Anticancer activities of alkaloids extracted from the *Ba lotus* seed in human nasopharyngeal carcinoma CNE-1 cells

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Abstract. To investigate the anticancer activities of alkaloids from the *Ba lotus* seed (BLSA) in human nasopharyngeal carcinoma (NPC) CNE-1 cells, an MTT assay, flow cytometry, reverse transcription-polymerase chain reaction and western blotting were performed. BLSA was found to significantly reduce CNE-1 cell proliferation in a dose-dependent manner at all concentrations compared with the control ($P<0.05$). In addition, flow cytometry analysis identified that BLSA treatment significantly increased the sub-G1 content in CNE-1 cells ($P<0.05$). Following BLSA treatment, the mRNA and protein levels of a number of apoptosis-related factors, such as caspase family members (caspase-3, -8 and -9), B-cell lymphoma (Bcl)-2-associated X protein, Fas and Fas ligand were significantly increased compared with the control ($P<0.05$). This was accompanied by a significant decrease in anti-apoptotic Bcl-2 and Bcl-extra large protein expression compared with the control ($P<0.05$). Furthermore, BLSA treatment was determined to modulate CNE-1 cell expression of nuclear factor (NF)-κB and NF-κB inhibitor α. The results of the present study indicate that BLSA has anticancer activity through inducing cellular apoptosis. In addition, these results suggest that BLSA can be used as a therapeutic agent in NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is a type of cancer with a high prevalence rate (2.8/100,000 and 1.9/100,000 people/year in men and women, respectively, in 2008) in Southeast China, particularly in the Guangxi, Guangdong, Hainan and the Hong Kong Special Administrative Region (1). NPC is characterized by a high metastatic potential, frequent initial dissemination to regional lymph nodes and distant metastases, causing patients to succumb to NPC (2). Early diagnosis of NPC and chemoradiotherapy treatment enables the best outcome. The overall five-year survival rate is associated with the NPC stage at diagnosis, ranging from between 58% at stage IV and 90% in stage I. However, in the advanced stages of NPC chemo-radiotherapy is impractical (3,4). Therefore, the induction of NPC cell apoptosis is a strategy to control NPC and other malignancies in clinical therapy (5).

B-cell lymphoma 2 (Bcl-2) is part of the Bcl-2 protein family, which regulates cell death by inducing or inhibiting apoptosis. The Bcl-2 family is divided into anti-apoptotic factors, including Bcl-2, Bcl-extra large (Bcl-xL) and Bcl-2-like protein 2, and pro-apoptotic factors, such as Bcl2-associated X protein (Bax), Bcl-2-associated death promoter, Bcl-2-interacting mediator of cell death (Bim), Bcl-2 homologous antagonist/killer and p53 upregulated modulator of apoptosis (6). The extrinsic apoptosis signaling pathway is mediated by receptor-ligand binding. In this signaling pathway, the Fas receptor, Fas ligand (FasL), Fas-associated death domain (FADD) and caspase-8 mediate apoptosis. Alternatively, apoptotic stimuli can cause the depolarization of the inner mitochondrial membrane, leading to the release of cytochrome *c* (Cyt *c*) into the cytosol (7). Cyt *c* molecules

induce the activation of apoptotic protease activation factor-1 and procaspase-9. Activated caspase-8 and -9 cleave and activate the final executioner of apoptosis, caspase-3, resulting in chromatin condensation and DNA fragmentation (8-10).

The seed of *Nelumbo nucifera* (Gaertn), also known as the lotus, is traditionally used in Chinese folk medicine. A number of previous studies have reported that the lotus seed exhibits numerous health benefits and pharmacological effects, such as anti-ischemic (11), antioxidant (12-14), hepatoprotective (12), antiproliferative (15-19), anti-inflammatory (20), anti-infertility (21), anti-arrhythmic (22-26), antifibrotic (27) and antiviral (28) activities. In the present study, the anticancer activity of alkaloids extracted from the *Ba lotus* seed (BLSA), a new variety of *Nelumbo nucifera,* which only grows in Chongqing, a city located in the southwest of China, was investigated in human NPC CNE-1 cells. In addition, the mechanism underlying this activity was examined.

Materials and methods

Chemical reagents. TRIzol, OligodT_{18} primer, murine Maloney leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, ethidium bromide (EtBr) and agarose were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All other chemical reagents were of analytical grade and purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Preparation of alkaloids from BLSA. Fresh BLSA was purchased from Chongqing Enterprise Engineering Research Center of Ba-lotus Breeding and Deep Processing (Chongqing, China), freeze-dried and ground into a fine powder. Alkaloids were extracted from powdered *Ba louts* seed (100 g) twice with 1,000 ml of ethanol (80% vol/vol) at 50˚C for 1 h. Following filtering, the extraction solution was loaded into a 80 cm cation exchange resin 732 column at 50˚C and the filtrate collected 3 h later. Distilled water was used to wash away water-soluble impurities and then an ethanol solution of BLSA extract (80%, v/v) at 3 ml/min was used to elute the alkaloids. The collected ethanol elucent was eluted by 80% ethanol solution (containing 2% of ammonia water) and finely condensed using a vacuum rotary evaporator at 37˚C, then freeze-dried and stored at -80˚C until required.

Cell culture. Human NPC CNE-1 cells were obtained from the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The cells were routinely maintained in Roswell Park Memorial Institute-1640 medium, supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin, at 37˚C in a humidified 5% CO_2 incubator at 95% relative humidity (model 3154; Forma Scientific, Inc., Marietta, OH, USA).

Cell viability assay. Cell viability was measured using the MTT assay. CNE-1 cells were seeded in 96-well plates (Nunc, Rochester, NY, USA) at a density of $1x10^4$ cells/well. Following incubation for 24 h, cells were treated with a number of concentrations (50, 100 and 200 μg/ml) of BLSA for a further 24 h. Then, 0.5 mg/ml of MTT reagent (100 μl; Ekear, Shanghai, China; cat. no. M0105) was added to each well and the cells incubated for 4 h at 37˚C. The formazan crystals formed was dissolved in dimethyl sulfoxide (100 μl/well). Then, the absorbance of the wells at 540 nm was measured using a micro plate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry analysis. BLSA-treated CNE-1 cells were collected following digestion with trypsin, washed twice with cold phosphate buffered saline (PBS) and re-suspended in 2 ml PBS. Then, the DNA of BLSA-treated cells was stained with propidium iodide using a Cycletest Plus DNA Reagent Kit (BD Biosciences; Franklin Lakes, NJ, USA; cat. no. 340242), according to the manufacturer's protocol. Fluorescence intensity was determined using a FACSCalibur flow cytometer and the data analyzed using Cell Quest Pro software (version 5.2.1) (both BD Biosciences).

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed for the following genes: Caspase-3, -8 and -9, Bax, Bcl-2, Bcl-xL, Fas, FasL, NF-κB, IkB-α and GADPH. Total RNA was isolated from BLSA-treated CNE-1 cells using TRIzol reagent, according to the manufacturer's recommendations, and centrifuged at 12,000 x *g* for 15 min at 25˚C following the addition of chloroform. Isopropanol was added to the supernatant in a 1:1 ratio and the RNA pelleted by centrifugation (12,000 x *g* for 15 min at 4˚C). The RNA was washed with ethanol, solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using a UV-1750 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). RNA (1 μg) was reverse transcribed using a PCR master mix [1X reverse transcriptase buffer, dNTPs (1 mM), oligo$(dT)_{18}$ primers (500 ng), MMLV reverse transcriptase (140 units) and RNase inhibitor (40 units)] for 45 min at 42˚C. Then, cDNA (2 μl) was mixed with 1 μl of each primer (10 μM) and 16 μl of DNase-free water in a PCR premix tube (AccuPower PCR PreMix; Bioneer Corporation, Daejeon, Korea) and PCR was performed in an automatic thermocycler (Bioneer Corporation, Daejeon, South Korea) for 40 cycles of 94˚C for 5 min, 58˚C for 30 sec, and 72˚C for 90 sec, followed by a 10 min cycle at 95˚C. Sequences of the primers used in PCR are presented at Table I. The PCR products were separated on 2% agarose gels and visualized by EtBr staining. GAPDH was used for normalization of the results. Gene expression was quantified using ImageJ software (version 1.44; National Institutes of Health, Bethesda, MD, USA) and results presented as fold change compared to the control group.

Protein extraction and western blot analysis. For protein extraction, BLSA-treated CNE-1 cells were washed with ice-cold PBS, homogenized with ice-cold radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 13,000 x *g* for 30 min at 4˚C. Protein concentrations were determined using the Bradford Protein Assay kit (Bio-Rad Laboratories, Inc.; cat. no. 5000001). For Western blot analysis, 30 μg of protein extract was separated by SDS-PAGE (10% gel) and then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA). Blocking and antibody treatment were conducted in 10% skimmed milk for 2 h at 4˚C. The blots were incubated for 4 h at 4˚C with primary

Table I. RT-PCR primer sequences.

Gene name	Primer sequences
Caspase-3	Forward: 5'-CAA ACT TTT TCA GAG GGG ATC G-3' Reverse: 5'-GCA TAC TGT TTC AGC ATG GCA-3'
Caspase-8	Forward: 5'-CCC CAC CCT CAC TTT GCT-3' Reverse: 5'-GGA GGA CCA GGC TCA CTT A-3'
Caspase-9	Forward: 5'-GGC CCT TCC TCG CTT CAT CTC-3' Reverse: 5'-GGT CCT TGG GCC TTC CTG GTA T-3'
Bax	Forward: 5'-AAG CTG AGC GAG TGT CTC CGG CG-3' Reverse: 5'-CAG ATG CCG GTT CAG GTA CTC AGT C-3'
Bcl-2	Forward: 5'-CTC GTC GCT ACC GTC GTG ACT TGG-3' Reverse: 5'-CAG ATG CCG GTT CAG GTA CTC AGT C-3'
Bcl-xL	Forward: 5'-CCC AGA AAG GAT ACA GCT GG-3' Reverse: 5'-GCG ATC CGA CTC ACC AAT AC-3'
Fas	Forward: 5'-GAA ATG AAA TCC AAA GCT-3' Reverse: 5'-TAA TTT AGA GGC AAA GTG GC-3'
FasL	Forward: 5'-GGA TTG GGC CTG GGG ATG TTT CA-3' Reverse: 5'-TTG TGG CTC AGG GGC AGG TTG TTG-3'
NF-κB	Forward: 5'-CAC TTA TGG ACA ACT ATG AGG TCT CTG G-3' Reverse: 5'-CTG TCT TGT GGA CAA CGC AGT GGA ATT TTA GG-3'
IκB-α	Forward: 5'-GCT GAA GAA GGA GCG GCT ACT-3' Reverse: 5'-TCG TAC TCC TCG TCT TTC ATG GA-3'
GAPDH	Forward: 5'-CGG AGT CAA CGG ATT TGG TC-3' Reverse: 5'-AGC CTT CTC CAT GGT CGT GA-3'

RT-PCR, reverse transcription polymerase chain reaction; Bax, Bcl2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, Bcl-extra large; FasL, Fas ligand; NF-κB, nuclear factor-κB; IκB-α, nuclear factor-κB inhibitor α.

antibodies against caspase-3 (rabbit monoclonal; 1:1,000; cat. no. 14220S), caspase-8 (rabbit monoclonal; 1:1,000; cat. no. 9478S) and caspase-9 (rabbit monoclonal; 1:1,000; cat. no. 9508S), and Bax (rabbit monoclonal; 1:1,000; cat. no. 14796S), Bcl-2 (rabbit monoclonal; 1:1,000; cat. no. 4223S), Bcl-xL (rabbit monoclonal; 1:1,000; cat. no. 2764S), Fas (mouse monoclonal; 1:1,000; cat. no. 8023S), FasL (rabbit polyclonal; 1:1,000; cat. no. 4273S), NF-κB (mouse monoclonal; 1:1,000; cat. no. 13681S), IκB-α (rabbit monoclonal; 1:1,000; cat. no. 4812S) and β-actin (mouse monoclonal; 1:1,000; cat. no. 12262S) (all Cell Signaling Technology, Inc., Danvers, MA, USA). Following washing with PBS containing 0.05% Tween 20 (PBS-T), the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 7074S) or horse anti-mouse antibodies (cat. no. 7076S) at a dilution of 1:5,000 (both Cell Signaling Technology, Inc.) for 1 h at room temperature. Then, blots were washed three times with PBS-T and antibody binding visualized by enhanced chemiluminescence (ECL Western Blotting Detection kit; GE Healthcare Life Sciences, Little Chalfont, UK; cat. no. RPN2108). Protein expression was quantified using ImageJ software (version 1.44; National Institutes of Health).

Statistical analysis. Results are presented as the mean ± standard deviation. Differences between the mean values of groups were assessed by one-way analysis of the variance, followed by a post-hoc Duncan's new multiple range test. $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Growth inhibition of human NPC CNE-1 cells by alkaloids of BLSA evaluated by the MTT assay.

Treatment (μg/ml)	OD_{540}	Inhibitory rate (%)
0	0.471±0.005[a]	-
50	0.376±0.010[a]	20.2±0.2[a]
100	0.249±0.014[b]	47.1±0.3[b]
200	0.088±0.012[b]	81.3±0.2[b]

Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]$P<0.05$, [b]$P<0.01$ vs. the control group. NPC, nasopharyngeal carcinoma; BLSA, *Ba lotus* seeds; OD_{540}, optical density at 540 nm.

SAS software (version 9.1; SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis.

Results

BLSA decreases CNE-1 cell proliferation. BLSA was found to significantly reduce CNE-1 cell proliferation *in vitro*, in a dose-dependent manner, at all concentrations tested compared with the control group ($P<0.05$; Table II). The highest dose of BLSA (200 μg/ml) showed the greatest inhibitory activity (81.3±0.2%; Table II).

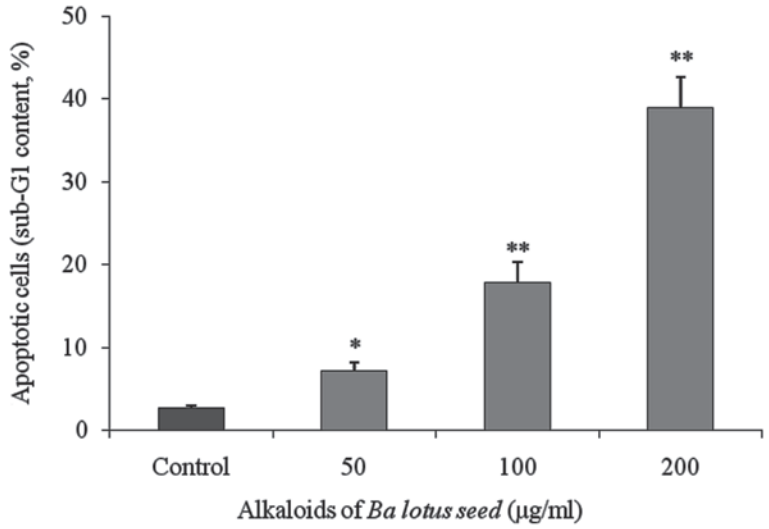

Figure 1. Level of apoptosis (sub-G1 content) induced by alkaloids of BLSA in human NPC CNE-1 cells, evaluated using flow cytometry. Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. *P<0.05, **P<0.01 vs. the control group. BLSA, *Ba lotus* seeds; NPC, nasopharyngeal carcinoma.

BLSA induces apoptosis in CNE-1 cells. Flow cytometry analysis identified that BLSA increased apoptosis in CNE-1 cells in a dose-dependent manner. BLSA treatment (50, 100 and 200 μg/ml) significantly increased the sub-G1 DNA content of CNE-1 cells to 7.1 (P<0.05), 17.8 (P<0.01) and 38.9% (P<0.01), respectively, compared with 2.6% in the untreated control group (Fig. 1).

BLSA increases Fas and FasL protein expression in CNE-1 cells. The effect of BLSA on mRNA and protein levels of specific genes was determined by RT-PCR and western blot analysis, respectively. BLSA treatment was identified to significantly increase mRNA and protein levels of Fas and FasL in CNE-1 cells, in a dose-dependent manner, at all concentrations tested compared with the control (P<0.05; Fig. 2). The highest dose of BLSA (200 μg/ml) significantly up-regulated Fas (2.9 fold) and FasL (1.7 fold) mRNA levels, and Fas (1.5 fold) and FasL (3.3 fold) protein levels compared with the control group (all P<0.01; Fig. 2C and D).

BLSA increases caspase-3, -8 and -9 expression in CNE-1 cells. BLSA treatment significantly increased mRNA and protein expression levels of caspase-3, -8 and -9 in CNE-1 cells, at all concentrations tested compared with the control (P<0.05; Fig. 3). The highest dose of BLSA (200 μg/ml) significantly increased mRNA and protein levels of caspase-3 (26.7 and 1.4 fold, respectively; Fig. 3C), -8 (3.3 and 1.5 fold, respectively; Fig. 3D) and -9 (5.3 and 1.6 fold, respectively; Fig. 3E) compared with the untreated control group (all P<0.01).

BLSA modulates Bcl-2, Bcl-xL and Bax expression in CNE-1 cells. Compared with the control group, BLSA treatment significantly decreased expression of Bcl-2 and Bcl-xL mRNA and protein, and increased expression of Bax mRNA and protein, in a dose dependent manner, in CNE-1 cells, at all concentrations tested (P<0.05; Fig. 4). At the highest dose (200 μg/ml), BLSA significantly reduced mRNA and protein levels of Bcl-2 (90 and 94%, respectively) and Bcl-xL (81 and 75%, respectively) compared with the control group (P<0.01; Fig. 4D and E). In contrast, 200 μg/ml BLSA enhanced mRNA (10.6 fold; P<0.01) and protein (1.6 fold; P<0.05) levels of Bax in CNE-1 cells (Fig. 4C).

BLSA modulates NF-κB and IκB-α expression in CNE-1 cells. BLSA treatment significantly decreased NF-κB mRNA and protein expression, and increased IκB-α mRNA and protein expression, in a dose dependent manner in CNE-1 cells, at all concentrations tested (P<0.05 vs. the control group; Fig. 5). Following treatment with 200 μg/ml BLSA, NF-κB mRNA and protein levels were significantly decreased by 78% and 35%, respectively, compared with the control group (P<0.01; Fig. 5C). In addition, BLSA increased mRNA and protein levels of IκB-α by 32.7 and 2.3-fold, respectively, compared with the control group (P<0.01; Fig. 5D).

Discussion

Alkaloids, isolated from herbs, may possess anti-cancer activities, including induction of cell cycle arrest, apoptosis, autophagy, and inhibition of angiogenesis and metastasis (29). A recent study reported that a number of alkaloids isolated from *N. nucifera* Gaertn. cv. *Rosa-plena* exhibited antioxidant and anticancer activity *in vitro* (30). In the present study, BLSA exhibited anti-cancer effects, associated with the induction of apoptosis, in CNE-1 cells. BLSA significantly reduced CNE-1 cell proliferation and promoted transition into the sub-G1 phase. These results indicate that the anti-CNE-1 effects of BLSA are associated with apoptosis.

In the current study, mRNA and proteins expression levels of a number of apoptosis-associated genes in BLSA-treated CNE-1 cells were investigated using RT-PCR and western blotting, respectively. Following treatment for 24 h with BLSA, mRNA and protein levels of Fas and FasL were significantly increased compared with untreated cells. Fas and FasL are inducers of apoptosis that serve a primary role in death receptor-mediated apoptosis (31). Activation of Fas/FasL

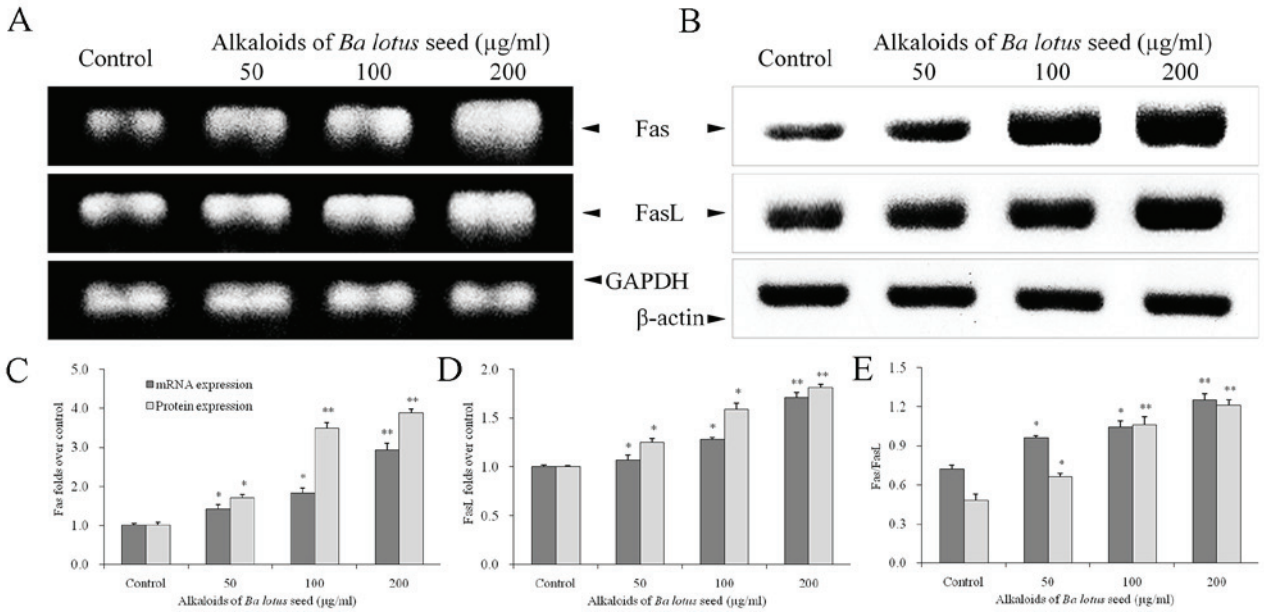

Figure 2. Effect of alkaloids of BLSA on the (A) mRNA and (B) protein expression levels of Fas and FasL in human NPC CNE-1 cells, measured by RT-PCR and western blotting, respectively. Fold change of (C) Fas, (D) FasL and (E) Fas/FasL ratio. Fold change over the control for mRNA was calculated as mRNA expression/GAPDH expression. Fold change over the control for protein was calculated as protein expression/β-actin expression. Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. *P<0.05, **P<0.01 vs. the control group. BLSA, *Ba lotus* seeds; NPC, nasopharyngeal carcinoma, FasL, Fas ligand.

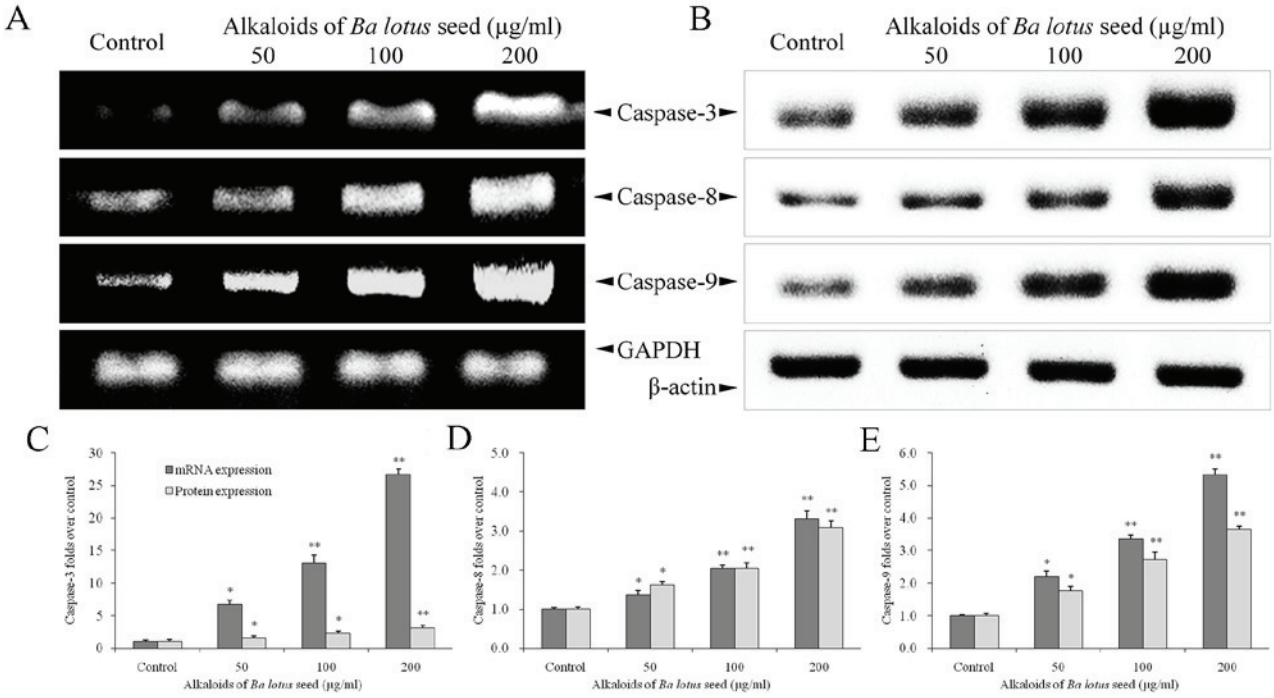

Figure 3. Effect of alkaloids of BLSA on the (A) mRNA and (B) protein expression levels of caspase-3, -8 and -9 in human NPC CNE-1 cells. Fold change of (C) caspase-3, (D) caspase-8 and (E) caspase-9 mRNA and protein expression over the control. Fold change over the control for mRNA was calculated as mRNA expression/GAPDH expression. Fold change over the control for protein was calculated as protein expression/β-actin expression. Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. *P<0.05, **P<0.01 vs. the control group. BLSA, *Ba lotus* seeds; NPC, nasopharyngeal carcinoma.

recruits FADD and the death domain, which subsequently induce the activation of caspase-8, -9 and -10, key regulators that promote cellar apoptosis (32).

The results of the present study determined that mRNA and protein levels of caspase-3, -8 and -9 were significantly increased in BLSA-treated CNE-1 cells compared with the control group. The caspase signaling cascade is a key event in extrinsic and intrinsic apoptosis, which is characterized by the activation of caspase-8 and -9, respectively (9). Caspase-8, the initiator caspase in Fas signaling, is recruited to the acti-

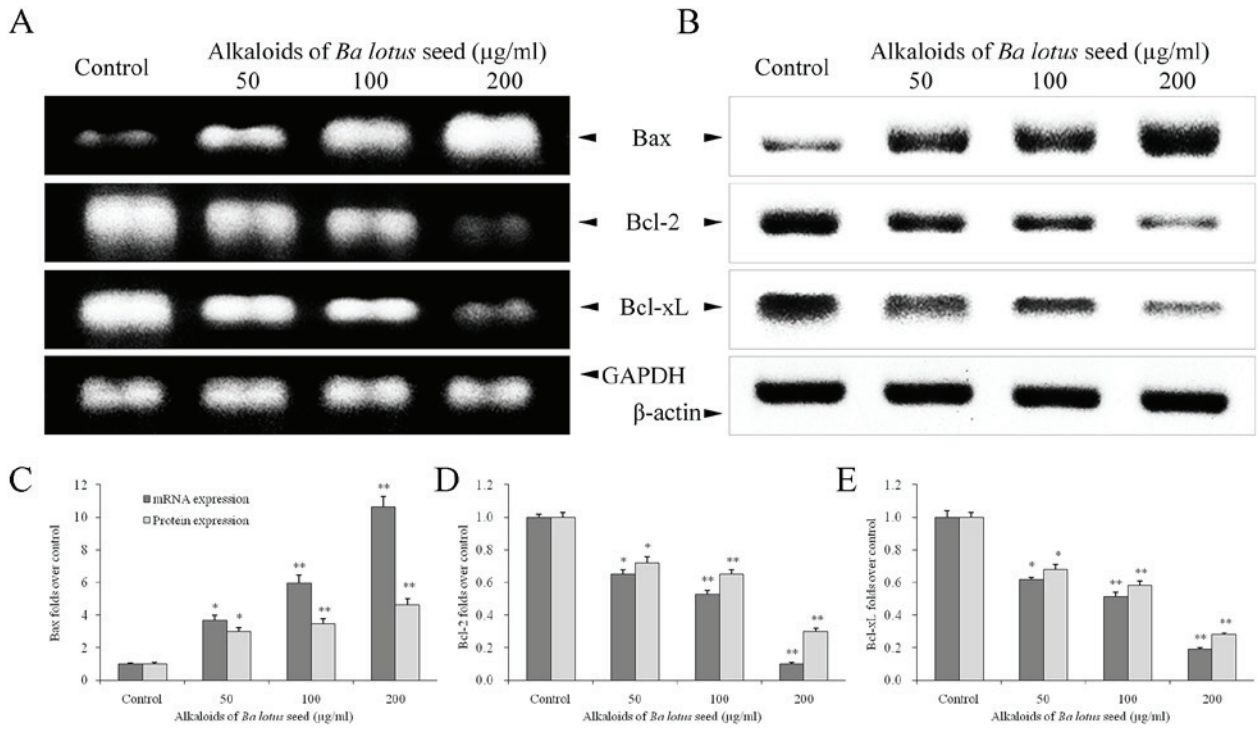

Figure 4. Effect of alkaloids of BLSA on the (A) mRNA and (B) protein expression levels of Bax, Bcl-2 and Bcl-xL in human NPC CNE-1 cells. Fold change of (C) Bax, (D) Bcl-2 and (E) Bcl-xL mRNA and protein expression over the control. Fold change over the control for mRNA was calculated as mRNA expression/GAPDH expression. Fold change over the control for protein was calculated as protein expression/β-actin expression. Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. *P<0.05, **P<0.01 vs. the control group. BLSA, *Ba lotus* seeds; NPC, nasopharyngeal carcinoma; Bax, Bcl2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large.

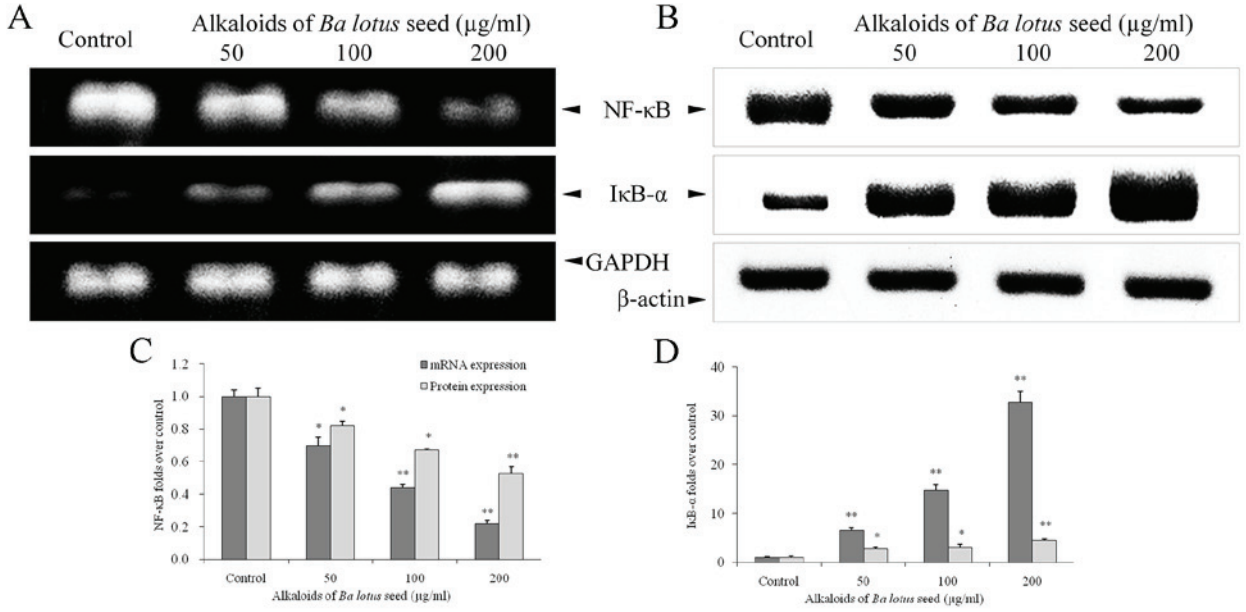

Figure 5. Effect of alkaloids of BLSA on the (A) mRNA and (B) protein expression levels of NF-κB and IκB-α in human NPC CNE-1 cells. Fold change of (C) NF-κB and (D) IκBα mRNA and protein expression over the control. Fold change over the control for mRNA was calculated as mRNA expression/GAPDH expression. Fold change over the control for protein was calculated as protein expression/β-actin expression. Results are presented as the mean ± the standard deviation of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. *P<0.05, **P<0.01 vs. the control group. BLSA, *Ba lotus* seeds; NPC, nasopharyngeal carcinoma; NF-κB, nuclear factor-κB; IκB-α, nuclear factor-κB inhibitor α.

vated Fas receptor and facilitates death receptor-mediated apoptosis (33). Caspase-9, an apoptotic effector molecule in intrinsic apoptosis, initiates programmed cell death following activation (10). Activated caspase-8 and -9 activate caspase-3, an executioner caspase that subsequently induces apoptosis (8). These results indicate that BLSA induces CNE-1 cell apoptosis

through activating extrinsic (Fas/FasL) and intrinsic apoptotic signaling pathways.

The Bcl-2 family, a well-known family of apoptosis regulators, serves a primary role in intrinsic apoptosis (34). Bcl-2 and Bcl-xL are typically anti-apoptotic factors that block the release of Cyt *c* from mitochondria and thus promote cell survival. Bcl-2 can reduce the release of Cyt *c* from the mitochondria, thus inhibiting apoptosis (6). In contrast, Bax is a pro-apoptotic factor that promotes apoptosis (6,7). The balance between anti- and pro-apoptotic factors influences the occurrence of apoptosis, and is associated with the success rate of chemotherapy in cancer patients (35). In the present study, BLSA treatment significantly increased mRNA and protein levels of pro-apoptotic Bax, and reduced mRNA and protein levels of anti-apoptotic Bcl-2 and Bcl-xL in CNE-1 cells. Activated Bax is directly engaged by Bim to promote apoptosis (36). In addition, caspases-8 may activate Bax and induce the release of Cyt *c* from the mitochondria, causing the cleavage of caspase-9 and contributing to the activation of caspase-3 (6,37). The results of the current study suggest that BLSA modulates the ratio of anti-apoptotic to pro-apoptotic factors, in particular by enhancing the expression Bax to promote the apoptosis of CNE-1 cells.

NF-κB reduces tumor necrosis factor (TNF)-α-induced cell apoptosis (38) and is an important negative regulator of apoptosis in cancer cells (39). Deregulation of NF-κB expression has been found in a number of human cancers (40,41). Overexpression of NF-κB promotes cell proliferation and reduces cell death (42). In addition, NF-κB can directly activate Bcl-xL (43) and suppress a number of anti-apoptotic factors, such as inhibitor of apoptosis, caspase-8-like FADD-like interleukin-1β-converting enzyme inhibitory protein, TNF receptor associated factor 1 (TRAF1) and TRAF2, to regulate apoptosis (44). Following treatment with BLSA, mRNA and protein levels of NF-κB were significantly reduced in CNE-1 cells. In addition, BLSA treatment significantly increased mRNA and protein levels of IκB-α. Increasing IκB-α levels is a therapeutic strategy to reduce cancer cell growth in clinical chemotherapy (45-47).

In conclusion, the results of the present study indicate that BLSA suppresses the proliferation of human CNE-1 NPC cells *in vitro*. In addition, the results indicate that BLSA induces apoptosis, through reducing the ratio of anti-apoptotic (Bcl-2 and Bcl-xL) to pro-apoptotic (Bax) factors, increasing mRNA and protein expression levels of Fas/FasL and promoting cleavage of caspase-3, -8 and -9 in CNE-1 cells. BLSA, as an inducer of apoptosis, may have future applications as an adjuvant in clinical therapy for NPC patients.

Acknowledegments

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References

1. Cao SM, Simons MJ and Qian CN: The prevalence and prevention of nasopharyngeal carcinoma in China. Chin J Cancer 30: 114-119, 2011.
2. Lo KW, To KF and Huang DP: Focus on nasopharyngeal carcinoma. Cancer Cell 5: 423-428, 2004.
3. Wang J, Guo LP, Chen LZ, Zeng YX and Lu SH: Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. Cancer Res 67: 3716-3724, 2007.
4. Su J, Xu XH, Huang Q, Lu MQ, Li DJ, Xue F, Yi F, Ren JH and Wu YP: Identification of cancer stem-like $CD44^+$ cells in human nasopharyngeal carcinoma cell line. Arch Med Res 42: 15-21, 2011.
5. Wong RS: Apoptosis in cancer: From pathogenesis to treatment. J Exp Clin Cancer Res 30: 87, 2011.
6. Ola MS, Nawaz M and Ahsan H: Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 351: 41-58, 2011.
7. Martinou JC and Youle RJ: Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 21: 92-101, 2011.
8. Peter ME and Krammer PH: The CD95 (APO-1/Fas) DISC and beyond. Cell Death Differ 10: 26-35, 2003.
9. Cullen SP and Martin SJ: Caspase activation pathways: Some recent progress. Cell Death Differ 16: 935-938, 2009.
10. Zhao Y, Lei M, Wang Z, Qiao G, Yang T and Zhang J: TCR-induced, PKC-θ-mediated NF-κB activation is regulated by a caspase-8-caspase-9-caspase-3 cascade. Biochem Biophys Res Commun 450: 526-531, 2014.
11. Kim JH, Kang M, Cho C, Chung HS, Kang CW, Parvez S and Bae H: Effects of *Nelumbinis* semen on contractile dysfunction in ischemic and reperfused rat heart. Arch Pharm Res 29: 777-785, 2006.
12. Sohn DH, Kim YC, Oh SH, Park EJ, Li X and Lee BH: Hepato-protective and free radical scavenging effects of *Nelumbo nucifera*. Phytomedicine 10: 165-169, 2003.
13. Rai S, Wahile A, Mukherjee K, Saha BP and Mukherjee PK: Antioxidant activity of *Nelumbo nucifera* (sacred lotus) seeds. J Ethnopharmacol 104: 322-327, 2006.
14. Ling ZQ, Xie BJ and Yang EL: Isolation, characterization, and determination of anti-oxidative activity of oligomericprocyanidins from the seedpod of *Nelumbo nucifera* Gaertn. J Agric Food Chem 53: 2441-2445, 2005.
15. Liu CP, Tsai WJ, Lin YL, Liao JF, Chen CF and Kuo YC: The extracts from *Nelumbo nucifera* suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. Life Sci 75: 699-716, 2004.
16. Liu CP, Tsai WJ, Shen CC, Lin YL, Liao JF, Chen CF and Kuo YC: Inhibition of (S)-armepavine from *Nelumbo nucifera* on autoimmune disease of MRL/MpJ-lpr/lpr mice. Eur J Pharmacol 531: 270-279, 2006.
17. Liu CP, Kuo YC, Shen CC, Wu MH, Liao JF, Lin YL, Chen CF and Tsai WJ: (S)-armepavine inhibits human peripheral blood mononuclear cell activation by regulating Itk and PLCgamma activation in a PI-3K-dependent manner. J Leukoc Biol 81: 1276-1286, 2007.
18. Xiao JH, Zhang YL, Feng XL, Wang JL and Qian JQ: Effects of isoliensinine on angiotensin II-induced proliferation of porcine coronary arterial smooth muscle cells. J Asian Nat Pro Res 8: 209-216, 2006.
19. Yu J and Hu WS: Effects of neferine on platelet aggregation in rabbits. Acta Pharm Sin 32: 1-4, 1997 (In Chinese).
20. Lin JY, Wu AR, Liu CJ and Ying S: Suppressive effects of lotus plumule (*Nelumbo nucifera* Geartn.) supplementation on LPS-induced systemic inflammation in a BALB/c mouse model. J Food Drug Anal 14: 273-278, 2006.
21. Mazumder UK, Gupta M, Pramanik G, Mukhopadhyay RK and Sarkar S: Antifertility activity of seed of *Nelumbo nucifera* in mice. Ind J Exp Biol 30: 533-534, 1992.
22. Li G, Li X and Lü F: Effects of neferine on transmembrane potentials of guinea pig myocardium. Zhongguo Yao Li Xue Bao 10: 406-410, 1989 (In Chinese).
23. Li GR, Li XG and Lu FH: Effects of neferine on transmembrane potential in rabbit sinoatrial nodes and clusters of cultured myocardial cells from neonatal rats. Zhongguo Yao Li Xue Bao 10: 328-331, 1989 (In Chinese).
24. Li GR, Qian JQ and Lü FH: Effects of neferine on heart electromechanical activity in anaesthetized cats. Zhongguo Yao Li Xue Bao 11: 158-161, 1990 (In Chinese).
25. Wang JL, Nong Y and Jiang MX: Effects of liensinine on haemodynamics in rats and the physiologic properties of isolated rabbit atria. Yao Xue Xue Bao 27: 881-885, 1992 (In Chinese).
26. Wang JL, Nong Y, Xia GJ, Yao WX and Jiang MX: Effects of liensinine on slow action potentials in myocardium and slow inward current in canine cardiac Purkinje fibers. Yao Xue Xue Bao 28: 812-816, 1993 (In Chinese).

27. Xiao JH, Zhang JH, Chen HL, Feng XL and Wang JL: Inhibitory effect of isoliensinine on bleomycin-induced pulmonary fibrosis in mice. Planta Med 71: 225-230, 2005.
28. Kuo YC, Lin YL, Liu CP and Tsai WJ: Herpes simplex virus type 1 propagation in HeLa cells interrupted by *Nelumbo nucifera*. J Biomed Sci 12: 1021-1034, 2005.
29. Lu JJ, Bao JL, Chen XP, Huang M and Wang YT: Alkaloids isolated from natural herbs as the anticancer agents. Evid Based Complement Alternat Med 2012: 485042, 2012.
30. Liu CM, Kao CL, Wu HM, Li WJ, Huang CT, Li HT and Chen CY: Antioxidant and anticancer aporphine alkaloids from the leaves of *Nelumbo nucifera* Gaertn. cv. *Rosa-plena*. Molecules 19: 17829-17838, 2014.
31. O' Reilly LA, Tai L, Lee L, Kruse EA, Grabow S, Fairlie WD, Haynes NM, Tarlinton DM, Zhang JG, Belz GT, *et al*: Membrane-bound Fas ligand is essential for Fas-induced apoptosis. Nature 461: 659-663, 2009.
32. Waring P and Müllbacher A: Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol 77: 312-317, 1999.
33. Scaffidi C, Medema JP, Krammer PH and Peter ME: FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J Biol Chem 272: 26953-26958, 1997.
34. Volkmann N, Marassi FM, Newmeyer DD and Hanein D: The rheostat in the membrane: BCL-2 family proteins and apoptosis. Cell Death Differ 21: 206-215, 2014.
35. Czabotar PE, Lessene G, Strasser A and Adams JM: Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nat Rev Mol Cell Biol 15: 49-63, 2014.
36. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S and Korsmeyer SJ: Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2: 183-192, 2002.
37. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG and Green DR: Bax-induced caspase activation and apoptosis via cytochrome *c* released from mitochondria is inhibited by Bcl-xL. J Biol Chem 274: 2225-2233, 1999.
38. Van Antwerp DJ, Martin SJ, Verma IM and Green DR: Inhibition of TNF-induced apoptosis by NF-κB. Trends Cell Biol 8: 107-111, 1998.
39. Ohshima K, Sugihara M, Haraoka S, Suzumiya J, Kanda M, Kawasaki C, Shimazaki K and Kikuchi M: Possible immortalization of Hodgkin and Reed-Sternberg cells: Telomerase expression, lengthening of telomere and inhibition of apoptosis by NF-kappaB expression. Leuk Lymphoma 41: 367-376, 2001.
40. Rayet B and Gélinas C: Aberrant rel/nfkb genes and activity in human cancer. Oncogene 18: 6938-6947, 1999.
41. Dolcet X, Llobet D, Pallares J and Matias-Guiu X: NF-kB in development and progression of human cancer. Virchows Arch 446: 475-482, 2005.
42. Ghosh S and Karin M: Missing pieces in the NF-kappaB puzzle. Cell 109 (Suppl): S81-S96, 2002.
43. Chen C, Edelstein LC and Gélinas C: The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 20: 2687-2695, 2000.
44. Lin A and Karin M: NF-κB in cancer: A marked target. Semin Cancer Biol 13: 107-114, 2003.
45. Tergaonkar V, Bottero V, Ikawa M, Li Q and Verma IM: IkappaB kinase-independent IkappaBalpha degradation pathway: Functional NF-kappaB activity and implications for cancer therapy. Mol Cell Biol 23: 8070-8083, 2003.
46. Lee CH, Jeon YT, Kim SH and Song YS: NF-kappaB as a potential molecular target for cancer therapy. Biofactors 29: 19-35, 2007.
47. Gilmore TD and Garbati MR: Inhibition of NF-κB signaling as a strategy in disease therapy. Curr Top Microbiol Immunol 349: 245-263, 2011.