The aqueous extract of *Brucea javanica* suppresses cell growth and alleviates tumorigenesis of human lung cancer cells by targeting mutated epidermal growth factor receptor

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Abstract: As a practical and safe herbal medicine, the seeds of *Brucea javanica* (L.) Merr., were used to cure patients suffering from infectious diseases such as malaria. Recent advances revealed that the herb could also be a useful cancer therapy agent. The study demonstrated that aqueous *B. javanica* (BJ) extract attenuated the growth of human non-small-lung cancer cells bearing mutant L858R/T790M epidermal growth factor receptor (EGFR). The reduced cell viability in H1975 cells was attributed to apoptosis. Transfection of EGFR small hairpin RNA reverted the sensitivities. When nude mice were fed BJ extract, the growth of xenograft tumors, as established by H1975 cells, was suppressed. Additional histological examination and fluorescence analysis of the resected tissues proved that the induced apoptosis mitigated tumor growth. The work proved that the BJ extract exerted its effectiveness by targeting lung cancer cells carrying mutated EGFR while alleviating tumorigenesis. Aqueous BJ extract is a good candidate to overcome drug resistance in patients undergoing target therapy.

Keywords: *Brucea javanica*, target therapy, epidermal growth factor receptor, human lung, herbal medicine, apoptosis

Introduction

A number of medicinal herbs exhibit anticancer activities ranging from promoting cell death to reverting multidrug resistances.¹ Among them, the evergreen shrub *Brucea javanica* (L.) Merr. (*Simaroubaceae*), growing in Southeast Asia and Northern Australia, exhibits diverse biological activities. The fruits serve as traditional medicine in Indonesia, Myanmar, Thailand, and China.² Efficient without significant side effects, the medication has been used to treat patients suffering from parasite infections and malaria in East Asian countries.³ Many studies⁴–⁷ have reported that the medication serves as a potent anticancer therapeutic. The ethanol extract from the fruit has been shown to effectively repress growth of pancreatic cancer cells.⁴ By combining with conventional chemotherapy drugs, the emulsion of *B. javanica* oil provided an alternative nontoxic antiproliferative agent in treating patients with lung adenocarcinoma and advanced gastric cancer.⁵,⁶ In addition, the ethyl acetate extract of the seeds has been shown to heal patients with diseases related to inflammation and allergy.⁷ Despite numerous reports on the versatility of the fruit in treating various types of illness, the effectiveness of aqueous *B. javanica* (BJ) extract in cancer therapy is not completely understood.
Tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are the first-line therapeutic agents used for treating patients with non-small cell lung cancer (NSCLC) harboring mutated epidermal growth factor receptor (EGFR). The status of EGFR mutation provides a predictive biomarker of the response to gefitinib treatment. EGFR mutation at L858R is a prerequisite for drug sensitivity. This specific mutation site appears in a restricted subset of NSCLC patients that includes those of East Asian ethnicity, women, and nonsmoking individuals. However, patients receiving tailored target therapy gradually develop secondary mutations in EGFR, which results in relapse. The acquired somatic mutations at amino acid at 790 of EGFR (T790M) block steric binding of gefitinib and trigger resistance. Thus, to improve treatment, new developments aimed at overcoming the resistance stemming from double mutant EGFR at L858R/T790M in NSCLC patients, are needed to complement first-line target therapy.

To address this issue, the current study aims to find out if the aqueous BJ extract regulates the proliferation and the growth of the established xenograft tumors in H1975 cells carrying double mutant EGFR. The purpose is to identify more therapeutic approach among conventional medicines to override drug resistance in the course of progressive somatic EGFR mutation during target therapy.

Materials and methods

Cell culture

Human NSCLC cells, including H1975 (two mutations in EGFR, L858R/T790M, erlotinib-insensitive), H3255 (one mutation in EGFR, L858R, erlotinib-sensitive), A549, H1299, and H460, were acquired from American Type Culture Collection (Manassas, VA, USA) and cultured in 75 cm² tissue culture flasks. The cells were grown in Dulbecco’s Modified Eagle’s Medium with supplementation of 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 unit/mL penicillin and 100 μg/mL streptomycin, and maintained at 37°C in the environment of humidified incubator with 5% CO₂. The cell media was replaced every 3 or 4 days and subcultured. Cells reaching 80%–90% confluence were used for experiments.

Chemicals and reagents

Sun Ten Pharmaceutical (Taichung, Taiwan) provided the aqueous extracts of the whole plant following the published procedures. In brief, the collected materials samples were mixed with sterile water before boiling. The supernatant following centrifugation was filtered, concentrated, and adjusted to a final concentration of 1 g/mL before storage.

The chemicals propidium iodide (PI), ribonuclease A, trypsin blue, Tris–HCl, and Triton X-100 were from Sigma-Aldrich Chemical (St Louis, MO, USA); and penicillin–streptomycin, glutamine, trypsin–ethylenediaminetetraacetic acid, and Dulbecco’s Modified Eagle’s Medium from Thermo Fisher Scientific.

Liquid chromatography/mass spectrometry analysis and instrumental conditions

The liquid chromatography/mass spectrometry (LC/MS) method was used to identify the major markers of bioactive substances. The system for analysis consisted of a LC-20AD UFLC system (Shimadzu, Kyoto, Japan) linked to a LCMS-8040 triple quadrupole mass spectrometer. The running condition was designed as follows: gradient elution by the mixture of mobile phases A (0.1% formic acid and 1 g/L solution of ammonium acetate in water) and B (0.1% formic acid and 1 g/L solution of ammonium acetate in methanol) at minutes 0–40 with the ratio of 100%–70% in A and 0%–30% in B; at minutes 40–70 with the ratio of 70%–0% in A and 30%–100% in B; at minutes 70–70.1 with the ratio of 0%–100% in A and 100%–0% in B; and at minutes 70.1–80 with the ratio of 100% in A and 0% in B. The flow rate was fixed at 0.4 mL/min and column temperature kept at 40°C. The injection volume was adjusted at 30 μL and the analytical column used a Shim-pack XR-ODS II column (2.2 μm, 2×100 mm, Shimadzu). Dual ion modes (electrospray ionization, [ESI] [+] and [–]) were used in MS detection and the transmission of (M+H)⁺ and (M–H)⁻ was set as the optimum condition. The MS detection was arranged as full scan range (400–800 amu). The interface voltages were set at 4.5 kV for ESI (+) and −3.5 kV for ESI (−). With nitrogen as nebulizing and drying gas, the flow was set at 3.0 and 10 L/min, respectively. Argon as collision-induced dissociation gas was kept at 230 kPa. Desolvation lines temperature was set at 150°C and heat block temperature maintained at 400°C.

Cell viability assay

Cell viabilities were determined using trypan blue exclusion assay. To estimate viability changes, 1×10⁴ human lung carcinoma cells were added with various concentrations of aqueous BJ extract and incubated at 37°C for 12 hours, while water alone served as control group. Cells as collected by trypsin–ethylenediaminetetraacetic acid were stained with trypan blue. Cells with exclusion of the dye were counted as viable and measured with a Countess™ counter (Thermo Fisher Scientific). The viability was determined by comparing cell numbers of treated cells with those of water control as
percentages. The inhibition concentration, IC$_{50}$, defined as concentration that inhibited 50% of cell proliferation, was calculated from a calibration curve by linear regression using Microsoft Excel.

**Flow cytometry by PI staining**

Cells were analyzed with FACSCalibur™ flow cytometer (BD Bioscience, San Jose, CA, USA) for determination of cell cycle distribution. A total 2×10^6 cells were treated with various concentrations of aqueous BJ extract along with water for 12 hours at 37°C. For sample preparation, both medium and the trypsinized cells were centrifuged and the supernatant removed. The collected cells were washed with phosphate-buffered saline (PBS) and mixed with 70% alcohol and PBS mixture, and stored at −20°C for 24 hours. Before analysis, the cells were labeled with 10 μg/mL PI (Sigma-Aldrich) that was dissolved in PBS containing 10 μg/mL ribonuclease A (ICN Pharmaceutical; Costa Mesa, CA, USA) for 30 minutes in darkness. Data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

**Flow cytometry by double labeling with Annexin V/fluorescein isothiocyanate and PI**

Cells seeded in 12-well plates at 2×10^5 cells per well for 16 hours were incubated with various concentrations of BJ extract or water for 12 hours at 37°C. The trypsinized cells were labeled with a mixture of 1:1 ratio Annexin V/fluorescein isothiocyanate (FITC) (20 μg/mL) and PI (50 μg/mL) at room temperature for 30 minutes in darkness. The flow cytometer FACSCalibur™ (BD Bioscience) was used to determine the distribution of early and late phases of apoptotic cells. Data were analyzed with FlowJo software. The application of Annexin V/FITC (An) along with PI divides four population distributions, including the viable (An negative/PI negative), the early apoptotic (An positive/PI negative), the late apoptotic (An positive/PI positive), and the necrotic (An negative/PI positive) cells. The sums of early and late stage percentages were counted as apoptotic cell populations.

**Western blotting analysis of cell lysates**

Cells as plated (1×10^6 per well) in serum-free media were treated with various concentrations of aqueous BJ extract for 12 hours. The collected medium was centrifuged to remove cell debris before being lysed. The protein lysate concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin as standard. The lysates as resolved by electrophoresis were transferred to nitrocellulose membrane. The blotted membrane blocked with 0.2% skim milk for 30 minutes at room temperature was incubated overnight at 4°C with the primary antibodies of 1:2,000 dilution in 5% skim milk (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence Western blotting substrate (Pierce, Rockford, IL, USA). The signals were recorded with enhanced LAS-4000 (FUJIFILM) apparatus and the band intensities of images analyzed using ImageJ software.

**TUNEL assay**

The apoptotic cell death was evaluated by terminal deoxyuridine triphosphate-mediated dUTP nick-end labeling (TUNEL) staining. Cells treated with various concentrations of aqueous BJ extract for 12 hours were permeabilized in 10 mM citrate buffer, pH 6.0. After blocking the nonspecific labeling with PBS mixture containing 2% bovine serum albumin and 0.5% NP-40, cells were incubated in TUNEL reaction solution mixed with 9 mM dUTP, 1 mM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 2.5 mM cobalt chloride, 100 mM Tris pH 7.6 and 0.3 U/μL terminal deoxynucleotidyl transferase for 1 hour at 37°C in a humidified atmosphere. The sections were washed with PBS and incubated with a 1:200 dilution of horseradish peroxidase-conjugated digoxigenin antibody (Roche). With removal of unbound antibody, cell images were taken by fluorescence or confocal microscope. All colored TUNEL images were converted to black and white by Photoshop software, before being quantitated with Multi Gauge software (version 2.1, FUJIFILM). Sections of positively stained cells containing 50 nuclei were counted. Five slides of each concentration and control sections were recorded and three independent experiments carried out.

**In vivo xenograft tumor study**

Four- to six-week-old athymic female nu/nu BALB/c mice (BALB/cAnN) were purchased from the National Applied Research Laboratories (Taipei, Taiwan). The animals were housed under aseptic and ventilated conditions free of pathogens with a 12-hour light–dark cycle. All experiments were performed in accordance with the Animal Care and Use Protocols, and the study was approved by the Animal Committee of National Taiwan Normal University, Taipei, Taiwan. A total 200 μL of H1975 cells (5×10^6 cells/mL) mixed with Matrixgel™ Basement Membrane Matrix (Corning Incorporated, Corning, NY, USA) was injected into the dorsal leg hypodermic area in
each mouse. Once the xenograft tumors were palpable and no less than 0.1 cm³ in volume, the mice were randomly divided into two treatment groups comprising six mice each, plus a control group. The other two groups were administered orally with aqueous BJ extract at 1 and 2 g/kg body weight, respectively, for 7 consecutive days. The control animals received equivalent volumes of water. The health and body weights of the animals were monitored regularly. The tumor size at each time points was measured before gavage feeding. The dimensions of the xenografts (longitudinal length and transverse width) were measured using an electronic digital caliper, and the measurements converted to the xenograft volume (π/6 × width² × length). Sixteen days following final feeding, the mice were sacrificed under CO₂ and the collected tumor samples resected for analysis.

Statistical analysis
All data represent mean ± standard deviation (SD) of three individual experiments. The statistical analysis using Student’s t-test were performed using GraphPad Prism 5.0 for Windows (GraphPad, San Diego, CA, USA). Statistical differences were considered significant with P<0.05.

Results
LC/MS analysis of composition in aqueous BJ extract
A typical LC/MS chromatographic fingerprint profile of aqueous BJ extract was obtained (Figure 1). Twelve major components were identified from the chromatogram. The well-separated peaks with retention times of <40 minutes were identified as 1) Bruceoside D (PubChem CID: 10484578), 2) Bruceine E (PubChem CID: 122785), 3) Bruceine F (17.5 minutes), 4) Bruceine D (PubChem CID: 441788), 5) Bruceine B (PubChem CID: 161496), and 6) Bruceine I (PubChem CID: 196839), respectively. The distinct peaks above 40 minutes included 7) Bruceine J (PubChem CID: 23656476), 8) Yadanzioside F (PubChem CID: 3000798), 9) Bruceantinol B (PubChem CID: 23656477), 10) Brusatol (PubChem CID: 73432), 11) Bruceine A (PubChem CID: 160006), and 12) Bruceoside E (PubChem CID: 3000803).

Low concentrations of aqueous BJ extract inhibited growth of NSCLC cells bearing double mutant EGFR
H1975 cell line is an ideal model system for studying L858R/T790M EGFR-related TKI resistance in human NSCLC cells. Cell viabilities in different human lung cancer cells were determined using aqueous BJ extract from 1 to 5 mg/mL. Among the cell lines studied, H1975 cells were sensitive to BJ treatment after 12 hours and showed concentration-dependent growth inhibition. The acquired IC₅₀ value of 2 mg/mL of BJ in H1975 cells (Figure 2) was not detected in erlotinib-sensitive H3255 cells that carry EGFR of mutated L858R. Cells of wild-type EGFR were unaffected by aqueous BJ extract. The results suggested that the effectiveness of BJ extract is specific in NSCLC cells harboring L858R/T790M EGFR.

BJ extract induced sub-G₁ cell distribution, Annexin V FITC-positive populations and apoptotic characteristics in H1975 cells
The palpable cytotoxicity of H1975 cells was reached as the extract concentration was increased to 2 mg/mL. Thus,
cells were transfected with small hairpin RNA (shRNA) to understand how apoptotic death was started. H1975 BJ-induced apoptotic cell death in mutant EGFR having L858R/T790M. Knocking down egFr impaired apoptotic cell death in H1975 cells was at the expense of PARP and procaspase-3 together with formation of active caspase-3 fragment implied apoptotic feature.

Western blot analysis (Figure 4A). The ameliorated EGFR phosphorylation of both EGFR and the reduced pEGFR were significant with increasing BJ concentrations in comparison with water control. Furthermore, analysis of Annexin V-FITC/PI doubly labeled H1975 cells showed that, as the extract concentration reached 5 mg/mL following 12 hours treatment, populations of early and late apoptotic phase were increased to 11% and 15%, respectively (Figure 3C and D). The data implied that the induced apoptotic cell death contributed to the reduced viable H1975 cells when exposed to aqueous BJ extract.

Protein lysates of H1975 cells were subjected to Western blot analysis. BJ extract attenuated EGFR and decreased phosphorylation of both EGFR Y1068 and Akt S473 as shown in Western blot analysis (Figure 4A). The ameliorated EGFR and the reduced pEGFR Y1068 were significant with increasing BJ concentrations in comparison with water control (Figure 4B). Cleavage of poly(adenosine diphosphate ribose) polymerase (PARP) and procaspase-3 together with formation of active caspase-3 fragment implied apoptotic feature development. The results demonstrated that the accelerated apoptotic cell death in H1975 cells was at the expense of mutant EGFR having L858R/T790M.

Knocking down EGFR impaired BJ-induced apoptotic cell death in H1975 cells
To understand how apoptotic death was started, H1975 cells were transfected with small hairpin RNA (shRNA) targeting exon 4 of EGFR (RNA sequence: GAGAAUGUGGAAUACCUAAGG) along with nonspecific shRNA as control (RNA sequence: CCGGACACUGCAGCACUUUUGG). The recovered viable cells transfected with EGFR shRNA were more than cells with nonspecific shRNA following BJ treatment (Figure 5A). Aqueous BJ extract accentuated diminution of phosphorylated EGFR Y1068 in H1975 cells following EGFR shRNA transfection (Figure 5B). The reduced PARP cleavage signified that knocking down EGFR incapacitated BJ sensitivity. The results implied that BJ targeted intrinsic EGFR with double mutation and orchestrated cell death.

Orally administrated BJ reduced xenograft tumor growth of H1975 cells in nude mice
The sizes of xenograft tumors of H1975 cells were diminished when feeding mice with BJ extracts. The continuous tumor development was repressed in animals when fed with 2 g BJ/kg compared with those with water (Figure 6A). No comparable body weight loss in nude mice orally administered with aqueous BJ extract was seen relative to those administered water treatment during experiment durations (Figure 6B). The increased BJ dosage proportionately reduced weights of the dissected tumors (Figure 6C).

The increased apoptosis and the regressed EGFR in xenograft tumors
As shown in hematoxylin–eosin staining of the histological sections, mice administered with aqueous BJ extract induced apoptotic cell death in xenograft tumors. The microscopic images of tumor sections in mice fed with water showed packed viable cells, whereas those with 2 g BJ/kg exhibited increasingly apoptotic characteristics, including cytoplasm condensation, pyknotic nuclei, and interstitial space enlargement. Some apparent engulfed apoptotic bodies were visible (Figure 7A). Cytochrome c release from mitochondria were visualized by confocal microscopy analysis. In tumor section images, the induced fluorescent puncta with coalesced green mitochondria marker mitotracker and the released red cytochrome c suggested progressive apoptotic cell death of H1975 tumors in mice given increasing BJ extract (Figure 7B). The increasingly administered BJ depleted fluorescent EGFR (Figure 7C) and pEGFR Y1068 (Figure 7D) as evidenced by antibody detection of tumor dissections.

The enriched TUNEL fluorescence in the resected specimen implied induced apoptotic death in H1975 xenograft tumors.
tumors in mice administered with BJ extract (Figure 8A). The increased fluorescence intensities meant enhanced apoptotic index relative to groups with water (Figure 8B). On the other hand, BJ extract inhibited tumor proliferation as suggested by the reduced mitotic index of proliferating cell nuclear antigen (PCNA) (Figure 8C). The decreased nucleus PCNA staining as shown suggested that tumor growth alleviation is attributed to apoptosis (Figure 8D).

**Discussion**

The indigenous ethnic medicines have received wide attention and been identified with new anticancer prospect. Recent efforts have advanced herbal medicine research to complement cancer therapy treatment. An important objective of drug development is to uncover more potent regimens that overcome the arising resistance during target therapy. The emulsified formula of BJ has been approved to treat various...
types of cancer, such as lung cancer diagnosed at middle or late stage.\textsuperscript{17,18} To extend the scope, the study reported that the aqueous BJ extract inhibited the growth of human lung cancers with somatic mutation of \textit{EGFR}. The findings offered new dimension for the existing herbal medicine to override the evolving resistance during target therapy.

Being a transmembrane protein endowed with kinase activity on downstream effectors, \textit{EGFR} binds to ligand that leads to receptor dimerization and phosphorylation of tyrosine residues at the cytosolic domains. The phosphorylated tyrosine residues function as a kinase that activates downstream intracellular signals, such as extracellular-regulated kinase.
Mutated EGFR frequently occurred in lung adenocarcinoma patients, especially those of Asian ethnicity, females, and nonsmokers. Both gefitinib and erlotinib serve as the first-line TKI therapy to treat advanced NSCLC patients harboring EGFR mutation by preventing dimerization and phosphorylation of the receptor. As first choice for target therapy, gefitinib and erlotinib bind to L858R EGFR of lung cancer cells, thereby making treatment successful. Treatment of TKI significantly prolonged survival rates of lung cancers patients with the specifically mutated EGFR. Despite their distinct response, lung cancer patients receiving target therapy eventually develop resistance due to the evolved secondary T790M mutation. The rate arises at a median of 10–13 months that occurs in more than 50% of the patients. In order to overcome the high concentration toxicity and resistance because of the accumulated somatic mutation of T790M EGFR, new development of safer and more useful medication following target therapy is imperative. The study showed that BJ suppressed cell viabilities only in H1975 cells. The diminished EGFR intensities and the repressed EGFR\textsuperscript{Y1068} phosphorylation account for the growth rate reduction.

BJ increased sub-G\textsubscript{1} cells and Annexin V-positive populations in H1975 cells. More work showed that the aqueous extracts attenuated Akt signaling and induced procaspase-3 cleavage. Active caspase-3 is required to elicit and execute apoptosis by cleaving cellular proteins at appointed aspartate residues. Western blot analysis showed that both PARP and procaspase-3 were cleaved following BJ treatment that triggered subsequent apoptosis. The appearance of apoptotic caspase-3 fragment corroborated the decreased viable cells. Knocking-down EGFR in H1975 cells inhibited PARP cleavage, reduced subsequent apoptotic cell death and suppressed BJ sensitivity. The results supported the vital role of the mutant EGFR in H1975 cells during BJ-induced apoptosis.

More experiments showed that orally administered aqueous BJ extract inhibited the growth of the established xenograft H1975 tumors without affecting the healthiness of mice. Aqueous BJ administration diminished EGFR intensities...
and depressed phosphorylation levels in the collected tumor resections. The increment of cytochrome c and TUNEL staining plus the declined PCNA marker as shown in confocal images further validated that BJ eliminated H1975 tumor growth by apoptotic cell death. Overall, the aqueous extract of BJ effectively overcome drug resistance by targeting EGFR with secondary mutation during therapy. The results suggested that aqueous BJ extract rich in various forms of quassinoids prompted apoptosis in H1975 cells with L858R/T790M EGFR mutation in NSCLC cells.

**Conclusion**

The discovery underscored the potency of aqueous BJ in eliminating cancer cell growth and alleviating tumor growth. BJ extract targeted somatic EGFR mutation in NSCLC cells.
by inducing apoptosis and suppressing tumorigenesis. The study highlighted the value and potential application of BJ as an alternative to treat human lung cancer refractive to target therapy. The findings promise more innovative prospect of the conventionally available herbal medicine to improve relapse and prolong survival rates of cancer patients.

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Disclosure
The authors report no conflicts of interest in this work.
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