Flavonoid Extract from *Houttuynia Cordata* Thunb Induces Lung Cancer Cell Apoptosis through Cell Cycle Arrest at S and G2 Phases

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Abstract. *Houttuynia cordata* Thunb (HCT) is a species of saururaceae plant that has been analyzed for its potential healing properties. This study investigates the antitumor activity of flavonoids from HCT in human lung cancer A549 cells. Flavonoids enriched by macroporous resin adsorption technology from HCT were analyzed with ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-MS). MTT assay was utilized to evaluate cell viability. Annexin-V/PI staining was used to identify the apoptotic cells, and flow cytometry was used for cell cycle analysis. The results showed that ten flavonoids (rutin/ quercetin-3-O-β-D-robinoside, kaempferol-3-O-β-rutinoside, Quercitrin, Hyperoside/Isoquercitrin, Kaempferol-3-O-rhamnoside, Quercetin, Kaempherol, Houttuynoid D/E, Houttuynoid G and Houttuynoid A) were extracted and identified from HCT. Morphological changes of A549 cells treated with this extract of flavonoids included cell rounding and shrinkage. Flavonoids exhibited dose- and time-dependent inhibitory activities against A549 cells. These effects included the induction of apoptosis, secondary necrosis, and cell cycle arrest at the S and G2 phases. Our research data demonstrates that flavonoids from *Houttuynia cordata* Thunb might have the potential anti-lung cancer activity and the mechanism of proliferation inhibition is involved apoptosis and cell cycle.

Keywords: *houttuynia cordata* thunb; LC-MS; flavonoids; apoptosis; cell cycle

1. Introduction

*Houttuynia cordata* Thunb (HCT), a flowering plant, is named Yuxingcao in China. It is one of the most popular traditional Chinese medicines and is listed in the Chinese Pharmacopoeia. It is widely distributed in southern China, Japan, Korea, and Southeast Asia [1]. Pharmacological studies and clinical application have demonstrated that HCT possesses various biological properties, including antitumor, immunomodulation, antibacterial, antiviral, anti-inflammatory and anti-oxidation [2]-[7]. Flavonoids and volatile oils are the major active components which are responsible for the aforementioned properties of HCT [8]. Flavonoids are polyphenols that are naturally a part of many types of plants and have been shown to play a role in preventing cell proliferation, inducing apoptosis, and enhancing tumor suppressor gene activity to inhibit gene expression. However, the effects of flavonoids from HCT on lung cancer cells, including the A549 cell line, have yet to be investigated [9]-[11].
Here, we explored the potential use of flavonoids from HCT as a therapeutic adjuvant for lung cancer by utilizing the A549 cell line as a model to evaluate growth inhibition efficacy and to elucidate the molecular mechanisms involved in antitumor transformation.

2. Experimental Procedure

2.1. Chemicals and Materials

HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Formic acid of HPLC grade was obtained from CNW Technologies GmbH (Germany). Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Doxorubicin was obtained from Beijing Huafeng United Technology Co. Ltd. Fetal bovine serum (FBS) and DMEM medium were acquired from Gibco BRL (CA, USA). AnnexinV-FITC/FITC double staining kit was obtained from Pharmingen Company. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan).

The dried aerial parts of HCT were collected from Sichuan province of China. The samples were authenticated by Professor Jizhu Liu from Guangdong Pharmaceutical University. All the samples were preserved in our laboratory. Rutin was extracted, isolated, and purified from HCT in our laboratory. The purity of the isolated rutin was shown to be higher than 98% by HPLC.

2.2. Plant Material and Sample Preparation

400 g dried Herba Houttuyniae powder was extracted under ultrasound two times with 8000 mL ethanol for 40 min each time. The solutions were filtered, pooled, and evaporated in a vacuum to obtain an EtOH extract. The concentration of 0.434 mg/mL EtOH extract was suspended in distilled water and adjusted to a pH of 5. The water fraction was absorbed onto a D-101 macroporous resin column and eluted with water and 70% EtOH. The 70% EtOH fraction formed the flavone extract of HCT. The flavone extract of HCT was dissolved in methanol: water (1:1, v/v) and filtered through 0.22 µm membranes for LC-MS analysis.

2.3. Flavonoids Fraction Determination and Samples in Solution Preparation

Precision pipette rutin solutions of 0.00, 0.20, 0.40, 0.80, 1.20, 1.60 and 2.00 mL were placed in 10 mL volumetric flasks, respectively. Distilled water was added to 2 mL with 0.6 mL of 5% sodium nitrite solution and mixed well. After 6 min, 0.6 mL of 10% aluminum nitrate solution was added and mixed well. After 6 min, 5.0 mL of 4% NaOH solution was added and distilled water was added to 10 mL and incubated for 15 min. Without rutin reagent blank, absorbance was measured at 510 nm wavelength with a UV spectrophotometer. Linear regression was used to analyze the relationship between concentration and absorbance. The regression equation was $Y=12.012x -0.0068$, $r^2=0.9996$. The extract of flavonoids fraction was weighed and dissolved completely with serum-free medium, and 6.4 mg/mL concentration was prepared as storage solution, which acted as the experimental samples after membrane filtration.

2.4. Flavonoids Fraction from Herba Houttuyniae Determination

A Waters Acquity Ultra Performance LC system was used with a binary solvent delivery system and an autosampler. Samples were separated on an ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm, Waters Co., MA, USA) at a flow rate of 0.3 mL/min and a temperature of 30°C. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile (B) with a gradient elution: 0-12 min, 90% A-0% A, 13-15 min, 0% A-0% A. The injection volume was 5 µl.

A Waters Micromass Q-TOF micro system was used with a LockSpray and ESI interface operating in negative ion modes. The source parameters were electrospray capillary voltage 3.5 kV, source temperature 110°C, and desolvation temperature 350°C. The cone voltage was set at 30 V. Nitrogen and argon were used for cone and collision gases, respectively. Data were collected from m/z 100 to 1000, using independent reference lock-mass ions via the LockSpray interface to ensure mass accuracy and reproducibility. The solution of leucine enkephalin at 500 ng/mL was used as lock-mass, with an [M-H] ion at m/z 554.2615.

2.5. Cell Proliferation Assays
Lung cancer cell line A549 cell culture was according to ATCC methods. Cell proliferation was achieved by MTT assay. A549 Cells (0.5x10^5 cells/mL) were plated onto 96-well plates with increasing concentrations of Doxorubicin and extracts of flavonoids of 0.02, 0.1, 0.5, 2.5, and 5 mg/mL. After incubation for 24, 48, and 72 h, 5 mg/mL of MTT solution was added to each well then incubated for an additional 4 h at 37°C. The formazan crystals were solubilized with 100 μl/well of DMSO. A microplate reader (Bio-Rad) was used to evaluate the viability of A549 cell at 570 nm. The IC50 values were calculated.

2.6. Annexin-V/PI Double-Staining Analysis by Flow cytometry

Identification of apoptotic cells was determined by Annexin-V/PI staining. A549 cells (2.5x10^5 cells/mL) were plated onto 6-well plates and treated with different concentrations (0.5 mg/mL, 1 mg/mL, and 2 mg/mL) of flavonoids for 48 h. Annexin-V/PI staining was performed according to the instructions provided by the manufacturer. Samples were analyzed by flow cytometry with an excitation wavelength of 488 nm. Annexin-V and PI emissions were monitored at wavelengths of 525 nm and 630 nm, respectively.

2.7. Cell Cycle Analysis

Cells were harvested, washed with ice-cold PBS, and stained with 250 μl PBS containing 50 μg/mL PI and 40 μg/mL RNase. Cells were fixed gently in 70% ethanol and then stored at 4°C overnight. Cell cycle transitions were collected and analyzed by flow cytometry.

2.8. Statistical Analysis

The data were showed by the means ± standard error (SD). Two-tailed Student’s t-test was used to detect the differences between two groups. A P-value < 0.01 was considered to be statistically significant.

3. Result

3.1. LC-MS Analysis of Flavonoids from HCT

The constituents of flavonoids from HCT were analyzed using LC-MS. The chromatogram was shown in Fig. 1. The components were identified by comparing the mass spectra results with the data in the literature. The deviation of the elemental constituents was within 10 ppm. The relative content of each component was determined by area normalization, and the results were shown in Table 1. All the relative peak areas were calculated by the extracted ion chromatogram (EIC).

![Fig. 1: The base peak ion (BPI) chromatograms of flavonoids fraction from HCT obtained by LC-MS in negative mode.](image)

3.2. Effect of Flavonoids from Herba Houttuyniae on A549 Cell Morphology

Flavonoid concentrations up to 1 mg/mL caused cell shrinkage and evidence of cell death after 24 hours of treatment. Flavonoid concentrations up to 2 mg/mL caused a significant increase in cell death after 24 hours (Fig. 2).

3.3. Growth Inhibition Induced by Flavonoids against A549 Cells

Results demonstrated that flavonoids showed dose- and time-dependent growth inhibition activity against A549 cells after 24, 48, and 72 h incubations (Figure 3A). Furthermore, the effects of flavonoids on cell proliferation were compared with Doxorubicin, which is an inhibitor of A549 cells. Cells were separately cul-
tured with increasing concentrations of flavonoids and Doxorubicin, and cell growth was assessed by the MTT assay after 48 h (Fig. 3B). The results demonstrated that the growth of A549 cells was slightly inhibited by Doxorubicin, and cell viability was maintained at approximately 80% with 5 mg/mL of Doxorubicin. In contrast, flavonoids significantly inhibited the growth of A549 cells in a dose-dependent manner with IC$_{50}$ values of 0.7±0.1 mg/mL (n=5). A549 cell growth was inhibited by 48.2% after 48 h in the presence of 0.5 mg/mL of flavonoids.

![Fig. 2: Morphologic observation of the A549 cells treated with flavonoids from HCT after 48 hours. (A) Negative control with no flavonoids. (B) Treated with 0.5 mg/mL 1.0 mg/mL (C) and 2.0 mg/mL of flavonoids (D).](image)

### Table 1: Identification of the flavonoids fraction in HCT

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<th>Serial number</th>
<th>$t_R$(min)</th>
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<th>Calculated mass (m/z)</th>
<th>Error (ppm)</th>
<th>Formula</th>
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<th>Relative content (%)</th>
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![Fig. 3: (A) Effects of flavonoids from HCT on the growth of A549 cells after incubation for 24, 48, and 72 h. (B) The effects of flavonoids from HCT and Doxorubicin on the growth of A549 cells after 48 h treatment. The percentage of cell inhibition was quantified by the MTT assay. Data represent the mean ± SD of at least three independent experiment.](image)
3.4. Detection of A549 Cell Apoptosis by Annexin-V-FITC/PI Staining

A dose-dependent increase in the percentage of apoptotic cells was observed following incubation with different concentrations of flavonoids for 48 h compared with the vehicle control (Fig. 4). The addition of 0.5 mg/mL of flavonoids resulted in 87.08% apoptotic cells, and the percentage of apoptotic cells increased to 98.09% and 99.40% at concentrations of 1.0 and 2.0 mg/mL, respectively.

Fig. 4: Induction of apoptosis by flavonoids in A549 cells evaluated by Annexin-V-FITC/PI staining. (A) Quadrants represent the following populations: non-apoptotic, early apoptotic, late apoptotic or necrotic cells and mechanically injured cells. (B) Data represent the mean ± SD of at least three independent experiments. P < 0.01 versus control.

3.5. Effect of Flavonoids on A549 Cell Cycle Progression

To evaluate the effects of flavonoids on cell-cycle progression, A549 cells were treated with multiple concentrations of flavonoids (0, 0.5, 1.0, and 2.0 mg/mL). Fig. 5A and 5B show that flavonoids caused a dose-dependent accumulation of cells in the S- and G2-phases and a reduction of cells in the G1-phase. As shown in Fig. 5B, the cell cycle distribution of G1 after treatment with flavonoids (0, 0.5, 1.0, and 2.0 mg/mL) was 52.6%, 42.1%, 40.0%, and 27.1%, respectively. Furthermore, the S-phase cell population was significantly increased to 59.8% after treatment with 2.0 mg/mL of flavonoids compared to 30.2% observed in the control. These results indicate that flavonoids caused significant inhibition of cancer cell growth.

Fig. 5: Effect of flavonoids from HCT on A549 cell cycle. (A) Percentages of cells in the G1, S, and G2 phases are presented in a histogram. (B) The experiments were performed in triplicates (n=3). Data shown are representative of three separate experiments. *P < 0.05, **P< 0.01, ***P< 0.001 versus control.
4. Discussion

Chen YF, et. al. reported on the antitumor and apoptotic activity of ethanol extract prepared from HCT on the A549 human lung cancer cell line. They concluded that HCT induced G0/G1 phase arrest; however, there is no research elucidating the specific compounds that are responsible for these antitumor properties [16]. HCT contains the flavonoids quercetin and isoquercitrin, which have been shown to possess antitumor activity [17]. Youn HS, et. al. showed that quercitrin caused significant growth inhibition of H460 cells by inducing apoptosis through the NF-κB pathway [18].

The total of ten flavonoids extracted from HCT were detected and tentatively identified using LC-MS. MTT assay showed that the extract of flavonoids significantly inhibited the proliferation of A549 cells. The extracts of flavonoids caused significant inhibition of proliferation Our experiments also indicated that these extracts of flavonoids could induce apoptosis in both the early and late stages and cell cycle arrest at both the S and G2 phases, and these results are consistent with those observed.

In this study, we explored the antitumor activity of flavonoids from HCT in human lung cancer cells by Tsui KC, et. al.[19]. However, Chen YF, et. al. reported that the cell cycle arrest of A549 was at the G0/G1 phase [16]. A possible explanation for this disparity may be due to the use of ethanol extracts of HCT, which contain many other compounds in addition to flavonoids, by Chen YF, et. al., while this study and Tsui KC, et. al. used extracts of flavonoids from different herbs.

The subsequent investigation into the antitumor effects with much cell lines for broader analysis. *in vivo* investigation with an animal model should be underent for the pharmacodynamics and molecular mechanisms.

5. Conclusions

The flavonoids extracted from HCT might have antitumor activity in lung cancer cells by modulating S- and G2-phase arrest in human lung cancer A549 cells.

6. Acknowledgements

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7. References


