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RESEARCH ARTICLE

Brucea javanica Leaf Extract Induced Apoptosis in Human Oral Squamous Cell Carcinoma (HSC2) Cells by Attenuation of Mitochondrial Membrane Permeability

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Abstract

BACKGROUND: *Brucea javanica* extract has been reported to have anti-proliferative and cell death induction activities. *B. javanica* extract was reported to induce apoptosis through caspase cascade. Most of investigated *B. javanica* extracts were derived from seeds and fruits, or commercially available oil emulsion. Therefore we conducted a study on *B. javanica* leaf extract (BJLE) in oral cancer cells.

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. Human oral squamous cell carcinoma (HSC)-2 cells were fasted for 12 hours and treated with BJLE in various concentrations for 24 hours. Cells were then quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay, demonstrated with 4',6'-diamidino-2-phenylindole (DAPI) staining. To find out mitochondrial membrane permeability (MMP), mitochondrial membrane potential (ΔΨM) was analyzed.

RESULTS: BJLE reduced percentage of viable HSC-2 cells in a concentration dependent manner. BJLE induced apoptosis in HSC-2 cells. With treatment of 50 μg/ml BJLE, fragmented nuclei were seen. ΔΨM of HSC-2 cells treated with 50 μg/ml BJLE were shifted to the left, meaning that BJLE induced reduction of ΔΨM and attenuation of MMP.

CONCLUSION: Our results suggested that BJLE could induce apoptosis by attenuating MMP.

KEYWORDS: *Brucea javanica*, leaf, apoptosis, HSC-2, MTT, DAPI, mitochondria, permeability

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Introduction

Brucea javanica (L.) Merr. is an evergreen shrub belonging to Simaroubaceae family.(1) The seeds of *B. javanica*, named as *Ya-dan-zi* in traditional Chinese medicine, are listed in Chinese Pharmacopoeia for the treatment of dysentery, malaria, warts and corns.(1,2) *B. javanica* oil emulsion formulation has already been developed as commercially available drug for anti-tumor clinical applications in China, and its clinical indications include lung cancer, prostate cancer, and gastrointestinal cancer.(3-7)

B. javanica extract has been reported to have anti-proliferative and cell death induction activities.(8-16) There were numerous cancer lines have been tested with *B. javanica* extract, including breast cancer cell lines, such as MCF-7 (8-10), HTB126 (9) and MDA-MB231 (11); cervical cancer cell lines, such as CaSki (9), C33A (9) and HeLa (9); pancreatic cancer cell lines, such as PANC-1 (12,13), SW1990 (12,13), CAPAN-1 (12,13) and CAPAN-2

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(14); non-small cell lung cancer cell line, such as A549 (8,11); prostate cancer cell line, such as LNCaP (9); hepatic cancer cell line, such as Hep3B (11); oesophageal cancer cell line, such as SLMT-1 (11); bladder cancer cell line, such as T24 (15); colon cancer cell line, such as HCT116 (16); oral cancer cell lines, such as KB (17) and ORL-48 (17). All reports showed that *B. javanica* extract was cytotoxic for cancer cell lines.(8-17)

B. javanica extract was shown to have several mechanisms in inducing cell death.(9,11-15) Apoptosis induction by *B. javanica* extract was reported, in both p53-dependent and -independent pathways.(9) *B. javanica* extract was also reported to induce apoptosis through caspase cascade.(11-15) The activated caspases were correlated with p38 mitogen-activated protein kinases (MAPK) (13), B cell lymphoma 2 (BCL2) (14), Cyclooxygenase 2 (COX-2) (15) and nuclear factor κB (NFκB) (15).

Most of investigated *B. javanica* extracts were derived from seeds and fruits, or commercially available oil emulsion.(8,9,11-16) However, other parts of *B. javanica* are also resourceful, like leaves, twigs, fruits and stems.(1) However, these parts were not well investigated, only one report showing active compounds of *B. javanica* extract derived from twigs, leaves and and inflorescence.(10) Therefore we conducted a study on *B. javanica* leaf extract (BJLE). We hypothesized that BJLE have a potential in apoptosis induction in oral cancer cells.

Methods

Plant Extraction

B. javanica leaves were collected from West Kutai, East Borneo. The leaves were identified and extracted in Mulawarman University. Briefly, the leaves were minced and dried at room temperature. The dried material was extracted with distilled ethanol at room temperature for 24 hours, filtered and then evaporated using rotatory evaporator to produce crude ethanol extract of *B. javanica*. Resulted BJLE was stored at 4°C.

Cell Culture

Human oral squamous cell carcinoma (HSC)-2 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 cells were incubated in a humidified incubator with 5% CO2, 37°C.

Cell Viability

Cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay (Sigma) as described previously.(18,19) The MTT assay provides a quantitative measurement of viable HSC-2 cells by determining the amount of formazan crystals produced by metabolically active cells. Briefly, $1x10^4$ cells were seeded into each well of 96-well plates in medium containing active agent, hydrogen peroxide (H_2O_2) or medium merely. Cells were incubated for 24 hours. Ten µl of 5 mg/ml MTT in phosphate buffer saline (PBS) was added to each well. The plate was then incubated for 4 hours, and then the medium was discarded and formazan crystals were dissolved in 100 µl of 0.1N HCl. The absorbance of cells was measured at 570 nm by a microplate reader.

4',6'-diamidino-2-phenylindole (DAPI) Staining

DAPI staining was performed as described previously.(20,21) HSC-2 cells were seeded onto coverslips, treated with BJLE, and fixed with 50%, 70%, 90%, and 99.5% ethanol for 3 minutes. After washed in PBS, the fixed HSC-2 cells were treated with 0.1% BSA and 0.1% Triton X-100 for 5 minutes. The cells were stained by applying 1:150 diluted DAPI (Calbiochem, San Diego, CA, USA) for 5 minutes. Then, the coverslips were rinsed with PBS, mounted with glycerol, and evaluated under fluorescence microscope.

Mitochondrial membrane potential (ΔΨM) analysis

ΔΨM was performed as described previously.(20,21) After treatment with BJLE, cells were harvested and suspended in 250 µl of 20 nM 3,3'-Dihexyloxacarbocyanine Iodide ($DiOC_6$) in PBS and incubated in an incubator for 15 minutes. ΔΨM was measured using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Results

BJLE reduced percentage of viable HSC-2 cells

With treatment of 0.0014% H_2O_2 for 24 hours, percentage of viable HSC-2 cells were only 10.8 (Figure 1). Meanwhile with treatment of dimethyl sulfoxide (DMSO), there were 78% viable HSC-2 cells. Treatment of BJLE in various concentrations (5, 10, 20 and 50 µg/ml), showed that cell viability percentage was decreased in BJLE concentration dependent manner (85.8%, 82.2%, 69.2%, 49.4%, respectively).

BJLE induced apoptosis in HSC-2 cells

Nuclei of HSC-2 cells were stained with DAPI. Clear bluish round nuclei were observed in Figure 2A, showing healthy HSC-2 cells population. Meanwhile, with treatment of 50 μg/ml BJLE, fragmented nuclei were seen, marked by green arrow (Figure 2B). Cells with fragmented nuclei were apoptotic cells.

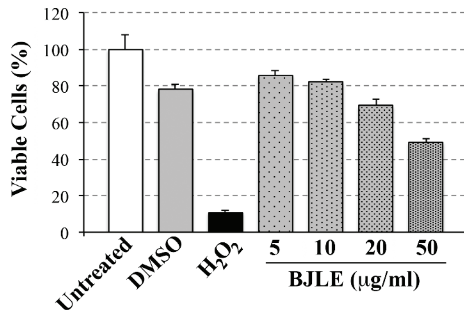

Figure 1. BJLE reduced percentage of viable HSC-2 cells. After HSC-2 cells were seeded and fasted for 12 hours, BJLE was added in various concentrations for 24 hours. Cells were quantified using MTT assay as described in "Method". For positive control, the cells were treated with 0.0014% H_2O_2. DMSO was used as negative control, since BJLE was suspended in DMSO. Amount of DMSO as negative control was equaled to the amount of DMSO in 50 μg/ml BJLE.

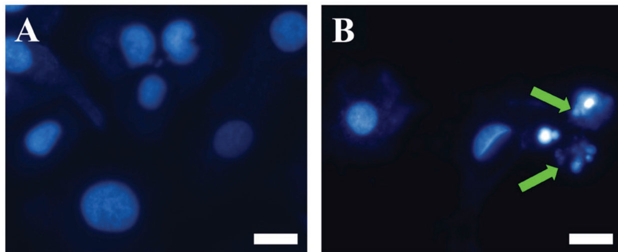

Figure 2. BJLE reduced percentage of viable HSC-2 cells. After HSC-2 cells were seeded and fasted for 12 hours, 50 μg/ml BJLE was added for 24 hours (**B**). For negative control, the cells were treated with DMSO merely for 24 hours (**A**). Cell nuclei were demonstrated with DAPI staining as described in "Method". Red arrow: necrotic cell; Green arrow: apoptotic cells; White bar: 4 μm.

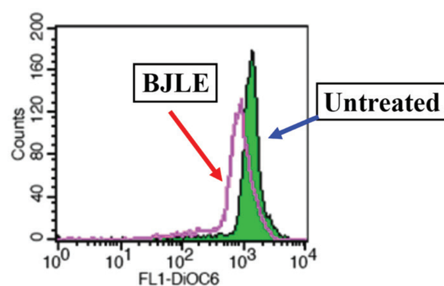

Figure 3. BJLE induced MMP. After HSC-2 cells were seeded and fasted for 12 hours, 50 μg/ml BJLE was added for 24 hours. MMP was measured using ΔΨM analysis as described in "Method".

BJLE attenuated mitochondrial membrane permeability (MMP)

Recorded ΔΨM in untreated HSC-2 cells were labeled by DiOC6, observed and marked as green area. ΔΨM of HSC-2 cells treated with 50 μg/ml BJLE were shifted to the left, marked by purple line (Figure 3). This meant BJLE induced reduction of ΔΨM and attenuation of MMP.

Discussion

B. javanica fruit and seed-derived extracts or commercially available oil emulsion have been investigated.(8,9,11-16) Currently, we investigated on BJLE as another possible potential source for anti-cancer agent. We found that treatment of BJLE reduced cell viability percentages in BJLE concentration dependent manner. Cell viability reduction was caused by apoptosis induction by BJLE, demonstrated as fragmented nuclei. Our results were in concordance to previous reports showing that *B. javanica* fruit and seed extracts induced apoptosis.(9,11-17)

B. javanica extract was mostly reported to induce apoptosis through caspase cascade.(11-15) *B. javanica* seed extract was shown to activate caspase 3, 8 and 9 (11-15) These caspases were correlated with mitochondrial apoptotic pathway. Therefore, BCL2 family was involved in this pathway (13,14), which then lead to attenuation of MMP.(14) Our results showed that MMP of HSC-2 cells was attenuated by treatment of BJLE. Upon MMP attenuation, ΔΨM will be disrupted, then apoptotic factor, such as cytochrome-c, will be released to cytoplasm. Other reported apoptotic mechanisms were p38-MAPK (13), COX-2 (15), and NFκB (15).

Combined with chemotherapy, *B. javanica* extract could improve quality of life in non-small cell lung cancer (NSCLC) patients without any increase in toxicity.(22) *B. javanica* oil emulsion (BJOE) combined with chemotherapy could be considered as a safe and effective regimen in treating patients with advanced gastric cancer (23) and advanced lung adenocarcinoma (24,25). BJOE can improve the quality of life and reduce the possibility of some adverse effects.(24,25) BJLE could also be important in treating cancer patient, therefore further study should be pursued. Taken together, our results suggested that BJLE could induce apoptosis by attenuating MMP. Further studies are necessary to explore BJLE mechanism, safety and efficacy.

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