Original Article
The anti-proliferation effect of triterpenoid extracted from Actinidia Chinese plant root

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Abstract: Objective: Actinidia Chinese plant root extract exhibits an antitumor effect, particularly on gastrointestinal tumors. Different methods and reagents may result in the extraction of different constituents. This study aimed to observe the antitumor effect of ethanol-derived triterpenoids from Actinidia Chinese plant (TACP) and to determine its mechanism on human colonic cancer cells. Method: MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay was used to verify the inhibitory effect of TACP. Hochest33258 staining was employed to observe the morphological changes of the cells. Annexin V/PI double staining was used to observe the apoptosis of SW480 cells, and PI single staining was employed to observe the cycle of SW480 cells. Western blot and real-time PCR analyses were performed to detect the expression of apoptosis/cell-cycle-related mRNA and protein levels in SW480 cells treated with TACP. Results: MTT results showed that TACP could inhibit the proliferation of SW480 and LOVO cells in a dose- and time-dependent manner. Hochest 33258 showed that the SW480 cells exhibited apoptotic features after TACP treatment. FCM results showed that the apoptosis rate of SW480 cells increased 48 h after TACP treatment. This result might be connected with the up regulation of caspase-3, caspase-7, and Fas expression levels. Moreover, TACP induced the cell cycle arrest of SW480 cells at the sub-G1 phase, which might have down regulated the expression of cyclinD1 and up regulated the expression of P53 and P21. Conclusion: The ethanolic extract of Actinidia Chinese plant root displayed strong antitumor effects against SW480 cells and could induce tumor cell apoptosis in vitro. Its mechanism of action might involve cell cycle arrest (G0/G1); induction of apoptosis through the up regulation of Fas, caspase-3, and caspase-7 protein levels, as well as P53 and P21 gene levels; and the down regulation of CyclinD1 expression. These findings provided a molecular theoretical basis for the clinical application of TACP.

Keywords: Triterpenoid, colorectal carcinoma cell, anti-proliferation effect

Introduction
As environmental pollution worsens and human lifestyle changes, the morbidity rate of tumors increases rapidly. Colorectal carcinoma is the most common gastrointestinal tumor [1], with surgical operation as the effective treatment. However, most colorectal carcinoma patients fail to undergo surgery because the early symptoms are not obvious. Under this situation, drug therapy plays an important role in the treatment of colorectal carcinoma. Traditional drug therapy usually refers to chemotherapy, but Chinese herb preparation of adjuvants is also used during actual clinical treatment to enhance treatment efficiency.

Over the centuries, natural compounds extracted from plants such as Chinese herbs have been used in medicinal traditions to treat various diseases including cancer [2]. The root of Actinidia Chinese plant is one of the traditional Chinese herbs commonly used to treat tumors [3], particularly gastrointestinal tumors [4]. Different extraction methods and reagents yield different constituents with differ-
ent effects. The chemical constituents of Actinidia Chinese plant root are flavonoids, triterpenoid, steroids, anthraquinone, phenolic acids, and many others. Triterpenoid is an active compound in traditional Chinese medicine and has been proven to exhibit antitumor properties in many studies [5]. For example, ursolic acid is a type of triterpenoid isolated from the roots of Actinidiachinese Planch; this acid displays an in vitro antitumor activity in human NSCLCCs and exerts a potent and preferential cell growth inhibitory effect on NCI-H460 cell lines [6].

This experimental study was performed for the first time to confirm the inhibitory effects of TACP on the growth of human colorectal carcinoma cells in vitro and to elucidate the possible mechanism of its activity.

Materials and methods

Plant materials

Actinidia Chinese Plant was collected in Guangxi Province and identified by Guangxi institute of botany. The triterpenoid of Actinidia Chinese Plant root (TACP) was extracted, isolated and analyzed as described in detail in previous research [7]. The TACP was dissolved by DMSO and diluted to different concentrations by medium. The content of DMSO in each concentration was below 1‰.

Cell culture

The human colonic cancer cell line SW480 and LOVO were obtained from the tumor pathology research center of Guilin medical University. Cells were cultured in RPMI 1640 with 10% fresh fetal bovine serum (Gemini company, American) and 1% antibiotic/antimycotic mix (Solarbiocompany, Beijing) at 37°C in 5% CO₂.

Cell viability assay

The SW480 and LOVO cells were treated in the following procedures: cells in the exponential phase of growth were seeded in 96-well plates (3.5×10³ cells/well) with 200 ul medium. Added different concentrations of TACP (0, 10, 20, 40, 80, 160, 320 mg·mL⁻¹) after the cells were adhered. The control group was examined with the same concentration in all cases with DMSO (below 0.1%) involved. Cultured the cells for 24, 48 and 72 h at 37°C in 5% CO₂. Added the thiazoly blue tetrazolium bromide (MTT) solution (0.5 mg/ml) into the wells and cultured the cells for another 4 h. Then removed the medium and added DMSO into the wells. Measured absorbance values at 490 nm by the microplate reader (MLDEL680, BIO-RAD). The column without cells was used as blank group. Calculated the viability (%) by the following equation:

\[
\text{Cell viability (\%)} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100\%
\]

Hoechst 33258 staining

Put the cover glasses which soaked in the 70% alcohol overnight into the 6-well plates. Seeded SW480 cells into the plates 10⁵ cells/well and cultured the cells at 37°C in 5% CO₂. After 24 h treated the cells with complete medium as control group and different concentrations of TACP (0, 40, 80 μg·mL⁻¹) as experimental group and then cultured the cells at 37°C in 5% CO₂ for 48 h. Then discarded the medium and fixed the cells with 500 ul fixative for 30 min. Washed the cover glasses three times with PBS and added Hoechst 33258 staining solution into the wells. Cultured the cells for 30 min at room temperate in dark. Then washed the glasses three times with PBS and added Antifade Solution onto the cover glasses. Viewed the cover glasses under a UV microscope and took pictures. Performed three times.

Annexin V/PI double staining method to observe the apoptosis of SW480 cells

The control group and the experimental groups were the same as the ‘Hoechst 33258 staining’ section. Three parallel samples were set up for each group. The four groups cells were harvested, centrifuged (1000 rp/s) and aspirated the supernatant. Then washed the cells with cold PBS twice and centrifuged (1000 rp/s) five min and aspirated the supernatant. According to the manufacturer’s instructions of Annexin V-FITC apoptosis kit (Becton Dickinson, American), mixed the cells of each groups with 100 ul buffer and then with 5 ul Annexin-V and 5 ul PI. Then put the samples in dark for 30 min. After staining, resuspended the cells in 300 µl PBS and filtration the cells with filters of
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**Table 1.** The primer sequences and their product lengths in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Length of product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21</td>
<td>F: CCT CAT CCC GTG TTT TCC TTT&lt;br&gt;R: GTA CCA CCC AGC GGA CAA GT</td>
<td>97</td>
</tr>
<tr>
<td>P53</td>
<td>F: TCA ACA AGA TGT TTT GGC AAC TG&lt;br&gt;R: ATG TGC TGT GAC TGC TTG TAG ATG</td>
<td>118</td>
</tr>
<tr>
<td>Cyclind1</td>
<td>F: TAT TGC GCT ACC GTT GA&lt;br&gt;R: CCA ATA GCA GCA AAC AAT GTG AAA</td>
<td>87</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: TGC ACC ACC TGC TTA GC&lt;br&gt;R: GCC ATG GAC TGT GGT CAT GAG</td>
<td>87</td>
</tr>
</tbody>
</table>

200 mesh. FCM (BD, Biosciences) was adopted to detect the apoptotic rate.

**PI Single staining method to observe the cycle of SW480 cells**

The procedures were the same as ‘Annexin V/PI Double Staining Method to observe the apoptosis of SW480 cells’ until washed the cells with cold PBS twice and centrifuged (1000 r.p/s) five min and aspirated the supernatant. Resuspended the cells with pre-cold 75% ethanol and put them at 4°C overnight. According to the Cell Cycle and Apoptosis Analysis Kit (Beijing 4A Biotech Co., Ltd), stained the cells like the manufacturer’s instructions mentioned. Resuspended the cells and filtration them with filters of 200 mesh. FCM (BD, Biosciences) was adopted to detect the cycle of SW480 cells.

**Western blot**

Treated SW480 cells with complete medium as control group and different concentrations of TACP (0, 40, 80 µg·mL\(^{-1}\)) as experimental group and then cultured the cells at 37°C in 5% CO\(_2\). 48 h later, added 300 ul RIPA in the plate of the 4 groups cells for 30 min on ice. Then centrifuged the cells liquid at 12000 g for 10 minutes at 4°C. Got the supernatant to measure the protein concentration. Resuspended the protein in loading buffer and then separated on a 12% SDS-polyacrylamide. The transferred the protein on the PVDF membranes (0.22 mm). Then blocked the membranes with TBS included 5% milk for 2 h. Then washed the membranes with TBST and incubated the membranes with anti-p53/anti-p21/anti-cyclind1 and anti-GAPDH at 4°C overnight. Then wash the membranes with TBST three times and incubated the membranes in 5% milk with secondary antibody for 2 h at normal temperature. Washed the membranes three times. Let the membranes reacted with ECL for one minute and then put into the Gel Imaging System (BIO-RAD, American) to get the results.

**RT-PCR**

Grouping and treated the SW480 cells as mentioned above. Extracted the total RNA as the manufacturer’s instructions of RNA simple Total RNA Kit (TIANGEN Biotech, Beijing, DP419). According to the manufacturer’s instructions of reverse Transcriptase M-MLV kit (Invitrogen, Beijing) to synthesized the First-strand cDNA. RT-qPCR was run in the ABI 7500 Fast Real-Time PCR System (ABI company, American) machine using Power SYBR® Green PCR Master Mix (ABI company, American, 4367659) with the following protocol: 2 min at 50°C, 1 min at 94°C, 1 min at 55°C and 1 min at 72°C cycling 40 cycles and then extended 5 min at 72°C. Data were analyzed according to the 2-ΔΔ Ct method. The expression level of cycle/apoptosis were normalized to GAPDH. The primer sequences and their product lengths in RT-PCR (**Table 1**). Three separate experiments were performed for each group.

**Statistical analysis**

All experiments were performed three times. Data are presented as means ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA and two-way ANOVA on spss19.0 and Graph pad prism 5. P < 0.05 indicates statistical significance.

**Result**

**Inhibitory effect of TACP on SW480 and LOVO cells**

We chose the human colonic cancer cell lines SW480 and LOVO to confirm the inhibitory effect of TACP. Results showed that TACP could inhibit the proliferation of these two cell lines in a dose- and time-dependent manner (**Figure 1**). The half maximal inhibitory concentrations (IC\(_{50}\)) at 24, 48, and 72 h of SW480 cells were 122.52, 78.18, and 39.96 µg·mL\(^{-1}\), respectively, whereas those of LOVO cells were 197.3,
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147.26, and 83.18 µg·mL\(^{-1}\), respectively. On the basis of this result, we chose SW480 cells treated with different concentrations of TACP (0, 40, and 80 µg·mL\(^{-1}\)) for 48 h in subsequent experiments.

**TACP-induced apoptosis of SW480 cells**

Three groups of SW480 cells were treated with different concentrations of TACP (0, 40, and 80 µg·mL\(^{-1}\)) for 48 h. Then we used hochest33258 staining to observed the morphological changes in SW480 cells. As the concentration of TACP was increased, the cells began to show apoptotic features, such as nuclei karyopyknosis, karorrhexis, and karyolysis (**Figure 2A**).

**Apoptosis rate of SW480 cells**

FCM detected the apoptosis rate of SW480 cells and showed that TACP mainly induced early and late apoptosis (**Figure 2B**). The apoptosis rates were 10%, 19.5%, and 48.3%.

Then, we detected the expression levels of three apoptosis-related proteins, namely, caspase3, caspase7, and Fas, by Western blot analysis. As shown in **Figure 2C**, the groups treated with 40 and 0 µg·mL\(^{-1}\) TACP showed no distinct difference in the expression levels of caspase-related proteins capase-3 and caspase-7. However, treatment with 80 µg·mL\(^{-1}\) TACP distinctly improved the expression levels of capase-3 and caspase-7. At the same time, the expression of fas/fasl-related protein improved with increasing concentrations of TACP, which demonstrated that the Fas death receptor pathway might have contributed to the apoptosis of SW480 cells.

**Inhibitory effect of TACP on the cell cycle progression of SW480 cells**

The inhibition of cell proliferation is usually caused by the induction of cell apoptosis and cell cycle arrest. After studying the effects on cell apoptosis, we observed changes in cell cycle after treatment with different concentrations of TACP (0, 40, and 80 µg·mL\(^{-1}\)) for 48 h. FCM detection showed that the cell cycle was mainly arrested at the G1/G0 phase in SW480 cells (**Figure 3A**). As the TACP concentration was increased (0, 40, and 80 µg·mL\(^{-1}\)), the G1/G0 phase became 49.12%, 57.83%, and 72.47%.

**TACP-induced expression changes of cell cycle-related mRNA in SW480 cells**

RT-PCR results (**Figure 3B**) showed that the mRNA expression levels of P53 and P21 were up regulated, whereas that of cyclinD1 was down regulated.

**TACP-induced expression changes of cell cycle-related protein in SW480 cells**

To verify the results of RT-PCR, we detected the protein expression levels of P53, P21, and cyclinD1. The protein expression levels of P53 and P21 were up regulated, whereas that of

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**Figure 1.** The MTT staining showed that both of the SW480 cells and lovo cells were inhibited after treated by the TACP (*P < 0.05, ***P < 0.01).
cyclinD1 was downregulated (Figure 3C). These results are generally the same as those of RT-PCR.

**Discussion**

Colorectal carcinoma typically occurs in older patients close to 70 years old [8]. More than 90% are diagnosed 50 years old. However, the incidence of colorectal carcinoma among young adults aged 20 to 39 years has increased over the past 20 to 30 years, even as incidence rates have declined in the overall US population [9, 10]. Chemotherapy drugs could improve the survival of Colorectal carcinoma patients, but the side effects also occur, including gastrointestinal reaction, bone marrow suppression, damage to liver function and so on, which decrease treatment related tolerance and compliance [11]. Thus, it is urgent to develop novel drugs which are more effective and fewer side effects.
In this study, we first verified the inhibitory effect of TACP against colorectal carcinoma cells through the MTT assay. TACP significantly inhibited the proliferation of SW480 and LOVO cells in a dose- and time-dependent manner. Moreover, SW480 cells underwent apoptosis after TACP treatment based on the typical morphologic changes.

Fas/FasL death receptor plays an important role in cell apoptosis. Upon binding to FasL, Fas trimerizes and induces apoptosis through the

Figure 3. The effect of TACP on cell cycle of SW480 cells. A. The cell cycle was detected by PI Single Staining on FCM (*P < 0.05). B. RT-PCR to detect expression changes of cell cycle related mRNA in SW480 cells. With the increasing concentrations of TACP, the P53 and P21 mRNA was high expression while the CyclinD1 was low expression (**P < 0.05, ***P < 0.01). C. Western-bolt to detect expression changes of cell cycle related protein in SW480 cells (*P < 0.05, ***P < 0.01).
cytoplasmic death domain (DD), which interacts with signaling adaptors such as Fas-associated DD (FADD). FADD carries a death effector domain (DED), which recruits the DED-containing procaspase-8 protein in its inactive state. Procaspase-8 is proteolytically activated to caspase-8, and FADD helps in the activation of caspase-10. Upon activation, caspase-8 and caspase-10 cleave and activate downstream effector caspases, including caspase-3, -6, and -7. However, reports have also shown that harmol activates a key element of the Fas signaling pathway independent of Fas/FADD activation [12]. Therefore, Fas, FasL, and two key downstream effector caspases (activated caspase-7 and caspase-3) were detected to reveal the involvement of the Fas/FasL signaling pathway in the apoptosis induction of SW480 cells upon TACP treatment. Surprisingly, the expression of Fas was upregulated, whereas those of caspase-7 and caspase-3 were only slightly activated in SW480 cells. The activity of caspase-3 was also detected in the colorectal carcinoma cells. As expected, caspase-3 activity increased in a dose-dependent manner. Blocking Fas/FasL signaling using an anti-Fas antibody blocked caspase-3 and caspase-7 activation. These results implied that the Fas/FasL signaling pathway was initiated and involved in SW480 cell apoptosis. However, whether or not the intrinsic apoptotic pathway also contributes to the apoptosis of SW480 cells treated with TACP remains unknown. This issue still needs to be verified in the future.

Moreover, TACP exerted a strong anti-proliferative effect against SW480 cells by inducing G1/G arrest. The cyclins are a family of proteins that control the progression of cells through the cell cycle by activating cyclin-dependent kinases (Cdks) [13]. Cyclins themselves have no enzymatic activity but have binding sites for specific substrates; thus, Cdks are easily recruited to specific subcellular locations. Cyclins can be divided into four classes based on their behavior in the cell cycle, with different cyclin classes having roles in specific segments of the cell cycle. In general, a Cdk without a corresponding cyclin exhibits a weak kinase activity, and only the cyclin-Cdk (such as cyclinE-Cdk2, cyclin D1-Cdk4) complex is an active kinase.

The tumor suppressor p53 and Cdk inhibitor p21 are important regulators of the cell cycle. In our study, p53 was markedly up regulated. P21, whose gene is tightly controlled by p53, binds to the cyclinE/Cdk2 complex, preventing pRb phosphorylation and progression through G1/S transition [14, 15]. Interestingly and paradoxically, p21 is required for the assembly of cyclinD/Cdk complexes when expressed at low levels, whereas the down regulation of p21 in tumor cells promotes apoptosis [16]. The mRNA expression of p21 can be markedly up regulated by TACP through the p53-independent pathway. Meanwhile, TACP can decrease the protein level of p21. Our results are congruent with those of previous studies, as we observed a marked up regulation of p21 mRNA and a down regulation of p21 protein. Overall, these findings indicate that TACP can inhibit tumor cell proliferation by affecting p21 levels through a p53-independent pathway.

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Disclosure of conflict of interest

None.

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