Hui Fang¹ and Hongmei Ji²,*

¹Department of Thoracic Surgery, The Fourth Affiliated Hospital of China Medical University, Shenyang, 110032, China; ²Department of Endocrinology, The Fourth Affiliated Hospital of China Medical University, Shenyang, 110032, China

Abstract: Background and Objective: The fruit of Fructus liquidambaris, which is recently being used for cancer treatment, has a history to be used as a traditional medicine in China for thousands of years.

Materials and Methods: Ten kg of dried F. liquidambaris was obtained with 70% alcohol-water solution under reflux for three times. The condensed extract was obtained from petroleum ether, ethyl acetate and N-butyl alcohol, respectively. Ethyl acetate extract was subjected to silica gel column, Sephadex LH-20, ODS column chromatography and RP-HPLC column chromatography to yield a new compound (1). The structure was identified through intensive analysis of NMR and MS spectra. The antitumor mechanism of the furanocoumarin A on human lung cancer A549 cells was confirmed by detecting the apoptosis-related proteins.

Results: Furanocoumarin A (1), a novel furanocoumarin constituent was isolated and identified from F. Liquidambaris. The IC₅₀ value of furanocoumarin A on A549 cell lines was 65.28±5.36μM obtained by the method of MTT. The compound could induce the apoptosis of A549 cells by inducing 21.5% early apoptosis and 32.4% late apoptosis at the concentration of 60μmol/L. Western blot analysis indicated that protein expressions of p53, caspase 3 and Bax increased in a dose-dependent manner between the concentrations from 40 to 80μM. The protein expression of Bcl-2 decreased the concentration of 60 and 80μM. The ratio of Bcl-2 to Bax was inversely proportional to the dose concentration.

Conclusion: Furanocoumarin A could be a novel anticancer agent from herbal medicine.

Keywords: Fructus liquidambaris, furanocoumarin, A549, apoptosis, caspase 3, bax.

1. INTRODUCTION

Lung cancer is one of the most common diseases of cancer-related deaths in the world. It has the second-highest morbidity rate among the malignant tumors in China. Non-small cell lung cancer accounts for more than 80% of lung cancer [1]. Surgery, radiotherapy and chemotherapy are the dominant treatments for lung cancer, however, a number of side effects are associated with them [2]. Traditional Chinese medicine has a long history with a number of advantages, with fewer side effects.

The fruit of Fructus liquidambaris has been used as a traditional medicine in China with the name of “Lulutong”.

Betulonic acid is considered as a major bioactive constituent in Fructus liquidambaris with obvious anticancer effects on ovarian cancer, prostatic cancer, liver cancer, etc. [3-6]. There is not much information on the antitumor bioactivities of the herb. In this study, the isolation and structural elucidation of a novel furanocoumarin from Fructus liquidambaris were reported, and the antitumor activity and mechanism on A549 cell lines were studied, subsequently. The expression of apoptosis-related proteins P53, Bax, Bcl-2 and Caspase-3 was detected after the confirmation of the inhibitory effect of furanocoumarin on A549 proliferation.

2. MATERIALS AND METHODS

2.1. General Experimental Procedures

NMR spectra including 1D and 2D spectra were recorded on a Bruker AV-400 FT-NMR spectrometer, and TMS was used as an internal standard. Agilent 6550 Q-TOF LC-MS system was used to determine the molecular weight of the compound. RP-HPLC separation was performed on an Agilent 1100 chromatograph apparatus equipped with a UV detector, and the flow cytometry system was from Bio-Rad Co., Ltd. Melting point was determined by a Jingke WRS-1B melting point apparatus (Shanghai, China).

2.2. Chemicals and Reagents

F. liquidambaris was obtained from Liaohe Pharmacy of Shenyang. Dimethyl Sulphoxide (DMSO) and chloroform-d were obtained from Sigma-Aldrich Inc. All the organic solvents such as methanol, alcohol, petroleum ether, ethyl acetate and N-butyl alcohol were purchased from Tianjin Kermel Chemical Co. (Tianjin, China). Normal phase silica gel (200–400 mesh) was obtained from Qingdao Marine Chemical Factory (Qingdao, China), whereas Sephadex LH-20 and ostade-cylsilane (ODS, 40–70μm) were purchased from Greenherb Chemical Co., Ltd. (Beijing, China). Fetal Bovine Serum (FBS), and RPMI-1640 were purchased from Gibco, Annexin V Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI) double staining kit was purchased from KeyGene Inc. (Nanjing, China).
2.3. Extraction and Isolation

Ten kg of dried *F. liquidambaris* was powdered and extracted with 70% alcohol-water solution under reflux for three times. About 1.1kg of the concentrated extract was obtained after evaporating the extract solution under reduced pressure. Subsequently, the extract was suspended in H2O and extracted with petroleum ether, then the extract solution was concentrated under reduced pressure. About 1.1kg of the condensed extract was obtained after evaporation.

2.4. Inhibitory Effect of Furanocoumarin A on A549 Proliferation

The *in vitro* experiment was approved by The Fourth Affiliated Hospital of China Medical University Clinical Research Ethics Committee [EC-2019-KS-013(YJ)]. Furanocoumarin A was dissolved in DMSO to make a series of test solutions firstly and stored at 4°C before use. MTT method was used to test the viability of the cells treated with furanocoumarin A according to the reference with minor modification [7]. A549 cell lines were seeded in a 96-well plate at a density of 5×10^3 cells per well. Following 24h incubation, the cells were treated with furanocoumarin A at the concentrations of 0, 10, 20, 40, 60, 80 and 100μM in the final test system and the concentration of DMSO in the final test system was 0.1% (v/v). After 24h incubation, 10μL of MTT solution (5mg/ml) was added per well and incubated for another 4h, then the medium was removed and 150μL of DMSO was added to solubilize the MTT formazan salt. The absorbance of the solution in the 96-well plate was measured by a microplate reader at 570nm. The inhibition rate of furanocoumarin A on A549 cell lines was calculated.

Inhibition rate (%) = (1- drug group OD_{570}/control group OD_{570}) × 100%

2.5. Apoptosis Analysis

Annexin V FITC apoptosis kit was used to analyze the apoptotic rates of the A549 cells induced by furanocoumarin A with flow cytometry. 1×10^6 of A549 cells were seeded into six-well plates, and then the cells were treated with furanocoumarin A (65μM) for 24h when the cell density reached about 80%. The staining was performed according to the instructions of the manufacturer and the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, USA).

2.6. Expression of Apoptosis-Related Proteins P53, Bax, Bel-2 and Caspase-3

The cells were mixed with different concentrations of furanocoumarin A for 24h. After washing the cells with cold PBS, A549 cells treated with furanocoumarin A were centrifuged at 4°C for 3min, 1500 rpm. The upper liquid was poured and 1×loading buffer was added to each sample to make it fully mixed. The mixture was boiled at 100°C for 10min. After transmembrane electrophoresis, the PVDF membrane was added in 50g/L skim milk powder at 4°C for 1h and then washed with PBST for 3 times. The first antibody was prepared, the membrane was removed and put into the antibody bag so that the liquid came in contact with the membrane evenly. The membrane was kept at 4°C in the refrigerator for incubation overnight. The membrane was removed and washed with PBST at room temperature for 3 times, each for 10min. The second resistance was prepared as per the instruction. The membrane was put in the second antibody solution and incubated in the shaking table at room temperature for 1h. The membrane was removed and washed with PBST at room temperature for 3 times, each for 10min. The ECL developers A and B were mixed in a 1:1 ratio. The membrane was placed in a petri dish and placed in the dark for 2min. The membrane was fully combined with the markers on the secondary resistance, then the excess ECL liquid was washed away. The film was placed on the developing plate for further development.

2.7. Real-time PCR Analysis

Total RNA samples were prepared by using PureZOL RNA isolation reagent (Bio-Rad, Hercules, CA, USA) as per the instructions. After spectrophotometric quantification, 1μg of total RNA was used only for Reverse Transcription (RT) in a final volume of 20μL with iScript cDNA Synthesis Kit (Bio-Rad) according to the instructions of the manufacturer. The gene expression levels were established by quantitative real-time RT-PCR conducted using the Applied Biosystems ViiA™ 7 Real-Time PCR system (Life Technologies, USA). The primers used in the experiments are listed in Table 1. The cDNA in a 10μL reaction volume was denatured at 95°C for 30s followed by 40 cycles of PCR stage (95°C, 5s, 60°C, 30s), while human GAPDH was measured to calibrate the original mRNA concentration. Each quantitative PCR was performed in triplicate and independently repeated three times.

2.8. Statistical Analysis

SPSS 18.0 was used for statistical data analysis and processing. One-way Anova was used for the comparison between multiple groups of samples, and t-test was used for each group. The experiments in each group were repeated three times. When *P*<0.05, the data showed to have statistical significance.
The inhibition of furanocoumarin A on A549 cells at 24h is shown in Fig. (2). Furanocoumarin A exhibited a dose-dependent inhibitory effect on A549 cells. With the increase of concentration and time, the inhibitory rate increases significantly. The IC50 value of furanocoumarin A on A549 cells was 65.28±5.36μM. According to the experimental results, three concentrations 40, 60 and 80μM were used for the subsequent experiments.

A549 cells were treated with furanocoumarin A at a concentration of 60μM, which was approximate to the value of IC50, for 24h. Then, apoptosis analysis of furanocoumarin A on A549 cells was achieved by the method of double-stained with anti-Annexin V-FITC antibody and PI (Fig. 3). The total apoptosis ratio of A549 cells was 53.9%, including 21.5% early apoptosis and 32.4% late apoptosis. This apoptosis ratio increased significantly as compared with that of the control group (5.6%).

Table 2. ¹H and ¹³C NMR data of compound 1 in chloroform-d (400 MHz).

<table>
<thead>
<tr>
<th>No.</th>
<th>¹H</th>
<th>¹³C</th>
<th>No.</th>
<th>¹H</th>
<th>¹³C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>160.7</td>
<td>9</td>
<td>-</td>
<td>144.5</td>
</tr>
<tr>
<td>3</td>
<td>6.28 (1H, d, J= 9.8 Hz)</td>
<td>112.9</td>
<td>10</td>
<td>-</td>
<td>107.7</td>
</tr>
<tr>
<td>4</td>
<td>8.12 (1H, d, J= 9.8 Hz)</td>
<td>139.6</td>
<td>2'</td>
<td>7.62 (1H, d, J= 2.3 Hz)</td>
<td>145.2</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>144.5</td>
<td>3'</td>
<td>6.99 (1H, d, J= 2.3 Hz)</td>
<td>105.2</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>114.7</td>
<td>1''</td>
<td>5.23 (1H, m, J= 8.3 Hz)</td>
<td>75.1</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>150.9</td>
<td>2''/3''</td>
<td>1.21 (6H, d, J= 8.3 Hz)</td>
<td>21.0</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>127.0</td>
<td>OCH₃</td>
<td>4.17 (3H, s)</td>
<td>60.9</td>
</tr>
</tbody>
</table>

3. RESULTS AND DISCUSSION

Compound 1 was obtained as light yellow (MeOH). HR-ESI-MS gave the molecular ion peaks for [M+Na]⁺ of 279.0739, indicating the molecular formula of C₁₅H₁₄O₅. Positive ferric hydroxamic acid reaction suggested a lactone moiety in compound 1. There were 9 degrees of unsaturation according to the molecular formula of C₁₅H₁₄O₅. However, in the ¹³C NMR spectrum, only eleven sp² carbon signals (including one carboxyl signal and ten double bond carbon signals) were observed. Thus, the structure of 1 was deduced as a furanocoumarin, whose ¹³C NMR features, including the presence of 9 degrees of unsaturation and lactone moiety, comply with that of the phellopterin. The ferric hydroxamic acid reaction indicated a lactone moiety; there were still two rings to be elucidated. In the ¹H NMR spectrum, two groups of proton signals assignable to the double bonds in the lactone and furan rings, respectively, were observed at δH 6.28 (1H, d, J = 9.8 Hz, H-3)/8.12 (1H, d, J = 2.3 Hz, H-2'). Furthermore, δH 4.17 (3H, s) suggested a methoxyl group, while δH 5.23 (1H, m, J = 8.3 Hz) and δH 1.21 (6H, d, J = 8.3 Hz) revealed the presence of an isopropyl group, leading to the assignment of all the sp³ carbon signals in the ¹³C NMR spectrum. Therefore, all substituents of the furanocoumarin skeleton were also determined, and 1 was considered to be a methoxyl and isopropyl substituted furanocoumarin. This deduction was confirmed by comparing the NMR data to those of the phellopterin [8], which is a methoxyl and isopentenyl di-substituted furanocoumarin. The results showed that all the NMR data of 1 were almost identical to those of phellopterin, except for the isopentenyl moiety that was replaced by an isopropyl group in 1. To further substantiate the deduced structure, the HMBC experiment was performed. In the HMBC spectrum, different correlations from H-4 to C-2/9; from -OCH₃ to C-5; from H-3' to C-5/7; from H-2'' to C-1''/3'' were observed (Fig. 1A and B). Thus, the structure of 1 was finally established as shown in Fig. (1A) and named as furanocoumarin A.

The inhibition of furanocoumarin A on the proliferation of A549 cells at 24h is shown in Fig. (2). Furanocoumarin A exhibited a dose-dependent inhibitory effect on A549 cells. With the increase of concentration and time, the inhibitory rate increases significantly. The IC₅₀ value of furanocoumarin A on A549 cells was 65.28±5.36μM. According to the experimental results, three concentrations 40, 60 and 80μM were used for the subsequent experiments.

A549 cells were treated with furanocoumarin A at a concentration of 60μM, which was approximate to the value of IC₅₀, for 24h. Then, apoptosis analysis of furanocoumarin A on A549 cells was achieved by the method of double-stained with anti-Annexin V-FITC antibody and PI (Fig. 3). The total apoptosis ratio of A549 cells was 53.9%, including 21.5% early apoptosis and 32.4% late apoptosis. This apoptosis ratio increased significantly as compared with that of the control group (5.6%).

For the research mechanism of furanocoumarin A induced apoptosis, the effects of furanocoumarin A on the protein expression of P53, Bax, Bcl-2 and Caspase-3, which are significant apoptosis-associated proteins in A549 cell lines, were analyzed. In this study, A549 cells were treated with 40, 60 and 80μM for 24h and then the four proteins were analyzed by western blot analysis.

Caspase 3 and p53 increased as a dose-dependent manner within the concentrations of 40 and 80μM, respectively (Figs. 4 and 5), which were different or significantly different from the blank group (*p<0.05, **p<0.01). Bax protein in 60 and 80μM groups also showed a dose-dependent manner (Fig. 6A and B), which was significantly different from that of the blank group (**p<0.01). Bcl-2 protein in 60, 80μM/L groups were significantly decreased as compared with that of the blank control group (**p<0.01) (Fig. 6A and C). The ratio of Bcl-2 to Bax was inversely proportional to the dose concentration (Fig. 6D), indicating that furanocoumarin A initiated the apoptosis of A549 cells. mRNA analysis indicated that A549 treated with furanocoumarin A could regulate the mRNA
Fig. (3). Effect of furanocoumarin A on the apoptosis of A549 cells.

Fig. (4). Furanocoumarin A treatment regulated the expression of P53.

Fig. (5). Furanocoumarin A treatment regulated the expression of Caspase-3.

Fig. (6). Furanocoumarin A treatment regulated the expression of Bcl-2 and Bax.
expression of P53, Bax, Bcl-2 and Caspase-3. mRNA expressions of P53, Bax and Caspase-3 are proportional to the concentration of furanocoumarin A, while the mRNA expression of Bcl-2 is inversely-proportional to the concentration of furanocoumarin A (Fig. 7).

Furanocoumarin A (1): light yellow. b.p. 129.2°C. IR (KBr) ν max: 3053, 2935, 2890, 1718, 1582, 1309, 1064, 990 cm -1. 1H- and 13C-NMR data: HR-ESI-MS (m/z): 279.0739 ([M+Na]+) (Table 2).

CONCLUSION

Coumarins are the characteristic bioactive ingredients in the herb plant with various pharmacological functions [9]. In the present study, a novel furanocoumarin ingredient, furanocoumarin A, was isolated from Fructus liquidambaris, which exhibited cytotoxic activities on A549 cell lines in a dose-dependent manner. The mechanism study result indicated that furanocoumarin A could induce the apoptosis in human lung cancer cell lines A549 by regulating the apoptosis-related proteins P53, Bax, Bcl-2 and Caspase-3. Therefore, the present study could be a support for the bioactive agent in the field of natural products.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by The Fourth Affiliated Hospital of China Medical University Clinical Research Ethics Committee [EC-2019-KS-013(YJ)].

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

