Scopoletin potentiates the anti-cancer effects of cisplatin against cholangiocarcinoma cell lines
Introduction

Cholangiocarcinoma (CCA) is a fatal adenocarcinoma with poor prognosis, and a health problem worldwide (Gores, 2003). Though surgical removal is the only curative approach (Kuhlmann, 2012), the recurrence rate is approximately 60% even after resection (Aljiffry et al., 2009). Chemotherapy with cisplatin, 5-fluorouracil and gemcitabine either alone or in combination has been practiced to improve survival of patients with cholangiocarcinoma (Thongprasert, 2005). Treatment with cisplatin has some drawbacks, including toxicity, adverse effects and resistance of tumors (Siddik, 2003). These limitations warrant the desperate search for relatively non-toxic natural products as chemopreventive agents (Pan and Ho, 2008).

Several natural products and their derivatives have been found to exhibit immense pharmacological and biological properties including molecular interventions in carcinogenesis (Saha and Khuda-Bukhsh, 2013). The ability of natural products to bind a variety of protein domains and folding motifs makes them effective modulators of cellular processes such as immune responses, signal transduction, mitosis and apoptosis (Peczuh and Hamilton, 2000). It was reported that coumarin inhibited the growth of HeLa cells (Chuang et al., 2007) and cholangiocarcinoma cells (Prakobwong et al., 2011). Phenolic compound scopoletin (7-hydroxy-6-methoxycoumarin) is ubiquitous in the plant kingdom (Peterson et al., 2003). Studies demonstrated that scopoletin exhibited significant pharmacological activities including antitumor, antiangiogenic (Zhou et al., 2012) and anti-inflammatory activities (Kang et al., 1999; Moon, 2007). Currently, a number of natural products are being evaluated in vitro and in vivo to demonstrate their additive or synergistic interaction with clinically approved anti-cancer drugs. However, there is no report on combined effects of scopoletin and cisplatin.
Therefore, we aimed to evaluate potential of scopoletin for enhancing anti-cancer effects of cisplatin in cholangiocarcinoma cells.

Materials and Methods

Reagents and media

Cisplatin and scopoletin were purchased from the Sigma-Aldrich® (USA). Culture media RPMI-1640, DMEM and supplements including antibiotics and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (USA). FITC Annexin V apoptosis detection kit was purchased from BioLegend Inc. (San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich (USA). Cisplatin was dissolved in normal saline (0.18 mg/mL) and kept in 4°C. Scopoletin was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The final concentration of DMSO was less than 0.5% (v/v), which was also present in the corresponding controls.

Cell lines and cell culture

In this study, two cholangiocarcinoma cell lines, KKU-100 (poorly differentiated) and KKU-M214 (moderately differentiated), and a human bile duct epithelial cell line (H69 cells) were used. Cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) (Gibco-BRL). H-69 cells were cultured in enriched Dulbecco’s Minimum Essential Medium (DMEM) (Gibco, Invitrogen, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Invitrogen), 100 U/mL penicillin (Gibco, Invitrogen), 100 µg/mL streptomycin (Gibco, Invitrogen), 25 µg/mL adenine (Sigma, USA), 5 µg/mL insulin (Gibco, Invitrogen), 1 µg/mL epinephrine (Sigma), 13.6 ng/mL T3, T4 triiodothyronine (T3) (Sigma), 8.3 µg/mL holotransferrin (Gibco, Invitrogen), 0.62 µg/mL hydrocortisone (Sigma) and 10 mg/mL epidermal growth factor (EGF; CytoLab Ltd., Israel). The cells were incubated at 37°C in a humidified atmosphere with 5% CO2.

Cell viability assay

Cell growth inhibition was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as described elsewhere (Senawong et al., 2013). Briefly, cells were seeded at a density of 8 × 10^4 cells/well into 96 well plates in triplicate and incubated for 24 hours for adherence. After 24 hours, these cells were treated with different concentrations of cisplatin and scopoletin for 24, 48 and 72 hours. For combination study, the subtoxic dose for each agent against each cell line alone and in combination was used. Vehicle control group was exposed to DMSO (final concentration of 0.5%) or isotonic saline. After desired period, the medium was removed and cells were incubated with MTT (0.5 mg/mL in PBS) for 2 hours. Formazan formed after MTT reduction was dissolved in DMSO, and the absorbance at 550 nm was measured with a microtiter plate reader (Bio-Rad Laboratories, USA). The absorbance at 655 nm was used as a reference wavelength. The number of viable cells is proportional to the production of formazan. Cell viability was determined as a percentage by the following equation:

\[
\% \text{Cell viability} = \frac{\text{Sample O.D.}}{\text{Control O.D.}} \times 100
\]

Analysis of drug interaction

To study drug interaction, cells were seeded into a 96-well plate and allowed to adhere for 24 hours. After that, cells were treated with cisplatin and scopoletin alone or in combination for 72 hours. Then cell viability was determined by MTT assay and the half maximal inhibitory concentration (IC50 value) in combination was calculated. Nature of interaction between cisplatin and scopoletin was evaluated according to the median-dose effect principle described by Chou and Talalay (1984) and Chou (2006). The combination index (CI) and dose reduction index (DRI) reflect the nature of drug interaction and the magnitude of dose to be reduced as a favorable response to interaction without hampering killing efficiency, respectively. For 50% growth inhibition, the combination index (CI) values for agents with mutually non-exclusive mode of actions were calculated based on the equation stated below:

\[
CI = \frac{(D_1)}{(D_{x1})} + \frac{(D_2)}{(D_{x2})} + \frac{(D_1)(D_2)}{(D_{x1})(D_{x2})} \alpha
\]

where (D1) and (D2) are doses of drug 1 (cisplatin) and drug 2 (scopoletin) in combination that inhibit 50% of cell proliferation. (Dx1) and (Dx2) are the doses of drug 1 (cisplatin) and drug 2 (scopoletin) alone that also inhibit 50% of cell proliferation (Chou and Talalay, 1984; Chou, 2006). \(\alpha = 1\) for mutually non-exclusive modes of drug action. CI < 0.9 indicates synergism; CI = 0.9-1.1 indicates an additive relationship, and CI >1.1 indicates antagonism. The dose reduction index (DRI), which determines the magnitude of dose reduction allowed for each drug in a combination as compared to the concentration of single agent required to achieve the same effect, was also calculated (Chou and Talalay, 1984; Chou, 2006). The equation for dose reduction is as follows:

\[
\text{DRI}_d = \frac{(D_{x1})}{(D_1)}
\]

Flow cytometric analysis of cell cycle profile

For cell cycle analysis, KKU-100 and KKU-M214 cells were seeded at the density of 2.5 × 10^5 cells/mL in a 4.5-cm culture dish. After 24 hours, cells were treated with cisplatin and scopoletin alone and in combination and incubated for 24 hours. After exposure to 24 hours, the
percentages of cells distributed in different phases of cell cycle were determined by flow cytometry using propidium iodide (PI) staining. Briefly, cells were harvested, washed with PBS and centrifuged at 3,000 rpm for 3 min. Pellets were fixed in 70% cold ethanol at 4°C for 1 hour. After fixation, cells were washed with PBS twice and incubated with 0.5 mg/mL RNase (RNase A type I; Sigma) for 1 hour to avoid double stranded RNA staining. Lastly, nuclear DNA staining was carried out using propidium iodide (50 µg/mL) in PBS solution under subdued light for 40 min at room temperature. The DNA histograms reflecting cell cycle distribution were determined using BD FACanto II Calibur flow cytometer (Becton-Dickinson, UK).

Flow cytometric analysis of apoptosis
Apoptosis induction of KKU-100 and KKU-M124 cells was analyzed by flow cytometer using the Annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions. Briefly, cells were seeded at the density of 2.5 x 10⁵ cells/mL in a 4.5-cm culture dish and cultured for 24 hours. Cells were treated with cisplatin and scopoletin alone and in combination for 24 hours. After 24 hours of exposure, cells were harvested by trypsinization, washed with cold PBS and centrifuged at 3,000 rpm for 3 min. The pellets were resuspended in the annexin binding buffer. Cell density was determined and diluted in the Annexin binding buffer to 10⁶ cells per assay. Cells were incubated with annexin V-FITC and propidium iodide (PI) at room temperature for 15 min. Following the incubation, cells were analyzed by flow cytometry using a BD FACanto II calibur flow cytometer (Becton-Dickinson, UK). The flow cytometry results were compared with conventional cell count and morphology under a fluorescence microscope.

Statistical analysis
All experiments were repeated independently at least three times. Data were expressed as means ± standard deviation (SD) from three independent experiments. Test for significant differences between sample-treated and solvent-treated cells was carried out using one-way ANOVA with Duncan’s post hoc test. The criterion for statistical significance was set at p<0.05.

Results and Discussion
To evaluate the therapeutic efficacy of scopoletin and cisplatin in cholangiocarcinoma cell lines, cell viability of KKU-100 and KKU-M214 cells was assessed. Results from dose-response curve revealed that cisplatin inhibited proliferation of KKU-100 and KKU-M214 in a dose- and period-dependent fashion, with IC₅₀ values of 7.1 ± 0.7 µM and 12.8 ± 0.5 µM at 72 hours, respectively. Likewise, scopoletin treatment resulted in significant reduction of cell viability in KKU-100 and KKU-M214 in a concentration- and time-dependent manner when treated at 0-500 µM concentrations (Figure 1). The estimated IC₅₀ values reflecting sensitivity against cell lines were 486.2 ± 1.5 µM and 493.5 ± 4.7 µM for KKU-100 and KKU-M214 cells for 72 hours exposure, respectively.

In agreement with our findings, several researchers documented the cytotoxic effects of cisplatin and scopoletin on various cancer cells. Cisplatin has been reported to produce cytotoxic effects against NSCLC cells (Chougule et al., 2011). A similar type of finding was reported by Li et al. (2012) who demonstrated that cisplatin-induced cytotoxicity in SC-M1 gastric cancer cells in a dose-dependent manner. Scopoletin and its several derivatives developed by a systematic combinatorial chemical approach have been reported to show anti-tumor potential against mammary (MCF-7 and MDA-MB231) and colon (HT-29) carcinoma cell lines (Liu et al., 2012). Treatment with scopoletin was found to reduce the viability of fibroblast-like synoviocytes (Li et al., 2009) and suppress proliferation of PC3 cells (a human prostate adenocarcinoma cell line) (Liu et al., 2001) and HL-60 cells (a promyeloleukemic cell line) (Kim et al., 2005) in a dose- and time-dependent manner. It is important to report that some synthetic derivatives of scopoletin exerted profound cytotoxic effects than the prototype scopoletin in both tumor cells and human umbilical vein endothelial cells (Cai et al., 2013). Besides, some other coumarin compounds have also been reported to have anti-cancer effects. Resveratrol, a plant compound called polyphenol, has been demonstrated to inhibit the proliferation of SK-ChA-1 cells (Roncoroni et al., 2008) and KKU-100 and KKU-M156 cells (Hahnvajanawong et al., 2011).

The bar graphs (Figures 1E and 1F) depict the effects of concurrent co-treatments of cisplatin and scopoletin on antiproliferative activity of KKU-100 and KKU-M214 cells. As demonstrated, the combined exposure of cisplatin (3 µM) and scopoletin (250 µM) resulted in cell viability reduction of 41.1 ± 2.0% in KKU-100 while cisplatin (3 µM) alone reduced cell viability to 71.9 ± 3.3% and scopoletin (250 µM) reduced cell viability to 72.4 ± 4.3%. In KKU-M214 cells, the cell viability decreased to 42.6 ± 2.1% for the combination of cisplatin (6 µM) and scopoletin (250 µM), which was significantly lower than cisplatin (73.2 ± 1.7%) and scopoletin (73.3 ± 4.8%) alone. The inhibition of cell growth by this combination was additive in that its effect was equal to the sum of the effects of the two compounds separately. For 50% growth inhibition, the combination index (CI) values (Table I), determined using median effect principle, reflect the additive interaction between cisplatin and scopoletin against both cancer cell lines, which leads to positive dose reduction.

Our results are congruous with findings of several researchers where they documented additive to synergistic interaction between cisplatin and some natural compounds. Synergistic anti-cancer effects between noscapine (an alkaloid) and cisplatin have been
Figure 1: Effect of cisplatin and scopoletin alone or in combination on the cell viability of CCA cells. KKU-100 and KKU-M214 cells were treated with various concentrations of (A, B) cisplatin, (C, D) scopoletin alone or (E, F) in combination for 24, 48 and 72 hours and analyzed by MTT assay. For combination study: (E) KKU-100 cells were treated with cisplatin alone (3 µM), scopoletin alone (250 µM), and combination of cisplatin (3 µM) and scopoletin (250 µM); (F) KKU-M214 cells were treated with cisplatin alone (6 µM), scopoletin alone (250 µM), and combination of cisplatin (6 µM) and scopoletin (250 µM). Data were expressed as mean ± SD of three individual experiments. The letter “a” indicates significant difference between drug and solvent control treatments while “b” indicates significant difference between combination and single drug treatments (p<0.05)

Table I

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Drug combination</th>
<th>Combination Index (CI)</th>
<th>Dose reduction index (DRI)</th>
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<tr>
<td></td>
<td>Cisplatin + Scopoletin</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>KKU-100</td>
<td></td>
<td></td>
<td>Scopoletin</td>
</tr>
<tr>
<td></td>
<td>Cisplatin + Scopoletin</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>KKU-M214</td>
<td></td>
<td></td>
<td>Scopoletin</td>
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reported in lung tumor NSCLC cells and these syner-
gistic effects are through induction of apoptosis via
intrinsic and extrinsic pathways, activation of growth
inhibitory, and inhibition of survival proteins (Chou-
gule et al., 2011). The cytotoxicity of cisplatin in H460
cells was modulated in a synergistic manner by sabaru-
bigicin (Bigioni et al., 2008). Several other studies have
provided evidence that enhanced tumor growth inhibi-
tion can be achieved by combining cisplatin with other
natural compounds. Escoleutin (6,7-dihydroxycouma-
rin) potentiates anti-cancer activity of cisplatin in
DMBA-induced mammary tumor in rat (Tikoo et al.,
2011). The cytotoxic interactions between curcumin and
cisplatin were synergistic in nature (Notarbartoloa et
al., 2005).

To further justify the therapeutic application of drug
combination, the impacts of this combination on proli-
feration of a non-cancer cell line (H69 cells) was exa-
mined. For combination study, the highest subtoxic
doses used against KKU-100 and KKU-M214 cells for
each agent were selected to evaluate. Figure 2 shows
the antiproliferative activity of cisplatin and scopoletin
alone and in combination in H69 cells. Dose response
curve demonstrates that cisplatin and scopoletin were
relatively ineffective against H69 cells, with IC50 values
of 15.1 ± 0.6 µM and >500 µM, respectively, for 72 hours
exposure. The combination of cisplatin (6.0 µM) with
scopoletin (250 µM) reduced the viability to 63.7 ± 1.9%
for 72 hours exposure while cisplatin (6.0 µM) reduced
the viability 80.0 ± 1.7% and scopoletin (250 µM) to 85.6
± 2.1% (Figure 2). The cell viability of a non-cancer cell
line for the combination was still higher when
compared with cancer cell lines, KKU-100 (39.1 ± 2.0%)
and KKU-M214 (42.6 ± 2.1%) cells after concurrent
treatment with cisplatin and scopoletin. It has been
reported that scopoletin and its derivatives have lower
cytotoxic effects on PBMC than tumor cells (Cai et al.,
2013). A prenylated coumarin, diversin, has been obser-
vied to exert considerable cytotoxic effects in bladder
carcinoma 5637 cells, but not on normal human fibro-
blast cells (Haghighitalab et al., 2014). In PBMC, only
large concentrations of the coumarin compounds elic-
ted a cytostatic action (Lopez-Gonzalez et al., 2004).

To investigate the mechanism underlying the cytotoxic
potentiation of cisplatin with scopoletin, we aimed to
analyze the cell cycle profile of propidium iodide (PI)
stained cells using flow cytometry. The percentage of
cells distributing in different phases of the cell cycle
were determined based on the cellular DNA content at
different cell cycle phases. As presented in Table II, in
the case of KKU-100 cells, data from flow cytometry
revealed that cisplatin (10 µM) produced growth arrest
at G1 check point leading to higher percentage(57.9 ±
3.3%) of cells at G0/G1, preventing G1/S entry.
Scopoletin (500 µM) induced significant G0/G1 arrest
with 71.0 ± 1.3%, and concomitant reduction of S phase
cells (by 20.2%) when compared with control (36.6 ±
1.3%). But when the cells were exposed to combination
treatment, they underwent both G0/G1 arrest and

Figure 2: Effect of cisplatin and scopoletin alone or in combina-
tion on the cell viability of a non cancer cell line (H69 cells).
Cells were treated with (A) cisplatin alone, (B) scopoletin alone
and (C) combination of cisplatin and scopoletin for 24, 48 and
72 hours, and analyzed by MTT assay. For combination study,
cells were treated with cisplatin alone (6 µM), scopoletin alone
(250 µM), and combination of cisplatin (6 µM) and scopoletin
(250 µM). Data were expressed as mean ± SD of three individu-
al experiments.
apoptosis as evidenced from more sub-G1 number. In the case of KKU-M214 cells, the cell cycle profile showed no growth arrest at any particular phase when compared with treatment and control. Single agent and combination treatment resulted in increased sub-G1 fraction where the combination produced the highest percentage (25.2 ± 1.8%) followed by cisplatin (18.4 ± 1.9%) and then scopoletin (8.9 ± 1.1%).

Our findings on cell cycle arrest are corroborated by some earlier studies. Cisplatin mediated cell cycle arrest at the G1/S checkpoint with an increase of cells in the G0/G1 phase and reduction of cells in the S phase (Sato et al., 2006). Cisplatin has been demonstrated to retard cell cycle progression of lung cancer cells by accumulation in the G1 phase and the concomitant reduction of cells in the S phase of the cell cycle (Wang et al., 2004).

### Table II

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Treatments</th>
<th>Cell cycle distribution (%) ± SD</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Sub-G1</td>
</tr>
<tr>
<td>KKU 100</td>
<td>Solvent control</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Cisplatin (10 µM)</td>
<td>12.5 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Scopoletin (500 µM)</td>
<td>4.9 ± 0.6</td>
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<tr>
<td></td>
<td>Cisplatin (10 µM) + Scopoletin (500 µM)</td>
<td>16.3 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KKU M214</td>
<td>Solvent control</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Cisplatin (10 µM)</td>
<td>18.4 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Scopoletin (500 µM)</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Cisplatin (10 µM) + Scopoletin (500 µM)</td>
<td>25.2 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

**Figure 3**: Representative DNA histograms on cell cycle profile of CCA cells. KKU-100 (A) and KKU-M214 (B) cells treated with DMSO (0.5%; v/v) as a solvent control, cisplatin alone (10 µM), scopoletin alone (500 µM) and combination of cisplatin (10 µM) and scopoletin (500 µM) for 24 hours were subjected to cell cycle analysis by flow cytometry using propidium iodide (PI) staining. Histograms showed a number of cells per channel (vertical axis) vs DNA content (horizontal axis).
Scopoletin prevented proliferation of human prostate tumor PC3 cells by arresting cell cycle at G0/G1 and S phases (Liu et al., 2001). Diversin was found to produce considerable cytotoxic effects in bladder carcinoma 5637 cells by blocking G2 phase of the cell cycle (Haghighi-talab et al., 2014). It has been reported that coumarin and 7-hydroxycoumarin inhibited cell growth by inducing cell cycle arrest in the G1 phase in lung carcinoma cell lines (Lopez-Gonzalez et al., 2004). Resveratrol was observed to significantly inhibit CCA cell growth in a dose- and time-dependent manner by inducing cell cycle arrest at the G0/G1 phase in KKU-100 cells and the S and G2 phases in KKU-M156 cells (Hahnvajana-wong et al., 2011). Representative DNA histograms of cell cycle profile are depicted in Figure 3.

After evaluation of the cytotoxic effects, we further...
aimed to explore the mechanisms of cytotoxicity enhancement of cisplatin by scopoletin by examining the induction of apoptosis in KKU-100 and KKU-M214 cells after exposure to scopoletin and cisplatin alone or in combination for 24 hours. The percentage of cell undergoing apoptosis upon treatment was determined based on detection of phosphatidylserine externalization by flow cytometry using annexin V-FITC staining. Consistent with sub-G1 group in cell cycle analysis, as can be seen in Figure 4, the combination of scopoletin with cisplatin induced more cells to undergo apoptosis than cisplatin or scopoletin either alone, which suggest additive interaction between the drugs in both cholangiocarcinoma cells. Cisplatin (10 µM) exposure to KKU-100 and KKU-M214 cells increased apoptosis to 14.3 ± 1.4% and 12.3 ± 0.6% and scopoletin (500 µM) produced 12.6 ± 0.7% and 10.8 ± 0.8%, respectively while combination of both agents led to 27.8 ± 1.1% and 23.3 ± 0.9% which are significantly higher than that of single drug controls.

Our results comply with the several research findings where scopoletin, its derivatives and some natural coumarin are reported to induce apoptosis. It has been reported that scopoletin increased apoptosis in human prostate tumor PC3 cells (Liu et al., 2001) and in human leukemia cell line HL-60 (Kim et al., 2005). Scopoletin has been suggested being a possible candidate for chemoprevention as scopoletin treatment for carcinoma-induced skin papilloma in mice produced apoptosis by increasing apoptosis regulator p53 (Bhattacharyya et al., 2010). Treatment with synthetic coumarin has unequivocally been demonstrated to induce up-regulation of p53 and apoptotic proteins Bax in DMBA-administered mice that favored apoptosis and antagonized tumor formation (Bhattacharyya et al., 2009). Antineoplastic action of scopoletin has been observed to be associated with increased apoptosis of HaCaT cells in vitro (Bhattacharyya et al., 2008). Scopoletin remarkably induced apoptosis in a dose-dependent manner in fibroblast-like synoviocytes, which was accompanied by up-regulated Bax mRNA expression and down-regulated mRNA level of Bcl-2(Li et al., 2009). It has been documented that resveratrol inhibits growth of the KKU-100 and KKU-M156 cells by inducing mitochondrial-dependent apoptosis (Hahnvajanawong et al., 2011). Apoptosis was observed in adenocarcinomas with coumarin compounds. Coumarins in combination with other anti-neoplastic drugs might increase the effectiveness of NSCLC treatments (Lopez-Gonzalez et al., 2004). Noscapine and cisplatin combination caused higher percent of apoptotic cell death in H460 and A549 NSCLC cells in comparison to single agent treatment, reflecting synergistic interaction between noscapine and cisplatin (Chougule et al., 2011). Curcumin potentiated the antitumor and apoptotic effects of cisplatin on human hepatic cancer HA22T/VGH cells in a sequence dependent way (Notarbartolo et al., 2005). Taken together, it can be concluded that scopoletin can be used to improve the efficacy of cisplatin in cancer treatments.

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Conflict of Interest

Authors declare no conflict of interest.

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References


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