Original Research

# Warangalone Induces Apoptosis in HeLa Cells via Mitochondria-Mediated Endogenous Pathway

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## ABSTRACT

Cervical cancer as one of the major malignant tumors seriously threatens women's health. More than 270,000 women die of cervical cancer each year. Warangalone is an isoflavone compound isolated from *Cudrania tricuspidata* with excellent antitumor activity. In this research, we investigated the molecular mechanism of warangalone-induced apoptosis in HeLa cells. The results show that warangalone can selectively and effectively inhibit HeLa cells proliferation. Warangalone can effectively inhibit the invasion and migration of HeLa cells. Furthermore, warangalone was confirmed to activate p53 and mitogen-activated protein kinase (MAPK) family signaling pathways to cause apoptosis. In this case, the expression of the B-cell lymphoma-2 (Bcl-2) family is regulated, and caspase-3 is eventually cleaved, finally triggering the mitochondrial apoptosis. In conclusion, warangalone can induce HeLa cells apoptosis via a mitochondria-mediated endogenous pathway, which represented the potential therapeutic effect of warangalone on cervical cancer.

## 1. INTRODUCTION

Cervical cancer is one of the most common gynecologic malignancies, ranking first in terms of incidence in developing countries of all diseases, and it is also the second leading cause of cancer death in women aged 20 to 39 years [1–4]. According to the World Health Organization data report, more than 270,000 people die each year from this disease [4]. At present, the traditional therapies for cervical cancer mainly include hysterectomy, radiation therapy, chemotherapy and traditional Chinese medicine treatment. However, traditional hysterectomy does not provide patients with a good prognosis; chemotherapy and radiation therapy will induce severe toxic side effects and resistance problems. Therefore, it is extremely important to find an anti-cervical cancer drug with good curative effect and low toxicity. Plant natural products play a significant role in the development of drugs for the treatment of malignant tumors [5, 6]. According to reports, over 40% of the more than 150 small molecule antitumor drugs developed from 1940 to 2006 were natural plant extracts or their derivatives [7]. In particular, the prevention and treatment of tumors by isoflavones has been widely investigated, and breakthrough in the research progress has been made [8, 9]. The literature indicates that isoflavones have a wide range of biological activities, such as anti-oxidation, anti-inflammatory, antitumor [10–13]. Moreover, due to its 2-phenylchromogen structure and estrogen effect, isoflavones presented great advantages of the prevention and treatment of

breast cancer [14, 15].

At present, the research on isoflavones anti-cervical cancer signaling pathways mainly focuses on the apoptosis signaling pathway, extracellular regulated protein kinases (ERK) / MAPK and PI3K / Akt signaling pathway [16, 17]. The occurrence of apoptosis is mainly regulated by two molecular signaling pathways: death receptor-mediated exogenous pathway and mitochondrial-mediated endogenous pathway [18]. Mitochondrial apoptosis pathway is one of the important pathways of apoptosis. The bcl-2 family of proteins are considered as the essential factor regulating programmed cell death. The bcl-2 family of proteins are divided into proapoptotic proteins (Bax, Bad, Bak, and so on.) and antiapoptotic proteins (Bcl-2, Bcl-XL, and so on). What is remarkable that various apoptosis-stimulating signals cause Bax protein to transfer to the outer membrane of the mitochondria to form membrane channels, and stimulate the mitochondria to release Cytochrome C (Cyt C). Cyt C then forms apoptotic bodies with caspases-9 via Apaf-1 factor, activating the cascade of downstream caspase family proteins. Studies have shown that isoflavones can induce cancer cell apoptosis through the mitochondrial pathway [16, 17, 19, 20]. So, we have paid attention to some excellent flavonoids in *Cudrania tricuspidata*.

*Cudrania tricuspidata* is a plant widely distributed in the Asia-Pacific region. Its roots, stems, leaves and fruits contain a large amount of flavonoids, xanthone, and isoflavones [21]. Antitumor compounds from *Cudrania tricuspidata* are used for the treatment

of different tumors, of which 78 types of flavonoids and xanthones are considered as potential antitumor drugs [22]. Among them, warangalone isolated and purified from the fruits of *Cudrania Tricuspidata* is an isoflavone compound with antitumor biological activities [23]. At the same time, our previous studies have found that warangalone has excellent antioxidant activity and a good inhibitory effect against breast cancer cells MDA-MB-231 and MCF-7. Based on the advantages of warangalone with low toxicity and good antitumor activity, it is interesting to explore the specific mechanism of warangalone against cervical tumors.

In this study, we isolated and purified warangalone from the *Cudrania tricuspidata*, comprehensively investigated the antitumor effects and related molecular mechanism on HeLa cells. This finding will provide the basis for the development of warangalone as a potential antineoplastic drug.

## 2. MATERIALS AND METHODS

### 2.1. Extraction and identification of warangalone

The compounds were extracted by methanol from dried fruits of the *Cudrania tricuspidata*. After centrifugation, the obtained supernatant was extracted with ethyl acetate and concentrated by a rotary evaporator to obtain an ethyl acetate extract. Subsequently, using a petroleum ether/ethyl acetate solvent system (100:3 - 0:100), the extracted ethyl acetate extract was subjected to gradient elution with a macroporous resin, and one portion was collected per 150 mL, and 160 fractions were collected finally.

The collected fractions were compared with a standard control point, and concentrated vanillin was used as a color developing agent, and the thin—layer chromatography analysis was combined to obtain 10 total fractions which may contain warangalone. Then all the 10 fractions were separated and purified by Sephadex LH-20 column chromatography and recrystallization, and the target compound - warangalone (30 mg) was isolated. Eventually, the obtained crystalline compound was identified by nuclear magnetic resonance (NMR), mass spectrometry (MS), X single crystal diffraction.

### 2.2. Cell viability assay, colony formation assay, cell scratch test, and uncoated transwell assay

All cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units / mL penicillin and 50 units / mL streptomycin under 5% $CO_2$, 37ºC incubator conditions. And all cells used in this experiment came from the Medical Experimental Center of Jinan University. SiHa, Caski, HeLa, HepG2, LO2, M231, MCF-7 and Chem-5 were seeded overnight in 96-well plates ($1 \times 10^4$ cells/well). The cells were then treated with indicated concentrations of warangalone. Cell viability was measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). The absorbance of the samples was measured by a Microplate Reader (Infinite F50, TECAN) at 570 nm.

For colony formation, 250 cells well were seeded in 6-well plates. The cells were allowed to form colonies for 10 days. After removing the medium, the cells were fixed for 15 min with 75% ethanol and stained with crystal violet for visualization.

For cell scratch test, cells were firstly seeded in 6-well plates at $4 \times 10^5$ cells/well overnight. A 200 $\mu$L pipette was used to draw across the middle of each well, and the suspended cells were removed by washing three times with cold PBS buffer. Subsequently, the cells were treated with indicated concentrations of warangalone containing 1% FBS. The cells were evaluated under the microscope at each time point.

Cell invasion was assessed by matrigel transwell (Corning Costar, Corning, NY, USA). Cells ($1 \times 10^5$/mL, 200 $\mu$L) were suspended in serum-free medium and seeded in the upper chamber of the transwell unit, whereas the bottom chamber contained 500 $\mu$L medium with 10% FBS. The chamber was fixed with 4% formaldehyde for 10 minutes, then the cells were stained with Giemsa stain, washed 3 times with PBS and wiped the upper chamber with a cotton swab. After naturally dried, it was observed under an inverted microscope. The experiment used 1 $\mu$M cisplatin as a positive control and set the waranglone concentration to 1 $\mu$M, 2 $\mu$M and 4 $\mu$M.

### 2.3. Cell morphology

HeLa cells were cultured to logarithmic phase and seeded in 6-well plates ($1 \times 10^6$ cells/well). After attaching, they were exposed to warangalone at concentration of 0, 10, 20, 30 $\mu$M. The changes in cell morphology at 12 h, 24 h, and 48 h were observed with a microscope and photographed.

### 2.4. Detection nucleus damage by immunofluorescence staining

Hoechst 3342 staining was used to observe the apoptotic morphology of cells. Briefly, HeLa cells were seeded in 6-well plates ($2 \times 10^5$ cells/well). Afterwards, the cells were treated with warangalone at different concentrations (0, 10, 20, and 30 $\mu$M). Then, the cells were stained with Hoechst 3342 (10 $\mu$g/mL) for 15 min. Finally, after washing with PBS, morphological changes were observed under a fluorescence microscope and photographed.

### 2.5. Annexin V-FITC double dyeing method for detection of HeLa cell apoptosis

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in cells using an Annexin V-FITC kit (Beyotime, Shanghai, China). Briefly, after digesting with trypsin, a single cell suspension with a density of $1 \times 10^5$ cells/mL was seeded in a 6-well plate with 2 mL per well. Then, cells were treated with warangalone at doses of 0, 10, 20, and 30 $\mu$M. Afterwards, cells were collected and treated according to the manufacturer's instructions. Finally, the cells were analyzed with FITC/PI staining using a flow cytometer (Beckman Coulter, USA), and WinMDI 2.8 software was used to calculate the percentage of apoptotic cells.

## 2.6. Detection of mitochondrial membrane potential in HeLa cells by JC-1 staining

The logarithmic phase of HeLa cells was digested with trypsin to prepare a single- cell suspension with a density of $1 \times 10^5$ cells/mL, and seeded in a 6-well plate with 2 mL per well. After adherent growth, mediums containing different concentrations of warangalone at 0, 10, 20, 30 $\mu$M were added. Then the medium was washed twice with PBS, then trypsinized, centrifuged at 1500 rpm for 5 min, and washed twice with serum-free DMEM. After washing, the cells were resuspended in serum-free DMEM, and the cell density was adjusted to $1 \times 10^6$ cells/mL. JC-1 stock solution was added to a final solution concentration of 2 $\mu$M. After incubation at 37°C in the dark for 30 min, the cells were detected by flow cytometry.

## 2.7. Determination of intracellular reactive oxygen species (ROS) content

After the cell density of HeLa was $4 \times 10^4$ cells/mL, the cells were adherently grown and the medium were replaced with warangalone medium at the concentrations of 0, 10, 20, and 30 $\mu$M. After treated for 48 h, the medium was replaced by serum-free medium with 10 $\mu$M dilution of DCFH-DA solution and incubated at 37 °C for 30 min. Then the dye was removed, and washed twice with serum-free cell culture medium to remove DCFH-DA remaining on the cell surface. Fluorescence microplate reader was used to detect the fluorescence intensity every 20 minutes. The excitation wavelength was set to 488 nm and the emission wavelength was set to 525 nm.

## 2.8. Fluorescent detection of protein activity

HeLa cells treated with different concentrations of warangalone for 48 h were collected after centrifugation at 12,000 rpm for 5 min and washing twice with PBS. After added RIPA cell lysate, the cells had been put into the -20°C refrigerator overnight. And the protein was fully cleaved and centrifuged at 12,000 rpm for 30 min. Then the supernatant protein was taken and the protein concentration was measured by BCA method. 100 $\mu$g of protein was added to each well in a 96-well plate, and 5 $\mu$L of caspase substrate was added to replenish the total volume to 100 $\mu$L. After incubating at 37 °C for 2 h, the fluorescence intensity was measured by fluorescence microplate reader at excitation wavelength: 380 nm and emission wavelength: 460 nm. Finally, the caspase activity was calculated.

## 2.9. Western blotting

Cells were seeded in culture dishes at a density of $1 \times 10^4$ cells/mL. Afterwards, cells were treated with warangalone at different concentrations (0, 10, 20, and 30 $\mu$M) for 48 h. Then, the isoflavone-containing medium was removed, trypsinized, and the debris was removed by centrifugation at 1500 rpm for 5 minutes. Then the cell lysate was removed by centrifugation at 12,000 rpm for 30 min and the cytosolic fraction was prepared. The protein concentrations of supernatant were determined with the BCA Protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h, incubated with primary antibodies for 2 h at room temperature, washed by TBST for three times and incubated with secondary antibodies for another 1 h. Finally, protein bands were visualized by enhanced chemiluminescence system and analyzed by Image J. Primary antibodies for $\beta$-actin, MAPKs and p53 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for p38 and caspase family were obtained from Abcam (Cambridge, UK). Secondary antibodies were obtained from Proteintech Group (Wuhan, China).

## 2.10. Statistical analysis

All data were repeated 3 times and statistically analyzed using GraphPad Prism 5 software and are shown as mean $\pm$ standard deviation (mean $\pm$ SD), $p < 0.05$ was considered statistically significant.
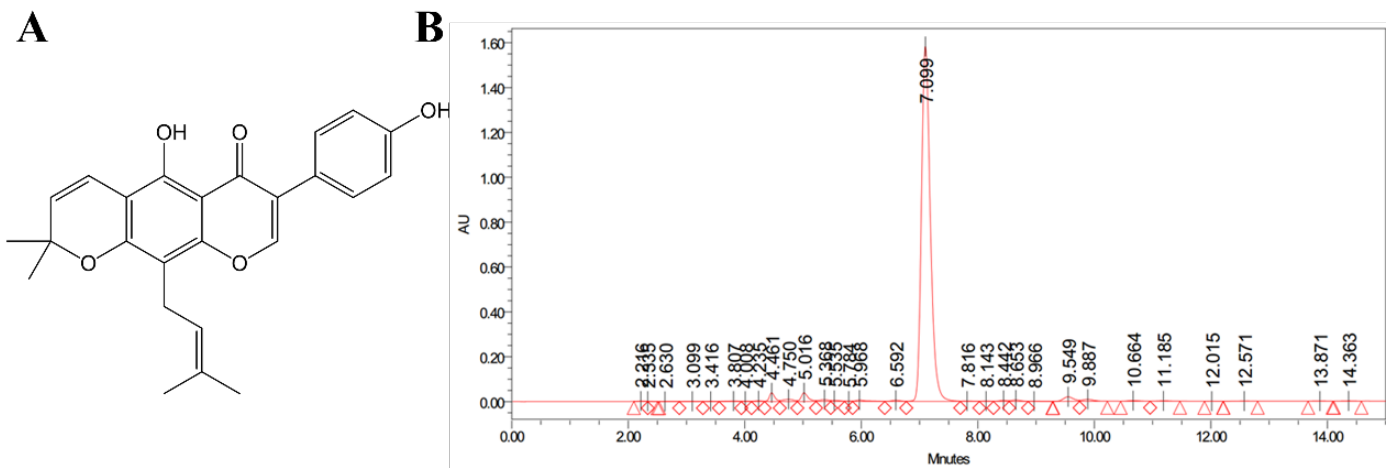
## 3. RESULTS

### 3.1. Identification of warangalone

All data were obtained from Xcalibur diffractometer using Mo K$\alpha$ radiation with a graphite monochromatic ratio as the light source ($\lambda$ = 0.71073 Å). At 293 (2) K, 8082 strong reflection data were collected with a scan of $\omega$ / $2\theta$ and a scan of $\theta_{max}$ = 29.39°, in which there were 4806 independent diffraction points (R$_{int}$ = 0.0158, R$_{sigma}$ = 0.0343). The structure is directly solved by the Sapphire3 program as shown in **Figure 1** and the purity of warangalone is 95.8%. The result shows that warangalone is triclinic crystal, and the molecular formula of it is C$_{25}$H$_{24}$O$_5$. Space group P-1, a= 6.2059(4) Å, b =8.1905(4) Å, c = 21.1807(14) Å, V = 1033.66(11) Å$^3$, Z = 2, d$_x$ = 1.299 g/cm$^3$, F (000) = 428.0, $\mu$ (Cu K$\alpha$) = 0.090 mm$^{-1}$. The main crystal structure data is shown in table 1. The crystal data has been uploaded to the CCDC library numbered 15208.

### 3.2. Warangalone regulates HeLa cells survival and proliferation

The growth of tumor cells treated with warangalone was inhibited to varying degrees. According to the results of treatment for 72 hours as showed in table 2, it can be seen that the IC$_{50}$ range of warangalone for SiHa, Caski, HeLa, HepG2, M321 and MCF-7 is between 13.3-29.6 $\mu$M, which is significantly lower than Normal liver cells LO2 (IC$_{50}$ = 69.5$\pm$5.63 $\mu$M) and glial cells Chem-5 (76.9$\pm$5.64 $\mu$M). Among them, HeLa had the smallest IC$_{50}$ value of 13.3$\pm$1.36 $\mu$M. The results showed that warangalone selectively inhibits tumor cells, and HeLa as one of cervical cancer cell is the most sensitive to warangalone. Therefore, HeLa was selected as the research object to further study.

MTT assay was used to evaluate the cell growth of HeLa cells treated with different concentrations of warangalone (0, 5, 10, 15, 20, 25, 30 $\mu$M) for 12 h, 24 h, 48 h and 72 h. The results are shown in **Figure 2A**. The growth inhibition effect of warangalone on HeLa cells was dose-

**A**

**B**



**Figure 1** | Extraction and identification of warangalone from the fruits of the *Cudrania tricuspidata* . (A) The chemical structure of warangalone. (B) The purity of warangalone was measured by HPLC.

**Table 1** | Crystal data and structure refinement for warangalone.

| Crystal data | Warangalone |
|---|---|
| Molecular formula | $C_{25}H_{24}O_5$ |
| Relative molecular mass | 404.463 |
| Measuring temperature /K | 293(2) |
| Measuring wavelength /$\lambda$ | 0.71073 |
| Crystal system | Triclinic Crystal |
| Space group | P-1 |
| a/Å | 6.2059(4) |
| b/Å | 8.1905(4) |
| c/Å | 21.1807(14) |
| $\alpha$/° | 97.550(5) |
| $\beta$/° | 96.446(5) |
| $\gamma$/° | 101.863(5) |
| Crystal volume /Å$^3$ | 1033.66(11) |
| Z | 2 |
| Density mg/mm$^3$ | 1.299 |
| m/mm$^{&#8209;1}$ | 0.090 |
| F (000) | 428.0 |
| Data collection scan range $2\theta$ | 5.88 to 58.78° |
| Minimum and maximum diffraction index | -8 ≤ h ≤ 7, -10 ≤ k ≤ 8, -28 ≤ l ≤28 |
| Total diffraction number | 8082 |
| Number of independent diffraction spots | 4806[$R_{int}$ = 0.0158, $R_{sigma}$ = 0.0343] |
| R,wR [I>=2$\sigma$ (I)] | $R_1$ = 0.0497, $wR_2$ = 0.1104 |
| R,wR [All data] | $R_1$ = 0.0777, $wR_2$ = 0.1288 |
| Fit /S | S = 1.025 |

**Table 2** | Effects of warangalone on the cell proliferation of human cancer cells.

| Cell line | Classification | IC$_{50}$ ($\mu M$) |
|---|---|---|
| SiHa | Cervical cancer cells | 26.0 + 4.92 |
| Caski | Cervical cancer cells | 24.7 + 2.84 |
| HeLa | Cervical cancer cells | 13.3 + 1.36 |
| HepG2 | Liver cancer cells | 29.6 + 2.84 |
| Chem-5 | brain glima cells | 76.9 + 5.64 |
| M231 | Breast cancer cells | 20.5 + 1.92 |
| MCF-7 | Breast cancer cells | 27.8 + 2.43 |
| LO2 | Normal liver cells | 69.5 + 5.63 |

To investigate the effect of warangalone on the morphology of apoptotic cells, hoechst 3342 staining was used (**Figure 2C**). After treatment with various concentrations (10, 20, and 30 $\mu$M) of warangalone, the crenation, condensation, and fragmentation of chromatin morphology were observed in HeLa cells at concentrations of 20 $\mu$M and 30 $\mu$M.

## 3.3. Warangalone inhibits invasion and migration of HeLa cells
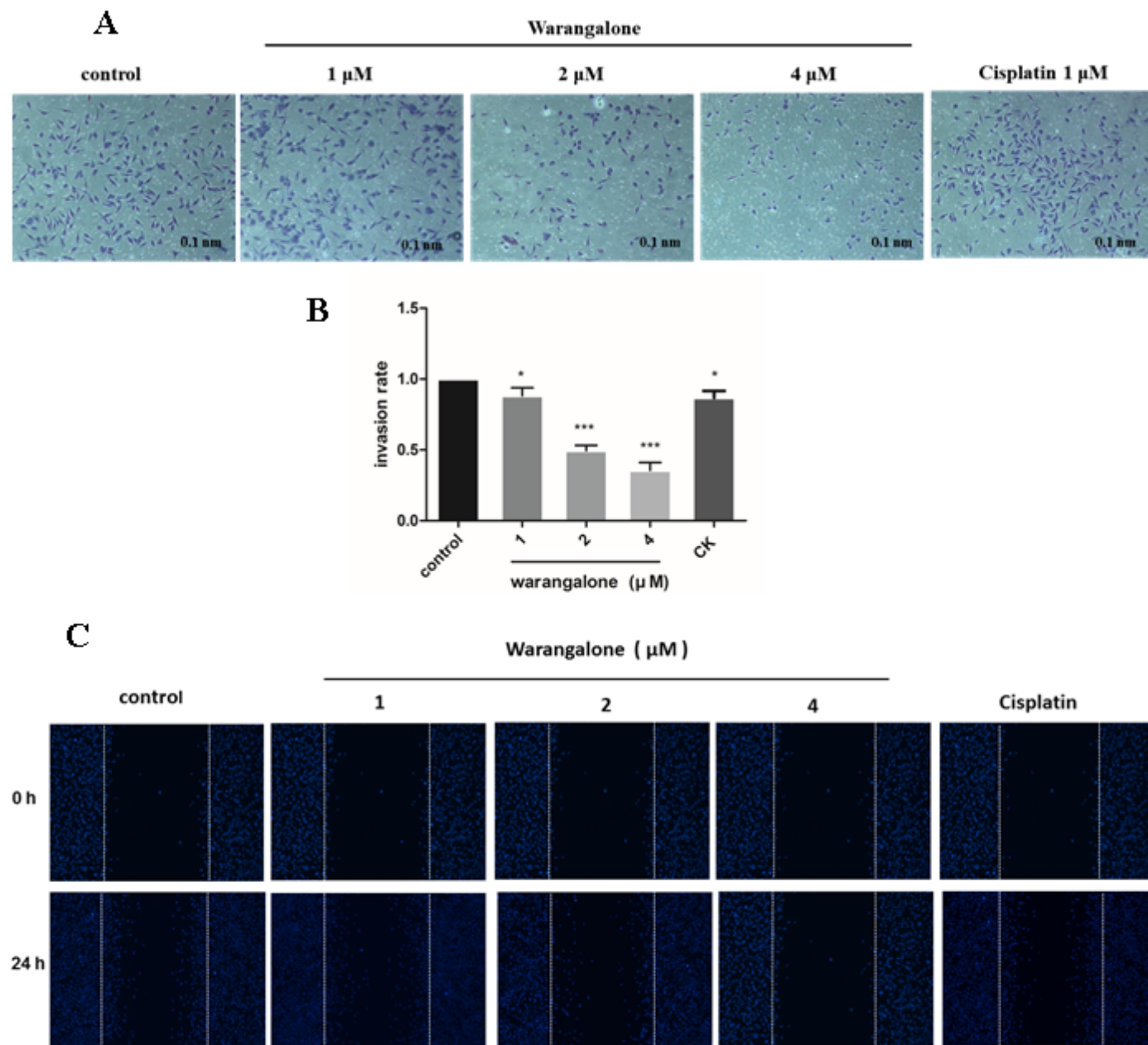
As the concentration of the warangalone increased, the number of cells penetrating from the transwell chamber to the lower chamber gradually decreased in a dose-dependent manner (**Figure 3A** and **3B**). Compared with the positive control group (cisplatin)-86.76%, the inhibition rate of cell invasion was 88.33%, 49.48%, 35.59%, after treatment with 1 $\mu$M, 2 $\mu$M, 4 $\mu$M, respectively.

The scratch test results are shown in **Figure 3C**. Compared with the control group, the migratory ability of cells to the scratched area was gradually weakened as the concentration of the warangalone increased, and the cell migration was inhibited at concentration of 2 $\mu$M and 4 $\mu$M. The results showed that warangalone significantly inhibited the invasion and migration of HeLa cells.

dependent. The IC$_{50}$ values of inhibition of HeLa cell proliferation at 12 h, 24 h, 48 h and 72 h were 37.82 $\pm$ 0.42 $\mu$M, 20.97 $\pm$ 0.55 $\mu$M, 17.38 $\pm$ 0.44 $\mu$M, and 13.31 $\pm$ 0.26 $\mu$M, respectively.

The morphology of HeLa cells was examined by phase contrast microscopy (**Figure 2B**). As the concentration of warangalone increased, the number of HeLa cells decreased, and the shape were also changed significantly. These results indicated that Hela cells proliferation was inhibited by warangalone in a dose-dependent manner.

**Figure 2** | Warangalone regulates HeLa cells survival and proliferation. (A) Cervical cancer HeLa cells were incubated with warangalone for 12–72 h, the cells viability was tested by MTT assay. Each value represents means±SD (n=3). (B) Morphological changes of HeLa cells under different concentrations of warangalone for 24 h and 48 h were shown (200×). (C) Nuclear morphometry of HeLa cells with warangalone by Hoechst 3342 (200×), and arrows indicate abnormally shaped nuclei.

**A**



**B**



**C**



**Figure 3** | Warangalone inhibits invasion and migration of HeLa cells. (A) and (B) Transwell experiment of warangalone on HeLa cells for 24h. The concentration of warangalone was adjusted to 1 $\mu$M, 2 $\mu$M, 4 $\mu$M, and 1 $\mu$M cisplatin was used as a positive control. The drug treatment time was 24 h. *$p<0.05$ and ***$p<0.0001$ compared to the control group. (C) Cell scratch test of HeLa cells treated with warangalone.

## 3.4. Warangalone induces apoptosis in HeLa cells

Flow cytometry was used to detect the effects of different concentrations of warangalone (10 $\mu$M, 20 $\mu$M, 30 $\mu$M) on HeLa cells for 48 h. The results are shown in **Figure 4A**. Compared with the control group, the apoptosis rates of HeLa cells treated with 10 $\mu$M, 20 $\mu$M and 30 $\mu$M warangalone for 48 h were 6.63%, 22.2%, and 31.5%, respectively. As the results shown in **Figure 4B**, warangalone was able to induce apoptosis in HeLa cells and dose-dependent with increasing concentration.

The initiation of cells apoptosis is often accompanied by a decrease in mitochondrial membrane potential (MMP) that can easily trigger the course of an apoptotic cascade [24, 25]. Therefore, the MMP changes of HeLa cells under warangalone treatment for 24 h and 48 h were determined. As shown in **Figure 4C**, the MMP level was dramatically increased responded to a higher concentration after both 24 h and 48 h treatment. Compared with the control group, as the increased concentration of warangalone, the proportion of green fluorescence and the dissipation of mitochondrial membrane were regarded to enhance gradually. Moreover, the dissipation of mitochondrial membrane potential was significantly higher than that of the 24 h at 48 h after warangalone treatment. The results showed that treatment of cells with warangalone destroyed the membrane potential of mitochondria and decreased the mitochondrial membrane potential of HeLa cells in a dose-dependent manner, which indicated that mitochondria were involved in the process of warangalone-induced apoptosis of HeLa cells.

Additionally, it was found that the mitochondrial membrane potential of warangalone treated HeLa cells decreased in a dose-dependent manner. Therefore, it is speculated that warangalone mainly induces HeLa cells apoptosis through the mitochondrial pathway. On the other hand, the Bcl-2 family plays a very important role in regulating the mitochondrial pathway of apoptosis. The expression of Bcl-2 family proteins was effective way to regulate changes of mitochondrial membrane potential. As shown in **Figure 4D** and **4E**, after treatments of warangalone, the expression of pro-apoptotic proteins Bad and Bax was up-regulated, but the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL was down-regulated.

## 3.5. Warangalone induces apoptosis in HeLa cells mainly through mitochondria-mediated endogenous pathways

Cysteine aspartic proteases are a class of proteases found in the cytoplasm. In most cases, the ultimate implementation of cell apoptosis depends on the activation of the caspases cascade. In order to verify whether warangalone activates caspase-3, caspase-8 and caspase-9, we detected its activity by fluorescence. As shown in **Figure 5A**, after different concentrations of warangalone treated, the activities of the cleaved forms of caspase-3, caspase-8 and caspase-9 were dose-dependent with the concentration of warangalone. There was no significant change in caspase-3, caspase-8, caspase-9 activity after treatment with 10 $\mu$M of warangalone.

However, the activity of the cleaved forms of caspase-3 and caspase-9 was significantly improved at 20 $\mu$M and 30 $\mu$M when compared with the control group, while caspase-8 only showed a significant improvement at 30 $\mu$M. It was further shown that warangalone mainly induced apoptosis of HeLa cells through mitochondria-mediated endogenous pathways.

PARP is the most important cleavage substrate for caspase-3 and plays an important role in the process of apoptosis [26]. After 48 h of HeLa cells in warangalone, a significant PARP cleavage band (89 kDa) appeared in the warangalone-treated group, and the degree of PARP cleavage increased with increasing warangalone concentration, as shown in **Figure 5C**. The results indicated that warangalone induced cleavage of intracellular PARP, thereby promoting apoptosis. To further prove that warangalone induces protein apoptosis mainly through the mitochondrial pathway, we examined the expression levels of caspase-3, caspase-8 and caspase-9 as shown in **Figure 5B**. The increase of the concentration of warangalone increased the expression of the cleaved forms of caspase-3 and caspase-9 in a dose-dependent manner, and there was no significant change in the expression of caspase-8 after dosing.
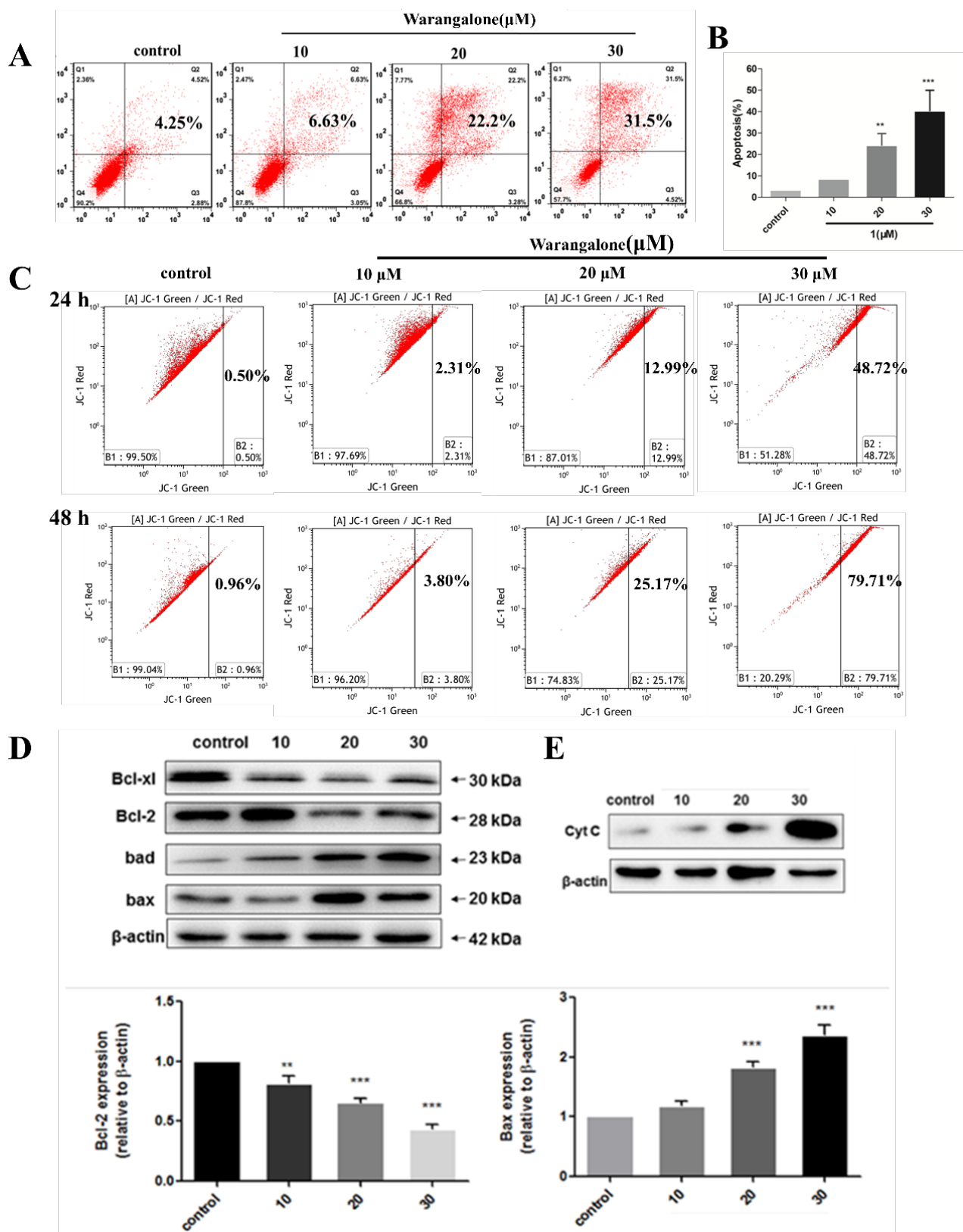
## 3.6. Warangalone causes oxidative damage in HeLa cells and induces apoptosis through the MAPK pathway

According to the results of the previous western blotting experiments (**Figure 4D** and **4E**), it was found that the expressions of Bcl-2 family proteins was changed and regulated by p53.. Akt and ERK have been confirmed to play an important role in cell apoptosis and drug resistance. ERK can inhibit the occurrence of apoptosis by activating anti-apoptotic protein and inhibiting the activation of caspase. Akt promotes apoptosis by inhibiting the expression of pro-apoptotic proteins such as p53 and Bad. MAPK family proteins promote apoptosis by promoting phosphorylation of c-Jun N-terminal kinase (JNK) and p38 under external stimulation. So, we detected p53, p38 and MAPK signaling pathway-related proteins by western blotting as shown in **Figures 6A**-**D**. Warangalone with different concentrations in HeLa cells inhibited the phosphorylation of ERK in a dose-dependent manner, and simultaneously promoted the phosphorylation of p38 and p53. However, it had no significant effect on the expression of Akt and JNK. The results indicated that warangalone may induce HeLa cells apoptosis by activating MAPK signaling pathway.
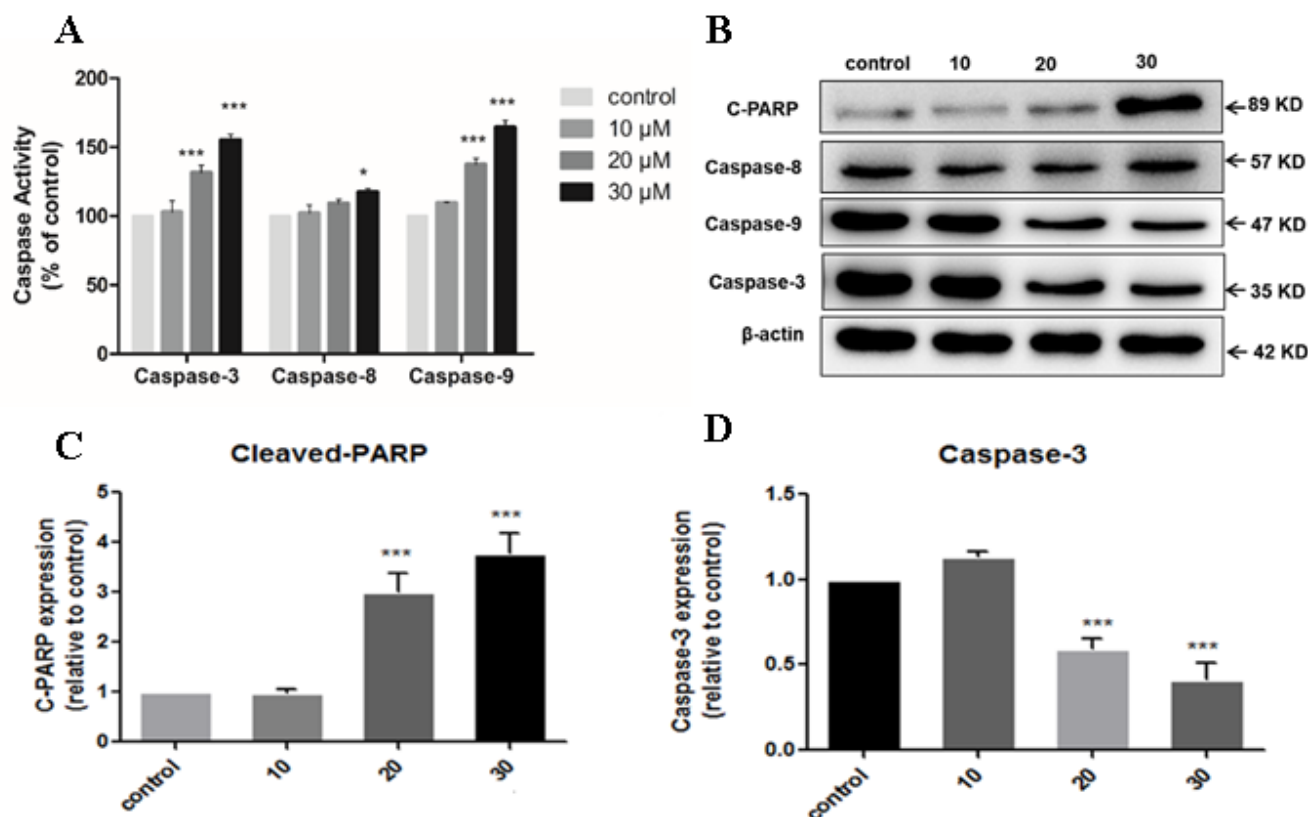
## 4. DISCUSSION

This study identified the antitumor effects of warangalone in human cervical cancer cells *in vitro*, based on an isoflavone extraction of nature plant - *Cudrania tricuspidata*. The specific mechanism of cells apoptosis about Hela cell treated by warangalone was explored by determining proliferation and expression of the apoptosis-related factor.

Isoflavones as an important class of antitumor natural products has received extensive attention [27, 28]. In recent years, it has been found that the flavonoids and isoflavones abundant in *Cudrania tricuspidata* exhibited health-promoting benefits,

**Figure 4** | Warangalone induces apoptosis in HeLa cells. (A) and (B) Annexin V-FITC/PI apoptosis detection on HeLa cells with warangalone (Mean $\pm$ SD, n = 3, **p < 0.01 vs. control, ***p < 0.001 vs. control). (C) Effects of different concentrations of warangalone for 24 h and 48 h on mitochondrial membrane potential in HeLa cells. (D) and (E) Effects of warangalone on Bcl-2 family and cytosolic cytochrome c expressions by western blot analysis. (Mean $\pm$ SD, n = 3. ***p < 0.001 vs. control).
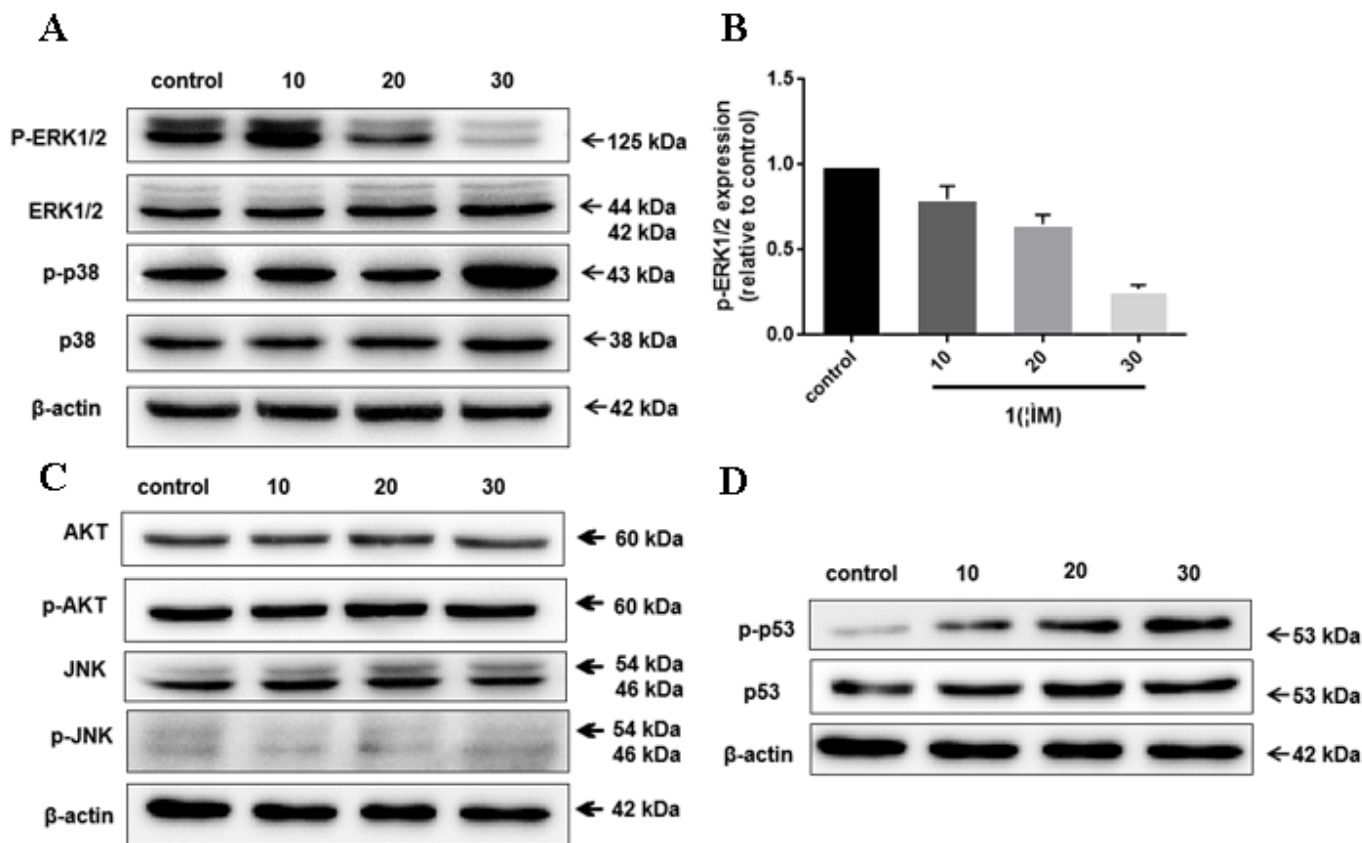
**Figure 5** | Warangalone induces apoptosis in HeLa cells mainly through mitochondria-mediated endogenous pathways. (A) Caspase activity in warangalone-induced apoptosis of HeLa cells (Mean ± SD, n = 3. *p < 0.05 vs. control, ***p < 0.001 vs. control). (B-D) The expression of caspases-3, caspase-8, caspase-9 and PARP were determined by western bolt after the HeLa cells were exposed to warangalone for 48 h. (Mean ± SD, n = 3. ***p < 0.001 vs. control).

including antioxidant, anti-inflammatory, antitumor and neuroprotection [29, 30]. Among the compounds, the crystal structure of warangalone has been identified and uploaded to the CCDC library by our group numbered 15208. Previous studies have found that it has a good anti-oxidation effect and has a certain inhibitory effect on breast cancer cell lines.

One of the important characteristics of malignant tumor cells is their ability to migrate and invade more than normal cells. After tumor cells shed from the primary focus and enter blood vessels, some cells are carried by blood to other organs. These cells grow and multiply where they stay, and eventually form the same tumor as the primary tumor. This is the process of tumor invasion and migration [31, 32]. Therefore, , tumor cell metastasis can be well suppressed by effectively inhibiting tumor cell invasion and migration ability. Studies have shown that the impact of cervical cancer cell invasion and metastasis is one of the key factors for patient efficacy and prognosis [33]. So, finding a method inhibited the invasion and metastasis is of great significance for improving the efficacy and quality of life of patients with cervical cancer [34]. Recently, various Chinese medicines can restrain tumor metastasis by inhibiting tumor cell invasion and migration [35–37]. According to the results of cell scratch test and transwell experiment, warangalone has a significant inhibitory effect on HeLa cell migration and invasion. In comparison to positive control group (1 $\mu$M), the warangalone

(1 $\mu$M) had been shown effectively to inhibit the proliferation, thereby inferring its potential application in inhibiting cervical cancer cells. Due to the less toxic to normal cells, warangalone further demonstrates its potential to develop antitumor drugs. Similarly, its mechanism of antitumor cells is also essential.

Researches have shown that both traditional cytotoxic drugs and targeted drugs are required to exert their antitumor effects by inducing apoptosis of tumor cells [38]. Isoflavone caused apoptosis in cancer cells is possible to produce antitumor effects. Therefore, it is necessary to evaluate whether the warangalone has antitumor effect by inducing tumor cell apoptosis. The apoptosis was observed in HeLa cells treated with warangalone. Apoptosis signaling pathways mainly include death receptor-mediated exogenous apoptotic pathways and mitochondrial-mediated endogenous apoptotic pathways [39, 40]. In the research results of apoptosis, the study of mitochondrial apoptotic pathway remains a hot topic in the field of life [17]. To investigate whether the apoptosis of cervical cancer cells induced by warangalone is related to the mitochondrial pathway, we used JC-1 staining flow cytometry to detect different concentrations of warangalone. According to the experimental results, we suspect that warangalone may induce HeLa cells apoptosis through the mitochondrial pathway.

**Figure 6** | Evaluation of warangalone on the apoptosis in HeLa cells through the p38-introduced MAPK pathway. (A, B) Measurement of the protein expression levels of ERK, p-ERK, p38 and p-p38. (C, D) Measurement of the protein expression levels of p-Akt, Akt, p- JNK, JNK, p53 and p-p53, which were detected and analyzed the density of gray bands by Image J; (Mean ± SD, n = 3. **p < 0.01 vs. control, ***p < 0.001 vs. control).

Caspase-8 and caspase-9 as initiators of apoptosis mediate death receptor apoptosis signaling pathway and mitochondrial apoptosis signaling pathway, respectively. Otherwise, caspase-3 is the ultimate performer of apoptosis in caspase family proteins [41]. The caspase family proteins including caspase-3, -8, -9 play an important role in the process of apoptosis [42]. Warangalone treatment of HeLa activated the activity of caspase-3, -9 in mitochondrial pathway, but had no significant effect on caspase-8, further indicating to the mechanism of apoptosis depended on mitochondrial pathway.

MAPK family proteins play an important role in the process of inducing apoptosis. MAPK regarded as a type of serine / threonine protein kinase exists in cells and involves in many different signaling. MAPK family proteins mainly include extracellular regulated protein kinases (ERK), JNKJ, p38, etc [43]. MAPK family proteins may be stimulated by extracellular signals to activate and eventually induce apoptosis [44, 45]. This study found that warangalone's treatment on HeLa cells led to activate phosphorylation of p53. The activation of p53 further affect the expression of related proteins in the MAPK signaling pathway. MAPK family proteins promote the phosphorylation of p38 and inhibit the phosphorylation of ERK, but has no significant effect on the phosphorylation of JNK and Akt. All in all, warangalone mainly inhibits the proliferation of HeLa cells by inducing apoptosis through mitochondrial-mediated signaling pathways. Warangalone can cause the activation of p53 and MAPK family signaling pathways, leading to cells occurrence of apoptosis.

## 5. CONCLUSION

Warangalone, as an isoflavone, can selectively inhibit the proliferation of cervical cancer cells - HeLa, and has little toxicity on human normal liver cells LO2 and Chem-5. Migration and invasion ability of HeLa cells are effectively inhibited after the treatment of warangalone. Warangalone causes the activation of p53 and MAPK family signaling pathways by promoting the phosphorylation of p38 and inhibiting the phosphorylation of ERK, consequently regulating Bcl-2 family expression and triggering the apoptosis of HeLa cells. Specifically, warangalone down-regulated the expression of Bcl-2, Bcl-XL and up-regulated the expression of Bax, Bad. At the same time, the expression of Bcl-2 family proteins leads to a decrease in the membrane potential of mitochondria, releasing cytochrome C, thereby activating the cascade of the caspase family, including caspase-9, and caspase-3. Warangalone mainly inhibits the proliferation of HeLa cells by inducing apoptosis through the mitochondria-mediated signaling pathway. Taken altogether, the results of this study indicate that warangalone can induce apoptosis in HeLa cells and thus contribute to the treatment of cervical cancer. Further studies should be performed in the future to examine dose-dependent effects in animals.

## CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

## AUTHORS' CONTRIBUTION

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