

RESEARCH ARTICLE

N-Butanol Extract of *Capparis spinosa* L. Induces Apoptosis Primarily Through a Mitochondrial Pathway Involving mPTP Open, Cytochrome C Release and Caspase Activation

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Abstract

Background: *Capparis spinosa* L., a Uygur medicine, had been shown to have anti-tumor activity in our early experiments with an N-butanol extract (CSBE) as its active fraction. However, the mechanisms responsible for its effects are not clearly understood. Here, we report that treatment of SGC-7901 cells with CSBE resulted in dose-dependent reduction of cell viability and induction of apoptosis. **Materials and Methods:** To observe the inhibitory and killing effects of CSBE on SGC-7901, the SRB method was adopted, apoptosis being observed by electron microscopy. To clarify the mechanisms of apoptosis, Western blot and enzyme-labeled methods were used to examine the release of cytochrome c (Cyt c) and the activation of the caspase cascade. **Results:** By electron microscopy, apoptotic morphologic changes were detectable after CSBE administration. In this study, it was also demonstrated that CSBE induced apoptosis in SGC-7901 cells by inhibiting mPTP open, mitochondrial cytochrome c release, caspase-9 and caspase-3 activation. **Conclusions:** The findings indicated that CSBE induces apoptosis through mitochondrial pathway.

Keywords: *Capparis spinosa* L. - n-butanol extract - mitochondrial pathway - apoptosis

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Introduction

Capparis spinosa L., the caper bush, is a perennial winter-deciduous plant. The plant is best known for the fruit, which is usually used in the manufacture of medicines and aliments. *Capparis spinosa* L. has been shown to have anti-oxidative (Germano et al., 2002; Tlili et al., 2010; Siracusa et al., 2011), anti-inflammatory (Issac et al., 2011), anti-bacterial (Boga et al., 2011), anti-diabetic (Huseini et al., 2013), anti-hepatotoxic (Gadgoli and Mishra, 1999; Aghel et al., 2007), anti-proliferative properties (Wu et al., 2003). In earlier studies, we have shown that topical treatment of n-butanol extract of *Capparis spinosa* L. (CSBE) to SGC-7901 cells resulted in inhibition of tumor cell growth. However, the molecular mechanisms associated with the anti-tumor effects of CSBE are not clearly and systematically understood. We therefore initiated studies to determine whether CSBE-induced apoptosis in SGC-7901 and if so whether mPTP, cytochrome c and caspases were involved in the process.

Materials and Methods

Materials

The dry mature fruits of *Capparis spinosa* L. were collected from Xinjinag Urumqi and identified by Prof. Q. H. Wang from institute for drug control of Hei

longjiang, Harbin, China. Petroleum ether (60-90°C), chloroform, ethyl acetate, methanol, n-butanol, acetone and all chemicals and reagents used were analytically pure. Human gastric carcinoma cell line SGC-7901 was cultured by institute of materia medica of Harbin University of Commerce, which was cultured in RPMI-1640 medium with 10% calf serum in a 5% CO₂ incubator at 37°C. SRB (Sigma); RPMI-1640 (Gibco company); FCS (Hyclone); Caspase-9 and Caspase-3 (Beyotime company); MPTP Reagent (GENMED company); monoclonal antibody of β-actin, Cyt-c (Santa Cruz).

SRB Test

SRB assay was used according to the method as previously described (Skehan et al., 1990). SGC-7901 Cells were seeded at the density of 4×10⁵/mL in 96-well plates and grown for 24 h. The OD₄₉₀ of un-treated cells was measured at 490 nm. The cells were then treated with test drugs. After 72h, cells were fixed with 50% trichloroacetic acid (1 h at 4°C), stained for 30 min with 100 μl of 0.4% SRB solution at room temperature. The cells were quickly rinsed five times with 1% acetic acid to remove un-bound SRB. After air-drying, protein-bound SRB was dissolved in 200 μl of 10 mM Tris base solution (pH=10.5) for 5 min on a gyratory shaker. The pink SRB was quantified by measuring the optical density at 540 nm. Growth inhibition and cytotoxic effect of drugs were

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calculated according to the formula reported (Monks et al., 1991): $[(OD_{treated} - OD_{zero}) / (OD_{control} - OD_{zero})] \times 100\%$, when $OD_{treated}$ is $>$ to OD_{zero} ; $[(OD_{treated} - OD_{zero}) / OD_{zero}] \times 100\%$, when $OD_{treated}$ is $<$ to OD_{zero} . If $OD_{treated}$ is above OD_{zero} , test drug has induced a cytostatic effect, whereas if $OD_{treated}$ is below OD_{zero} , cell killing has occurred. GI_{50} was the drug concentration when $[(OD_{treated} - OD_{zero}) / (OD_{control} - OD_{zero})] \times 100\% = 50\%$. TGI was the drug concentration when $OD_{treated} = OD_{zero}$. LC_{50} was the drug concentration when $[(OD_{treated} - OD_{zero}) / OD_{zero}] \times 100\% = 50\%$.

Electron microscopy

Logarithmic growth phase cells were inoculated in 6-well plates that were cultured in $37^{\circ}C$, $5\% CO_2$ incubator. After 24h, CSBE were added with 15, 30, 60 $\mu g/mL$ respectively. The final concentration of positive control group (HCPT) was 5 $\mu g/mL$, while negative control group received the same volume of culture medium (the cell culture and administration of following tests were as same as this test). After 72h, cells were digested by 0.25% pancreatin. The cell suspension was fixed by the addition of an equal volume of 2% glutaraldehyde for 2 h on ice. Then samples were postfixed in 1% osmium tetroxide and dehydrated in a graded ethanol series (Kerr et al., 1972). The fixed cells were embedded by Epon/Araldite. Thin sections (60 nm) were cut with a diamond knife, and subsequently stained with uranyl acetate and lead citrate for examination on electron microscope.

Determination of mPTP opening

Cell culture and administration were operated as above descriptions. Inner mitochondrial membrane permeability to the fluorescent dye calcein AM indicated the mPTP opening in intact cells. According to the previous report (Petronilli et al., 1999), the mPTP opening was directly assessed by co-loading with calcein AM and $CoCl_2$ in high conductance mode. After 24 h, cells of all groups were loaded for 15 min with 1 $\mu mol/L$ calcein AM at $37^{\circ}C$ and then washed free of calcein and $CoCl_2$. Release of calcein from mitochondria was excited with a 480nm laser and visualized with a 520 nm filter using a laser scanning confocal microscope.

Western blot analysis

Cell culture and administration were operated as above descriptions. Total protein was extracted after the treatment of test drugs for 24 h, and protein content was calculated by Bradford method. For electrophoresis, 50 μg of protein were loaded on a 10% Tris-HCl SDS polyacrylamide gel. Protein was electrotransferred to a NC membrane and then blocked with 5% nonfat dry milk in 20 mM of TBS with 0.1% Tween at $4^{\circ}C$ for 2 h. After blocking, the membrane was incubated overnight at $4^{\circ}C$ with anti-cyt-c primary antibody (1:200), and with the corresponding secondary antibody (1:500) at room temperature for 2 h. Cell membrane was took out, and colored with BCIP/NBT color agent. Picture was collected with Tannon gel imaging system, and hybrid band was analyzed quantitatively with Gel-Pro Analyzer 3.1 density analysis software.

Caspase-9 and Caspase-3 activity

Caspase-9 and caspase-3 activity was determined by measuring the release of AFC fluorophore from the caspase substrate Z-DEVD-AFC. SGC-7901 cells were plated at the density of 2×10^6 cells/ml in six well plates and grown for 24 h. Cell culture and administration were operated as above descriptions. The medium was replaced with medium containing test drugs and incubated for 24 h. The cells were subjected to caspase-3 assay according to manufacturer instructions. Each well was supplemented with 2 mM of the caspase-3 substrate Ac-DEVD-pNA (or the caspase-9 substrate Ac-LEHD-pNA). Plates were incubated for 2 h at $37^{\circ}C$, and caspase-3 (or caspase-9) activity was measured using Bio-Rad microplate Reader at 405 nm wavelength.

Statistical analysis

Data were presented as mean \pm SD from three independent experiments, and analyzed with one-way ANOVA. In all cases, $p < 0.05$ was considered statistically significant ($*p < 0.05$ and $**p < 0.01$).

Results

In vitro proliferation assay on SGC-7901 cells of n-butanol extract of Capparis spinosa L

SGC-7901 cells were exposed to n-butanol extract from *Capparis spinosa* L. (CSBE) for about 72h and cytotoxicity was determined with SRB assays. The cytotoxicity of CSBE on SGC-7901 cells was obvious, while GI_{50} was $31.785 \mu g \cdot mL^{-1}$, LC_{50} $40.146 \mu g \cdot mL^{-1}$, TGI $45.864 \mu g \cdot mL^{-1}$. The GI_{50} , LC_{50} and TGI of HCPT were respectively $0.097 \mu g \cdot mL^{-1}$, $5.57 \mu g \cdot mL^{-1}$ and $16.11 \mu g \cdot mL^{-1}$. The effect of CSBE against SGC-7901 cells (concentration range 1~100 $\mu g/ml$) was showed a decrease in percent cell viability in a dose-dependent manner, as compared with that of the control. It was demonstrated that CSBE dose-dependently inhibited the proliferation of SGC-7901 cells in SRB assays.

Apoptotic morphology evaluated by electron microscope

As a test to confirm the cytotoxicity of CSBE, SGC-7901 cells were incubated with different concentrations of CSBE and their morphological alterations were verified via electron microscope. Numerous cells with morphologic changes consistent with apoptosis were

Table 1. Inhibition rate of CSBE on SGC-7901 by SRB Assay

Group	Concentration ($\mu g \cdot mL^{-1}$)	Rate of inhibition % ($\bar{x} \pm s$)	GI_{50} ($\mu g \cdot mL^{-1}$)	LC_{50} ($\mu g \cdot mL^{-1}$)	TGI ($\mu g \cdot mL^{-1}$)
Control	-	0	-	-	-
CSBE	1	$8.323 \pm 4.998^{**}$			
	5	$18.047 \pm 5.336^{**}$			
	25	$40.513 \pm 5.122^{**}$	31.785	40.146	45.864
	50	$61.183 \pm 2.744^{**}$			
	75	$82.012 \pm 2.824^{**}$			
	100	$96.588 \pm 2.152^{**}$			
HCPT	0.01	$12.088 \pm 0.017^{**}$			
	0.1	$50.549 \pm 0.019^{**}$	0.097	5.57	16.11
	1	$64.286 \pm 0.006^{**}$			
	10	$70.147 \pm 0.006^{**}$			

* $p < 0.05$; ** $p < 0.01$ vs control group

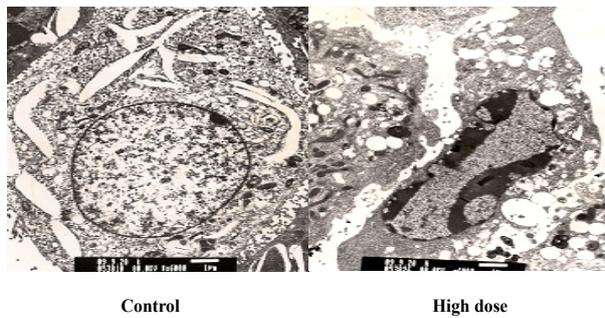


Figure 1. Morphological Appearance of SGC-7901 Cells by Electron Microscope

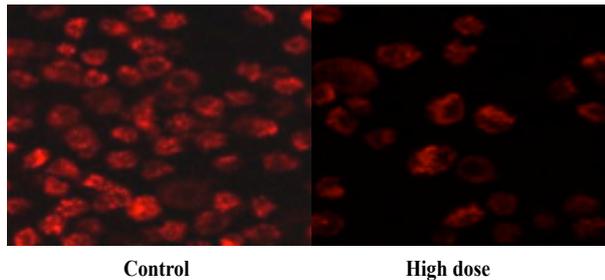


Figure 2. Effect of N-butanol Extract from *Capparis Spinosa* L. on Variation of [MPTP] in SGC-7901 Cells

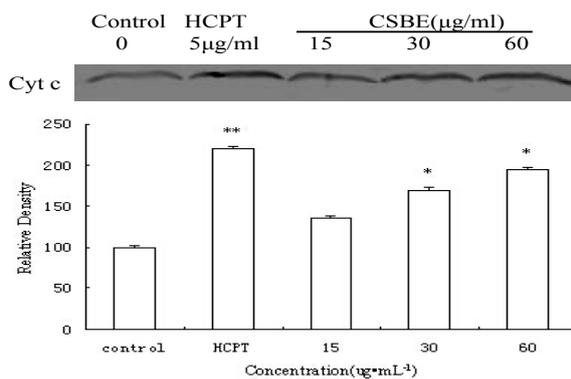


Figure 3. Effects of N-butanol Extract from *Capparis Spinosa* L. on Cyt c Expression

detected with transmission electron microscopy in three administration groups (Figure 1).

CSBE induces mPTP open in SGC-7901 cells

Figure 2 showed representative fluorescence image of calcein AM in SGC-7901 at absence and 24 h after exposure to CSBE. The fluorescence intensity of cells from the CSBE-treated group was weaker (H: 59.7%, M: 76.4%, L: 86.5%) than in the control group (107.2%, $p < 0.01$).

CSBE induces cytochrome c release in SGC-7901 cells

By western blot analysis, we found that treatment of SGC-7901 cells with CSBE (15–60 μg/ml) resulted in a dose-dependent increase in cytochrome c release from mitochondria compared with control cells (shown in Figure 3).

CSBE lead to the activation of caspase-9 and caspase-3

As shown in Figure 4, the significant increase in caspase-9 and caspase-3 basal activities could be observed in CSBE groups. There is a striking difference between control group and CSBE groups.

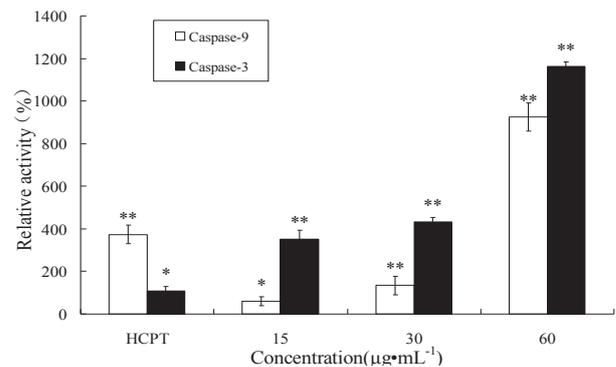


Figure 4. Statistic Graph of Caspase-9 and Caspase-3 activities. * $p < 0.05$; ** $p < 0.01$ vs Control Group

Discussion

In earlier studies, we have shown that n-butanol extract of *Capparis spinosa* L. (CSBE) had anti-tumor activity and significantly inhibited S180 sarcoma in terms of tumor weight (Li et al., 2014). However, the molecular mechanisms of the anti-tumor effects of CSBE are still not clearly understood. Induction of apoptosis may be one of the possible mechanisms of tumor prevention (Banjerdpongchai et al., 2008; Rasul et al., 2011; Inan et al., 2012; Hossein et al., 2013; Banjerdpongchai and Khaw-On, 2013; Alabsi et al., 2013). Apoptosis is induced by activation of a series of intracellular signaling events such as mitochondrial cytochrome c release, and activation of caspase proteases (Thompson, 1995; Chiarugi and Moskowitz, 2002). In our continuing efforts, we are trying to use SGC-7901 cell as an *in vitro* model to reveal that the treatment with CSBE results in induction of apoptosis which is associated with the open of mPTP, the release of apoptogenic molecules (cytochrome c) into cytosol and activation of caspase cascade.

The electron microscope forms a common analysis method in observation of cell ultrastructure (Searle et al., 1975; Arends and Wyllie, 1993). All those show that CSBE can induce characteristic apoptosis, such as cell shrinking of cytoplasm, condensation of nuclear chromatin and its segregation into sharply delineated masses against nuclear membrane, karyorrhexis or occurrence of apoptotic bodies. In three administration groups, the SGC-7901 cells are clearly apoptotic as judged from structural criteria such as chromatin condensation, margination and nuclear lobulation, which seem to cell undergoing apoptosis.

Mitochondria are essential for cell survival and play important roles in the regulation of cell death. When the channel is open, mPTP allows low-molecular-weight molecules to enter mitochondrial matrix, which will lead to imbalance of osmotic pressure, swell of matrix, rupture of outer mitochondrial membrane, and cell apoptosis ultimately (Bernardi et al., 1992; Crompton et al., 1999). Calcein AM fluorescence in cytosol is quenched by cobalt chloride, and staining is more diffuse when mPTP is closed than when mPTP is open. From the results, CSBE induces calcium overload and oxidative stress, which leads to mPTP open combined with other factors. The mPTP opening is a main mechanism mediating cytochrome c release and PARP cleavage, activating caspase cascade

and then inducing SGC-7901 cells apoptosis.

Cytochrome c, one of the key signalling molecules of cell apoptosis, normally resides between the inner and outer mitochondrial membrane. Cytochrome c translocates from mitochondria to cytosol, where it activates factor-1 together with ATP/dATP and a procaspase 9. Cytosolic cytochrome c also can interact with Apaf-1 and activate caspase-3 and PARP. This efflux of cytochrome c is a critical event in the activation of intracellular apoptosis signal. It results in a cascade of caspase activation, which leads to apoptosis (Kluck et al., 1997; Yang et al., 1997). CSBE can increase the release of cytochrome c from mitochondria to cytoplasm. The cytochrome c may interact to Apaf-1, lead to the activation of caspases-9 and finally lead to the activation of caspase-3, which subsequently leads to the apoptosis of SGC-7901 cells.

At the concentration that CSBE induces mPTP open and cyt-c releasing, it also increases caspase-9 activity, and increases caspase-3 activity ultimately. The caspase cascade is an index of apoptosis. We therefore measure the activities of caspase-9 and caspase-3 in SGC-7901 cells. We thus have confirmed the activation of those apoptosis effector enzymes induced by CSBE. Cytosolic cytochrome c may bind with procaspase-9 and Apaf-1 in the presence of dATP, which results in caspase-9 activation (Li et al., 1997). Then it will lead to activation of caspase-3, which may result in DNA fragmentation and cell apoptosis.

In summary, our data have demonstrated that CSBE can inhibit the proliferation of SGC-7901 cells *in vitro* via apoptosis induction in dose-dependent manner. In this study, it is found that treatment of CSBE results in the open of mPTP, the translocation of cytochrome c from mitochondria to cytosol and the activation of caspase-9 and caspase-3, which will lead to the apoptosis in SGC-7901 cells.

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