

ORIGINAL ARTICLE

Sinensetin flavone exhibits potent anticancer activity against drug-resistant human gallbladder adenocarcinoma cells by targeting PTEN/PI3K/AKT signalling pathway, induces cellular apoptosis and inhibits cell migration and invasion

Bo Huang*, Min Zhai*, Ancheng Qin, Jianwu Wu, Xinwei Jiang, Zhiming Qiao

Department of General Surgery, Nanjing Medical University, Affiliated Suzhou Hospital, Suzhou Municipal Hospital, Gusu, Suzhou, Jiangsu 215000, P.R. China

*These two authors contributed equally to this work.

Summary

Purpose: The main focus of the current research work was to unveil the anticancer activity of the naturally occurring Sinensetin flavone against aggressive gall bladder cancer adenocarcinoma (GBAC) TJ-GBG2 cell line. Its effect of inducing apoptosis mediated via targeting PTEN/PI3K/AKT signalling pathway were also examined along with cell migration and invasion.

Methods: Cell proliferation was tested by MTT cell viability assay. Fluorescence microscopy was utilized to carry out apoptosis related studies via DAPI staining along with flow cytometry using annexin V/propidium iodide (PI) assay. Further, western blotting analysis was carried out to examine the effects of Sinensetin on the expressions of apoptosis-related proteins and Bax Bcl-2 along with PTEN/PI3K/AKT signalling pathway. The impact of the test molecule on cell migration and invasion was studied through wound healing assay and transwell cell invasion assay respectively.

Results: The results showed that Sinensetin treatment caused a significant retardation in cell viability, in a dose-

dependent fashion. DAPI staining assay and annexin V/PI assay revealed that the cell viability of GBC cells was retarded due to induction of apoptosis. It was also associated with downregulation of Bcl-2 and upregulation of Bax levels. Further, wound healing assay and transwell cell invasion assay revealed that cell migration as well as cell invasion of cancer gallbladder cells was decreased in a concentration-dependent fashion. It was further seen that Sinensetin treatment resulted in inhibition of matrix metalloproteinase (MMP)-2 and enhancement of MMP-9 protein expressions. Results also showed that the tested molecule had the potential to inhibit PTEN/PI3K/AKT signalling pathway.

Conclusion: In conclusion, the current study indicated that Sinensetin flavone has the potential to be developed as a candidate drug against gallbladder adenocarcinoma provided more toxicological and in vivo studies are carried out.

Key words: Sinensetin, gallbladder cancer, apoptosis, flow cytometry, cell invasion

Introduction

Gallbladder adenocarcinoma (GBAC) ranks as fifth most frequent carcinoma associated with biliary tract, characterized with distant metastases and primary lymph node invasion [1-3]. GBAC is more prevalent in certain specific areas in the

world, and the widespread variation in ethnicity, cultural, and geographical differences in the occurrence of this malignancy symbolises the involvement of key environmental and genetic factors related to its growth and progression [4,5]. It spreads

Corresponding author: Zhiming Qiao, MD. Department of General Surgery, Nanjing Medical University, Affiliated Suzhou Hospital, Suzhou Municipal Hospital, no.26 Dacquin street, Gusu district, Suzhou, Jiangsu, 215000, P.R. China.
Tel/Fax: +86 0512 62364155, Email: EmilValentineif@yahoo.com
Received: 07/07/2019; Accepted: 21/07/2019

early and tends to be an aggressive tumor, while about 90% of GBAC patients are diagnosed at an inoperable advanced stage [6,7]. Early GBAC does not show any characteristic symptoms or demonstrates only a symptom of abdominal distress. This asymptotic behaviour results in poor prognosis of GBAC, and the lack of efficient sensitive screening tests further adds to its poor prognosis [8]. There is only one method to cure GBAC that is surgical resection, however only 10% of patients diagnosed with this malignancy are surgically curative [9]. Even among the patients who are suitable for surgical resection, the anatomical complexity associated with portobiliary hepatic system results in tumour spread, and ultimately high mortality rate [10]. Patients who are not eligible for surgical resection have a very high relapse rate. The overall 5-year survival rate in this lethal malignancy is very low (about 5%) [11]. Hence, there is a pressing need to recognise novel consistent biomarkers and drugs for monitoring both advancement and treatment of GBAC.

As plants disclose a substantial potential to manufacture an immense range of chemical entities, they are tremendously sophisticated natural chemical factories. Flavonoids, plant products, belonging to a class of polyphenols, are divided into six subclasses i.e. flavones, flavanols, flavanones, isoflavones, and anthocyanidins [12,13]. Recent studies regarding flavonoids have unveiled some beneficial biological and pharmacological properties like antiallergic, antiinflammatory, antioxidant, antiviral, and antitumor activities [14-17]. Additionally, researchers have also reported that flavones promote apoptosis in carcinoma cells thereby inhibiting tumor growth [17]. Sinensetin, a methylated flavone, found in orange oil and *Orthosiphon stamineus*, shows antibacterial and anticancer effect and it was also reported to inhibit oral carcinoma cells [18,19].

The current study was performed in order to explore the anticancer effects of Sinensetin on drug-resistant human gallbladder adenocarcinoma cells along with studying its effects on PTEN/PI3K/AKT signalling pathway, cellular apoptosis and cell migration and invasion.

Methods

MTT cell proliferation assay

Sinensetin drug sensitivity was assessed via MTT assay. Briefly, gallbladder cancer cell line TJ-GBC2 as well as normal gallbladder primary cell line 36126-53N were trypsinized and placed at a density of 4×10^4 cells onto 96-well plates (Corning, USA). Culturing of the cells was performed for 12 h and then fresh Dulbecco's Modified Eagle's Medium (DMEM) was reloaded containing various concentrations, i.e. control, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μ M of Sinensetin for 12 h. Subsequently, 10 ml of MTT (Sigma-Aldrich) were dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml and were directly added to each 96-well plates. Afterwards, the plates were incubated for 4 h at 37°C, causing the formation of formazan crystals. After removal of the supernatant, these crystals were dissolved in 100 μ l of dimethyl sulfoxide (DMSO). The optical density (OD) was evaluated at 490 nm using a microplate reader (Bio-Tek, Wiskonsin, VT05404, USA).

Apoptosis analysis through DAPI staining and Annexin V/PI assay

TJ-GBC2 gallbladder cancer cells were harvested at a density of 3×10^4 cells/well using 96-well plates. These cells were then incubated with different doses of Sinensetin i.e. control, 6.12, 12.5 and 25 μ M and incubated for 24h. After incubation, the cells were stained with DAPI (4',6-diamidino-2-phenylindole) followed by further incubation. Next, the cells were washed with PBS and fixed using PBS and formaldehyde (10%) respectively. The DAPI-stained blue colour cells were then investigated under fluorescence microscope. For annexin V/PI assay a parallel method as for DAPI was followed, except

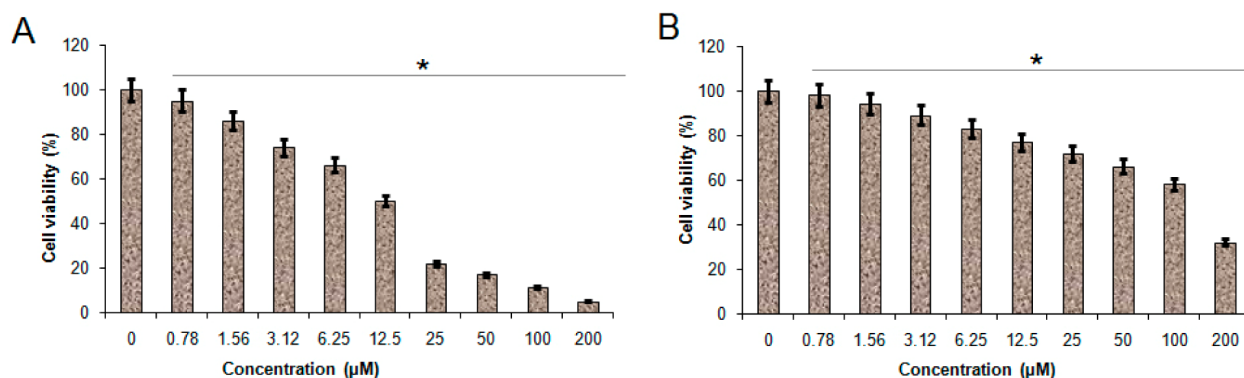


Figure 1. Sinensetin prevents the proliferation of gallbladder carcinoma cells *in vitro*. TJ-GBC2 (A) and 36126-53N (B) cells were treated with changing concentrations of Sinensetin, and the cell proliferation was determined by MTT assay. The experiments were performed in triplicate and shown as mean \pm SD (*p<0.05).

staining was performed using annexin V/PI and studied through flow cytometry.

Wound healing assay for cell migration analysis

TJ-GBC2 gallbladder cancer cells (1×10^4 cells/well) were placed into 24-well plates and 200 μ l pipette tip was used for scraping a wound. Dethatched cells were removed through washing with PBS. These cells were then put into complete growth DMEM holding different concentrations of the current test molecule, i.e. 0 and 12.5 μ M, followed by incubation at 37°C for 24h. A phase-contrast microscope at 100x magnification was used to examine cell migration at 0 and 24 h after induction of injury. Image J software version 1.50 (National Institutes of Health, Bethesda, MD, USA) was utilised to quantify and measure the number of migrated cells in the denuded area of six arbitrary fields.

Transwell cell invasion assay

Cancer cell invasion was studied through transwell cell invasion assay. In the ratio of 1:5 Matrigel/RPMI-1640 medium was coated onto the upper chambers and 3×10^4 cells/well were cultured in 500 μ L of RPMI-1640 medium with varying doses of Sinensetin molecule, i.e. control, 6.12, 12.5 and 25 μ M. In the lower chamber only 700 μ L of RPMI-1640 medium with 10% fetal bovine serum (FBS) was placed. Both chambers were then incubated at 37°C for 36h and thereafter scrubbing of upper cham-

bers was performed by using a cotton swab to wipe out non-migrated cells. The migrated cells were then fixed with formalin for 15 min, followed by staining with 0.1% crystal violet for 20 min. Further, using a microscope with 100x magnification, 5 random fields were captured and examined.

Western blotting analysis

The TJ-GBC2 gallbladder cancer cells were exposed to different doses of Sinensetin molecule, i.e. control, 6.12, 12.5 and 25 μ M. Then, these cells were lysed, the lysate was collected and quantified for protein content. Quantified protein lysates were then subjected to electrophoresis in 10% SDS-polyacrylamide gel (SDS-PAGE), followed by transference to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then subjected to primary antibody treatment overnight at 4°C. Thereafter, washing was performed twice with Tris-buffered saline, 0.1% Tween 20 (TBST buffer), and the PVDF membranes were incubated with secondary antibody for 1 h. Finally, chemiluminescence method by using the Western blot image-forming system (Tanon 5200, China) was utilised to visualise proteins. All of the experiments were performed in triplicate.

Statistics

All significant values are shown as mean \pm SD and were studied by the Student's *t*-test via SPSS version 13.0 software. A *p* value of <0.05 was considered significant.

Results

Inhibition of cell viability/proliferation of human TJ-GBC2 gallbladder cancer cells by Sinensetin

The proliferation of TJ-GBC2 gallbladder cancer cells and normal gallbladder cell line 36126-53N was evaluated through MTT assay. The results showed that cell viability was altered by exposure to test molecule in a dose-dependent manner. In case of cancer cells, the viability decreased significantly from 100% to near about 5% with increasing drug concentration, i.e. control, 0.78, 1.56, 3.12, 6.2, 12.5, 25, 50, 100 and 200 μ M (Figure 1A) and in case of normal gallbladder cells the viability was not affected significantly (Figure 1B). The IC_{50} value of the tested molecule was much higher in case of normal cells in contrast to cancer cells where it showed lower IC_{50} value indicating its selective toxicity against cancer cells.

Apoptosis induction by Sinensetin in TJ-GBC2 gallbladder cancer cells

To investigate whether the antiproliferative effects of Sinensetin molecule on human TJ-GBC2 gallbladder cancer cells were due to apoptosis induction, we performed DAPI staining using fluorescence microscopy. The results revealed that the current tested molecule induced apoptosis in hu-

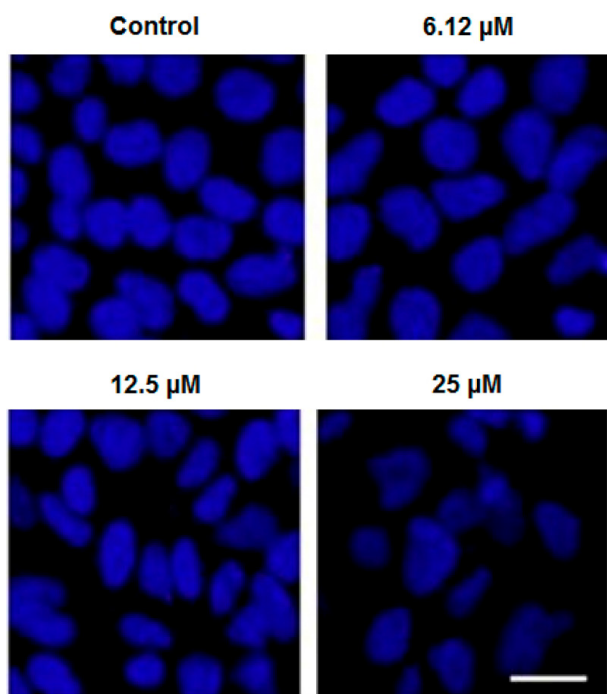


Figure 2. Sinensetin induces apoptosis in TJ-GBC2 gallbladder carcinoma cells. Apoptotic morphological changes such as cell shrinkage, abnormal nuclear morphology, and reduction in cell number with apoptotic body formation, were induced by Sinensetin (6.12, 12.5 and 25 μ mol/L) treatment for 24 h, were observed by DAPI staining using fluorescence microscopy. The experiments were performed in triplicate.

man TJ-GBC2 gallbladder cancer cells as demonstrated by the formation of cell blebbing and apoptotic crops (Figure 2). Furthermore, the apoptosis inducing capability of the test molecule was found to be dose-dependent and the percentage of apoptotic cells increased significantly with increased doses of Sinensetin. Moreover, annexin V/PI staining depicted that the population of apoptotic cells increased tremendously with increasing doses, i.e. control, 6.12, 12.5 and 25 μ M of the studied molecule (Figure 3). Further, western blotting assay revealed that the expression of apoptosis-related

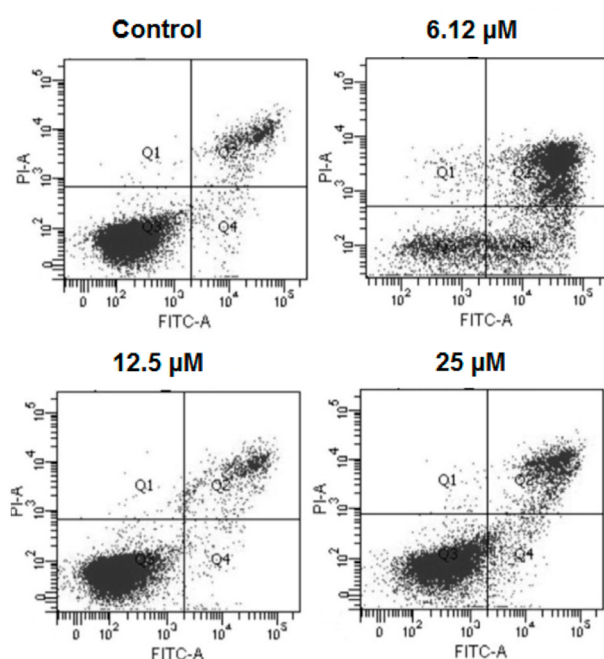


Figure 3. Flow cytometric measurement of apoptotic cell population. Cells were incubated with Sinensetin (6.12, 12.5 and 25 μ mol/L) for 24 h, followed by staining with annexin-V/PI. Cell apoptosis was assessed by flow cytometry. The experiments were performed in triplicate.

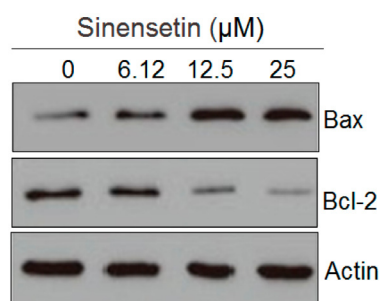


Figure 4. Sinensetin induces the activation/deactivation of apoptosis-related proteins in TJ-GBC2 gallbladder carcinoma cells. After treatment with Sinensetin (6.12, 12.5 and 25 μ mol/L) for 12 h, cell lysates were prepared and western blot analysis was performed against Bcl-2 and Bax. Actin was used as a loading control. The results shown are illustrative of at least three independent experiments.

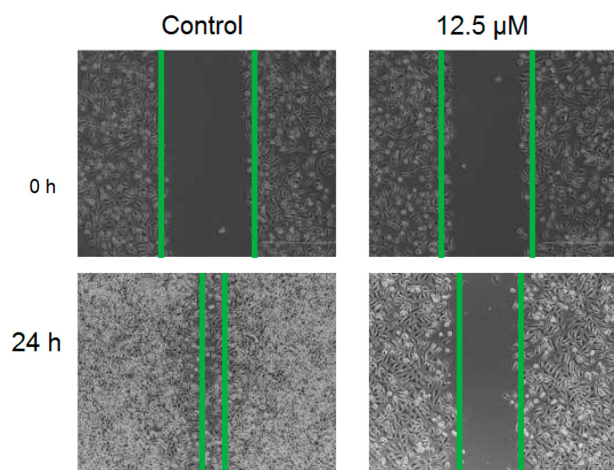


Figure 5. Effect of Sinensetin on the mobility of TJ-GBC2 gallbladder carcinoma cells. Prior to the migration assay cells were treated with untreated control and 6.12 μ M of Sinensetin for 24 h. Cell monolayers were wounded by scratching with a 200 μ l pipette tip.

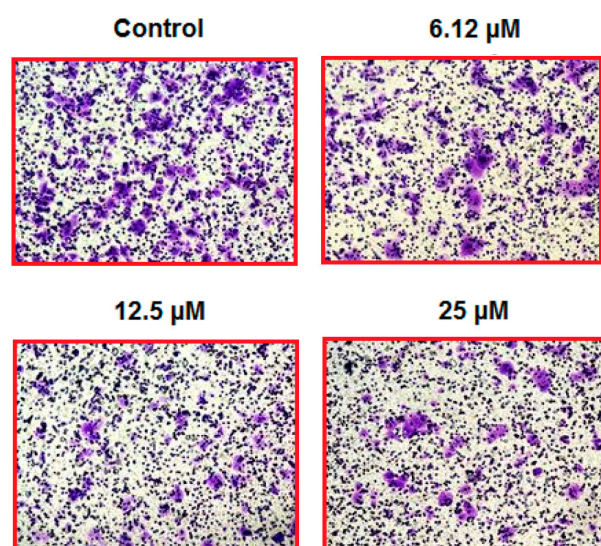


Figure 6. Effect of Sinensetin on invasive potential of TJ-GBC2 gallbladder carcinoma cells. Prior to the cell invasion assay cells were treated with control, 6.12, 12.5 and 25 μ M of Sinensetin for 24 h, and there was a dose-dependent decrease in cell invasion after 12 h treatment.

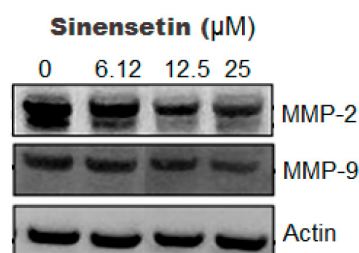


Figure 7. Sinensetin affects the expression of matrix metalloproteinases (MMPs). The protein expression of MMP-2 and MMP-9 was detected by western blotting after treated with Sinensetin for 24 h. The results shown are illustrative of at least three independent experiments.

proteins was significantly altered by Sinensetin exposure. It resulted in declining the expression of Bcl-2 and enhancing the levels as well as expression of Bax (Figure 4). These results indicate that the antiproliferative effects of the current tested molecule were mediated through apoptosis induction.

Inhibition of cell migration and cell invasion by Sinensetin via targeting MMP-2 and MMP-9 expressions

In vitro wound healing and transwell invasion assays were used to study the effect of the test molecule on the cell migration and invasion, respectively. Wound healing assay revealed that cell migration was retarded significantly on exposure with the test molecule in a dose dependent-manner (Figure 5). After treatment of cultured cancer cells with different concentrations of Sinensetin, i.e. control and 12.5 μ M for 12 h, antimigratory effects were seen by the wound width. Sinensetin-treated cells showed significant inhibition of cancer cell migration. Moreover, transwell invasion assay depicted that Sinensetin treatment of TJ-GBC2 gallbladder cancer cells resulted in inhibition of cell invasion ability of cancer cells in a dose-dependent manner (control, 6.12, 12.5 and 25 μ M) (Figure 6). It was further confirmed by western blotting analysis, which further revealed significant decrease in MMP-2 and MMP-9 expression levels in a concentration-dependent manner (Figure 7).

Sinensetin targets PTEN/PI3K/AKT signalling pathway through hampering the expressions of this pathway associated proteins

It was further depicted by western blotting

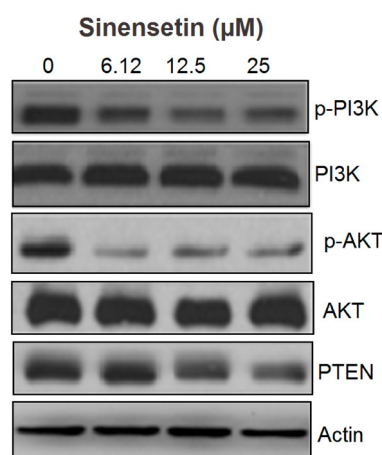


Figure 8. Effect of Sinensetin on the PTEN/PI3K/AKT protein expression in TJ-GBC2 gallbladder carcinoma cells at indicated doses estimated through western blotting. Sinensetin treatment resulted in a dose-dependent decrease in the levels of PTEN, p-PI3K, and p-AKT.

analysis that Sinensetin targets the PTEN/PI3K/AKT signalling pathway. The results revealed that treated cancer cells showed significant alteration in the expression of this pathway-related proteins resulting in decreasing expression of PTEN, p-PI3K and p-AKT, while the expressions of PI3K and AKT remained almost constant, as the Sinensetin dose increased from control to 6.12, 12.5 and 25 μ M (Figure 8).

Discussion

Gallbladder adenocarcinoma is among the major malignancies associated with biliary tract. Poor prognosis, asymptotic behaviour, lack of effective screening tests and low overall 5-year survival rate increases its lethality. Due to the side effects of conventional chemotherapeutic agents, there is an immediate need to switch to new potential anticancer agents. Natural products can be a major source of such agents. Flavones are naturally occurring compounds, and previous studies have revealed their different pharmacological activities like antibacterial and anticancer [20-22]. Herein, we verified Sinensetin flavone for its anticancer activity on drug-resistant human GBAC cells by targeting PTEN/PI3K/AKT signalling pathway, which induces cellular apoptosis and inhibits cell migration and invasion. Multiple biological processes like cell proliferation, cell growth, metabolism and apoptosis are regulated via PTEN/PI3K/AKT signalling pathway. PTEN, a dual lipid/protein phosphatase, whose key substrate is phosphatidyl-inositol, 3,4,5 triphosphate (PIP3), is the product of PI3K. Proliferation in PIP3 employs AKT to the membrane where it gets stimulated by further kinases also reliant on PIP3. Several components associated to this pathway have been designated as underlying forces in cancer [23]. Cytotoxicity of the current test molecule was estimated through MTT assay and revealed significant dose-dependent and time-dependent inhibition of cell viability of Sinensetin-treated cells without affecting much the normal gallbladder cells. Further, we studied whether the antiproliferative impact on gallbladder cancer cells is due to apoptosis induction, and for that we performed DAPI staining and Annexin V/PI assay, the results of which showed that along with development of cell blebbing and apoptotic crops the number of apoptotic cells increased after test molecule exposure, indicative of apoptosis induction. Next, cell migration and invasion analysis were performed, and the results were quite significant as both cell migration and invasion were retarded/stopped upon exposure to current test

molecule in a dose-dependent manner. Finally, western blotting analysis was performed to establish the impact of Sinensetin on PTEN/PI3K/AKT signalling pathway of TJ-GBC2 gallbladder cancer cells. The results unveiled that Sinensetin treatment leads to increase of PI3K and AKT levels, while the expression levels of PTEN, p-PI3K, and p-AKT remained constant, suggestive of blocking this pathway. Thus, by all the above examinations and discussions it is evidenced that Sinensetin is a potent anticancer agent against gallbladder carcinoma cells and can be developed as a promising drug candidate.

Conclusion

In conclusion, it is clear that the current test molecule (Sinensetin) is a potential anticancer agent against human gallbladder adenocarcinoma cells. The anticancer effects are induced through antiproliferative effect, apoptosis, retarding of cell migration and invasion, and blocking PTEN/PI3K/AKT signalling pathway of TJ-GBC2 gallbladder cancer cells.

Conflict of interests

The authors declare no conflict of interests.

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