Anticancer Activity and Apoptotic Effects of *Bulnesia sarmienti* Against Human Lung Cancer H460 Cells

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Bulnesia sarmienti (BS), a traditional South American herbal medicine native to Gran Chaco, has been used to treat various human ailments. The effects of BS aqueous extract (100, 200, and 400 µg/ml) on H460 cell lines were investigated. High-performance liquid chromatography (HPLC) confirmed that BS contains catechins as major compound. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell cycle analysis, DNA fragmentation, apoptosis, and immunoblot analysis on cells were carried out. BS has strong cytotoxic activity on the H460 cell lines (IC_{50}; less than 100 µg/ml) in MTT assay. Flow cytometry indicated that BS arrested the cell cycle in the sub-G_1 phase. When BS was treated on H460 cells, DNA fragmentation was increased, and early apoptotic cells were shown to be positive by annexin V staining. Also, the expressions of the p53 and Bax were increased and Bcl-2 protein was downregulated with BS treatment. These results indicated that the BS has anticancer activity on H460 cells and BS may be useful in future therapeutic applications for developing anticancer agents.

Key words: *Bulnesia sarmienti*; Cytotoxicity; Anticancer; Apoptosis; H460 cells

INTRODUCTION

Lung cancer is the leading cause of cancer death in the world and classified into four major types: squamous cell, adenocarcinoma, large and small cell. Non-small cell lung carcinoma comprises the first three types and accounts for approximately 75–85% of lung cancer patients (1–3). More than 75% of patients prove to be potential candidates for chemotherapy (4). However, efficacy of chemotherapy seems to have reached a plateau, and the current prognosis for most lung cancer patients remains poor, with an overall survival rate of 5 years for only 10–15% of people (5). Therefore, there is a need to develop compounds that can effectively treat cancer.

Numerous cancer patients have been considered for alternative treatments for malignancies, including herbal therapies. Several studies have demonstrated that some herbal mixtures could inhibit cancer cells proliferation in vitro and in vivo (6,7).

In recent years, several studies have demonstrated that plant extracts and Chinese herbal medicines such as *Phyllanthus urinaria* (8), *Polygonatum zanlanscianense Pamp* (9), and *Hemsleya amabilis* (10) possess various biological effects including anticancer activity (11,12). *Bulnesia sarmienti* (BS) is a tree that inhabits a part of the Gran Chaco area in South America, around the Argentinian–Bolivian–Paraguayan border. Its common name is palo santo, and it is also known as Vera wood in trade. BS is appreciated for the skin-healing properties of its essence, and natives of the Chaco region employ the bark to treat gastrointestinal problems. Some intensive researchers have studied BS for cytological (13) and biochemical characters (14). BS has shown antioxidant and antitumor effects on the HT-29 colon carcinoma cells (15). It also has been shown to have biosafety, lipid-lowering effect in high-fat diet-fed rats and streptozotocin-induced insulin-dependent and insulin-independent diabetic rats (16–20). However, the definite mechanisms of BS effects on cancer have not yet been elucidated.

We applied BS aqueous extract to the human lung cancer H460 cells with expectation of its anticancer activity. As we expected, BS aqueous extract showed anti-

cancer activity in the growth of human lung cancer H460 cell lines as non-small cell carcinoma.

MATERIALS AND METHODS

Preparation of Bulnesia sarmienti Aqueous Extract

The barks of *Bulnesia sarmienti* were provided by Lucky Pharmaceutics Co. (Daegu, Korea). The 300 g of BS was washed, segmented, added to distilled water (10 ml/g), and boiled at 95°C for 3 h, cooled to room temperature, then filtered and lyophilized. The yield of lyophilized residue corresponded to 22.6%. The extract was dissolved directly in distilled water and stored at −20°C until use. The voucher specimen has been deposited in Kyungpook National University, Laboratory of Veterinary Toxicology.

High-Performance Liquid Chromatography (HPLC) Analysis

Typical HPLC chromatograms of BS aqueous extract were analyzed on Waters 2487 HPLC system (Waters Co., MA, USA) using a C-18 column. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) as the following gradient: A/B (80:20). B was gradually increased to 8–20% at 20 min at a flow rate of 0.8 ml/min. Column temperature was set at 30°C. The column was then reequilibrated with the initial conditions for 5 min before the next injection. The UV detection wavelength was monitored at 280 nm. Identification of compounds was performed on the basis of the retention time, coinjections, and diode array spectral matching with standard.

Cell Lines and Culture

The human lung cancer H460 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in an RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 100 μg/L streptomycin, and 100 U/ml penicillin. All the cells were incubated at 37°C in 5% CO_2 and 95% air.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Cell viability was determined using the conversion of MTT to formazan via mitochondrial oxidation. H460 cells were seeded at a density of 1.5×10^4 cells/ml in a sterile 96-well plate. After overnight incubation, serial concentrations of BS extract (0, 100, 200, and 400 μg/ml) were treated and incubated for 24 or 48 h, then 100 μl of MTT (Sigma, St. Louis, MO, USA) solution was added to each well and incubated at 37°C for 2 h. The media were removed and formazan was dissolved in DMSO and the optical density was measured at 570 nm using a microplate reader (VERSAmax, Molecular Devices, USA). Three replicates per condition were assayed, and data averages from three to five separate experiments are presented. Data are expressed as the percentage of control values.

Morphological Evaluation of the Differentiated Cells

The cells were seeded at a density of 2×10^5 cells per well to six-well tissue culture plates in RPMI-1640 supplemented with 10% FBS in the absence or presence
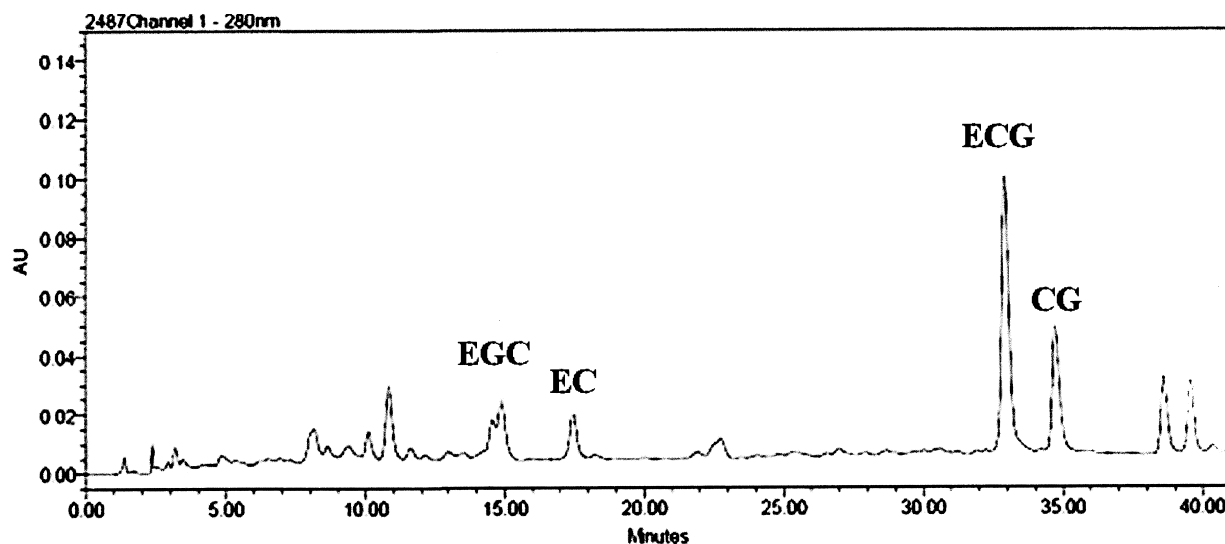

Figure 1. HPLC chromatogram of *Bulnesia sarmienti* aqueous extracts. (−)-Epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (+)-catechin gallate (CG).
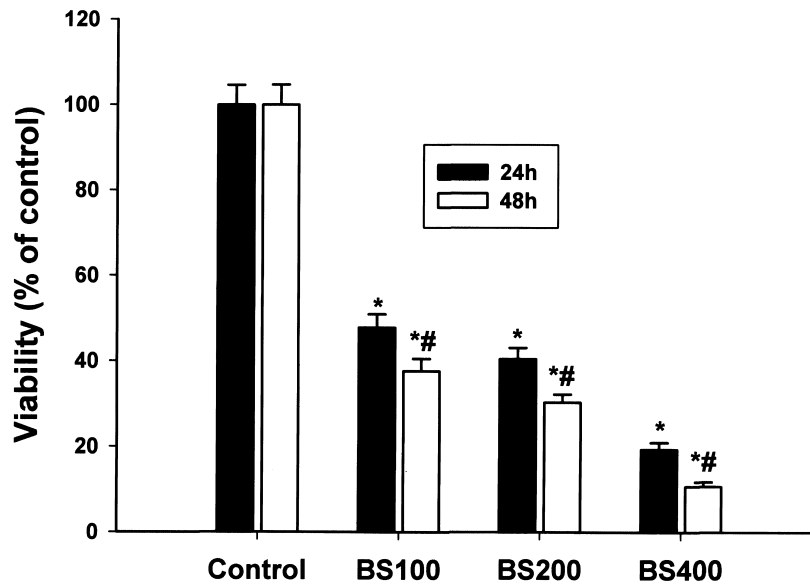
Figure 2. The antiproliferative effect of *Bulnesia sarmienti* extract in H460 cells. Cells were treated with various concentrations (0, 100, 200, and 400 µg/ml) of BS extract for 24 and 48 h when the cell viability was determined by the MTT assay. The growth inhibition was calculated as a percentage of inhibition compared with the control. Each data point represents the mean ± SD of four wells from three independent experiments ($n = 4$, $*p < 0.05$).

of BS extract at concentrations of dilution in the culture mediums. The cultures were maintained in incubation at 37°C for 48 h; the cells were harvested, stained with Wright-Giemsa solution, and observed with a light microscope. Differentiated cells were identified on the basis of cytoplasmic protrusions and the nuclear patterns.

DNA Fragmentation

The fragmented DNA was isolated from cultivated cells. In brief, cells (2×10^5) were treated with the BS extract for 48 h and then collected by centrifugation ($2,000 \times g$, 10 min). The plates were resuspended in a 0.5 ml DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 8.5). The lysate was immediately incubated with 0.1 mg/ml proteinase K and then incubated for 3 h at 37°C. After addition of isopropanol, DNA was precipitated with 70% ethanol. Then the suspension was centrifuged, and DNA was treated with 100 µl of 10 mM Tris-HCl and 0.5 mg/ml RNase A (Biosesang, Korea) at 37°C for 24 h. The sample was then loaded into a 2% agarose gel containing ethidium bromide and electrophoresed. The DNA bands were visualized under ultraviolet illumination.

Cell Cycle Analysis

H460 cells (2×10^5 per well) were seeded in six-well culture plates. After overnight culture, 0, 100, 200, and 400 µg/ml of BS extract was added and the cells were

incubated for 48 h. After treatment, the cells were harvested and washed twice with PBS. Then, 1.5 ml of 75% ethanol was added to fix the cells at 4°C overnight. After fixation, the cells were washed with PBS and resuspended in 400 µl of PBS, 50 µl of RNase A (10 mg/ml), and 10 µl of propidium iodide (PI, 2 mg/ml in PBS). The cells were further incubated at 37°C for 30 min. Finally, the samples were analyzed by FACSort flow cytometry (Becton Dickinson, Cowley, Oxford, UK) using Cell Quest software. The cell population was chosen by forward scatter (FSC) light and side scatter (SSC) light. The signals were detected by the FL2 channel for PI with a log scale.

Annexin V Staining

To quantify apoptosis, H460 cells were stained with annexin V kit (Assay Designs, USA), following the manufacturer's protocol. Briefly, 1×10^5 cells were treated with 0, 100, 200, and 400 µg/ml of BS for 48 h; cells were then collected by trypsinization and washed twice with PBS. Cell pellets were resuspended in 100 µl 1× annexin binding buffer, 1 µl of annexin V-FITC working solution was added to each 100 µl of cell resuspension. The cells were incubated on ice for 10 min in the dark. The cell suspension was diluted to 250 µl with 1× binding buffer and the stained cells were placed on a glass slide and covered with a glass cover slip. The cells were observed under a fluorescence microscope using a filter set for FITC.
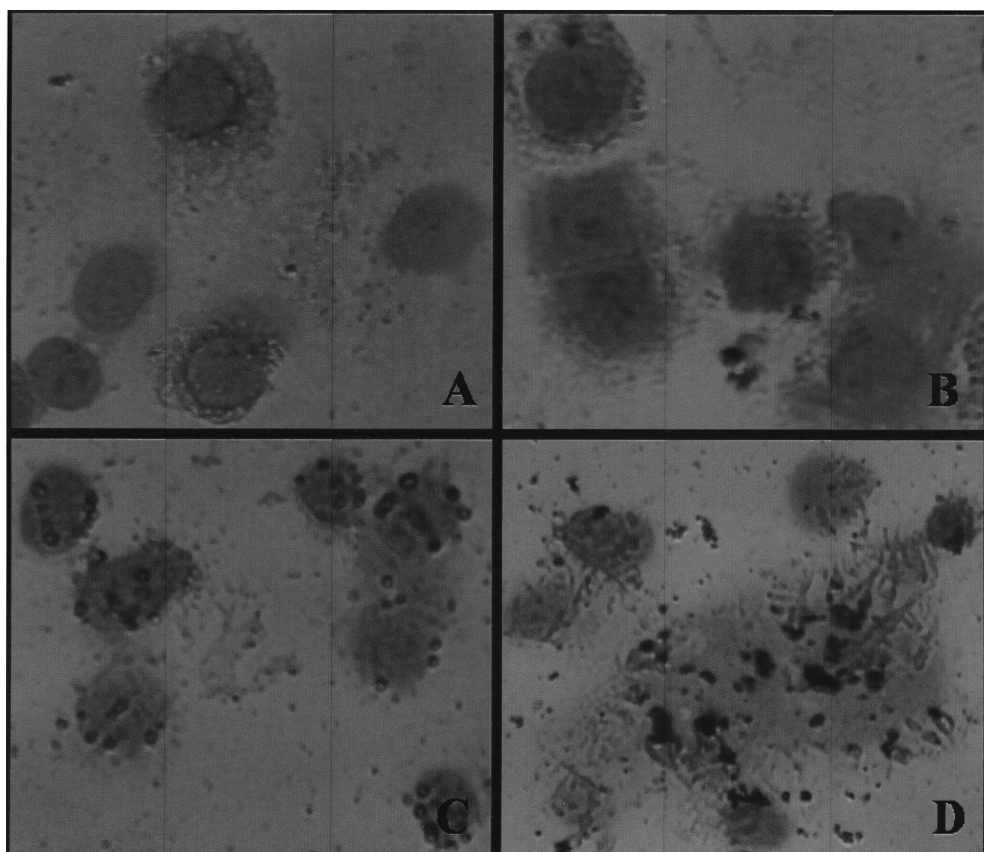
Figure 3. Effect of *Bulnesia sarmienti* extract on differentiation of H460 cells. The cells were treated with various concentration of BS extract. (A) Control cells without BS extract treatment; (B) cells treated with 100 µg/ml of BS extract; (C) cells treated with 200 µg/ml; (D) cells treated with 400 µg/ml of BS extract treatment for 48 h, then the differentiation criteria were studied by Wright-Giemsa staining.

Immunoblot Analysis

After treatment with 0, 100, 200, and 400 µg/ml of BS extract for 48 h, H460 cells were lysed in lysis buffer and the protein concentration was determined by using a protein measurement solution (iNtRON biotech, Korea) with bovine serum albumin as the standard. Samples with equal amounts of protein were analyzed by 12% SDS-PAGE. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a transfer buffer at 70 V for 3 h. The membranes were incubated with blocking buffer (1× TBS-T and 5% skim milk) overnight at room temperature. Then membranes were incubated with antibodies against p53 (1:250), Bax (1:1,000), Bcl-2 (1:1,000), and β-actin (1:5,000) (Assay Designs, USA) for 3 h at room temperature with constant shaking. The membranes were washed three times in a 1× TBS-T buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 2–3 h. The membranes were washed and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA).

Statistical Analysis

The data were shown as mean ± SD. The statistical difference was analyzed using the Student's *t*-test. Values of $p < 0.05$ were considered significant.

RESULTS

HPLC Analysis

We analyzed compounds of BS extract using HPLC separations (Fig. 1). Separations of BS extract show that main ingredients were found as various types of catechins, such as (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (+)-catechin gallate (CG).

Inhibition of Cell Viability

MTT assay was performed to determine the cell viability of H460 cells after treatment of BS extract (Fig. 2). The BS extracts inhibited the H460 cells concentration and time dependently. The 50% inhibitory concentration (IC_{50}) was less than 100 µg/ml of BS extract after
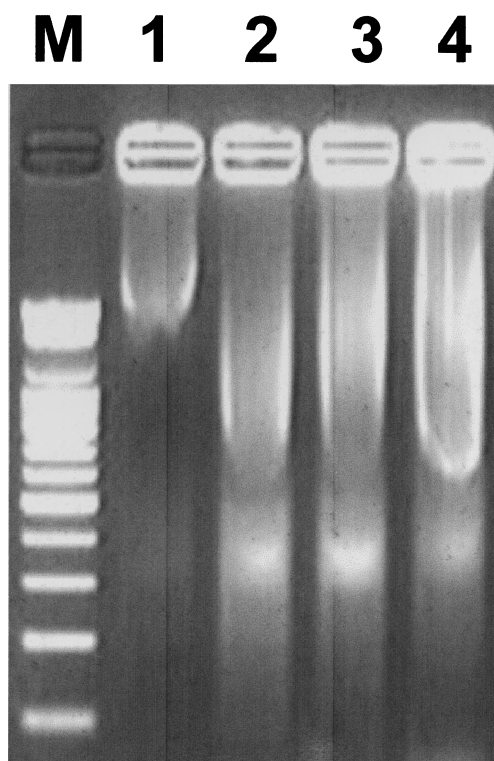
Figure 4. Effect of *Bulnesia sarmienti* extract on DNA fragmentation in H460 cells. M: 100-bp DNA marker, lane 1: control without BS extract treatment, lanes 2, 3, 4: 100, 200, and 400 µg/ml of the BS extract for 48 h. DNA laddering typical for apoptotic cells is visible for the cells treated with the BS extract.

incubation for 24 h. A treatment of 400 µg/ml of BS extract showed maximum inhibition of 80.62% in cell viability for 24 h. This result shows that BS extract has a potent cytotoxic effect on H460 cells. The selected concentrations (100, 200, and 400 µg/ml) of BS in our study were based on pilot test using a wider range of concentrations (50, 100, 200, 400, 800, and 1,600 µg/ml). A result of the pilot test showed that concentrations higher than 400 µg/ml of BS extract has similar cytotoxicity.

Cell Differentiation

The cell morphology affected by BS extract was examined. Wright-Giemsa staining was performed using the BS extract treatment (Fig. 3). The morphological features such as enlargement of nuclear size and cytoplasmic mass were clearly evident after 48-h treatment of BS extract relative to the control cells. The malformations of H460 cells with BS extract were observed. The degrees of abnormalities were raised as increase of the BS concentrations.

DNA Fragmentation

To confirm the effects of BS extract on the induction of apoptosis in the H460 cells, the DNA was prepared from 2×10^5 cells that had been incubated in the absence (lane 1) or presence (lanes 2, 3, and 4) of BS extract for 48 h. The integrity of the DNA was then assessed by agarose gel electrophoresis (Fig. 4). The internucleosomal DNA fragmentation was increased in the cells treated with BS extract. It is widely accepted that DNA fragments detected in conventional agarose gel electrophoresis as a DNA ladder is a hallmark feature of apoptosis (21). In this study, DNA fragments were detected when H460 cells were treated with BS extract at concentrations of 100, 200, and 400 µg/ml for 48 h. These results indicate that the BS extract might cause apoptosis in the cells.

Flow Cytometry

The appearance of a sub-G_1 (M1 fraction) peak in the cell cycle analysis was an indication of apoptosis (Fig. 5). The sub-G_1 peak increased with higher concentrations of BS extract. The percentage of cells in sub-G_1 phase were 16.12, 31.79, and 65.64 when treated with 100, 200, and 400 µg/ml of BS extract for 48 h, respectively. Apoptotic cells with degraded DNA, mostly located below the G_1 peak in the DNA histogram (M1), were estimated from Figure 5A to be 4.11% in control, while the percentage of apoptotic cells increased in a concentration-dependent manner after 48 h. The results of flow cytometry analysis indicated that BS extract arrested the cell cycle in the sub-G_1 phase; it was obvious that the BS extract induces inhibiting proliferation of H460 cells.

Apoptosis in H460 Cells

Phosphatidylserine (PS) translocation occurs early in apoptosis when cell membrane integrity is still intact, and annexin V can bind to PS with high affinity. The H460 cells after BS treatment showed massive apoptosis, which was confirmed by fluorescent microscopic analysis with annexin V-FITC staining (Fig. 6). These results showed that BS induced significantly increased apoptosis on H460 cells in a concentration-dependent manner.

Changes of p53, Bax, and Bcl-2 Proteins in H460 Cells

To determine whether tumor suppression factors such as p53 and Bax and antiapoptotic protein such as Bcl-2 were involved in antiproliferative effect of BS extract on H460 cells, the levels of protein were analyzed by immunoblot (Fig. 7). Expressions of p53 and Bax pro-
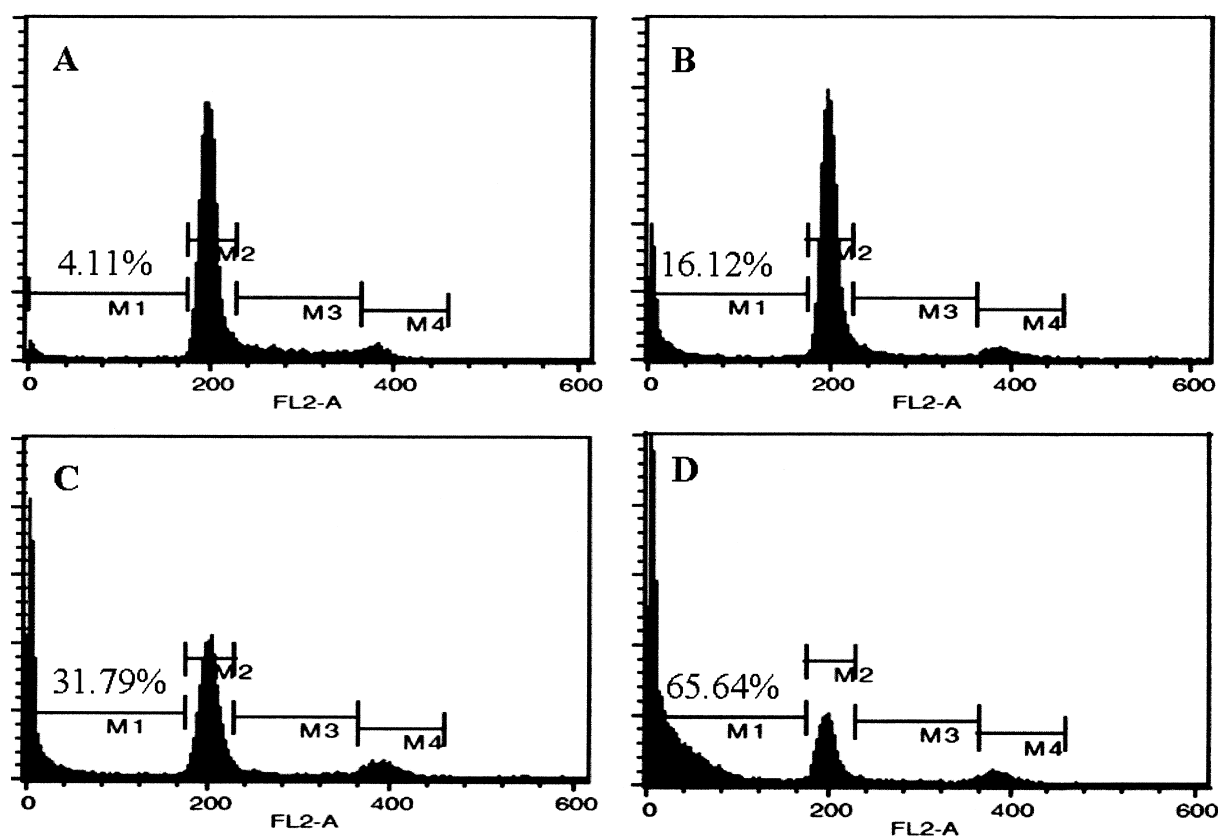
Figure 5. Flow cytometric analysis of apoptosis on H460 cells by *Bulnesia sarmienti* extract treatment. (A) Control cells without BS extract treatment; (B) cells treated with 100 µg/ml; (C) cells treated with 200 µg/ml; (D) cells treated with 400 µg/ml of BS extract for 48 h. Increased sub-G_1 peaks can be detected in 100, 200, and 400 µg/ml of BS extract treatment.

tein significantly increased in the cells with BS treatment in a concentration-dependent manner. In contrast, Bcl-2 protein expression was remarkably downregulated with BS treatment.

DISCUSSION

Bulnesia sarmienti (BS) has been shown to have inflammation-inhibitory effect on lipopolysaccharide-stimulated RAW 264.7 cells (22), and to have antioxidant and antitumor effects on HT-29 colon carcinoma cells (15). No evidence of BS extract-related toxicity was identified in the treated animals (16). HPLC analysis of BS extract confirmed catechins as one of the major compounds. Catechins are a group of compounds that naturally occur in some plants, and that show strong biological activity in some aspects, including apoptosis, cell growth inhibition, etc. (−)-Epigallocatechin-3-gallate has demonstrated anticarcinogenic activities in human and animal models, including cancer of breast, prostate, stomach, esophagus, colon, pancreas, skin, and lung (23–25). Catechins are might be a marker compound of

BS extract. Further investigations are needed to study the overall mechanism in anticancer effect of BS.

Inhibition of tumor growth has been a continuous effort in cancer treatment. Reduction in cell growth and the induction of cell death are two major means to inhibit tumor growth (8). In this study, it was demonstrated that BS extract could cause significant growth inhibition in H460 cells at low concentrations with IC_{50} less than 100 µg/ml. The effects of BS on cell viability were demonstrated by MTT assay. It was found that the viability of H460 cells were inhibited by treatment of BS extract.

Necrosis and apoptosis are involved in cell death. There are some key features for apoptosis that are common to different cell death pathways. Apoptosis includes cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (26). Disturbance of the cancer cell cycle is one of the therapeutic targets for development of new anticancer drugs (27).

In this study, DNA fragments were detected when

H460 cells were treated with BS extract. In addition to inhibiting tumor cell proliferation, BS also showed a good ability to induce cell apoptosis. Early apoptotic cells were defined as annexin V positive and PI negative, while late apoptotic cells as annexin V positive and PI positive. Annexin V preferentially binds phosphatidylserine, which is exposed during early apoptosis because of cell surface phospholipids asymmetry disruption. In the present study, most H460 cells treated with BS extract were positive to the annexin V-FITC staining and found to be in the early apoptotic stage.

The upregulation of p53 and Bax by BS extract together would have played a key role in the induction of apoptosis in cancer cells. Normal p53 plays a crucial role in inducing apoptosis and cell cycle checkpoints in human and murine cells following DNA damage (28). This has been further supported by the finding that p53 is the most commonly mutated tumor suppressor gene. The p53 protein plays an important role in cell cycle control and induction of apoptosis during the treatment period in cancer patients (29). Moreover, the sensitivity of cancer cells to chemotherapeutic agents is greatly influenced when the function of p53 is abrogated (26). Bax expression is stimulated by transcriptional activator p53, a tumor suppressor protein that induces apoptosis caused by damage to DNA. Apoptosis is modulated by antiapoptotic and proapoptotic effectors, which involve a large number of proteins. The proapoptotic and antiapoptotic members of the Bcl-2 family act as a rheostat in regulating programmed cell death and as a target of anticancer therapy (30). The ratio of death antagonists (Bcl2, Bcl-xL) to agonists (Bax, Bcl-xs, Bad, Bid) determines whether a cell will respond to an apoptotic stimulus. The proapoptotic Bcl-2 family protein Bax and the antiapoptotic protein Bcl-2 play important roles in the regulation of apoptosis (31). When Bcl-2 is produced in excess, cells are protected from Bax-induced apoptosis. On the other hand, when Bax expression is high, the
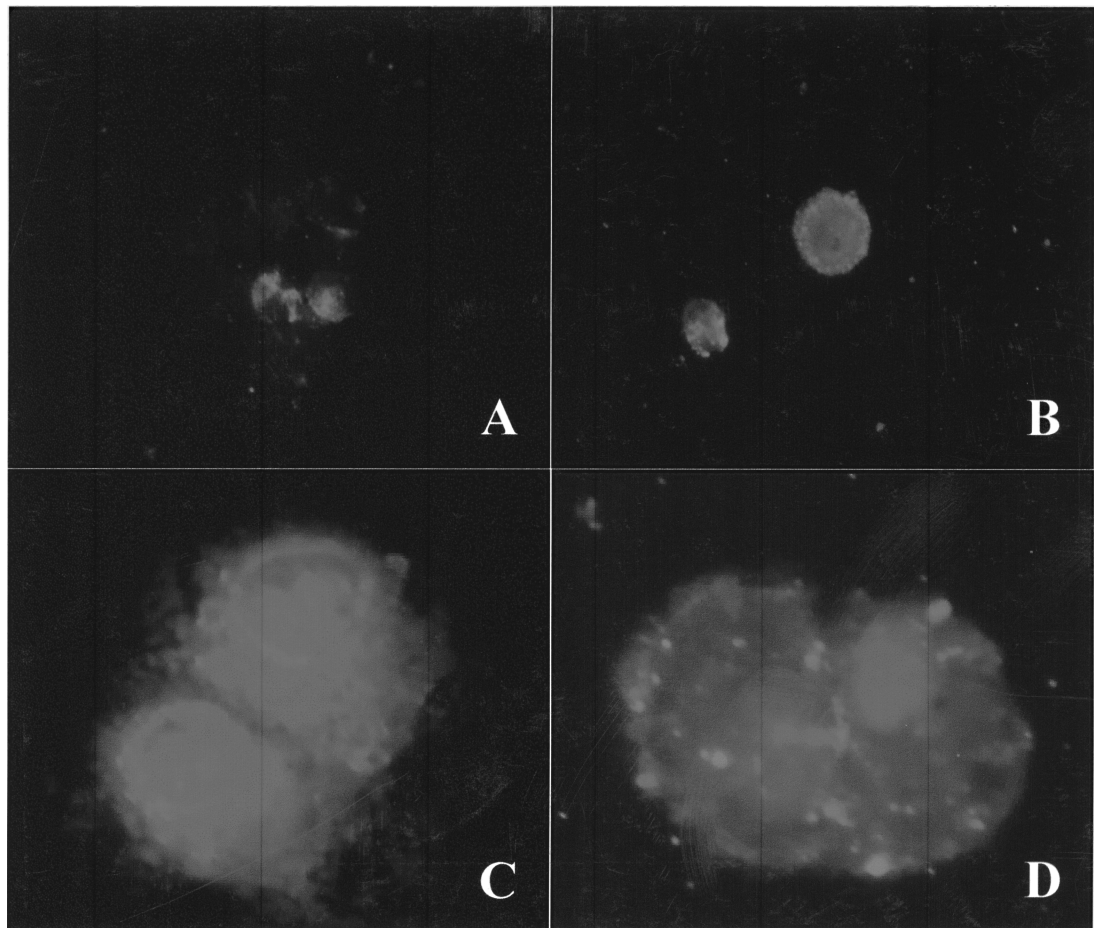

Figure 6. Observation of apoptotic cells with annexin V-FITC staining under a fluorescence microscope. H460 cells cultured and BS extracts treatment for 48 h. (A) Control cells without BS treatment; (B) cells treated with 100 µg/ml; (C) cells treated with 200 µg/ml; (4) cells treated with 400 µg/ml.
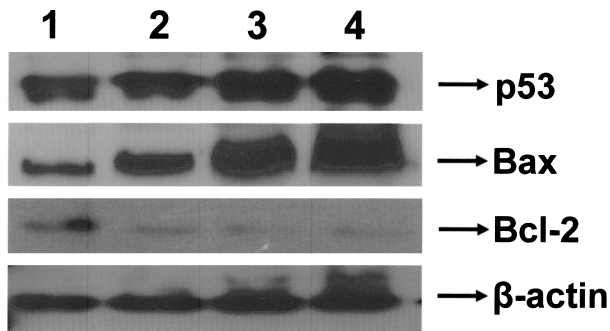
Figure 7. Immunoblot analysis of expression levels of the p53, Bax, and Bcl-2 protein on H460 cells after *Bulnesia sarmienti* extract treatment. Lane 1: control cells without BS treatment, lane 2: cells treated with 100 μg/ml; lane 3: cells treated with 200 μg/ml; lane 4: cells treated with 400 μg/ml of BS extract for 48 h. The values below the figure represent changes in protein expression of the bands normalized by β-actin.

cells proceed into apoptosis. Therefore, the ratio between Bcl-2 and Bax will determine whether or not cells undergo apoptosis. In this study, BS downregulated Bcl-2 protein expression while at the same time upregulated Bax protein. In addition, these results indicate that BS induces p53 protein expression in H460 cells. The basic mechanisms of inhibition were due to cytotoxic and cell apoptosis effects. These results may improve our understanding of the pharmacological mechanism of BS in the treatment of cancer.

In conclusion, the catechins were identified as possible significant biomarkers to determine the anticancer effects. The results of this study demonstrated that the BS extract has strong cytotoxic activity, cell cycle arrest in sub-G_1 phase, and induction of apoptosis. The upregulation of the proapoptotic genes and downregulation of the prosurvival genes suggests that BS may prove to be an effective therapeutic agent. These results indicated that BS may be useful in future practical applications for developing anticancer agents.

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