RESEARCH ARTICLE

Brucea javanica Leaf Extract Activates Caspase-9 and Caspase-3 of Mitochondrial Apoptotic Pathway in Human Oral Squamous Cell Carcinoma

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Abstract

BACKGROUND: We previously reported *Brucea javanica* leaf extract (BJLE) induced apoptosis in human oral squamous cell carcinoma (HSC2) cells by attenuation of mitochondrial membrane permeability. However, further underlying mechanism is not known yet. Therefore, we conducted a study to investigate activation of Caspases related to attenuation of mitochondrial membrane permeability in BJLE-treated human oral squamous cell carcinoma.

METHODS: *B. javanica* leaves were collected, identified, minced, dried, extracted with distilled ethanol at room temperature for 24 hours, filtered and evaporated. Resulted BJLE was stored at 4°C. HSC-2 and HSC-3 cells were fasted for 12 hours and treated with BJLE in various concentrations for 24 hours. Treated HSC-2 and HSC-3 cells were lysed and subjected to western blot, to detect cleaved-Caspase-9, cleaved-Caspase-3 and β-actin. All visualized bands were captured and quantified.

RESULTS: Low numbers and morphological alterations of adherent HSC-2 and HSC-3 cells were observed in the group of cells treated with 500, 100 and 10 µg/mL BJLE. Numbers of adherent HSC-2 and HSC-3 cells treated with BJLE were shown decreased along with the increase of BJLE concentrations. Meanwhile, numbers of floating HSC-2 and HSC-3 cells were increased. Bands of cleaved-Caspase-9 and cleaved-Caspase-3 were observed in HSC-2 and HSC-3 cells treated with 500 and 100 µg/mL BJLE. Higher-density bands of cleaved-Caspase-9 and cleaved-Caspase-3 were observed in HSC-2 and HSC-3 cells treated with 500 µg/mL BJLE than 100 µg/mL BJLE.

CONCLUSION: BJLE could induce apoptosis by activation of Caspase 9 and Caspase 3 of mitochondrial apoptotic pathway in human oral squamous cell carcinoma.

KEYWORDS: *Brucea javanica*, leaf, apoptosis, HSC-2, HSC-3, Caspase 9, Caspase 3

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Introduction

Many anticancer drugs are obtained from natural sources. Among them, reports in herbs have shown various potential for medicine.(1,2) The herbal medicine *Brucea javanica* (*B. javanica*) has demonstrated a variety of anti-cancer, anti-malarial, and anti-inflammatory properties. As an anti-cancer, *B. javanica* has been investigated in lung (3), gastrointestinal (3), cervical (4), non-small cell lung (5), hepatocellular (5), breast (5), oesophageal (5), pancreatic (6-8) and bladder (9) cancers. The anti-cancer property of *B. javanica* is specific, since there are significant differences in cytotoxicity between cervical cancer cells (CaSki, C33A, and HeLa) and normal keratinocyte (PHKs).(4)

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B. javanica extract induces cancer apoptosis. Apoptosis proceeds through a mitochondrial dependent pathway associated with Caspase 3 activation in MDA-MB231 breast cancer cells.(5) Weak activation of Caspase 8 was observed upon *B. javanica* extract treatment in MDA-MB231 cells.(5) On the other hand, Caspase 3 activity was highly activated in *B. javanica* extract-treated MDA-MB231 cells compared to untreated controls.(5) Study in T24 bladder cancer cells has shown that *B. javanica* significantly induced the expressions of Caspase-3 and Caspase-9 in T24 cells, whereas the expressions of nuclear factor (NF)-κB and cyclooxygenase (COX)-2 proteins were inhibited.(9) In addition the p53-dependent and p53-independent activities contribute to *B. javanica* extract-induced apoptosis.(4) Under treatment of *B. javanica* extract, p53 level of cervical cancer HeLa cells was increased and associated with apoptosis.(4)

We previously reported *B. javanica* leaf extract (BJLE) induced apoptosis in human oral squamous cell carcinoma (HSC2) cells by attenuation of mitochondrial membrane permeability.(10) However, further underlying mechanism is not known yet. Therefore, we conducted a study to investigate activation of Caspases related to attenuation of mitochondrial membrane permeability in BJLE-treated human oral squamous cell carcinoma.

Methods

Plant Extraction

BJLE was isolated as described by Wicaksono, *et al*.(10). Briefly, collected leaves were identified, minced, dried and extracted with distilled ethanol at room temperature for 24 hours. Further the solution as filtered and evaporated using rotatory evaporator. Resulted BJLE was stored at 4°C.

Cell Culture and BJLE Treatment

Human oral squamous cell carcinoma (HSC)-2 and HSC-3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HSC-2 and HSC-3 cells were incubated in a humidified incubator with 5% CO_2, 37°C. After HSC-2 and HSC-3 cells were seeded and fasted for 12 hours, BJLE was added in various concentrations. For a positive control, doxorubicin was added.

Western blot

Treated HSC-2 and HSC-3 cells were lysed with lysate buffer containing 50 mM Hepes/NaOH, 0.5 M NaCl, 5 mM EDTA, 20 mM β-mercaptoethanol, 0.2% Triton X-100, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium orthovanadate and 1 mM p-amidinophenyl methanesulfonyl fluoride. Then 15 µg lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride sheet. After blocking with 5% skimmed milk in phosphate buffered saline (PBS), the sheets were probed with the following 1:1000 diluted antibodies: rabbit polyclonal anti-cleaved-Caspase-9 (Asp315) (Cell Signaling, Beverly, MA, USA) and rabbit polyclonal anti-cleaved-Caspase-3 (Asp175) (Cell Signaling) antibodies. The secondary antibody was 1:2000 diluted horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (Amersham, Buckinghamshire, UK) or anti-mouse (Amersham) IgG antibody. The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories, Inc.). Membrane was then stripped with Seppro stripping buffer (Sigma-Aldrich), blocked with 5% skim milk in PBS, probed with mouse monoclonal anti-β-actin (C-4, Santa Cruz Biotechnology) antibodies), bound with anti-mouse IgG secondary antibody and visualized with chemiluminescent kit. All visualized bands were captured using Alliance 4.7 (UVItech, Ltd., Cambridge, UK) and quantified using UVIband software (UVItech, Ltd.).

Results

Numbers and Morphological Alteration of BJLE-treated Cells

High numbers and normal appearances of adherent HSC-2 (Figure 1A) and HSC-3 (Figure 2A) cells were shown in the untreated group. High numbers and unaffected appearances of adherent HSC-2 (Figure 1B) and -3 (Figure 2B) cells were also shown in the group of 1% DMSO-treated cells. In contrast, low numbers and morphological alterations of adherent HSC-2 cells were observed in the group of cells treated with 3 µM Doxorubicin (Figure 1C), 500 µg/mL BJLE (Figure 1D), 100 µg/mL BJLE (Figure 1E) and 10 µg/mL BJLE (Figure 1F). Similarly, low numbers and morphological alterations of adherent HSC-3 cells were also observed in the group of cells treated with 3 µM Doxorubicin (Figure 2C), 500 µg/mL BJLE (Figure 2D), 100 µg/mL BJLE (Figure 2E) and 10 µg/mL BJLE (Figure 2F).

Numbers of adherent HSC-2 (Figure 1D-E) and HSC-3 (Figure 2D-E) cells treated with BJLE were shown decreased along with the increase of BJLE concentrations.

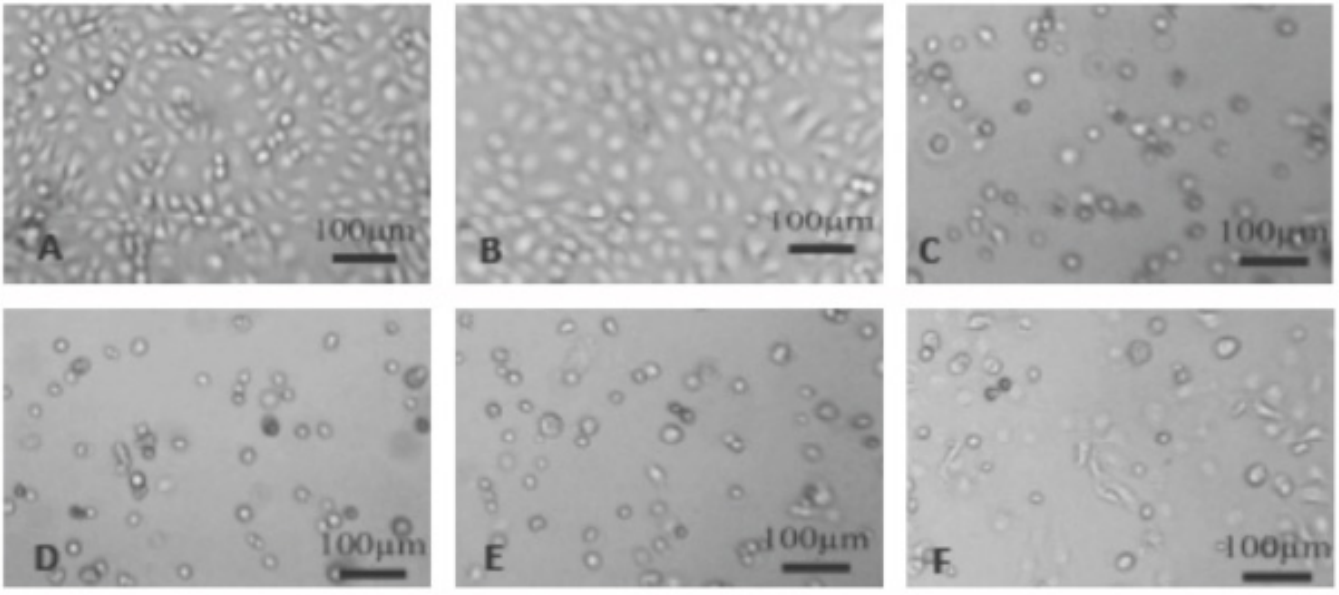

Figure 1. BJLE reduced number of HSC-2 cells. HSC-2 cells were untreated (A) or treated with 1% DMSO (B), 3 µM Doxorubicin (C), 500 µg/mL BJLE (D), 100 µg/mL BJLE (E) and 10 µg/mL BJLE (F) for 24 hours. Cells were documented under inverted microscope. Size bars were provided as indicated in the picture.

In addition, increasing numbers of floating HSC-2 (Figure 1D-E) and HSC-3 (Figure 2D-E) cells were observed along with the increase of BJLE concentrations as well.

Activation of Caspase-9 and Caspase-3 in BJLE-treated Cells

Bands of cleaved-Caspase-9 and cleaved-Caspase-3 were observed in HSC-2 (Figure 3A) and HSC-3 (Figure 3B) cells treated with doxorubicin, 500 µg/mL BJLE and 100 µg/mL BJLE. Meanwhile, the bands were not observed in untreated, 1% DMSO or 10 µg/mL BJLE-treated HSC-2 and HSC-3 cells. Higher-density bands of cleaved-Caspase-9 and cleaved-Caspase-3 were observed in HSC-2 and HSC-3 cells treated with 500 µg/mL BJLE than 100 µg/mL BJLE. Similar β-actin band densities were observed in all samples of HSC-2 and HSC-3 cells.

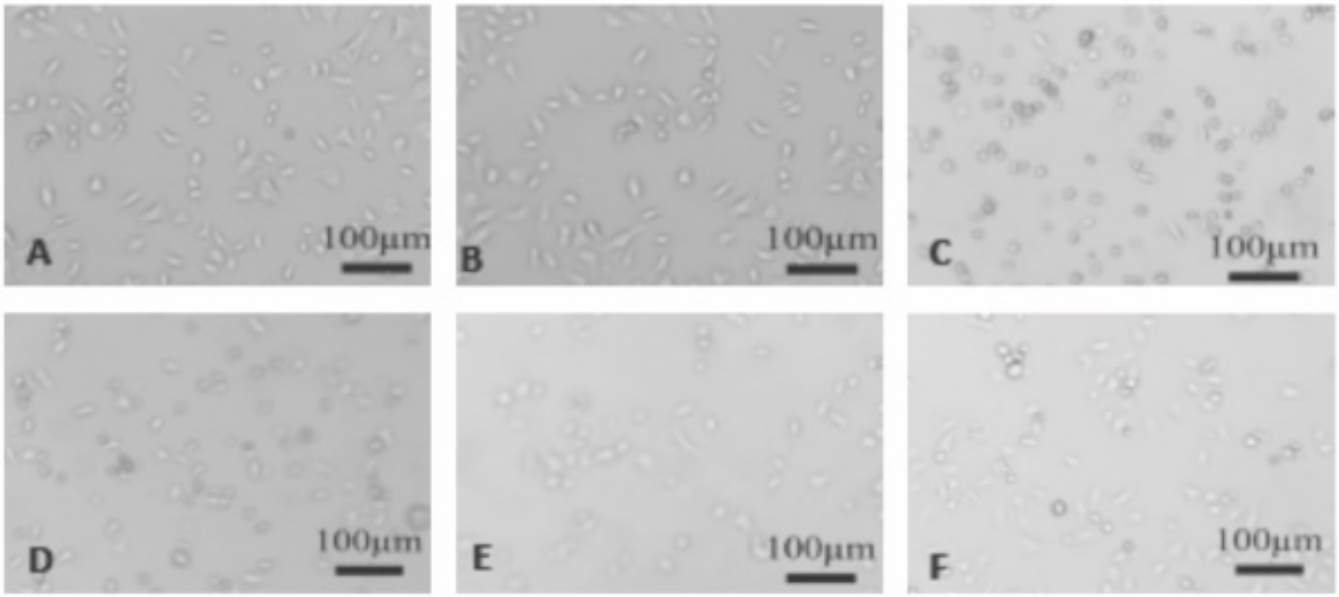

Figure 2. BJLE reduced number of HSC-3 cells. HSC-3 cells were untreated (A) or treated with 1% DMSO (B), 3 µM Doxorubicin (C), 500 µg/mL BJLE (D), 100 µg/mL BJLE (E) and 10 µg/mL BJLE (F) for 24 hours. Cells were documented under inverted microscope. Size bars were provided as indicated in the picture.

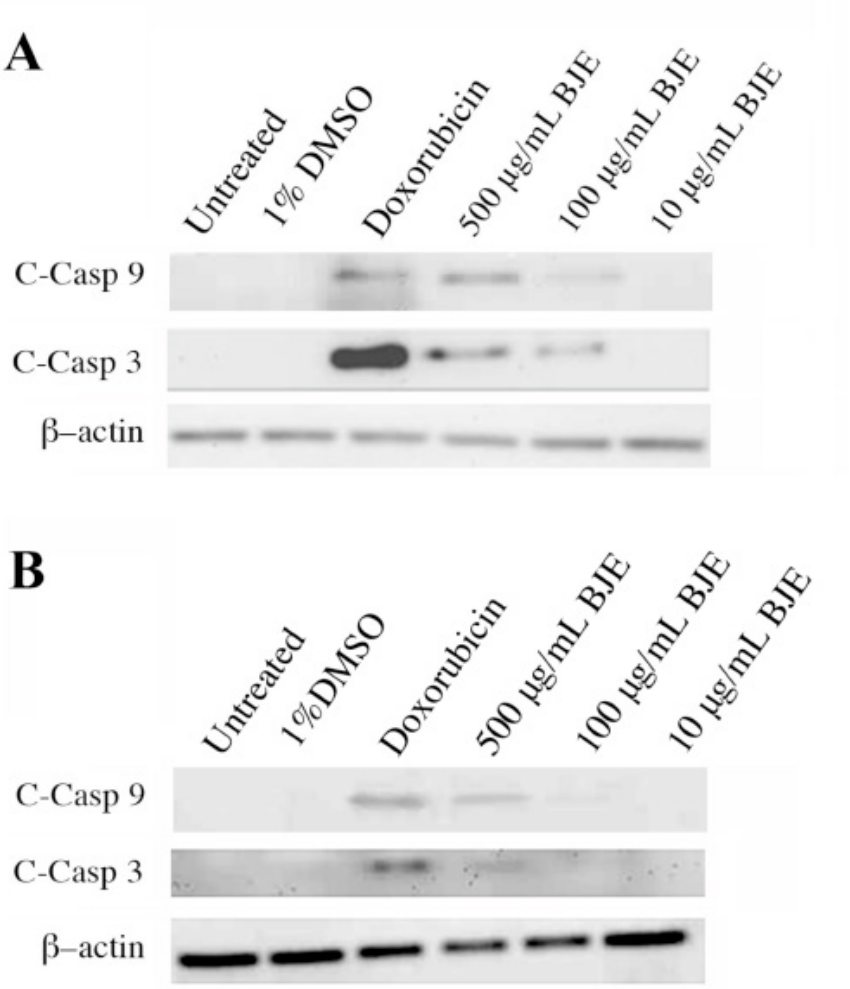

Figure 3. BJLE induced activation of Caspase-9 & -3. HSC-2 (A) and -3 (B) cells were treated with 1% DMSO, 3 µM Doxorubicin, 500 µg/mL BJLE, 100 µg/mL BJLE or 10 µg/mL BJLE as indicated in the panel, for 24 hours. Cells were collected, lysed, further processed for western blot analysis as described in Methods. C-Casp9: cleaved-Caspase 9; C-Casp3: cleaved-Caspase 3.

Discussion

Apoptosis has been suggested as a strategy for cancer chemotherapy.(11-14) Apoptotic morphological changes involve cytoplasmic shrinkage, plasma membrane blebbing, chromatin condensation and apoptotic bodies formation. (12) Double-strand cleavages of DNA occur in apoptotic cells.(11-16) Natural products have played a major role in apoptosis induction of osteosarcoma cells.(17,18) Due to the apoptosis induction by these natural products, several apoptosis-related proteins were induced, including Caspase 3, Caspase 7, Caspase 8 and Caspase 9, B cell lymphoma (Bcl)-2 and Survivin, Bcl-2-associated x protein (Bax), p53 upregulated modulator of apoptosis (Puma) and Fas.(18)

In our current study, we pursued a further investigation on BJLE-induced apoptosis mechanism related to attenuation of mitochondrial membrane permeability as we found earlier in our previous study. Current results show that treatment of BJLE decreased numbers of adherent HSC-2 and HSC-3 cells, and increased floating HSC-2 and HSC-3 cells. This suggests that BJLE could decrease viable HSC-2 and HSC-3 cells by induction of apoptosis. This result is in accordance to our previous results(10) and other reports. (4-9) We also found that under treatment of BJLE, Caspase 9 and Caspase 3 were activated in HSC-2 and HSC-3 cells, marked by high density of cleaved-Caspase 9 and cleaved-Caspase 3. Among all BJLE concentrations tested, we found that cleaved-Caspase 9 and cleaved-Caspase 3 were clearly seen in HSC-2 and HSC-3 cells treated with 500 µg/ml BJLE and 100 µg/ml BJLE. These results are in accordance to lower numbers of adherent HSC-2 and HSC-3 cells, and

higher numbers of floating HSC-2 and HSC-3 cells. When we look closely, densities of cleaved-Caspase 9 and cleaved-Caspase 3 were higher in HSC-2 and HSC-3 cells treated with 500 µg/ml BJLE compared to the ones treated with 100 µg/ml BJLE. Under treatment of 10 µg/ml BJLE, cleaved-Caspase 9 and cleaved-Caspase 3 in HSC-2 and HSC-3 cells were not detected. Along with that, numbers of adherent HSC-2 and HSC-3 cells were high, and numbers of floating HSC-2 and HSC-3 cells were low. This suggests that BJLE induced apoptosis of HSC-2 and HSC-3 in a concentration dependent manner.

Most of Caspases are apoptotic second messengers in signal transduction in cancer,(19) Inactivated Caspases are in the quiescent zymogen procaspase form and activation of pathway leads to cleavage (activation) and signal transduction.(20) In vitro, upstream Caspases can be activated by dimerization, while the executioner caspases require proteolytic cleavage.(20,21) The upstream Caspases are activated through association with activation complexes, leading to conformational change and cleavage. (20) Downstream Caspases can only be activated by proteolytic cleavage of pro-domains by activated upstream Caspases (20,22). Activated Caspases result in intracellular proteolysis of cytoskeletal components and proteins vital for organelle integrity towards cell execution (apoptosis). There are two main apoptotic signaling pathways, the extrinsic (death receptor-mediated) and intrinsic (mitochondrial) pathways.(20,23)

Since our current results showed that BJLE induced Caspase 9 and Caspase 3 in HSC-2 and HSC-3 cells in a concentration dependent manner, which lead to apoptosis. Therefore,e suggest that BJLE could be an alternative potential agent for induction of apoptosis in HSC-2 and HSC-3 cells. However, we also realize that additional conformational study related to the specificity of Caspase 9 and Caspase 3-dependent pathway should be performed. It has been reported that combined with chemotherapy, B. javanica extract could improve quality of life in non small small cell lung cancer (NSCLC) patients without any increase in toxicity.(24) This shows a potential of BJLE as an alternative anti-osteosarcoma agent in the future.

Conclusion

Taken together, our results suggested that BJLE could induce apoptosis by activation of Caspase 9 and Caspase 3 of mitochondrial apoptotic pathway in human oral squamous cell carcinoma.

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