Ethyl acetate fraction in ethanol extract from root of "Dai-Bai-Jie" (Marsdenia tenacissima): anti-tumor activity in A549 cancer cells

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Supported by the Grant from the Basic Scientific Research Special of the Central Public Welfare Research Institutes of IMPLAD, Chinese Academy of Medical Sciences: Study on anti-lung cancer chemical constituents and mechanism of "Dai-Bai-Jie" (No. YZYN1501) and Study on the resources of "Dai-Bai-Jie" (No. YZYN-10-02)

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Abstract

OBJECTIVE: To evaluate the anti-tumor activity of ethyl acetate fraction (EFA), extracted with ethanol from the root of "Dai-Bai-Jie" in A549 cancer cells and its underlying mechanism.

METHODS: "Dai-Bai-Jie" was extracted with 95% ethanol-aqueous (DBJ-1), 50% ethanol-aqueous (DBJ-2), and water (DBJ-3) by reflux method. 95% ethanol-aqueous extract was separated by ethyl acetate (EFA) and n-butyl alcohol (DBJ-5), consecutively. The SRB method was used to evaluate the cytotoxic activity. Annexin V-FITC staining was applied to observe the apoptosis and analyze the cell cycle activated by EFA in A549 tumor cell. Western blot was used to detect the apoptosis-related proteins expressions. A549 tumor cells bearing nude mice model was employed to measure the tumor volume, mice weight, and tumor inhibition ratio in order to verify the antitumor activity in vivo.

RESULTS: DBJ-1 and EFA showed better cytotoxic activity on A549 tumor cells with IC50 25 and 3.5 μg/mL, respectively. EFA can exhibit the proliferation, arrest cell cycle at G0/G1 phase, and induce apoptosis in A549 tumor cells in vitro. The mechanisms of apoptosis induced by EFA may be associated with decreasing Bcl-2 protein expression and increasing p53, Bax, Caspase-3, and Caspase-8 protein expression. EFA also possessed significant anti-tumor efficacy in nude mice, and little toxicity was observed in the host.

CONCLUSION: EAF could induce A549 tumor cells apoptosis and G0/G1 cell cycle arrest. A549 tumor cells apoptosis induced by EAF may be associated with the decrease in the ratio of Bcl-2 and Bax mRNA levels, and increase in the expression of p53, Caspase-3, and Caspase-8 proteins.

INTRODUCTION

Cancer, a global public health issue, is a leading cause of morbidity and mortality. Lung cancer is the most common incident cancer and the leading cause of cancer/related deaths in China.1,2 Presently, chemotherapy and/or radiation therapy are the primary modes of treatment for lung cancer, accompanied by side-effects...
such as nausea and vomiting. Traditional Chinese medicine (TCM) has a unique theoretical system with a lengthy history of cancer treatment and has demonstrated efficacy in the lung cancer treatment.\textsuperscript{16} TCM focuses on multiple targets in lung cancer treatment that differ from Western Medicine.\textsuperscript{17} The anti-tumor drugs, such as vinblastine, vincristine, and paclitaxel,\textsuperscript{18} separated from TCM have played a critical role as anti-cancer agents.\textsuperscript{19} Recently, an increasing number of studies are focusing on finding and developing anti-cancer drugs from natural plants for its little toxic effects.\textsuperscript{20,21} Thus, it is necessary to explore new anti-lung cancer drugs with high efficiency and low toxicity.

"Dai-Bai-Jie" is the root of Marsdenia tenacissima (Roxb.) Moon (Asclepiadaceae), which is a perennial climber extensively distributed in the Yunnan province of China (Figure 1). It is a Dai traditional medicine, also known as "Ya-Jie-Xian-Da" in Dai language, which indicates its detoxifyin ability of hundreds of poisons. It is widely used traditionally for treating detoxification, decreasing swelling, and alleviating pain. The results of "The Fourth National Survey of Chinese Materia Medica Resources" showed that "Dai-Bai-Jie" is widely used for cancer and virus patients cure in Dai nationality region; however, the origin is not clear. The study of "Dai-Bai-Jie" origin displayed that the plant was different from Degeasinensis Hemsl. recorded in "Standard of Chinese Medicinal Materials in Yunnan Province—Dai Medicine" and "Chinese Dai Medicine Colorful Illustrations". While the characters match to Marsdenia tenacissima (Roxb.) Moon recorded in "Flora of China", the illustration in this book was incorrect. Although "Dai-Bai-Jie" was used as the core medicinal material for a series of preparations such as "Ya-jie" tablets and "Bai-jie" capsules in China, modern pharmaceutical research is lacking. The chemical constituents' study indicated that the main compounds were polyoxypregnane glycosides, which possessed various biological activities and pharmacological effects.\textsuperscript{21,22} A previous study showed that 95% extract and ethyl acetate fraction showed a robust cytotoxicity activity in vitro and in vivo and elucidate the potential mechanism of ethyl acetate extract of "Dai-Bai-Jie", the subsequent study was performed.

\textbf{MATERIALS AND METHODS}

\textbf{Reagents}

"Xiao Ai-ping" tablets, paclitaxel, and cyclophosphamide were purchased from Shanxi Medicine (Xianyang, China), Melonepharma (Dalian, China), and Pudepharma (Datong, China), respectively. RPMI1640 cell culture medium, fetal bovine serum (FBS), and trypsin were obtained from Gibco BRL Co., Ltd. (Grand Island, NY, USA). p53, Bcl-2, Bax, Caspase-3/8, and β-tubulin antibodies were bought from Abcam (University of Cambridge, Cambridge District, Cambridge County, UK). Sulforhodamine B was purchased from Sigma-Aldrich (St. Louis, LA, USA), Propidium iodide was obtained from Roche (Basel, CH), and ApoScreen Annexin V Apoptosis Kit was bought from SouthernBiotech (Birmingham, AL, USA).

All solvents used for the preparation of extracts and fractions were of analytical grade. The solvents were purchased from Tianjin Chemical Reagent Factory and Chengdu Kelong Chemical Reagent Factory (Tianjin, China).

\textbf{Equipments}

The following equipment was utilized: CO\textsubscript{2} incubator (Thermo Forma, Waltham, MA, USA); inverted microscope (Olympus, Japan); enzyme-labeled meter (Molecular Devices, Silicon Valley, CA, USA; vertical electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China); developer and fixer (Huqiu image equipment Co., Ltd., Suzhou, China), transblot apparatus (YuanPingHao Biological Technology Co., Ltd., Beijing, China).

\textbf{Plant material and extraction}

The roots of Marsdenia tenacissima (Roxb.) Moon ("Dai-Bai-Jie") materials were collected from Xishuangbanna prefecture, Yunnan province, China, in March 2013 and the sample was identified by Haitao Li at Yunnan Branch of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. A voucher specimen (D20100720008) was deposited at the Herbarium Yunnan Branch of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.
Three portions of air-dried and powdered materials of "Dai-Bai-Jie" (50 g) were extracted by 10-fold 95% ethanol (DBJ-1), 50% ethanol (DBJ-2), and aqueous (DBJ-3), respectively, with reflux method. The extracts were concentrated to residues by a rotary vacuum evaporator at 60 °C. The yields were 12.4%, 17.3%, and 16.2% respectively. Before the pharmacological assays, the extracts were solubilized in DMSO-distilled water to produce different concentrations in order to investigate the anti-tumor activity in vitro and select the best extract solvent.

Another 400 g "Dai-Bai-Jie" dry powder was extracted by 10-fold 95% ethanol using reflux method to produce the original extract. The extract was concentrated to residues by a rotary vacuum evaporator at 60 °C. The residue was solubilized in distilled water and extracted with ethyl acetate and butyl alcohol sequentially; ethyl acetate fraction (EFA), butyl alcohol fraction (DBJ-5), and aqueous fraction (DBJ-6) were obtained. The fractions were dissolved in DMSO-distilled water to produce different concentrations in order to investigate the anti-tumor activity in vitro to select the best active fraction. EFA was used to evaluate the underlying mechanism.

**Cell lines and culture**

A549 (human lung tumor cells) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were grown and maintained in Ham’s F12K supplemented with 2 mM L-glutamine, 90% 1.5 g/L sodium bicarbonate, and 10% FBS in an incubator at 37 °C, with 90% humidity and 5% CO₂.

**Animals**

4-6-week-old BALB/c-nude mice (weight 20-22 g) were bought from National Institutes for Food and Drug Control (Protocol 11400500008524; Beijing, China). The mice were housed in pathogen-free autoclaved cages and maintained under controlled conditions at 40%-60% relative humidity and (24 ± 2) °C. Healthy male mice were selected for experiments. The animal protocols were approved by the Institute of Laboratory Animal Sciences, CAMS & PUMC in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the EEC Directive of 1986 (86/609/EEC).

**Tumor cell growth inhibition assay (SRB assay)**

*In vitro* cytotoxicity of the extracts and fractions of "Dai-Bai-Jie" was determined by SRB assay[15,17]. The A549 tumor cells were grown in tissue culture flasks at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. 100 µL cell suspension was added to each well of 96-well plates, and the cell density was adjusted to 1 × 10⁴ cells/well, followed by incubation as above for 24 h. EFA was dissolved with 0.1% DMSO at concentrations of 0.1, 0.3, 1, 3, 10, 33, 100, 333, 1000 µg/mL, followed by the addition of 100 µL to the 96-well cell culture plates. This was repeated 3 times for each concentration.

The plates were incubated at 37 °C for 72 h, and 50 µL chilled 50% TCA was added to each well. The plates were then incubated at 4 °C for 1 h to fix the cells attached to the bottom of the wells. Then, the plates were washed 5-6 times with distilled water and air-dried; 100 µL SRB dye (0.4% w/v in 1% acetic acid) was added and incubated at room temperature for 30 min. Subsequently, the plates were washed with 1% acetic acid and air-dried, and 100 µL Tris buffer (10 mM, pH 10.5) was added to each well. The wells containing media but no cells served as blanks and wells containing cells, but no test samples were controls. The optical density (OD) of the plate was measured with SpectraMax 190 at 540 nm. The inhibition ratio was calculated as follows:

\[
\text{Inhibition ratio (\%)} = \left( \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100
\]

**Estimation of apoptosis by annexin V7-AAD staining assay**

A549 cells in the logarithmic phase growth were seeded into 6-well culture plates (5 × 10⁴ cells/well) and treated with a medium for 24 h at 37 °C in a humidified incubator with 5% CO₂. Then, the cells were treated with different concentrations of EAF (1,75, 3.5, and 7.0 µg/mL) for 48 h. Next, the cells were washed with cold PBS twice and resuspended in the 200 µL binding buffer; 10 µL Annexin V-FITC was added and
Cells in the logarithmic growth phase were treated with the above method for 24 h and then treated with different concentrations of EAF (1.75, 3.5, 7.0 µg/mL) for 48 h. The cells were harvest and lysed in RIPA lysis buffer (Beyotime). The protein concentrations were determined using Enhanced BCA Protein Assay Kit (Beyotime). The samples were subjected to electrophoresis on 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, USA). The membranes were blocked with Blotto and agitated for 2h. The membranes were probed with primary antibodies against Bax, Bcl-2, Caspase-3, and Caspase-8 wasexamined by Western blotting. Cells in the logarithmic growth phase were treated with the above method for 24 h and then treated with different concentrations of EAF (1.75, 3.5, 7.0 µg/mL) for 48 h. The cells were harvest and lysed in RIPA lysis buffer (Beyotime). The protein concentrations were determined using Enhanced BCA Protein Assay Kit (Beyotime). The samples were subjected to electrophoresis on 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, USA). The membranes were blocked with Blotto and agitated for 2h. The membranes were probed with primary antibodies against Bax, Bcl-2, Caspase-3, Caspase-8, p53, and β-tubulin (Gibco) at 4 ℃ for 24 h. Then, the membranes were washed with TBST 3 times, 5min each, and immersed in horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies, respectively. Finally, the membranes were washed with TBST as above, and the immunoreactive bands were visualized with Western Lightning™ - ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer, NEL.100001EA).

**Anti-tumor activity in vivo**

The anti-tumor activity of EFA was further assestedin A549 tumor cells bearing nude mice model. The mice, except the normal control group, were injected 0.1 mL A549 tumor cells (approximately 5.0 × 10^4 cells/mL) in the left axilla. Seven days after inoculation, the inoculated mice were randomly divided into seven groups consisting 6 mice each. The experimental groups were treated with different doses of EFA (50, 100, and 200 mg/kg, respectively), which was solubilized in 0.5 mL of 0.5% CMC by gastric infusion for 19 d. The positive groups were treated with cyclophosphamide (50 mg/kg) by peritoneal injection and "Xiao-Ai-ping" pellets (1.2 g/kg) by gastric infusion, and the negative group was treated by administration of equivalent saline. The tumors' volume was measured by Vernier calipers, and sizes calculated as follows: Tumor volume (mm³) = (length × width × height)/2. On day 19, all the animals were sacrificed and the tumors excised and measured. The tumor inhibition ratio (%) was calculated as follows: Tumor inhibition ratio (%) = [(C-T)/C] × 100%. Where, C is the tumor size average of the blank control (normal saline), and T is the tumor size average of the treated group.

**Statistical analysis**

Experimental values were expressed as mean± standard deviation of at least three experiments in triplicate. Data were analyzed using one-way analysis of variance and Student's t-test. P < 0.05 was the statistically significant level. All data were analyzed using SPSS 13.0. (SPSS Inc. SPSS for Windows, Version 13.0. Chicago, IL, USA).

**RESULTS**

**In vitro cytotoxic activity**

The IC₅₀ were 25 and 3.5 µg/mL, respectively, "Xiao Ai-ping" pellets and paclitaxel served as positive control drugs, which were widely used in practice in China. The rank order of extracts based on activity was DBJ-1 > DBJ-2 > DBJ-3 and that of fractions was EFA > DBJ-5 > DBJ-6 (Table 1).

**Assessment of EAF-induced apoptosis on A549 tumor cells in vitro**

Compared to 3.28% apoptotic cells in control, A549 tumor cells were treated with EAF 1.75, 3.5 and 7.0 µg/mL for 48 h, and the cells populations in late apoptotic phase increased to 5.0 ± 2.2, 8.5 ± 5.7, and 11.2 ± 4.8 respectively.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>In vitro anti-tumor results of extracts and fractions from &quot;Dai-Bai-Jie&quot; on A549 tumor cells (5 × 10⁴/mL, n = 3, x ± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>DBJ-1</td>
</tr>
<tr>
<td>IC₅₀ (µg/mL)</td>
<td>25.13±0.39</td>
</tr>
</tbody>
</table>

Notes: DBJ-1: 95% ethanol extract of Marsdenia tanacessisa; DBJ-2: 50% ethanol extract of Marsdenia tanacessisa; DBJ-3: aqueous extract of Marsdenia tanacessisa; EFA: Ethyl acetate fraction of 95% ethanol extract; DBJ-5: Butyl alcohol fraction of 95% ethanol extract; DBJ-6: aqueous fraction of 95% ethanol extract.
*A549 cell cycle arrest treated with EAF*

The cells were treated with different concentrations of EFA, and the results showed that the percentage of cells in G0/G1 phase increased from 71.46% to 78.05%, in control, which was treated with 7.0 µg/mL of EAF; the percentage of cells in G2-M and S phase were decreased (Figure 3).

*Cell cycle and apoptosis-related proteins expression in A549 tumor cells*

The results showed that the expression level of pro-apoptotic Bcl-2 expression decreased in a dose-dependent manner ($P < 0.05$), whereas the expressions of anti-apoptotic p53, Bax, Caspase-3, and Caspase-8 increased ($P < 0.05$, Figure 4).

*Anti-tumor activity of EAF in vivo*

The anti-tumor activities in vivo of EFA were studied for its prevailing anticancer activities in vitro. No mice deceased when treated with EFA during the experiment. The tumor volumes of A549 tumor-bearing mice with EFA treatment showed significant decreases from 10th day of treatment ($P < 0.01$, Table 3, Figure 5). After 19 d, the inhibitory rates were 51.75%, 73.63%, and 85.39% at the doses of 50, 100, 200 mg/kg, respectively. The weight of A549 tumor cells in mice showed no significant differences compared to the control group at different doses (Figure 5).

**DISCUSSION**

The study showed that EFA had significant anti-tumor activity, *in vitro* and *in vivo*, in A549 human lung cancer cells. The findings also suggest that EFA could inhibit tumor cell growth, causing cell cycle arrest in G0/G1 phase and promote apoptosis *in vitro* by downregulating the expression of Bcl-2 and upregulating the expression of Bax, p53, Caspase-3, and Caspase-8. Recent studies confirmed that cancer progression is associated with apoptotic pathways. Apoptosis is the programmed cell death, which maintains the healthy survival/death balance in metazoan cells, thereby playing a critical role in the development and homeostasis in normal tissues. Although "Dai-Bai-Jie" has an extensive history of clinical usage in cancer patients, little is known about its active fractions and effect on tumor cells.

It has been affirmed that some genes played important roles in apoptotic pathway. p53 protein, "the guardian of genome", is a tumor suppressor, which can arrest cell cycle by activating p21 and inducing cell death.
Inhibitory effect of EFA innude mice bearing A549 tumor cells (activating caspase-3, 5, and 7, which in turn activates Caspases-9 and -3.6) The caspase family is the common executor of apoptosis. Caspases-8 and -9 are considered as initiators at upstream, which can activate the executioners such as Caspases-3, -6, and -7 downstream.7 Caspase-8 and -3 form the core in apoptosis progress, which is the key step and the common pathway of all the apoptotic signals transduction.

In the present study, cell cycle analysis indicated that EAF induced A549 tumor cells apoptosis and G0/G1 cell cycle arrest. The expression patterns of Bcl-2, Bax, Caspase-3, Caspase-8, and p53 displayed that in the presence of EFA, the levels of Bax, Caspase-3, Caspase 8 and p53 are upregulated and that of Bcl-2 is down-regulated. These studies suggested that EFA inhibits the growth of A549 human lung cancer cells through mitochondrial pathway-induced apoptosis. However, anti-tumor activity of EFA in vitro did not predict the activity in xenograft models and against clinical cancer.8 Animal models have been used for predicting the efficacy and potential toxicities of anticancer drugs. The nude mice strains allow established in vitro human cell lines to be propagated subcutaneously, reconstituting solid tumors. Human tumor tissue explants obtained from biopsy or autopsy can be transplanted directly into mice.9 In order to further confirm the antitumor activity of FEA, tumor volume, mice weight, and tumor image were assessed in tumor-bearing BALB/C-nude mice. The results of anti-tumor activity in vivo showed a significant reduction in tumor volume (Table 3, Figure 5) and little side-effects.

### Table 2 Inhibitory effect of EFA in nude mice bearing A549 tumor cells (n = 6, x ± t)

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor volume (mm³)</th>
<th>Tumor inhibition ratio (%)</th>
</tr>
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<tbody>
<tr>
<td>&quot;Xai Ai-ping&quot; negative control</td>
<td>824.0 ± 217.6</td>
<td>-</td>
</tr>
<tr>
<td>Cyclophosphamide negative control</td>
<td>805.7 ± 82.7</td>
<td>-</td>
</tr>
<tr>
<td>&quot;Xiao Ai-ping&quot; (12000 mg/kg)</td>
<td>111.8 ± 27.0</td>
<td>86.4 ± 2.7</td>
</tr>
<tr>
<td>Cyclophosphamide (50 mg/kg)</td>
<td>80.5 ± 17.8</td>
<td>90.0 ± 1.4</td>
</tr>
<tr>
<td>EFA (50 mg/kg)</td>
<td>397.6 ± 91.1</td>
<td>51.8 ± 2.2</td>
</tr>
<tr>
<td>EFA (100 mg/kg)</td>
<td>217.3 ± 29.1</td>
<td>73.6 ± 5.7</td>
</tr>
<tr>
<td>EFA (200 mg/kg)</td>
<td>120.4 ± 25.2</td>
<td>85.4 ± 4.8</td>
</tr>
</tbody>
</table>

Notes: Negative groups ("Xai Ai-ping" negative control, Cyclophosphamide negative control) treated with 0.5% CMC and physiological saline, positive groups ("Xiao Ai-ping" (12000 mg/kg), Cyclophosphamide (50 mg/kg)) were treated with 12000 mg/kg "Xiao Ai-ping" and 50 mg/kg Cyclophosphamide, experimental groups were treated with 50, 100, and 200 mg/kg FEA three weeks respectively. FEA: Ethyl acetate fraction of 95% ethanol extract. Compared with "Xiao Ai-ping" negative control group, P < 0.01.
In conclusion, EAF possessed anti-tumor activity in vitro and in vivo without obvious toxicity. EAF could induce A549 tumor cells apoptosis and G0/G1 cell cycle arrest. A549 tumor cells apoptosis induced by EAF may be associated with the decrease in the ratio of Bcl-2 and Bax mRNA levels, and increase in the expression of p53, Caspase-3, and Caspase-8 proteins.

REFERENCES


