Xie, 1999). (1) The intra- and extra-membrane ion concentration tends toward

equilibrium, the transmembrane  $H^+$  gradient disappears, and the respiratory chain is uncoupled; (2) The flow of intra- and extra-membrane ions toward equilibrium leads to a hypertonic mitochondrial matrix, resulting in change in the volume of the mitochondrion. Since the surface area of the folded inner membrane of mitochondrion is larger than that of the outer membrane, this would lead to the rupture of the outer membrane.

These two events respectively lead to two consequences. Since a large number of  $Ca^{2+}$  have accumulated in mitochondria, the concentration of  $Ca^{2+}$  in mitochondria is much higher than that of  $Ca^{2+}$  in the cytoplasm. The occurrence of the first event would lead directly to the flow of  $Ca^{2+}$  from mitochondria to cytoplasm, resulting in a rapid increase in the concentration of  $Ca^{2+}$  in the cytoplasm. Therefore, in the ensuing study, we used TMRE staining and Fluo-3 staining to observe the changes caused by solanine in the mitochondrial membrane potential and  $[Ca^{2+}]_i$  in HepG2 cells. The results of the experiment show that solanine in all dosage groups (0.08, 0.4, and 2  $\mu\text{g/mL}$ ) leads to decreases in membrane potential and increases in the concentration of  $Ca^{2+}$ , suggesting that the decrease in the membrane potential of mitochondria and the increase in the concentration of  $Ca^{2+}$  are two closely associated processes in HepG2 cells. Since decrease in mitochondrial membrane potential reflects the opening of the PT channels on mitochondria (Lv and Li, 1999), we deduce that solanine first leads to the opening of PT on mitochondria, to be followed by an increase in the concentration of  $Ca^{2+}$ .

The occurrence of the second event means that, with the breaking up of the outer membrane of mitochondria, cytochrome C originally inside mitochondria is released into cytoplasm, activating enzymes related to caspases, thus resulting in apoptosis of the cells. At the same time, the expression levels of Bcl-2 and Bax proteins help to regulate the amount of cytochrome C flowing from inside mitochondria to cytoplasm, with high expression of Bcl-2 and low expression of Bax tending to inhibit the permeability of mitochondrial membrane and thus the release of cytochrome C.

In the ensuing experiment, we observed the effect of solanine on the expression of Bcl-2 and Bax protein and on caspase-3 activity in HepG2 cells.

In this experiment, LCSM (an indirect immunological fluorescence method) was used to measure the expression of Bcl-2 and Bax protein in HepG2 cells. The results show that solanine could decrease the protein expression of Bcl-2 in HepG2 cells and increase that of Bax, resulting in a decrease in the Bcl-2/Bax ratio. The results are consistent with our previous research achievements (Ji *et al*, 2008).

The colorimetric method was used to measure the effect of solanine on the relative activity of caspase-3 in HepG2 cells, with the results showing that solanine could increase the relative activity of caspase-3 in HepG2 cells.

From the above, we believe that solanine clearly has antitumor activity with a rather good inhibitory effect on human hepatocarcinoma. The inhibitory effect of solanine is achieved through lowering Bcl-2/Bax ratio, which leads to an increase in the concentration of cytoplasmic  $Ca^{2+}$  and the activation of the enzymes of the caspase family, thereby inducing the apoptosis of HepG2 cells.

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