

Original Research Article

Gentiopictin exerts anticancer effect on human colon cancer cells via caspase-dependent apoptosis, cell cycle arrest, and inhibition of cell migration and invasion

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Abstract

Purpose: To investigate the anticancer effect of gentiopictin on human colon cancer (HT-295) cells, and its effects on caspase-mediated cellular apoptosis, cell cycle, cell migration and cell invasion.

Methods: MTT assay and clonogenic assay were used to study the effect of gentiopictin on cell viability and cancer colony formation, respectively, while the apoptotic effects of gentiopictin were determined using fluorescence microscopy and Western blotting. The effect of gentiopictin on cell cycle was evaluated by flow cytometry, while Transwell assay was used to study its effects on cell migration and invasion.

Results: Gentiopictin exerted potent and dose-dependent suppression of cell proliferation and colony formation, and produced pro-apoptotic effects on HT-295 colon cancer cells. Treatment of HT-295 cells with gentiopictin resulted in up-regulated expressions of caspase-3, caspase-8, caspase-9 and Bax, while Bcl-2 expression was downregulated. Moreover, gentiopictin dose-dependently induced cell cycle arrest in HT-295 cells at the G2/M phase, and inhibited cell migration and invasion.

Conclusion: Gentiopictin exerts potent anticancer effects on human colon cancer cells via cell apoptosis and G2/M phase cell cycle arrest. In addition, it suppressed the migration and invasion of HT-295 cells. These findings provide useful basis for further in vivo research of gentiopictin on colon cancer.

Keywords: Colon cancer, Glycosides, Gentiopictin, Caspase, Apoptosis, Cell cycle

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INTRODUCTION

Plants yield a huge diversity of natural products (phytochemicals) with enormous structural diversity. These phytochemicals are known as secondary metabolites, and in contrast with primary metabolites, they were previously considered as waste products [1-3].

Phytochemicals produce huge pharmacological and biological effects. Glycosides, a major class of phytochemicals, are considered as bioactive compounds in terms of medicinal and biological applications [4]. Glycosides have been conventionally used in traditional medicine for treating heart disorders, due to their selective inhibition of Na⁺-K⁺-ATPase and enhancement of

intracellular Ca^{2+} concentrations [5]. Glycosides modulate carcinogenesis via several death and survival pathways [6]. Potential inhibitors of Na^+ - K^+ -ATPase may target cancer cells by promoting autophagy, apoptosis, intracellular oxygen radical species and inhibition of cell cycle [7]. Studies have demonstrated that glycosides exert anticancer effects against lung cancer, breast cancer, leukemia, lung cancer and prostate cancer [8-11]. Moreover, glycosides, especially cardiac glycosides, have been shown to exhibit anti-colon cancer effects with high specificity and effectiveness [12].

Colon cancer is a serious human malignancy associated with high morbidity and mortality. It was very rare in the past few decades. However, the last decade has witnessed sudden increases in colon cancer cases. Indeed, colon cancer has become a leading malignant disorder accounting for about 10 % of all cancer-related mortalities in western countries [13]. The increased frequency of colon cancer in developed countries may be attributed to harmful dietary habits and increases in ageing populations. The major risk factors for colon cancer include obesity, poor physical exercise and smoking [14]. Despite improvements in treatment strategies, mortality from colon cancer constitutes a huge challenge. The currently-used methodologies for colon cancer management include palliative and neoadjuvant chemotherapy, radiotherapy, aggressive resection of metastatic tissues (pulmonary and liver metastasis), and laparoscopic surgery at early stages [15]. Notwithstanding these strategies, the long-term survival and cure rates associated with colon cancer remain very poor. Therefore, to tackle colon cancer, there is need for novel treatment strategies and efficient chemotherapeutics.

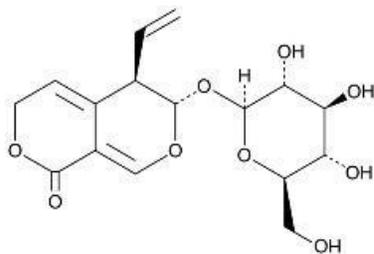


Figure 1: Chemical structure of gentiopicrin

Gentiopicrin (also known as gentiopicroside (Figure 1)) is a glycoside with huge bioactivity potential. A study has shown that gentiopicrin induced anticancer effects on human ovary cancer via suppression of the NF- κ B signaling pathway, induction of loss of mitochondrial membrane potential, and apoptosis [16]. In this study, the anticancer effect of gentiopicrin was

investigated in human colon cancer cells, in addition to the its effect on caspase-dependent apoptosis, cell cycle arrest, and cell migration and invasion.

EXPERIMENTAL

Cell proliferation

Cell proliferation was determined with MTT assay. The cells were seeded in 96-well plates at a density of 1.5×10^5 cells/well and pre-cultured at 37 °C for 24 h in a humidified incubator with 95 % air and 5 % CO_2 . Subsequently, the HT-295 cells were exposed to gentiopicrin at doses of 6, 12, 24, 48 and 96 μM for 24 h and 48 h. Thereafter, the HT-295 cells were treated with MTT solution (10 mg/mL), followed by incubation for additional 4 h. The medium was removed, and the formazan crystals formed were dissolved in 250 μL DMSO. Finally, the absorbance of the formazan solution was read at 490 nm in a microplate reader (ELX 800; Bio-tek Instruments, Inc., Winooski, United States).

Cell colony formation assay

The HT-295 cells were seeded in 6-well plates at a density of 300 colonies/ well. These colonies were left untouched for 12 h to let them adhere. Thereafter, they were treated with gentiopicrin at doses of 12, 48 and 96 μM for 48 h. Untreated colonies served as control. Gentiopicrin treatment was followed by replacement of medium with a fresh one, and the cells were cultured for the next 14 days. Thereafter, HT-295 cell colonies were fixed in 4 % paraformaldehyde, followed by staining with crystal violet. Finally, the cell colonies were analysed under a light microscope (Nikon, Japan). Colonies with diameter > 0.5 mm were counted.

AO/EB staining assay

To determine apoptotic cell death in gentiopicrin-treated HT-295 cells, AO/EB staining was performed. The HT-295 cells were cultured at a density of 1.5×10^4 cells/well in 6-well plates for 24 h, followed by gentiopicrin exposure at different doses viz 12, 48 and 96 μM for 48 h. Untreated cells served as control. The gentiopicrin-treated colon cancer cells were centrifuged for 20 min, followed by washing thrice in PBS. Thereafter, 10 μL each of AO and EB were added to the cell suspension. Finally, the stained HT-295 cells were loaded on glass slides and analyzed under a fluorescence microscope.

Cell cycle analysis

Cell cycle analysis of the HT-295 carcinoma cells was done using flow cytometry. The cells were treated with gentiopicrin at doses of 12, 48 and 96 μM for 24 h. The treated cells were harvested at 90 % confluence, followed by fixation in 70 % ethanol. Then, the fixed HT-295 cells were rinsed twice in PBS and subjected to PI staining for 30 min using a solution containing PI (50 $\mu\text{g}/\text{mL}$) and RNase (25 $\mu\text{g}/\text{mL}$). Thereafter, the cells were subjected to flow cytometric analysis in a cytometry (FACS Calibur).

Transwell chamber assay

The effect of gentiopicrin on the migration and invasion of HT-295 cells was determined using transwell chamber assay. The HT-295 cells at exponential growth phase were used at a density of 1×10^4 cells/well. The cells were placed on the upper transwell chamber containing different doses of gentiopicrin viz 12, 48 and 96 μM , followed by 48 h of incubation. Untreated cells served as control. The lower chamber contained medium only. Thereafter, the culture medium was replaced with fresh serum-free medium. The upper and lower transwell chambers were incubated for 24 h. The migrated cells were fixed in methanol, while un-migrated cells were cleaned off. Subsequently, the fixed migrated cells were stained with crystal violet for 20 min, examined under a microscope, and photographed at x200 magnification. A similar procedure was used for determination of the effect of gentiopicrin on cell invasion of HT-295 cells, except that the transwell chambers were coated with Matrigel.

Western blotting assay

The expressions of apoptosis-related proteins in gentiopicrin treated HT-295 cells were assayed using Western blotting assay. The HT-295 cells were treated with gentiopicrin at doses of 12, 48 and 96 μM for 24 h. Then, total protein was extracted from the treated HT-295 cells through lysis, and protein content was determined using BCA assay. Then, 40- μg protein samples from the lysates were separated by subjecting them to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. The membranes were blocked for 2 h at room temperature with 5 % non-fat milk in TBST buffer. The blocked membranes were then incubated overnight at 4 $^{\circ}\text{C}$ with primary antibodies against caspase-3, caspase-8, caspase-9, Bax and Bcl-2, followed by rinsing in TBST. Thereafter, the membranes were treated with HRP-conjugated secondary antibody in TBST (1:20000 dilution). Finally, the

protein bands were developed using enhanced chemiluminescence (ECL) kit.

Statistical analysis

Each experiment was repeated three times, and results are presented as mean \pm standard error of mean (SEM). The gentiopicrin-treated groups were compared using ANOVA, followed by Newman-Keuls post hoc test. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Gentiopicrin induced cytotoxicity in HT-295 cells

The cytotoxic effect of gentiopicrin against HT-295 cell line was determined using MTT assay. Gentiopicrin induced dose- and time-dependent cytotoxicity against HT-295 cells. The viability in controls was taken as 100 %. Gentiopicrin at a concentration of 6 μM , reduced viability to almost 90 and 80 % after 24 and 48 h exposure, respectively. When higher gentiopicrin doses were applied, viability was reduced to 20 and 10 % after 24 and 48 h exposure, respectively (Figure 2). These results demonstrate that gentiopicrin induced strong cytotoxicity in HT-295 cells.

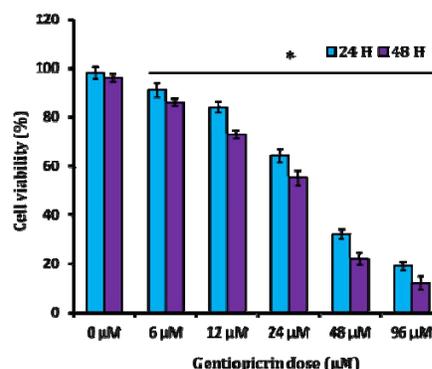


Figure 2: Effect of gentiopicrin on proliferation of colon HT-295 carcinoma cells. The cells were incubated with different gentiopicrin doses as indicated for 24 h. Results showed dose- as well as time-reliant inhibition of cellular proliferation. Data from triplicate experiments are presented as mean \pm SEM; $p < 0.05$

Gentiopicrin induced anti-clonogenic effects on HT-295 cell colonies

The effect of gentiopicrin on HT-295 cell colonies was determined with clonogenic assay. After exposure of these cells to gentiopicrin for 14 days, there was significant suppression of treated cell colonies, relative to untreated

controls (Figure 3A). Three hundred (300) colonies of HT-295 cells were counted in untreated controls, in contrast to treated cells which showed significant decrease in the number of colonies. The number of colonies was reduced to about 70 on application of a higher gentiopicrin dose of 96 μM (Figure 3B).

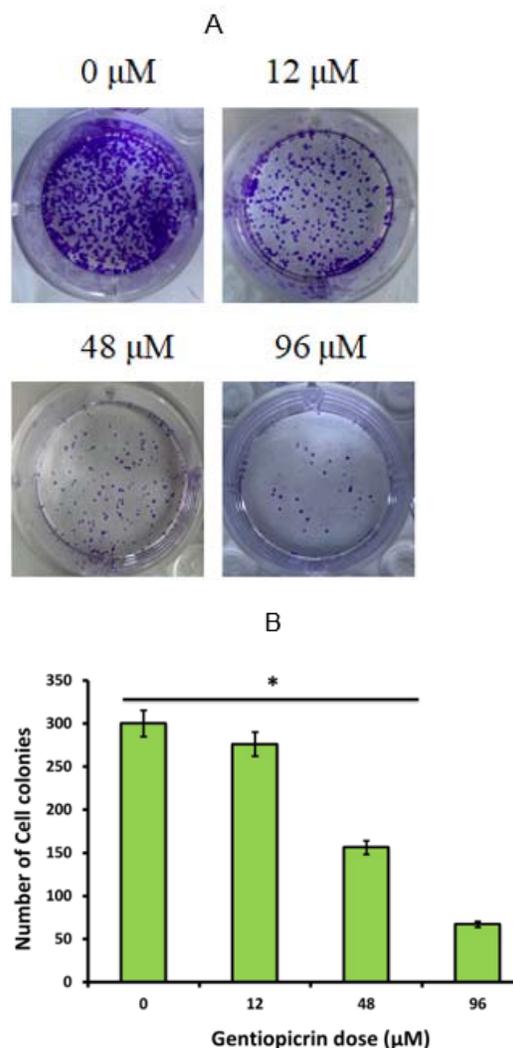


Figure 3: Effect of gentiopicrin on colony formation of HT-295 cells. **A)** Untreated cells served as control. Blue stains represent colonies after two weeks of gentiopicrin treatment. Each experiment was repeated thrice. **B)** Graphical representation of number of HT-295 cell colonies after treatment with gentiopicrin for two weeks. Data from triplicate experiments are presented as \pm SEM; $p < 0.05$

Gentiopicrin induced apoptosis in HT-295 cells

Results from AO/EB staining assay showed that gentiopicrin induced significant apoptotic effects on HT-295 cells. There was an increase in the percentage of apoptotic (early and late) and

necrotic HT-295 cells post-gentiopicrin exposure (Figure 4). The increase in percentage apoptosis was dose-dependent. Results from Western blotting assay validated the pro-apoptotic effect of gentiopicrin. The results showed that, compared with control group, the expressions of caspase-3, caspase-8, caspase-9 and Bax were up-regulated in gentiopicrin-treated groups, while the expression of Bcl-2 was down-regulated (Figure 5).

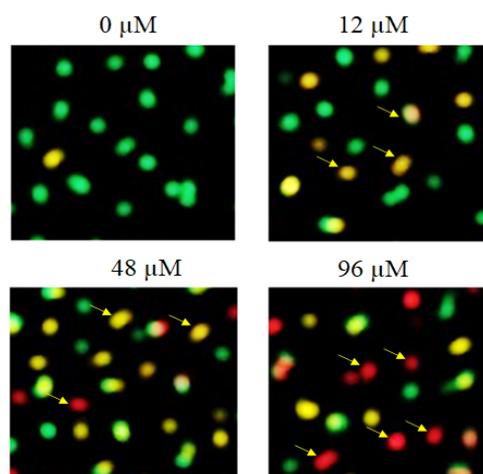


Figure 4: Results of AO/EB staining assay showing apoptotic cells after gentiopicrin exposure. There were marked dose-dependent increases in numbers of early apoptotic, late apoptotic and necrotic cells. Each experiment was done in triplicate

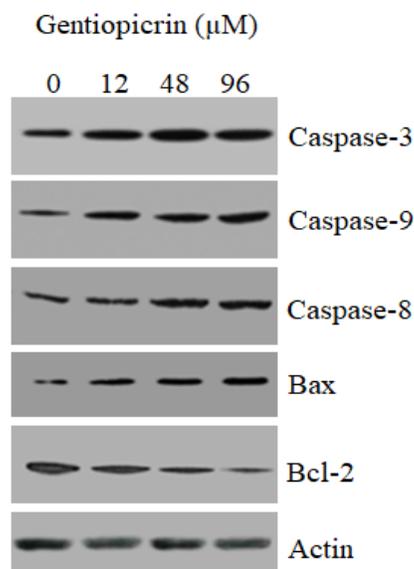


Figure 5: Effect of gentiopicrin on protein expressions of caspase-3, caspase-8, caspase-9, Bax and Bcl-2 in HT-295 cells, as assayed using Western blotting. The experiments were repeated thrice

Gentiopictin inhibited cell cycle in HT-295 cells

Gentiopictin inhibited the G2/M-phase of the cell cycle. This was evident in the increase in the population of accumulated cells in the G2/M phase at enhanced gentiopictin concentrations. At higher gentiopictin doses, the number of cells in G2/M-phase reached almost 37 (Figure 6).

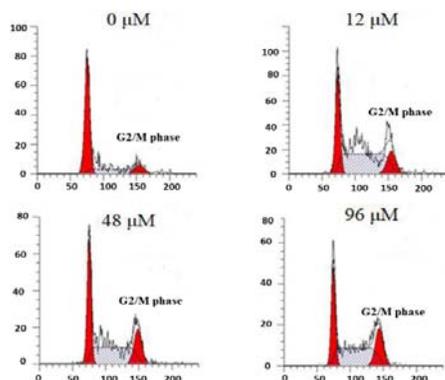


Figure 6: Flow cytometric analysis for determination of the effect of gentiopictin on HT-295 cells at various check points in the cell cycle. The number of G2/M phase cells increased significantly in response to exposure of HT-295 cells to increasing doses of gentiopictin. Data from triplicate experiments are presented as mean \pm SEM; $p < 0.05$

Gentiopictin inhibited migration and invasion of HT-295 cells

Cell migration and invasion were determined in gentiopictin-treated HT-295 cells through transwell chamber assay. The migration potential of HT-295 cells was suppressed significantly in a concentration-dependent fashion (Figure 7). The invasion of HT-295 cells was reduced significantly by gentiopictin in a concentration-reliant fashion, when compared to controls (Figure 8).

DISCUSSION

Colon cancer is a life-threatening malignancy and a leading global cancer burden. Unfortunately, colon cancer cases have increased at an alarming rate. Due to lack of treatment options and poor outcomes associated with currently-used chemotherapy, long term survival of patients remains poor [17]. Therefore, novel chemo-preventives are needed to tackle colon cancer and improve patients' overall survival. Apoptosis is an essential and organized biochemical mechanism in multicellular organisms.

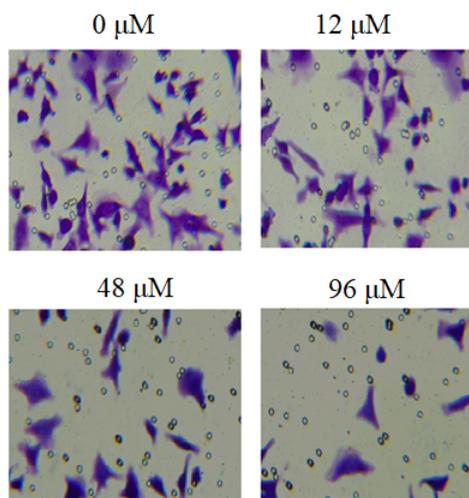


Figure 7: Effects of gentiopictin on cell migration of HT-295 cells after 48 h of treatment. The figure demonstrates the number of migrated cells at each treatment concentration of gentiopictin. The experiments were repeated thrice

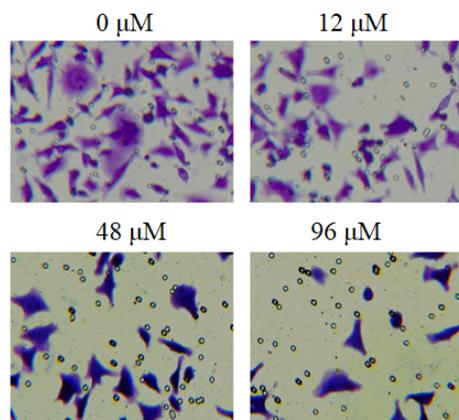


Figure 8: Effects of gentiopictin on cell invasion of HT-295 cells after 48 h of treatment. The figure demonstrates the number of invasive cells at each treatment concentration of gentiopictin. The experiments were repeated thrice

It is a programmed cell death process which occurs through intrinsic and extrinsic signaling pathways. Any imbalance in the regulation of apoptosis results in serious disorders, including carcinogenesis [18,19]. Several morphological alterations in cells are indicators of apoptosis. These features include apoptotic bodies, DNA and nuclear fragmentation, cell shrinkage, and membrane blebbing. There is an urgent medical need for novel chemo-preventives that stimulate and induce apoptosis in cancer cells [20,21]. Mitochondria, which are also known as "power house of the cell" regulate several biochemical processes, including apoptosis. In addition,

mitochondria modulate different biochemical processes such as levels of pro- and anti-apoptotic proteins, discharge of caspase stimulators, and mitochondrial membrane potential [22].

The present research was designed to investigate the anticancer effects of gentiopiricin on human colon cancer. The results of obtained indicate that gentiopiricin suppresses the proliferation of colon HT-295 cancer cells in a concentration- and time-dependent fashion. The HT-295 colony-formation potential was suppressed significantly. The underlying mechanism of action of gentiopiricin was studied by determining its effect on apoptosis. Results indicated that gentiopiricin induced apoptotic cell death which was associated with increased caspase activity. Cell cycle examination through flow cytometry revealed G2/M-phase cell cycle arrest in HT-295 cells after gentiopiricin treatment. Moreover, it was observed that cell migration and invasion potential of HT-295 cells were significantly inhibited by gentiopiricin.

CONCLUSION

Gentioipiricin inhibits cell proliferation in colon cancer cells via caspase-dependent apoptosis, cell cycle arrest, and suppression of cell migration and invasion. Therefore, gentioipiricin may be a potent anti-colon cancer agent. However, more *in vivo* and clinical investigations are required to validate this assertion.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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