Typhonium blumei extract inhibits proliferation of human lung adenocarcinoma A549 cells via induction of cell cycle arrest and apoptosis

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A B S T R A C T

Ethnopharmacological relevance: Typhonium blumei Nicolson & Sivadasan is a traditional Chinese medicinal herb endowed with detumescence, detoxification, anti-inflammation activities, and has been used as a folk prescription on anticancer in Taiwan.

Aim of the study: The purpose of this study is to investigate the inhibitory effect of Typhonium blumei (Tb) extract on the viability of different cancer cells and the apoptotic effect of this extract on A549 lung cancer cells.

Materials and methods: Human A549 cell line and other cancer cell lines were treated with different concentrations of Tb extract at different time intervals. Growth inhibition was determined by MTT assay. Apoptosis was detected by cell morphologic observation, cell cycle analysis, and immunoblot analysis on the expression of protein associated with cell death. GC–MS were used to determine the chemical constituents of this extract.

Results: The Tb extract had cytotoxicity toward A549 lung cancer cells (IC₅₀ = 97.7 μg/ml), LNCaP prostate cancer cells (IC₅₀ = 124.5 μg/ml) and MCF-7 breast cancer cells (IC₅₀ = 125.8 μg/ml). Conversely, the adverse effects of Tb extract on normal embryonic lung fibroblast MRC-5 cells (IC₅₀ = 245.5 μg/ml) and embryonic kidney fibroblast HEK293 cells (IC₅₀ = 251.1 μg/ml) were comparatively low. Cytometric analysis results demonstrate that A549 cells were arrested at the G2/M phase by treatment with Tb extract. The extract induced A549 cell apoptosis via the mitochondrial pathway by down-regulating Bcl-2 and Bcl-xl protein expression, up-regulating Bax, Bad and Bak protein expression, and activating caspase-9 and caspase-3. Experimental results of bioactive compound analysis indicate that dibutyl phthalate, -linolenic acid, phytol, campesterol, stigmasterol and -sitosterol were the major bioactive ingredients of Tb extract. Although all these compounds had good anti-proliferative effects on A549 cells, campesterol (IC₅₀ = 2.2 μM for 24 h treatment) and -sitosterol (IC₅₀ = 1.9 μM for 24 h treatment) displayed the greatest inhibitory activity.

Conclusions: Experimental results of this study suggest that the Tb extract exerts potential anticancer activity through the growth inhibition and the apoptosis on A549 cells.

1. Introduction

Some cancer chemotherapeutic agents can abate or reverse cancer development and/or progression. As an important source,
survival rates and enhance the quality of patient life (Kinghorn et al., 2003; Li et al., 2009).

Lung cancer, a common cancer-related death worldwide, ranked first and second in 2009 among the ten leading forms of cancer in Taiwan for both genders, respectively (Department of Health, 2009). More than difficult to cure, lung cancer also has a high incidence of recurrence (Khiuri et al., 2001; Li et al., 2004; Molina et al., 2006). The non-small cell lung cancer (NSCLC) constitutes a majority of lung cancers. Either cisplatin or platinum-based chemotherapy is the most common regimen for treating patients with advanced NSCLC. However, these drugs are highly toxic with a low survival profile (De Petris et al., 2006). Cancer cells may resist current chemotherapeutic regimens, dominate a cell population and ultimately cause mortality, necessitating the improvement of clinical management against NSCLC. Recently targeting apoptosis, a programmed cell death, is an effective means of treating cancer, including NSCLC (Singhal et al., 2005).

Apoptosis, an important process in cell development and maintenance of tissue homeostasis, plays an essential role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells (Kauffmann and Hengartner, 2001; Schuchmann and Galle, 2004). Apoptosis is characterized by particular morphological changes, including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Wyllie et al., 1980). The relationship between apoptosis and cancer has been a recent focus. Apoptosis provides a number of useful clues when generating effective therapies, and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Schuchmann and Galle, 2004). Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective (Kundu et al., 2005).

Belonging to the Araceae family (Huang, 2000), Typhonium blumei Nicolson & Sivadasan (formerly known as Typhonium divaricatum (L.) Decne) is a traditional Chinese medicinal herb endowing with detumescence, detoxification, anti-inflammation, antivirus, and anticancer bioactivities. However, its bioactivities have seldom been investigated. Luo et al. (2007) separated lectin, a protein with 48 kDa that can recognize and bind to polysaccharides or glycoproteins expressed on cell surfaces, from Typhonium blumei. This lectin displayed significant antiviral activity against HSV-II of Vero cell line and an anti-proliferative effect on human cancer cell lines, including Pro-01 prostate cancer cells, Lu-04 lung cancer cell lines and Br-04 breast cancer cells. In Taiwan, the leaf of Typhonium blumei has been administered as a folk prescription on anticancer (swallow 15 pieces of leaves each time weekly), especially for treating lung cancer and prostate cancer. However, to our knowledge, its anticancer activity has not been verified.

This study investigated how Typhonium blumei (Tb) extract affects cell growth and apoptosis of A549 human NSCLC cells. The anticancer mechanism of Tb extract was also elucidated by analyzing expressions of apoptosis-related molecules, including caspases and Bcl-2 family proteins. Finally, several bioactive ingredients in Tb extract were determined.

2. Materials and methods

2.1. Materials and chemicals

The raw materials of Typhonium blumei were bought from a local herb store in Kaohsiung City and were deposited in the Herbarium of I-Shou University (Kaohsiung City, Taiwan). RPMI and DMEM medium were from Gibco (Grand Island, New York, USA). RNase A was purchased from Gentra Systems Inc. (Minneapolis, MN, USA). Annexin V-FITC apoptosis detection kit was obtained from Strong Biotech Co. (Taipei, Taiwan). Antibodies against procaspase-9, caspase-3, -8, -9, Bcl-2, Bcl-xl, Bax, Bad, Bak, and β-actin were purchased from BD Bioscience (Franklin Lakes, NJ, USA). Anti-actin antibody was from Santa Cruz Biotechnology ( Delaware, California, USA). 5-Fluorouracil (5-FU) was from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of reagent or analytical grade.

2.2. Authentication of plant material

The morphological characteristics of Typhonium blumei were identified by Prof. Ming-Hong Yen (Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan). Sequences of internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were used to authenticate this plant. DNA isolation, polymerase chain reaction (PCR) and DNA analysis were conducted according to Chou et al. (2007) with some modifications. The genus of Typhonium blumei was determined using three representative plants. For each plant, 150 mg of dried leaves were ground to a fine powder and mixed with 1.5 ml of pre-warmed extraction buffer (100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 1 M NaCl, 1% CTAB, 1% PVP–40). The mixture was incubated in a 65 °C water bath with gentle shaking for 20 min, then the sample solution was extracted with an equal volume of chloroform: isooamyl alcohol (24:1) and centrifuged at 11,000 × g for 20 min at 4 °C. The supernatant was carefully transferred to a new eppendorf tube and 2 volumes of 95% ethanol with one tenth volume of 3 M sodium acetate were added to precipitate the DNA. After centrifugation at 12,000 × g for 20 min, the DNA pellet was washed with 0.5 ml, 70% ethanol, dried and dissolved in 50–100 μl TE buffer. Polymerase chain reaction was performed using forward primer (5′-GGCGGAAGTCCTCAGAACA-3′) and reverse primer (5′-GGCGGTTCTTCGACTAAT-3′) with 3 μl dimethyl sulfoxide (DMSO) in 50 μl reaction solution. The mixture for PCR was denatured at 94 °C for 10 min, then subjected to 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s with an extension period of 10 min at 72 °C. The PCR products were examined on a 1.2% agarose gel and sequenced. The obtained sequences were analyzed with DNAMAN software (Lynnon Corporation, Quebec, Canada) and blasted with GenBank databases.

2.3. Preparation of Tb extract

Dry leaves of Typhonium blumei (1.8 kg) were crushed and drenched in 201 ethanol for one day, and extracted three times with 201 ethanol. After filtration by medicinal gauze, the filtrates were collected, concentrated with a vacuum evaporator and dried by a freeze-dryer. The yield was 3.34%.

2.4. Cancer cell lines and culture

Human A549, H661 (lung cancer), LN CaP, DU145 (prostate cancer), Hep 3B, Hep G2 (hepatoma), MDA-MB-231, MCF-7 (breast cancer), GBM (brain glioma), HEK293 (embryonic kidney cells) and MRC-5 (normal lung fibroblast cells) cell lines were purchased from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). The cultivation of A549, DU145, Hep 3B, Hep G2, MDA-MB-231, MCF-7, HEK293 and MRC-5 cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM –F12), while cell lines of H661, LN CaP and GBM were grown in RPMI 1640 medium. Both of these two media were supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin and 1.5 g/l sodium bicarbonate.

2.5. Determination of cytotoxicity of cancer cells

The cytotoxicity was determined according to the method of Swamy and Tan (2000) with some modifications. Cells (5 × 10^3)
were seeded in 96-well plates containing medium with various concentrations of Tb extract. The cells were cultured at 37 °C with 5% CO₂ and 95% air and in 100% relative humidity. After various durations of cultivation, the medium solution was removed. An aliquot of 100 μl of medium containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was loaded to the plate. The cells were cultured for 2 h, and then the medium solution was removed. An aliquot of 100 μl of DMSO was added to the plate, which was shaken until the crystals dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 570 nm in an ELISA reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). Cytotoxicity of each sample is expressed as IC₅₀ value. The IC₅₀ value is the concentration of test sample that cause 50% inhibition of cell growth, averaged from three to five replicate experiments, and was obtained by plotting the percentage inhibition versus concentration of test sample. 5-FU was used as the positive control which is an effective anticancer drug.

2.6. Cellular morphology analysis

A549 cells (5 × 10⁴) grown on 24-well plates were treated with Tb extract at different doses of 0, 50, 100 and 200 μg/ml for 6, 12 and 24 h. The morphological changes were observed under an inverted microscope (Nikon Eclipse TS100, Japan).

2.7. Flow cytometric analysis on cell cycle

To investigate the effect of Tb extract on the cell cycle distribution, A549 cells (1 × 10⁵ cells/ml) were treated with various Tb extract concentrations and cultured for 6 and 24 h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and fixed in 75% ethanol at 4 °C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase A (Genta Systems Inc., MN, USA), followed by shaking at 37 °C for 30 min. The stained cells were analyzed with flow cyrometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA).

2.8. Measurement of apoptotic ratio of A549 cells

The apoptotic effects of Tb extract and 5-FU on A549 cells were determined by the Annexin V-FITC staining method and flow cytometry. The A549 cells (1 × 10⁵ cells/ml) were treated with various Tb extract concentrations and cultured for 6 h. The treated cells were harvested, washed with PBS, and then treated with trypsin/EDTA solution. The suspended cells were centrifuged at 200 × g for 10 min. The cell pellet was added in 100 μl of Annexin V-FITC staining-solution (Strong Biotech Co., Taipei, Taiwan) and incubated for 10–15 min at 25 °C. The cells were then analyzed with flow cyrometer and the data were calculated with WinMDI 2.9 software.

2.9. Western blot analysis

Western blotting was performed to detect the proteins of procaspase-9, caspase-9, -8, -3 and Bcl-family. A549 cells (1.5 × 10⁵) were seeded onto 100-mm culture dishes in the presence or absence of Tb extract, and were treated for 12 h. The medium was removed and the cells were washed with PBS (0.01 M, pH 7.2) for several times. Following removal of the supernatant solution, the cells were lysed with lysis buffer (0.1 ml lysis buffer/each plate) for 20 min. The composition of lysis buffer was 1 ml RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 1% tert-octylphenoxy poly(oxyethylene)ethanol (IGEPAL), 4 μl of 0.25 M sodium vanadat, 20 μl of 0.1 M EGTA, 10 μl of 0.1 M PMSF, 2 μl of 5 mg/ml aprotinin, 2 μl of 5 mg/ml leupeptin, and 2 μl of 0.5 M EDTA. The supernatants were collected by centrifugation at 10,000 × g for 5 min at 4 °C, and were used as the cell protein extracts. The harvested protein concentration was measured using a protein assay kit (Bio-Rad). The same amounts of proteins from each extract were applied to 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Immunoblot; Millipore, Billerica, MA, USA), and then blocked for 1 h using 10% skim milk in water. After washing in PBS containing 0.1% Tween 20 for 3 times, primary antibodies against procaspase-9, caspase-9, -8, -3, Bcl-2, Bcl-xl, Bak, Bad, Bak or B-actin were added at a v/v ratio of 1:1000. After overnight incubation at 4 °C, the primary antibodies were washed away and secondary antibodies were added for 1 h incubation at room temperature. Finally, the Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham Biosciences, Buckinghamshire, UK) were used to develop the signal of the membrane.

2.10. Gas chromatography–mass spectrometry

GC–MS analysis was performed using Varian 450-GC and 240-MS system (Varian, USA) with the electron impact mode (70 eV) injector, and a Varian data system. The GC column was VF-5ms capillary column (30 m × 0.25 mm, film thickness 0.25 μm, FactorFour™, USA). Injector and detector temperatures were set at 250 °C and 290 °C, respectively. Oven temperature was kept at 50 °C for 5 min, then raised to 120 °C by a rate of 5 °C/min, kept at 120 °C for 8 min, then raised to 300 °C by a rate of 10 °C/min. The carrier gas was helium at a flow rate of 1 ml/min. Diluted samples of 1.0 μl were injected manually and in the splitless mode. The percentages of the compounds were calculated by the area normalization method without considering response factors. The compounds were identified by comparison of their mass spectra with the NIST MS 2.0 database (Gaithersburg, MD, USA). The compounds used in the cytotoxicity experiment on A549 cells were purchased from Sigma–Aldrich.

2.11. Statistical analysis

All experiments were conducted for three to five independent replicates. The data are expressed in terms of mean and standard deviation. Statistical differences were calculated using the Student’s t test with a significance level of p < 0.05–0.01. The experimental data were analyzed using Microsoft Excel software (Microsoft Software Inc., USA).

3. Results

3.1. DNA sequence identification of Typhonium blumei

Plant species are generally categorized based on their appearance, and from tissue slides of flowers, stems, roots, and seeds. However, morphological and histological characterization of herbal medicines often fails to distinguish between closely related species that are not as effective or have potential side effects (Shaw et al., 2002). Sequence comparisons of ITSs are becoming the prevailing method for authentication of medicinal plants at the DNA level; that is, DNA sequence analysis has become a powerful tool in plant species identification (Lau et al., 2001; Zhao et al., 2006). Fig. 1 shows the nucleotide sequences of ITS1, 5.8S and ITS2 of rDNA from Typhonium blumei, which were deposited in GenBank under the accession number HM362769. Blast searches on the NCBI’s database revealed that no species closely related to
During treatment exceeding 6 h, the cytotoxicity of Tb extract was effective against A549 cells (IC50 = 97.7 ± 0.3 g/ml), indicating that our sequence results are valuable for use as the Typhonium blumei DNA barcode (Chen et al., 2010).

### 3.2. Cytotoxicity of Tb extract on various cancer cell lines

To investigate the potential effect of Tb extract against cancer cells, various cancer cell lines were utilized to assess the cytotoxicity of Tb extract. Normal cell lines HEK293 and MRC-5 were also tested for comparison. According to Table 1, the Tb extract was effective against various cancer cell lines, such as normal lung fibroblast cells MRC-5 and HEK293, but only had a minor effect on other cancer cells. Conversely, the adverse effect of Tb extract on MRC-5 (IC50 = 245.5 ± 0.4 μg/ml) normal cells was comparatively low.

### 3.3. Inhibitory effect of Tb extract on A549 cell growth

Fig. 2 shows the inhibitory effect of Tb extract on the viability of A549 cells after treatment with various concentrations (0–250 μg/ml) of Tb extract under different incubation durations (1.5–120 h). The experimental results indicate that Tb extract inhibited cell viability in a dose-dependent manner (Fig. 2A). Notably, optimal treatment time was found at 6 h (IC50 = 41.3 μg/ml; Fig. 2B). During treatment exceeding 6 h, the cytotoxicity of Tb extract decreased as treatment time increased, indicating that Tb extract has a short-duration cytotoxicity on A549 cells.

### 3.4. Apoptotic effects of Tb extract on A549 cells

#### 3.4.1. Cell morphology

Fig. 3 shows morphological changes to A549 cells after Tb extract treatment for 6–24 h. Phase-contrast micrographs reveal that Tb extract induced cell shrinkage, apoptotic vacuoles, membrane blebbing and forming the floating cells in a dose- and time-dependent manner. These changes are suggestive of apoptosis.

#### 3.4.2. Cell cycle regulation

To determine whether Tb extract induced apoptosis was related to arrest of cell cycle progression in A549 cells, flow cytometry was used to quantitate the cell cycle distribution under treatment with different Tb extract concentrations for various durations (6 and 24 h). The number of cells in the G2/M phase increased, and that in the G0/G1 phase decreased in Tb extract treated cells when the Tb extract dose increased (Fig. 4). This experimental finding implies that Tb extract induced apoptosis on A549 cells via G2/M cell cycle arrest. However, this apoptotic effect did not increase as treatment time increased. This may be due to the short-duration cytotoxicity of Tb extract on A549 cells, as mentioned.

The Annexin V-FITC apoptosis detection kit was then employed to examine the influence of Tb extract on A549 cell death by flow cytometry. Fig. 5 shows that the dots were dispersed and shifted to the lower right (LR) side in a dose-dependent manner when A549 cells were treated with Tb extract, indicating that the cells moved to the early apoptotic stage. When the Tb extract dose increased,

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>IC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>A549</td>
<td>97.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>H661</td>
<td>251.9 ± 0.1</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>LNCaP</td>
<td>124.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>DU145</td>
<td>226.9 ± 0.5</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>Hep 3B</td>
<td>269.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Hep G2</td>
<td>232.9 ± 0.4</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>MDA-MB-231</td>
<td>230.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>MCF-7</td>
<td>125.8 ± 0.1</td>
</tr>
<tr>
<td>Brain glioma</td>
<td>GBM</td>
<td>298.5 ± 0.1</td>
</tr>
<tr>
<td>Embryonic kidney cells</td>
<td>HEK293</td>
<td>251.1 ± 0.2</td>
</tr>
<tr>
<td>Normal lung fibroblast cells</td>
<td>MRC-5</td>
<td>245.5 ± 0.4</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SD of 5 experiments.*

### Additional Information

- **Fig. 1.** Nucleotide sequences of the ITS region of Typhonium blumei Nicolson & Sivadasan. Putative borders are in bold and the 5.8S region is shaded. The nucleotide sequence was deposited in the GenBank database with accession number HM362769.

- **Typhonium blumei**, indicating that our sequence results are valuable for use as the *Typhonium blumei* DNA barcode (Chen et al., 2010).

- **Fig. 2.** Effects of different Tb extract doses and treatment durations on A549 cell growth. (A) Variation in viability under treatment with different Tb extract doses for 24 h. The 5-FU of 100 μg/ml was used as a positive control. (B) Variation in IC50 values under different treatment durations.

- **Fig. 3.** Shows morphological changes to A549 cells after Tb extract treatment for 6–24 h. Phase-contrast micrographs reveal that Tb extract induced cell shrinkage, apoptotic vacuoles, membrane blebbing and forming the floating cells in a dose- and time-dependent manner. These changes are suggestive of apoptosis.

- **Fig. 4.** Shows cell cycle regulation to determine whether Tb extract induced apoptosis was related to arrest of cell cycle progression in A549 cells, flow cytometry was used to quantitate the cell cycle distribution under treatment with different Tb extract concentrations for various durations (6 and 24 h). The number of cells in the G2/M phase increased, and that in the G0/G1 phase decreased in Tb extract treated cells when the Tb extract dose increased.

- **Fig. 5.** Shows that the dots were dispersed and shifted to the lower right (LR) side in a dose-dependent manner when A549 cells were treated with Tb extract, indicating that the cells moved to the early apoptotic stage. When the Tb extract dose increased,
Fig. 3. Apoptotic morphological changes of A549 lung cancer cells by treatment with Tb extract. Cells were treated with DMSO only (vehicle) or media containing 50, 100 or 200 μg/ml Tb extract for 6, 12 and 24 h. Photomicrographs were taken directly from culture plates with a phase contrast microscope (magnification 40×).

Fig. 4. Cell cycle analysis of A549 cells treated with different Tb extract concentrations by flow cytometry. A549 cells (5 × 10⁴ cells/ml) were incubated with 0–200 μg/ml of Tb extract as indicated in each graph for (A) 6 h and (B) 24 h. The percentage of each phase was analyzed by WinMDI 2.5 software. 5-FU of 50 μg/ml was as a positive control.

cell dots on the upper right (UR) side increased, indicating that cells had progressed gradually to the late apoptotic stage. These experimental results demonstrate that Tb extract induced apoptosis of A549 cells.

3.4.3. Pro-apoptotic and anti-apoptotic proteins expression

To elucidate the molecular effector pathway of Tb extract mediated apoptosis, this study determined whether caspases are involved as downstream effectors. The Tb extract increased cleavage of procaspase-9 accompanied by an increase in caspase-3 and caspase-9 protease expression in a dose-dependent manner (Fig. 6A). However, caspase-8 expression did not differ significantly when Tb extract concentration increased. This implies that apoptosis was not via the extrinsic pathway.

Several cytoplasmic proteins, particularly members of the Bcl-2 family, are critical to apoptosis regulation. In which, the pro-apoptotic subgroup, including Bax, Bad and Bak, promotes cell death; while the anti-apoptotic subgroup, including Bcl-2 and Bcl-xL, inhibits apoptosis (Srivastava et al., 1998; Catz and Johnson, 2003). Western blot analysis in this study shows that the expression of Bcl-2 and Bcl-xL decreased, while Bax, Bad and Bak increased as the Tb extract concentration increased (Fig. 6B).

The Bad/Bcl-xL and Bax/Bcl-2 ratios in a cell regulate susceptibility to apoptosis (Vander Heiden and Thompson, 1999; Hsu et al., 2008). This study demonstrates that the Bad/Bcl-xL and Bax/Bcl-2 ratios increased significantly when Tb extract concentration increased (Fig. 6C), correlating strongly with Tb extract induced apoptosis in A549 cells. These experimental findings suggest that Tb extract induced apoptosis of A549 cells via the mitochondrial pathway.
3.5. Cytotoxicity of main ingredients on A549 cells

The GC–MS analysis results show that at least 32 compounds existed in Tb extract, of which 16 were identified using mass spectrometry (Fig. 7). The mass spectra of these compounds were matched with those found in the NIST spectral database. Dibutyl phthalate (retention time, RT = 30.2 min; relative content, RC = 26.8%), α-linolenic acid (RT = 34.6 min; RC = 13.7%), phytol (RT = 34.9 min; RC = 3.2%), campesterol (RT = 46.7 min; RC = 1.5%), stigmasterol (RT = 46.9 min; RC = 3.2%) and β-sitosterol (RT = 47.8 min; RC = 1.3%) were confirmed by comparing their mass spectral data with the NIST mass spectral library and commercially available products. Fig. 8 shows their chemical structures.

Table 2 lists the cytotoxicities of these compounds on A549 cells under different treatment times. Although all of these compounds had anti-proliferative effects on A549 cells, campesterol (IC₅₀ = 2.2 μM for 24 h treatment) and β-sitosterol (IC₅₀ = 1.9 μM for 24 h treatment) had the most significant inhibitory activities.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ in μg/ml (μM)</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Dibutyl phthalate</td>
<td>ND</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>ND</td>
</tr>
<tr>
<td>Phytol</td>
<td>52.7 (177.9)</td>
</tr>
<tr>
<td>Campesterol</td>
<td>0.90 (2.2)</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>20 (48.5)</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>0.77 (1.9)</td>
</tr>
<tr>
<td>5-FU</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, effect undetectable.

Dibutyl phthalate, α-linolenic acid and phytol exhibited cytotoxicity in a time-dependent manner. However, the cytotoxicity of campesterol, stigmasterol and β-sitosterol weakened as treatment progressed. This finding may explain why Tb extract had a short-term cytotoxic effect on A549 cells.

Fig. 5. Effects of Tb extract on A549 cell apoptosis: (A) no treatment, (B) treated with vehicle, (C) treated with 50 μg/ml Tb extract, (D) treated with 100 μg/ml Tb extract, (E) treated with 200 μg/ml Tb extract, (F) treated with 50 μg/ml 5-FU. Treatment time was 6 h. Lower left (LL) quadrant: viable cells; lower right (LR) quadrant: early apoptotic cells; upper right (UR) quadrant: late apoptotic cells.
4. Discussion

This study is the first to examine the effect of Tb extract on anti-proliferative activity toward lung cancer cells. Experimental results show that Tb extract had good cytotoxicity against A549 cells, but was less sensitive to normal human cells, including human embryonic kidney cell HEK293 and normal lung fibroblast cell MRC-5, based on the high IC_{50} value (Table 1). The high sensitivity of malignant cells may in part reflect the difference in growth rates between malignant and non-malignant cells. Experimental results imply that the cytotoxic behavior of this extract toward A549 cells was selective. Additionally, Tb extract also demonstrated significant cytotoxicity on human prostate cell LNCaP and human breast cancer cell MCF-7 with IC_{50} values of 124.5 and 125.8 μg/ml, respectively, at 24 h treatment.

The Tb extract treatment inhibited A549 cell growth in a dose-dependent manner (Fig. 2A). However, this inhibitory activity did not increase as treatment time increased. The highest cytotoxicity on A549 cells occurred at 6 h (Fig. 2B), indicating that the extract has a short-term effect. Based on morphological changes identified by phase-contrast inverse microscopy, typical morphological characteristics of apoptosis, such as cell shrinkage, apoptotic vacuoles, and forming majority of the floating cells, were observed (Fig. 3).
Cell cycle control is a major event in cellular division. Deregulation of cell cycle has been proven to be associated with numerous carcinogenic processes (Kamesaki, 1998). Analytical results indicate that a G2/M phase arrest existed in Tb extract treated A549 cells, implying that cell death was caused by either apoptosis or necrosis (Fig. 4). In comparison, 5-FU arrested A549 cells at G0/G1 phase in a dose-dependent manner (data not shown). The presence of phosphatidylserine on the outer leaflet of apoptotic cells membrane was then assessed using Annexin-V staining (Hsu et al., 2009) to quantify the amount of cells in the early and/or late stage of apoptosis.

The numbers of early apoptotic cells increased in a dose-dependent manner for A549 cells (Fig. 5). Notably, the apoptotic rate by Tb extract was faster than that by 5-FU during the early stage of drug treatment. All these results suggest that Tb extract treatment can induce cell death of A549 cells via apoptosis.

Generally, apoptosis can occur via two fundamental pathways: (1) the mitochondrial or intrinsic pathway; and, (2) the death receptor or extrinsic pathway (Yoon and Gores, 2002). The intrinsic pathway is triggered by release of mitochondrial proteins, such as cytochrome c, which bind with Apaf-1 and procaspase-9 in a dATP-dependent manner to form the apoptosisome (Mignotte and Vayssiere, 1998). The apoptosisome can induce activation of caspase-9, thereby initiating apoptotic caspase cascades (Hengartner, 2000; Sun et al., 2004). Conversely, the extrinsic pathway is initiated by the interaction of ligands with their respective death receptors, sequentially leading to cleavage of initiator caspase-8. The active caspase-8 cleaves executioner caspase-3, resulting in apoptosis (Scaffidi et al., 1998). The apoptic proteas in the intrinsic and extrinsic pathways are caspase-9 and caspase-8, respectively. Activated caspase-9 and -8 further initiate activation of the caspase cascade, leading to biochemical and morphological changes associated with apoptosis (Li et al., 1997; Tepper et al., 2000). Caspase-3 is a well-known downstream adaptor caspase which can be proteolytically activated by caspase-9 or -8 via mitochondrial or cell death receptor signaling pathways (Cohen, 1997; Zhang et al., 2004; Hsu et al., 2008). Thus caspases have been shown to be activated during apoptosis in many cells and play critical roles in both initiation and execution of apoptosis (Liu et al., 2009). This study reveals that Tb extract significantly elevated the expression of caspase-3 and caspase-9 on A549 cells, as compared with that of untreated cells. On the other hand, the caspase-8 expression was not significantly different under the Tb extract treatment (Fig. 6A). These findings confirm that Tb extract can induce apoptosis in A549 cells by stimulating enzymes that have central roles in apoptosis initiation. This experimental result suggests that intrinsic pathway was involved in Tb extract induced apoptosis. Sharma et al. (2009) reported that tumors arise more frequently through intrinsic pathway than the extrinsic pathway because of the sensitivity.

The intrinsic pathway of apoptosis is regulated by the Bcl-2 family of proteins. Anti-apoptotic (e.g. Bcl-2 and Bcl-xl) and pro-apoptotic (e.g. Bad, Bak and Bak) are two of the major members in Bcl-2 family (Zhong et al., 1993; Wolter et al., 1997; Bruce-Keller et al., 1998). Anti-apoptotic Bcl-2 and Bcl-xl inhibit apoptosis by sequestering proforms of caspases or by preventing the release of mitochondrial apoptogenic factors (Tsujimoto, 1998; Adams and Cory, 2007). Bad, Bak and Bak inhibit Bcl-2 activity and promote apoptosis (Reed, 1995). In this study, Tb extract treatments altered the expression of anti-apoptotic (Bcl-2 and Bcl-xl) and pro-apoptotic (Bad, Bak and Bak) proteins, resulting in A549 cells apoptosis (Fig. 6B). Furthermore, the increased intracellular ratios of Bax/Bcl-2 and Bax/Bcl-xl occur during increased apoptotic cell death (Fig. 6C); therefore, the increased Bax/Bcl-2 and Bax/Bcl-xl ratios promote apoptosis (Zha and Reed, 1997; Vander Heiden and Thompson, 1999; Hsu et al., 2008). These experimental findings suggest that Tb extract induced A549 cells apoptosis via the mitochondrial pathway. However, further detailed investigations of this mechanism are warranted to obtain definite conclusions.

In this study, six major bioactive ingredients were identified by GC–MS analysis and their anti-proliferative activities on A549 cells were elucidated. Campesterol, β-sitosterol and stigmasterol displayed remarkable cytotoxic activity against A549 cells. These in vitro data support findings that a mixture consisting of these three sterols exerted cytotoxic activity against cancer cells (Lai et al., 2010). Notably, β-sitosterol induced G2/M arrest, endoreduplication, and apoptosis on U937 lymphoma cells and HL60 promyelocytic leukemic cells through the Bcl-2 and PI3K/Akt signaling pathways (Moon et al., 2008). Stigmasterol maintained a non-dose-responsive inhibition of growth (around 40–50% inhibition between 1.6 and 50 μg/ml) of H5578T breast cancer cells (Lai et al., 2010). Additionally, β-sitosterol and phytol exhibited good cytotoxicities against various cancer cells, including KB nasopharyngeal epidermoid carcinoma cells, MCF-7 breast cancer cells, CaSki cervical carcinoma cells, HCT 116 colon carcinoma cells and A549 cells (Malek et al., 2009). Phytol exhibited high cytotoxicity on Hela (human cervix carcinoma), WI-38 (human lung fibroblast) and HL-60 (human promyelocytic leukemia) cells (IC50 = 13.8–16.4 μg/ml) (Block et al., 2004). This study verified that these compounds had a strong anti-proliferative effect on A549 cells.

5. Conclusions

This study demonstrates that Tb extract significantly inhibited A549 cell proliferation, while this extract affected the sensitivity of normal lung fibroblast cells to a lesser extent. Treating A549 cells with Tb extract led to a cell cycle arrest at the G2/M phase and induced apoptosis of A549 cells by down-regulating Bcl-2 and Bcl-xl protein expression and up-regulating Bax, Bad and Bak expression. Additionally, dibutyl phthalate, α-linolenic acid, phytol, campesterol, stigmasterol and β-sitosterol were the major effective bioactive compounds in Tb extract. Further studies are needed to fully elucidate the mechanisms involved in cancer cell death and to ensure that this extract is safe for human consumption.

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