The ethyl acetate extraction of *Pileostegia tomentella* (ZLTE) exerts anti-cancer effects on H1299 cells via ROS-induced canonical apoptosis

FAN Qiu-Mei\(^1\,\,2\), ZHAO Wen-Tong\(^3\), YUAN Renyikun\(^3\), WANG Qin-Qin\(^1\,\,2\), ZHANG Li-Feng\(^4\), GAO Hong-Wei\(^1\,\,2\)*, LENG Jing\(^4\,\,5\)*, YANG Shi-Lin\(^1\,\,2\)

\(^1\)College of Pharmacy, Guangxi University of Chinese Medicine, Nanning 530000, China; \(^2\)Guangxi Engineering Technology Research Center of Advantage Chinese Patent Drug and Ethnic Drug Development, Nanning 530200, China; \(^3\)State Key Laboratory of Innovative Drug and Efficient Energy-Saving Pharmaceutical Equipment, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China; \(^4\)Guangxi Key Laboratory of Translational Medicine for Treating High-Incidence Infectious Diseases with Integrative Medicine, Nanning, Guangxi 530200, China; \(^5\)Guangxi Key Laboratory of Efficacy Study on Chinese Materia Medica, Nanning 530200, China

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**[ABSTRACT]** Lung cancer is the leading cause of cancer death and the most common malignant tumor, the long-term survival of which has stagnated in the past several decades. *Pileostegia tomentella* Hand. Mazz is a traditional Chinese medicine called “Zhongliuteng” (ZLT) in the pharmacopeia, which has been proved to possess a potent anti-tumor effect on various cancers. In this study, the effects of ZLT N-butanol extraction (ZLTN) and ZLT ethyl acetate extraction (ZLTE) on the viability of non-small cell lung cancer cell (NSCLC) lines H1299 and A549 were evaluated. Here, we firstly reported that ZLTE significantly inhibited H1299 cells growth without affecting the release of lactate dehydrogenase (LDH). In addition, ZLTE induced caspase-dependent apoptosis in a concentration-dependent manner and increased the expression cleaved-PARP and decreased pro-caspase-3, pro-caspase-7, pro-caspase-8, and pro-caspase-9. Moreover, ZLTE increased the level of cellular reactive oxygen species (ROS) in H1299 cells to lead to apoptosis, which was reversed by N-acetyl-cysteine (NAC). Taken together, our results revealed that ZLTE induced caspase-dependent apoptosis via ROS generation, suggesting that ZLTE is a promising herbal medicine for the treatment of NSCLC.

**[KEY WORDS]** Pileostegia tomentella; Lung cancer; Apoptosis; ROS

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**Introduction**

Lung cancer, the most common cause of cancer-related mortality in humans, was responsible for 1.8 million deaths each year \(^1\). Generally, lung cancer is classified into two categories based on histological type, namely non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) \(^2,\,3\). NSCLC presents approximately 85% of all lung cancer cases \(^4\). Surgery, radiotherapy, and chemotherapy have been widely used to treat lung cancer \(^5,\,6\). However, the decreasing mortality and improving the life quality of lung cancer patients remains a huge therapeutic challenge. Apoptosis, one classical programmed cell death, is also called type I cell death, which plays a pivotal role in keeping homeostasis in various organisms \(^7\). When a cell experiences apoptosis, the morphological change is pretty significant such as membrane blebbing, cell shrinkage, and chromosomal DNA fragmentation \(^7,\,8\). Generally, the protease activity of caspases is essential for the biochemical hallmarks of apoptosis. Caspases-dependent apoptosis can be classified into two pathways the extrinsic pathway and the intrinsic (also called mitochondrial) pathway \(^7\). The extrinsic pathway requires external stimula-
tion via the death receptor to bind with ligand to activate caspases, resulting in cleavage of caspase and cell death. For the intrinsic pathway, mitochondria play an important role in initiating and developing apoptosis in a cell. Mitochondria is always initially activated to release cytochrome c to form an apoptosome to activate caspases 9, 3, and 7, resulting in rapid cell death [7].

When the intrinsic pathway is activated, mitochondria will produce a deluge of reactive oxygen species (ROS) [9]. In addition, the level of ROS is always higher in cancer cells, which facilitates cancer cell growth [10]. However, excessive ROS level would lead to oxidative damage to cancer cells that could experience apoptosis [10]. Cancer cells are damaged by the higher level of ROS induced by compounds. Therefore, the development of novel therapies for the management of lung cancer that increases the ROS level of cancer cells is an alternative strategy.

_Pileostegia tomentella_, a traditional Chinese medicine, is also called Zhongluteng (ZLT) in Yao medicine, which activates blood circulation to dissipate blood stasis [11]. ZLT is always used as an anti-cancer herb in Yao medicine, of which the major constituents mainly included flavonoids, saponins, and coumarins [11]. The previous study indicated that ZLT has an inhibitory effect on H22 tumor and Hela cells [11]. However, quite few studies involved in lung cancer are found. In the present study, using non-small cell lung cancer cells H1299, we investigated the anti-cancer effect and mechanism of ZLT in vitro.

**Materials and Methods**

**Reagents and antibodies**

_N-acetyl-cysteine_ (NAC) was purchased from Sigma Aldrich (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH2-DA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS) was purchased from Life Technologies/Gibco Laboratories (Grand Island, NY, USA). N-benzoyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-vad-fmk) was from Selleckchem (Houston, TX, USA). Antibodies against caspase-3, caspase-7, caspase-8, caspase-9, poly (ADPribose) polymerase (PARP), cleaved-PARP and NADPH oxidase 4 (Nox4) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against caspase-3, caspase-7, caspase-8, caspase-9, poly (ADPribose) polymerase (PARP), cleaved-PARP and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA).

**Preparation of ZLTE**

In this study, ZLT (2 kg) was powered into 100 mesh, which was extracted by 95% ethanol to yield ZLT extraction (200 g). ZLT extraction was extracted by petroleum ether, ethyl acetate, and n-butyl alcohol, respectively to yield petroleum ether extraction (42.5 g), ethyl acetate extraction (ZLTE 22.4 g), and n-butyl alcohol (ZLTN 36.8 g). The quality control of ZLTE was performed on HPLC, which contained oleaonic acid (0.259%) and syringin (2.388%) (Supplementary information)

**Cell lines**

A549 and H1299 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and the cell bank of the Chinese Academy of Sciences (Shanghai, China), respectively. A549 and H1299 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained in humidified air with 5% CO2 at 37 °C.

**Cell viability assay**

Cells (5 × 10^4 cells/well) cultured in 96-well plates were treated with ZLTE (125, 250, 500 μg·mL⁻¹) for 24 h. After adding 100 μL of MTT mixture and culturing for another 4 h, formazan was solubilized with DMSO and the absorbance at 570 nm was detected using a multilabel microplate reader (Vermont, Bio Tek, USA).

**LDH release assay**

Cells (1 × 10^5 cells/well) cultured in 24-well plates were treated with ZLTE (125, 250, 500 μg·mL⁻¹) for 24 h. 100 μL medium was collected and released LDH in the cell culture supernatant was detected using LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer’s protocol.

**Apoptosis assay**

Apoptosis was examined by double staining Annexin V-PE/7AAD kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. 2.0 × 10^4 cells were seeded in a 6-well plate, followed by the treatment of ZLTE with different concentrations. Cells were harvested at the indicated time points followed by incubation with Annexin V-PE/7AAD solution for 15 min at room temperature in the dark [10]. The samples were immediately analyzed by flow cytometry using flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). At least 1 × 10^4 cells were analyzed for each sample.

**Measurement of intracellular ROS**

H1299 cells were seeded in 12-well plates with a density of 1.0 × 10^5 cells per well and cultured overnight. Cells were treated with ZLTE at different concentrations for 24 h. Then the cells were incubated with DCFH-DA (1 μmol·L⁻¹) for 30 min at 37 °C. The level of ROS was determined by a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

**Western blot analysis**

H1299 cells were seeded into a dish and fostered overnight. Cells were treated with ZLTE (125, 250, 500 μg·mL⁻¹) for 24 h. Total proteins were extracted using RIPA (1% PMSF and 1% cocktail) and the protein content was determined with a BCA protein assay kit (Thermo fisher, Waltham, MA, USA). The denatured proteins were then separated by 12% or 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking the PVDF membrane with 5% nonfat milk for 1 h, the PVDF membrane was incubated with primary antibodies (1 : 1000) at 4 °C for more than 12 h. After a wash with TBST and incubation with secondary antibody (1 : 5000) for 2 h at room temperature, the membranes were analyzed with ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).
Analysis of MMP

H1299 cells were seeded in 96-well plates with a density of $3 \times 10^3$ cells/well overnight. Cells were treated with ZLTE (500 μg·mL$^{-1}$) for 4 and 8 h, then labeled with JC-1 (5 μg·mL$^{-1}$) for 30 min. Fluorescence images were captured by fluorescence microscopy (Leica, Wetzlar, Germany).

Statistical Analysis

Data are presented as means ± SD. All experiments were repeated at least three times. Data were normally distributed and analyzed by one-way-ANOVA by GraphPad Prism 8 software (Microsoft, Seattle, WA, USA). A $P$ value < 0.05 was considered to be statistically significant.

Results

The quality control of ZLTE

The HPLC chromatogram of ZLTE was shown in Fig. S1. Quantitative analysis indicated that the contents of oleanolic acid and syringin in ZLTE were 0.259% and 2.388%, respectively.

The cytotoxicity of ZLT extractions on NSCLC cells

To examine the cytotoxicity of ZLTN and ZLTE on NSCLC cells, A549 and H1299 cells were treated with different concentrations of ZLTN and ZLTE for 24 h. The MTT results indicated that ZLTN (Fig. 1A) and ZLTE (Fig. 1B) exerted no effects on A549 cells. In addition, ZLTN exhibited no cytotoxicity in H1299 cells (Fig. 1C). Interestingly, ZLTE significantly showed a detrimental effect on H1299 cells in a concentration-dependent manner (Fig. 1D). Therefore, the subsequent study of ZLTE’s anti-cancer was developed in H1299 cells.

ZLTE induces caspase-dependent apoptosis

To confirm whether apoptosis is responsible for ZLTE-induced cell death, double staining Annexin V-PE/7-AAD was employed in our study. The proportion of apoptosis was negligible for control cells, whereas 24 h of exposure of cells to ZLTE resulted in a dose-dependent increase of early apoptotic cells (Figs. 2A and 2B). Generally, necrotic cell death would increase the release of LDH. However, apoptotic cell death could not increase the release of LDH$^{[13]}$. In our study, we found that ZLTE did not lead to an increase in LDH release in H1299 cells (Fig. 2C). That indicated that ZLTE did not destroy the cell membrane structure, which further provided favorable evidence for ZLTE-induced apoptosis in H1299 cells. In addition, apoptosis inhibitor z-vad-fmk was used to detect which pathway was involved in ZLTE-induced cell death. Our results indicated that z-vad-fmk reversed ZLTE-induced death in H1299 cells (Fig. 2D). Furthermore, ZLTE markedly activated caspase-3, caspase-7, caspase-8, caspase-9, and PARP cleavage (Figs. 2E and 2F), suggesting that caspase plays an important role in ZLTE-induced cell death. Overall, these results corroborated that ZLTE induced the cell death via caspase-dependent apoptosis.

ZLTE caused mitochondrial dysfunction

Mitochondrial dysfunction is a very important signal in...
the intrinsic pathway of apoptosis. JC-1 is an ideal fluorescent probe widely used to detect mitochondrial membrane potential (MMP). The fluorescence of JC-1 stained cells changes shifted from red to green during the process of apoptosis. In our study, after ZLTE treatment, the red fluorescence significantly decreased, whereas green fluorescence significantly increased in H1299 cells (Fig. 3A), suggesting that ZLTE caused MMP loss.

**ZLTE induced caspase-dependent apoptosis through ROS generation**

It has been reported that the increase in ROS production may induce caspase activation, resulting in the induction of apoptosis. Therefore, we investigated whether ZLTE increased ROS levels in H1299 cells. Using DCFH-DA to investigate ROS production, we found that the generation of ROS increased following ZLTE treatment of H1299 cells in a concentration-dependent manner (Figs. 4A and 4B). To investigate whether ROS affected the cell viability, NAC (ROS scavenger) was employed to detect the cell viability of ZLTE-treated H1299 cells. In comparison with the ZLTE-treated group, the NAC plus ZLTE-treated group showed a dramatic recovery in the cell viability (Fig. 4C). In addition, as shown in Figs. 5A and 5B, our results indicated that NAC blocked the ZLTE-induced ROS generation. To further con-

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**Figure A**

![Graph showing apoptosis and cell viability](image)

**Figure B**

![Graph showing double positive cells](image)

**Figure C**

![Graph showing LDH ratio](image)

**Figure D**

![Graph showing cell viability](image)
firm the role of ROS in ZLTE-induced cell death, we used the Annexin V-PE/7AAD assay to detect whether ROS could reverse ZLTE-induced apoptosis in H1299 cells. Our results indicated that NAC significantly reversed ZLTE-induced apoptosis in H1299 cells (Figs. 5C and 5D), suggesting that ZLTE-induced apoptosis via ROS generation.

Discussion

Despite the prognosis of localized lung cancer has markedly improved due to new therapeutic developments, long-term survival has stagnated in the past several decades [18-20]. Neoteric drugs are needed for better improvement of prognosis in lung cancer patients. ZLT has been used as traditional medicine for thousands of years in China, which has the potential to be used for cancer chemotherapy [11, 21, 22]. ZLT is one of the most hopeful medicine as it is reported to have anticancer effects on a variety of tumors [23]. The study of ZLT against human lung cancer, however, has not previously been reported. Therefore, the anti-cancer activity of ZLT in lung cancer and its underlying mechanisms were examined in the present study. We investigated the anti-cancer effects of ZLTN and ZLTE in A549 cells and H1299 cells. Surprisingly, our data indicated that ZLTE has anti-cancer potential in H1299 cells instead of A549 cells (Fig. 1). We have speculated the reason led to the difference between ZLTE’s anti-cancer effects on H1299 and
A549 cells. Although, H1299 and A549 cells are both called non-small lung cancer cells, yet the origin is different. A549 cells are original from the lung organ of a tumor patient, while H1299 cells are transferred to the lung from the lymph node of a tumor patient[24]. In addition, in contrast to A549 cells, H1299 cells are in the absence of p53 protein, leading to H1299 cells more sensitive to drugs[24]. The previous study also indicated that H1299 cells are more sensitive to a drug than A549 cells[25]. Taken together, ZLTE exerted a more significant effect on H1299 than A549 cells, suggesting that ZLTE is worthy to have a deep study.

Tumor growth and inhibition are closely related to apoptosis[26, 27]. To inhibit tumor cells’ proliferation, apoptosis is progressing to happen in tumor cells[28, 29]. Meanwhile, apoptosis is also a major route to eradicate cancers[30, 31]. The present results indicated that the anti-cancer activity of ZLTE

Fig. 3  The ethyl acetate extraction of Zhongliuteng (ZLTE) induced mitochondrial membrane potential (MMP) loss. Cells were treated with ZLTE (500 μg·mL⁻¹) for 4 or 6 h. The probe of JC-1 (5 μg·mL⁻¹) was labeled cells for 20 min (n = 3). Images were captured by fluorescence microscopy

Fig. 4  ROS participated in the ethyl acetate extraction of Zhongliuteng (ZLTE)-induced death in H1299. (A) H1299 cells were treated with ZLTE for 6 h and the ROS levels were measured by a flow cytometer with a fluorescence probe DCFH₂-DA (1 μmol·L⁻¹) (n = 3). (B) The statistical analysis of fluorescence intensity. (C) H1299 cells pretreated with NAC (10 mmol·L⁻¹) for 1 h with were co-cultured with ZLTE for 24 h. The cell viability was detected by MTS assay (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 vs control
against human lung cancer cells through apoptosis induction. The LDH leakage exhibited no change to exclude ZLTE-induced cell death via necrosis. In addition, Annexin V-PE/7AAD double-staining assay revealed that ZLTE induced cell death via apoptosis, which was further confirmed by an apoptosis inhibitor (Fig. 2). In addition, ZLTE increased the cleavage of caspase-3, caspase-7, caspase-8, caspase-9, and PARP (Fig. 2), suggesting that ZLTE induced caspase-dependent apoptosis.

Previous reports showed that mitochondrial dysfunction facilitates excessive production of ROS [32-34], leading to the failure of energy supplementation and cell death [35]. MMP is also an important response to cancer cells [36-38], implying that the reduction of MMP can also lead to ROS generation to kill cancer cells [39]. Our results indicated that ZLTE significantly reduced MMP (Fig. 3). Previous studies demonstrated that ROS plays an important role in regulating cell viability via apoptosis [40-42]. In this study, we observed that ZLTE increased ROS production in H1299 cells in a concentration-dependent manner. Furthermore, the ROS scavenger (NAC) effectively reversed the
ZLTE-induced ROS generation and cell death in H1299 cells, which was further confirmed by the Annexin V-PE/7AAD staining (Figs. 4 and 5). Collectively, we concluded that ZLTE induced caspase-dependent apoptosis via the ROS generation in H1299 (Fig. 6).

In conclusion, we firstly reported that ZLTE exhibited an anti-cancer effect on lung cancer H1299 cells via mediating ROS generation to induce caspase-dependent apoptosis, suggesting that ZLTE could potentially be used as herbal medicine for the treatment of NSCLC.

Fig. 6 The schematic of the signaling pathways of ZLTE-induced apoptosis

References


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