

RESEARCH COMMUNICATION

Xanthoxyletin, a Coumarin Induces S Phase Arrest and Apoptosis in Human Gastric Adenocarcinoma SGC-7901 Cells

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Abstract

This study was conducted to explore the novel anticancer compounds from Chinese herbs. During the process of screening, to evaluate the potential chemopreventive effect of natural compounds, Xanthoxyletin was isolated from *Erythrina variegata*. It has been reported that Xanthoxyletin possesses antibacterial, fungicidal, and algicidal properties. In this study, we examined the antiproliferative effects of Xanthoxyletin against SGC-7901 cells and its ability to induce apoptosis and cell cycle arrest for the first time. We observed that its inhibitory effects on cells were associated with the DNA damage, apoptosis through mitochondrial dysfunction, and cell cycle arrest at S phase in a dose-dependent manner. Additionally, Xanthoxyletin also increased the production of reactive oxygen species in SGC-7901 cells. These results suggest that Xanthoxyletin may be promising anticancer agent and has worth for further mechanistic and therapeutic studies against gastric cancer.

Keywords: Xanthoxyletin - coumarin - SGC-7901 cells - apoptosis - S phase arrest

Asian Pacific J Cancer Prev, **12**, 1219-1223

Introduction

Stomach cancer is the fourth most frequently diagnosed cancer after lung, breast, and colorectal cancers. It is the second most cause of cancer-related deaths worldwide (738,000 deaths, 9.7%), after lung cancer (Ferlay et al., 2010). According to world estimate cancer survey report 2002, more cases of gastric cancer were found in less developed countries, only in the Eastern Asia 56% new cases were observed. On the whole, 65–70% of new cases and deaths from gastric cancer occur in less-developed countries (Hernandez et al., 2010). In 2005, the incidence rate of gastric cancer (0.3 million deaths and 0.4 million new cases) ranked third among the most common cancers in China (Yang et al., 2005).

Presently, the main therapy of gastric cancer is surgical treatment. The chances to cure with surgical removal are only less than 33% of patients. The 5-years survival rate is only 30–40% with a poorer prognosis for advanced tumors. Thus, there is a massive need for novel and promising agents for the treatment of gastric cancer. In the past several decades numerous dietary and botanical natural compounds have been identified, which are potential chemopreventive anticancer agents (Amin et al., 2009). Natural compounds are one of most important sources of potential anticancer agents (Cragg and Newman, 2005; Gordaliza, 2007).

This study was conducted to explore the novel anticancer compounds from Chinese herbs. For this purpose, we screened the ethanol extract of 300 Chinese herbs against human gastric cancer using SGC-7901 cells.

We found that ethanol extract of several Chinese herbs specifically inhibited the cell growth of SGC-7901 cells. Then, we isolated the target compounds from Chinese herbs and the Xanthoxyletin was one of them which induced the cell growth inhibition of SGC-7901 cells. Previously, the Xanthoxyletin was isolated from different plants, such as *Zanthoxylum americanum* (Ju et al., 2001), stem bark of the plant *Afraegle paniculata* (Tsassi et al., 2010), *Clausena guillauminii* (Rutaceae) (Nakamura et al., 2009), *Clausena excavate* (Sunthitikawinsakul et al., 2003), *Plumbago zeylanica* (Lin et al., 2003), and *Citrus grandis* (Teng et al., 1992). In the present study, Xanthoxyletin was isolated from *Erythrina variegata*.

Previous studies demonstrated that Xanthoxyletin possesses antibacterial, fungicidal, algicidal (Tsassi et al., 2010), and anti-inflammatory properties (Nakamura et al., 2009). During the screening process, to evaluate the potential chemopreventive effect of natural compounds, Xanthoxyletin was isolated from the medicinal plant *Erythrina variegata*. We found that Xanthoxyletin had the activity of inhibiting tumor-cell proliferation, especially in SGC-7901 cells for the first time. Furthermore, we investigated the cell-growth inhibitory effects of Xanthoxyletin and examined its cytotoxic activity against Human gastric adenocarcinoma SGC-7901 cells.

Materials and Methods

Chemicals and reagents

Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Culture

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medium (DMEM), methylthiazolyldiphenyl-tetrazolium (MTT), propidium iodide (PI), and dimethyl sulfoxide (DMSO) were purchased from Sigma. Annexin V-FITC apoptosis detection kit and Reactive oxygen species assay kit were purchased from Beyotime Institute of Biotechnology Jiangsu China.

Extraction, isolation, and identification of Xanthoxyletin

Xanthoxyletin was isolated from Chinese herbal plant *Erythrina variegata* via fractionation of *Erythrina variegata* extract as we previously described (Shawi et al., 2011). Briefly, the herbal plant was crushed and extracted in Soxhlet extractor with alcohol for more than 12 cycles to achieve maximum extraction of its ingredients. The ethanol extract was hemi-dried using rotary evaporator and then dissolved in 80% methanol. After centrifugation at 12000 rpm for 15 minutes, the supernatant was separated and filtered with filter paper. Starting from the first peak to the end of the last peak, the extracted material was divided into 80 fractions on the basis of time (30 seconds per fraction) using HPLC. The fractions were dried and dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mg/mL stock solution. These fractions were subjected to screening for cytotoxicity against human gastric SGC-7901 cells and were analyzed by MTT assay. Then the cells were microscopically examined to detect morphological changes. Materials around the positive fractions were collected and further fractionated by preparative HPLC (Solvent phase: Methanol and water with 0.02% acetic acid). Single positive compound was purified by further fractionation guided by anti-tumor activity. An active single compound with 99.6% purity was characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) to determine its chemical structure.

Cell culture

Human gastric adenocarcinoma SGC-7901 cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5% CO₂ and 95% air. Cells were plated at 10 cm culture dish and allowed to grow to approximately 70% confluence before experimentation.

Cell proliferation assay

The growth inhibitory effects of the Xanthoxyletin on the viability of cells were determined by the MTT assay. Briefly, SGC-7901 cells were plated at a density of 1×10⁴ cells per well in 96-well plates. After 20 h, cells were treated with 100 µl of complete culture medium containing 30, 60, 125, 250, 500, and 1000 µM of Xanthoxyletin with a negative control. Each concentration of Xanthoxyletin was repeated in four wells. After incubation for 48 h, cell viability was determined. We added 10 µl MTT (5 mg/ml) to each well and incubated them for 4 h. After careful removal of the medium, 150 µl dimethyl sulfoxide (DMSO) was added to each well and shaken carefully. The absorbance was recorded by the microplate reader (ELX 800, BIO-TEK Instruments, Inc.) at a wavelength of 570 nm. The effect of Xanthoxyletin on cell growth inhibition was measured and rate of inhibition (I %) was calculated

using the following equation (Yu et al., 2007):

$$I\% = [A_{570}(\text{control}) - A_{570}(\text{treated})] / A_{570}(\text{control}) * 100$$

Cell Cycle Analysis

SGC-7901 cells were seeded in 12-well plates and then treated with 200 and 400 µM concentrations of Xanthoxyletin for 48 h. After treatment, the percentages of cells in different phases of the cell cycle were evaluated by determining the DNA content after propidium iodide staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1500 rpm at 4 °C for 5 min. Pellets were fixed in 70% ice-cold ethanol overnight. After fixation, cells were centrifuged again, washed twice with PBS, and incubated in PBS containing RNase (1 mg/ml) for 10 min at room temperature. Finally, samples were stained with propidium iodide (1 mg/ml) for 30 min. Data acquisition was done by flow cytometry (EPICSXL-MCL, Beckman Coulter, US) using Cell Quest software.

Determination of apoptosis

The apoptotic rate of SGC-7901 cells was measured using flow cytometry with the double staining Annexin V-FITC/PI method (Brumatti et al., 2008). Briefly, SGC-7901 cells were seeded in 6-well plates and allowed to attach overnight. After 20 h, cells were treated with 200 and 400 µM concentrations of Xanthoxyletin for 48 h. Then, cells were harvested, washed with pre-chilled PBS, trypsinized and collected in 10 ml centrifuged tubes. The cell suspension was centrifuged at 1500 rpm for 5 min. Apoptotic cell death was identified by double survival staining with recombinant FITC (fluorescent isothiocyanate)-conjugated Annexin V and PI, using the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed in flow cytometry using Cell Quest software. DNA fragmentation was observed with the propidium iodide (PI) staining by fluorescence microscopy.

Measurement of intracellular ROS generation

Reactive oxygen species' level was measured by DCF-DA detection kit according to the manufacturer's instructions. After treating with 200 and 400 µM of Xanthoxyletin for the indicated time periods, the cells were harvested and the pellets were suspended in 1 mL of PBS and incubated with 10 mM DCF-DA at 37 °C for 15 min. The intracellular ROS mediated the oxidation of DCF-DA to the fluorescent compound 2', 7'-dichlorofluorescein (DCF). Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by flow cytometry.

Determination of mitochondrial transmembrane potential

Rhodamine 123 was used to evaluate perturbations in mitochondrial transmembrane potential in SGC-7901 cells by flow cytometry (Ji et al., 2009). SGC-7901 cells were plated in 6-well plates and cells were treated with 200 and 400 µM of Xanthoxyletin for 48 h. Cells were collected in centrifuge tube and re-suspended in 500 mL

PBS. Then, incubated with the rhodamine 123 ($10 \mu\text{M}$) at 37°C for 20 min. Cells were centrifuged at 1500 rpm for 5 min, the supernatant was removed, pellets were gently rinsed once with PBS, and then re-suspended in 800 mL PBS. After filtration (300 apertures), the suspension was analyzed by flow cytometry.

Statistical analysis of the data

Data were analyzed by statistical method and expressed as mean \pm SD (standard deviation). Statistical comparisons were made by Student's t-test and $p < 0.05$ was considered statistically significant. Three independent experiments were done for confirmation of presented data and results.

Results and Discussion

The present study was initiated by the screening of HPLC fractions of ethanol extract of *Erythrina variegata* to evaluate its inhibitory effect on cell growth of human gastric adenocarcinoma SGC-7901 cells. Then, the target compound was isolated by using HPLC. The analytical HPLC chromatogram of ethanol extract of raw medicine is shown in figure 1A. The single compound, with inhibitory activity on SGC-7901 cells, was isolated via subfractionation by preparative HPLC and the purity of that compound was determined by analytical chromatogram that is shown in the figure 1B. The compound was identified by comparing its physical, spectroscopic (^1H NMR, ^{13}C NMR), and MS data with the data already reported in the literature (Lin et al., 2003; Su et al., 2009). The structure of the compound was determined as Xanthoxyletin as shown in the figure 1C. We initially treated SGC-7901 cells with various doses of Xanthoxyletin for 48 h and morphological changes were observed under the phase-contrast microscopy. Cell proliferation was measured by MTT assay. The results indicated that Xanthoxyletin inhibited cellular proliferation of SGC-7901 cells in a dose-dependent manner (Fig. 1D). The cytotoxic activity of Xanthoxyletin

is similar to that of other compounds of coumarin as reported previously (Chuang et al., 2007). Based on our MTT results, we selected moderate concentrations of 200 and $400 \mu\text{M}$ for mechanistic study of Xanthoxyletin. Accumulated data indicated that cell cycle regulators are frequently deregulated in most of the common malignancies and results in hyperproliferation (Abraham, 2001; Kastan et al., 1995; Molinari, 2000). It has been documented that control of cell cycle progression in cancer cells is a successful strategy to halt tumor growth (Grana and Reddy, 1995; Pavletich, 1999). To elucidate the effects of Xanthoxyletin on the cell cycle, we treated SGC-7901 cells with 200 and $400 \mu\text{M}$ of Xanthoxyletin for 48 h and then subjected for cell cycle analysis as described in materials and methods. The results showed that Xanthoxyletin arrested the cell cycle of SGC-7901 cells at S phase. The percentages of cells were increased from 25.58% (in untreated cells) to 37.52% and 58.61% in cells treated with 200 and $400 \mu\text{M}$ of Xanthoxyletin respectively for 48 h. The ratio of S phase significantly increased in the Xanthoxyletin treated groups as compared to the negative control group (Figure 2). It showed that one of the mechanisms by which Xanthoxyletin inhibited the proliferation of SGC-7901 cells was by arresting cell cycle progression. This study also demonstrated that Xanthoxyletin arrested the cell cycle as a result of the DNA damage and indicated that Xanthoxyletin induced inhibitory cell growth was likely to involve the modulation of cell cycle progression in SGC-7901 cells. Recently, many chemotherapeutic and chemopreventive agents have been shown to impart antiproliferative effects via arresting cell division at certain checkpoints in the cell cycle (Mantena et al., 2006; Srivastava and Gupta, 2006). The control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth (Ortega et al., 2002; Pavletich, 1999). In the present study, it was found that Xanthoxyletin inhibited the cell growth of SGC-7901 cells in a dose-dependent manner.

Apoptosis is fundamental process for maintenance of

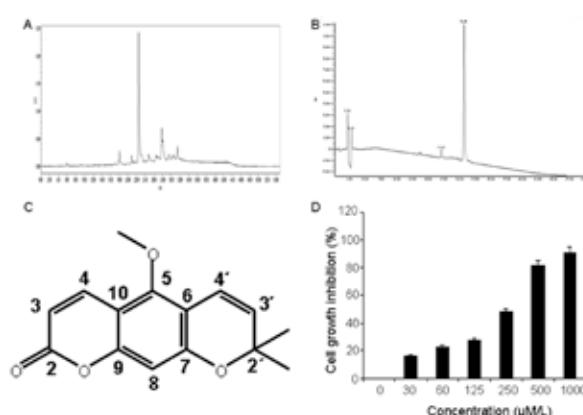


Figure 1. Isolation of Xanthoxyletin and Its Cytotoxic effects: (A) Analytical HPLC chromatogram of raw ethanol extract of *Erythrina variegata*, (B) analytical HPLC chromatogram of Single compound, (C) chemical structure of compound. (D) SGC-7901 cells were treated with various concentrations of Xanthoxyletin for 48 h and cell viability was determined by MTT assay. Data shown are mean \pm SD ($n = 3$).

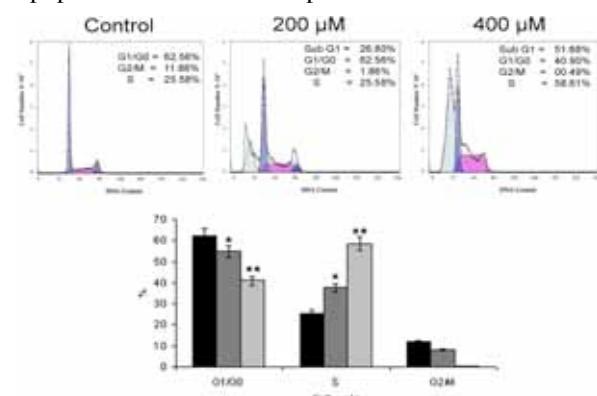


Figure 2. Effect of Xanthoxyletin on the cell cycle distribution: SGC-7901 cells were treated with 0, 200, and $400 \mu\text{M}$ of Xanthoxyletin for 48 h and were stained with PI for flow cytometric analysis. Histograms show number of cells/channel (y-axis) vs. DNA content (x-axis) and the values indicate the percentage of cells in the indicated phases of cell cycle. The data shown are representative of three independent experiments with the similar results. * $p < 0.05$ and ** $p < 0.01$ compared with the control group.

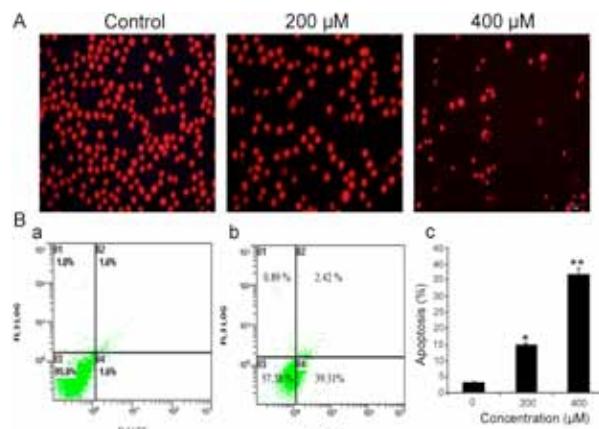


Figure 3. Apoptosis induced by Xanthoxyletin in SGC-7901 cells: (A) SGC-7901 cells stained with PI after treating with 0, 200, and 400 μ M of Xanthoxyletin for 48 h and DNA morphological changes were examined under the fluorescence microscopy. (B) Cells were stained with Annexin V-FITC/PI and were analyzed by flow cytometry. Cell populations shown in the lower right (Annexin V+/PI-) represents apoptotic cells, upper right (Annexin V+/PI+) represents necrotic cells.

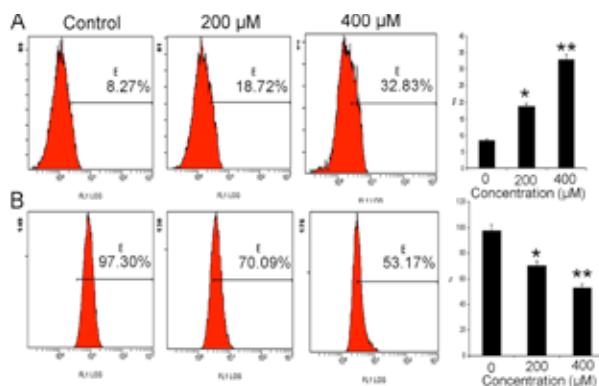


Figure 4. The effects of Xanthoxyletin on ROS level and mitochondrial membrane potential: (A) Effects of Xanthoxyletin on intracellular ROS levels of SGC-7901 cells. (B) Effects of Xanthoxyletin on mitochondrial transmembrane potential of SGC-7901 cells. The values represent the percentages of ROS level and mitochondrial membrane potential in each condition. The data shown are representative of three independent experiments with the similar results. * $p<0.05$ and ** $p<0.01$ compared with the control.

homeostasis and elimination of damaged or unwanted cells. There are many chemopreventive agents, which involved in cancer cell death by induction of apoptosis (Hu and Kavanagh, 2003; Kelloff et al., 2000). Previous studies have shown that coumarins have a potent growth inhibition in many tumor cell lines through induction of apoptosis (Lopez-Gonzalez et al., 2004; Murata et al., 2008). To test whether Xanthoxyletin-induced cell death resulted in the induction of apoptosis, flow cytometric analysis was performed. Incubation of fixed and permeabilized cells with propidium iodide (PI) resulted in the quantitative PI binding with total cellular DNA and the fluorescence intensity of PI-labeled cells was relative to the DNA content. Apoptotic nuclei with hypodiploid DNA content harmonized to sub G1 peak (Fig. 2).

In the next step, we determined whether treatment with Xanthoxyletin resulted in the apoptotic or necrotic cell

death, the marked morphological changes of cells were observed such as nuclear fragmentation, cell shrinkage, condensation of chromatin, and apoptotic bodies in treated cells with Xanthoxyletin for 48 h by using the PI staining under fluorescence microscopy (Fig. 3A). For apoptosis and necrosis confirmation, the apoptosis inducing effect of Xanthoxyletin was evaluated by Annexin V-FITC and PI staining. As shown in Figure 3B, Xanthoxyletin induced apoptosis in SGC-7901 cells and percentage of apoptotic cells was 41.47% after the exposure of 400 μ M of Xanthoxyletin for 48 h as compared to 2.6% in the control group. These results suggested that Xanthoxyletin induced apoptotic cell death in SGC-7901 cells.

Previously it was reported that some of coumarins are involved in the production of reactive oxygen species (Chuang et al., 2007). To examine whether Xanthoxyletin aroused ROS production in SGC-7901 cells, the intracellular ROS level was measured using the ROS-detecting fluorescence dye 2, 7 -dichlorofluorescein diacetate (DCF-DA). After treating the SGC-7901 cells with 200 and 400 μ M of Xanthoxyletin for 48 h, ROS production was detected and the ratios of DCF-positive cells were 18.66% and 32.86% respectively as compared to 8.27% in the control group (Fig. 4A)

Several reports have been documented that the chemotherapeutic agents that cause the enhancement of oxidative stress may particularly be more toxic to the cancer cells than normal cells. They can provoke various biological processes such as cell cycle arrest, DNA repair and apoptosis (Kang et al., 2010; Mounjaroen et al., 2006; Pelicano et al., 2004). This study disclosed that, Xanthoxyletin involved in the stimulation of ROS production, which might arbitrated the apoptosis and cell arrest in SGC-7901 cells.

It has been shown that apoptosis involves a dysfunction of mitochondrial membrane integrity, which leads to apoptotic cell death (Kang et al., 2010; Narvaez and Welsh, 2001). To determine the loss of mitochondrial membrane potential, induced by Xanthoxyletin, Rhodamine 123 was used to quantify the mitochondrial membrane potential. The decrease of Rhodamine 123 fluorescence was directly proportional to the loss of mitochondrial membrane potential. Interestingly, this coumarin induced the loss of the mitochondrial membrane potential. The fluorescence intensity was 97.30%, 70.09%, and 53.17% after treatment with 0, 200, and 400 μ M of Xanthoxyletin respectively for 48 h (Fig. 4B). These findings supported the previously reported studies about coumarin (Chuang et al., 2007; Murata et al., 2008). These results suggested that Xanthoxyletin induced cell growth inhibition and apoptosis in SGC-7901 cells through mitochondrial dysfunction which might be mediated by the production of ROS. The exact mechanism needs further investigation.

Acknowledgements

This work has been supported by Ministry of Education of Pakistan and China Scholarship Council of China. The authors would like to acknowledge Teacher Wang Xiu-Li for technical assistance during flow cytometry experiments and Syed Manzar Abbas Shah for language editing.

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