Nonselective Cell Necrosis Mediated by the Total Flavones of *Penthorum Chinensis* Pursh and Thonningianin-A in Human Hepatic and Hepatoma Cells

Xin Shen¹*, Zekun Li²,³*, Zhifang Guo², Yanan Wang², Tongtong Li⁴ and Guohui Li¹

Abstract

*Penthorum chinensis* Pursh (PCP), family Penthoraceae, has been used for hundreds of years in China. With the launch of PCP tablets, clinical applications focused on liver fibrosis and hepatocarcinoma. The purpose of this research was to explore the selectivity and toxicity of the active pharmacodynamic ingredients of PCP in vitro. The total flavones of PCP (TFPCE) and thonningianin-A (Th-A), a major flavone in TFPCE, were investigated on the cell death patterns in human hepatoma cells (HepG2) and human hepatic cells (LO2), followed by a concentration detection of LDH in the supernatants. Apoptosis and necrosis detection kits were used to validate the patterns of cell death caused by TFPCE and Th-A. Finally, the cytotoxicity of both TFPCE and Th-A were reproduced in the colorectal adenocarcinoma cells (NCI-H716). The results indicated that TFPCE inhibits the cell viability of HepG2 cells at a concentration lower than 25 μg/mL. Alternatively, the cell viability of LO2 cells dramatically decreased in the treatment of TFPCE at 25 μg/mL. The effects of Th-A on the cell viability of HepG2 cells and LO2 cells were consistent with TFPCE. LDH detection indicated that TFPCE and Th-A increased the LDH concentration of the supernatants in a dose-dependent way, indicating the pattern of cell necrosis. Fluorescence staining verified the necrosis cell death caused by TFPCE and Th-A. A dose-dependent tendency was obtained in NCI-H716 cells, indicating that the cell viability of NCI-H716 cells was significantly suppressed with the treatment of TFPCE and Th-A. Our results bring the potential toxicity of PCP to the forefront of public attention. Therefore, the clinical application of *P chinensis* is required to focus more on its cytotoxic effect.

Keywords

*Penthorum chinensis* Pursh, total flavones of *Penthorum chinensis* Pursh, cell necrosis, thonningianin-A, HepG2

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Introduction

*Penthorum chinensis* Pursh (Gan-Huang-Cao, PCP), a valuable traditional Chinese medicine, is widely distributed in eastern Asia, including China, Japan, and Korea,¹ and is used for the treatment of liver disease.² Both clinical and basic research have observed that the extract of *P chinensis* (PCE) is responsible for the prevention and treatment of hepatitis B, hepatitis C, hepatocarcinoma, and other related liver damage.³⁶ With the launch of Ganhunagcao tablet in the market, clinical application rapidly increased, especially in the treatment of liver fibrosis and hepatocarcinoma.⁷⁻⁹

PCP clinically centers on liver diseases, including hepatocellular carcinoma, hepatic virus infections, alcoholic liver disease, nonalcoholic fatty liver disease, and liver fibrosis. Pharmacological studies have revealed that PCP exerts multiple effects in pro-apoptosis of hepatoma cells, cell cycle arrest, inhibition of virus replication, and inhibition of hepatic stellate cells activation. Moreover, toxicology studies have shown that PCP has no obvious toxicity or side effects in either animals

¹National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
²Shijiazhuang Yiling Pharmaceutical, Shijiazhuang, China
³The Pennsylvania State University, University Park, PA, USA
⁴College of Integrated Chinese and Western Medicine, Hebei University of Chinese Medicine, Shijiazhuang, China
*Both authors contribute equally.

Corresponding Author:
Guohui Li, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China.
Email: lgh0603@cicams.ac.cn
or humans. These studies indicate that either PCP or its extract is an ideal medicine for the treatment of hepatocellular carcinoma for its selective cytotoxicity on hepatoma cells. However, the lack of understanding of the mechanisms of action on the selectivity on the hepatoma cells and normal hepatocytes needs further attention.

Phytochemical studies of PCP have identified some main bioactive components including flavonoids, polyphenols, and steroids. Based on previous studies, flavonoids and phenols are usually considered to be responsible for the bioactivities of PCP. Flavonoids, being the major bioactive constituents of PCE, are known to have a wide spectrum of properties, including anti-inflammatory, antioxidant, and anti-apoptotic effects. Current knowledge has revealed that flavonoids play a protective role in various models of chemical-induced liver injury. Thonningianin A (Th-A), an ellagic tannin flavonoid found in relatively high content in PCP, exhibits strong hepatoprotective and anti-hepatocarcinoma properties. Although many chemical and pharmacological studies have been carried out on PCP, the underlying mechanisms of anti-hepatotoxicity and anti-fibrotic effects are still poorly understood. Moreover, the possible toxicity of natural herbal products cannot be ignored. Compelling evidence has shown that some herbal medicines cause serious organ damage, which constrains their clinical application.

The anti-fibrotic and anti-hepatocarcinoma properties may rest with either the suppression of cell proliferation or the induction of cell death, which would be reflected by a change in cell viability. The ultimate objective of this study was to investigate the cell death patterns induced by TFPCE and Th-A. Furthermore, the difference between somatic cells and cancer cells enables us to evaluate the selectivity and safety in the treatment of hepatocarcinoma and predict any serious side effects.

**Result and Discussion**

**Th-A is the Dominant Flavone of PCP and Responsible for its Biological Activities**

Flavonoids have been identified to be the active compounds in PCP. Th-A is the active flavonoid responsible for the antioxidant and anti-hepatocarcinoma properties, and exhibits strong hepatoprotective activity. HPLC analysis showed the dominant presence of Th-A in TFPCE. The retention time of Th-A (31.736 min) was the same as that of the Th-A standard.

**Cell Viability of HepG2 Cells and LO2 Cells Were Suppressed by TFPCE**

To illustrate whether TFPCE effectively influenced hepatic carcinoma, we treated human hepatocellular carcinoma cells (HepG2) with TFPCE and monitored cell proliferation through MTS assay. HepG2 cells were divided into 8 groups and treated with TFPCE of different concentrations for 24 h. All groups experienced similar cell states at the start of treatment and physical conditions during the incubation period. Although the proliferation of HepG2 cells treated with TFPCE was slightly improved in concentrations lower than 12 μg/mL, the cell viability of HepG2 cells was significantly suppressed in a dose-dependent manner within the concentration of 25 μg/mL-50 μg/mL, shown by the relative growth rate by MTS assay. The lowest cell viability (22.6%) was obtained at 50 μg/mL (Figure 1A). When the concentration of TFPCE increased from 12 μg/mL to 25 μg/mL in the LO2 cell line, the cell viability dramatically decreased (Figure 1B). A sharp drop in cell viability was obtained at a concentration of 25 μg/mL when the inhibition rate reached its highest (96.6%); the half-maximal inhibitory concentration (IC50) was determined to be 30.7 μg/mL on HepG2 (Figure 1C), and 11.8 μg/mL on LO2 cells (Figure 1D). The results of the current study clearly indicated that the proliferation of HepG2 cells was significantly suppressed by TFPCE in a dose-dependent way. However, surprisingly, TFPCE also showed a significant antiproliferative effect on LO2 cells with a lower IC50 (Figure 1), which indicates a possible cytotoxic effect.

**Cell Viability of LO2 Cells and HepG2 Cells Were Suppressed by Th-A**

Since Th-A, to some extent, represents the major biological activities of TFPCE, and the content of Th-A accounts for 39.5% of TFPCE, a further assay for Th-A on cell viability was carried out. To investigate further whether Th-A is the constituent responsible for the anti-hepatocarcinoma properties of TFPCE, we applied 24 h treatment of Th-A on 2 groups of the same cells, LO2 cells and HepG2 cells, and measured the relative growth rate. To compare with groups treated with TFPCE, each group experienced similar conditions. Based on the result of MTS assay, the cell viability of HepG2 cells treated with Th-A was first determined. Proliferation was slightly accelerated as the concentration of Th-A was lower than 1 × 10^{-6} M and then the cell viability of HepG2 cells sharply decreased to 20.9% when the concentration of Th-A was above 1 × 10^{-6} M (Figure 2A). The cell viability of LO2 cells treated with Th-A was remarkably reduced to 1.7% at the same concentration (1 × 10^{-6} M) of Th-A, as shown in Figure 2B. However, Th-A showed negligible toxic effects on LO2 cells until the concentration achieved 1 × 10^{-6} M (Figure 2B). The inhibition rate of Th-A on both cells showed similar tendencies in that the rate dramatically increased at a concentration of 1 × 10^{-6} M. In addition, the IC50 values of Th-A on HepG2 cells and LO2 cells were determined to be 8.30 μM and 2.82 μM (Figure 2C and D), which indicated that Th-A exerts a nonselective cytotoxic effect on both hepatic and hepatoma cells, suggesting that the pattern of cell death caused by Th-A was most likely to be necrotic.
To avoid the histological origin, the cytotoxic effects of TFPCE and Th-A were reevaluated on colorectal adenocarcinoma cells (NCI-H716). We treated these cells in 2 separate groups with either TFPCE or Th-A in a concentration gradient for 24 h. The cells in all groups were in a similar state at the start of treatment and the physical conditions were the same during the incubation period. TFPCE treatment indicated that the cell viability of NCI-H716 cells was significantly suppressed in a dose-dependent manner within the concentration range of 0 μg/mL to 320 μg/mL, and then the inhibition rate gradually increased from 0% to 100%. When the concentration of TFPCE achieved 320 μg/mL, a negative value for cell viability was detected at a concentration of 320 μg/mL. Basically, all cells died and the phenomenon was also proved by observation under a microscope (Figure 3A). The second group of NCI-H716 cells treated with Th-A gave relatively high cell viability that was greater than 90% within the concentration range of 5 × 10^{-10} M to 5 × 10^{-7} M. After the concentration had reached 5 × 10^{-5} M, the lowest cell viability (12.5%) was obtained (Figure 3B). The IC_{50} was determined to be 89.2 μg/mL for the TFPCE treatment group and 10.6 μM for the Th-A treatment group (Figure 3C and D). This result indicated that TFPCE and Th-A were also effective in reducing the cell viability of other cancer cells.

**TFPCE-Induced Cytotoxicity in HepG2 Cells was Caused by Necrosis**

Cell death modes include programmed cell death (apoptosis, necrotizing apoptosis, autophagy, pyroptosis, and ferroptosis) and non-programmed cell death (necrosis). Lactate dehydrogenase (LDH) activity is an available biomarker of cell necrosis. To investigate the specific impact of *P. chinensis* on HepG2 cells, we measured LDH release by applying the nonradioactive cytotoxicity assay. HepG2 cells was divided into 2 individual groups treated with either TFPCE or Th-A. The LDH release level of both the TFPCE and Th-A treatment groups was increased in a dose-dependent manner within the concentration range of 0 μg/mL to 320 μg/mL, and then the inhibition rate gradually increased from 0% to 100%. When the concentration of TFPCE achieved 320 μg/mL, a negative value for cell viability was detected at a concentration of 320 μg/mL. Basically, all cells died and the phenomenon was also proved by observation under a microscope (Figure 3A). The second group of NCI-H716 cells treated with Th-A gave relatively high cell viability that was greater than 90% within the concentration range of 5 × 10^{-10} M to 5 × 10^{-7} M. After the concentration had reached 5 × 10^{-5} M, the lowest cell viability (12.5%) was obtained (Figure 3B). The IC_{50} was determined to be 89.2 μg/mL for the TFPCE treatment group and 10.6 μM for the Th-A treatment group (Figure 3C and D). This result indicated that TFPCE and Th-A were also effective in reducing the cell viability of other cancer cells.
executed by means of cell necrosis. Notably, Th-A has been reported to trigger cell DNA damage and induce cell apoptosis by an increase in intracellular ROS, which is not shown in this study.

Cell Viability of RDF Cells was Negatively Adjusted by TFPCE and Th-A

As mentioned above, PCP exerts therapeutic effects for the treatment of liver fibrosis. The aqueous extract of PCP (25 μg/mL-100 μg/mL) has been found to significantly suppress the expressions of fibrotic markers, including collagen I and α-smooth muscle actin, in vitro. The polyphenols of PCP are the active compounds by inhibiting the proliferation of HSC-T6 cells. To determine further the protective effects of PCP on liver fibrosis, we treated dermal fibroblasts cells (RDF) with either TFPCE or Th-A in 2 separate groups. The TFPCE treatment indicated that the cell viability of RDF cells was significantly decreased to 13.2% at the concentration of 50 μg/mL (Figure 6A). The second group of RDF cells treated with Th-A showed stable cell viability that was about 100% within the concentration range of 5×10^{-9} M-5×10^{-6} M. When the concentration reached 5×10^{-5} M, the lowest cell viability, 4.8%, was obtained (Figure 6B). The IC_{50} of TFPCE was determined to be 23.9 μg/mL and 3.2 μM for Th-A. (Figure 6C and D). Our results revealed that Th-A not only dominated TFPCE, but was also responsible for its cytotoxicity, which brought both PCP and its extract to the forefront of public attention. Although the cytotoxic effects help eliminate cancer cells, nonselective damage to normal cells and the pattern of necrotic cell death cause severe side effects, with even life-threatening consequences. Because of the lack of animal validation, these results might only raise a warning for the future application of PCP in the clinic, indicating the need for further in vivo studies in this regard.

Materials and Methods

Preparation of Total Flavones of PCP and Thonningianin-A

PCP was obtained from Shijiazhuang Yiling Pharmaceutical Co., Ltd. A UPLC fingerprint of PCE showed its dominant component to be Th-A (39.5%). The herb (50 kg) was smashed into pieces and extracted 2 times with 70% ethanol for 1.5 h each time by refluxing (1:10, v/v; 1:8, v/v). The extract was vacuum concentrated under 60 °C and the concentrated solution was mixed with ethanol (95%) to reach 80% ethanol in the mixture, and then left for 12 h. The supernatant was centrifuged and vacuum

Figure 2. The anti-hepatocarcinoma effect of Th-A on HepG2 and LO2 cells. Th-A suppressed cell viability of HepG2 cells (A) and LO2 cells (B). The cell growth inhibition rate was upregulated by Th-A in a concentration-dependent manner on HepG2 cells (C) and LO2 cells (D).

*P<.05 and **P<.01, treated group versus control group.

Abbreviations: HepG2: human hepatoma cells; LO2: human hepatic cells; Th-A: thonningianin-A; TFPCE: total flavones of PCP.
concentrated to obtain a final concentration of 0.2 g/mL. Concentrated supernatant was applied to a polyamide resin column and eluted with a gradient flow rate of 2 BV/h (5 BV water, 3 BV 5% ethanol). Finally, the sample was eluted with 90% ethanol, and this final eluent was dried under reduced pressure to produce the TFPCE. The TFPCE (25 mg) was dissolved in methanol (20 mL) in a flask and sonicated for 40 min. The supernatant (5 mL) was transferred to a measuring flask (50 mL) and diluted with methanol.

HPLC was applied to samples with a 10 μL injection volume using a Symmetry C18 column (4.6 x 250 mm, 5 μM) with a gradient elution program at a flow rate of 1.0 mL/min. The mobile phase was composed of acetonitrile (A) and 0.01% aqueous phosphoric acid (B), and the following gradients were applied: 0 to 10 min, 15-20% A; 10 to 15 min, 20% to 25% A; 15 to 18 min, 25% to 40% A; 18 to 30 min, 40% to 45% A; 30 to 40 min, 45% to 100% A. The column temperature was maintained at 30 °C and the detection wavelength was 254 nm. To investigate further the properties of Th-A, the pure compound was purchased from National Institutes for Food and Drug Control (purity = 97.3%). In further experiments, TFPCE and Th-A were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and diluted to suitable concentrations in DMEM.

**HepG2 Cell Line and RDF Cells**

The HepG2 cell line and the RDF cells were purchased from the American Type Culture Collection (ATCC), the LO2 cell line, and NCI-H716 cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen), supplemented with 15% fetal bovine serum (FBS) (Excell Bio USA) and 1% penicillin/streptomycin (P/S) (Invitrogen) at 37 °C in a 5% CO2 humidified environment. Cells were grown in 96-well plates (Corning) with serum-free DMEM containing 0.5% bovine serum albumin (BSA) (Solarbio) to achieve 70% to 80% confluence 12 h before treatment.

**Measurement of Cell Viability**

MTS assay was performed to study the cytotoxic effects of TFPCE and Th-A on HepG2, LO2, NCI-H716 and RDF cells. Cells were seeded in 96-well plates (Corning, New York, NK, USA) at a density of 1 x 10^5 cells per well. After 24 h incubation, cells achieved approximately 70% to 80% confluence when they were treated with different concentrations of either TFPCE or Th-A for another 24 h. To determine the cell viability, cells were incubated with MTS solution (Promega) (5 mg/
mL) for another 2 h at 37 °C. Finally, cell viability was detected by measuring the absorbance at 490 nm and 630 nm using a microplate reader (TECAN).

Nonradioactive Cytotoxic Assay
Nonradioactive cytotoxicity assay was performed to evaluate cell damage by quantitatively measuring LDH release. Cells were cultured in 96-well assay plates (Corning, New York, NK, USA) at a density of $2 \times 10^5$ cells per well and incubated at 37 °C in 5% CO$_2$ until approximately 70% to 80% confluence. After treatment with different concentrations of either TFPCE or Th-A for 2 h at 37 °C, 50 μL aliquots were transferred from all wells to a fresh 96-well flat-bottom plate (Corning, New York, NK, USA). To each well was added 50 μL of CytoTox 96 Reagent (Promega) and covered with foil to protect it from light and incubated for 30 min at room temperature. After adding 50 μL of stop solution to each well, the absorbance at 490 nm was recorded by a microplate reader (TECAN).

Apoptosis and Necrosis Detection
An apoptosis and necrosis detection kit (Beyotime) was used to determine the type of cell damage indicated by different fluorescent colors. Cells were seeded on 96-well plates at a density of $1 \times 10^5$ cells per well. When confluence achieved approximately 80%, cells were treated with different concentrations of either TFPCE or Th-A. All cells were harvested after treatment with the indicated drug for 24 h, centrifuged (1000 g) for 5 min and then washed with PBS (Invitrogen). To each well was added 100 μL detection solution and incubated at 37 °C for 10 min in the dark. Finally, the 2 types of fluorescence were observed by a fluorescence microscope (TECAN) at the specific wavelengths (green fluorescence: Ex/Em = 491/509 nm and red fluorescence: Ex/Em = 535/617 nm).

Statistical Analysis
Data were obtained from more than 3 biological replicates and statistically analyzed by the Student’s t-test using GraphPad Prism 9 (GraphPad Software). All results are reported as mean ± SEM. For multiple comparisons between control and experimental groups, a one-way analysis of variance (ANOVA) was applied. Differences were considered statistically significant when the $P$ value was less than .05.

Declaration of Conflicting Interests
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ORCID iD
Guohui Li https://orcid.org/0000-0003-0391-744X

Figure 4. Cytotoxic effect of TFPCE and Th-A on NCI-H716 cells. TFPCE (A) and Th-A (B) stimulated cell death in a concentration-dependent manner on NCI-H716.

* $P < .05$ and ** $P < .01$, treated group versus control group.
Abbreviations: NCI-H716: colorectal adenocarcinoma cells; Th-A: thonningianin-A; TFPCE: total flavones of PCP.
Figure 5. Pro-necrotic effect of TFPCE and Th-A in HepG2 cells. TFPCE (A) and Th-A (B) decreased the cell proliferation by prompting necrosis in a concentration-dependent manner. (Green fluorescence: apoptosis and red fluorescence: necrosis).

Abbreviations: HePG2: human hepatoma cells; NCI-H716: colorectal adenocarcinoma cells; Th-A: thonningian-A; TFPCE: total flavones of PCP.
Figure 6. The anti-fibrosis effect of TFPCE and Th-A on RDF cells. Proliferation of RDF cells was downregulated by TFPCE (A) and Th-A (B). TFPCE (C) and Th-A (D) increased the cell growth inhibition rate in a concentration-dependent manner on RDF cells.

*P < .05 and **P < .01, treated group versus control group.

Abbreviations: RDF: dermal fibroblast cells; Th-A: thornningianin-A; TFPCE: total flavones of PCP.

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