Different Apoptotic Effects of Triterpenoid Saponin-Rich Gypsophila oldhamiana Root Extract on Human Hepatoma SMMC-7721 and Normal Human Hepatic L02 Cells

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Cancer is a major systemic disease worldwide with high incidence and mortality.1) Most of the anticancer drugs, because of their side effects and general toxicity, are life-threatening and have poor prognosis for patients.2) Therefore, the need for expediting the discovery of drugs with lower side effect is all the more urgent. Natural products, found in many plant extracts and herbs, provided a promising approach for cancer therapy owing to their potentially low toxicity and potential effectiveness.3,4) Recently, triterpenoid saponins, as a kind of fairly large structural diversity of natural products, have attracted much attention due to cytotoxicity and chemopreventive activity. Many triterpenoid saponins exhibit obvious antitumor activity, due to their ability to stimulate apoptotic process in tumor cells.5–7) Thus, the longstanding interest in the investigation of triterpenoid saponins for the development of cancer therapeutics is increasing. Triterpenoid saponins are produced abundantly in the plants of Caryophyllaceae family. Especially, the genus Gypsophila is highlighted, since species in this genus contain abundant amounts of triterpenoid saponins.8) Previous studies have mostly been focused on the anticancer and cytotoxic properties of Caryophyllaceae saponins.9) A prominent example is saponinum album (Merck), a complex mixture of triterpenoid saponins from G. paniculata L. It was shown that the combination of saponinum album (Merck) and saponin-base chimeric anti-tumor toxins (SA2E) resulted in a 94% tumor regression in mice.10) G. oldhamiana Miq. is a small perennial herb widely distributed in the north of China. Its roots have been usually used as a folk medicine to treat fever, consumptive disease, and infantile malnutrition syndrome in China.11) Previous chemical researches on its roots have revealed the occurrence of saponins, sterols, and fatty acid,11–15) and triterpenoid saponins and saponin-rich extract of its roots showed obvious anticancer activity in vitro and in vivo.16–18) However, there is no report on the mechanisms responsible for the anti-proliferative effects.

The current study was therefore initiated with the following objectives: (1) to prepare a triterpenoid saponin-rich G. oldhamiana Root extract (TGOE), whose phytochemical characterization was performed using LC-electrospray ionization (ESI)-MS*, for biological studies and (2) to investigate the anti-proliferative effects and possible mechanisms of TGOE on human hepatoma SMMC-7721 and normal human hepatic L02 cells.

MATERIALS AND METHODS

Plant Material and Preparation of G. oldhamiana Root Extract The roots of G. oldhamiana Miq. (Caryophyllaceae) were obtained from Lianyungang city, Jiangsu Province, China. The botanical origin of material was identified by Prof. Minjian Qin, China Pharmaceutical University, Nanjing, China. And the voucher specimens (No. 20110616) were deposited at the Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing, China. Dried roots (100 g) were extracted twice with 800 mL pure water.
The total triterpenoid saponin content of TGOE was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The logarithmic growth cells were collected and seeded in 96-well plates at a density of 5×10^4 cells per well in 200 µL medium for 24 h at 37°C. Cells were then exposed to TGOE at different concentrations (0–50 µg/mL) for 48 h. 5-Fluorouracil (5-FU, ≥99% pure, KeyGen, Nanjing, China) was used as a positive control with different concentrations (0–50 µg/mL). After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and cultured for 4 h. Then the supernatant was discarded and dimethyl sulfoxide (DMSO) was added (150 µL/well). Absorbance was measured at 570 nm by a Universal Microplate Reader (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA, U.S.A.).

**Cell Morphological Assessment** Both SMMC-7721 and L02 cells were seeded in 6-well tissue culture plates (2×10^5 cells/well) and treated with TGOE (12, 24, 36 µg/mL). 5-FU was used as a positive control with the concentrations of 10 µg/mL for SMMC-7721 cells and 15 µg/mL for L02 cells. After incubation for 48 h, the cell morphology was examined under an inverted microscope (Olympus IX-70, Tokyo, Japan).

**Apoptosis Analysis by Flow Cytometry** Apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining and flow cytometry analysis. Both SMMC-7721 and L02 cells were incubated with TGOE (12, 24, 36 µg/mL) for 48 h, then harvested and resuspended in PBS. 5-FU was used as a positive control with the concentrations of 10 µg/mL for SMMC-7721 cells and 15 µg/mL for L02 cells. Apoptotic cells were determined by FITC-annexin V apoptosis detection kit (KeyGen, Nanjing, China) according to the manufacturer’s instructions. Percentages of apoptosis cells were determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, U.S.A.).

**Apoptosis Assessment by Hoechst 33258 Staining** Apoptotic morphological changes were observed by Hoechst 33258 staining. Both SMMC-7721 and L02 cells were seeded in 6-well plates at a concentration of 2×10^5 cells/well and treated with TGOE (12, 24, 36 µg/mL). 5-FU was used as a positive control with the concentrations of 10 µg/mL for SMMC-7721 cells and 15 µg/mL for L02 cells. After incubation for 48 h, the attached cells were washed twice with PBS and fixed with 4% formaldehyde for 20 min, then stained with Hoechst 33258 (Beyotime, Haimen, China) for 10 min. After being washed with PBS, the stained cells were observed under a fluorescence microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

**Western Blot Analysis** For caspase-3 and mitogen activated protein kinases (MAPKs) detection, both SMMC-7721 and L02 cells cultured in RPMI 1640 at the mid-log phase were exposed to TGOE (0, 12, 24, 36 µg/mL). For extracellular signal-regulated kinase (ERK) inhibition studies, SMMC-7721 cells pre-treated with or without ERK inhibitor (PD98059, 10 µM, Cell Signaling Technology, Beverly, MA, U.S.A.) were incubated in the absence or presence of TGOE (36 µg/mL). After treatment for 48 h, cells were collected and washed with PBS, then lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF). After centrifugation, protein concentration was determined using the BCA protein assay kit (Beyotime, Haimen, China). Protein samples (50 µg) were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). After being incubated with 5% nonfat dry milk in TBST for 2 h to block nonspecific binding, the membranes were probed with primary antibodies capase-3, cleaved caspase-3, p38, p-p38, c-Jun N-terminal kinase (JNK),
p-JNK, ERK, p-ERK and β-actin (1:1000, Cell Signaling Technology, Beverly, MA, U.S.A.) overnight at 4°C. After being washed, the blots were incubated with secondary antibodies (1:2000, Cell Signaling Technology, Beverly, MA, U.S.A.) for 2 h. Finally, Enhanced Chemiluminescence (ECL) Detection Reagents (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) were used to develop the signal of the membrane.

**Statistical Analysis** Data are expressed as means±S.D. from triplicate parallel experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) or Student’s t-test. Significance of difference was defined as p<0.05 for all tests.

**RESULTS**

**Phytochemical Analysis of TGOE** Since the triterpenoid saponin-rich extract of *G. oldhamiana* root showed obvious anticancer activity *in vitro* and *in vivo*, the TGOE was prepared and identified by HPLC-MS, and total saponins content of TGOE was determined. After several calibrations, a standardized triterpenoid saponin-rich *G. oldhamiana* root extract (TGOE, 2.97±0.53 g, n=3) was achieved, which possessed triterpenoid saponins content of 71.61±2.6% (n=3). In Fig. 1, the HPLC-ELSD chromatogram corresponding to TGOE is shown, of which the five main peaks 1–5 were identified as saponins 1–5 (Fig. 2) by LC-ESI-MS, according to the pseudo-molecular ions [M−H]− at m/z 1643, 1393, 1525, 1231 and 1495, respectively, and the characteristic fragment ions (Table 1) in the negative mode.

**Different Growth Inhibition of SMMC-7721 and L02 Cells Induced by TGOE** The cytotoxic effects of TGOE on the growth of SMMC-7721, HepG2, NCI-H460 and L02

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Table 1. HPLC-ESI-MS Data for Identification of Triterpenoid Saponins 1–5 in TGOE

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>t_R (min)</th>
<th>[M−H]− (m/z)</th>
<th>MS² (m/z)</th>
<th>MS³ (m/z)</th>
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<tr>
<td>1</td>
<td>19.71</td>
<td>1643</td>
<td>955</td>
<td>823, 579, 485, 405</td>
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<tr>
<td>2</td>
<td>23.06</td>
<td>1393</td>
<td>807, 583</td>
<td>763, 627, 479</td>
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<tr>
<td>3</td>
<td>24.23</td>
<td>1525</td>
<td>939, 469</td>
<td>807, 563, 469, 157</td>
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<tr>
<td>4</td>
<td>27.41</td>
<td>1231</td>
<td>807, 627</td>
<td>763, 645, 627, 469</td>
</tr>
<tr>
<td>5</td>
<td>28.66</td>
<td>1495</td>
<td>939</td>
<td>807, 563, 469</td>
</tr>
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</table>
cells were evaluated in our study. The IC\textsubscript{50} of TGOE for SMMC-7721, HepG2, NCI-H460 cells were 19.50±3.63, 29.20±2.09 and 33.37±2.63 µg/mL, respectively, and for normal human hepatic L02 cells was 40.48±3.74 µg/mL, whereas the IC\textsubscript{50} of the positive control 5-FU was 10.38±2.31 µg/mL for SMMC-7721 cells and 14.63±2.71 µg/mL for L02 cells. Therefore, subsequent experiments were performed on SMMC-7721 cells and normal human hepatic L02 cells. The morphological changes of cells were observed by light microscopy. After treatment with TGOE, SMMC-7721 cells shrunk and were transformed into semi-suspended and sphere-shaped cells. In addition, protuberances or “blebs” at the plasma membrane were observed with increasing doses of TGOE. However, almost no morphological change was observed in the L02 cells exposed to the same doses of TGOE. After treatment with 5-FU (10 µg/mL for SMMC-7721 cells and 15 µg/mL for L02 cells), which was used as a standard, both cell lines showed significant morphological changes (Fig. 3).

**Different Apoptotic Effects of SMMC-7721 and L02 Cells Induced by TGOE**

In order to explore the underlying basis for the growth inhibitory activity of TGOE, apoptosis analysis was performed by FITC labeled annexin V/PI staining (Fig. 4B). Viable cells were negative for both annexin V and PI (lower left quadrant); early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic cells displayed positive for both annexin V and PI (upper right quadrant); dead cells which underwent necrosis were positive for PI and negative for annexin V (upper left quadrant). As shown in Fig. 4B, SMMC-7721 cells treated with TGOE presented a dose-dependent increase of apoptosis ratios compared with the control group. Quantitative results showed that the percentage of apoptotic SMMC-7721 cells treated with 0, 12, 24, and 36 µg/mL of TGOE was 5.53%, 13.50%, 24.45% and 48.00%, respectively. But L02 cells, treated with 12 and 24 µg/mL of TGOE, exhibited no significant changes compared with the control group. Although the apoptosis ratio of L02 cells increased to 12.19% at the concentration of 36 µg/mL, it was almost a quarter of the apoptosis ratio in SMMC-7721 cells. The apoptotic rate produced by the positive control 5-FU was 24.24% for SMMC-7721 cells and 23.15% for L02 cells. To further verify the TGOE-induced apoptosis, the cell nuclear morphology was detected by fluorescence microscopy after Hoechst 33258 staining (Fig. 4A). TGOE-treated SMMC-7721 cells demonstrated condensed and fragmented chromatin, characteristic of cell apoptotic death, in a dose dependent manner. However, only slight nucleolus pyknosis of the treated L02 cells was observed, with no significant phenomena of apoptosis. As the positive control, both of the 5-FU-treated cells exhibited fragmented nuclei with brilliant blue staining.

**TGOE-Induced Apoptosis through the Caspase-3 and MAPKs Signaling Pathways**

To explore the potential molecular mechanisms of TGOE-induced apoptosis in SMMC-7721 and L02 cells, the expression of caspase-3 and cleaved caspase-3 were examined by Western blot analysis. As shown in Fig. 5, caspase-3 was proteolytically cleaved, showing the characteristic shift of the protein band from 35 to 19 kDa in a dose-dependent manner in SMMC-7721 cells after treatment with TGOE. However, in L02 cells, the levels of the two proteins showed no significantly difference. These results suggested that TGOE invoked apoptosis in SMMC-7721 cells via caspase-3 activation. To examine whether MAPKs were involved in the processing of TGOE-induced apoptosis, levels of p38, ERK, JNK and their phosphorylation status (p-p38, p-ERK, p-JNK) were investigated (Fig. 6). TGOE markedly up-regulated the phosphorylation of ERK and JNK in SMMC-7721 cells, while no effects on L02 cells. The phosphorylation levels of p38 were reduced by TGOE in SMMC-7721 cells, but elevated in L02 cells. Furthermore, pre-treatment with ERK inhibitor PD98059 evidently reduced the expression of the cleaved caspase-3 in TGOE-treated SMMC-7721 cells (Fig. 7). These findings suggested that MAPKs played an important role in the process of TGOE-induced apoptosis in SMMC-7721 cells.

**DISCUSSION**

Recently, increasing efficacy and decreasing side effects without causing excessive damage to normal cells have become a capital and urgent goal for the anticancer drug research. Triterpenoid saponins and saponin-rich extract of *G. oldhmiana* root have been suggested to exhibit cancer chemopreventive and chemotherapeutic properties.\(^{16–18}\) Therefore, our study was performed to research the anti-proliferative effects and possible mechanisms of TGOE on human hepatoma SMMC-7721 and normal human hepatic L02 cells for the first time.

Results showed that TGOE preferentially inhibited the growth of SMMC-7721 cells in a dose-dependent manner,
Fig. 4. Different Apoptotic Effects of SMMC-7721 and L02 Cells Induced by TGOE
(A) Cell nuclear morphological changes after treatment with TGOE (12, 24, 36 µg/mL) for 48 h in SMMC-7721 and L02 cells by Hoechst 33258 staining under fluorescence microscopy (×400); (B) Flow cytometric analysis of annexin-V/PI double-staining assay after incubation with TGOE (12, 24, 36 µg/mL) for 48 h. 5-FU (10 µg/mL for SMMC-7721 cells and 15 µg/mL for L02 cells) was used as a positive control. The results are expressed as means±S.D. (n=3). Asterisks (**p<0.01, ***p<0.001) illustrate significant difference compared control. Octothorpes (#p<0.05) illustrate significant difference compared between the two items linked.

Fig. 5. TGOE-Induced Apoptosis through the Caspase-3 Signaling Pathway by Western Blot Analysis
Protein expression of caspase-3 and cleaved caspase-3 in SMMC-7721 and L02 cells incubated with TGOE (12, 24, 36 µg/mL) for 48 h. β-Actin was used as a loading control. The results are expressed as means±S.D. (n=3). Asterisks (**p<0.01, ***p<0.001) illustrate significant difference compared control. Octothorpes (#p<0.05) illustrate significant difference compared between the two items linked.
while not exhibiting significant cytotoxicity on L02 cells, suggesting that TGOE had selective cytotoxicity between hepatoma and normal cells. The selectivity of TGOE to hepatoma cells compared to non-cancerous cells increases its prospect as a potential chemopreventive agent.

Apoptosis seems to be a reliable marker for the appraisal of potential agents for cancer prevention, and a wide variety of natural products have been known to have the ability to induce apoptosis in various human tumor cells. Therefore, to further investigate the mechanisms of anti-proliferative effects of TGOE on SMMC-7721 and L02 cells, apoptosis analysis was determined. The results of the present study demonstrated that apoptotic SMMC-7721 cells significantly increased in a dose-dependent manner after treatment with TGOE, whereas TGOE-treated normal liver cells were not affected at lower concentrations except for slight apoptosis at highest concentration (Fig. 4B). In addition, the conspicuous changes in the hepatoma cell nuclear morphology could be the direct reflections of cell apoptosis (Fig. 4A). It seemed that TGOE could specifically induce apoptosis in hepatoma cells.

Caspase-3 plays a very important role in apoptosis and is considered to be the terminal event preceding cell death. This current study demonstrated that caspase-3 was dose-dependently activated by TGOE in SMMC-7721 cells while it was not activated in L02 cells (Fig. 5). These results suggested that caspase-3 might be a predominant target involved in TGOE-induced apoptosis in SMMC-7721 cells, due to the fact that caspase-3 can be activated by proteolytic processing at internal aspartate residues when cells receive an apoptosis-inducing signal, and TGOE preferentially killed hepatoma cells.

Mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38, play an essential role in the regulation of cell proliferation and apoptosis. MAPKs, involved in multiple intracellular signaling cascades, can phosphorylate and activate their target proteins after activation. In this study, we found that phosphorylation levels of ERK and JNK were significantly elevated by TGOE in SMMC-7721 cells, but showed no statistical changes in L02 cells. TGOE decreased phosphorylation of p38 in SMMC-7721 cells while increased in L02 cells. ERK are usually associated with cell proliferation and differentiation. However, there were growing evidences suggested that activation of ERK also contributes to cell death, which was concluded from our results that pre-treatment with ERK inhibitor PD98059 could decrease the cleavage of caspase-3 in TGOE-treated SMMC-7721 cells. Since ERK and JNK activation play an
active role in mediating apoptosis in cancer cells, and the decrease in p38 phosphorylation and the inhibition of p38 by its specific inhibitor promote apoptosis in cancer cells, we infer that ERK, JNK and p38 pathways selectively participate in TGOE-induced apoptosis in SMMC-7721 cells, while not in L02 cells.

Cross-talk between MAPKs and caspase-3 signaling pathways has been certified by the evidence that the JNK and ERK specific inhibitors can block caspase-3 activation while p38 specific inhibitors can evidently activate caspase-3. Therefore, we conjecture that MAPKs and caspase-3 play an active role in mediating TGOE-induced apoptosis and MAPKs function upstream of caspase-3 activation to initiate the apoptotic signal. However, further investigations into the precise mechanisms of TGOE-induced apoptotic effects are needed to verify the validity of this hypothesis.

Triterpenoid saponins have been demonstrated to have cytotoxic activities against cancer cell lines. As the major constituents of TGOE, triterpenoid saponins (71.61% of TGOE) or the sum of interactions between them and other compounds (28.39% of TGOE) might be responsible for the cytotoxic effects of TGOE on SMMC-7721 cells. Some triterpenoid saponins, of which the structures are similar with saponins 1–5, induce apoptosis via caspase-3 signaling pathway against cancer cells. The anti-proliferative activity and possible mechanisms of individual active components and interactive effects between these saponins and other phytochemicals from TGOE are ongoing in our lab.

In conclusion, our study indicated that TGOE, whose main constituents were proved to be triterpenoid saponins by HPLC-ELSD and LC-ESI-MS, had an effective and selective anti-proliferative activity against hepatoma cells. The anti-proliferative activity might be associated with apoptosis induction through MAPKs and caspase-3 signaling pathways. Altogether, TGOE may be a novel therapeutic agent in hepatocellular carcinoma treatment, and deserves to be studied further.

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