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Isoorientin from *Gypsophila elegans* induces apoptosis in liver cancer cells *via* mitochondrial-mediated pathway

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ABSTRACT

Ethnopharmacological relevance: *Gypsophila elegans* has been used as a traditional herbal medicine for treating immune disorders and chronic liver diseases in China. The aim of this study is to isolate an active ingredient from this herb and investigate its anti-tumor activity.
Materials and methods: An active ingredient was isolated from the ethanol extract using bioassay-guided screening. And its anti-tumor activity was analyzed by testing the cytotoxicity, lactate dehydrogenase (LDH) release, clonogenecity and migration in HepG2 cells. To investigate its potential mechanism, cell apoptosis, cell cycle arrest, reactive oxygen species (ROS), cytochrome c, mitochondria membrane potential (MMP) and caspase level were determined in liver cancer cell line HepG2.
Results: A flavonoid glycoside, i.e., *G. elegans* isoorientin (GEI), was isolated from this herb and identified as Isoorientin-2″-O-α-L-arabinopyranosyl. Our results showed that GEI significantly inhibited the viability and proliferation of HepG2 cells in a dose- and time-dependent manner, and its cytotoxic effect was also confirmed by the elevated level of LDH. GEI treatment could markedly inhibit the clonogenicity and migration of HepG2 cells. Moreover, GEI induced remarkable apoptotic death of HepG2 cells through cell cycle arrest at G1 phase *via* the regulation of cell cycle-related genes, such as cyclin D, cyclin E and CDK2. Further study showed that GEI treatment significantly elevated ROS formation, followed by attenuation of MMP *via* up-regulation of Bax and down-regulation of Bcl-2, accompanied by cytochrome c release to the cytosol. In addition, GEI treatment resulted in a significant dose-dependent increase in caspase-3 and -9 proteolytic activities.
Conclusion: The present study demonstrates that the ability of GEI to induce apoptosis against HepG2 cells mediated by mitochondrial-mediated pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with very high morbidity and mortality rates, and a poor prognosis. The high incidence of this kind of liver cancer has been caused by factors such as persistent infection with hepatitis B virus and contact with hepatocarcinogens such as nitrosamines, aflatoxins and alcohol (Henry et al., 2002). Despite extensive efforts by many investigators, systemic chemotherapy for HCC has been quite ineffective, as demonstrated by low response rates and no survival benefits (França et al., 2004; Rougier et al., 2007).

Apoptosis, or programmed cell death, is thought to play a key role in the development and regulation of growth of both normal and cancerous cells. The hallmark of cancer cells is the dysregulation of cell proliferation and apoptosis. The tumor growth depends on the cell proliferation rate and apoptosis. Therefore, induction of apoptosis of tumor cells has become a strategy in cancer treatment (Youn et al., 2008; Tan et al., 2009). Generally, apoptosis may occur *via* the mitochondrial (intrinsic) pathway or the death receptor (extrinsic) pathway (Li et al., 1997; Nagata, 1997). In the mitochondrial (intrinsic) pathway, the regulation of apoptosis involves a large set of proteins including Bcl-2, an anti-apoptotic protein (Luna-More et al., 2004), which disrupts the mitochondria membrane potential, resulting in release of apoptogenic factors from the mitochondria to the cytoplasm.

Abbreviations: GEI, *G. elegans* isoorientin; ROS, reactive oxygen species; LDH, lactate dehydrogenase; FBS, fetal bovine serum; DCFH-DA, 2′,7′-dihydrofluorescein-diacetate; MMP, Mitochondria membrane potential; TBS, Tris-buffer saline

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Natural products, a rich source of compounds with enormous structural diversity, have been extensively explored in the field of drug discovery. A large number of different sources of natural compounds present anti-cancer effects both *in vitro* and *in vivo* (Huang et al., 2012a; Lu et al., 2012, 2013). Therefore, it is vital to screen efficient natural compounds for hepatocellular carcinoma treatment. Caryophyllaceae *Gypsophila elegans* has been widely used in traditional Chinese medicine with a record of safety and efficacy in the treatment of immune disorders and liver diseases (Cao, 2007). In our study, a flavonoid glycoside, i.e., *G. elegans* isoorientin (GEI), was isolated from this herb and identified as Isoorientin-2″-O-α-L-arabinopyranosyl. Our results revealed that GEI significantly attenuated liver injury and fibrosis induced by chronic alcohol. The preliminary exploration of the underlying mechanisms indicated its protection against hepatic injury by radical scavenging action, antioxidant activity, as well as its ability to attenuate HSCs activation (Huang et al., 2012b).

To further understand the anti-tumor effects of GEI, the present study was designed to investigate the effects of GEI on proliferation and apoptosis of human hepatocarcinoma cells (HepG2 cell). We found that GEI induces apoptosis in HepG2 cells, and the mitochondrial-mediated pathway may contribute to GEI-induced apoptosis.

2. Materials and methods

2.1. Materials

G. elegans herb was purchased from Nanning Qianjinzi Chinese Pharmaceutical Co. Ltd (Nanning, China). Fetal bovine serum was purchased from Life Sciences (Grand Island, NY). MTT (3-(4, 5-dimethylthiazol-2-yl)−2, 5-diphenyltetrazolium bromide) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, U.S.A). The antibodies including cytochrome c, Bcl-2, Bax and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences Pharmigen (San Diego, CA).

2.2. Preparation of GEI

GEI was isolated from *G. elegans* according to our previous study (Huang et al., 2012b) with some modification. In the present study, the result indicated that the yield of GEI was higher than that of our previous study. Briefly, the dried powder of *G. elegans* herb (10 kg) was extracted with 80 L 70% ethanol under reflux for 2 h two times. The extract was negative-pressure filtered, and the filtrate was evaporated to a fluid extract by removing the ethanol under reduced pressure in a rotary evaporator at 60 °C. The crude extract was successively extracted with ethyl acetate and water-saturated n-butanol, and the later extract was then subjected to chromatography on a silicagel column (200–300 mesh, Yantai, PR China; Ø10 cm × 300 cm) eluting with a gradient mixture of $CHCl_3$ and MeOH (0–100% MeOH, 1500 mL each fraction). The CH_3Cl_3–MeOH (35: 65) fraction yielded a yellow powder after concentration, which was purified by Sephadex LH-20 and preparative HPLC to produce compound (27.82 g). Its structure was elucidated on the basis of physicochemical properties and spectral data: ESI-MS(m/z): 604 $[M+Na]^+$; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.32, 7.29, 6.88, 6.49, 6.40, 4.98, 4.37; ^{13}C NMR(125 MHz, CD_3OD) δ: 165.9, 104.7, 185.0, 159.8, 110.3, 166.1, 95.9, 163.7, 105.9, 123.0, 114.8, 148.0, 152.1, 117.9, 121.2, 73.9, 82.2, 74.8, 73.1, 83.1, 63.9, 110.2, 71.9, 81.1, 68.3, 66.8. These results indicated that the compound is isoorientin-2″- O-α-L- arabinopyranosyl, i.e., *G. elegans* isoorientin (GEI), with a chemical formula of $C_{26}O_{15}H_{28}$ and a molecular weight of 580.24. Its chemical structure is shown in Fig. 1.

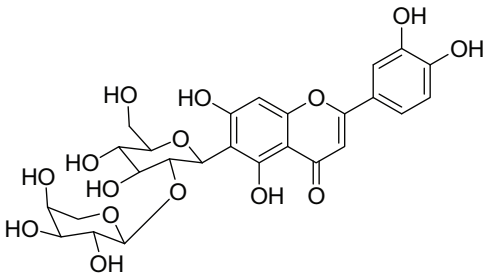

Fig. 1. The chemical structure of GEI.

2.3. Cell line and cytotoxicity assay

Human liver carcinoma cell line (HepG2 cells) was obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China) and maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in a humidified incubator with 5% CO_2 and 95% oxygen at 37 °C.

Cytotoxicity was measured by MTT method. Briefly, exponentially growing cells were seeded in 96-well plates and cultured until 50% confluence. Then, various concentrations of GEI were added and the final concentration in each well was 5, 10, 20, 40, 80 and 160 μmol/L, respectively. Each treatment was tested in tetrad wells and the untreated control group was administered culture medium containing no drug. All of the above plates were placed in a CO_2 humidified-atmosphere incubator at 37 °C for 24, 48 and 72 h. At the end of exposure, 20 μL MTT (5 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 h. Then, all culture medium supernatant was removed from the wells and replaced with 200 μL DMSO. The absorbance of each well was measured by standard enzyme-linked immunosorbant assay at 570 nm. The cell viability was calculated based on the following formula: cell viability (%)=average A570 nm of treated group/average A570 nm of untreated control group × 100% (Xie et al., 2012).

2.4. Lactate dehydrogenase (LDH) release assay

LDH released into the culture supernatants was measured using Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific™, Pittsburgh, PA, USA) following the manufacturer's instructions. In brief, cells were seeded in 96-well plates and then treated with GEI at different concentrations (5, 10 or 20 μmol/L) for 48 h. The supernatant was transferred into 96-well plate to assess the LDH activity. Triton X-100 (2%) served as a positive control was used to completely lyse the cells and release the maximum LDH. Next, the LDH reaction solution (100 μL) was added to the cells for 30 min The optical density of the color generated was determined at a wave length of 490 nm using a Microplate Reader (Tecan, Männedorf, Switzerland). The results were expressed as percentage of Triton X-100-induced LDH release.

2.5. Clonogenicity assay

The clonogenicity test was performed according to the previous study (Franken et al., 2006). HepG2 cells were seeded at a density of 1×10^6 cells in 6-well plates. After 24 h of incubation, the cells were treated with 5, 10 or 20 μmol/L of GEI for 144 h. The cells were fixed and stained with 0.1% crystal violet to visualize colonies.