Inhibition of human liver cancer cell growth by evodiamine involves apoptosis and deactivation of PI3K/AKT pathway

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Abstract
Evodiamine is an active alkaloid member found in Traditional Chinese Herb (TCH) Evodia rutaecarpa. It has been reported to exhibit remarkable biological and medicinal activities including anticancer and anti-inflammatory. This study was designed to investigate the anticancer effects of evodiamine against human liver cancer and evaluate its effects on cell migration, cell invasion, cellular apoptosis and PI3K/AKT pathway. The results showed that evodiamine exhibits potent antiproliferative effects against two human liver cancer cell lines (HepG2 and PLHC-1) with an IC₅₀ of 20 µM. Nonetheless, the cytotoxic effects of evodiamine were comparatively low against the normal cells as evident from the IC₅₀ of 100 µM. The growth inhibitory effects of evodiamine were found to be due to the induction of apoptosis as revealed by the DAPI, AO/EB and annexin V/PI staining assays. The induction of apoptosis was also associated with upregulation of Bax and downregulation of Bcl-2 expression in a concentration dependent manner. The wound healing and transwell assay revealed that evodiamine caused a significant decline in the migration and invasion of the HepG2 and PLHC-1 cells. Investigation of the effects of evodiamine on the PI3K/AKT signalling revealed that evodiamine inhibited the phosphorylation of PI3K and AKT proteins. Taken together, the results showed that evodiamine inhibits the growth of human liver cancer via induction of apoptosis and deactivation of PI3K/AKT pathway. The results point towards the therapeutic potential of evodiamine in the treatment of liver cancer.

Keywords: Alkaloids, Liver cancer, Evodiamine, Apoptosis, PI3K/AKT pathway

Introduction
Traditional Chinese herbs (TCH) are considered as promising and novel sources of chemotherapy adjuvants and antitumor remedies. Additionally, TCH assists in improving the efficiency of chemotherapy and eliminating/lowering its hazardous side-effects. Over the years, researchers have extensively undertaken clinical and experimental investigations to augment the effectiveness of chemotherapy. Interestingly, several bioactive molecules have been extracted from TCH which exhibit potent antitumor activity in epidemiological as well as experimental models. *Evodia rutaecarpa* is a popular TCH, locally known as “Wu-Chu-Yu”. It has been an important constituent of Traditional Chinese Medicine from a long period of time prescribed for treatments of several ailments including postpartum hemorrhage, headache and gastrointestinal disorders [2, 3]. *Evodia rutaecarpa* is rich source of alkaloid which is believed to be responsible for its bioactivities [3]. The total content of evodiamine in *Evodia* species ranges from 0.072 to 2.52% [4]. Alkaloids have been reported to exhibit a spectrum of biological activities including antiroliferative and antitumor activities against an array of human cancer cell lines [5].

Evodiamine is one of the key alkaloids isolated from *Evodia rutaecarpa*, which has been shown to exhibit remarkable bioactivities [6]. Evodiamine has been reported to show uterotonic, thermoregulatory, vasodilatory, anti-obesity, anti-inflammatory,
antinociceptive, catecholamine secretion and testosterone secretion effects [7, 8]. Studies have also reported cytotoxic effects of evodiamine against colon, prostate and liver cancer [9]. The antitumor activity of evodiamine have been attributed to its potential of proliferation inhibition, apoptosis initiation, invasion inhibition and metastasis suppression against several human cancer cells including lung cancer, colon cancer, cervical cancer, melanoma, leukemic T-lymphocyte, prostate cancer and breast cancer cells [10].

Liver cancer is one of the dangerous and prevalent types of primary liver malignancies across the globe. Currently, it is ranked as second among high mortality cancers and with each passing year there is an alarming increase in liver cancer incidences across the globe [11]. The key treatment options for liver cancer include surgical resection, organ transplant and radio-frequency ablation. The only proven potential anti-liver cancer agent for chemotherapy is sorafenib, which amplifies the patient's survival [12]. However, high cost and low availability of these treatments generate an emergency for novel and efficient agents that can assist us with better outcome against liver cancer.

A previous study has reported the anticancer effects of evodiamine against human hepatocellular carcinoma cells [13]. However, the anticancer effects of evodiamine against human liver cancer cells via modulation of PI3K/AKT pathway and its effects on liver cancer cell migration and invasion have not been studied. Against this backdrop, the present study was designed to investigate the anticancer effects of evodiamine against human liver cancer cells (PLHC-1 and HepG2) and to evaluate its effects on PI3K/AKT signalling, cell migration and invasion.

Materials and methods

Cell culture, chemicals and cultural conditions

Human normal liver THLE-2 and liver cancer HepG2 and PLHC-1 cells were collected from the Cell Bank of Type Culture Collection of Chinese Academy of Science, Shanghai, China. All three cell lines were seeded in RPMI 1640 cultural medium containing 10% of fetal bovine serum (GIBCO BRL) and potential antibiotics streptomycin (100 μg/ml) and penicillin (100 U/ml). Seeding of the cell lines was performed in an incubator under an atmosphere of 5% CO₂, 95% air and 37°C of temperature. Equivalent amounts phosphate buffered saline (PBS) was used as vehicle control. All the chemicals and reagents involved in this study were bought from Sigma-Aldrich including evodiamine (>98% purity).

The viability assay

The effects of evodiamine on cell viability of liver cancer HepG2 and PLHC-1, and normal liver THLE-2 cells were determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, 1.4 × 10⁵ cells/well of each cell line was cultured separately overnight using 96-well plates. Following overnight incubation, each well plate was supplied with different doses of evodiamine (0 to 640 μM) for a time interval of 24 h. After drug treatment, all cell lines were stained with 50 μl of MTT solution of concentration 5 mg/ml, and resultant formazan crystals were dissolved using DMSO (dimethyl sulfoxide). Finally, microplate spectrophotometer (BioTek Instruments, Inc., Winooski, United States) at 570 nm was used to record absorbance for optical density calculations.

Apoptotic assay

The apoptosis was detected by 4’,6-diamidino-2-phenylindole (DAPI), acridine orange/ethidium bromide (AO/EB) and annexin V/PI staining assays. The cancerous HepG2 and PLHC-1 cells were harvested at exponential phase of growth followed by loading onto 24-well plate. After the incubation period, HepG2 and PLHC-1 cells were subjected to evodiamine treatment at different doses (0 to 640 μM) for 48 h. In case of DAPI staining assay, cells were harvested, washed with PBS and fixed at room temperature with 80% ethanol for 30 min. Thereafter, fixative was discarded and cells were rehashed with PBS thrice prior to 50 min of incubation in dark at 25°C with 1 μg/ml of DAPI solution. In case of AO/EB staining assay, cells were deprived of fixation and were loaded with 100 μl of freshly prepared AO/EB solution (100 μg/ml). Both DAPI and AO/EB stained HepG2 and PLHC-1 cells were immediately loaded onto Nikon fluorescence microscope (Nikon Inc., Japan) for apoptosis measurements. Annexin V/PI staining assay was used to determine the percentage of the apoptotic liver cancer cells as described previously [14].

Transwell assay

Cell invasion assay was executed using transwell chambers coated with Matrigel (BD Biosciences) bearing membranes of 8 μm of pore size (Corning Co., NY, United States). Fresh cell culture of HepG2 and PLHC-1 cells were placed onto upper transwell chambers maintain serum free cultural medium with a concentration of 4.2 × 10⁴ cells. These cells were supplemented with different evodiamine doses (0 to 640 μM). Lower transwell were only filled with RPMI medium (600 μl) and FBS (20%). Afterwards, transwell chambers were placed under incubation for 24 h followed by removal of non-invasive
cells by scrubbing. Invaded cells were fixed with paraformaldehyde fixative and then stained with crystal violet (10%). Finally, invaded cells were numbered under a microscope (Olympus, Japan).

**Wound healing assay**

HepG2 and PLHC-1 cells were seeded in fresh culture media till 85% confluence. Thereafter, a plastic scraper was used to create a wound followed by PBS washing. The cultural medium was completely removed and replaced with a fresh one to maintain different concentrations of evodiamine (0 to 640 μM). Then cells were incubated for 48 h at 37 °C followed by two times of washing using PBS. Finally, the wound was investigated under a light microscope (Nikon, Tokyo, Japan).

**Western blotting**

The effects of evodiamine on the apoptosis and PI3K/AKT pathway allied proteins were examined by western blotting. After treatment of HepG2 and PLHC-1 cells with variant evodiamine doses (0 to 640 μM), cells were subjected for lysing using RIPA buffer (Beyotime, Beijing, China). Bicinchonic Acid assay was performed to monitor the protein content among each lysate. About 35 μg of proteins was subjected to separation via SDS-PAGE followed by electrophoretic transferal to PVDF membranes (Millipore Corp, Atlanta, GA, United States). These membranes were blocked using non-fat dry milk (5%) as blocking agent for 1 h at room temperature. Afterwards, membranes were treated overnight at 4 °C with indicated primary antibodies. Followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. In the end, the protein signals were recorded using ECL (enhanced chemiluminescence) reagent (Pierce, Rockford, United States).

**Statistical analysis**

The data from independent triplicate experiments were indicated as mean ± SD. For statistical comparison, student’s t-test was used for each assay. A probability value of P < 0.05 was taken as significant.

**Results**

Evodiamine exerted antiproliferative effects on HepG2 and PLHC-1 cells

The antiproliferative effects of evodiamine (Fig. 1a) were evaluated by using MTT assay. The results showed that evodiamine caused a significant (P < 0.05) decrease in the viability of the human HepG2 and PLHC-1 liver cancer cells (Fig. 1b and IC). The antiproliferative effects of evodiamine on the two liver cancer cell lines and with exceptionally low toxicity against the normal liver cell lines. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05)

![Fig. 1](https://example.com/fig1.png)
effects of evodiamine against the liver cancers cells exhibited a dose dependent pattern. The IC\textsubscript{50} of evodiamine against the human liver cancer cells (HepG2 and PLHC-1) was found to be 20 μM. Nonetheless, the antiproliferative effects of evodiamine were found to be less profound against the normal THLE-2 normal liver cells as evident from the IC\textsubscript{50} of 100 μM (Fig. 1d). Taken together, these results indicate selective anticancer effects of evodiamine against the liver cancer cells.

**Evodiamine induced apoptotic cell death in HepG2 and PLHC-1 cells**

Several assays were used to determine if evodiamine exerts antiproliferative effects in liver cancer cells via induction of apoptosis. The results of the DAPI staining revealed that evodiamine caused alterations in the nuclear morphology of the HepG2 and PLHC-1 cells such as nuclear condensation and fragmentation suggestive of apoptosis (Fig. 2). AO/EB staining assay results revealed that evodiamine treatment increased the number of early and late stage apoptotic cells along with necrotic cells (Fig. 3). Further, annexin V/PI showed that apoptotic cell percentage increased from 0.92% in control group to 35.99% in evodiamine treated PLHC-1 cells and from 2.46% to 30.4% against HepG2 cells (Fig. 4a). Evodiamine induced apoptotic cell death in both HepG2 and PLHC-1 cells were further supported by western blotting data. The results of western blotting showed considerable increase in Bax protein levels and downregulation of Bcl-2 levels (Fig. 4b).

**Evodiamine retarded migration and invasion of HepG2 and PLHC-1 cells**

To analyze the effects of evodiamine on migration and invasion of HepG2 and PLHC-1 cells, wound healing and transwell chambers assays were performed. Herein, it was observed that evodiamine inhibited the migration of the HepG2 and PLHC-1 cell lines. The wound width of treated groups showed decreased considerably compared to that of control group (Fig. 5). In case of PLHC-1 cells, the invasion percentage of the HepG2 and PLHC-1 cells was significantly (P<0.05) decreased upon evodiamine treatment and exhibited in a dose dependent pattern (Fig. 6).

**Evodiamine blocked PI3K/AKT pathway in HepG2 and PLHC-1 cells**

Effects on PI3K/AKT pathway by evodiamine were studied by using western blotting assay. The results indicated that the expression levels of p-PI3K, p-AKT and PI3K decreased and those of AKT remained almost constant (Fig. 7).

**Discussion**

Natural products provide a vast variety of chemical entities many of which prove to novel drugs and lead molecules [15]. Since time immemorial, natural products have served humanity in one or the other way to improve life as well as health issues. The exploration of plant-based drugs has led to the development of several antitumor drugs from alkaloids [16]. Alkaloids are mostly plant secondary metabolites and predominantly found in certain species of blooming plants [5]. **Evodia rutaecarpa**

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*Fig. 2* Evodiamine induced morphological changes in nuclei of liver cancer cells. DAPI staining was used to study nuclear morphology of the HepG2 and PLHC-1 cells after treatment with indicated doses of evodiamine. The experiments were performed in triplicate.
is rich in alkaloid content with evodiamine alkaloid as a key constituent responsible for its high medicinal value [3]. Although, evodiamine has been reported to induce apoptosis in human hepatocellular carcinoma cells [13], the anticancer effects of evodiamine on liver cancer cells via deactivation of PI3K/AKT signalling cascade has not been studied. Additionally, the effect of evodiamine on the migration and invasion of liver cancer cells is still unclear. The results of the present study evodiamine induced dose-dependent toxicity against liver cancer HepG2 and PLHC-1 cells with an IC_{50} of 20 µM. Nonetheless, the cytotoxicity of evodiamine against the normal liver cells were significantly lower than the normal cells. There results indicate selective anticancer effects of evodiamine and are consistent with previous studies wherein evodiamine has been reported of exert anticancer effects against lung cancer, colon cancer, cervical cancer and melanoma cells [17, 18]. Previous studies have shown that evodiamine exerts anticancer effects by inducing apoptotic cell death in a number of human cancer cell lines such as murine L929 fibroblastoma, breast NCI/ADR-RES cells, prostate LNCaP, DU145 and PC-3 cancer cells, leukemic U937 cells and A375-S2 melanoma cells via alterations in balance of proapoptotic Bax and antiapoptotic Bcl-2 protein levels [19]. Consistently, in the present study, it was found that evodiamine induced apoptosis in the HepG2 and PLHC-1 liver cancer cells via induction of apoptosis [13]. Western blotting indicated that evodiamine downregulated Bcl-2 and upregulated the Bax protein levels in both HepG2 and PLHC-1 cells in a concentration-dependent manner. Next the effects of evodiamine were also investigated on the migration and invasion of the liver cancer cells. It was found that evodiamine inhibited the liver cancer cell migration and invasion. These results suggest that evodiamine may prove to be beneficial for the treatment of metastatic cancers. Additionally, these results are in agreement with a previous study wherein evodiamine has been found to suppress the migration and invasion of the liver cancer cells [20]. The PI3K/AKT pathways has been shown to be aberrantly activated in several cancer types and is responsible for the growth, development, and tumorigenesis of different human cancers [21]. As such it is an important drug target for the management of human cancer including liver cancer. Interestingly, in the present study it was found that evodiamine could deactivate the PI3K/AKT pathway in liver cancer cells indicative of its potential as lead molecule for the treatment of liver cancer.

**Conclusion**

The results of the present investigation revealed potent anticancer activity of evodiamine alkaloid against human liver cancer cells. Evodiamine induced anticancer effects via stimulation of apoptosis and blocking of PI3K/AKT signalling pathway. Evodiamine also suppressed the migration and invasion of liver cancer cells. Taken together, evodiamine may prove a lead
Fig. 4  Induction of apoptosis in liver cancer cells by evodiamine and quantification of apoptotic cell percentage of evodiamine treated HepG2 and PLHC-1 cells by Annexin V/PI staining assay. Results showed that apoptotic cell percentage of the liver cancer cells increased remarkably with increasing evodiamine doses. 

b Western blotting assay showing increase in Bax and decrease in Bcl-2 expression in HepG2 and PLHC-1 cells. The experiments were performed in triplicate.

Fig. 5  Evodiamine inhibits the migration of the liver cancer cells. Wound healing assay was executed to monitor HepG2 and PLHC-1 cell migration ability after evodiamine treatment. Results showed that migratory capability of HepG2 and PLHC-1 cells was arrested by test drug. The experiments were performed in triplicate.
molecule in the development of liver cancer chemotherapy. However, further in vivo research endeavors are recommended.

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Authors' contributions
JJ and JZ designed the study. JJ, KK, and YL carried out bulk of the experiments and collect the data. JJ and JZ performed the statistical analysis. JZ supervised the work and drafted the manuscript. All authors read and approved the final manuscript.

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Fig. 6 Evodiamine inhibits the invasion of the liver cancer cells. Transwell invasion assay was executed to monitor HepG2 and PLHC-1 cells invasion ability following evodiamine treatment. Results showed that invasiveness of HepG2 and PLHC-1 cells was suppressed upon evodiamine treatment. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

Fig. 7 Evodiamine deactivates the PI3K/AKT signaling pathway. Western blotting assay was performed to determine the expressions of PI3K/AKT pathway linked proteins. Results indicated the expression of p-PI3K, p-AKT and p3K decreased whereas the expression of AKT remained intact. The experiments were performed in triplicate.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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